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Inhibitors and Mechanism of Phospho-N-acetylmuramyl-pentapeptide Translocase (*Escherichia coli*).

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ABSTRACT

FACULTY OF SCIENCE
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Bacterial drug resistance is an increasingly serious problem facing public health. Consequently there is a continuing need for the development of novel antibacterial agents as therapeutic antibiotics. Bacterial peptidoglycan biosynthesis is a good target for antimicrobial agents since there is no equivalent in mammalian cells.

The first step in the membrane cycle of reactions of bacterial peptidoglycan biosynthesis is transfer of phospho-MurNAc-pentapeptide from UMP to a membrane bound carrier molecule, undecaprenyl phosphate. This step remains an unexploited potential target for antibiotics. This reaction is catalysed by the integral membrane enzyme, phospho-N-acetylmuramyl-pentapeptide translocase (translocase I). The gene coding for this enzyme in Escherichia coli, mraY, has recently been cloned. Two novel classes of antibiotics, the mureidomycins and liposidomycins, have recently been characterised as potent and specific inhibitors of translocase I.

Translocase I (E. coli) has been overexpressed 30-fold in E. coli and has been solubilised from particulate membranes with retention of catalytic activity. A continuous fluorescence-based assay for translocase I activity has been developed based on the work of Weppner and Neuhaus (W.A. Weppner and F.C. Neuhaus 1977, J. Biol. Chem. **252**, 2296-2303). Mureidomycin A is a potent slow-binding inhibitor of translocase I activity with K_i and K_i^* values of 36 nM and 2 nM respectively. Liposidomycin B also appears to be a slow-binding inhibitor with a K_i^* value of 143 nM.

Evidence has been obtained for a covalent enzyme-linked intermediate involved in the reaction pathway implying a ping pong type mechanism. The intermediate was labile to hydroxylamine suggesting an acyl-phosphate in the active-site.

Sequence alignments have revealed an evolutionary superfamily of phospho-aminosugar transferase enzymes, of which translocase I is a member. Three aspartate residues conserved through this class of enzymes have been investigated by site-directed mutagenesis as possible active-site nucleophiles. The results are discussed in the context of rational drug design.

To all the people and things who have made my life so rich.

For my parents especially.....

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I would like to thank the following people without whose help it would not have been possible to produce this thesis: Tim Bugg for putting up with me and for being the voice of reason in the madness; Bob Southgate and John Lonsdale without whose support both personally and on behalf of SB Pharmaceuticals this project would not have got off the ground; Martin Burnham for expression vectors; Mick Black for teaching me the basics of mol. biol.; Masatoshi Inukai for supplying us with mureidomycin A; Ken-ichi Kimura for supplying us with liposidomycin B; Mark Bradley and Denise Rennex for invaluable discussion; people in Biochemistry that I hassled for this and that in the early days and finally the founder members of the Wolfson lab, Magic, Ems, Mr. Merrick, Balders, Millsey, Roon, Bry, Proby, Winnie, Flemster & Hels..... "ooh look, half past five...staff club?"

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List of Abbreviations.

EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid
CHAPS	3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane-sulphonate
MES	2-(N-Morpholino)ethanesulphonic acid
MOPS	3-(N-Morpholino)propanesulphonic acid
Tris	Trishydroxymethylaminomethane
UMP	Uridine 5'-monophosphate
UDP	Uridine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
DMF	Dimethylformamide
rt	Room temperature
tlc	Thin layer chromatography
CMC	Critical micellar concentration
BSA	Bovine serum albumin
HPLC	High pressure liquid chromatography
MALDI-TOF-MS	Matrix assisted laser desorptive ionisation time of flight mass spectroscopy
BCA	Biconchininic acid
DAP	diaminopimelic acid
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Δ	Change in
ex.	Fluorescence excitation at
em.	Fluorescence emission monitored at
FEA	Fluorescence enhancement assay
DTT	Dithiothreitol
M_r	Relative molecular mass
TE	10 mM Tris pH 8.0, 1 mM EDTA
TBE	10 mM Tris-borate pH 8.3, 2 mM EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine

Chapter 1. Introduction.

1.1 Bacteria, antibiotics and drug resistance.

The bacterial kingdom represents a huge and diverse part of life on earth. They are essential in our ecosystem. Along with fungi, they cause the decay of organic material and the subsequent recycling of nutrients. Bacteria are involved in energy assimilation in the carbon cycle (e.g. photosynthetic bacteria in marine organisms) as well as consumption of oxygen and production of carbon dioxide in aerobic respiration. Bacteria form part of the nitrogen cycle, fixing atmospheric nitrogen to nitrate in the root nodules of some plants. We ourselves play host to a variety of bacteria which aid digestion of food in the gut. At the same time they are the causative agent of many infectious diseases. In the past, and in the developing world today, bacterial infections were and are a major cause of death. With the advent of 'wonderdrugs' such as penicillin infectious disease in the developed world was beaten into submission; but the ever-adapting bacteria began to acquire resistance to the antibiotics present in their environment. Today bacterial drug resistance is an increasingly serious problem in hospitals and other shared facility establishments. For example, methicillin-resistant *Staph. aureus* (MRSA) is an all too real threat to post-operative patients, and tuberculosis is returning to haunt the hospitals of the West.[1]

Figure 1.1 presents a brief overview of some of the significant events in the history of antibacterial agents and bacterial antibiotic resistance. It is a fact that bacteria will, in time, develop resistance to harmful agents in their environment. General methods by which bacteria survive in the presence of antibiotics are by degrading the antibiotic, by actively transporting it out of the cell, by increasing the cellular amounts of the protein affected (e.g. by gene duplication) or by substituting the affected protein with a new one not sensitive to the antibiotic. Eventually, the genes required for resistance are incorporated into plasmids or transposons and become transferable to other strains and genera of bacteria.

1863 - Pasteur demonstrates the microbial origin of putrefaction.
1910 - Discovery of salvarsan for treatment of syphilis.
1913 - Introduction of acriflavin (an antiseptic).
1926 - Fleming discovers penicillin.
1932 - Discovery of Prontosil rubrum (progenitor of the sulphonamides).
1941 - Demonstration of the therapeutic capacity of penicillin.

1943 - Discovery of PAS (*para*-aminosalicylic acid) as an antibacterial agent.
1943-6 - Discovery and development of streptomycin.
1947 - Appearance of clinical streptomycin resistance.
1948 - One in three PAS-treated patients carried resistant strains.
1950's - Emergence of penicillin resistance via ß-lactamases.
1951 - Discovery of isoniazid.
1952 - Appearance of clinical isoniazid resistance.
1960's - Introduction of glycopeptide antibiotics (e.g. vancomycin) to treat ß-lactam resistant strains.
1970's - Development of cephalosporins and other novel ß-lactam antibiotics.
- Discovery of clavulanic acid - a ß-lactamase inhibitor.
- Emergence of methicillin-resistant *Staph. aureus* (resistant to all ß-lactam antibiotics)
1985 - Admission of a patient with tuberculosis resistant to all known anti-TB drugs.
1988 - Emergence of plasmid-mediated vancomycin in *Enterococcus* resistance.
- Reports of resistance to fluoroquinolones.
1989 - 15% of all nosocomial (patient derived) isolates of *Staph. aureus* in U.S.A. methicillin resistant.
1989 - 0.4 % of *Enterococcus* isolates resistant to vancomycin, increased to 13.6 % in 1993.
1992 - Demonstration that plasmid-based vancomycin resistance could be transferred from *E. faecalis* to *S. aureus*.

Figure 1.1. A brief history of antibacterial agents and bacterial resistance to them.[1-6]

There is therefore an increasing need for compounds against which the bacteria have no defence. One target for development of such compounds is cell wall biosynthesis.

1.2. Biopolymers of the bacterial cell periphery.

In 1884 Christian Gram described a staining method for the classification of bacteria.[7] The Gram procedure involves treatment of bacteria with crystal violet followed by Gram's iodine. When solutions of these are mixed a gold coloured precipitate forms. The cells are treated with ethanol and are repeatedly washed in ethanol until the supernatant is clear. Bacteria whose cells retain the precipitate are classified gram-positive and those whose cells do not are classified gram-negative. It is generally accepted that differences in the staining response are attributable to differences in the structural cell wall.[8, 9] Typical gram-negative bacteria such as *Escherichia coli* or *Salmonella typhimurium* have a thin layer of peptidoglycan (PG)

between inner and outer membranes consisting of proteins and lipids or proteins, lipids and lipopolysaccharide respectively (fig. 1.2). Typical gram-positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus* have no outer membrane and a thick PG layer often supplemented with secondary polymers like teichoic or teichuronic acids.

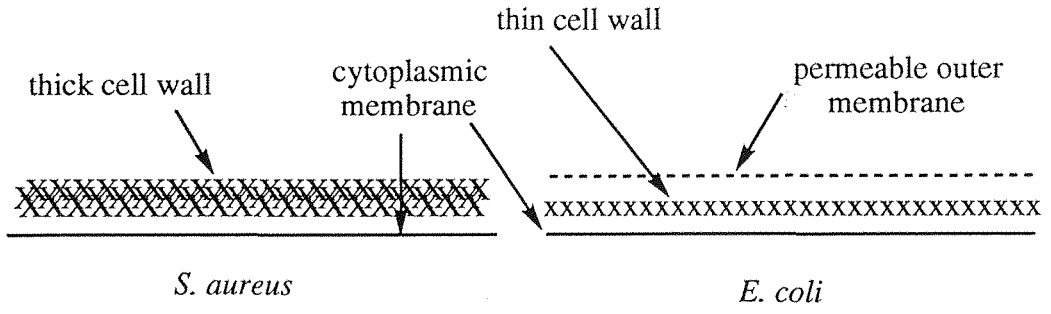


Figure 1.2. Cell peripheries of typical gram-negative (*E. coli*) and gram positive (*S. aureus*) bacteria.

This section is intended as a broad introduction to the types and structures of biopolymers which give bacteria shape and integrity.

1.2.1 Peptidoglycan.

Peptidoglycan, also commonly called murein, is a polysaccharide consisting of a repeating ß1→4 linked N-acetylmuramic acid (MurNAc)/N-acetylglucosamine (GlcNAc) disaccharide backbone with a pentapeptide side-chain attached to alternate MurNAc sugar residues. The structure of the cell wall polymer of *S. aureus* Copenhagen (the strain used in most early studies of the bacterial cell wall) was initially investigated by chemical hydrolysis with trichloroacetic acid.[10, 11] These studies were later refined using purified hydrolytic enzymes; an amidase which cleaved the amide bond between muramic acid and L-Ala, and an acetylhexosaminidase which cleaved the ß1→4 glycoside bond.[12] Strength is increased by cross-linking between strands via transpeptidation reactions. The two terminal residues of the pentapeptide side-chain are D-amino acids as is the second residue, glutamic acid. Consequently peptidoglycan is not degraded by the cell's L-specific peptidases or by mammalian peptidases. The *meso*-diaminopimelic acid unit has its L-centre in the peptide chain with the D-centre involved in cross-linking. When *m*-DAP is substituted with lysine or other amino acids in peptidoglycan in other organisms, an L-amino acid is found. The linkage between the D-Glu and *m*-DAP residues is unusual in that it is γ-linked. Thus the γ-carboxy group of D-Glu is

linked to the amino group of the *m*-DAP residue. Its general structure in *E. coli* is shown in fig.1.3 and its chemical structure in fig. 1.4.

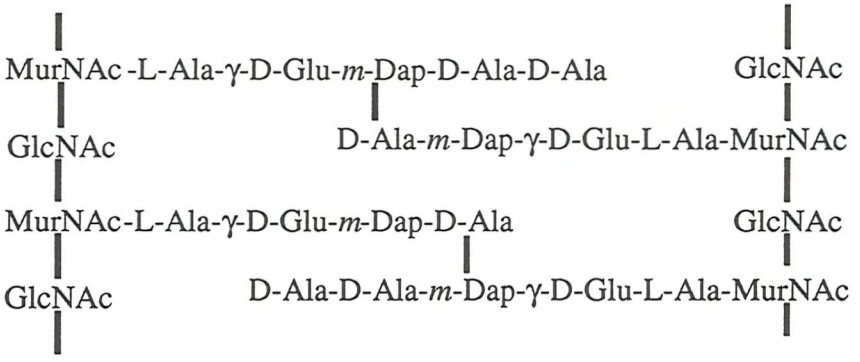


Figure 1.3. General structure of peptidoglycan in *E. coli*. MurNAc = N-acetylmuramic acid, GlcNAc = N-acetylglucosamine, *m*-DAP = *meso*-diaminopimelic acid.

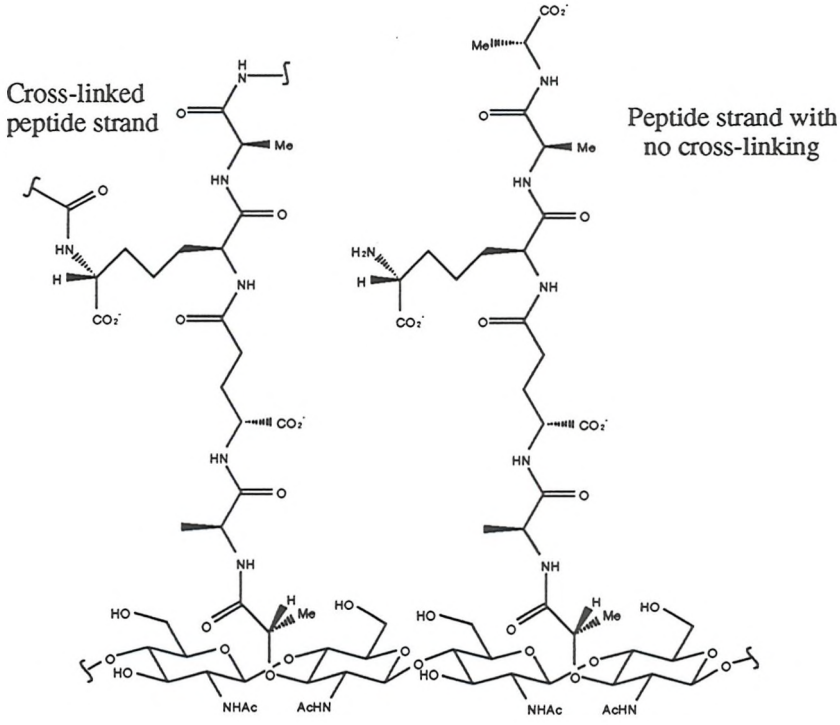


Figure 1.4. Chemical structure of peptidoglycan in *Escherichia coli*.

In gram-positive bacteria such as *B. subtilis* and *S. aureus* *m*-diaminopimelate is replaced by L-lysine, the difference being that *m*-DAP has an ε-carboxylic acid function that lysine does not.[13] Less commonly the third residue is L-ornithine, L,L-DAP or 3-hydroxy-ß-*meso*-DAP. In rare cases the 3-position amino acid unit is not

involved in cross-linking and is replaced by L-homoserine, L-Ala or L-Glu. In these cases cross-linking occurs via the side chain of the position-2 amino acid, D-Glu.

In *E. coli* the cross-linking between strands is a transpeptidation of the *m*-DAP ε-amino group with the terminal D-Ala of another strand. However in other bacteria there is often an interstrand linkage unit made up of one or more amino acid units. There is considerable variance in the linkage unit itself and the way in which the linker is constructed. For example, in *S. aureus* Copenhagen there is a pentaglycine bridge. Variations on this type of linear cross-link are oligopeptides consisting of glycine, L-alanine, L-serine and L-theonine. Interestingly the amino acid units that make up these bridges are derived from the corresponding amino-acyl-tRNA and are added sequentially to the amino group of the position-3 amino acid. Alternatively the linker can be made up of D-glutamic acid and D-aspartic acid. In these cases the amino acid is activated as the phosphate (ß-aspartyl phosphate and γ-glutamyl phosphate). These and other intracacies of peptidoglycan structure have been described at length by Schleifer and Kandler.[13]

During growth peptidoglycan is both synthesised and broken down to allow the cell to grow and to replace damaged polymer. In *E. coli* there are at least nine peptidoglycan hydrolases which release peptides (fig. 1.5).[14]

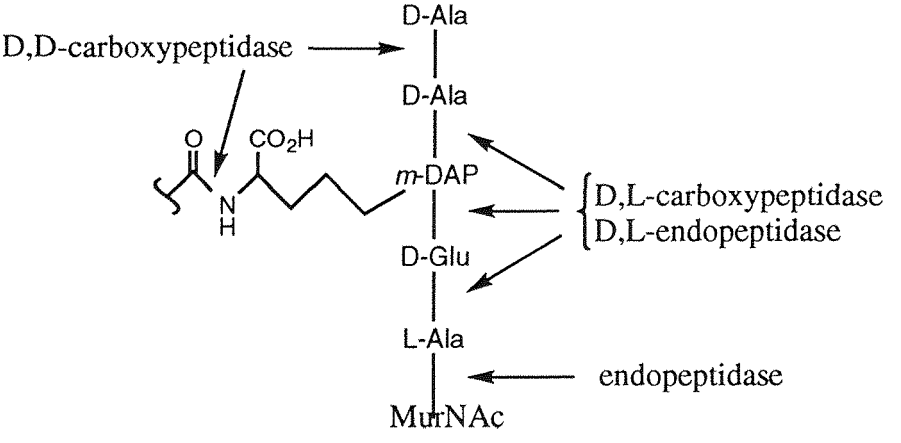


Figure 1.5. Peptidase activities known to degrade peptidoglycan.

It has been found that these peptides can be recycled; *E. coli* can take up radiolabelled L-Ala-γ-D-Glu-*m*-DAP from the surrounding medium and incorporate it into UDP-MurNAc-pentapeptide without prior degradation.[15] *E. coli* also contains two genetically distinct lytic transglycosylases which hydrolyse the ß1→4 glycosidic bond between MurNAc and GlcNAc to give a 1,6-dehydromuramyl species (fig. 1.6).[16]

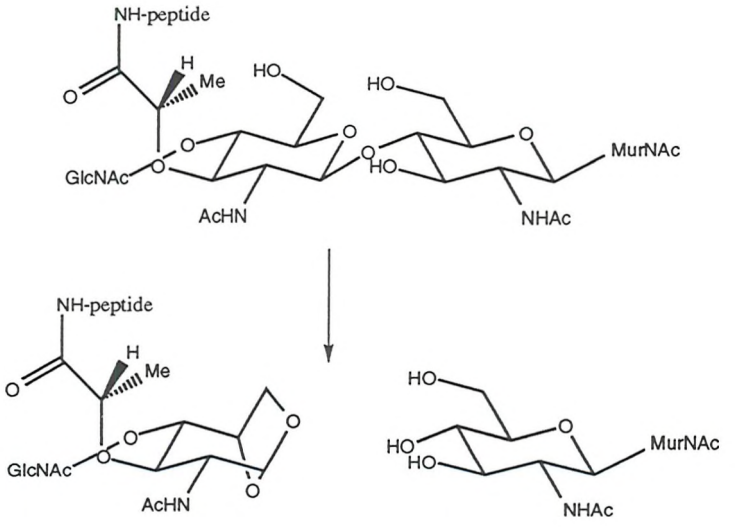


Figure 1.6. The reaction catalysed by *E. coli* soluble lytic transglycosylase.

Clearly the cell must maintain a careful balance between the constructive and destructive activities of the above enzymes. Perturbation of this balance by antibiotics which inhibit synthesis of peptidoglycan results in spheroplast formation and eventually lysis due to the low osmotic pressure of the surrounding medium.

1.2.2. Other polymers.

Many gram-positive bacteria have secondary polymers in addition to peptidoglycan such as teichoic and teichuronic acids. These serve to increase the strength of the structural cell wall in the absence of an outer membrane.

Teichoic acids are water soluble polymers containing carbohydrate, often 1-phospho-N-acetylglucosamine, D-alanine residues and either glycerol and/or ribitol phosphates (fig. 1.7). Teichuronic acids are less widespread and consist of glycosidically linked carbohydrate and uronic acid residues.[13] It does not contain phosphate and so some bacteria will change from synthesising teichoic acid to teichuronic acid in times of limiting phosphate. The point of attachment for these polymers is the 6-hydroxyl group of N-acetylmuramic acid.[4]

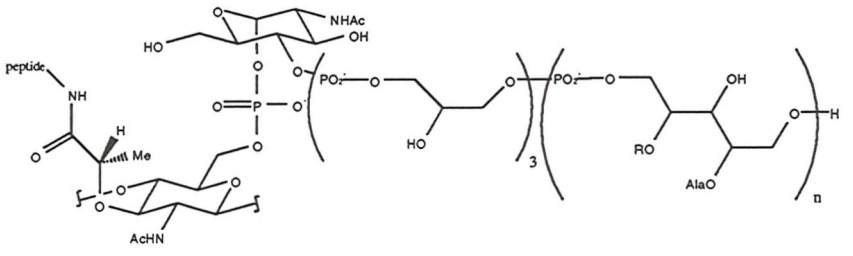


Figure 1.7. An example of the structure of a teichoic acid attached to peptidoglycan of *Staphylococcus aureus.*

Typical gram-negative bacteria such as *E. coli* and *S. typhimurium* contain a further outer membrane. The outer leaflet is made up from a complex molecule called lipopolysaccharide (fig. 1.8). LPS consists of three domains: a membrane anchor called lipid A, a core oligosaccharide and highly strain/species variable distal polysaccharide which protrudes into the surrounding medium. This distal region is called the 'O antigen' and is commonly used in serotyping on account of its variability. The outer membrane also contains proteins, e.g. porins whose function is to allow small hydrophilic molecules to enter the periplasm.

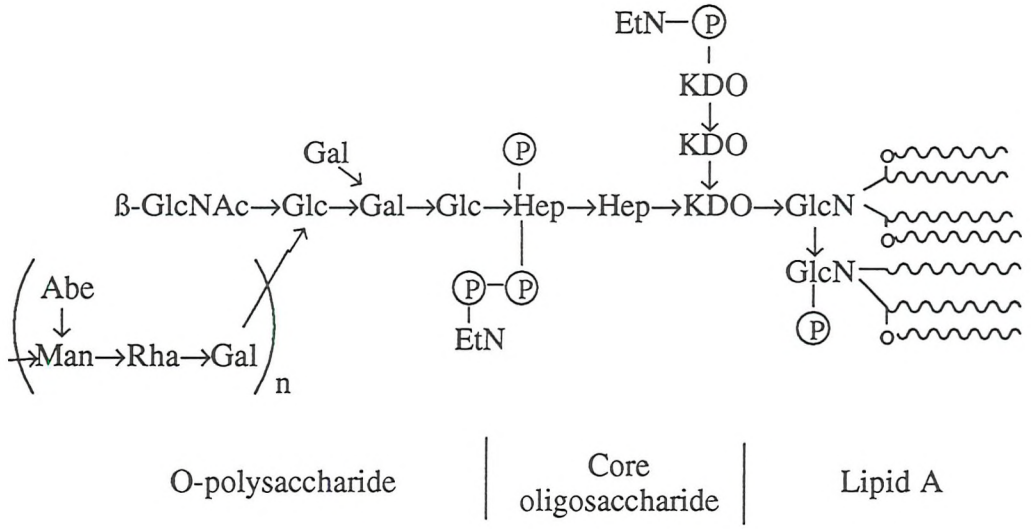


Figure 1.8. Structure of lipopolysaccharide of *Salmonella typhimurium* LT2. Abbreviations: Abe - abequose, Man - D-mannose, Rha - rhamnose, Gal - galactose, Glc - glucose, GlcNAc - N-acetylglucosamine, Hep - L-glycero-D-mannoheptose, GlcN - D-glucosamine, KDO - 2-keto-3-deoxyoctonic acid, EtN - ethanolamine and P - phosphate.

Another attachment found in *E. coli* and other enteric bacteria is lipoprotein which is attached to the *m*-DAP residue in place of the position-4 D-Ala i.e. tripeptide (fig. 1.9).[4]

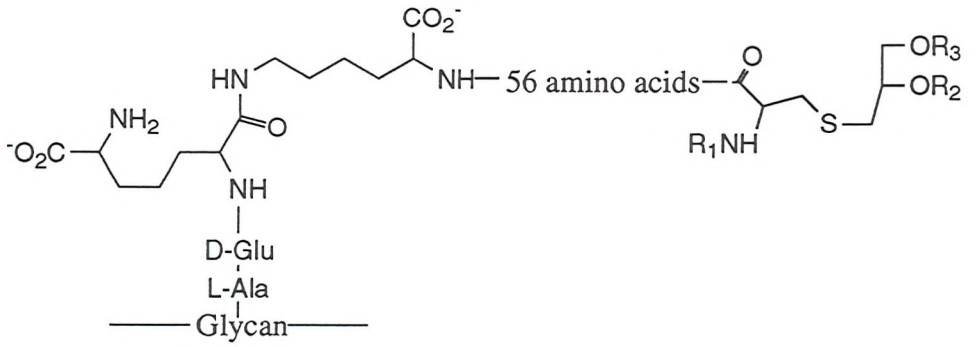


Figure 1.9. Structure of lipoprotein in *E. coli*. $R_{1\text{-}3}$ are fatty acyl chains.

1.3. Structural cell wall biosynthesis in *Escherichia coli.*

In this section the biosynthesis of cell wall polymers and exopolysaccharrides will be reviewed with an emphasis on the murein sacculus, peptidoglycan.

1.3.1 Peptidoglycan.

1. Cytoplasmic steps. The biosynthesis of peptidoglycan in *E. coli* can be divided up into three discrete stages: construction of UDPMurNAc-pentapeptide in the cytoplasm, translocation across the inner membrane, and finally polymerisation.

The first committed step in peptidoglyan biosynthesis is addition of phosphoenolpyruvate (PEP) to uridine diphospho-N-acetylglucosamine (UDP-GlcNAc) by UDPGlcNAc enol-pyruvyl transferase (*murA*).[18] Reduction by an NADPH-dependent reductase affords UDPN-acetylmuramic acid (UDPMurNAc) (fig. 1.10).

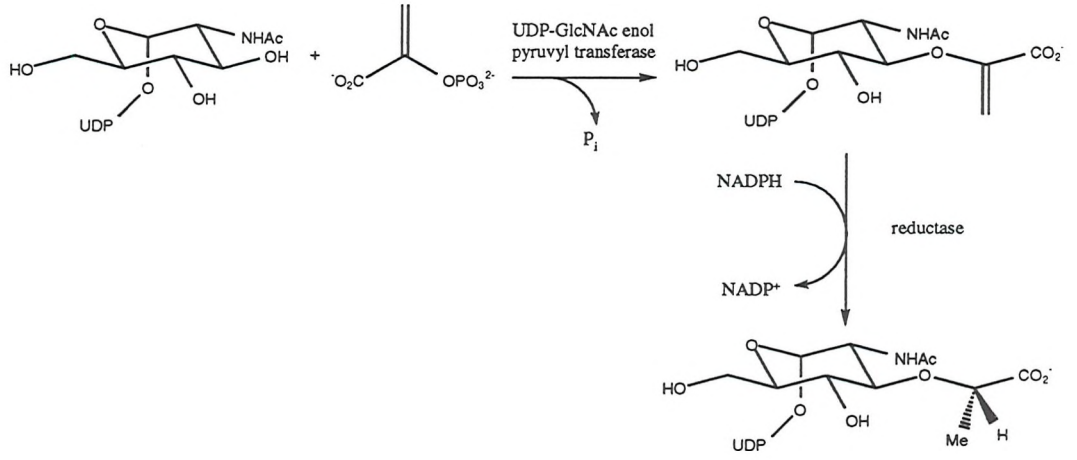


Figure 1.10. Transformation of UDPGlcNAc to UDPMurNAc.

L-alanine, D-glutamic acid (γ-linked) and *meso*-diaminopimelic acid are successively added to UDPMurNAc by three ATP-dependent ligase enzymes (coded

for by *murC, murD* and *murE* respectively in *E. coli*) to form the first advanced precursor UDPMurNAc-tripeptide.[19-23] Separately two D-alanine residues are condensed to the D-Ala-D-Ala dipeptide by D-Ala-D-Ala ligase (*ddlA* and *ddlB*). There are two genes coding for D-Ala-D-Ala ligase in *E. coli*. The encoded proteins have have very similar kinetic properties but have only 35 % sequence identity and differ in size (39 kDa and 32 kDa for DdlA and DdlB respectively). It has been suggested that there may be two separate peptidoglycan biosynthesis pathways, possibly with one utilising recycled degradation products of existing peptidoglycan.[24] An ATP-dependent D-Ala-D-Ala *adding* enzyme (*murF*) completes the synthesis of UDPMurNAc-pentapeptide and the cytoplasmic steps of peptidoglycan biosynthesis. These steps are summarised in figure 1.11.

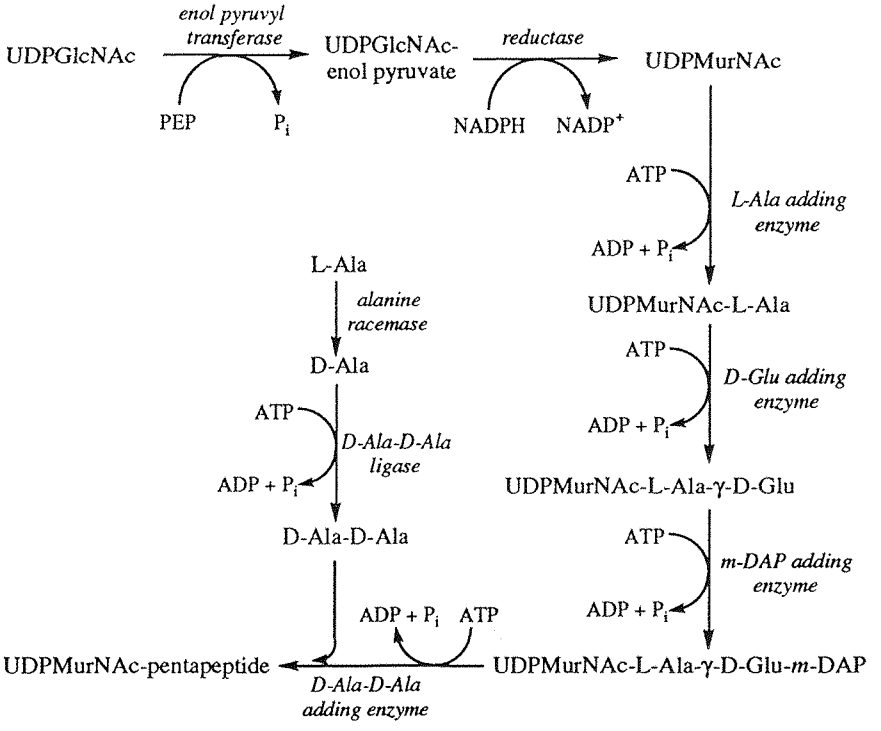


Figure 1.11. The cytoplasmic steps of peptidoglycan biosynthesis.

2. *Membrane steps.* The membrane cycle of reactions begins with the transfer of phospho-MurNAc-pentapeptide from the nucleotide carrier (UMP) to a membrane bound carrier, undecaprenyl phosphate, to form lipid-linked intermediate I (LI-I). This conversion is catalysed by translocase I (formally phospho-MurNAc-pentapeptide translocase, *mraY*) which is the focus of this project. A GlcNAc unit is transferred from UDPGlcNAc to the MurNAc C-4 hydroxyl of LI-I by translocase II (*murG*)[25] making a ß1→4 disaccharide, lipid-linked intermediate II. The pentaglycine bridge found in the peptidoglycan of *S. aureus* Copenhagen is added at this point.[26]

This is then flipped across the membrane bilayer ready for incorporation into the existing peptidoglycan.

As shown in fig. 1.12, translocase I is an integral membrane protein. This assignment is based on a hydropathy analysis of the primary sequence inferred from the cloned gene, *mraY*, which codes for translocase I in *E. coli*.[27] Translocase II however appears to be associated with the inner face of the membrane.[28]

It is not known how the translocation event occurs. It may be a protein assisted process or it may be a spontaneous event induced by the C_{55}-isoprenoid chain of the lipid carrier molecule.[14] Considerable efforts have been made to investigate the destabilisation of membrane bilayer structure by dolichyl and undecaprenyl phosphates and the effects of membrane potentials. Janas and co-workers demonstrated by voltammetry that membrane potential does play a role in the dynamics of dol-P in phospholipid bilayers. They suggested that membrane potential may facilitate formation of local non-bilayer structures accelerating movement of the polar part of the molecule across the bilayer.[29] NMR spectroscopic studies of dolichyl and undecaprenyl phosphates in phosphatidylethanolamine (PE)/ phosphatidylcholine (PC) vesicles also demonstrated disruption of local membrane bilayer structure by these molecules. The effect is five times greater with the phosphates than with the free alcohols.[30] These findings are supported by the demonstration of dolichyl phosphate-induced calcein (a fluorescein derivative which is retained within phospholipid vesicles unless they are permeabilised) leakage from PE/PC vesicles.[31]

Weppner and Neuhaus have studied the motion of dansylated undecaprenyl-diphospho-MurNAc-pentapeptide *in vitro* in native *Staphylococcus aureus* Copenhagen membrane fragments by fluorescence energy transfer experiments. They have found that the fluorophore has a high rotational relaxation time (>100 ns) and a large value of anisotropy (0.32). The results indicate that the rate of unassisted flipping back and forth across the bilayer is not nearly sufficient to account for the synthesis of nascent peptidoglycan.[32]

The final stage is polymerisation and transpeptidation. A membrane associated transglycosylase enzyme polymerises the disaccharide monomers, initially to produce a nascent strand still attached to the lipid carrier molecule, then into the existing peptidoglycan (fig. 1.13).[33] A transpeptidase then strengthens the peptidoglycan by cross-linking the peptide chains.[34] The terminal D-Ala unit of one peptide chain is displaced by the ε-amino group of the *m*-DAP residue of another peptide chain (fig. 1.13). Transpeptidase enzymes are the target for penicillin[4], and they are included in the class of proteins called penicillin binding proteins (PBP's). These proteins are so called because they can be visualised in non-denaturing polyacrylamide gel electrophoresis with [^{3}H]-penicillin.

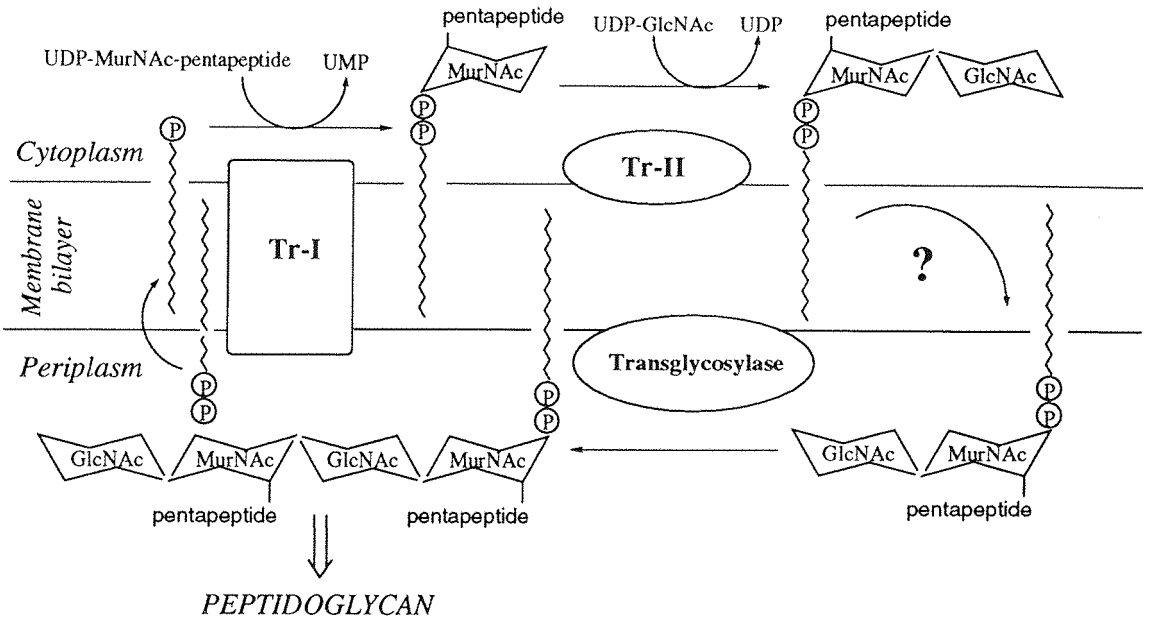


Figure 1.12. The membrane cycle of reactions in peptidoglycan biosynthesis.

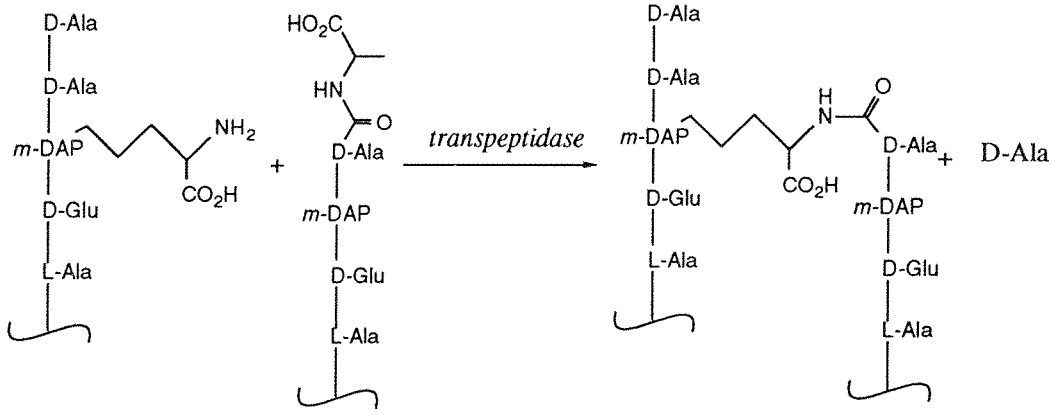


Figure 1.13. Transpeptidation.

The polymerisation steps regenerate undecaprenyl pyrophosphate on the periplasmic side of the membrane. It is recycled by a phosphatase enzyme back to undecaprenyl phosphate. It has been suggested that the level of phosphorylation of the lipid carrier pool is one method for regulation of the rate of peptidoglycan biosynthesis in *S. aureus* (fig. 1.14).[35]

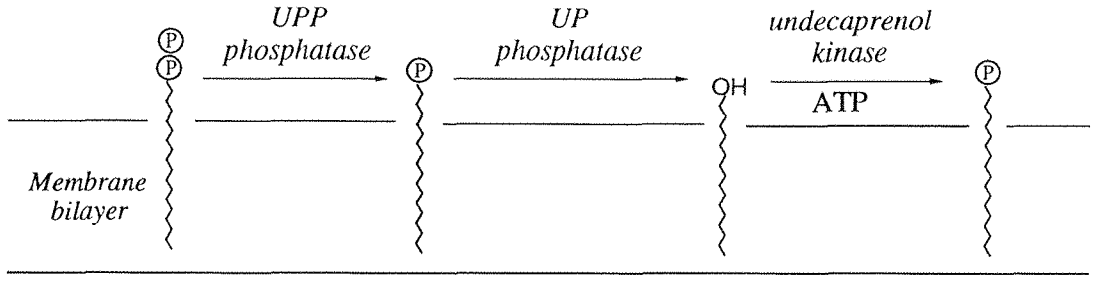


Figure 1.14. Phosphorylation and dephosphorylation of undecaprenol.

3. The lipid carrier molecule. Undecaprenyl phosphate is made in the cell by stepwise condensation of isoprene units beginning with a primer which can, *in vitro*, be from C_{10}- (geranyl) to C_{25}-pyrophosphate, but not dimethylallyl pyrophosphate. Synthases characterised to date (from *Lactobacillus plantarum, Micrococcus luteus* and *Bacillus subtilis*) exhibit a high specificity for the C_{55} final product.[36-38] The product of these enzymes is a polyprenyl pyrophosphate which is subsequently hydrolysed to the monophosphate and free polyprenol. Undecaprenyl pyrophosphate phosphatase has been purified from *M. lysodeikticus*.[39] Undecaprenol kinase has been purified from *S. aureus*. It is a membrane bound protein which is soluble in butanol.[40-42]

Ficaprenol from *Ficus elastica* (rubber plant), castaprenol-11 from *Aesculus hippocastanum* (horse chestnut) and undecaprenol from *Ailanthus altissima* all contain an ω-unit, three *trans* units and seven *cis* units.[43, 44] The *trans* units are almost certainly adjacent to the terminal ω-unit since all polyisoprenyl pyrophosphate synthases characterised to date add isoprene units with exclusively *cis* configuration.[36-38] Undecaprenol isolated from either *Lactobacillus plantarum* or *Micrococcus lysodeikticus* was found by NMR spectroscopy to contain two internal *trans* units and eight *cis* units. [45, 46] Thus there is a subtle difference in structure depending on the source organism.

4. Regulation of peptidoglycan biosynthesis. In *E. coli* it is not possible to accumulate high levels of UDP-MurNAc-tripeptide or -pentapeptide by treatment with antibiotics which block steps in peptidoglycan biosynthesis or by mutation. Consequently it has been suggested that regulation is exerted by feedback inhibition.[47] Indeed UDPMurNAc-pentapeptide does inhibit UDPGlcNAc enol pyruvyl transferase *in vitro*.[48] Conversely, in gram-positive bacteria such as *B. subtilis* and *S. aureus*, feedback inhibition does not seem to operate because UDPMurNAc-tripeptide or UDPMurNAc-pentapeptide rapidly accumulate in cells treated with D-cycloserine (which inhibits alanine racemase and D-Ala-D-Ala ligase)

or vancomycin (which inhibits utilisation of lipid-linked intermediate II) respectively.[49]

All of the genes coding for the enzymes responsible for the synthesis of UDPMurNAc-pentapeptide and lipid-linked intermediate II in *E. coli* have been cloned and sequenced. Many of these genes form a cluster situated at minute 2 of the *E. coli* chromosome (fig. 1.15). For a review see Bugg and Walsh.[24]

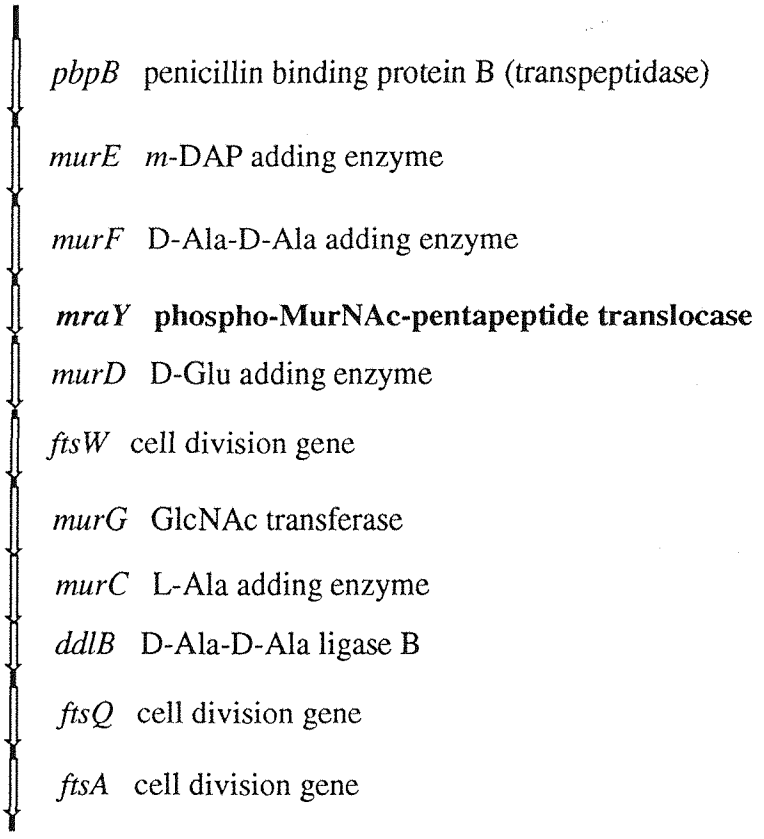


Figure 1.15. Organisation of genes involved in the biosynthesis of peptidoglycan.

1.3.2 Biosynthesis of other cell wall polymers.

In addition to peptidoglycan, teichoic acid and to a lesser extent teichuronic acid polymers are found in the cell walls of some gram-positive bacteria. Like peptidoglycan, these polymers are constructed with undecaprenol as a membrane carrier linked to the first sugar residue (N-acetylglucosamine) by a pyrophosphate linkage. The polysaccharide portion of lipopolysaccharide common to gram-negative bacteria is also assembled on undecaprenyl phosphate with a pyrophosphate linkage before transfer to lipid A. For a review of these polymers and their taxonomic significance see Schleifer and Kandler.[13] This feature is also part of the biosynthetic route for the exopolysaccharide succinoglycan.[50]

The theme of construction of a polysaccharide on a membrane-bound isoprenoid alcohol phosphate carrier molecule extends through both prokaryotic and eukaroytic systems i.e. bacterial peptidoglycan, lipopolysaccharide and exopolysaccharide biosynthesis and eukaryotic glycoprotein biosynthesis. Many eukaryotic proteins have complex carbohydrates attached to serine, threonine (O-linked) or asparagine (N-linked). N-linked carbohydrates are first constructed on a membrane bound carrier molecule, dolichyl phosphate.[51] Dolichol is a long chain polyisoprenol like undecaprenol, except that the isoprene unit adjacent to the alcohol terminus is saturated. Fig. 1.16 compares the relevant parts of peptidoglycan and glycoprotein biosynthesis. It is possible that these pathways share a distant common ancestry.[52]

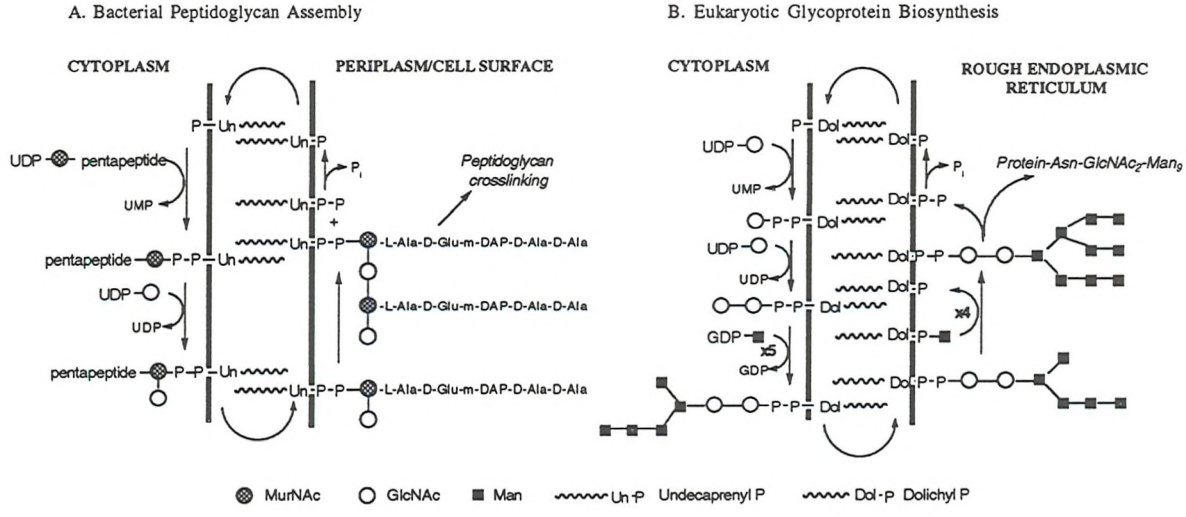


Figure 1.16. Schematic representation of lipid-linked cycles involved in bacterial peptidoglycan assembly (A) and eukaryotic glycoprotein biosynthesis (B). N-Acetylmuramic acid (MurNAc), N-acetyl-glucosamine (GlcAc), mannose (Man), undecaprenyl phosphate and dolichyl phosphate represented schematically as shown.

In each of these pathways, peptidoglycan, glycoprotein, lipopolysaccharide, succinoglycan biosynthesis, there is an enzyme which catalyses the initial transfer of a phospho-amino-sugar (or a derivatised phospho-amino-sugar as in the case of peptidoglycan) from a nucleotide carrier to the membrane bound lipid carrier releasing UMP. The reactions are analogous and it might be expected that they all proceed via the same basic mechanism. At this time though, the mechanism of catalysis is not known for any of these enzymes.

1.4 Antibiotics which inhibit peptidoglycan biosynthesis.

A large number of agents, both natural products and synthetic derivatives, inhibit the biosynthesis of bacterial peptidoglycan. In this section these compounds are reviewed with emphasis on those whose target is the reaction catalysed by translocase I. Table 1.1 summarises the compounds discussed and their respective sites of action. Structures of those compounds inhibiting the translocase reaction are given in figure 1.21.

Target/Step	Inhibitory compounds (or classes of)
Enol-pyruvyl transferase	Phosphonomycin (also fosfomycin)
Alanine racemase	Alafosfalin, D-cycloserine[53]
D-Ala-D-Ala ligase	D-cycloserine[53]
Translocase I	Mureidomycins, liposidomycins, tunicamycin, amphomycin, napsamycins, pacidamycins
Transglycosylase	Moenomycin, prasinomycin, enduracidin, vancomycin, ristocetin, teicoplanin[54], other glycopeptide antibiotics
Transpeptidation	ß-Lactams (penicillins/cephalosporins), vancomycin
Lipid recycling	Bacitracin

Table 1.1. Summary of antibiotics inhibiting various steps of peptidoglycan biosynthesis.

1.4.1. Natural products which target peptidoglycan biosynthesis.

Of the compounds listed in table 1.1 phosphonomycin (also fosfomycin), alafosfalin and D-cycloserine inhibit cytoplasmic steps whilst the majority inhibit processes associated with polymerisation and cross-linking of peptidoglycan.

Phosphonomycin irreversibly inactivates enol pyruvyl transferase (the first committed step in peptidoglycan biosynthesis) by forming a covalent adduct with an active-site cysteine residue (fig. 1.17).[55, 56]

Alafosfalin (L-alanyl-L-1-aminoethylphosphinic acid) was found to act as a prodrug *in vivo*.[57] In both gram positive and gram negative organisms alafosfalin was actively taken up from the medium by peptide permeases, and cleaved intracellularly to give L-1-aminoethylphosphinic acid which inhibits alanine racemase. This results in accumulation of UDPMurNAc-tripeptide and a decrease in the cellular level of D-

alanine. Atherton *et al;* also presented evidence that L-1-aminoethylphosphinic acid was a substrate for UDPMurNAc-L-ala synthetase.[57]

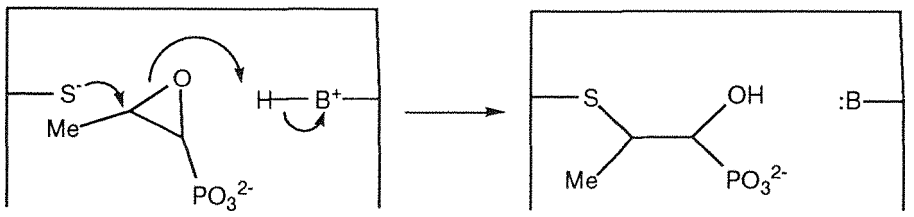


Figure 1.17. Inactivation of enol-pyruvyl transferase by phosphonomycin.

D-cycloserine is an analogue of D-alanine and inhibits both alanine racemase and D-ala-D-ala ligase. Inhibition of alanine racemase is mechanism-based and irreversible, whilst inhibition of D-Ala-D-Ala ligase is competitive and reversible acting as an analogue of D-alanine.[53]

Lugtenberg *et al;*[58] found that enduracidin, prasinomycin and moenomycin (members of a family of complex glycolipid antibiotics) all efficiently inhibited peptidoglycan synthesis *in vitro* but did not inhibit formation of lipid-linked intermediate II. Their mode of action was 'indistinguishable from that of vancomycin'. Moenomycin was later shown to specifically inhibit polymerisation of peptidoglycan precursors and to bind reversibly to membranes and membrane proteins indicating that it may be a bi-functional substrate analogue inhibitor (fig. 1.18).[59]

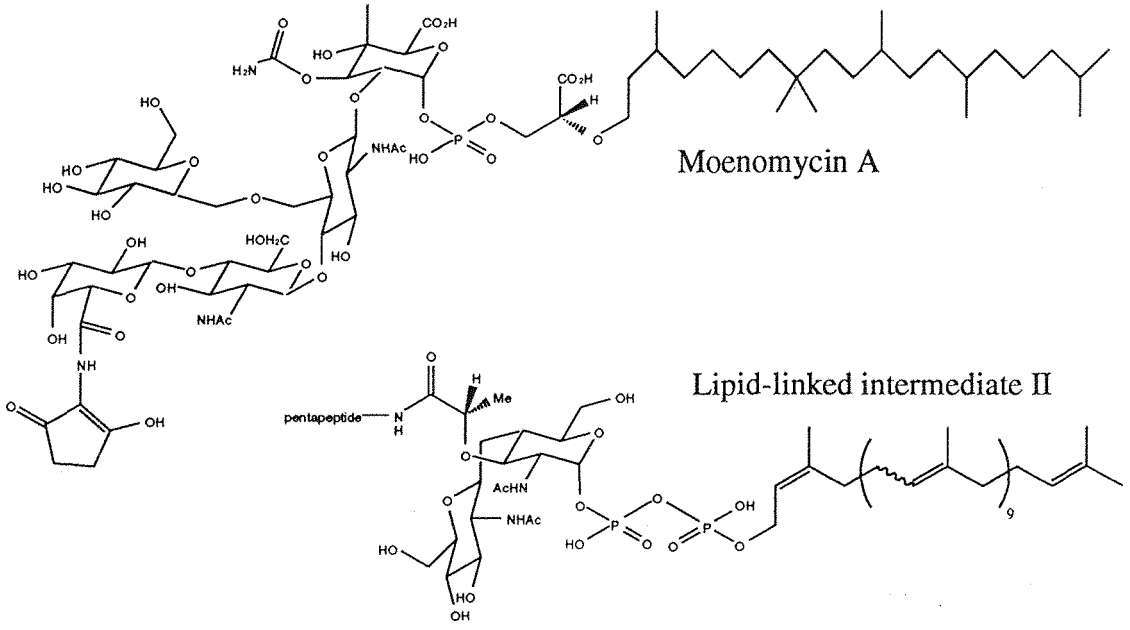


Figure 1.18. Structures of moenomycin A and lipid-linked intermediate II.

ß-Lactam antibiotics are well characterised as irreversible inhibitors of penicillin-binding proteins involved in transpeptidation (cross-linking) and this area has been reviewed.[60] The first ß-lactam antibiotic to be discovered was penicillin, from culture supernatants of *Penicillum notatum.* The penicillins have a general structure consisting of a ß-lactam ring fused to a five-membered The nitrogen attached to the ß-lactam ring is acylated (fig. 1.19). Variants are produced by chemical acylation of 6'-aminopenicillanic acid, which is in turn prepared by enzymatic hydrolysis of benzylpenicillin, penicillin G, the naturally produced variant. Some of the synthetic variants have significant advantages over penicillin G. For example, methicillin is much less susceptible to the ß-lactamase enzymes which inactivate penicillin in resistant bacteria.[4]

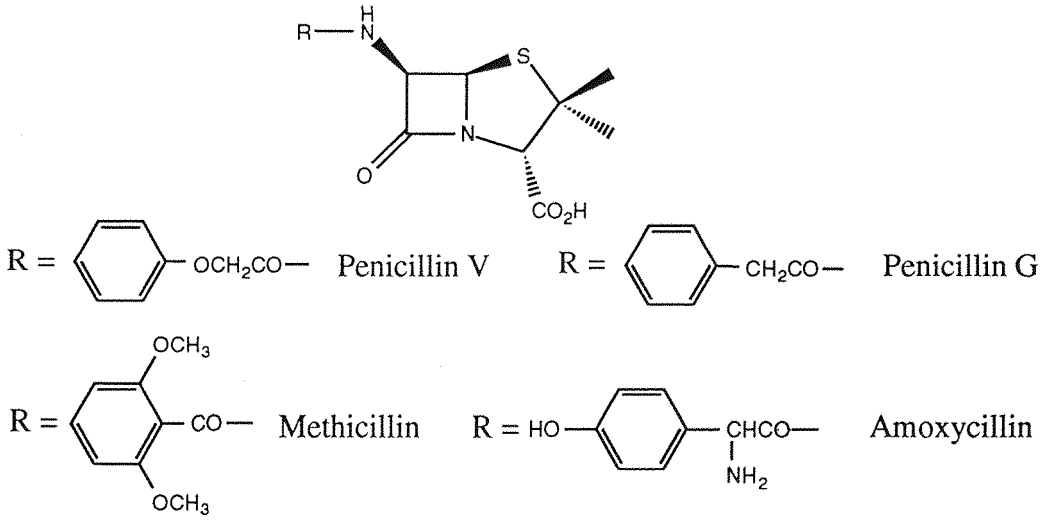


Figure 1.19. The general penicillin structure and some important variants.

Vancomycin and other glycopeptide anitibiotics such as ristocetin and teicoplanin have been shown to inhibit peptidoglycan biosynthesis by binding the D-Ala-D-Ala section of lipid-linked intermediate II so preventing transglycosylation and trnspeptidation.[54, 61, 62] The binding of vancomycin to peptidoglycan precursors is complex, involving dimerisation of the antibiotic.[63, 64] It was found that dimerisation of the antibiotic was associated with an increased affinity for the D-Ala-D-Ala terminus and *vice versa.* Modelling studies indicated that dimerisation would occur through hydrogen bonds between the 'back' side of the vancomycin molecules as illustrated in fig. 1.20.

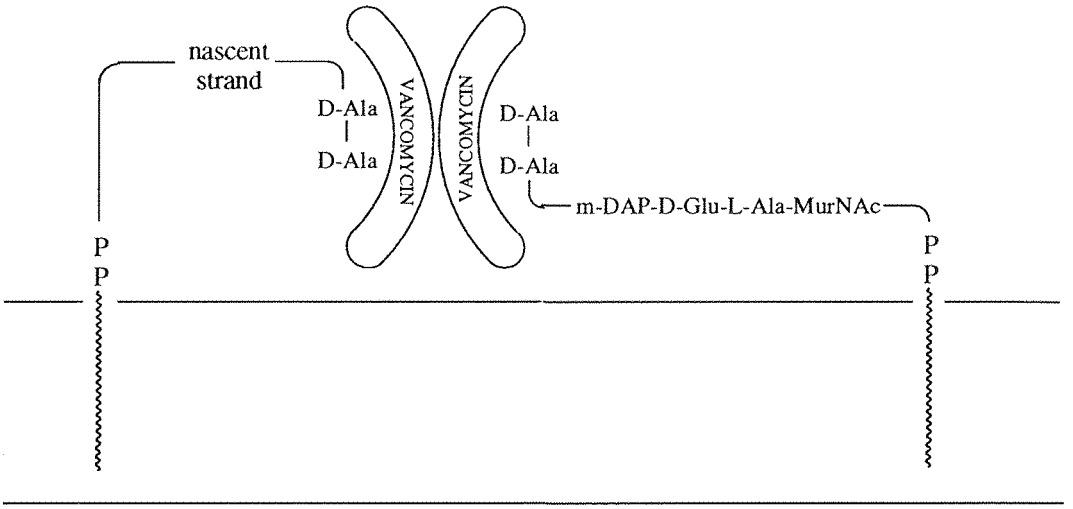


Figure 1.20. A model for *in vivo* dimerisation of vancomycin.

Bacitracin is a complex of cyclic polypeptides which chelates undecaprenyl pyrophosphate in the presence of calcium ions so preventing dephosphorylation back to undecaprenyl phosphate. It is this effective removal of lipid carrier which causes inhibition of peptidoglycan biosynthesis.[65, 66]

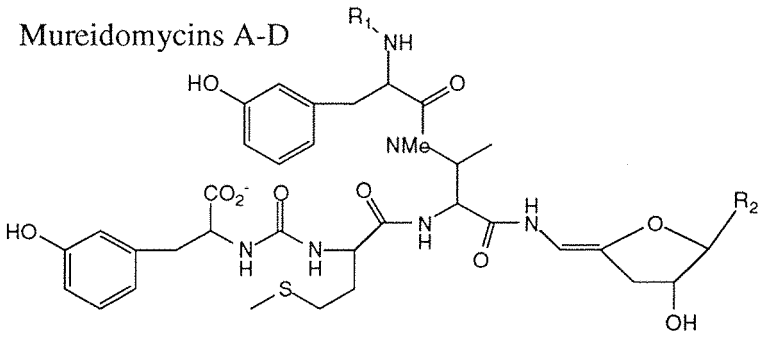
This section is not a complete summary of antibiotics which inhibit peptidoglycan biosynthesis, rather a selection of choice examples. For a fuller analysis the reader is referred to the book 'Antibiotic Inhibition of Bacterial Cell Surface Assembly and Function'.[67]

1.4.2. Natural products which target translocase I.

Since this project is focussed on the first reaction in the membrane cycle of peptidoglycan biosynthesis, the natural product groups acting at this point will be discussed in turn.

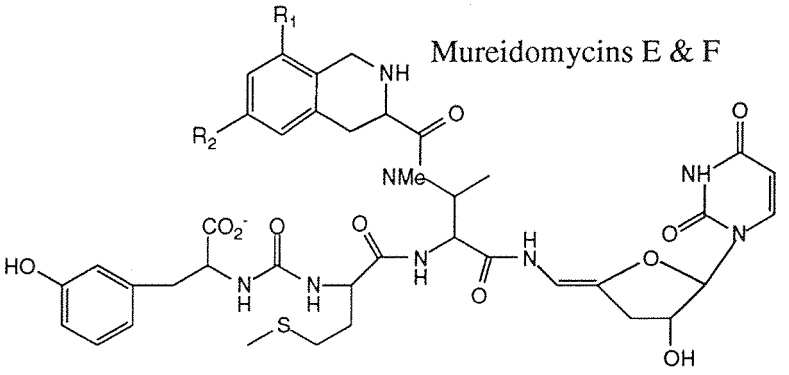
Tunicamycin is a complex of related uridine disaccharide-containing natural products isolated from *Streptomyces lysosuperficus* which differ in the length and saturation of their fatty acyl chains. The complex has been structurally characterised [68] and studied extensively[69]. In bacteria tunicamycin inhibits both the transfer of phospho-MurNAc-pentapeptide to undecaprenyl phosphate and the transfer of phospho-GlcNAc to undecaprenyl phosphate (the first step in teichoic acid biosynthesis), but not the addition of GlcNAc to lipid intermediate I by translocase II[70]. MIC values against gram positive bacteria range from 0.2 - 50 μg/mL.[70]

Mureidomycins A-D



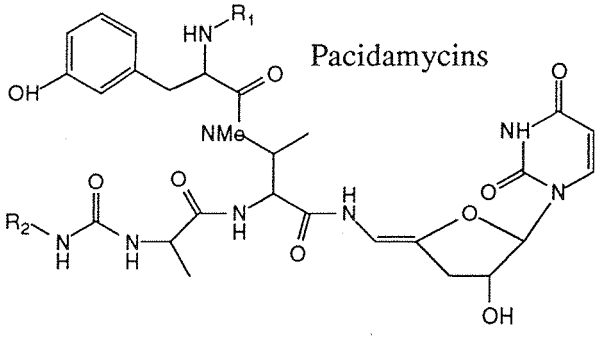
	R_1	R_2
A	H	uracil
B	H	dihydrouracil
C	Gly	uracil
D	Gly	dihydrouracil

Mureidomycins E & F



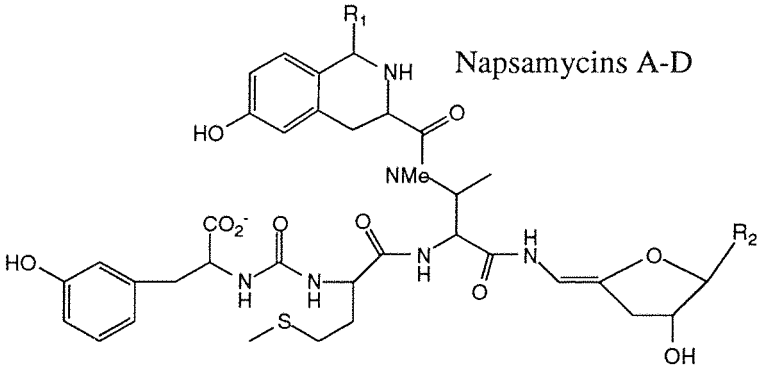
	R_1	R_2
E	OH	H
F	H	OH

Pacidamycins



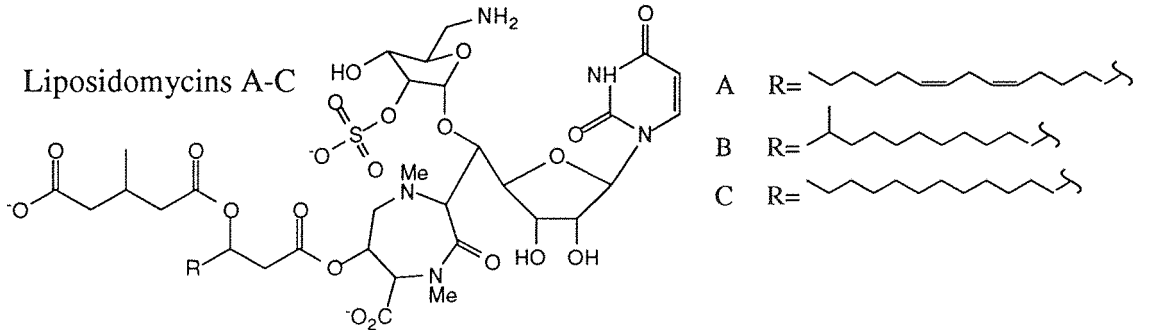
	R_1	R_2
1	Ala	Trp
2	Ala	Phe
3	Ala	Tyr
4	H	Trp
5	H	Phe
6	Gly	Trp
7	Gly	Phe

Napsamycins A-D



	R_1	R_2
A	H	uracil
B	Me	dihydrouracil
C	H	uracil
D	Me	dihydrouracil

Liposidomycins A-C



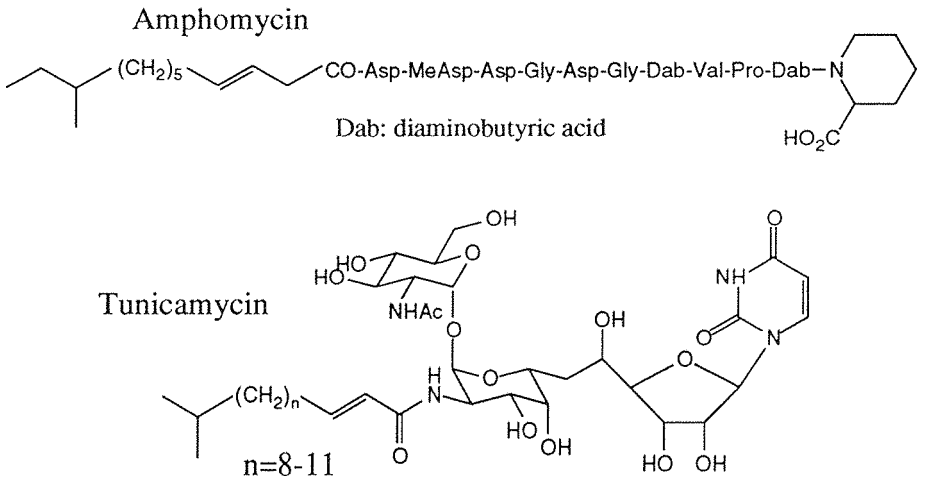


Figure 1.21. Structures of natural products which inhibit transfer of phospho-MurNAc-pentapeptide to undecaprenyl phosphate.

Tunicamycin is highly toxic in eucaryotic cells on account of its inhibition of glycoprotein synthesis. Here the transfer of phospho-GlcNAc from a nucleotide precursor onto dolichyl phosphate (a saturated analogue of undecaprenyl phosphate) is blocked. This reaction is analogous to the bacterial transfer reaction. In *Bacillus subtilis*, the GlcNAc transferase involved in teichoic acid biosynthesis is 5-fold more sensitive to tunicamycin than translocase I[70]. It has been suggested that this difference explains the observation that at low concentrations of tunicamycin *in vivo* synthesis of peptidoglycan is stimulated rather than inhibited. At low concentrations of tunicamycin, inhibition of the GlcNAc transferase makes more undecaprenyl phosphate available to translocase I thus increasing the rate of peptidoglycan synthesis[71].

The selectivity of tunicamycin towards the phospho-GlcNAc transferase enzymes has been described more recently[72]. However, considering the cellular roles of teichoic acid and peptidoglycan, it seems that the bacteriocidal activity of tunicamycin arises through inhibition of translocase I.

With respect to its structure, similarities with the substrates for these reactions are clear - the fatty acyl chain, the N-acetylated hexosamine, the ribose and uracil moeity. Heifetz and co-workers have carried out a study of the inhibition of solubilised, partially purified phospho-GlcNAc transferase from porcine aorta.[73] They conclude that tunicamycin is an irreversible inhibitor (based on the inability of dialysis to reverse the inhibition) which is noncompetitive with respect to both substrates. These conclusions are questionable and are discussed in detail in the

relevant section. Tunicamycin V and some analogues have recently been prepared synthetically.[74]

Liposidomycins A-C were isolated from *Streptomyces griseosporus* and characterised ten years ago by Isono *et al;*[75] They are nucleoside antibiotics containing uracil, a fatty acid, a seven-membered heterocyclic ring and a sulfated aminosugar. They were shown to inhibit *in vitro* peptidoglycan synthesis three orders of magnitude more potently than tunicamycin. At the same time it was shown that the liposidomycins did not elicit a toxic response in mice at 180 mg/kg. The *in vivo* activity of the liposidomycins is less striking with MIC values for typical gram positive and gram negative strains being ≥100 μg/mL. They were however highly active against a strain of *Mycobacterium, M. phlei* IFO 3158; MIC = 1.6 μg/mL for each antibiotic homologue.[75] The structure of liposidomycin B was determined by Ubukata *et al;*[76] revealing its structural complexity. The complete structures of liposidomycins A-C were reported by Ubukata *et al;* [77] Kimura *et al;* showed that liposidomycin C strongly inhibited translocase I (IC_{50} 0.03μg/mL) but only (comparatively) weakly inhibited translocase II and the transglycosylase enzyme (IC_{50} ~1μg/mL each) in the same particulate membrane preparations from *E. coli* Y10.[78] The heterocyclic ring moiety of liposidomycin has recently been prepared synthetically with a view to a total synthesis.[79]

Mureidomycins A-D were isolated and characterised by Isono, Inukai and co-workers.[80-82] They are peptidylnucleoside antibiotics containing a 3'-deoxy uridine moiety attached via an eneamide linkage to a peptide chain containing two *meta*-tyrosine residues. They were isolated in milligram (1 mg/L culture) quantities from *Streptomyces flavidoviridens* SANK 60486. They were selectively active against strains of *Pseudomonas* , MIC values ranging from 0.125 - 50 μg/mL,[81] and could protect mice from infection with *P. aeruginosa*, mureidomycin C being the most potent analogue. It was later shown that the mureidomycins specifically and selectively inhibit translocase I.[72, 83] The mureidomycins did not inhibit bacterial teichoic acid biosynthesis or mammalian glycoprotein biosynthesis, which tunicamycin does. Two minor components of the complex, mureidomycins E and F have also been structurally characterised.[84] They were less potent *in vivo* than mureidomycin A, but exhibited a similar spectrum of antibacterial activity.

Pacidamycins 1-7 are selective antipseudomonal agents produced by *Streptomyces coeruleorubidus* strain AB 1183F-64. They are structurally related to the mureidomycins,[85-87] and MIC values were similar to the mureidomycins (8 - 64 μg/mL against *Pseudomonas sp.*). However, unlike the mureidomycins, these compounds did not protect mice against *P. aeruginosa* infection.[87]

Napsamycins A-D are the most recently discovered subclass of compounds from the mureidomycin family.[88] They were isolated from *Streptomyces sp.* HIL Y-

82. They exhibited selective antipseudomonal activity like the mureidomycins. Napsamycin A is identical to mureidomycin F. MIC values were similar to the mureidomycins (6.25 - 100 μg/mL against *Pseudomonas sp.*). Further biological properties remain to be reported.

The mureidomycins, napsamycins and pacidamycins are closely related peptidylnucleoside antibiotics. The division into three named classes arises from the initial discoveries of complexes from separate strains of *Streptomyces*. As illustrated fully in fig. 1.21 the structural differences are subtle. The greatest variance is in the pacidamycins where the terminal peptide units change. Note that the N-terminal *meta*-tyrosine which is invariant in the mureidomycins and napsamycins is replaced with phenylalanine or tryptophan in the pacidamycins. Two other variations are the reduced uracil moiety present among some of the mureidomycins and napsamycins and the introduction of an extra carbon atom concomitant with C-terminal cyclisation.

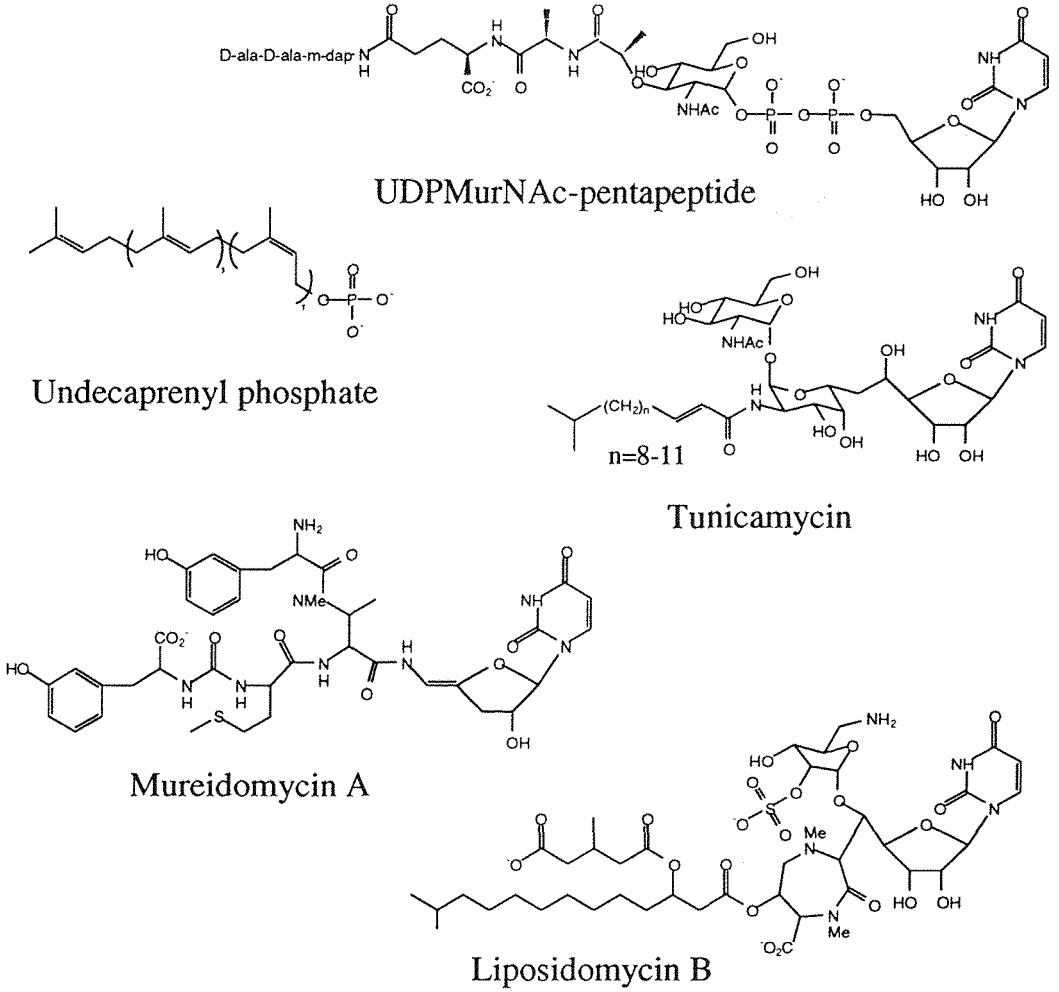


Figure 1.22. Comparison of the structures of the substrates of translocase I in *E. coli* and tunicamycin, mureidomycin A and liposidomycin B.

Amphomycin is an acyl-peptide which was originally believed to derive its bacteriocidal activity from inhibition of translocase I [89, 90]. This model was revised by Banerjee[91]. Amphomycin does inhibit this step of the pathway, but by complexation of undecaprenyl phosphate, the lipid-acceptor molecule, rather than binding to the enzyme. Amphomycin requires Ca^{2+} for complex formation and cannot chelate the free alcohol or the pyrophosphate (unlike bacitracin which complexes with the pyrophosphate only). It is reasonable to suggest that the role of the acyl chain of amphomycin is to anchor it to the lipid bilayer where undecaprenyl phosphate resides.

The common feature of the translocase I substrate UDPMurNAc-pentapeptide and the mureidomycins, etc;, the liposidomycins and tunicamycin is the nucleotide moiety (fig. 1.22). Nucleoside-containing antibiotics are a large class of natural products with broad-ranging biological activities. They have been classified and reviewed in detail by Isono.[92]

1.4.3 Mechanisms of resistance - some examples.

An inevitable consequence of using an antimicrobial agent is the emergence of strains which are resistant to that agent. An important part of progress in the treatment of bacterial infections is understanding the mechanisms by which the bacteria defend themselves against antibiotics. This section will outline some examples of resistance mechanisms pertaining to peptidoglycan biosynthesis.

The classic example of drug resistance is the case of the ß-lactam antibiotics. Soon after penicillin made its clinical debut, resistant strains appeared which contained ß-lactamase enzymes which destroyed the drug by hydrolysing the ß-lactam ring.[93] Spontaneous mutation plays an important role in ß-lactamase enzymes. These enzymes have no co-factor requirement (although some require metal ions) and have only one substrate binding-site. Thus a single base change in the gene for a ß-lactamase can change its substrate specificity. Bacteria have also become resistant to ß-lactams by producing PBPs (a generic term for proteins involved in peptidoglycan biosynthesis which bind penicillin) with reduced sensitivity to the antiobiotics, i.e. reduced affinity for penicillins.[94] Transfer of genes conferrring resistance to ß-lactams has become plasmid mediated and consequently resistance to ß-lactam antibiotics is now widespread.

In recent years several reports have been published describing mechanisms of resistance to vancomycin in strains of *Enterococcus*, *Lactobacillus* and *Leuconostoc*.[95-100] The resistance exhibited by *Lactobacillus caesi* and *Leuconostoc mesenteroides* is chromosomally based (intrinsic) whilst the resistance genes of *Enterococcus faecalis* and *Enterococcus faecium* are plasmid-based. It is possible that these strains have acquired these resistance genes from the intrinsically resistant

species by transposition/conjugation events. A very worrying scenario is that this resistance might be transmitted to a methicillin resistant *S. aureus* for which vancomycin therapy is the only treatment. However, for now at least, this plasmid cannot be transferred to *S. aureus*.[2]

Bugg *et al;* showed that high level vancomycin resistant *Enterococcus faecium* BM4147 was capable of making UDPMurNAc-pentapeptide precursor terminating in D-2-hydroxybutyrate instead of D-Ala. This altered pathway was mediated by plasmid derived proteins VanH and VanA. Vancomycin cannot bind this altered peptidoglycan precursor, hence the observed resistance.[95, 96] Handwerger *et al;* found that *Enterococcus faecalis* did indeed make peptidoglycan terminating in D-lactate and that intrinsically resistant species also did this (fig. 1.23).[97, 98]

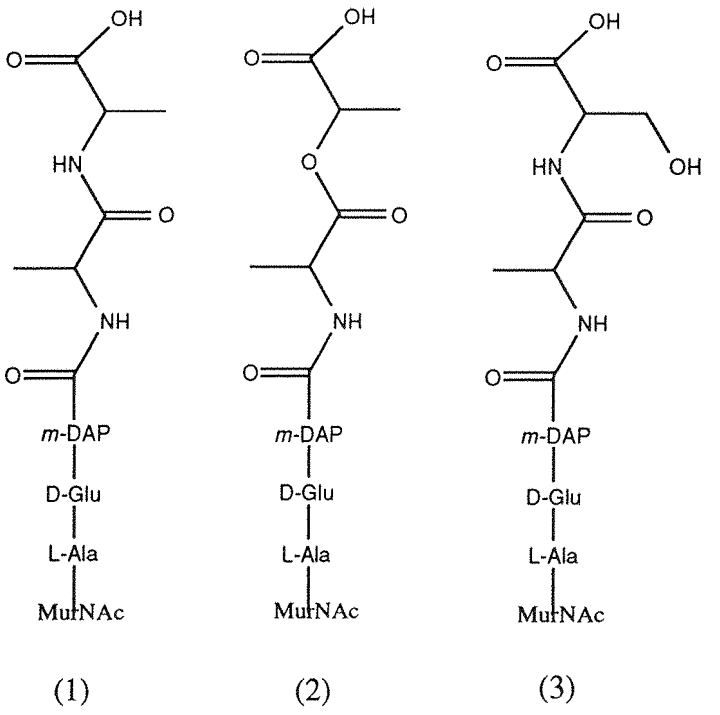


Figure 1.23. Altered peptidoglycan structures resulting in vancomycin resistance. (1) -D-Ala-D-Ala, (2) -D-Ala-D-lactate, (3) D-Ala-D-serine.

Reynolds *et al;* found that D-serine was the new terminal residue in low level vancomycin resistant strain *Enterococcus gallinarium* BM4174 and that this strain also contained activities which destroyed D-ala-D-ala and UDPMurNAc-L-Ala-γ-D-Glu-*m*-DAP-D-Ala-D-Ala using a D,D-peptidase activity thus preventing synthesis of vancomycin susceptible peptidoglycan precusors.[99] Billot-Klein *et al;* suggested that it is this ability to shut down the normal peptidoglycan biosynthetic pathway that distinguishes between low-level and high-level vancomycin resistance.

Bacitracin chelates undecaprenyl pyrophosphate and so disrupts peptidoglycan biosynthesis as well as other pathways using undecaprenyl phosphate. Cain *et al;* found that *E. coli* could increase *de novo* synthesis of undecaprenyl phosphate by increasing levels of a kinase which phosphorylates undecaprenol, thereby negating the effect of the bacitracin.[101] Other bacteria stopped making non-essential exopolysaccharides whose biosynthetic pathways involved undecaprenol phosphate presumably making more undecaprenyl phosphate available for essential peptidoglycan biosynthesis.[66]

Strains of *Staph. aureus* resistant to methicillin currently pose a considerable threat to post-operative and immunocompromised patients. There are several determinants for the mec^+ phenotype (resistant to all ß-lactam antibiotics). These include the *mecA* gene coding for PBP2α (an enzyme with low affinity for penicillin which may be involved in incorporation of nascent peptidoglycan into existing peptidoglycan[102]) and the *fem* genes (*A, B, C, D* and *X*). Loss of any one of these genes results in loss of resistance. It seems that *femA* and *femB* are involved in construction of the pentaglycine interpeptide bridge in the peptidoglycan of *S. aureus* since mutation of either the *femA* or the *femB* gene produces peptidoglycan with a 40 % reduction in glycine content or a greater proportion of monoglycyl and serine-containing muropeptides at the expense of pentaglycine respectively.[103, 104]

Recently Maki *et al;* have cloned and sequenced the *S.aureus* gene *llm* and showed it to be a determinant in methicillin resistance in a number of clinical isolates.[105] The authors suggest that this along with the hydrophobicity of the deduced protein sequence may mean that the gene product of *llm* may be involved in the membrane cycle of peptidoglycan biosynthesis.

The mechanisms by which bacteria develop resistance are as varied as the biochemical processes within their cells. The race to produce new antibiotics faster than bacteria can devise ways of coping with their effects is one which we seem to be losing at present.

1.5. Translocase I

Studies to date of translocase I have been conducted in the main with particulate (i.e. membrane bound) preparations from *Staphylococcus aureus* Copenhagen[106-111]. These studies have been hampered throughout by lack of purified or fully active solubilised enzyme, by the very low natural abundance, by the absence of a *continuous* assay and by the numerous technical problems associated with membrane proteins. This section will outline those studies from the identification of the transferase activity and the nature of the substrates up to the starting point for the current project.

1.5.1. Phospho-N-acetylmuramyl pentapeptide translocase.

Phospho-N-acetylmuramyl pentapeptide translocase is the name for translocase I which was first proposed by Struve *et al*;[111] when this distinct catalytic activity was formally identified. The substrate for cell-free peptidoglycan synthesis had already been identified as uridine diphospho-N-acetylmuramyl pentapeptide[112, 113]. Higashi *et al;* later showed [114] that the lipid moiety of lipid-pyrophosphoryl-disaccharide pentapeptide isolated from *S.aureus* was a C55-isoprenoid alcohol. Thus the translocase catalyses the reversible reaction illustrated in fig 1.24.

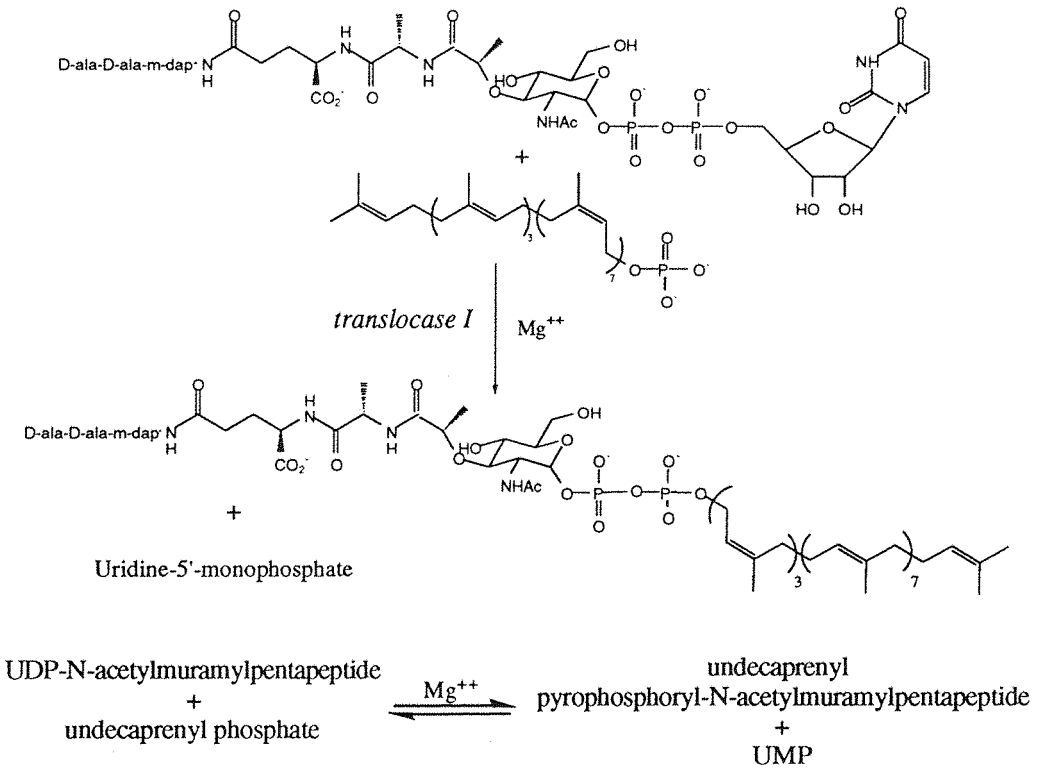


Figure 1.24. Reaction catalysed by translocase I.

In addition to this reaction, the enzyme also catalyses the exchange of radiolabelled UMP with the UMP moiety of UDPMurNAc-pentapeptide (fig. 1.25).[111]

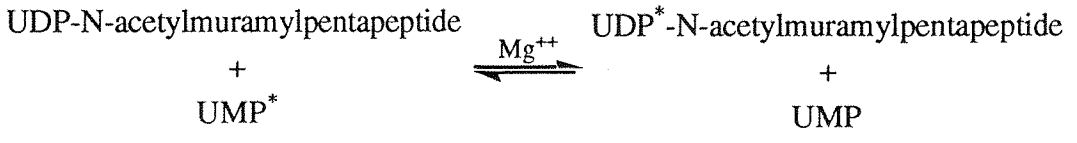


Figure 1.25. Isotope exchange catalysed by translocase I.

1.5.2. Assay methods.

Assay methods have been described which measure either formation of the lipid-linked product or the exchange of radiolabelled UMP with the UMP moiety of UDPMurNAc-pentapeptide (or *vice versa*). These are referred to in the literature as transfer and exchange assays respectively. A variety of protocols have been reported for both. The soluble substrate, UDPMurNAc-pentapeptide, can readily be isolated from antiobiotic-treated cells of *S. aureus* or *B. subtilis* [47, 111, 115]. The transfer assay uses radiolabelled UDPMurNAc-pentapeptide: the label has been ^{14}C or ^{3}H in various positions in the peptide chain prepared by inclusion of radiolabelled precursor in the growth medium[112] or by cell free enzymatic synthesis[116]. UDPMurNAc-pentapeptide has also been prepared with a ^{32}P radiolabel[116] although this has not been used for routine assay of enzyme activity. The radiolabelled lipid-linked product can either be extracted with organic solvents after acidification [90, 117] or precipitated with perchloric acid[112, 118].

In exchange assays, the radiolabel is either in UMP or the uridine moiety of UDPMurNAc-pentapeptide and is redistributed throughout the uridine pool as in fig. 1.25. The newly labelled product is separated from starting material by treatment with alkaline phosphatase (UMP hydrolysis) and ion-exchange chromatography. Both of these assay types are *stopped*, i.e. the reaction must be stopped to quantify the product. A potential *continuous* assay (product formation is monitored continuously without termination of the reaction) has been demonstrated by Weppner *et al;*[118]. The substrate in this assay is a dansylated analogue of UDPMurNAc-pentapeptide where the fluorophore is attached to the L-lysine side chain and is discussed in more detail in chapter 4.

1.5.3. Characterisation of *S. aureus* translocase I.

Translocase I from *S. aureus* has been characterised by Neuhaus and co-workers over the period 1965 - 1980.[106-111, 118, 119] With the particulate enzyme, the K_m values for UDPMurNAc-pentapeptide and UMP were 1.8μM and 27μM respectively. The equilibrium constant for the reaction (fig 1.24) was 0.25.[111] 5-fluoro-UMP and 5-fluoro-UDPMurNAc-pentapeptide were very poor substrates for translocase I and inhibited catalysis with K_i values 120μM and 50μM respectively (measured in the exchange reaction for 5-fluoro-UMP and in the transfer reaction for 5-fluoro-UDPMurNAc-pentapeptide).[119] Heydanek *et al;*[107] showed that membrane preparations from *S. aureus* could also catalyse the hydrolysis of UDPMurNAc-pentapeptide to UMP and 1-phospho-MurNAc-pentapeptide in a pseudo-first order fashion. This reaction was slower than the transfer of 1-phospho-MurNAc-pentapeptide to lipid acceptor. There was a lag observed in the formation of 1-phospho-MurNAc-pentapeptide which corresponded to the initial rapid formation of

lipid-linked product suggesting that the same enzyme (i.e. translocase I) was catalysing both reactions.

In the same paper, the observation was reported that the exchange reaction proceeded 6-24-fold faster than the transfer reaction depending on conditions. These observations can be rationalised by either of two models for the mechanism of catalysis by translocase I.

In the first case, undecaprenyl phosphate directly attacks the UDPMurNAc-pentapeptide pyrophosphate bond to give undecaprenyl-pyrophosphoryl-MurNAc-pentapeptide and UMP as in figure 1.26.

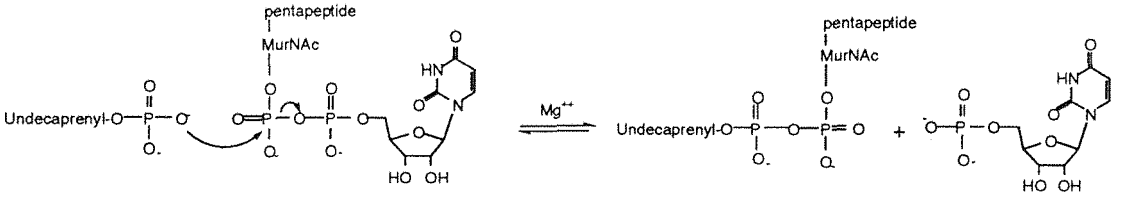


Figure 1.26. Direct displacement mechanism for translocase I.

If this mechanism is operating, then exchange of [^{14}C]-UMP would be explained by the reversibility of the overall reaction. If the rate-limiting step is dissociation of undecaprenyl-pyrophosphoryl-MurNAc-pentapeptide from the enzyme, then the rate of exchange would be faster than the rate of transfer. When undecaprenyl phosphate is depleted or absent water is the nucleophilic species and hydrolysis occurs yielding 1-phospho-MurNAc-pentapeptide. However, a word of caution is that hydrolysis of phosphodiesters is known to be very slow, hence the great stability of DNA in aqueous solution.

In the second case, a nucleophilic residue in the enzyme active-site attacks UDPMurNAc-pentapeptide to form a covalent enzyme-linked intermediate. Undecaprenyl phosphate attacks this species to give lipid-linked product (fig. 1.27).

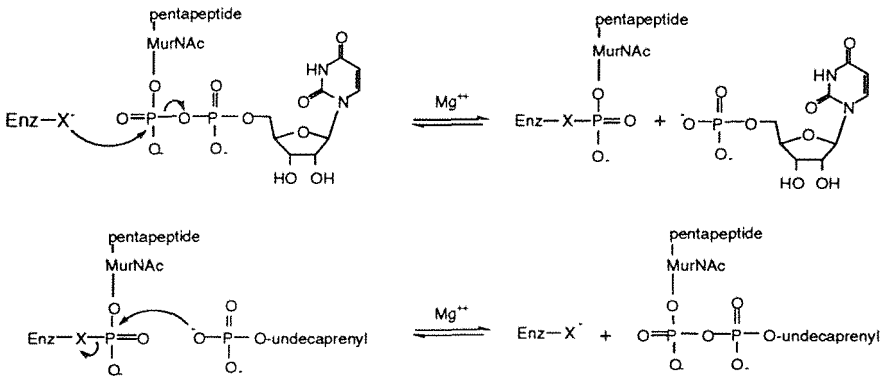


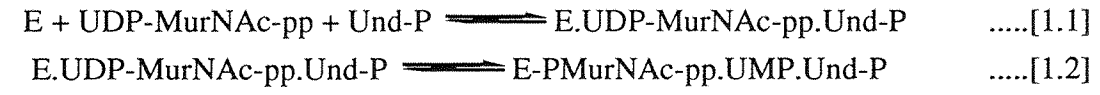
Figure 1.27. Double displacement mechanism for translocase I.

If this mechanism is operating, then exchange of [^{14}C]-UMP would be explained by the first partial reaction not involving undecaprenyl phosphate. If the second step is the rate-limiting step, then the rate of exchange would be faster than the rate of transfer. In the absence of undecaprenyl phosphate water can attack the intermediate species effecting substrate hydrolysis.

The accelerated rate of isotope exchange noted above favours a two-step mechanism, but it is not conclusive. Detection of exchange activity in the absence of undecaprenyl phosphate or derivatives would effectively rule out the direct displacement mechanism. However, inability to separate the enzyme from endogenous lipids with certainty has precluded interpretation of experiments in that respect.

Heydanek *et al;* also solubilised the enzyme from particulate fractions using potassium hydroxide, urea or one of several detergents including lauroyl sarcosinate, oleoyl sarcosinate and Triton X-100.[106] In each case apart from urea the ratio of exchange activity to transfer activity increased, transfer activity being only poorly solubilised. Not surprisingly, enzyme solubilised with 10 M urea was not active, but remarkably greater than 85 % of transfer activity was routinely recovered when the urea was removed by dialysis in the presence of buffer containing 1M KCl. This achievement represents one of, if not *the*, earliest examples of successful refolding of a membrane protein.

Pless *et al;* found that exchange activity of *S. aureus* translocase I solubilised with 1 % Triton X-100 was activated by phosphatides and by undecaprenyl phosphate/undecaprenyl-pyrophosphoryl-MurNAc-pentapeptide and that the effects were additive.[108] Stimulation of the exchange reaction by undecaprenyl phosphate/undecaprenyl-pyrophosphoryl-MurNAc-pentapeptide would be expected if the direct displacement mechanism (fig. 1.26) were taking place. If the double displacement mechanism was taking place (fig. 1.27) then the observed activation would imply a catalytic sequence more complex than an ordered ping pong bi bi mechanism. It could be the case that for full activity in the exchange reaction mediated by formation of an enzyme-linked intermediate, undecaprenyl phosphate must be present in the Michaelis complex (equations 1.1 and 1.2), i.e. there are distinct binding sites for UMP and undecaprenyl phosphate and both need to be occupied for maximum catalytic efficiency.



The authors draw the inference that if this were indeed the case then other undecaprenyl phosphate derivatives such as undecaprenol or undecaprenyl pyrophosphate should also activate the translocase in the exchange assay. No activation was seen with either ficaprenol (undecaprenol isolated from *Ficus elastica* rather than bacteria) of undecaprenyl pyrophosphate.

Hammes *et al;* studied the substrate specificity of particulate translocase I from *S. aureus* with respect to the peptide part of UDPMurNAc-pentapeptide.[109] The L-Ala and first D-Ala residues were important (as judged by glycine substituted analogs) whereas the second D-Ala was not. The L-Lys residue could be substituted with *meso*-diaminopimelic acid or L-ornithine with little consequence. UDPMurNAc-tetrapeptide was a 4-fold less efficient substrate (V_{max}/K_m) than the genuine substrate and UDPMurNAc-tripeptide was 77-fold less efficient.

Weppner *et al;* synthesised UDPMurNAc-L-Ala-γ-D-Glu-(N^{ε}-dansyl)-L-Lys-D-Ala-D-Ala by condensation of the natural substrate with dansyl chloride.[118] The fluorescent analog was a slightly better substrate (V_{max}/K_m and V_{max} values) than the genuine substrate, but was a poor substrate for the *in vitro* synthesis of peptidoglycan. In a particulate system, the quantum yield of the fluorescent lipid-linked product was six times that of the fluorescent precursor.

In summary, translocase I from *S. aureus* can catalyse both the transfer of phospho-MurNAc-pentapeptide to lipid acceptor and the exchange of radiolabelled UMP with the UMP moiety of UDPMurNAc-pentapeptide. It can be solubilised from membrane fragments but requires addition of a phosphatide and undecaprenyl phosphate for full exchange activity. The mechanism by which the enzyme catalyses these reactions remains unresolved and the enzyme has not been purified beyond crude extracts. Continuous assay of the enzyme has been shown to be possible.

1.5.6. Identification of the *mra*Y gene product.

The *mra* (murein synthetic gene cluster a) cluster of genes is found at minute 2 of the *E. coli* chromosome and contains ten of the genes required for peptidoglycan synthesis and cell division.[120] One of these genes, *mraY*, has been shown to be the structural gene for translocase I in *E. coli* since when the gene dose of *mraY* was increased by introduction into *E. coli* JM109 on a pUC118-derived plasmid the functional translocase I activity increased.[27]

Using the derived amino acid sequence Ikeda *et al*; also found by a hydropathy analysis that translocase I is likely to be an integral membrane protein. The derived sequence showed statistically significant (20.7% similarity) to ALG7, a yeast protein thought to be identical to GlcNAc-1-phosphate transferase.[121, 122] This enzyme catalyses the first committed step in eukaryotic glycoprotein biosynthesis (see sections 1.3.2 and 3.2).

The identification and cloning of the *mraY* gene makes overexpression of translocase I a possibility.

1.7. Aims, goals and strategy.

The aim of this project is to answer two questions:

1. What is the catalytic mechanism of translocase I?
2. What is the mode of inhibition of translocase I by mureidomycin A and liposidomycin B?

If these two issues can be understood, then we will be one step closer to the ultimate goal of this research, namely rational design of inhibitors of translocase I as novel antibacterial agents for use as therapeutic antibiotics.

The general strategy in approaching this research problem has been to define a series of goals directed towards developing the methodology for a detailed molecular study of translocase I. These goals are listed:

1. To assay the *E. coli* enzyme and to overexpress it the enzyme in *E. coli.*
2. To solubilise the enzyme in a stable and active form from *E. coli* membranes.
3. To develop a continuous assay methodology to permit a detailed kinetic assessment of the inhibitors.
4. To purify the enzyme as far as possible.

The following six chapters describe the successes, and sometimes failures, in pursuit of these goals, and the subsequent experiments which have given us a brief insight into the workings of this catalyst and two remarkable natural products.....

Chapter 2. Overexpression, solubilisation and preliminary characterisation of translocase I.

2.1. Introduction.

Central to the study of any enzyme is preparation of a cell-free extract containing enzyme activity which can be reliably and reproducibly measured. The first goal of the project was therefore to prepare such an extract and characterise it in terms of level of activity, stability, contaminating activities, etc;. Since translocase I is almost certainly a membrane protein,[27] the second goal was to extract translocase I from the membrane with retention of catalytic activity using detergents. This would give a solubilised preparation which could then be used for further studies. This chapter describes the experiments conducted towards these aims.

2.2. Radiochemical assay for translocase I activity.

This assay method measures incorporation of carbon-14 radiolabel from UDP-MurNAc-L-Ala-γ-D-Glu-*m*-DAP-D-Ala-D-Ala into butanol-extractable products. The substrate, UDPMurNAc-pentapeptide is turned over into lipid-linked product which can be separated by extraction with n-butanol. It is based on a transglycosylase assay used by our collaborators, SB Pharmaceuticals, and the method of Tanaka.[90] The radiolabelled material was supplied by SB Pharmaceuticals. It was generated from UDP-MurNAc-tripeptide using a cell-free extract of *E. coli* K235 with added ATP, pyridoxal phosphate and [^{14}C]-L-alanine (fig. 2.1).

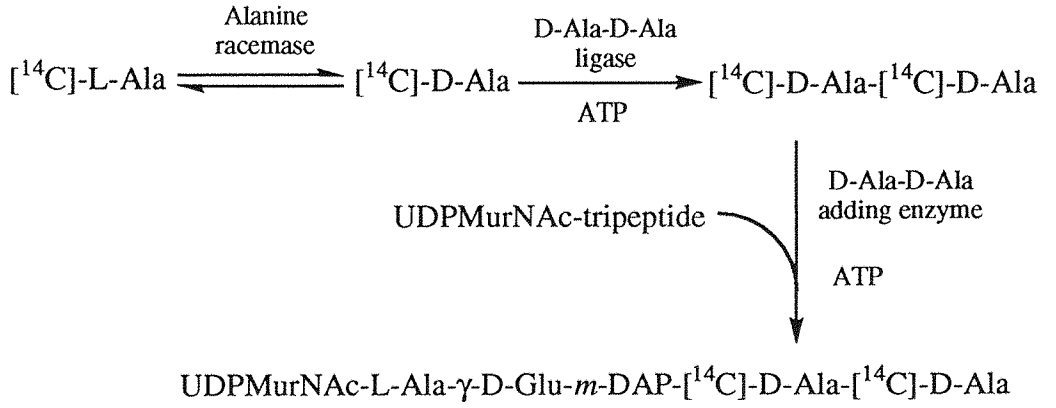


Figure 2.1. *In vitro* synthesis of UDPMurNAc-pentapeptide from UDPMurNAc-tripeptide and [^{14}C]-L-alanine using a cell-free extract of *E. coli* K235.

The specific activity of the stock used in experiments in this chapter and chapter 3 was 78 μCi/μmole.

With respect to the extraction procedure, 6M pyridinium acetate was used to acidify the reaction mixture in keeping with previously reported procedures. n-Butanol was the organic solvent of choice since being non-chlorinated it forms the upper layer facilitating removal for scintillation counting. After vortexing and separation of the layers by microcentrifugation, the volume of the upper layer was 120 μL and that of the lower layer 80 μL. 100 μL of the organic phase was taken for scintillation counting and the radioactivity measured multiplied by 1.2 to arrive at the total radioactivity extracted. This action also served to avoid the precipitate formed at the layer interface. Knowing the specific activity of the substrate stock used it was possible to calculate the number of picomoles of product formed.

It was found that the radiolabelled substrate, UDP-MurNAc-L-Ala-γ-D-Glu-*m*-DAP-[^{14}C]-D-Ala-[^{14}C]-D-Ala could be diluted one part to four with unlabelled material without reducing the certainty or reproducibility of the assay. This was done in order to conserve the valuable substrate.

The exact procedure used in each of the experiments below is described fully in the experimental chapter. The sensitivity of the assay was such that as little as 0.5 pmoles of product could be detected in a 50 μL assay volume.

2.3. Overexpression of translocase I.

The *mraY* gene coding for translocase I in *E. coli* was cloned and identified by Ikeda *et al;* in 1991.[27] Dr. Martin Burnham at SB Pharmaceuticals had constructed a series of plasmids containing genes coding for one of or combinations of each of translocase I, translocase II and PBP1b (transglycosylase). One of these, pBROC512, harboured in *E. coli* JM109 was given to us by Dr. Burnham. This is a single-copy plasmid derived from the expression vector, pMR100, and contains the *mraY* gene with its natural ribosome-binding site at a non-optimal distance (*circa* 120 bp) from the strong promoter, *trc* (appendix 1). This series of plasmids were not designed to be high level expression systems.

The level of expression of translocase I in *E. coli* JM109 pBROC512 was compared with that of *E. coli* JM105. *E. coli* JM109 pMR100 was not available. The two strains were grown in LB liquid medium, the *E. coli* JM109 pBROC512 being supplemented with kanamycin and IPTG as described in the experimental section. Membranes were isolated and diluted to the same protein concentration for assaying. Note that at this stage no lipid substrate is added; the assay relies on endogenous undecaprenol phosphate present in *E. coli* membranes. The assays were carried out as described in the experimental section. The time-course of incorporation of radiolabel into butanol-extractable product (fig. 2.2) indicated that the preparation from *E. coli*

JM109 pBROC512 contained approximately 15-fold more translocase I activity than that from *E. coli* JM105.

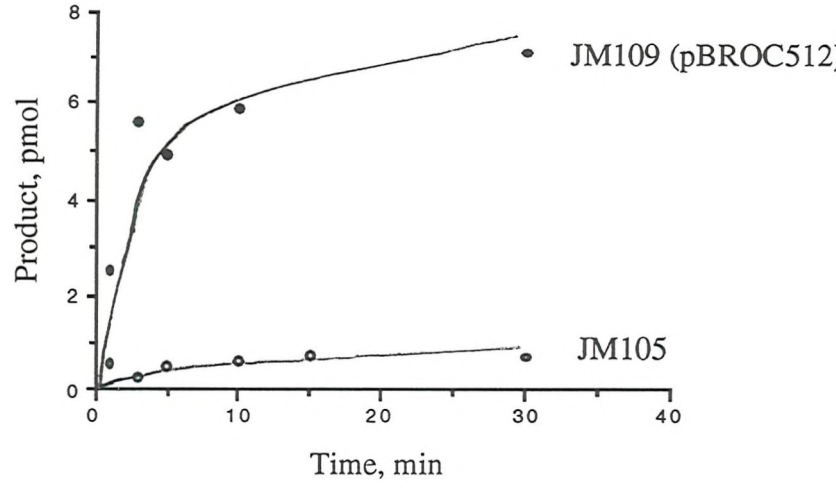


Figure 2.2. Time-course assay of membranes from *E. coli* JM109 pBROC512 and *E. coli* JM105.

2.4. Characterisation of pBROC512 expressed particulate enzyme.

At this stage, the possibility of substituting undecaprenol phosphate with a cheap shorter chain analogue was investigated. Farnesol phosphate (a mixture of *cis* and *trans* isomers) was prepared by the method of Danilov *et al;* (fig. 2.3) in 4 % yield as described in the experimental section.

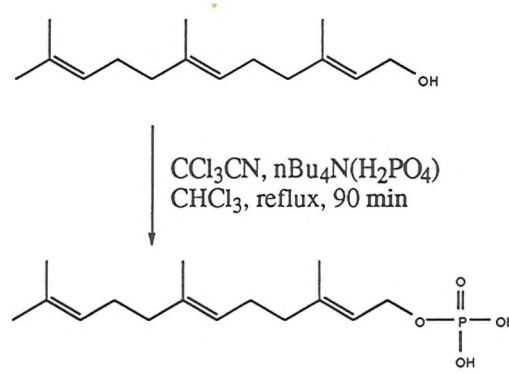


Figure 2.3. Chemical phosphorylation of farnesol.

Farnesol phosphate (FP) was added to the membrane preparations prior to assay from a concentrated stock solution in 1:1 chloroform/methanol such that the

assay concentration ranged from 7 -140 μM. Under these conditions there was no increase in incorporation of radioactivity into butanol-extractable product.

With hindsight this is not surprising since other enzymes using polyprenyl phosphates as substrates have exhibited a strict dependence on chain length.[52] Dolichyl-diphospho-GlcNAc synthesis from either *S. cerevisiae* or calf liver microsomes could not use dolichyl phosphate analogues with less than seven isoprene units, farnesyl phosphate having three.[123, 124] Alternatively, it is quite possible that the FP never partitioned into the membranes.

In some previous studies, bacterial alkaline phosphatase has been included in translocase I assays, the rationale being that hydrolysis of product UMP will drive the reaction to completion (fig. 2.4).[106-108]

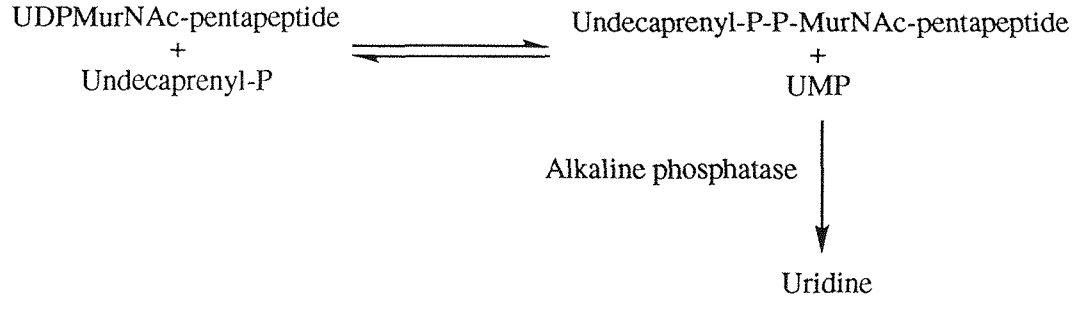


Figure 2.4. Hydrolysis of product UMP during translocase I assays.

When partially purified snake venom nucleotidase (Sigma), 20 milliunits, was included in the assay radioactivity incorporated increased by 18% from 495 cpm to 585 cpm after 30 min. This enzyme was not included in further assays.

Dr. John Lonsdale at SB Pharmaceuticals has found that 10 mM ATP increased production of nascent peptidoglycan from UDP-MurNAc-pentapeptide by 200% in a cell-free system using a membrane preparation from *E. coli* JM109 (pHK2414). Plasmid pHK2414 carries the *E. coli* PBP1b gene which codes for the major biosynthetic transglycosylase of this organism. It was suggested that ATP might be being used to increase the amount of undecaprenyl phosphate (undecaprenol kinase is a membrane bound enzyme in *S. aureus*[40]) in the membranes allowing increased throughput of UDPMurNAc-pentapeptide to nascent peptidoglycan. To investigate the effect of ATP in the present system, particulate enzyme was assayed in the presence of 0.1 - 10 mM ATP and butanol extractable product quantified at 1 min and 30 min (table 2.1).

In no case did ATP potentiate activity; indeed at higher concentrations ATP was inhibitory. ATP was not included in subsequent assays. It is not known why ATP

is inhibitory, but it may be that the ATP chelates the Mg^{2+} ions which translocase I requires for activity.

ATP, mM	Product, pmol	
	1 min	30 min
0	5.9	9.0
0.1	4.9	7.2
1	4.0	7.1
5	5.3	5.9
10	4.6	4.1
20	1.9	3.0

Table 2.1. Effect of ATP on particulate translocase I activity.

2.5. Overexpression of translocase I using pBROC525.

A second expression vector, pBROC525 (appendix 1), was constructed by Dr. Martin Burnham at SB Pharmaceuticals with the aim of maximising expression of translocase I. An 8.6 kb *KpnI/KpnI* fragment carrying the *mraY* gene was subcloned from Kohara phage 110[125] into the *KpnI* site of pUC19 to give pBROC508. A 1.3 kb blunt-ended *MluI/MluI* fragment containing the *mraY* gene was subcloned from pBROC508 into the *SmaI* site of pUC19 to give pBROC511 (appendix 1). The 1.3kb *AflIII/BamHI* fragment of pBROC511 was cloned into the *NcoI/BamHI* sites of the expression vector pTrc99A (Pharmacia Biotech) to give pBROC525. This construct contains the *mraY* gene at an optimal distance from the strong *trc* promoter. This construct also carries a ß-lactamase gene for selection purposes, so growth media for strain *E. coli* JM109 (pBROC525) contained 100 µg/mL ampicillin. A map of pBROC525 is included in the appendices.

To assess the level of expression in *E. coli* JM109 (pBROC525), this strain along with *E. coli* JM105 and *E. coli* JM109 (pBROC512) were used to prepare membrane extracts. The plasmid-carrying strains were induced with 0.2 mM IPTG at OD_{600nm} = 0.6 and grown for a further 4 hr at 37°C. Membranes were prepared and enzyme assayed (table 2.2) as desribed in the experimental chapter.

Whilst the specific activity of the *E. coli* JM109 (pBROC525) was greater than that of the *E. coli* JM109 (pBROC512) preparation, it was by no means the level of overproduction that had been hoped for.

Two experiments were conducted towards maximising the production of translocase I. Cultures of *E. coli* JM109 (pBROC525) were induced at OD_{600nm} = 0.6 or 1.7 (early or late logarithmic phase) with 0.2 mM IPTG and grown for a further 2 hr. The specific activities of particulate enzyme preparations were 204 and 370

pmol/min/mg protein from the early and late log phase-induced cultures respectively. This improvement was incorporated into subsequent protocols.

Strain	Specific activity, pmol/min/mg	Overexpression
E. coli JM105	20.6	-
E. coli JM109 (pBROC512)	141.6	7-fold
E. coli JM109 (pBROC525)	289.4	14-fold

Table 2.2. Translocase I activity in membranes of *E. coli* bearing no plasmid, pBROC512 or pBROC525.

The concentration of IPTG used to induce expression of translocase I was varied. In a similar experiment to the last, three cultures of *E. coli* JM109 (pBROC525) were grown at 37°C to OD_{600nm} = 1.7, induced with 0.05, 0.2 or 1.0 mM IPTG and grown for a further 90 min. Specific activities of particulate enzyme preparations were measured as before (table 2.3).

IPTG, mM	Specific activity, pmol/min/mg
0.05	343
0.2	519
1.0	348

Table 2.3. Effect of IPTG concentration on expression of translocase I in *E. coli* JM109 (pBROC525)

The most active extract was obtained with 0.2 mM, which is the concentration which was used in previous experiments, and all subsequent experiments with *E. coli* JM109 (pBROC525).

It was thought that the protein might be expressed in moderate amounts (it is probably not expressed in very large quantities because growth rate is not significantly affected by IPTG induction) but that it is not correctly folded or targetted into the membrane. Reduction of growth rate might alleviate this problem to an extent. A culture of *E. coli* JM109 (pBROC525) was cooled to room temperature (20 °C) just before induction and grown overnight on the bench with shaking. However, the yield of activity was not improved over the existing protocol.

It is also possible, even likely given the hydrophobic nature of the protein, that expressed protein will have aggregated inside the cell forming inclusion bodies.

Whole IPTG-induced cells of *E. coli* JM109 (pBROC525) were examined for their protein content by SDS-PAGE. No new protein bands were visible in the region 20 - 66 kDa up to 3 hr after induction making the presence of large quantities of aggregated protein unlikely.

The control strain used in these experiments was *E. coli* JM105 since strains *E. coli* JM109 (pMR100) and *E. coli* JM109 (pTrc99A), the empty expression vectors were not available at that time. However, in a later experiment described in chapter 6, activity in a solubilised preparation from *E. coli* JM109 (pBROC525) was compared with that in a similarly solubilised preparation from *E. coli* JM109 (pTrc99A) using the fluorescence enhancement assay. The overexpression measured was 28-fold thus validating *E. coli* JM105 as a control strain.

2.6. Solubilisation of translocase I activity.

2.6.1. A brief introduction.

A major goal of this project is to purify translocase I, to homogeneity if possible. A particulate, membrane bound preparation is not suitable for the chromatographic methods generally applied in protein purification, so the protein must first be solubilised. The criterion for solubilisation generally applied to membrane proteins is that the protein is not sedimented by centrifugation at $10^5 g$, the remaining supernatant being optically clear. This can be achieved by various methods, the efficiency of solubilisation varying from case to case. The agents used may be a simple chaotropic salt (e.g. guanidinium hydrochloride) or urea or alkali (e.g. KOH), but these agents commonly cause loss of functional activity.

Detergents on the other hand often have the ability to solubilise the membrane fragments without destroying functional activity. Detergents are amphipathic molecules. They have a hydrophilic region and a hydrophobic region and above a certain concentration, called the critical micelle concentration (CMC), the detergent molecules associate to form micelles (fig. 2.5).The aggregation number is the number of detergent monomers per micelle. The CMC and aggregation no. are key properties of any detergent.

Detergents are classified as either non-ionic, anionic, cationic or zwitterionic depending on their charge at neutral pH. The non-ionic detergents (e.g. Triton and Brij series) tend to have low cmc values and high aggregation numbers, whilst the ionic detergents (e.g. deoxycholate, dodecyltrimethylammonium bromide, CHAPS) tend to have high CMC values and lower aggregation numbers.

In some cases the hydrophobic part of the detergent molecule can 'invade' the hydrophobic core of the protein resulting in denaturation, which inevitably results in loss of activity. Thus detergents are further classified as either denaturing (e.g.

sodium dodecyl sulphate, SDS) or non-denaturing (e.g. Triton, CHAPS, octylglucoside).

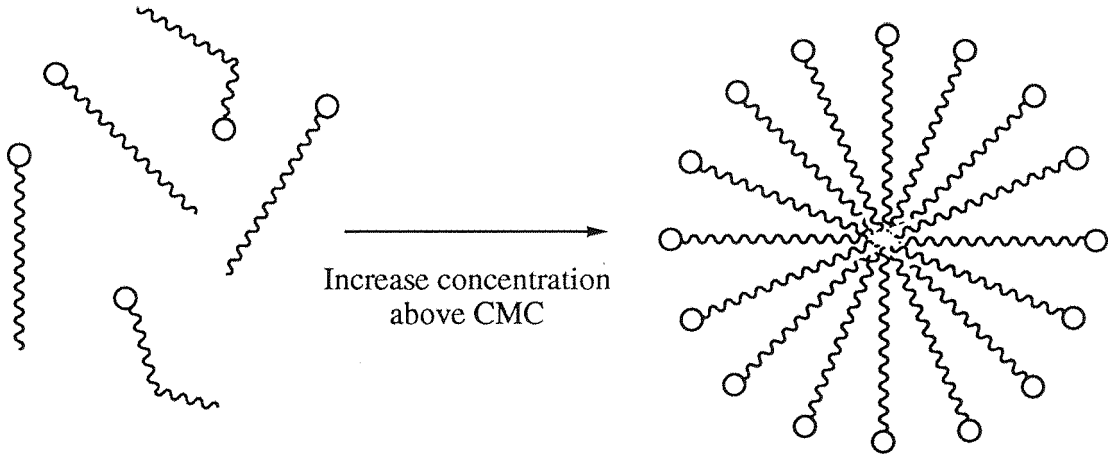


Figure 2.5. Micelle formation by detergents.

At present detergents and their solubilising effects are poorly understood, making description of their properties with respect to membrane proteins largely empirical. Consequently generalisations cannot readily be made and every case must be investigated with a degree of trial and error. An excellent introduction to detergents is included in Deutscher, section V.[126]

2.6.2. Solubilisation experiments.

For the results of the solubilisation experiments to be comparable, standard conditions must be maintained throughout. These are described in the experimental section. There is evidence in the literature that phosphate buffers are generally effective as good solubilisation buffers and that a concentration of 50 mM is usually enough to cope with pH changes induced by detergent.[126] In several cases elevated concentrations of phosphate buffer (0.1 - 0.5 M) markedly increased solubilisation, partially by virtue of higher ionic strength, but additionally for reasons not yet understood. The buffer chosen for initial experiments was therefore 100 mM potassium phosphate, 2 mM ß-mercaptoethanol, 25 mM $MgCl_2$.

It was found that inclusion of 25 mM $MgCl_2$ in preparative buffers for isolation of membrane fragments increased the yield of activity by about 50%. It is not known why this should happen, but it is possibly connected with the requirement of translocase I for Mg^{2+} ions. This was later adjusted to 1 mM $MgCl_2$ without a reduction in the yield of activity because on standing overnight the potassium phosphate buffer used in early experiments formed a precipitate in the presence of 25 mM $MgCl_2$.

Membrane preparations from *E. coli* JM109 (pBROC512) were extracted at 4 mg protein/mL with Triton X-100, sodium lauroyl sarcosinate, EDT-20 (N,N',N'-polyoxyethlyene(10)-N-tallow-1,3-diaminopropane), sodium deoxycholate or CHAPS (3-cholamidopropyldimethyl-ammonio-1-propane sulphonate) at 0.25 % to 3.0 % for 1 hr at 4 °C with occasional vortexing. The deoxycholic acid was recrystallised from hot ether prior to use to remove impurities. Sodium deoxycholate was prepared by mixing a solution of deoxycholic acid with an equimolar amount of NaOH. Extraction mixtures were centrifuged at 10^5 *g* for 1 hr at 4 °C and the supernatants assayed for activity (table 2.4). The salient properties of each of the detergents are listed in fig. 2.6.

Detergent and Concentration	Detergent/ protein ratio, mmol/mg	Protein extracted, %	Activity extracted	
			pmols/min at 30 °C	% of that in membranes
Lauroyl sarcosinate:				
Membranes	-	-	(23.5)	-
0.25 %	2.1	24	24	102
0.5 %	4.2	23	19.7	83
1.0 %	8.5	22	18.3	77
3.0 %	25.5	73	21.1	89
EDT-20:				
Membranes	-	-	(34.1)	-
0.25 %	nd	40	29.2	98
0.5 %	nd	42	30.1	84
1.0 %	nd	44	33.1	87
3.0 %	nd	51	34.8	96
Triton X-100				
Membranes	-	-	(66.5)	-
0.5 %	2	71	82	123
1.0 %	4	71	72.4	109
3.0 %	12	67	72.6	109
Sodium deoxycholate				
Membranes	-	-	(66.5)	-
0.5 %	3	31	59.4	89
1.0 %	6	30	61.2	92
3.0 %	12	60	63.8	96
Tween 20:				

Membranes	-	-	(30)	-
0.5 %	1	nd	3.3	11
1.0 %	2	nd	3.3	11
2.0 %	4	nd	2.3	8
CHAPS:				
Membranes	-	-	(42.3)	-
0.5 %	2	nd	22.4	53
1.5 %	6.1	nd	50.8	120

Table 2.4. Solubilisation of translocase I activity from membrane preparations of *E. coli* JM109 pBROC512.

Detergent	**Charge at pH 7.5**	**CMC**	**Dialysable?**
Lauroyl sarcosinate	anionic	13.7 mM	√
Triton X-100	non-ionic	0.24 mM, 0.02 %	x
Deoxycholate	anionic	4 mM, 0.21 %	√
EDT-20	cationic	?	x
CHAPS	zwitterionic	9 mM, 0.49 %	√
Tween 20	non-ionic	49 μM, 0.006 %	x

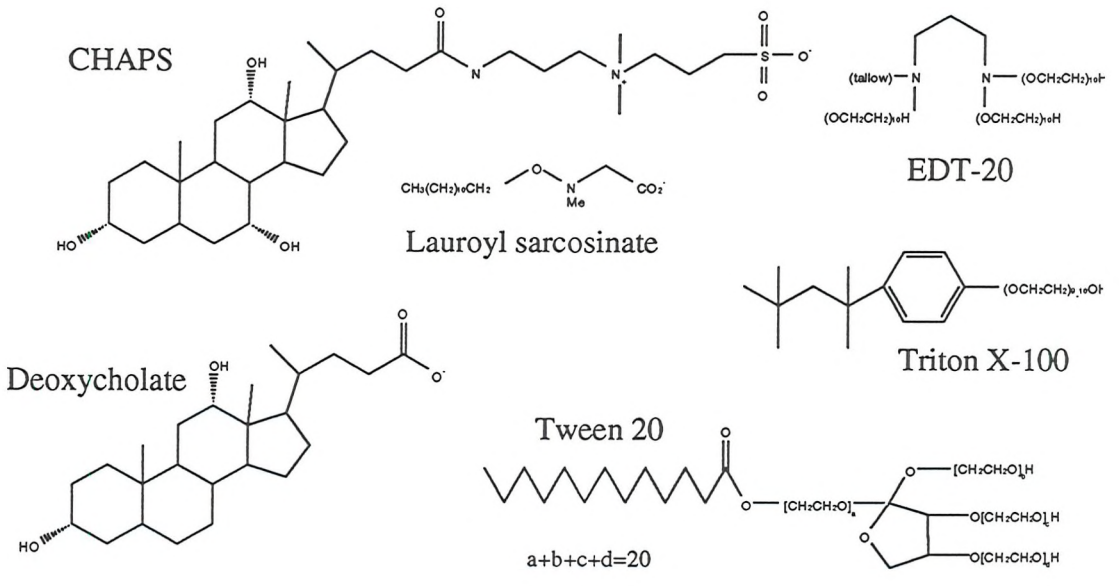


Figure 2.6. Structures and properties of detergents tested for ability to solubilise translocase I activity.

In any solubilisation experiment, the ratio of detergent to lipid is the crucial factor, but since lipid concentration is not easily measured, the detergent to protein ratio is quoted instead.

It must be pointed out that exogenous lipid acceptor is not added to assays of solubilised enzyme preparations. Thus we are reliant on the detergent co-extracting the endogenous lipid acceptor with the enzyme to be able to observe activity. At first sight this seems rather limiting, but in reality a detergent which is to be used in the purification and further study of the enzyme must be able to solubilise both the insoluble enzyme and the insoluble substrate.

All of the detergents listed except Tween 20 extracted comparable amounts of activity. The amount extracted is expressed as a percentage of the activity in the membranes used in each experiment so that the results from the six experiments can easily be compared.

As mentioned in the introduction chapter (section 1.5.3), lauroyl sarcosinate has been used to solubilise translocase I from membranes of *S. aureus* Copenhagen.[106] Heydanek and co-workers tested six detergents for their ability to solubilise translocase I, measuring both exchange and transfer activities (see section 1.5). We have concentrated on the transfer activity because we are primarily interested in studying the complete reaction rather than a partial reaction. In their study with the *S. aureus* enzyme, lauroyl sarcosinate was the best detergent for solubilising the exchange activity (43 % yield), but was less efficient at solubilising the transfer activity (14 % yield). In our study, lauroyl sarcosinate solubilised the transfer activity with a degree of selectivity, with a 100 % yield of activity, but only 24 % of protein being extracted at 0.25 % detergent.

The cationic detergent, EDT-20, was similarly efficient at solubilising the translocase, but little is known about this detergent, its cmc, aggregation number, spectral properties, etc;.

Triton X-100 is a non-ionic polyoxyethylene detergent. There is considerable precedent for its use in solubilisation of membrane proteins catalysing sugar transfer reactions to lipid acceptors (table 2.5). Of the six detergents tested by Heydanek and co-workers[106] deoxycholate, SDS and Triton X-100 (at 0.5, 0.5 and 3.0 μmoles detergent per mg protein) maintained the ratio of exchange to transferase activity (~24:1) upon solubilisation. This indicated that translocase I solubilised under these conditions was functionally similar to the particulate form, a criterion for selection of the most suitable detergent. In our study, Triton X-100 gave the best yield of activity at 0.5 % v/v.

Sodium deoxycholate, like lauroyl sarcosinate, showed a degree of selectivity in solubilising translocase I, giving a good yield (92 %) of activity whilst only extracting 30 % of protein at 1 % w/v. However, after carrying out this experiment, it

was found that deoxycholate is reported to form a gel below pH 8.0 and that its magnesium salt is insoluble[126], although at pH 7.5 no precipitate was observed and the supernatant from the detergent extraction was only viscous rather than gel-like. For these reasons deoxycholate was not investigated further.

Enzyme	Detergent/concentration	Reference
Translocase I (*S. aureus*)	Triton X-100, 0.5/3.0 μmoles/mg protein	Heydanek, 1969[106]
GDP-man:dol-P transferase (rabbit liver)		Schutzbach, 1976[127]
UDPGlcNAc:dol-P transferase (hen oviduct)	0.5 % sodium deoxycholate	Keller, 1979[128]
UDPGlcNAc:dol-P transferase (pig aorta)	0.5 % Nonidet P-40[a]	Kaushal, 1985[129]
UDPGlcNAc:dol-P transferase (soybean)	1 % Triton X-100	Kaushal, 1986[130]
UDPGlucose:dol-P transferase (mung bean)	1.5 % Triton X-100	Drake, 1991[131]

Table 2.5. Nucleotide-sugar: polyprenyl phosphate transferase enzymes solubilised with detergent. [a]Nonidet P-40 is the same as Triton X-100.

CHAPS efficiently solubilised activity at 1.5 % with a yield of activity comparable to that obtained with Triton X-100. Tween 20 gave low yields of activity and so was not investigated further.

Triton X-100 was selected for further studies over the other detergents tested because of its efficiency in solubilising translocase I, and because it is cheap and readily available, it is suitable for all types of chromatography commonly used and because of literature precedent.

2.6.3. Initial characterisation of solubilised translocase I.

The immediate problem beyond that of solubilisation is that of stability. Whilst membrane preparations have been stored at -20 °C for greater than three months without significant loss of activity, the Triton X-100 solubilised preparation lost all activity after freezing and thawing overnight.

Enzyme was held on ice in 100 mM phosphate buffer containing 2 mM ß-mercaptoethanol (to maintain a reducing environment), 1 mM $MgCl_2$ and either no additive, 5 %, 10 % or 20 % glycerol, or 20 % sucrose, 1 mM EDTA, 1 mM EGTA or

1 mM PMSF (phenylmethylsulphonylfluoride - a protease inhibitor). Activity remaining after one week on ice in the control was 60 % and for those samples with additives was 74 %, 78 %, 86 %, 81 %, 77 %, 68 % and 68 % respectively. Therefore 20 % glycerol was included in solubilisation buffers thereafter.

It had been planned to develop an assay based on hydrolysis of product UMP to uridine and inorganic phosphate and measurement of the phosphate using a colorimetric assay. Clearly a phosphate preparative buffer is not compatable with such an assay, so another common buffer, Tris (trishydroxymethylaminomethane) was tested for solubilisation and stability. 50 mM Tris pH 7.5 substituted 100 mM potassium phosphate, pH 7.5. The yields of protein and activity were 70 % and 118 % respectively. After six days on ice, 85 % of the original activity remained. These data are approximately the same as those data obtained with the phosphate buffer. In all subsequent experiments the buffer was Tris.

Enzyme activity versus pH was examined over the range 7.0 - 9.5 (fig. 2.7). The pH optimum is approximately 8.0.

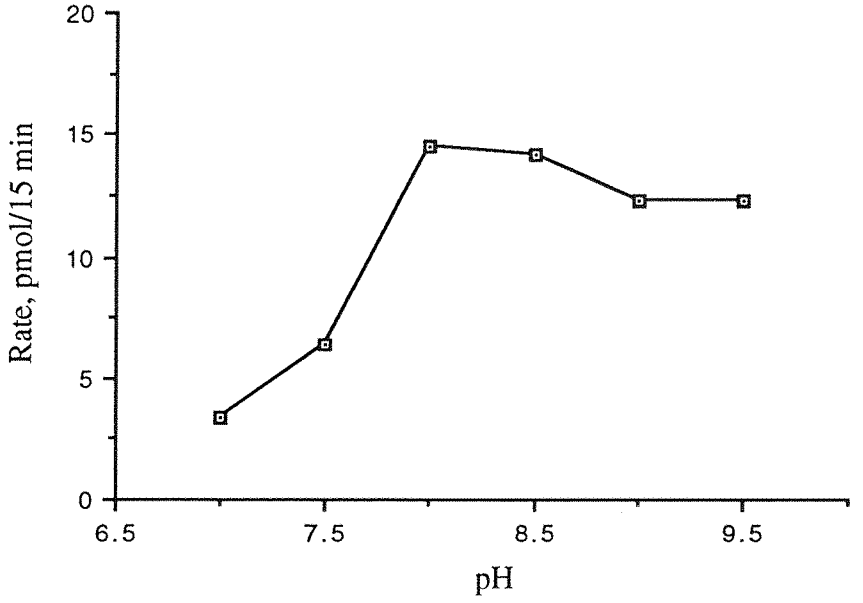


Figure 2.7. pH-rate profile of Triton X-100-solubilised translocase I.

When a protein is particularly robust, heating to 60 °C for a short time (e.g. 15min) can be a useful initial bulk fractionation step, less stable proteins denaturing and precipitating under these conditions.[126] Aliquots of Triton X-100 solubilised enzyme were held on ice, at 37 °C, 50 °C or 60 °C for 15 min and then assayed as described in the experimental chapter. Heat fractionation is not feasable in this case since incubation at 60 °C for 15 min resulted in complete loss of activity (table 2.6).

Incubation temperature, °C	Activity, pmol/15 min at 30 °C
ice	15.2
37	16.0
50	12.3
60	0

Table 2.6. Heat stability of Triton X-100-solubilised translocase I.

2.7. Characterisation of pyrophosphatase activity.

2.7.1. Too much product!

As discussed in chapter 1 (section 1.5.3), Neuhaus' group have demonstrated an activity in *S. aureus* membranes which hydrolysed the phosphodiester bond of UDPMurNAc-pentapeptide. They called this activity a 'pyrophosphatase' activity. In strict terms, the activity is a phosphodiesterase, a pyrophosphatase being an enzyme which catalyses the hydrolysis of inorganic pyrophosphate (PP_i) to inorganic phosphate (P_i). However, for consistency with the work of Neuhaus, the UDPMurNAc-pentapeptide hydrolytic activity will be referred to as 'pyrophosphatase' in this work.

In studying the translocase I reaction an attempt was made to titrate the endogenous undecaprenol phosphate present in the membrane preparations. However, it quickly became apparent that more butanol-extractable product was being formed than was consistent with the amount of undecaprenyl phosphate expected to be present, e.g. fig. 2.8.

Pless and Neuhaus[108] estimated the amount of undecaprenyl phosphate in membranes of *S. aureus* Copenhagen by extended incubation of membrane fragments with [^{14}C]-UDPMurNAc-pentapeptide in the presence of bacterial alkaline phosphatase. Inclusion of this enzyme hydrolyses UMP thus driving the reaction to completion with all the undecaprenyl phosphate being converted to lipid-linked intermediate I. They obtained a value of 2.76 pmol per μg of protein. Whilst no direct comparison can be made because we are studying *E. coli*, not *S. aureus*, the work of Pless and Neuhaus does at least give an idea of the amount of undecaprenyl phosphate likely to be present. Even if *E. coli* membranes contained 25 times more undecaprenyl phosphate per μg of protein, the end point in the above experiment (fig. 2.8) should still only be 1.7 nmoles of butanol-extractable product.

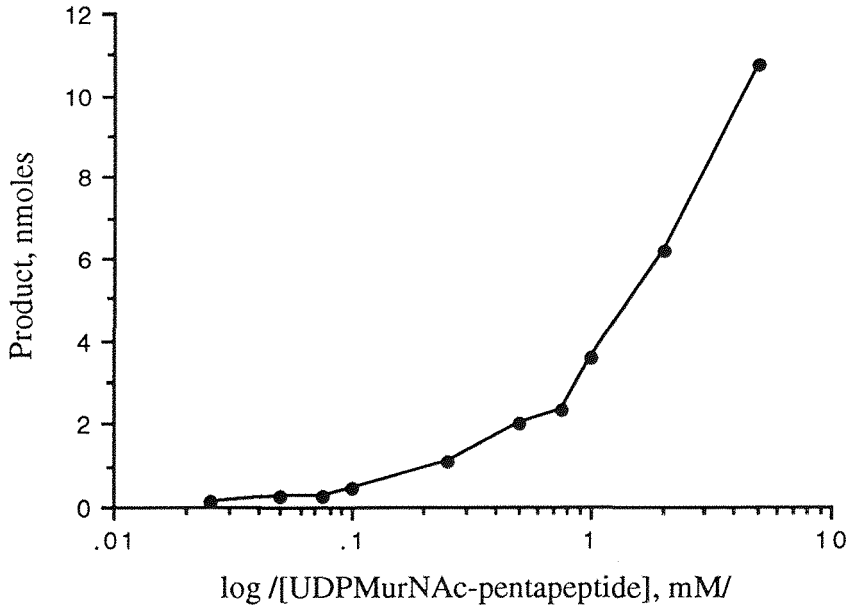


Figure 2.8. Butanol-extractable product after 12 hr incubation at room temperature with varying [UDPMurNAc-pentapeptide]. Assays contained 0.5 mg protein/mL of membrane fragments from *E. coli* JM109 (pBROC512) in a 50 µL volume.

Heydanek *et al;*[107] demonstrated production of 1-phospho-MurNAc-pentapeptide when UDPMurNAc-pentapeptide was incubated with membrane fragments from *S. aureus* (see section 1.5.3). There was a lag in the appearance of this species corresponding to the formation of lipid-linked product. As the authors point out, this suggests that this apparent pyrophosphatase activity associated with the membranes arises either from hydrolysis of lipid-linked product, or from hydrolysis of a putative covalent enzyme-linked intermediate (fig. 2.9), the rate of which increases as production of lipid-linked product slows.

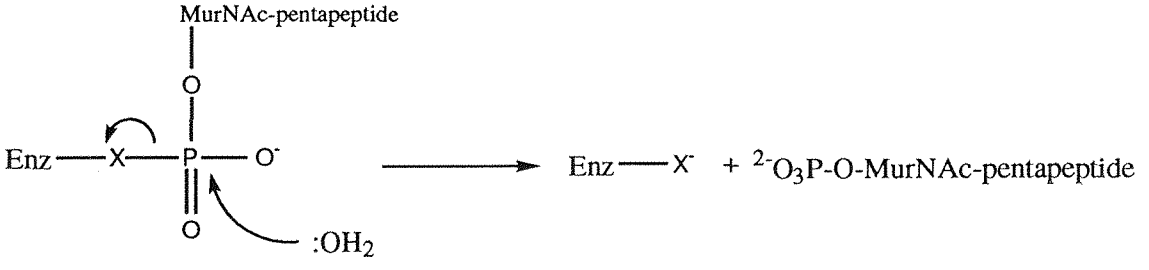


Figure 2.9. Hydrolysis of a putative covalent enzyme-linked intermediate resulting in release of phospho-MurNAc-pentapeptide.

With respect to our experiments, the question is raised - would 1-phospho-MurNAc-pentapeptide extract into butanol after acidification of the reaction mixture with 6M pyridinium acetate pH 4.2? Heydanek *et al;*[107] found that 1-phospho-MurNAc-pentapeptide migrated with $R_f \approx 0.3$ in tlc with solvent 1-butanol/acetic acid/H_2O (2:1:1 v/v). This suggests that this species would be at least partially extracted.

To try to consolidate these data, and to investigate the possibility of a non-radiochemical assay, attention was focussed on the development of the phosphate-release assay mentioned earlier in section 2.6.3.

2.6.2. Phosphate-release assay.

The second product of the reaction catalysed by translocase I is uridine-5'-monophosphate (UMP). UMP can be hydrolysed with commercially available snake venom 5'-nucleotidase releasing inorganic phosphate. Nanomolar (0 - 10 nmol) quantities of inorganic phosphate can be measured using the malachite green phosphate assay.[132] Methodology was developed to allow assay of UMP product (fig. 2.10).

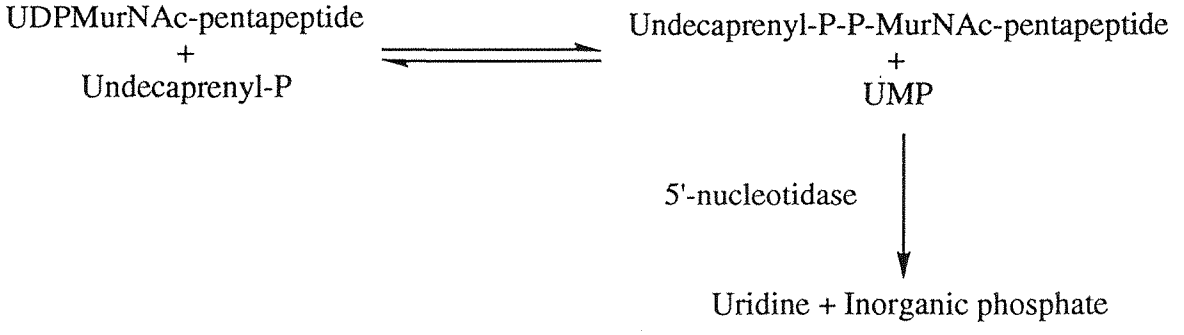


Figure 2.10. Phosphate-release assay.

Soluble 5'-nucleotidase (Sigma) is costly and relatively unstable, with only 1 % of activity remaining after storage for one month at 4 °C. An agarose-immobilised preparation is also available and was found to be stable. This form of the enzyme can also be recycled by sedimentation without significant loss of activity. This was used in the experiments reported below.

Quantitation of UMP was effected by taking a 50 µL aliquot of assay mixture, boiling for 1 min, plunging into an ice-bath and adding the aliquot to a pellet of agarose-immobilised 5'-nucleotidase (0.1 unit). This was vortexed and incubated at 37 °C for 5 min after which the 5'-nucleotidase was pelleted by centrifugation. (Pelleted enzyme was washed with pH 9.0 buffer and resuspended in buffer containing 0.5 M NaCl for storage). To compare the phosphate-release method with

the radiochemical method, an incubation was set up containing particulate enzyme and UDPMurNAc-pentapeptide (4 mM unlabelled, 2.8 μM (120 nCi) radiolabelled) in pH 9.0 buffer at 37 °C. 50 μL aliquots were withdrawn at 0, 1, 2, 4 and 8 hr and quantified by both methods (table 2.7).

Time, hr	Product, nmoles	
	Radiochemical method	**Phosphate-release method**
0	0	0
1	5.0	4.5
2	8.5	4.8
4	12.5	Out of range
8	19.0	Out of range

Table 2.7. Time-course assay of particulate translocase I by the radiochemical and phosphate-release methods. OOR = out of range.

Whilst the agreement between the two methods is only approximate, it does nonetheless confirm the levels of product seen in the radiochemical assays above (section 2.7.1). Two factors led us not to use this assay in future work. Firstly it is more troublesome to carry out than the radiochemical assay without offering any significant advantages. Secondly, it is a measure of UMP, which is not a measure of lipid-linked intermediate I, the physiologically relevant product.

2.7.3. Dissection of activities.

If as suspected particulate enzyme catalyses the fast production of lipid-linked product with subsequent slow hydrolysis of UDPMurNAc-pentapeptide, then the reaction time-course will be biphasic. This seemed to be the case when particulate enzyme was assayed in the absence or presence of 165 μM heptaprenyl phosphate (fig. 2.11), although a firm conclusion obviously cannot be drawn from a two-point time-course!

The point here is that the increase between the first and second time points is approximately the same in both cases, consistent with an increased amount of lipid-linked product due to the extra exogenous lipid substrate, but no change in the rate of UDPMurNAc-pentapeptide hydrolysis.

An alternative work-up for the transfer assay with particulate enzyme is acid precipitation of the lipid-linked product, as described by Hammes and Neuhaus.[109] Any phospho-MurNAc-pentapeptide produced would remain in solution and so would not be counted. Exactly this experiment was carried out with particulate translocase I from *E. coli* JM109 (pBROC512). A time-course assay revealed

approach to an equilibrium, rather than the biphasic production of large amounts of radiolabelled products (fig. 2.12).

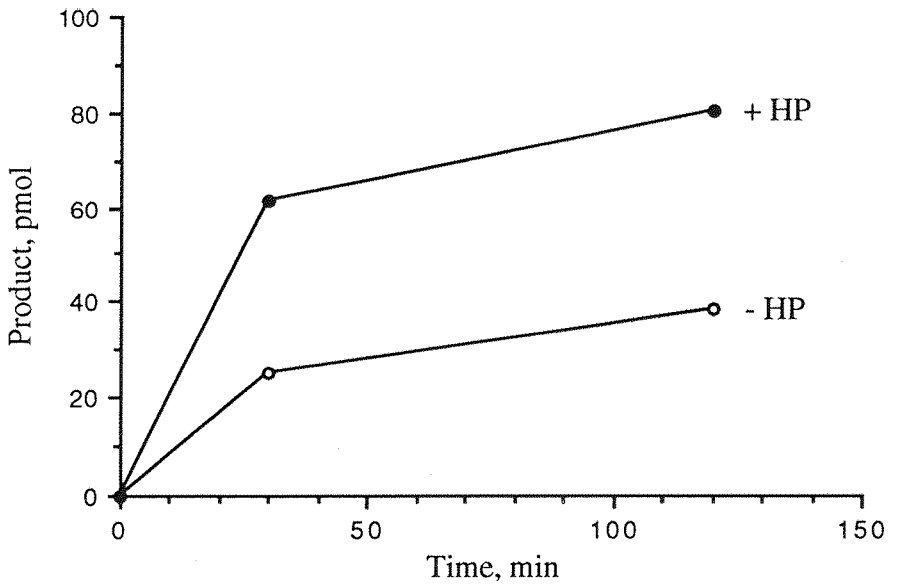


Figure 2.11. Assay of particulate enzyme in the absence or presence of 165 μM heptaprenyl phosphate.

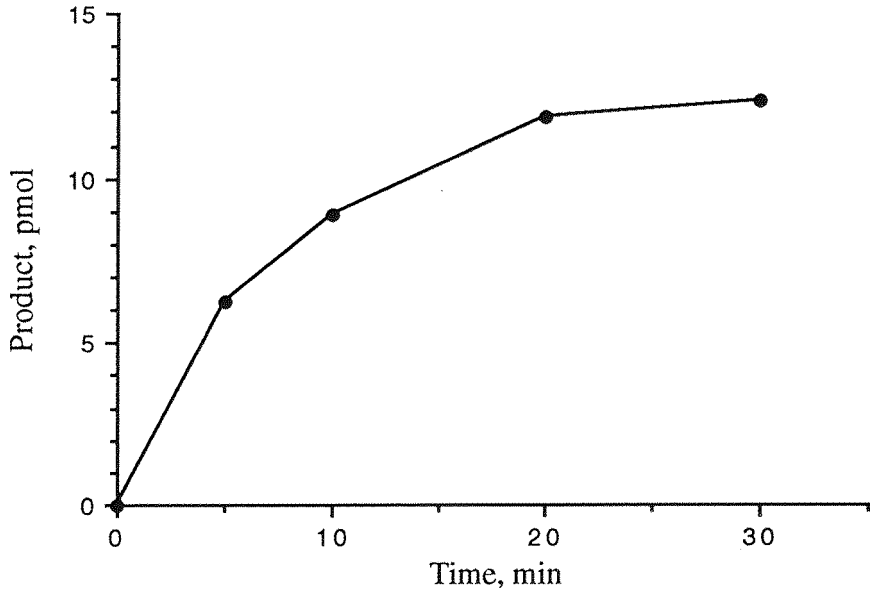


Figure 2.12. Time-course assay of appearance of acid-precipitable products in the assay of *E. coli* JM109 (pBROC512) particulate translocase I. Each reaction (50 μL) contained 700 pmoles (14 μM) UDPMurNAc-pentapeptide.

Thus it seems likely that our preparation contains a 'pyrophosphatase' activity consistent with the observations of Heydanek *et al;*.[107]

2.8. Reconstitution of solubilised enzyme with lipid acceptor.

Initial experiments indicated that, unlike particulate enzyme, Triton X-100 solubilised enzyme could not utilise exogenously added heptaprenyl phosphate to make lipid-linked product. It is possible that phospholipids not sufficiently solubilised by the detergent are required to reconstitute the enzyme with the exogenous lipid acceptor. The three major phospholipids of the *E. coli* inner membrane are phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL, also called diphosphatidylglycerol) (fig. 2.13).[133]

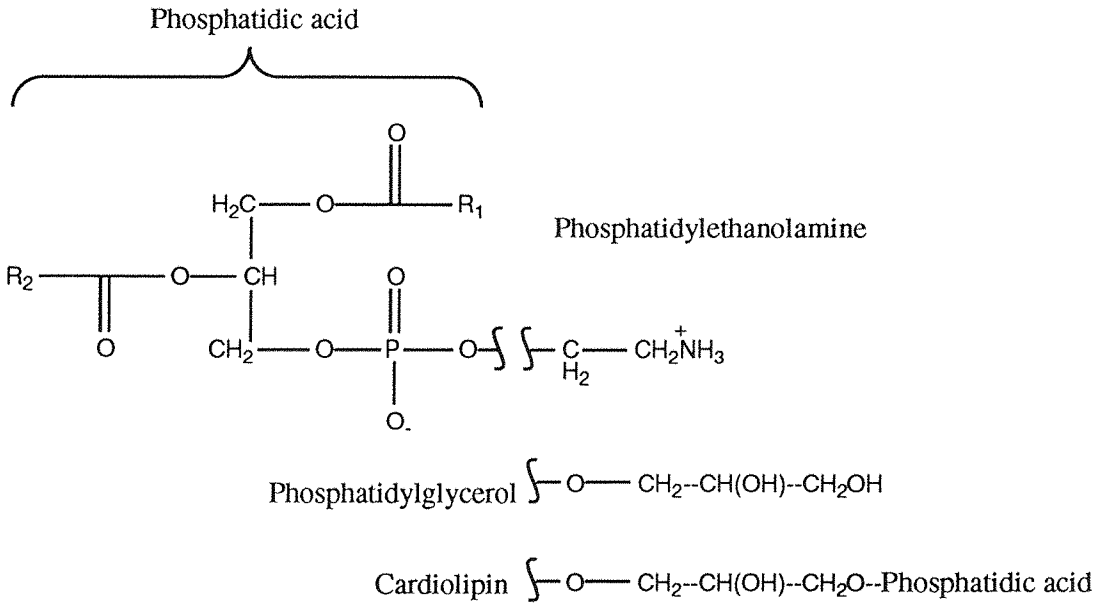


Figure 2.13. Structures of phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL).

PE makes up about 50 % of the *E. coli* inner membrane phospholipid, the remainder being a mixture of PG and CL and traces of other lipids (e.g. phosphatidylserine). The relative amounts of PG and CL depend on growth conditions and phase, PG dominating during logarithmmic growth and CL dominating in the stationary phase.[133] PE is neutral, whilst PG and CL are acidic; PE alone does not form a bilayer structure, rather a micelle, whereas PG forms a vesicle (bilayer structure). Mixtures of these lipids form vesicles with phase transitions (liquid crystalline to fluid state as temperature increases) similar to those of PG. It is quite possible that the relative amounts of the different lipids in the membrane serve to regulate membrane fluidity and hence function.

With this in mind, the effect of a 1:1 (w/w) mixture of PE and PG on the activity of Triton X-100 solubilised translocase I was examined (fig. 2.14).

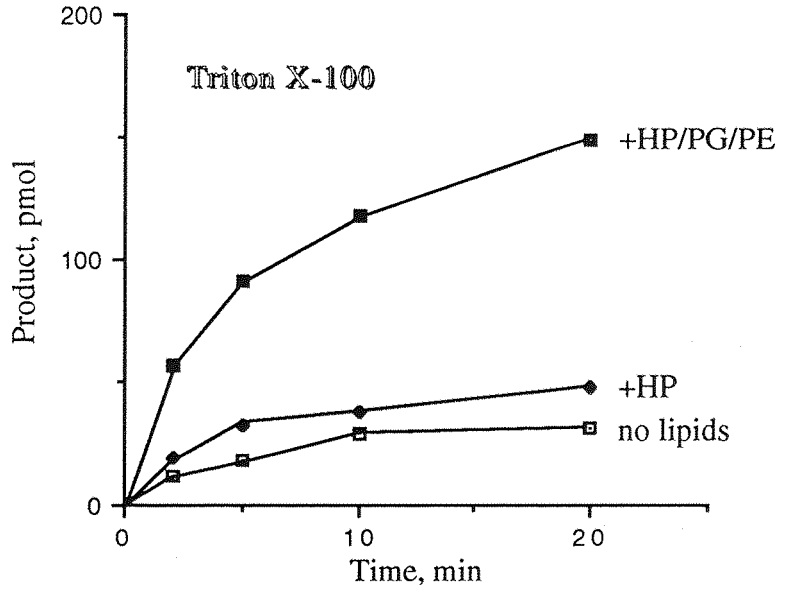


Figure 2.14. Effect of phospholipids on Triton X-100 solubilised translocase I from *E. coli* JM109 pBROC525. HP = heptaprenyl phosphate, PE = phosphatidylethanolamine, PG = phosphatidylglycerol. The assay concentrations of lipids were HP, 5.7 µg/mL (9.4 µM), PE and PG, 28.5 µg/mL each.

There was a clear enhancement of activity when PE and PG were included in the reaction mixture. It is not clear whether the activity seen in the absence of added HP or phospholipids is formation of lipid-linked intermediate I or the pyrophosphatase activity discussed in section 2.7. If the former is the case then it seems that the role of the phospholipids is delivery of the HP to the enzyme, rather than mediating the interaction between enzyme and lipid acceptor.

It was of interest to examine whether the same effect would be seen if translocase I was solubilised using a different detergent. The detergent used in this experiment was CHAPS (fig. 2.15) because its properties (CMC, aggregation number, structure, charge, etc;) are radically different to those of Triton X-100. Indeed, the lipid dependence seen with Triton X-100 is not so apparent with CHAPS (fig. 2.15).

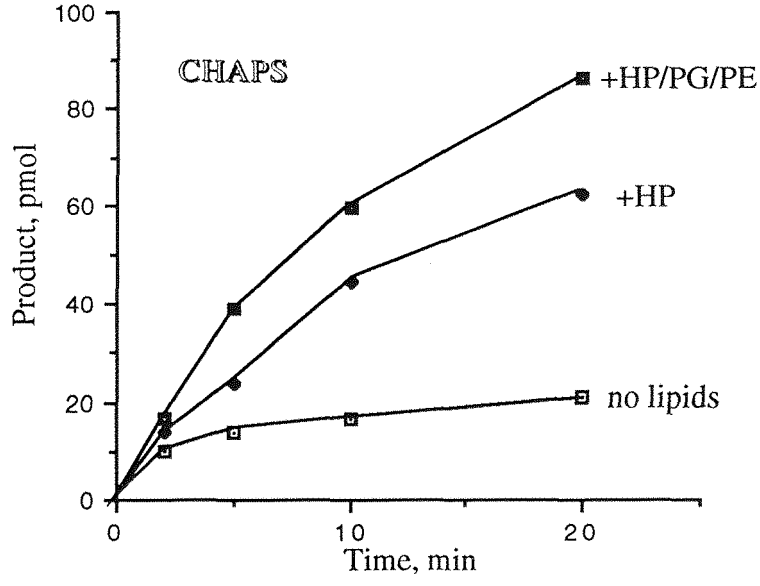


Figure 2.15. Effect of phospholipids on CHAPS solubilised translocase I from *E. coli* JM109 pBROC525. HP = heptaprenyl phosphate, PE = phosphatidylethanolamine, PG = phosphatidylglycerol. The assay concentrations of lipids were HP, 5.7 μg/mL (9.4 μM), PE and PG, 28.5 μg/mL each.

Thus it would appear that CHAPS is able to fulfil some delivery or interactive role that Triton X-100 cannot.

Lipid dependence was examined again after development of the continuous assay methodology described in chapter 4 (table 2.8).

Lipids added	**Rate, u/min**
None	0.02
HP, 4.6 μM	0.07
HP, 4.6 μM + PE, 10 μg/mL	0.13
HP, 4.6 μM + PG, 10 μg/mL	0.85
HP, 4.6 μM + PE, 10 μg/mL + PG, 10 μg/mL	0.45

Table 2.8. Effect of individual lipids on translocase I activity. Assays were by the fluorescence enhancement method as described in the experimental chapter. HP = heptaprenyl phosphate, PE = phosphatidylethanolamine, PG = phosphatidylglycerol.

The activation by phosphatidylglycerol in the fluorescence enhancement assay has been observed to be 4 - 11-fold. Thus it seems that this acidic phospholipid is responsible for the activiation, with PE having a slight inhibitory effect.

Comparable results were obtained by Kaushal and Elbein in their study of pig aorta GlcNAc-1-phosphate transferase, a member of the phospho-aminosugar transferase superfamily proposed in chapter 6.[129] They found that enzyme solubilised with Nonidet P-40 (identical to Triton X-100) was stimulated 5-fold by 100 μg/mL PG (optimal concentration), but was inhibited by >50 % by the same concentration of PE. The procedure used in their work and ours to introduce the lipids to the assay is to evaporate solvent from a mixture of the lipids in organic solvent and to disperse the residue with aqueous detergent. Thus co-solubilisation of the lipid acceptor (dolichyl phosphate in the case of Kaushal's work) with the phospholipids is a plausable explanation for the observed activation.

Pless and Neuhaus examined the effect of individual phospholipids on the exchange activity (see section 1.6.1) of translocase I from *S. aureus* after solubilisation with Triton X-100 and fractionation using DEAE-cellulose.[108] Exchange activity recovered after DEAE-cellulose chromatography was <2 % of that applied to the column. Phosphatidylglycerol at 75 μM activated exchange activity to approximately 15 % of that applied to the column. Only a total lipid extract prepared from *S. aureus* (containing undecaprenyl phosphate) could completely reactivate the exchange activity of the enzyme. It is difficult to compare these results with our study since Pless did not investigate the transferase activity of the solubilised enzyme with respect to phospholipids.

Since the goal of this part of the project was to purify the enzyme, it was decided to pursue this aspect rather than further characterise the crude extract.

2.9. Summary.

Translocase I from *E. coli* has been overexpressed using two expression vectors, pBROC512 and pBROC525, the latter giving the greater yield of activity (approximately 30-fold over wildtype), with specific activity 0.5 nmol/min/mg protein at 25 °C and pH 9.0. The enzyme preparation appeared to contain a pyrophosphatase activity consistent with previous studies.

Activity has been solubilised with several detergents, including Triton X-100. The enzyme solubilised with Triton X-100 is activated 4 - 11-fold by phosphatidylglycerol, but not by phosphatidylethanolamine. The specific activity was 1.30 nmol/min/mg protein at 25 °C and pH 8.5 .

Chapter 3. Efforts towards purification of translocase I.

3.1. Introduction.

Central to the study of any protein is partial or complete purification of that protein in sufficient quantities for the planned experiments. Four major reasons for this are (i) many proteins are greatly stabilised by purification (e.g. by removal of proteases), (ii) a pure source allows experiments to be carried out under well defined conditions, (iii) removal of contaminating activities which may make results awkward to interpret and (iv) to permit stoichiometric active-site studies. Fortunately, in the case of translocase I contaminating activities are unlikely to complicate matters other than D-ala-D-ala ligase (which could degrade UDPMurNAc-pentapeptide since the reaction catalysed by this enzme is reversible) and undecaprenyl phosphate phosphatase (which could hydrolyse the lipid substrate). D-ala-D-ala ligase is a very soluble enzyme which will be removed during the preparation of membrane fragments. UP phosphatase activity has been demonstrated in *S. aureus* , but not in *E. coli*.[39]

This chapter is dedicated to experiments directed towards the purification of translocase I. In short, purification of fully active enzyme beyond crude solubilised extracts was not achieved. However, a considerable amount was learned about translocase I in the process of trying. Since future workers will doubtless attempt, and hopefully succeed, in this endeavour, the purpose of this chapter is to document what has been discovered so far.

There are two basic methods for the fractionation of proteins; bulk methods such as precipitation, and chromatography, either columnwise or batchwise. The most common bulk method is precipitation by high concentrations of ammonium sulphate, but this is unsuitable for integral membrane proteins since it can effect phase separation of the detergent used to maintain the protein in solution. Other methods such as precipitation with polyethylene glycol, extraction/precipitation with organic solvents, or non-denaturing electrophoretic methods are available, but again were not investigated.

Chromatography can be subdivided according to the type of interaction with the resin - ion exchange, hydrophobic interaction, gel filtration and affinity (taking advantage of biological specificity). Efforts have been concentrated on such chromatographic methods. The requirement for detergents in buffer solutions to maintain translocase I in solution adds complications, different detergents having

different properties and thus affecting chromatographic steps in different ways. These factors will be discussed in the relevant sections.

Since this chapter is intended as a record and guide for future experimenters, the results are arranged by chromatographic type, rather than as a step by step historical account.

3.2. Ion exchange chromatography (IEC).

The isoelectric point, pI, of a protein is the pH at which the net charge of the protein is zero. Above its pI, a protein will have a net negative charge and can be adsorbed to a positively charged resin; below its pI, a protein will have a net positive charge and can be adsorbed to a negatively charged resin. Whether or not a protein will be retained by an ion exchange resin at a given pH is determined by a variety of factors (other than the pI of the protein) including temperature, buffer ionic strength, choice of detergent, etc; Four ion exchange resins were investigated as described below.

Enzyme solubilised in Triton X-100 at pH 7.5 did not bind to DEAE-sephadex (diethylaminoethyl-sephadex, anion exchange), 52 % of the applied activity appearing in the buffer wash fraction. Naively, a high ionic strength buffer, 100 mM phosphate, was used. This may have prevented efficient adsorption. Indeed, when enzyme solubilised in CHAPS at pH 8.2 with 50 mM Tris as buffer, only 20 % of the applied activity appeared in the flow-through and buffer wash fractions, whilst 60 % of the applied activity eluted when the column was washed with buffer containing 330 mM NaCl (table 3.1).

Fraction	Volume assayed, µL	Rate, pmol/min	Fraction volume, mL	Total activity, pmol/min	Specific activity, pmol/min/mg
Applied	5	4.1	1.0	820	303
Flow thro: and wash	5	0.19	5.1	194	112
Salt wash	5	0.68	3.1	422	296

Table 3.1. Fractionation of CHAPS-solubilised translocase I with DEAE-sephadex.

Even though there was a slight decrease in specific activity from the applied enzyme to the eluted enzyme (330 and 296 pmol/min/mg protein at 37 °C), a purification could be achieved with the use of a gradient and collection of fractions.

However, the above experiment was only performed on a trial scale: exploration on a large scale was prohibited by the high cost of CHAPS detergent.

Therefore anion exchange was again investigated using Triton X-100. This time a resin with a larger pore size, DEAE-sepharose, was used in case the large Triton X-100 micelles (aggregation number = 140, micelle M_r = 88 kDa) were interfering with adsorption. In a larger scale experiment carried out at 4 °C, enzyme was solubilised with Triton X-100 and diluted with buffer at pH 7.5 such that the final mixture contained 50 mM NaCl and 0.1 % Triton X-100. It has been suggested that inclusion of salt in the applicate buffer can improve purification by preventing initial adsorption of some protein. [126] The column was washed with the same buffer and enzyme eluted with a linear gradient of 50 - 600 mM NaCl (table 3.2).

Fraction no.	Total activity, units/min	Protein, mg
Crude extract	83.3	3.2
Flow through	1.7	-
Wash	5.9	-
1	0.4	<0.25
2	0.8	<0.25
3	1.1	<0.25
4	3.7	0.55
5	0	0.77
6	0	<0.25
7	0	<0.25
8	0	<0.25
9	0	<0.25

Table 3.2. Elution of translocase I from DEAE-sepharose. Activity was determined using the fluorescence enhancement assay described in chapter 4.

Some (9 %) of the applied activity appeared in the flow through and wash fractions. This was attributed to the reduction in temperature from ambient where trial scale experiments were carried out, to 4 °C where this experiment was performed. In the next experiment NaCl concentration was reduced to 30 mM. Activity no longer appeared in the flow through or wash fractions. The most active fraction is fraction 4 (approximately 100 - 150 mM NaCl). Fraction 5 contains the most protein but no translocase I activity indicating a degree of purification. But the amount of activity eluted is only a fraction of what should have been presented had activity been retained. The specific activity of fraction 4 was 0.6 units/min/mg protein compared to

26 units/min/mg protein (this measure of activity is described fully in chapter 4) for the applied enzyme suggesting that the enzyme was present but inactive. This suggest that either the enzyme has been separated from some stimulatory factor such as another protein, co-factor or phospholipid, or that the adsorption results in a loss of structural integrity (i.e. partial denaturation) with concommitant loss of activity.

A similar effect was observed by Kaushal and Elbein when purifying UDPGlcNAc-1-phosphate transferase (GPT) on a column of DEAE-cellulose.[129] GPT is a eukaryotic membrane protein involved in glycoprotein biosynthesis. It catalyses a reaction similar to that of translocase I (fig. 3.1).

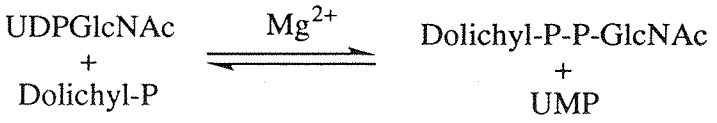


Figure 3.1. Reaction catalysed by UDPGlcNAc-1-phosphate transferase.

In that case, a stimulatory factor was eluted from the column by a higher salt concentration than the enzyme. The factor stimulated the partially purified enzyme five-fold. There was a basal activity in the absence of factor, indicating that either not all of the factor had been separated from the enzyme during chromatography, or that it was not an absolute requirement. The authors make no mention of this point. Whilst the factor was not identified, it was shown to be non-dialysable, not extractable with 1:1 $CHCl_3$/MeOH (i.e. not a lipid), neither protease nor RNAase sensitive, neither labile to heat nor weak acid (but was to strong acid) and was precipitated by 10 % trichloroacetic acid.

The DEAE-sepharose column experiment was repeated, this time with only 30 mM NaCl in the applicate buffer. Again fraction 4 contained the most activity, 3 % of that applied. This fraction was mixed in a 1:1 ratio with each of the other column fractions (crude enzyme, flow through, wash and fractions 1 - 15, a complete 30 - 500 mM NaCl gradient). In no case was activity greater than that expected from the sum of the two individual activities indicating that if a 'factor' had been separated from translocase I during chromatography, it could not simply be mixed back with enzyme to regenerate activity. It is possible as mentioned above that there is no 'factor', just that translocase I is inactivated during adsorptive chromatograhy. Indeed the enzyme can be desalted on small (3 mL) columns of sephadex-G10 without significant loss of activity.

Cation exchange at lower pH was also investigated. Enzyme solubilised with Triton X-100 at pH 7.5 was exchanged into a 10 mM MOPS buffer at pH 6.5 using a 3 mL column of sephadex-G10 equilibrated with the appropriate buffer as described

in the experimental section. This was applied to a small column of carboxymethyl(CM)-sephadex or cellulose-phosphate. Activity was recovered in the flow through and wash fractions with CM-sephadex. No activity was recovered from the cellulose-phosphate column. In a similar experiment with cellulose-phosphate where the detergent was CHAPS, 93 % of applied activity was recovered in the combined flow through and wash fractions. Cation exchange was not pursued further.

3.3. Hydrophobic interaction chromatography (HIC).

This type of chromatography takes advantage of differences in hydrophobicity of proteins. It relies on the associative interaction of protein hydrophobic groups or areas with an immobilised ligand (e.g. phenyl-, pentyl- or octyl-). The strength of this association is increased in the presence of water structure-forming salts (commonly called 'salting out' salts) such as ammonium sulphate. An excellent practical guide to this type of chromatography is available from Pharmacia, 'HIC - Principles and Methods'.

Although membrane proteins are by their very nature hydrophobic, it was hoped that some fractionation of translocase I might be achieved using the HIC resin phenyl-agarose. Enzyme was solubilised with Triton X-100, $(NH_4)_2SO_4$ added to 0.5 M, and 2 mL applied to a 1 mL column of phenyl-agarose in buffer also containing 0.5 M $(NH_4)_2SO_4$. No separation of detergent was observed at 0.5 M $(NH_4)_2SO_4$ (higher concentrations of this salt will cause phase separation). The column was washed with 2 mL of buffer followed by 1 mL of each of 0.4 M, 0.3 M, 0.2 M, 0.1 M and 2 mL of 0 M $(NH_4)_2SO_4$, and fractions assayed for activity (table 3.3).

Fraction	Volume, mL	Total activity, pmol/min	Specific activity[a]
Applied enzyme	2	387	60.5
Applied + 0.5 M $(NH_4)_2SO_4$	2	157	nd
Flow thro: + wash	4	18.2	nd
0.4 M	1	5.5	nd
0.3 M	1	7.3	nd
0.2 M	1	12.4	nd
0.1 M	1	23.1	55.0
0 M - 1	1	31.3	84.6
0 M - 2	1	36.5	91.3

Table 3.3. Elution of translocase I activity from phenyl-agarose. [a]pmol/min/mg protein at 37 °C; no lipids added.

Fractions were diluted tenfold in assay mixtures meaning that the highest concentration of $(NH_4)_2SO_4$ was 50 mM. As seen by comparing the assays of crude enzyme and enzyme containing 0.5 M $(NH_4)_2SO_4$, the presence of the salt reduced the observed activity by 60 %. Thus there was greater than 10 % of the applied activity in the combined flow through and wash fractions. Either the column was overloaded or the sample was applied too quickly. Also it would appear that the activity had not finished eluting from the column. Whilst there is a slight purification, the recovery of activity was low in this experiment (17.5 % in the no salt fractions). However, in conclusion, it seems that phenyl-agarose would be worth including in a future purification scheme.

3.4. Affinity chromatography.

3.4.1. Introduction to affinity chromatography.

This type of chromatography makes use of the biological specificities of proteins, the extreme example being where a monoclonal antibody is immobilised on a support matrix and used to purify a protein. Affinity chromatography has several advantages over conventional techniques. The first is obvious; the high specificity of the matrix for the protein of interest increases the purification factor. Consequences of this are that a smaller bed volume can be used, allowing elution of protein in a small volume (meaning that the step can be used a second time at the end of the purification protocol to concentrate the protein which is otherwise difficult to do with membrane proteins) and that the time taken to complete the step is reduced. A good affinity purification step might reduce the time taken to purify a protein from 5 columns over 5 -6 days to perhaps 2 columns in the same day. This is certain to increase the yield of protein. As for HIC, a useful publication on principles and methods in affinity chromatography is available from Pharmacia.

Many affinity resins are commercially available, but activiated matrices can also be purchased to allow preparation of custom media by the individual. As outlined below, several commercial and custom resins have been investigated.

3.4.2. Commercial resins.

There is precedent for the use of reactive dye-linked resins in the successful purification of enzymes whose substrates contain nucleotides, presumably due to the structural resemblence of the dye to a nucleotide cofactor or substrate. [134-136] Five

such resins, namely cibacron blue 3GA-agarose, reactive blue 4-agarose, reactive brown 10-agarose, reactive green 19-agarose, reactive red 120-agarose and reactive yellow 86-agarose (purchased as a kit from Sigma), were investigated on a trial scale. Triton X-100 solubilised enzyme was incubated batchwise with each resin for 1 hr at 4 °C and then poured into pasteur pipettes plugged with cotton wool. The flow through was collected and each column washed with a further 2 mL of buffer. Only in the case of reactive yellow 86-agarose did activity (31 % of that applied) appear in the flow through/wash fraction. Either the resins have inactivated the enzyme or it has bound to five of six resins. These resins remain to be investigated further. Reactive blue-3GA-agarose and reactive red-agarose were selected for further investigation. Again Triton X-100 solubilised enzyme was applied to the resins batchwise. Columns were poured and washed successively with buffer, 0.2 M and 0.4 M NaCl and 10 mM UMP. No activity was found in any fraction. Since it is very unlikely that the enzyme would remain bound under the elution conditions used, the conclusion from these experiments is that the process has inactivated the enzyme, possibly by the same mechanism as the loss of activity during DEAE-sepharose chromatography.

Also on the basis of the enzyme's substrate containing UMP/UDP, the resin UDP-agarose (attached through the ribose hydroxyl groups) was tested. With this resin, enzyme from *E. coli* JM109 pBROC512 solubilised with 0.5 % Triton X-100 was applied to a 2 mL column and was washed successively with buffer, and then with buffer containing 0.5 M or 1.0 M NaCl. Activity appeared in the wash fraction only (71 %).

3.4.3. Custom prepared resins.

The enzyme binds its substrates, UDPMurNAc-pentapeptide and undecaprenyl phosphate reversibly, and is inhibited by the antibiotic tunicamycin. It was supposed that if one of these molecules were immobilised on an inert matrix, that translocase I might bind reversibly to that matrix.

The resin used was epoxy-activated agarose which provides a 12 atom spacer when the ligand is attached (fig. 3.2).

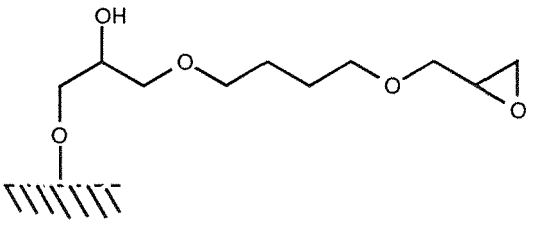


Figure 3.2. Epoxy-activated agarose.

UDPMurNAc-pentapeptide was coupled to the resin in carbonate buffer at pH 9.0 as described in the experimental chapter. Activated sites were present in approximately 2-fold molar excess over UDPMurNAc-pentapeptide and approximately 20 % of the UDPMurNAc-pentapeptide was judged to have coupled to the resin from measurement of nucleotide absorbance before and after the coupling reaction. Unreacted coupling sites were blocked with methylamine at pH 9.0. The most nucleophilic group of UDPMurNAc-pentapeptide is the primary amine of the *m*-DAP residue in the peptide part of the molecule. This is the most probable site of attachment (fig. 3.3).

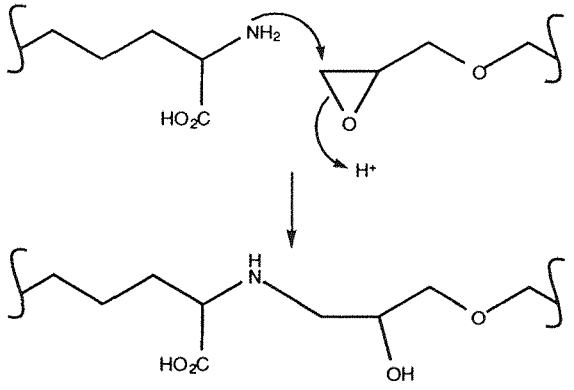


Figure 3.3. Possible coupling reaction of UDPMurNAc-pentapeptide *m*-DAP side chain with epoxy-activated agarose.

It was hoped that attachment in this part of the molecule would allow the enzyme still to bind.

Enzyme was solubilised in 1 % Triton X-100 and applied to the 2 mL column of UDPMurNAc-pentapeptide-agarose under gravity, washed with 2 mL of 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol, 0.1 % Triton X-100 and 3 mL of the same buffer containing 1M NaCl. Activity (>100 %) was recovered in the combined flow through and wash fractions. No activity was found in the desalted salt wash fraction. Thus the resin did not bind translocase I under these conditions.

A similar experiment involved tunicamycin-agarose. The activated sites were in approximately 5-fold molar excess over tunicamycin (of which 2 mg, 2 µmoles was used). Tunicamycin (fig.3.4) does not contain any primary amines, but does contain a number of hydroxyl groups. In this case the coupling reaction was carried out at pH 10.0

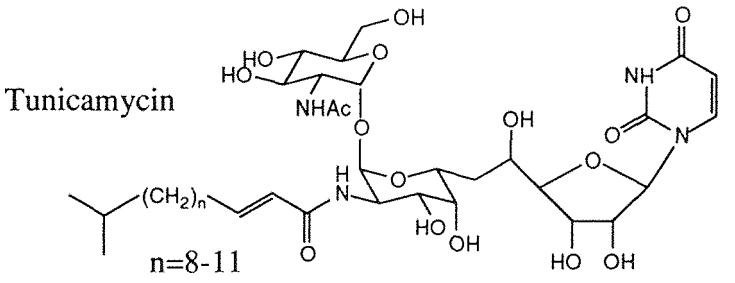


Figure 3.4. The structure of tunicamycin.

The tunicamycin was judged to have efficiently coupled to the resin, since tunicamycin could not be detected in the washings after coupling (by tlc/UV illumination). Unreacted sites were blocked with methylamine.

As with UDPMurNAc-pentapeptide-agarose, enzyme solubilised with Triton X-100 did not bind to this resin, with 107 % of the applied activity appearing in the flow through and wash fractions.

It has been reported in the literature that enzymes which use undecaprenyl- or dolichyl phosphate as substrates exhibit a specificity for alkyl chain length.[123, 124] This indicates that these enzymes and maybe translocase I recognise and bind parts of the prenyl phosphate molecule remote from the phosphate end. Thus it was supposed that C_{55}-prenol attached to an inert support matrix might act as a hydrophobic interaction resin with a certain specificity for translocase I.

Polyprenol-agarose was prepared by coupling of a crude mixture of prenols (mainly C_{50} and C_{55} homologues) with epoxy-activated agarose in 50/50 water/DMF with 1 equivalent of NaOH as described in the experimental chapter. Unreacted sites were blocked with methylamine. The amount of prenol coupled could not adequately be quantified, but in theory only a very small amount of ligand needs to be attached ot the resin if the interaction with the protein is suitably specific. Column experiments were performed on the assumption that sufficient prenol had coupled.

The detergent selected for these experiments was CHAPS rather than Triton X-100 because earlier experiments (presented in section 2.8) had indicated that enzyme solubilised in Triton X-100 could not interact properly with exogenous lipid substrate, requiring activation with phospholipids whereas enzyme solubilised in CHAPS could. Enzyme solubilised in CHAPS was incubated with the resin overnight at 4 °C; no ammonium sulphate was included as is the general case in HIC to avoid promotion of non-specific binding. The resin was poured into a column (plugged syringe, final bed volume, 2 mL) and washed with 5 mL buffer. The combined flow-through and wash fraction contained only 4 % of the activity applied, but apparently contained all of the protein applied (measured 2.8 mg applied, 3.2 mg recovered). Assuming that incubation with the resin did not inactivate the enzyme (a reasonable

one since no inactivation was seen in the other experiments with epoxy-activated agarose derived resins), the matrix has retained almost all of the translocase I, but apparently very little of the protein. If active enzyme could be eluted then this would provide a considerable purification.

Activity was not eluted by buffer containing either 10 % ethanol or 32 μM (20 μg/mL) heptaprenyl phosphate. In the process of doing these two experiments it was found that activity was also adsorbed to the column when the sample was applied columnwise under gravity, i.e. the overnight batchwise adsorption step was not necessary. It was decided to discontinue these experiments until the matter of loss of activity following adsorptive chromatography as described in section 3.2 had been resolved.

A possibility for the future is that enzyme may be eluted by a change of detergent. For example, Lee and co-workers purified a diacylglycerol lipase on octyl-sepharose. The protein was adsorbed in a buffer containing 5 mM (0.3 %) CHAPS and eluted in a 0 - 0.3 % gradient of Triton X-100.[137]

3.5. Gel filtration chromatography.

Gel filtration is not an adsorptive method. Gel filtration separates proteins on the basis of molecular size, which, to a reasonable approximation, means their molecular weight. The gel itself is made up of small (1 - 100 μM diameter) porous beads. The holes in the beads are of a defined size which is characterised by the exclusion limit of that resin. The exclusion limit is given by the manufacturer as a molecular weight. Proteins (or complexes of protein and detergent) above that molecular weight cannot enter the beads; they are excluded and elute at the solvent front. Proteins below that molecular weight will enter the beads to varying degrees and will be fractionated accordingly since they effectively have to pass through a larger column. A lower limit is also specified by the manufacturer below which solutes move equally slowly through the column.

Whilst gel filtration has not been investigated as a means of purification *per se*, experiments with mureidomycin A described in chapter 4 revealed that translocase I was stabilised by passage through a column of sephadex G75 (exclusion limit, 80 kDa). Translocase I solubilised with Triton X-100 elutes at or near the solvent front. The yield of activity was also routinely 150 - 200 % of that applied (possibly due to the removal of inhibitory factors).

It was considered that the earlier experiment with UDP-agarose was probably unsuccessful because of the mode of attachment to the resin (i.e. through the ribose hydroxyl groups). Also translocase I may have required more than 1 mM $MgCl_2$ in the column buffer to bind efficiently to the resin. To investigate this possibility, another resin, UDP-hexanolamine-agarose (attached to the matrix via the

hexanolamine group, fig. 3.5) was tested in conjunction with the above gel filtration step.

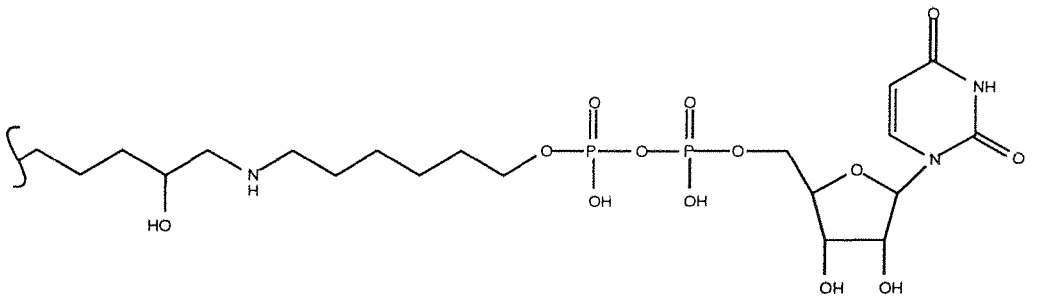


Figure 3.5. UDP-Hexanolamine-agarose.

In this experiment, 2 mL of enzyme solubilised in 1 % Triton X-100 as described in the experimental chapter was applied to the G75 column (30 mL) and eluted over 4 mL. The yield of activity was 193 %. The two active fractions were pooled and $MgCl_2$ added to 25 mM and applied to a 2 mL column of UDP-hexanolamine-agarose equilibrated with buffer containing 25 mM $MgCl_2$. The column was washed with 2 mL of the same buffer followed by two 2 mL aliquots of buffer without $MgCl_2$ containing 25 mM EDTA and 10 mM UMP. These two fractions were dialysed against 200 volumes of buffer containing 5 mM $MgCl_2$ to remove EDTA and UMP (other experiments described in chapter 4 had shown that the enzyme was stable to this treatment). They were assayed for activity along with the flow through and wash fractions. The flow through and wash fractions contained 50 % of the applied activity. There was no activity in either EDTA/UMP fraction. Thus UDP-hexanolamine-agarose is not a suitable resin for purification of translocase I under the conditions described.

3.6. Hexahistidine fusion protein.

3.6.1. Introduction.

An increasingly common application of molecular biology to protein biochemistry is the construction of fusion proteins. Rather than the purification protocol being tailored to the protein, the protein is tailored to the purification protocol. DNA coding for another protein or peptide is added to the 5' or 3' end of the gene coding for the protein of interest in a suitable expression vector. Thus the expressed proteins contain either an additional N- or C-terminal peptide or protein. The fusion protein can then be purified by a previously established procedure for the fusion partner.

Several expression vectors designed exactly this application are commercially available. Fusion partners include dihydrofolate reductase (DHFR) which allows purification on methotrexate-sepharose, glutathione-S-transferase (GST) which allows purification on glutathione-sepharose, and a peptide containing a polyhistidine motif which allows purification on a metal-chelating column (often with Ni^{2+}). These commercially available constructs also include in the fusion partner a protease cleavage site to allow removal of the fusion partner after purification, such as factor X or enterokinase.

3.6.2. Construction of pPB1.

When molecular biology facilities were made available to us by our collaborators SB Pharmaceuticals, it was decided to attempt expression/purification of a translocase I fusion protein for several reasons. At the time, the purification of translocase I by conventional methods was proving difficult, since activity was lost during purification by adsorptive techniques. Use of a fusion partner to purify translocase I would not necessarily rely on a functional assay of the catalytic activity to locate the protein, i.e. purification in one step and localisation by UV-absorbance and SDS-PAGE. Existing expression systems had given only modest levels of expression of translocase I (see chapter 2), thus we were keen to investigate alternative expression systems (most of the available fusion constructs carry the T7 ϕ10 promoter rather than an *E. coli* RNA polymerase directed promoter; this is described fully below). At the same time, a series of mutant translocase I enzymes were in preparation (see chapter 6). Expression of a translocase I fusion protein would allow differential purification of the mutants from contaminating chromosomal, wild-type enzyme.

Since DHFR and GST are highly soluble proteins it was considered that their bulk might interfere with the successful membrane insertion of translocase I, so the small polyhistidine-type fusion partner (<5 kDa) was selected. After this work was undertaken, Peters and co-workers reported the successful non-denaturing purification of a his-tagged membrane protein, the *S. aureus* lactose specific integral membrane protein of the phosphoenolpyruvate-dependent phosphotransferase system.[138]

The next consideration was which end of translocase I to attach the fusion partner to, C-terminus or N-terminus. Evidence is presented in chapter 6 (section 6.5) that the N-terminus of translocase I is exposed to the cytoplasm, with a possible α-helical structure between it and the membrane, whilst the C-terminus is at the periplasmic face of the membrane. Thus if the fusion peptide were attached to the N-terminus it would not have to cross the bilayer, giving a better chance of insertion into the membrane.

The source of the *mra*Y gene for construction of this vector was to be SB Pharmaceuticals plasmid pBROC511. This was constructed by Dr. M.Burnham by excision of a 1.3 kbp *mlu* I/*mlu* I fragment containing the *mra*Y gene from pBROC508 (pUC19 carrying an 8.5 kbp *Kpn* I/*Kpn* I fragment from Kohara phage 110^{125}), blunt-ending, and cloning into the *Sma* I site of pUC19. pBROC511 (appendix 1) has an *Afl* III recognition site (C↓CATGT) on the ATG start codon of the *mra*Y gene. This *Afl* III site gives a 4 bp overhang which is compatible with the *Nco* I overhang (C↓CATGG). Ligation destroys both sites. For this reason and the reasons described above, the Invitrogen pRSET N-terminal polyhistidine fusion system was selected.

The pRSET vectors contain the bacteriophage T7 promoter, followed by a ribosome binding site. The translated region contains the his^6 motif, an enterokinase cleavage site followed by a transcriptional terminator (fig. 3.6).

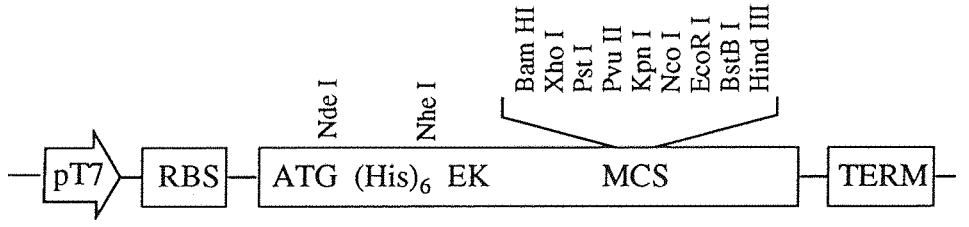


Figure 3.6. Part of the pRSET vector.

The vector also carries an ampicillin resistance gene which conveniently contains a unique *Sca* I site for restriction analyses.

A 1200 bp *Afl* III/*Hind* III fragment excised from pBROC511 containing the complete *mraY* gene was purified by gel electrophoresis. DNA was recovered from the gel by electroelution. The fragment was ligated into *Nco* I/*Hind* III digested pRSET-B and the ligation mixture used to transform *E. coli* MV1190 (a *rec A*$^-$ strain which did not contain the gene for T7 RNA polymerase) selecting with 100 μg/mL ampicillin. Five transformant colonies were selected at random and plasmid DNA's prepared from 1 mL LB cultures. Three of five showed the right fragments (1.15 and 2.95 kbp) when digested with *Hinc*II/*Sca*I. The original vector contains a *Sca*I site remote from the multiple cloning site and no *Hinc*II sites. One clone was further analysed with *Sca*I, *Sca*I/*Hinc*II and *Sca*I/*Eco*RI digests giving the expected 4.1, 1.15/2.95 and 1.55/2.55 kbp fragment sizes respectively and was denoted pPB1 (appendix 1). Since the operation was a simple cut and paste manipulation DNA sequencing of the new construct was not necessary.

3.6.3. Cytotoxicity of his^6-translocase I.

The T7 expression system developed by Studier and Moffatt[139] uses the bacteriophage T7 RNA polymerase to direct selective high level expression of cloned genes. The T7 RNA polymerase is highly specific for its own promoter[140] and so does not transcribe host (*E. coli*) genes. The T7 RNA polymerase is derived from a single copy of the T7gene *1* under the control of the *lac*UV5 promoter such that promotion of the polymerase can be induced with IPTG in *lac*I^q hosts. This copy of the T7 gene *1* is either present on the host chromosome (de3 element) or is delivered by phage infection (Lamda or M13). Figure 3.7 is a schematic of this expression system.

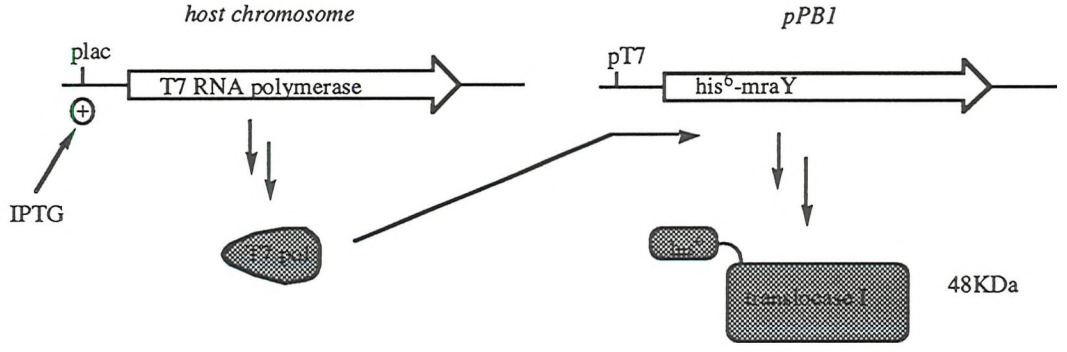


Figure 3.7. T7 expression system.

It rapidly became apparent that *his^6-mra*Y was a toxic gene. *E. coli* JM109 (de3) could not be transformed with pPB1, whilst transformation with pRSET-B was highly efficient. p PB1 could transform *E. coli* JM109 with similar efficiency.

E. coli C600 (pGP1-2) is another commonly used host for T7 expression vectors. The T7 RNA polymerase gene resides on a plasmid. Expression of the T7 RNA polymerase gene is induced by heat shock with subsequent expression of the desired gene on a second plasmid. This 'dual plasmid' system[141] is documented to have lower basal expression levels than *E. coli* JM109(de3) and is therefore better suited to toxic genes. This host could not be transformed with pPB1 either.

The plasmid pLysS carries the gene for T7 lysozyme and produces low levels of this protein in hosts producing T7 RNA polymerase. T7 lysozyme is a natural inhibitor of T7 RNA polymerase and its presence allows establishment and maintainence of toxic genes until induction.[142] Competent cells of *E. coli* BL21(de3)(pLysS) were efficiently transformed by pPB1, although it took a full 24 hr growth at 37 °C for colonies to reach a pickable size. Two colonies were taken to liquid culture. One grew well and was not affected by IPTG; the other grew slowly and growth was halted by 1 mM IPTG, consistent with expression of a toxic gene.

This was repeated with a further two colonies picked at random and grown overnight in liquid culture; one grew, one did not. Again, as with *E. coli* JM109(de3) transformants, it appears that some cell lines have neutralised the toxic effects of pPB1 to return to a healthy phenotype, possibly via a spontaneous recombination event. Indeed, the IPTG-sensitive transformant overnight culture was streaked out on LB/amp agar; a single colony picked from this plate was found to have reverted to a healthy phenotype and was no longer sensitive to IPTG. It was not known at this stage what, if anything, had happened to pPB1 during this apparent phenotypic change. Unfortunately facilities were not available at that time for preparation and restriction analysis or sequencing of plasmid DNA.

3.6.4. Assay of solubilised membranes from *E. coli* BL21(de3)(pLysS)(pPB1) for translocase I activity.

Membranes were prepared from the *E. coli* BL21(de3)(pLysS)(pPB1) cell line whose growth was halted by 1 mM IPTG in section 3.6.3. They were solubilised with 0.5 % Triton X-100 and assayed using the standardised fluorescence enhancement assay (chapter 4) as described in the experimental chapter. A similarly solubilised extract of *E. coli* JM109 (pTrc99A) was prepared as a control strain. The rates for *E. coli* JM109 (pTrc99A) and *E. coli* BL21(de3)(pLysS)(pPB1) extracts were 0.06 and 0.03 units/min respectively, i.e. if *his*^{6}-translocase I is present then it is inactive.

3.6.5. [^{35}S]-Methionine labelling of pPB1-derived protein.

One advantage of the T7 expression system is that T7 RNA polymerase is not sensitive to rifampicin, whereas *E. coli* RNA polymerase is.[144] This means that in the presence of rifampicin, only genes under control of T7 promoters will be expressed. If [^{35}S]-methionine is added to the cell culture under these conditions, it will only be incorporated into proteins derived from genes under the control of a T7 promoter. In the case of *E. coli* BL21(de3)(pLysS)(pPB1) this means his^6-translocase I (42.5 kDa) and a very small amount of the LysS protein (16.8 kDa[143]). pLysS has the weak ϕ3.8 T7 promoter immediately downstream from the *LysS* gene such that the T7 RNA polymerase must transcribe right around the plasmid to reach *LysS*. The protein content of the cell can then be analysed by SDS-PAGE followed by autoradiography. The protocol used was based on that described by Tabor and Richardson.[144]

E. coli BL21(de3)(pLysS) was transformed with each of pRSET-B (negative control), pRESTA/CAT (positive control, his^6-chloramphenicol acetyl transferase, 29 kDa) and pPB1. Transformant colonies were picked at random and grown overnight in LB. The following day cultures were diluted 1 in 40 in LB and grown to OD_{600nm} = 0.6. Cells were collected from 1 mL of culture, washed in 1 mL prewarmed

minimal medium (M9 salts + glucose + 18 amino acids, not methionine or cysteine) and then grown in 1 mL of that medium for 1 hr. IPTG was added to 1 mM and cells grown for a further 20 min to induce expression of the T7 RNA polymerase. Rifampicin was added to 200 μg/mL and cells grown for a further 30 min to arrest non-T7 directed protein synthesis. [^{35}S]-methionine (10 μCi) was added and cells grown for a further 5 min. Cells were collected and protein analysed by SDS-PAGE as described in the experimental chapter. The gel was dried and exposed to film for 5 days.

The negative control (empty expression vector) showed no radiolabelled proteins as expected. The positive control showed an intense band at 29 kDa confirming the validity of the protocol. The lane containing protein extract from the *E. coli* BL21(de3)(pLysS)(pPB1) experiment showed a faint band at 34 kDa. This result was reproducible.

Thus it would seem that either the plasmid pPB1 has been altered such that small amounts of a tolerable 34 kDa protein was produced, or that the correct protein was produced but efficiently and specifically proteolysed to give the observed product.

3.7. Summary.

The main obstacle to purification of translocase I at present is that activity is partially or completely lost following adsorptive chromatography. This aside, detergent-solubilised translocase I can be adsorbed to and eluted from phenyl-agarose (HIC) and DEAE-sepharose (IEC) with apparent purification. If the 'loss of activity' problem can be resolved, either by reconstitution with a 'factor' or phospholipid, or by using a different detergent, then the custom prepared matrix polyprenyl-agarose and the commercial dye-linked resins may well afford considerable purification, with luck to homogeneity. The possibility of a hexahistidine fusion protein was investigated, but it seems that the resulting protein was too toxic and that the expression vector may have been unstable.

Chapter 4. Kinetic Characterisation of Translocase I and its Inhibition by Mureidomycin A, Tunicamycin and Liposidomycin B.

4.1. Introduction.

A major goal of this project is to examine and characterise the inhibition of translocase I by mureidomycin A, tunicamycin and liposidomycin B. Purified mureidomycin A (20 mg) and liposidomycin B (1.14 mg) were obtained during the course of the project from Dr. M. Inukai, Sankyo Co. Ltd., Japan and Dr. K. Kimura, Snow Brand Milk Products Co. Ltd., Japan respectively. Tunicamycin is available as a mixture of closely related structures from commercial sources. The issues we wished to address using enzyme kinetics were: Is the inhibition reversible, irreversible or slow-binding? Is the inhibitor competitive, non-competitive or uncompetitive with respect to the individual substrates? What are the values of the kinetic constants associated with the inhibition? Further investigation of the inhibitors using protein chemistry would depend on the availability of pure enzyme.

In studying translocase I using the radiochemical assay, it rapidly became apparent that this assay, whilst in theory sufficient, was, in practice, not suitable for the detailed kinetic analysis which we had in mind. The radiochemical assay can give accurate measurements of rate over a time-course assay, but problems arise when inhibition is time-dependent and time-courses are non-linear. The other major practical restriction of the radiochemical assay is the time taken to complete a set of assays and to quantify the product was a minimum of two hours. The study of inhibition kinetics routinely requires the result of one assay to guide the conditions of the following assay. Consequently kinetic analysis of the inhibitors using the radiochemical assay would be extremely arduous and time-consuming, factors which would undoubtedly be to the detriment of the data produced.

A rapid continuous assay system was therefore required for kinetic studies. Weppner and Neuhaus [118] had already shown that translocase I from *S. aureus* could accept a substrate analogue which had the dansyl fluorophore attached to the ε-amino group lysine side-chain. Weppner demonstrated that this could in principle be used as a basis for continuous assay of translocase I since incorporation of the fluorophore into a membrane bound (hydrophobic environment) product resulted in a measurable change in fluorescence properties, namely a blue shift of the emission maximum and an increase in quantum yield. Therefore, the first aim of this part of the project was to

develop rapid continuous assay methodology based on the work of Weppner and Neuhaus.

4.2. Preparation and characterisation of dansyl-UDPMurNAc-pentapeptide.

4.2.1. Preparation of UDPMurNAc-pentapeptide.

UDPMurNAc-L-Ala-γ-D-Glu-*m*-DAP-D-ALa-D-Ala was isolated from antibiotic-treated cells of *Bacillus subtilis* W23 (NCIMB 11824). This strain was used because biosynthesis of UDPMurNAc-pentapeptide does not appear to be regulated in *Bacillus* as it does in *E. coli.*[47, 49] Consequently the metabolite can be caused to accumulate and can be isolated in milligram quantities. The method used was based on those of Lugtenberg *et al;* and Fluoret *et al;*. [47, 115]

Cells of *B. subtilis* W23 were grown to mid-logarithmic phase in a rich medium (phosphate/yeast extract/peptone), collected by centrifugation and resuspended in a 'cell wall synthesis medium' (CWSM). This medium contained 50 mg/L chloramphenicol to arrest protein synthesis and 12.5 mg/L vancomycin to block peptidoglycan synthesis at the level of lipid-linked intermediate II (hence accumulation of UDPMurNAc-pentapeptide). The medium also contained salts, uracil and amino acids (all listed in the experimental chapter) of the peptide side-chain of UDPMurNAc-pentapeptide. In our laboratory, 50 mg/L ampicillin seemed to improve the yield of UDPMurNAc-pentapeptide, although this antibiotic has not been included in any cell wall synthesis medium reported to date. Rapid cooling (ice-bath) of cells prior to centrifugation and resuspension in CWSM were crucial for a good yield. Cells were grown in CWSM for 45 min, collected by centrifugation, resuspended in ice-cold 5 % trichloroacetic acid (TCA) and left on ice for 30 min. Precipitate was collected by centrifugation and extracted twice more with 5 % TCA. The pooled supernatants were extracted with ether to remove the TCA. The aqeous phase was neutralised, concentrated, clarified and chromatographed on sephadex G15 or Biogel P2 eluting with water. Fractions containing UDPMurNAc-pentapeptide were identified by analytical reverse-phase HPLC by comparison with authentic material provided by Dr. J.T.Lonsdale of SB Pharmaceuticals. Fractions containing UDPMurNAc-pentapeptide free of other materials absorbing at 262 nm were pooled and lyophilised. Yield and purity was determined from the UV absorbance spectrum ($\varepsilon_{262nm} = 10^4\ M^{-1}cm^{-1}$). A typical yield after purification on sephadex G15 was 33 mg (67 % pure by nucleotide absorption) from 6.5 g cells, wet weight, starting from 2 L of mid-log phase culture. An NMR spectroscopy study conducted by colleagues at SB Pharmaceuticals found that the only major impurity in UDPMurNAc-pentapeptide made this way was water (UDPMurNAc-pentapeptide is very hygroscopic).

This protocol does lend itself well to scaling up. In a larger scale operation conducted using facilities made available by SB Pharmaceuticals, the yield was 192 mg (44 % pure by nucleotide absorbance) from 55 g cells, wet weight prepared by the same method. The yield is about half of that (mg/g cells) from the smaller scale experiment. This may have been due to the increased time taken to change media.

The possibility of a 'one pot' protocol was investigated. In this experiment cells were grown in the rich medium to mid-log phase before addition of antibiotics to the same final concentrations as in the above method. After 5 min, uracil and amino acids were added and cells grown for a further 40 min. UDPMurNAc-pentapeptide was extracted as above and pirified on a column of Biogel P2, again analysing fractions by reverse-phase HPLC. The yield of UDPMurNAc-pentapeptide was less than 2 mg from 2 L of culture.

Peptidoglycan isolated from *Bacillus subtilis* W23 has been analysed by gas chromatography (following hydrolysis with 6 M HCl and derviatisation to the C-isobutyryl, N-heptafluorobutyryl analogues) by Dr. J.T.Lonsdale and Mrs.J.Gillett of SB Pharmaceuticals for amino acid content. This study confirmed the presence of diaminopimelic acid rather than lysine as reported for *B. subtilis* and indeed the majority of strains of the genus *Bacillus* by Schleifer and Kandler.[13]

4.2.2. Dansylation of UDPMurNAc-pentapeptide.

UDPMurNAc-L-Ala-γ-D-Glu-(N$^{\varepsilon}$-dansyl)-*m*-DAP-D-Ala-D-Ala (fig. 4.1) was prepared by coupling UDPMurNAc-pentapeptide with dansyl chloride (96-fold molar excess) at pH 10.0 in carbonate buffered 50 % aq. acetone based on the method of Weppner and Neuhaus.[118] Dansyl-UDPMurNAc-pentapeptide was separated from dansic acid by chromatography on sephadex G15 or Biogel P2.

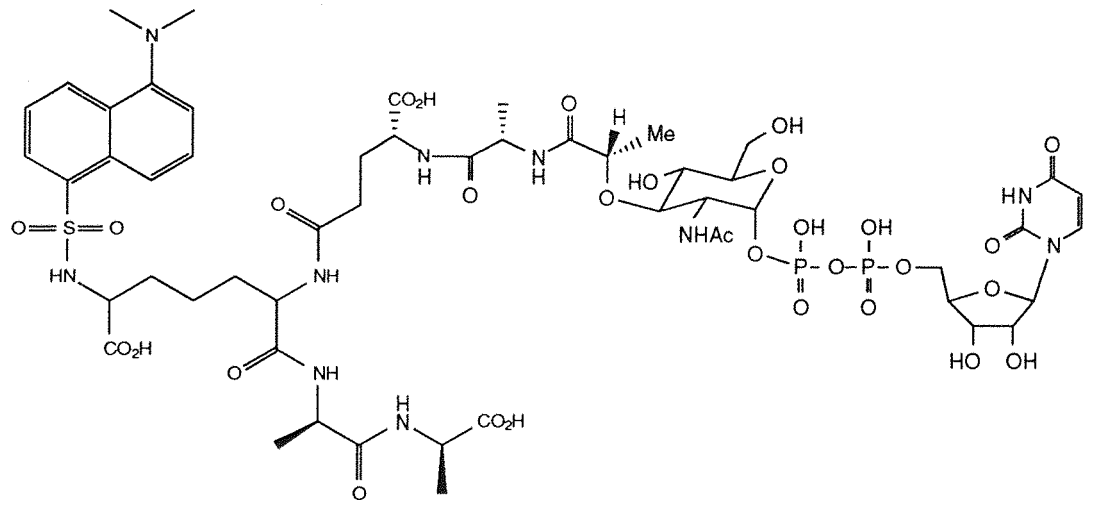


Figure 4.1. UDPMurNAc-L-Ala-γ-D-Glu-(N$^{\varepsilon}$-dansyl)-*m*-DAP-D-Ala-D-Ala.

The dansylated material was initially characterised by UV spectroscopy. The absorbance spectrum contained maxima at 249 nm and 327 nm. The ratio of absorbances 249/327 nm was 5.1. Negative ion MALDI-TOF MS was also used to analyse this material. The mass spectrum showed compounds with molecular weights 1025, 1431 and 1664, the 1431 peak being largest and the 1664 peak being the smallest (appendix 2). The spectrum was mass calibrated using a calibration spectrum recorded in positive ion mode; consequently the masses are not accurately calibrated. The molecular weight of dansyl-UDPMurNAc-pentapeptide is 1426. It is possible that some of the UDPMurNAc-pentapeptide coupled with a second dansyl moiety (perhaps at the muramic acid 6'-hydroxyl group or the imide NH of uridine). This didansylated material would have a molecular weight of 1659. Thus it is very likely that the 1431 and 1659 peaks are dansyl-UDPMurNAc-pentapeptide and didansyl-UDPMurNAc-pentapeptide respectively. It is possible that the peak at 1025 results from cleavage of the glycosidic bond of dansyl-UDPMurNAc-pentapeptide (fig. 4.2). Unfortunately MALDI-TOF MS does not give information about the relative amounts of compounds in a mixture since some molecules are ionised more readily than others.

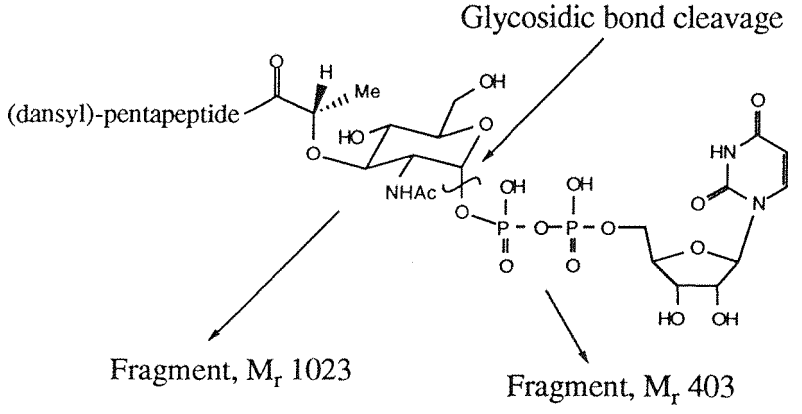


Figure 4.2. Possible cleavage of the glycosidic bond of dansyl-UDPMurNAc-pentapeptide during MALDI-TOF MS.

The dansylated material was also analysed by reverse-phase HPLC on an analytical C-18 column eluting with water at a flow-rate of 0.8 mL/min monitoring absorbance at 250 nm. It was found to contain only a small amount (< 5 %) of UDPMurNAc-pentapeptide (by comparison with authentic material), retention time, 3min. The major peak eluting at 17 min was assumed to be dansyl-UDPMurNAc-pentapeptide. This peak had a slight shoulder on each side indicating the presence of contaminants.

The absorbance and fluorescence properties of our material are the same as those reported by Weppner and Neuhaus for UDPMurNAc-L-Ala-γ-D-Glu-(N$^{\varepsilon}$-dansyl)-L-Lys-D-Ala-D-Ala (table 4.1).[118] Unfortunately, Weppner and Neuhaus did not quote absorbance maxima or estimate the purity of their material.

Property	Our material	Weppner, 1977
Absorbance 280/260 nm	0.29	0.29
Absorbance 250/260 nm	1.56	1.28
Fluorescence excitation maximum	340 nm	340 nm
Fluorescence emission maximum	565 nm	565 nm

Table 4.1. Absorbance and fluorescence properties of fluorescent translocase I substrates prepared in this study and by Weppner and Neuhaus.

Based on this characterisation and the HPLC data, the purity of the material was estimated at 90 ± 5 %. This was used to calculate an approximate stock concentration for the material derived from a larger scale preparation which was used in all of the experiments in this chapter except for the initial establishment of the assay protocol. Hence 75 mg of purified/lyophilised solid dissolved in 15 mL water (5 mg/mL) gave 3.15 mM ±0.15 mM. Further characterisation could be obtained in the future by preparative or semi-preparative scale purification by HPLC and accurately calibrated MALDI-TOF mass spectrometry.

4.3. Preliminary experiments with dansyl-UDPMurNAc-pentapeptide as a substrate for translocase I.

Before starting this piece of work it was considered very likely that the *E. coli* translocase I would accept dansyl-UDPMurNAc-pentapeptide as a substrate from the precedent of Weppner and Neuhaus.[118] We wished to determine how efficient a substrate dansyl-UDPMurNAc-pentapeptide would be and the fluorescence properties would change.

The experiments described in this section (4.3) were carried out up to the end of June, 1994 in a volume of 3 mL. Consequently availability of the substrate restricted the number of assays which could be performed, hence the lack of optimisation. Following my industrial placement (July - September, 1994), during which dPP was prepared on a 75 mg scale as described in 4.2.2, the fluorescence enhancement assay was used again. At this time (October, 1994) a change occurred in the enzyme preparation (to date we have been unable to determine what that

change was) which manifested itself as a 10 - 15-fold reduction in specific activity under the conditions of the assay at the time. A series of experiments were conducted to determine the root of the problem. The fluorescent substrate had not degraded during storage at -70 °C since the absorbance and fluorescence properties were identical to when it was fresh. All buffers and lipids (heptaprenyl phosphate and phosphatidylglycerol) were replaced without effect. The specific activity of the membrane preparation was unchanged as determined by the radiochemical assay, whereas this parameter was reduced approximately 10-fold when the enzyme was solubilised with either Triton X-100 or CHAPS. This reduction in specific activity correlates with the reduction in activity observed in the fluorescence enhancement assay. This forced us to optimise the assay and to invest in new apparatus to allow the assay to be performed on a 300 µL scale as opposed to 3 mL. A key factor in recovering the activity was increasing the concentrations of heptaprenyl phosphate/dodecaprenyl phosphate and phosphatidylglycerol. Thus it seems that the problem may have been connected with the interaction between enzyme and lipid acceptor. With luck, this matter will be resolved in the future when the enzyme is purified.

Whilst the absolute numbers for rates measured before the apparent loss of activity are not directly comparable with those measured afterwards, the results are. In any case, all the Michaelis-Menten and inhibition kinetics, which comprimise the bulk of the results in this chapter, were performed after these events (November, 1994).

The first experiment was to see if there was a change in fluorescence emission when dPP was incubated with translocase I in the presence of lipid acceptor. Translocase I was solubilised in 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol, 0.5 % Triton X-100 at 2 mg protein/mL. An incubation was set up in a 3 mL plastic cuvette at room temperature containing 100 mM Tris pH 8.5, 25 mM $MgCl_2$, 20 µM heptaprenyl phosphate (HP), 0.2 % Triton X-100, 25 µM dPP and 0.3 mL enzyme. The fluorescence spectrum was recorded (excitation at 340 nm) immediately and again after 2 hr (table 4.2).

Time, hr	**Emission maximum, nm**	**Fluorescence intensity, (arbitrary units)**
0	565	29.0
2	554	39.7

Table 4.2. Incubation of dansyl-UDPMurNAc-pentapeptide with translocase I and heptaprenyl phosphate.

There was a 50 % increase in fluorescence intensity and a relatively small blue shift of 11 nm in the emission maximum. In order to investigate the time-course of this reaction, the assay was repeated with either 0.3 mL or 0.6 mL of solubilised enzyme. All other constituents were present at the same concentration as above. ΔF_{554nm} (ex. 340 nm) was monitored over 0 - 30 min. The rates were 0.06 and 0.12 units/min (these are units of relative fluorescence intensity as recorded by the fluorimeter and are arbitrary; they will be expressed as u/min from here on) with 0.3 mL or 0.6 mL of enzyme respectively, and the time-courses were linear.

It had previously been found that phospholipids (PE and PG) stimulated the activity of translocase I in the presence of heptaprenyl phosphate (HP) using the radiochemical assay (section 2.8). Aliquots of these lipids and HP were mixed in a microfuge tube and solvent removed with a stream of nitrogen. The residue was solubilised directly in detergent-solubilised enzyme (0.3 mL) such that the final assay concentrations of the lipids would be 20 μg/mL PE and PG (each) and 9.2 μM HP. The other assay constituents were 100 mM Tris pH 8.5, 25 mM $MgCl_2$, 0.2 % Triton X-100, enzyme/lipids (0.3 mL) and 9 μM dPP. This gave a fluorescence enhancement rate of 0.83 u/min. Doubling the concentration of dPP gave 1.60 u/min; diluting the enzyme 5-fold gave 0.16 u/min.

The observation that reaction rate is proportional to substrate concentration implies that dPP is present below its K_m value. Actvity is proportional to enzyme concentration and stimulation of activity is seen in the presence of phospholipids, PE and PG.

The reader is reminded at this point that the fluorescence emission is not an absolute measure of product, rather a relative one. Numerous factors such as temperature, detergent concentration, ionic strength, dissolved oxygen, etc; affect and modulate the fluorescence emission. Indeed, it is this very sensitivity of the fluorophore to its solvent environment which makes this assay technique work. Hence these factors are rigorously maintained in the assays described in order that the changes in fluorescence can be interpreted as turnover of substrate.

The question of which phospholipid was responsible for the activation was addressed as has been discussed in section 2.8 (table 2.14). The acidic phosphatide, phosphatidylglycerol was found to be responsible for the activation with phosphatidylethanolamine having a slight inhibitory effect. This implications of this and literature precedent are discussed in 2.8.

The effect of temperature on the fluorescence emission of dPP was examined in a reaction mixture which had reached equilibrium (table 4.3).

Temperature, °C	Fluorescence intensity
25	10.6
31	10.25
38	9.77

Table 4.3. Relative fluorescence (ex. 340 nm, em. 554 nm) of a reaction mixture at equilibrium at temperatures, t.

As a general rule, fluorescence quantum yield (the number of photons emitted per photon absorbed) decreases with increasing temperature because of solvent quenching of the excited state due to a greater number of intermolecular collisions per unit time.[145] It was decided at this point to do all future assays at 30 °C for the simple reason that it would not always be possible to maintain the cuvette at 25 °C (e.g. during Summer months). Of course, the rate of enzymatic reaction will also be increased with increasing temperature.

To estimate the sensitivity of the fluorescence enhancement assay, Triton X-100 solubilised enzyme was assayed under comparable conditions (identical except for the substrate) in both fluorescence enhancement and radiochemical assays (fig. 4.3) as described in the experimental chapter.

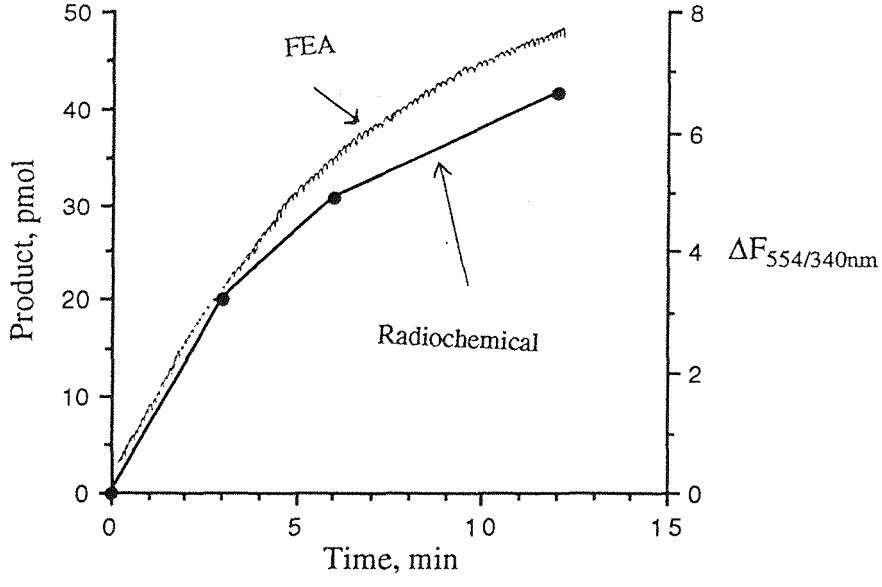


Figure 4.3. Comparison of fluorescence enhancement and radiochemical assays.

The specific activity in the radiochemical assay was 1.28 nmol/min/mg. The specific activity in the fluorescence enhancement assay (FEA) was 2.66 u/min/mg.

Thus the two assays can detect the same concentrations of enzyme activity, although the FEA consumes 60-fold more enzyme per assay (3 mL volume *vs.* 50 μL volume).

The FEA relies on the difference in hydrophobicity between the micellar and bulk aqueous compartments where the product and starting material reside respectively. Another detergent, CHAPS, was examined for its ability to provide this change in environment. Membranes were solubilised at 2 mg/protein/mL in 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol with either 1.5 % CHAPS or 0.5 % Triton X-100. The absolute specific activities of the solubilised preparations were determined using the radiochemical assay to be 0.75 nmol/min/mg with CHAPS and 0.64 nmol/min/mg with Triton X-100 under the conditions described in the experimental chapter. CHAPS was present in the radiochemical and fluorescence enhancement assays at 0.6 %, Triton X-100 at 0.2 %. The specific activities in the FEA were 1.07 u/min/mg with CHAPS and 2.97 u/min/mg with Triton X-100. Again the conditions for the radiochemical assay and FEA were identical except for the soluble substrate for each detergent. This means that Triton X-100 at 0.2 % gave a 3.25-fold greater fluorescence enhancement than CHAPS at 0.6 % per unit of enzyme activity. This result is consistent with the formation of large spheroid micelles with a discrete hydrophobic interior volume by Triton X-100 as compared with the small 'back to back' micelles formed by the bile acid steroid derivative, CHAPS.[126]

Lastly, in keeping with the radiochemical assay protocol, the fluorescent product, undecaprenyl-pyrophosphoryl-MurNAc-dansyl-pentapeptide, should be extractable into butanol. A reaction mixture was allowed to go to equilibrium. The absolute fluorescence (ex. 340 nm, em. 535 nm) increased from 9.0 to 20.0. This was extracted with 3 mL of n-butanol. The fluorescence (ex. 340 nm, em. 535 nm) of the butanol layer from this and an extraction of the same amount of dPP from 3 mL of water was 10.4 and 1.7 relative to butanol only respectively. This supports the premise that dPP is turned over into lipid-linked product.

4.4. Fluorescence enhancement assay (300 μL scale).

As reported and discussed at the begininng of section 3.3, the assay protocol developed above was superceeded by a more efficient 300 μL assay protocol following an apparent reduction in available enzyme activity. This section is concerned with the development and optimisation of the 300 μL scale assay which was then used in the kinetic analysis of the inhibitors mureidomycin A, tunicamycin and liposidomycin B.

Dansylated UDPMurNAc-pentapeptide prepared at SB Pharmaceuticals in September, 1994 was characterised in terms of fluorescence properties. The absorbance spectum was identical to previous preparations. This batch was used for

all the experiments described in the remainder of this chapter. An assay mixture minus enzyme/lipids was prepared containing 111 mM Tris pH 8.5, 28 mM $MgCl_2$, 0.17 % Triton X-100 and 39 μM dPP in 2.7 mL. The fluorescence excitation maximum was at 330 nm (em. 565 nm). The fluorescence emission maximum was at 563 nm (ex. 330 nm). Enzyme solubilised at 2 mg protein/mL in 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol, 0.5 % Triton X-100 with added PG and HP (0.3 mL) was added to start the assay (giving final concentrations of 10 μg/mL PG, 10 μM HP and 0.2 % Triton X-100). After 30 min at 30 °C, the fluorescence excitation maximum was at 340 nm (em. 565 nm) and the fluorescence emission maximum was at 535 nm (ex. 340 nm) . Thus in all of the kinetic experiments reported in the remainder of this chapter, change in fluorescence emission at 535 nm (ex. 340 nm) was monitored with time.

Assays carried out using enzyme solubilised at 2 mg protein/mL in 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol, 0.5 % Triton X-100 on a 3 mL scale gave 0.07 u/min with 10 μM HP (C_{35}) and 0.54 u/min with 10 μM dodecaprenyl phosphate (C_{60}) (average of two assays each). Potassium chloride stimulated activity up to 4-fold (fig. 4.4). This is consistent with stimulation of activity of particulate enzyme from *S. aureus* by K^+ or Cs^+ or Rb^+ or NH_4^+ with K^+ being the best.[146]

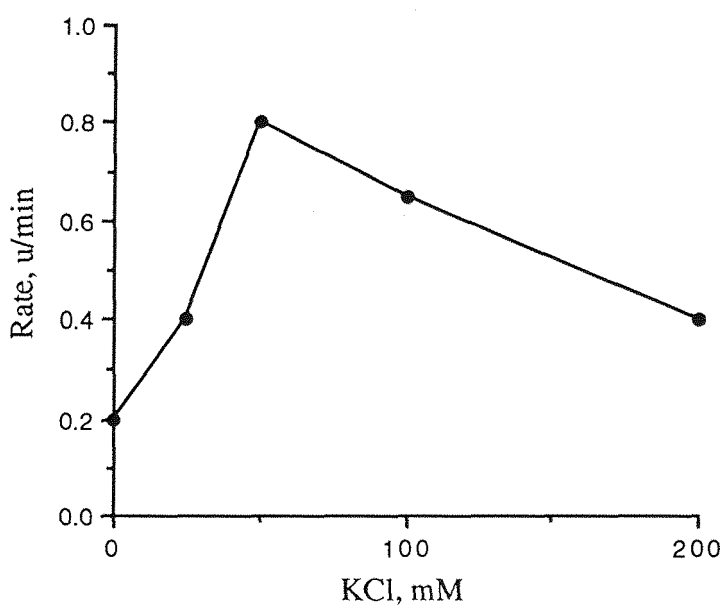


Figure 4.4. Stimulation of translocase I activity by KCl.

Activity at pH 7.5 was 62 % greater than at pH 8.5 (100 mM Tris buffer in both cases). The effect of pH on rate in the FEA has not been examined; neither has choice of buffering species or the presence of other additives such as glycerol, DTT,

ß-mercaptoethanol, ethanol, polyethyleneglycol, DMSO or surfactants except for Triton X-100 and CHAPS. Rates with DP (10 μM) in the presence of 0, 20 or 100 μg/mL PG (final assay concentrations) were 0.4, 0.6 and 1.4 u/min respectively. The use of higher concentrations of PG has not been investigated.

The concentration of enzyme activity in the enzyme preparation was doubled by solubilising enzyme at 4 mg protein/mL. The concentration of Triton X-100 was increased to 1 % to maintain the detergent/protein ratio. An 0.5 cm light path silica quartz cuvette and four plastic holder adaptors were purchased from Perkin Elmer in order to reduce the assay volume to 300 μL to reduce costs and make the method viable for routine assays. Conditions were determined experimentally to give the same absolute change in fluorescence from an 300 μL assay volume as from a 3.0 mL assay volume. Theoretically, a 2-fold increase in rate of product evolution in 300 μL would result in the same detected change in fluorescence because the light path has been halved. In practice, effects such as scattering, absorptive losses and general reduction of efficiency due to the reduced size of the cuvette mean that more than a 2-fold increase was required. The new optimised conditions were 100 mM Tris pH 7.5, 25 mM $MgCl_2$, 50 mM KCl, 0.2 % Triton X-100, 100 μg/mL PG, enzyme (60 μL) and substrates DP and dPP in a final volume of 300 μL at 30 °C monitoring $\Delta F_{535nm(ex.340nm)}$.

Later, the effects of varying concentrations of divalent metal ions and detergent concentration were examined (table 4.4). All the assays contained a basal concentration of 0.2 mM $MgCl_2$ derived from the salt present in the solubilisation buffer.

$[M^{2+}]$, mM	Mg^{2+}	Mn^{2+}	Ca^{2+}	Zn^{2+}	Ni^{2+}
0.2	0.4[a]	nd	nd	nd	nd
1	1.2	2.0	0	0	0
5	2.6	1.8	0	0	0
10	3.5	1.8	0	0	0
25	3.6	0.8	0	0	nd
40	5.1	0.7	nd	nd	0
80	3.8	nd	nd	nd	nd

Table 4.4. Dependence of translocase I activity on divalent metal ions. [a]In this assay activity diminished rapidly.

The optimal concentration of $MgCl_2$ was around 40 mM, but 25 mM continued as the standard concentration used for the sake of consistency. Mn^{2+} can

replace Mg^{2+} with a 2.5-fold smaller maximal activity. It is interesting to note that the optimal concentration of Mn^{2+} is ≤ 1 mM. The effect of varying detergent concentration was similarly examined (fig 4.5).

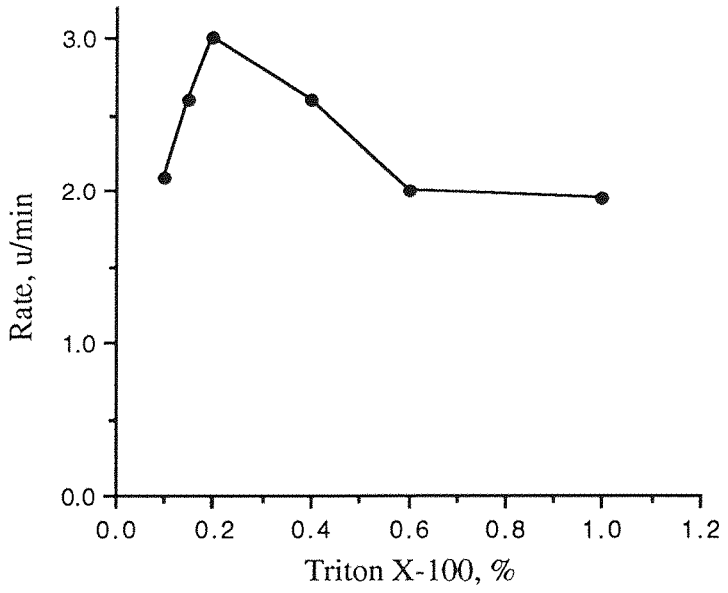


Figure 4.5. Effect of varying Triton X-100 concentration on translocase I activity.

The optimal concentration was 0.2 % as had always been the standard assay concentration! In reality this is probably a measure of the optimal detergent/lipid ratio, since above the CMC value, increasing the concentration of a detergent does not affect the constitution of the micelle. These assays contained 40 μM DP (K_m determined at 10 - 15 μM). Increasing detergent concentration will eventually reduce the measured rate by effective dilution of this substrate. K_m values for both substrates are reported in section 4.6.2.

4.5. Assay of the reverse reaction.

The reaction catalysed by translocase I is reversible. Weppner and Neuhaus demonstrated that the reverse reaction could be measured by the fluorescence method by allowing the forward reaction to go to equilibrium and adding an aliquot of UMP.[118] The same was true with our assay for solubilised enzyme. Decrease in fluorescence emission at 535 nm (ex. 340 nm) was initially linear and approached a new equilibrium. The initial rate was dependent on UMP concentration and approached a maximal rate implying Michaelis-Menten behaviour. An apparent K_m value was determined for UMP, 425 ± 40 μM (fig. 4.6). The value is apparent because the absolute concentrations of dansyl-lipid intermediate I and the 'products' are not known.

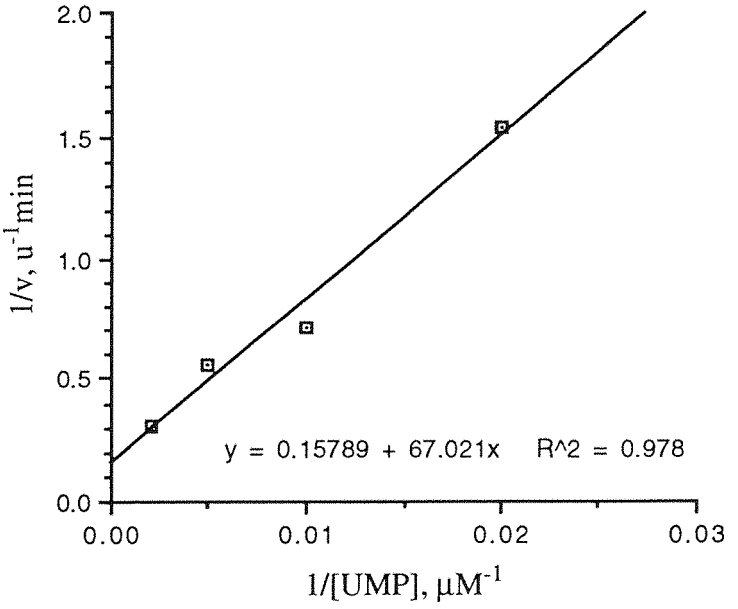


Figure 4.6. Determination of apparent K_m for UMP in the reverse reaction using the Lineweaver-Burk double reciprocal plot.

In doing these experiments it was realised that following attainment of equilibrium there was a gradual and time-dependent reduction in fluorescence at 0.05 u/min (the initial rate in this case was 3.0 u/min). This rate was unaffected by by 0.5 μM mureidomycin A when added after equilibrium had been reached (this concentration of mureidomycin A gave >95 % inhibition of the initial rate) suggesting that the change was not mediated by translocase I. The nature of this artefact remains unknown.

4.6. Mureidomycin A.

The mureidomycins are a class of peptidylnucleoside antibiotics whose properties are described in section 1.4.2. The most potent analogs are A and C. Mureidomycin A (mrdA, 20 mg, fig. 4.7) was a gift from Dr. Masatoshi Inukai of Sankyo Co. Ltd., Tokyo.

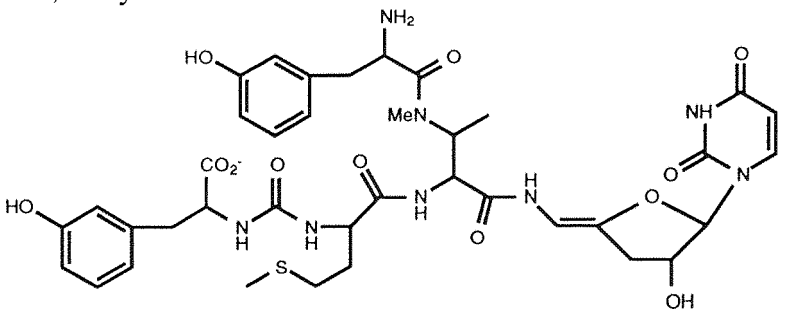


Figure 4.7. Mureidomycin A.

4.6.1. Verification of the antibacterial potency of mureidomycin A.

The potency of the sample of mrdA provided by Dr. Inukai was verified by its activity against cultures of *Pseudomonas putida* (ATCC 33015). The observed inhibition (fig. 4.8) was consistent with the reported MIC values for *Pseudomonas sp.*, <25 μg/mL.[81]

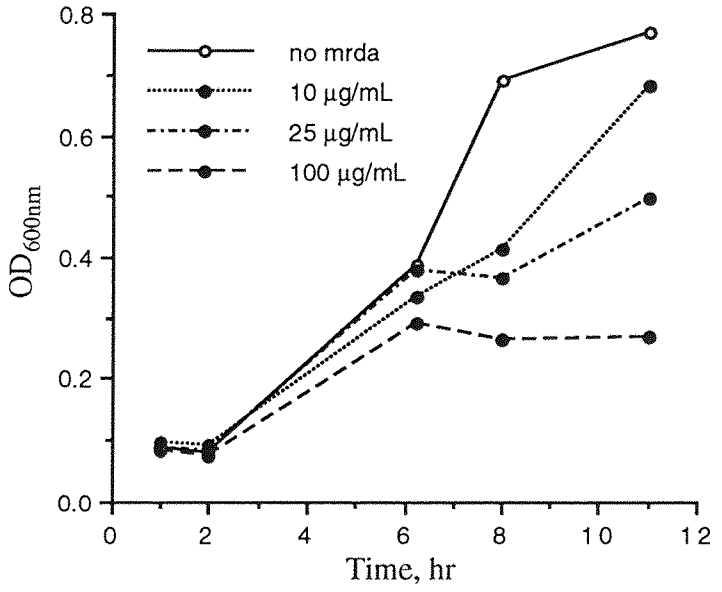


Figure 4.8. Antipseudomonal activity of mureidomycin A. Mureidomycin A was present at the indicated concentrations from the time of innoculation, t = 0.

4.6.2. Inhibition of translocase I activity by mureidomycin A in the radiochemical assay.

Enzyme solubilised at 4 mg protein/mL in 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol, 0.5 % Triton X-100 was assayed in the presence of 0 - 10 μg/mL mrdA as described in the experimental chapter (table 4.5).

MrdA, μg/mL	Rate, pmol/min	% Inhibition
0	2.42	-
0.1	0.38	80
1.0	0.30	88
10	0.24	90

Table 4.5. Inhibition of translocase I activity by mureidomycin A.

The M_r of mrdA is 840. Thus in this assay the IC_{50} <100 nM. This is consistent with the work of Isono *et al;* in which *in vitro* peptidoglycan synthesis by ether treated cells of *E. coli* NIHJ was inhibited by 91 % by 0.1 μg/mL mrdA.[147] Satisfied that the material given to us was authentic, we turned to the FEA for a detailed analysis of the inhibition.

4.6.3. Effect of mureidomycin A on translocase I activity.

Using the FEA it immediately became apparent that mrdA inhibited translocase I activity in a time-dependent fashion. It was also apparent that at lower concentrations of mrdA, the inhibition did not go to completion, rather it approached a steady-state final rate (fig. 4.9).

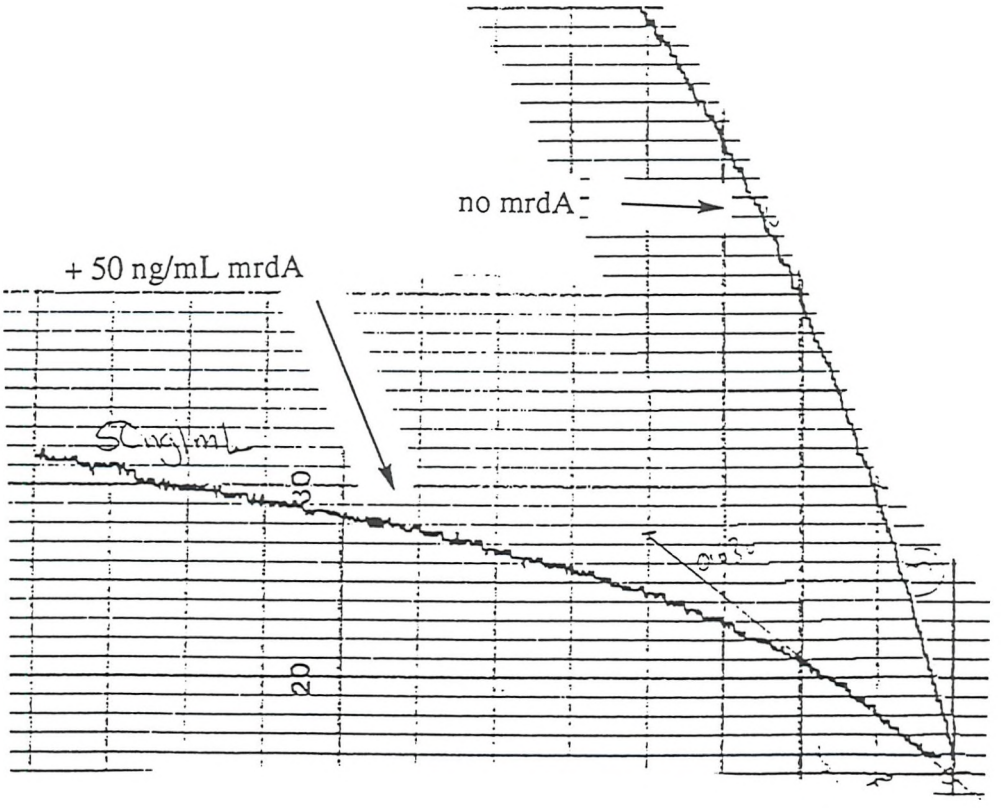


Figure 4.9. Typical fluorescence enhancement assay traces plus and minus mureidomycin A.

Given that inhibition was non-linear, we initially sought to determine whether inhibition was reversible or not. The rationale here is that enzyme is incubated plus and minus inhibitor for a fixed time and aliquots of both incubations are then removed, diluted into a second assay cuvette and assayed such that the inhibitor is effectively diluted out. If inhibition is reversible, then activity will return instantly; if inhibition is irreversible, activity, then will not return.

This experiment was carried out as described in the experimental section using 50 nM mrdA which gave completely inactive enzyme after 5 min. Enzyme treated this way, or from a control incubation with no mrdA, was diluted 5-fold into a fresh assay after 5 min (fig. 4.10). When mrdA was present in the first assay, the second assay mixture contained 10 nM mrdA carried through from the first incubation, so mrdA was added to 10 nM in the control second assay. The constituents of the control and experiment second assays were therefore identical. The results (table 4.6) confirmed that mrdA inhibition was time-dependent, and suggested that it was irreversible.

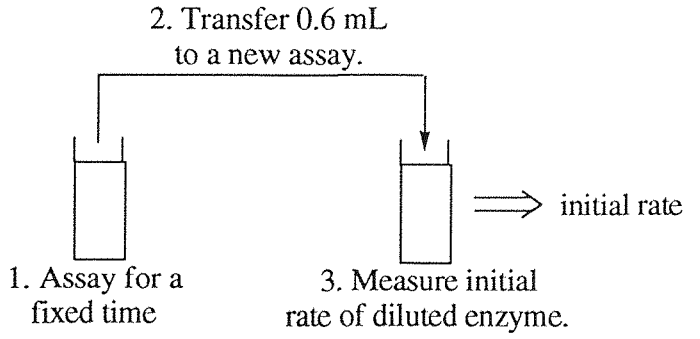


Figure 4.10. Schematic representation of the dilution of inhibitor experiment.

	Initial rate, u/min	
	t = 0 assay	**t = 5 min assay***
Control	3.2	0.7
+ 50 nM mrdA	1.7	0

Table 4.6. Dilution of inhibitor experiment with mureidomycin A. *Contains 20 % of the enzyme from the t = 0 assay.

This conclusion however was not entirely consistent with the previous observation that at concentrations of mrdA which gave <60 % reduction in initial rate, the time-dependent inhibition process did not go to completion. It was considered that mrdA might be a slow-binding inhibitor. Slow-binding inhibition is characterised by a reduced initial rate due to rapid equilibrium reversible inhibition (formation of the EI complex) followed by a 'slow' time-dependent appraoch to a steady-state final rate (formation of the EI* complex) (figs. 4.11 and 4.12).

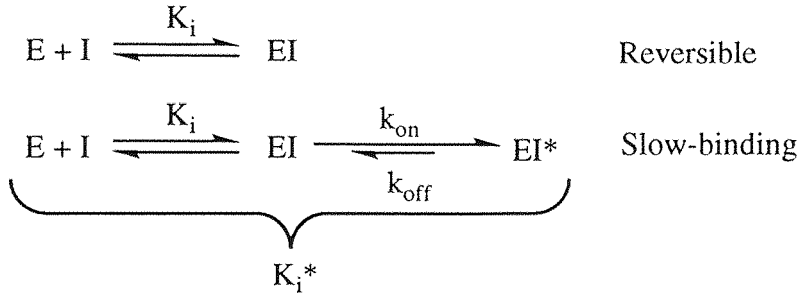


Figure 4.11. Reversible and slow-binding inhibition.

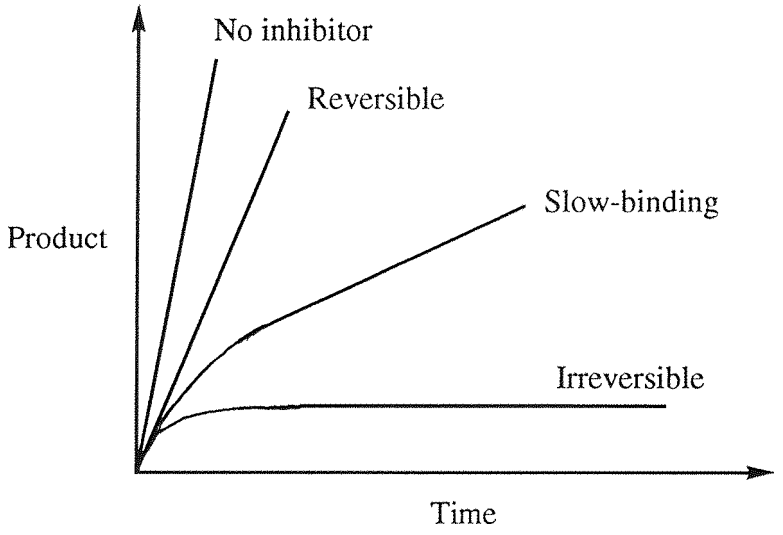


Figure 4.12. Illustration of the differences between reversible, slow-binding and irreversible inhibition.

When unbound inhibitor is removed, time-dependent return of activity should be observed as the EI* complex dissociates to E + I via EI. EI* can either be a tightly bound non-covalent complex arising from an isomerisation of the EI complex, or it can be a labile covalent complex. This class of inhibitors and the associated kinetics have been reviewed in detail by Morrison and Walsh.[148]

4.6.4. Substrate-dependence of inhibition by mureidomycin A.

If inhibition is mechanism-based, it may require the presence of one or both of the substrates for inhibition to take place. Two experiments were performed to determine whether or not the inhibition of translocase I by mrdA was dependent on the presence of one or both of the substrates. Enzyme was assayed plus and minus 300 nM mrdA. The time-dependent onset of inhibition resulting in a final steady-state rate characteristic of mrdA inhibition was seen: the initial rate was 1.2 u/min; the final rate was 0.15 u/min; the final steady-state was reached after 2 min. Enzyme was

incubated in an assay mixture in the fluorimeter cuvette without substrate for 135 s at which point substrate was added along with mrdA to a final concentration of 300 nM; the same time-course was seen as in the no-preincubation experiment. When mrdA was included in the preincubation (300 nM) only the final steady-state rate was seen. Thus inhibition was independent of dPP. An analogous experiment was performed with respect to DP as described in the experimental chapter. Inhibition was also independent of DP.

4.6.5. Time-dependence of inhibition by mureidomycin A in the reverse reaction.

The ability to measure the rate of the reverse reaction by adding UMP to a reaction mixture after *in situ* generation of lipid-linked product was used to examine the time-dependence of inhibition by mrdA. Solubilised enzyme was prepared and assayed as described in the experimental section in the presence or absence of 100 nM mrdA. This equilibrium position was very reproducible from assay to assay indicating that although the absolute concentration of the lipid-linked product is not known, it is constant. UMP was added to a final concentration of 0.4 mM at varying times after equilibrium had been reached and the reverse reaction monitored exactly as for the forward reaction. The results of this experiment are summarised in table 4.7.

Assay	Initial rate, u/min	Time before UMP, min	Reverse initial rate, u/min	Reverse rate, % of forward rate
no mrdA	3.3	7.3	2.6	79
no mrdA	3.3	7.5	2.8	85
no mrdA	3.0	49.5	2.6	87
+ mrdA	1.4	8.15	1.65	118
+ mrdA	1.9	21.4	2.0	105
+ mrdA	1.7	40.5	1.8	106

Table 4.7. Reverse initial rates for translocase I after different periods of incubation with mureidomycin A.

The rate of the reverse reaction was independent of time before addition of UMP over the time window examined. This indicates that the time-dependent inhibition by 100 nM mrdA reaches a steady-state which is not necessarily 100 % inhibition of activity.

To observe the decay of activity in the reverse reaction due to the presence of mrdA, the experiment was repeated, but mrdA was added to 200 nM 3 min after

starting the forward part of the assay and time before addition of UMP to 1 mM measured from this point (figs. 4.13 and 4.14).

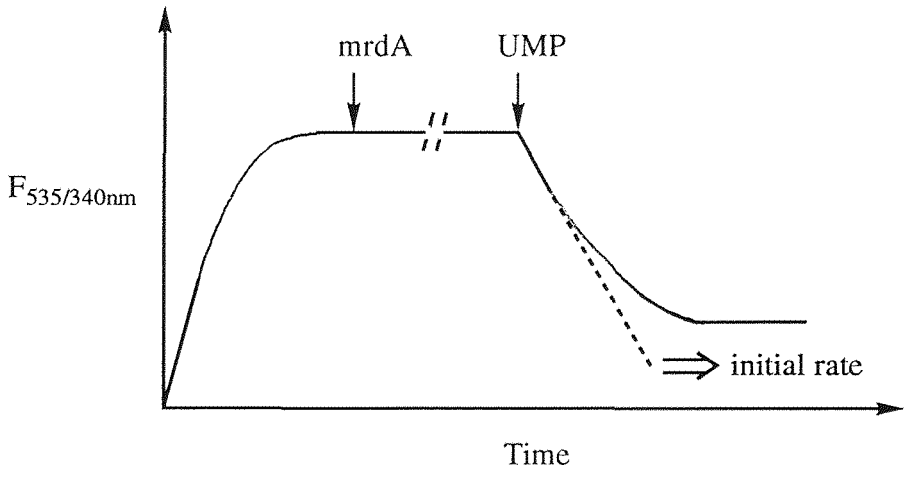


Figure 4.13. Experiment to observe decay of translocase I activity in the presence of mureidomycin A using the reverse reaction.

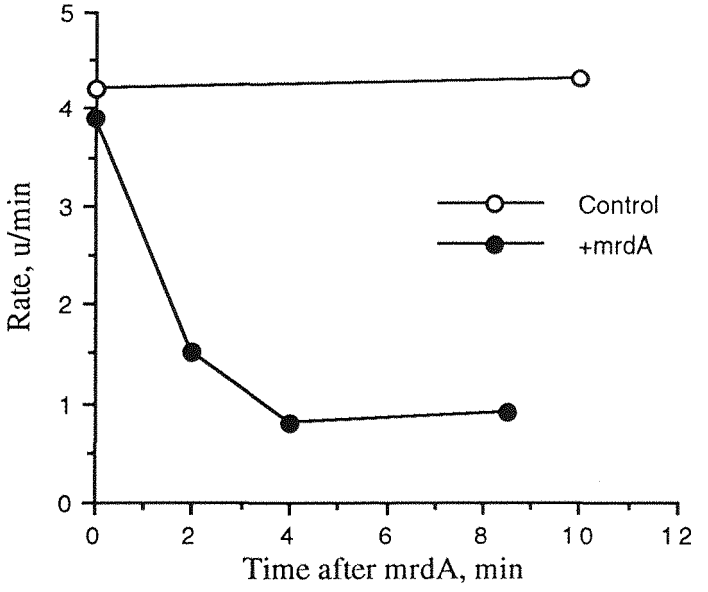


Figure 4.14. Decay of translocase I activity in the presence of mureidomycin A using the reverse reaction.

Thus it is clearly apparent that inhibition of translocase I by mrdA is time-dependent but does not go to completion consistent with slow-binding inhibition or the following special case of irreversible inhibition. The only scenario in which this could happen with an irreversible inhibitor is where the inhibitor is consumed during the time-course of the assay.

4.6.6. Does mureidomycin A lose potency upon incubation with translocase I?

If mrdA is an irreversible inhibitor which upon incubation with translocase I under assay conditions is itself inactivated, then in the case where the final rate is not zero, the mureidomycin A must have been consumed. In this case an aliquot of the reaction mixture would not inhibit a second aliquot of translocase I.

To test this enzyme was assayed in the presence of 300 nM mrdA for 3 min. This gave a steady-state final rate of 0.3 u/min, i.e. not zero; if mrdA is an irreversible inhibitor, this reaction mixture now contains no mrdA. A 100 µL aliquot was boiled for 1 min, centrifuged and 5-fold diluted enzyme assayed in the presence of 80 µL of the centrifugation supernatant as described in the experimental chapter. Controls were:

- the experiment without mrdA to find the activity expected if the mrdA were inactivated;

- the experiment where mrdA was missing from the first incubation, but added to the second to 80 nM to guage the effect if mrdA was unaffected by incubation with translocase I;

- the experiment where enzyme was missing from the first incubation to check that mrdA was not inactivated by the boiling process (which it was not).

Enzyme in the second incubation was inhibited by the 80 µL aliquot from the first incubation to the same extent as in the control where mrdA was not present in the first incubation, but was added to 80 nM in the second incubation. The conclusion then is that mureidomycin A is not inactivated by incubation with translocase I.

The above observations can only be rationalised if mrdA is a slow-binding inhibitor of translocase I activity.

4.6.7. Measurement of K_i for mureidomycin A.

The next goal was to determine the kinetic constants associated with the slow-binding inhibition of translocase I by mrdA. Initial rates were measured at varying concentrations of dPP and fixed DP (40 µM) in the presence of 0, 200 or 400 nM mrdA, and at varying concentrations of DP and fixed dPP (105 µM) in the presence of 0 or 300 nM mrdA. For each set of experiments the reciprocal of the initial rate was plotted against the reciprocal of the concentration of the varied substrate (fig. 4.15). In both cases the K_m values decreased with increasing inhibitor concentration, but V_{max} values stay the same. Thus mrdA is competitive with respect to both substrates. It was expected that this inhibitor would be competitive with dPP on account of the modified uridine moiety in mrdA being most likely to mimic the uridine moiety of dPP. The fact that it is competitive with the lipid acceptor substrate also indicates that the inhibitor interacts with the binding site for this substrate, a

property that could not be implied from the structure of mrdA (fig. 4.7). These results apply to the reversible formation of the EI complex from enzyme plus inhibitor, they do not necessarily apply to the isomerisation of EI to EI*.

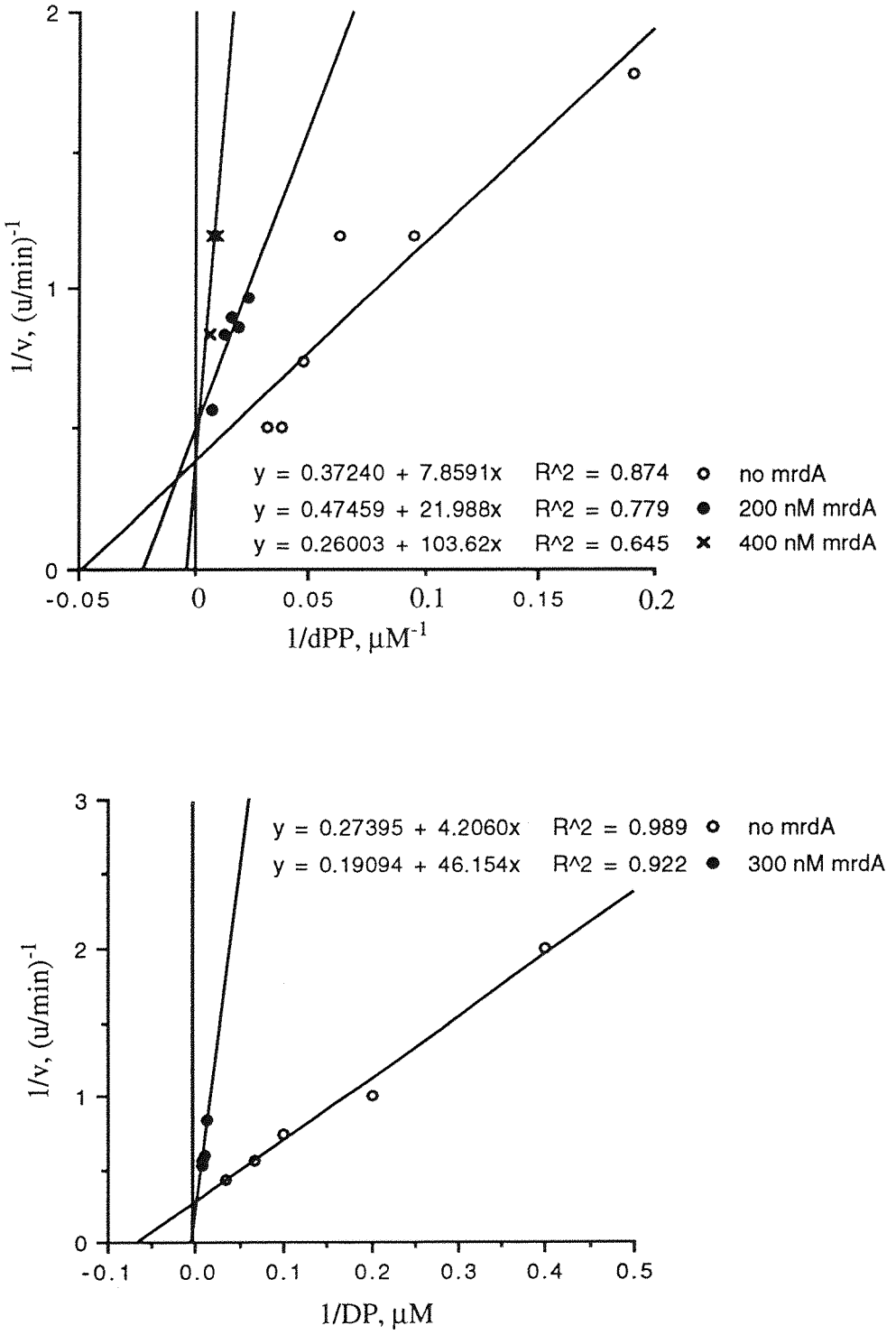


Figure 4.15. Lineweaver-Burk double reciprocal plots with varying [dPP] and fixed [DP] in the presence of 0, 200 or 400 nM mrdA and varying [DP] and fixed [dPP] in the presence of 0 or 300 nM mrdA.

To determine the K_m values for dPP and DP and the dissociation and rate constants for the slow-binding inhibition, two assumptions were made. The first was that the translocase I catalysed reaction follows a ping pong mechanism (fig. 4.16).

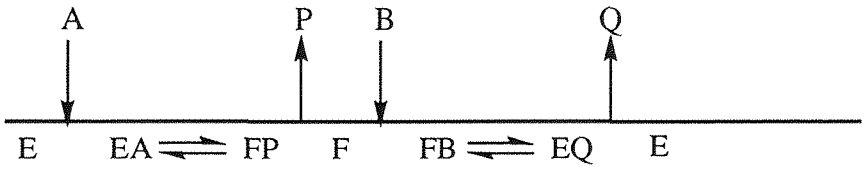


Figure 4.16. Ping pong mechanism assumed for the translocase I catalysed reaction for calculation of kinetic constants. A = dPP, B = DP, P = UMP, Q = lipid-linked product. E = free enzyme, F = enzyme-P-MurNAc-pentapeptide.

This is a reasonable assumption in the light of evidence presented in chapter 5 for the double displacement mechanism and literature precedent discussed in that chapter. The second assumption is that mrdA interacts only with free enzyme (E), acting as a classical dead-end inhibitor (fig. 4.17).

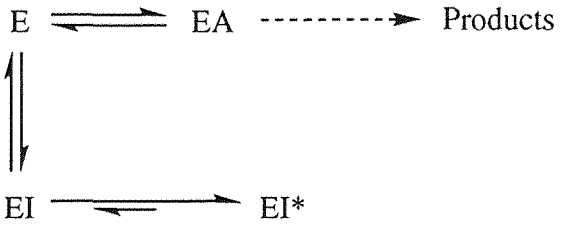


Figure 4.17. Classical dead-end inhibition of translocase I by mrdA assumed for calculation of kinetic constants.

This again is a reasonable assumption given that inhibition by mrdA is substrate independent.

The K_m value for DP was determined using the rearranged (double reciprocal) initial forward rate equation for a ping pong mechanism, equations [4.1], [4.2] and [4.3].[149]

$$\frac{V}{V_{max}} = \frac{[A][B]}{K_{m(B)}[A] + K_{m(A)}[B] + [A][B]} \qquad \text{.....[4.1]}$$

$$\frac{1}{V} = \frac{1}{V_{max}}\left(1 + \frac{K_{m(A)}}{[A]}\right) + \frac{K_{m(B)}}{V_{max}} \cdot \frac{1}{[B]} \qquad \text{.....[4.2]}$$

$$\frac{1}{V} = \frac{1}{V_{max}}\left(1 + \frac{K_{m(B)}}{[B]}\right) + \frac{K_{m(A)}}{V_{max}} \cdot \frac{1}{[A]} \qquad \text{.....[4.3]}$$

The value determined was 13 ± 2 μM† (fig. 4.18). This was an apparent K_m because the effective concentration in detergent micelles was not known, because there was a contaminating amount of endogenous undecaprenyl phosphate co-solubilised with the enzyme present in the assays, and because the ratio $K_{m(A)}/[A]$ used (0.15) was an estimated ratio based on previous experiments. The V_{max} was 4.7 ± 0.7 u/min.

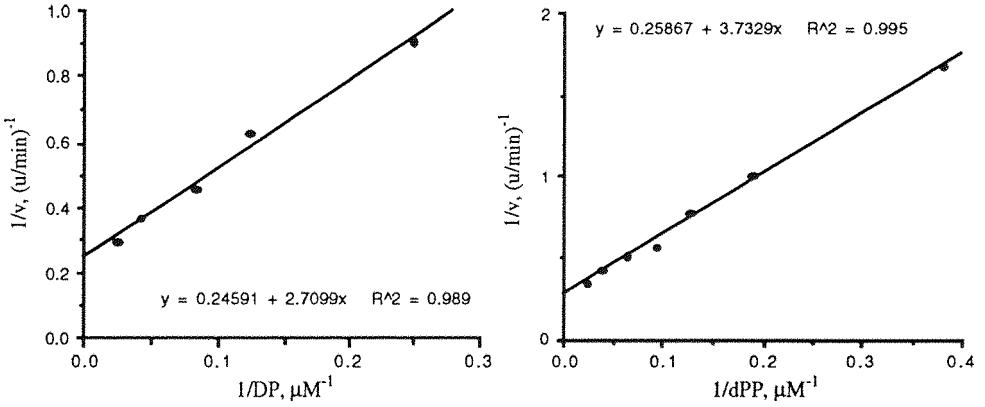


Figure 4.18. Determination of Km values for DP (left) and dPP (right).

The K_m for dPP was similarly determined, varying dPP concentration with fixed DP concentration (fig. 4.18). The value obtained was 19 ± 3 μM.

The K_i value, the dissociation constant of EI, for mrdA was determined by varying inhibitor concentration with fixed substrate concentrations and plotting the reciprocal of the initial rate against inhibitor concentration. The K_i was determined from the slope according to a rearrangement of the initial forward rate equation for a ping pong mechanism in the presence of a competitive dead-end inhibitor, equations [4.4] and [4.5].[149] The value obtained was 36 ± 8 nM (fig. 4.19).

$$\frac{V}{V_{max}} = \frac{[A][B]}{K_{m(B)}[A] + K_{m(A)}[B]\left(1 + \frac{[I]}{K_i}\right) + [A][B]} \qquad \text{.....[4.4]}$$

$$\frac{1}{V} = \left(\frac{1}{V_{max}} + \frac{K_{m(B)}}{[B]V_{max}} + \frac{K_{m(A)}}{[A]V_{max}}\right) + \frac{K_{m(A)}}{[A]V_{max}K_i}.[I] \qquad \text{.....[4.5]}$$

† Errors for kinetic constants were estimated assuming a 10 % error in measurement by hand of initial rates, and a 5 % error in the concentration of dPP. Percentage errors in slopes and intercepts taken from graphs were approximated from the R^2 best fit value. Total errors were estimated by compouding the errors of the parameters used in determination of that constant.

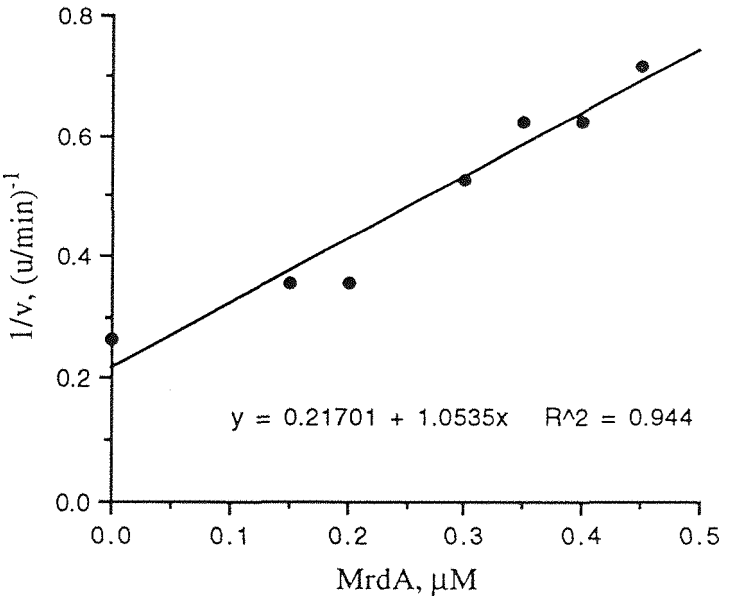


Figure 4.19. Determination of K_i for mureidomycin A.

4.6.8. Measurement of k_{on} and K_i* for mureidomycin A.

The rate constant for the isomerisation of EI to EI*, k_{on}, was determined via measurement of the observed rate constant for the onset of slow-binding inhibtion, k_{obs}, at different concentrations of inhibitor, mrdA. This was achieved by constructing a family of progress curves, an example of which is shown in fig. 4.20.

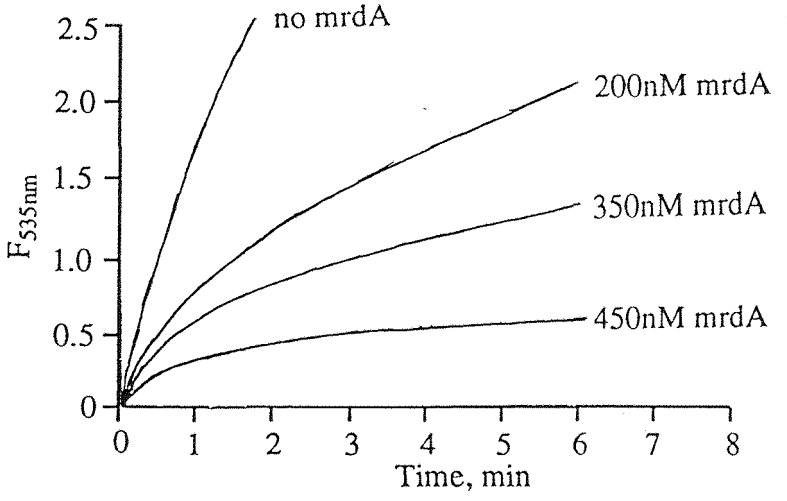


Figure 4.20. Progress curves in the presence of increasing concentrations of mureidomycin A.

The range of inhibitor concentration used was 50 nM - 425 nM. The approach towards the steady-state final rate is negatively exponential and is given by the equation [4.6] where v is the rate at time, t and v_o and v_s and the initial and steady-state final rates.[148] Rearranging and taking the natural logarithm of both sides gives equation [4.7].

$$v = v_s + (v_o - v_s)e^{-k_{obs}t} \qquad \text{.....[4.6]}$$

$$\ln|(v - v_s)(v_o - v_s)| = -k_{obs}t \qquad \text{.....[4.7]}$$

For each progress curve, v was measured (by ruler) at times, t and ln /(v - v_s)(v_o - v_s)/ plotted against t (appendix 3). The slope of the straight line is -k_{obs}. k_{obs} is related to k_{on} and k_{off} by equation [4.8].[148]

$$k_{obs} = k_{off} + k_{on}\left[\frac{[I]/K_i}{1+[A]/K_{m(A)}+[I]/K_i}\right] \qquad \text{.....[4.8]}$$

$$= k_{off} + k_{on}.f(I)$$

The values obtained for k_{obs} were plotted against f(I) using values of K_i and $K_{m(A)}$ as determined above (fig. 4.21).

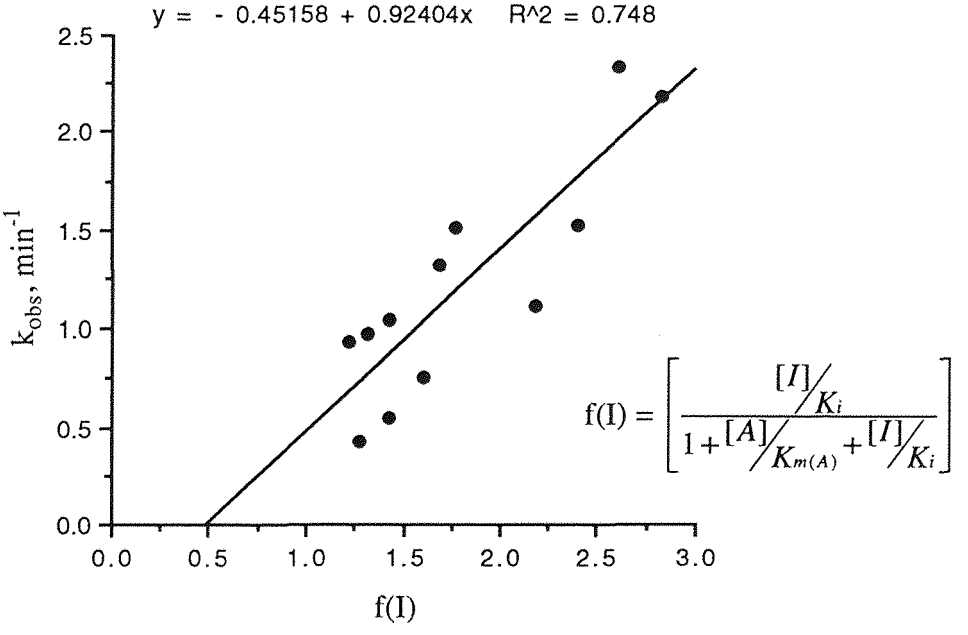


Figure 4.21. Determination of the rate constant, k_{on}, for mureidomycin A.

The slope is k_{on}, 0.92 ± 0.4 min^{-1}. The intercept, k_{off}, is negative. The sign of k_{off} is opposite to that of k_{on} since k_{off} is the rate constant for the isomerisation of EI* to EI, i.e. k_{off} reduces k_{obs}, rather than contributing to it (equation 4.9).

Observed rate of formation of EI*, $k_{obs} = k_{on}[EI] - k_{off}[EI^*]$ .....[4.9]

Whilst this plot could be used to derive a value for k_{off}, the rate constant for the regain of activity in the absence of unbound inhibitor, one is not quoted because this rate constant should be determined experimentally.

The family of progress curves was also used to derive a value for K_i^*, the dissociation constant for the EI* complex. The constant was determined as for K_i, but using steady-state final rates rather than initial rates (fig. 4.22). This is based on the assumption that mrdA is competitive with respect to dPP in the EI* complex.

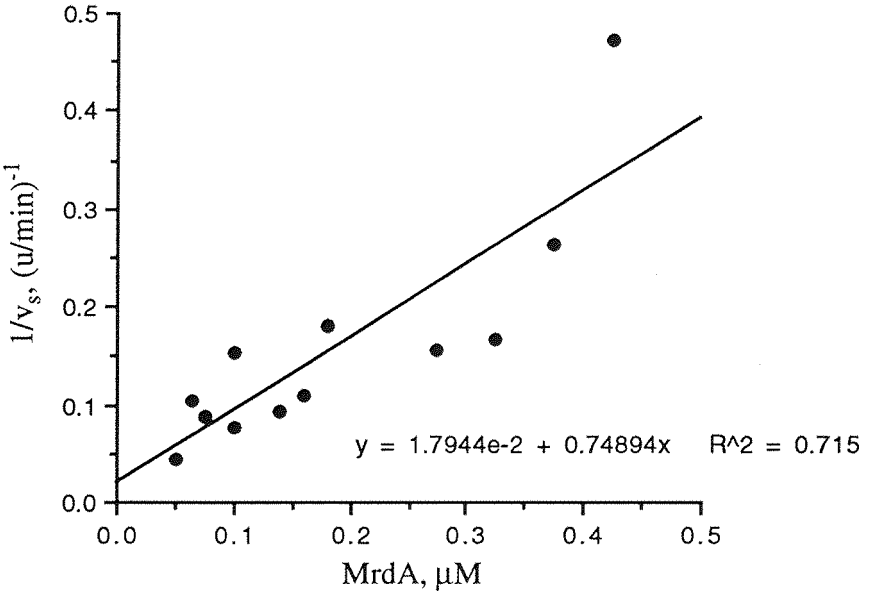


Figure 4.22. Determination of K_i^* for mureidomycin A. Since the family of progress curves was constructed using three different enzyme concentrations, rates are expressed as a percentage of the no inhibitor control initial rate. V_{max} was 125 % of that rate.

The value obtained for K_i^* was 2 ± 0.65 nM. This is 18-fold lower than K_i reflecting the tighter association in the EI* complex compared to the EI complex.

4.6.9. Measurement of k_{regain} for mureidomycin A.

The key feature of slow-binding inhibition which distinguishes it from irreversible inhibition is that if inactivated enzyme is separated from unbound

inhibitor, a time-dependent regain of activity is observed. Since no method is available for the quantitation of mrdA in a mixture containing protein, lipids, detergent, etc; these experiments were conducted by trial and error - carrying out a separation experiment and looking for time-dependent regain of activity.

Since no regain of actvity was seen after 30 min in the 'dilution of inhibitor' experiment (4.6.3), the first experiment was dialysis of mrdA-inactivated enzyme against 100 volumes of 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol, 0.2 % Triton X-100 for 3 hr at 4 °C. Enzyme not treated with mrdA was similarly dialysed. The activity of the dialysed mrdA-treated sample was 5 % of that of the control and no further activity returned in the next 72 hr as documented in the experimental section. It is possible that mrdA associates with the detergent micelles in which case it might not have been removed by dialysis, hence no return of activity.

Gel filtration of mrdA-treated translocase I on sephadex G75 equilibrated with 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 0.2 % Triton X-100 at 4 °C was tested as a method of separating enzyme from unbound inhibitor. The exclusion limit of this resin is 80 kDa for proteins. Triton X-100 can add at least 40 kDa to the apparent M_r of a protein due to the large size of the micelles.[126] Therefore, it was expected that translocase I would elute in the void volume (39.5 kDa + 40 kDa) which it did as determined in a control experiment. In this experiment translocase I was inactivated in 5 min with 100 nM mrdA on a 3 mL scale as described in the experimental chapter. When applied to the column, the activity of the mrdA-treated enzyme was 4 % of the initial activity of the control. Gel-filtered mrdA-treated enzyme had 25 % of the activity of the control immediately after gel filtration (approximately 20 min to complete). This activity did not increase at all over 21 hr on ice. Thus it seemed that the process had at least partially separated the inhibitor from the enzyme, but that the time-course of regain of activity (if indeed it was time-dependent) was complete after 20 min.

Therefore the rapid desalting method of Penefsky[150] was used to separate enzyme from unbound inhibitor on a 90 s timescale as described in the experimental chapter. Briefly, a 100 µL sample is applied to a 1 mL column (plastic syringe) of sephadex G50 equilibrated with 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 0.2 % Triton X-100 with centrifugation (30 s, 100 *g*) and eluted in 50 µL of the same buffer. Recovery of activity was quantitative. Enzyme was inactivated in a standard assay with 400 nM mrdA on a 600 µL scale. After 5 min the rate was zero and 100 µL aliquots were chilled in an ice-bath and desalted as described in 8.4.6.13. Desalted aliquots (50 µL) were assayed at 2, 5 and 49 min after application ot the spin-columns (chromatographed and held at 4 °C). The initial rate was zero in every case. Again, either unbound mrdA had not been efficiently separated from translocase

I, or the time window examined was not appropriate. It was considered that an impasse had been reached and this line of experiments was not continued, the most likely conclusion being that mrdA associates with the micelles.

4.7. Tunicamycin.

Tunicamycin is a complex of related uridine-containing antibiotics which differ in the chain length and saturation of the fatty acyl chain (fig. 4.23).

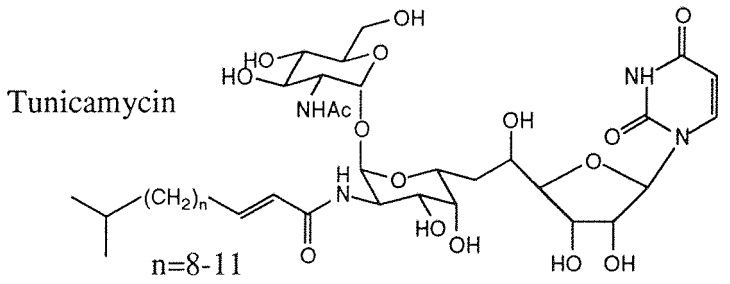


Figure 4.23. Tunicamycin.

A recent study by Inukai *et al;* compared mureidomycin A and tunicamycin for their ability to inhibit synthesis of lipid-linked intermediate I from [^{14}C]-UDPMurNAc-pentapeptide in ether treated cells of *P. aeruginosa.*[72] The IC_{50} values were 0.05 μg/mL for mrdA and 44 μg/mL for tunicamycin, a 600-fold molar difference. Conversely tunicamycin inhibited formation of lipid-linked GlcNAc (part of teichoic acid biosynthesis) with IC_{50} 0.8 μg/mL, whereas mrdA gave only 15 % inhibition at 100 μg/mL. This stark contrast in the specificities of these two antibiotics prompted us to characterise tunicamycin as an inhibitor of *E. coli* translocase I.

Tunicamycin was purchased from Sigma. This preparation was a complex of homologs A-D (3 % A, 36 % B, 38 % C and 20 % D). The concentrations quoted below refer to this complex, whose average M_r is 1000.

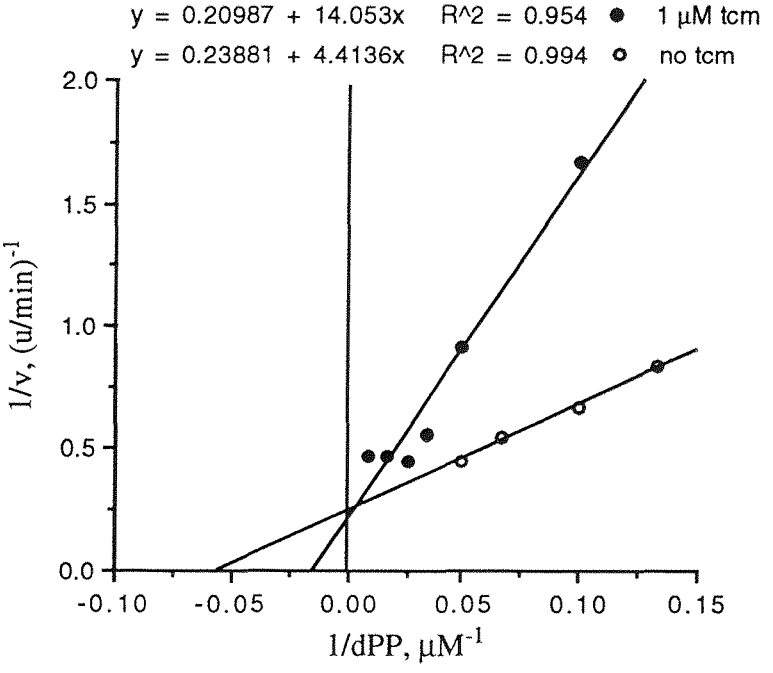
Assays of solubilised translocase I using the fluorescence enhancement assay under standard conditions revealed that assays in the presence of tunicamycin were linear with IC_{50} = 2 μM. To determine whether or not this inhibition was reversible, a 'dilution of inhibitor' experiment exactly analogous to that performed with mrdA in section 4.6.3 was carried out. Solubilised enzyme was assayed for 2 min plus or minus 2 μM tunicamycin; then 30 μL of this reaction mixture was transferred directly to a second assay in an adjacent cuvette, thus diluting enzyme and inhibitor 10-fold. In the control experiment, the second assay mixture contained 0.2 μM tunicamycin so that the second assay mixtures were identical for both control and experiment. The

initial rates were the same in the second assay indicating that inhibition was reversible (table 4.8).

	Initial rates, u/min	
	t = 0 assay	**t = 2 min assay***
Control	3.3	0.33
+ 2 μM tunicamycin	1.1	0.33

Table 4.8. Reversibility of inhibition of translocase I by tunicamycin. *Contains 20 % of the enzyme from the t = 0 assay.

The next step in characterising the inhibitor, tunicamycin, was to determine whether it was competitive, non-competitive or uncompetitive with respect to each substrate. Initial rates were measured at different concentrations of dPP with fixed DP (40 μM) in the presence of 0 or 1 μM tunicamycin and at different concentrations of DP with fixed dPP (105 μM) in the presence of 0, 1 or 2 μM tunicamycin. The data was analysed using the Lineweaver-Burk double reciprocal plot (fig. 4.24).



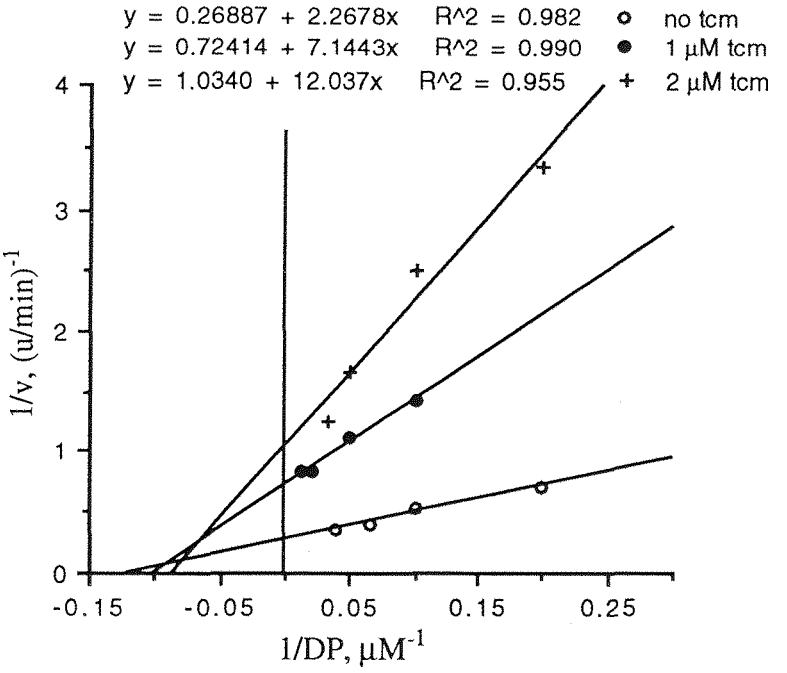


Figure 4.24. Lineweaver-Burk double reciprocal plots in the presence of tunicamycin with varying dPP, fixed DP or varying DP, fixed dPP.

For dPP, adding tunicamycin increased the K_m, but did not decrease V_{max}. Thus tunicamycin is a competitive inhibitor with respect to dPP. For DP, adding tunicamycin did not alter K_m, but did reduce V_{max}. Thus tunicamycin is a non-competitive inhibitor with respect to DP.

For a reversible competitive inhibitor, the K_i can be determined by plotting the slope of the double reciprocal plot against the inhibitor concentration (equation 4.10).

$$\frac{K_{m(app)}}{V_{max}} = \frac{K_m}{V_{max}}\left(1+\frac{[I]}{K_i}\right) = \frac{K_m}{V_{max}} + \frac{K_m}{V_{max}K_i}.[I] \qquad .....[4.10]$$

The K_i can similarly be obtained for a non-competitive inhibitor by plotting the abscissal intercept against the inhibitor concentration (equation 4.11).

$$\frac{1}{V_{max(app)}} = \frac{1+{}^{[I]}/_{K_i}}{V_{max}}\left(1+\frac{K_{m(A)}}{[A]}\right) = \frac{[A]+K_{m(A)}}{[A]V_{max}} + \frac{[A]+K_{m(A)}}{[A]V_{max}K_i}.[I] \qquad .....[4.11]$$

The values obtained for K_i (ordinate intercept = $-K_i$ in both cases) were 380 ± 80 nM from the dPP data set and 734 ± 160 nM from the DP data set (fig. 4.25).

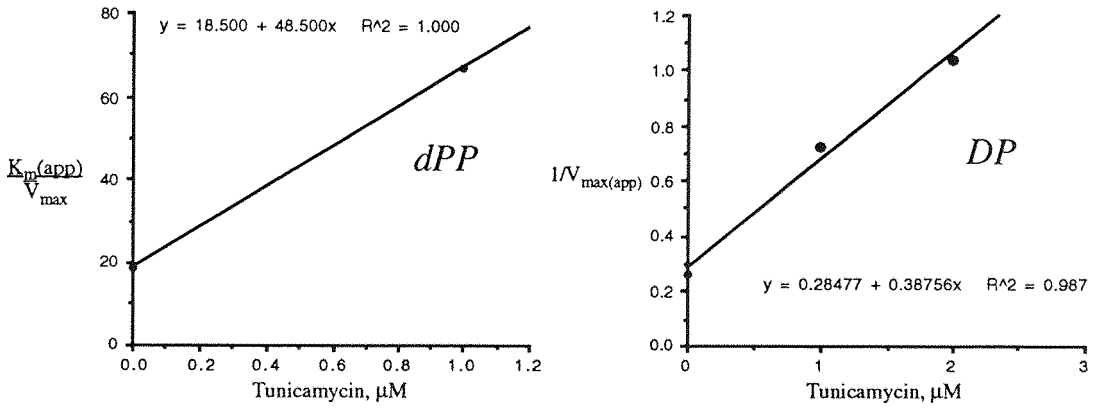


Figure 4.25. Replots to determine K_i for tunicamycin from dPP (left) and DP (right) double reciprocal plots.

Derivations used in the determination of K_i for competitive and non-competititive and non-competitive inhibitors in a two substrate ping pong system are given in appendix 4. The quality of the data obtained with varying DP, fixed dPP was somewhat better than with varying dPP, fixed DP indicating that the value for K_i, 734 nM is the more accurate one.

The only other study of tunicamycin as an enzyme inhibitor in greater detail than IC_{50} values is that of Heifetz and co-workers with the pig aorta GlcNAc-1-phosphate transferase.[73] This enzyme transfers GlcNAc-1-phosphate from UDPGlcNAc to dolichyl phosphate. It is proposed in chapter 6 that enzymes catalysing this reaction are part of an evolutionary superfamily of enzymes with analagous catalytic function of which translocase I is a member. This study found, using a radiochemical stopped assay, that tunicamycin was non-competitive with respect to both UDPGlcNAc and dolichyl phosphate and that inhibition was irreversible. This last conclusion was based on the inability to remove tunicamycin by dialysis or rapid desalting by gel filtration. Our study has found tunicamycin to be non-competitive with the lipid acceptor substrate also, indicating that the hydrophobic 'tail' of tunicamycin is more likely to be involved in localisation of the antibiotic to the membrane rather than binding at the active-site. Tunicamycin is then most probably micelle associated (Heifetz was using partially purified NP-40-solubilised enzyme) which makes it unlikely to be removed by dialysis or desalting, as may have been the case with mrdA. It may of course be that tunicamycin interacts differently with the two enzymes.

4.8. Liposidomycin B.

The liposidomycins are nucleoside antibiotics containing uracil, a fatty acid, a seven-membered heterocyclic ring and a sulfated amino-sugar (section 1.4.2). Liposidomycin B (fig. 4.26, 1.14 mg) was a gift from Dr. Ken-ichi Kimura of Snow Brand Milk Products Co. Ltd., Japan.

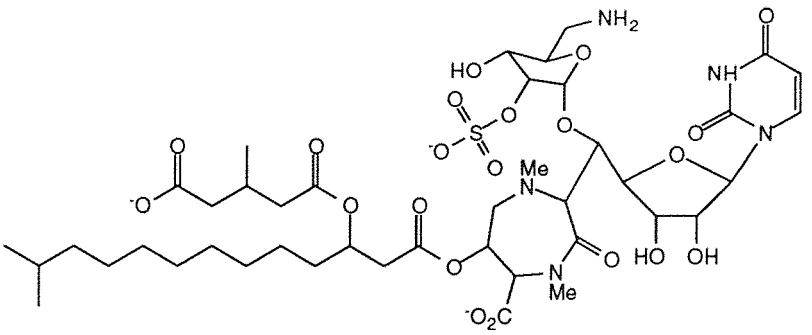


Figure 4.26. Liposidomycin B.

Dr. Kimura included HPLC data for this material so it was used in assays without further verification.

Assays of solubilised translocase I using the FEA under standard conditions revealed apparently linear inhibition by liposidomycin B (lipB) with an IC_{50} of 250 nM. To determine whether or not this inhibition was reversible, a 'dilution of inhibitor' experiment exactly analagous to that performed with mrdA in section 4.6.3 was carried out. Solubilised enzyme was assayed plus or minus 250 nM lipB for 2 min before 30 µL of the reaction mixture was transferred directly to a second assay in an adjacent cuvette thus diluting enzyme and inhibitor 10-fold. In the control experiment, the second assay contained 25 nM lipB so that the second assay mixtures were identical. The second assay initial rate in the positive experiment was less than that in the control (table 4.9) indicating that inhibition was only partially reversible.

	Initial rates, u/min	
	t = 0 assay	**t = 2 min assay***
Control	2.90	0.40
+ 250 nM lipB	0.45	0.15

Table 4.9. 'Dilution of inhibitor' experiment for liposidomycin B. *Contains 20 % of the enzyme from the t = 0 assay.

If the inhibition is not reversible, then we would expect to see time-dependent inhibition in a standard assay. That was not the case. However, upon re-examination of earlier assay traces, it was apparent that the first 5 - 10 s of the assays were sometimes not linear. It was unclear whether or not this was an artefact since the first few seconds of an assay are awkward to measure because the data from the fluorimeter is not immediately smooth (a common feature in spectroscopy-based assays).

Solubilised translocase I was assayed on a 600 µL (double) scale in the presence or absence of 500 nM lipB for 10 min. Enzyme treated this way was inactive after this time. The reaction mixtures were chilled on an ice-bath and dialysed against 100 volumes of 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol, 0.2 % Triton X-100 at 4 °C for 3 hr. The dialysed samples were placed on ice. There was no activity in the lipB-treated sample up to 18 hr after dialysis. It is quite likely that lipB is micelle associated on account of its fatty acyl chain. Since dialysis might not remove the inhibitor in this case, absence of activity in the dialysed lipB-treated sample does not mean that inhibition is not reversible.

The 'loss of potency' experiment described for mrdA was carried out with lipB. The protocol was identical to that used with mrdA in 4.6.6. LipB was not affected by the boiling step. Solubilised translocase I was incubated with 300 nM lipB for 5 min, a 100 µL aliquot boiled and centrifuged and 80 µL of the supernatant added to a standard assay of 5-fold diluted enzyme. The activity was identical to that of the control where lipB was introduced in the second part, not the first, indicating that lipB did not lose any potency upon incubation with translocase I.

We now suspected that inhibition of translocase I by lipB was time-dependent, but that a steady-state was reached after only 5 - 10 s, preventing us from detecting it with certainty. That a steady-state is reached rather than complete inhibition implies slow-binding inhibition, as was seen with mrdA. We wondered if the suspected time-dependence could be detected using the reverse reaction as with mrdA (4.6.5). A standard enzyme assay was allowed to proceed to equilibrium (3 min). LipB was added in 6 µL to 200 nM and at times after that UMP was added in 6 µL to 1 mM. The initial reverse rates were measured (fig. 4.27).

The result of this experiment is strikingly similar to that obtained with mrdA (fig. 4.14). Loss of activity is time-dependent but does not appear to go to completion. Thus it seems that lipB is a slow-binding inhibitor also. In this case however, the onset of inhibition is comparatively fast making it inaccessible to kinetic analysis with the tools in hand. This conclusion implies that the linear rates seen in the forward assay are steady-state final rates. In this case, a K_i determined for the inhibition would actually be the K_i^* constant.

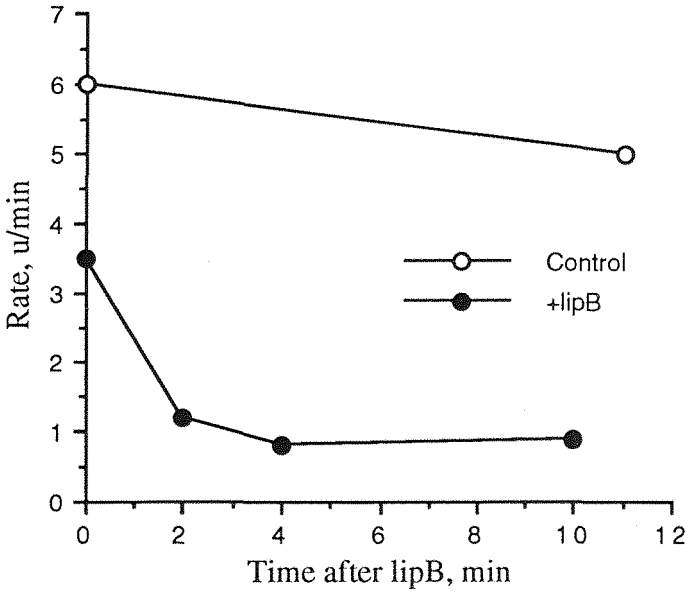


Figure 4.27. Decay of translocase I activity in the presence of liposidomycin B using the reverse reaction.

This analysis was carried out. Initial rates were measured at varying concentrations of DP with fixed dPP (105 μM) in the presence of 0, 100 or 200 nM lipB and at varying concentrations of dPP with fixed DP (40 μM) in the presence of 0, 100 or 200 nM lipB. The data was analysed using the Lineweaver-Burk double reciprocal plot (fig. 4.28).

With DP, increasing inhibitor concentration increased K_m, but V_{max} was unaffected. Thus lipB is a competitive inhibitor with respect to DP. With dPP, increasing inhibitor concentration decreased V_{max}, but K_m was unaffected. Thus lipB is a non-competitive inhibitor with respect to dPP. The results implt that lipB binds at the DP binding site, which is credible given the fatty acyl chain it contains. They also imply that it does not compete with dPP, which is odd given that lipB contains uridine as does dPP. However, as discussed above, it seems likely that we are considering the EI* complex rather than the EI complex (fig. 4.11). It may be that lipB is competitive with dPP in the reversible formation of EI from enzyme plus inhibitor, but as EI isomerises to EI*, lipB ceases to be competitive with dPP. Values of K_i* were determined from replots of the Lineweaver-Burk plots as for tunicamycin (fig. 4.29).

The values of K_i* obtained from the ordinate intercepts were 78 ± 16 nM and 207 ± 45nM from the DP and dPP data respectively. Since both data sets are of a high quality, an average of the two values was taken, i.e. 143 ± 30 nM. Thus liposidomycin

B is more potent than tunicamycin (K_i, 734 nM), but less potent than mureidomycin A (K_i, 36 nM).

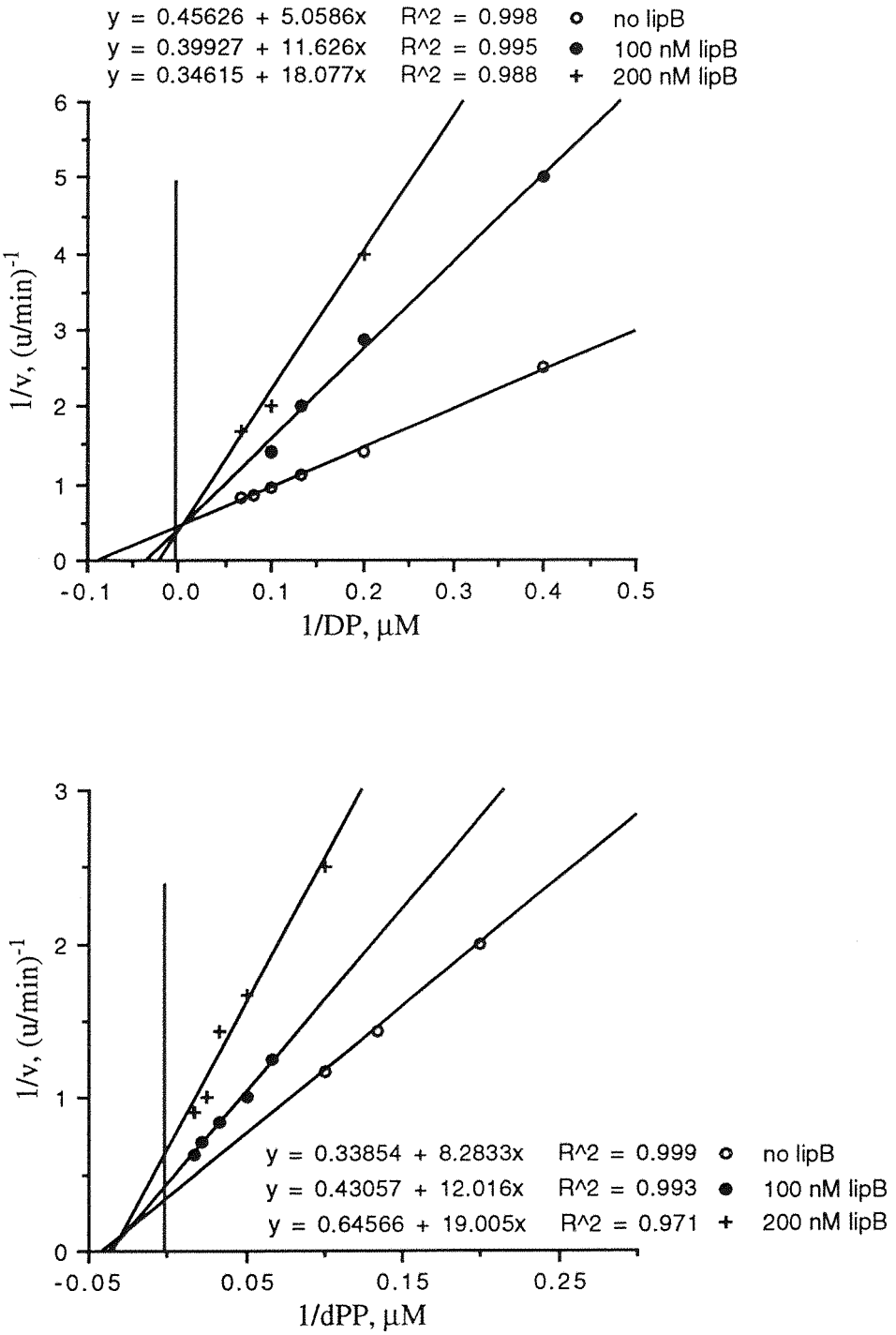


Figure 4.28. Lineweaver-Burk double reciprocal plots in the presence of liposidomycin B with varying DP, fixed dPP or varying dPP, fixed DP.

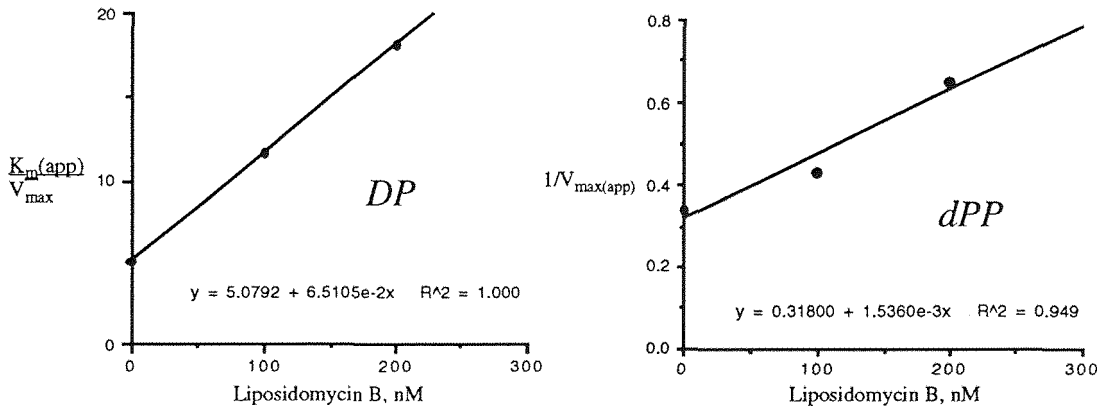


Figure 4.29. Replots to determine K_i* for liposidomycin B from double reciprocal plots for DP (left) and dPP (right).

4.9. Summary.

A continuous fluorescence enhancement assay for translocase I activity has been developed based on the work of Weppner and Neuhaus. It has been optimised to permit a kinetic evaluation of the inhibitors mureidomycin A, tunicamycin and liposidomycin B. The results of this work are summarised in table 4.10.

Inhibitor	**Type of inhibition**	**Type of competition**		**K_i, nM**	**K_i*, nM**
		wrt dPP	**wrt DP**		
Mureidomycin A	Slow-binding	comp.[a]	comp.[a]	36	2
Tunicamycin	Reversible	comp.	non-comp.	734	-
Liposidomycin B	Slow-binding	non-comp.[b]	comp.[b]	n.d.	143

Table 4.10. Inhibition of translocase I by mureidomycin A, tunicamycin and liposidomycin B. [a]For the reversible formation of EI. [b]For slow-binding formation of EI*. comp. = competitive, non-comp. = non-competitive.

All three natural products are potent inhibitors of the detergent-solubilised *E. coli* translocase I. Inukai and co-workers reported that in ether-treated cells of *P. aeruginosa*, mureidomycin A was 600-fold more potent than tunicamycin (mole for mole).[72] In our assay system, mureidomycin A is 20 to 370-fold more potent than tunicamycin. The slow-binding aspect of the mureidomycin inhibition makes comparison awkward, but there is an approximate agreement with the literature precedent. Liposidomycin B is more potent than tunicamycin, but less potent than mureidomycin A.

Examination of the slow-binding inhibition by mureidomycin A was hampered by the inability to separate enzyme from inhibitor. Since this seems likely to have been caused by interaction of the antibiotic with detergent (similar problems were encountered with tunicamycin and possibly liposidomycin B), it may be that the problem could be solved by changing detergent to one which forms low aggregation number micelles which have little or no effective interior, such as the bile acids. However, it is this property which makes these detergents (i.e. CHAPS) unsuitable for use in the fluorescence enhancement assay, at least for the present.

Availability of radiolabelled inhibitor (e.g. tritiated via exchange with T_2O) would allow the experimenter to know with certainty where the inhibitor was. This would reduce the number of unknowns in future investigations. This is perhaps wishful at present given that the mureidomycins and especially the liposidomycins are very precious.

The implications of the results in this chapter as to mechanisms of inhibition will be explored in chapter 7.

Chapter 5. Evidence for a Covalent Intermediate in the Reaction Catalysed by Translocase I.

5.1. Introduction.

Evidence for two possible mechanisms for the reaction catalysed by translocase I was discussed in chapter 1 (1.5.3) (fig. 5.1). The question addressed in this chapter is whether there is a covalent enzyme-linked intermediate or not. If there is then it is very likely that the reaction proceeds in two steps by the double displacement mechanism. In strict terms the chemical competence of an intermediate does not necessarily entail kinetic competence.

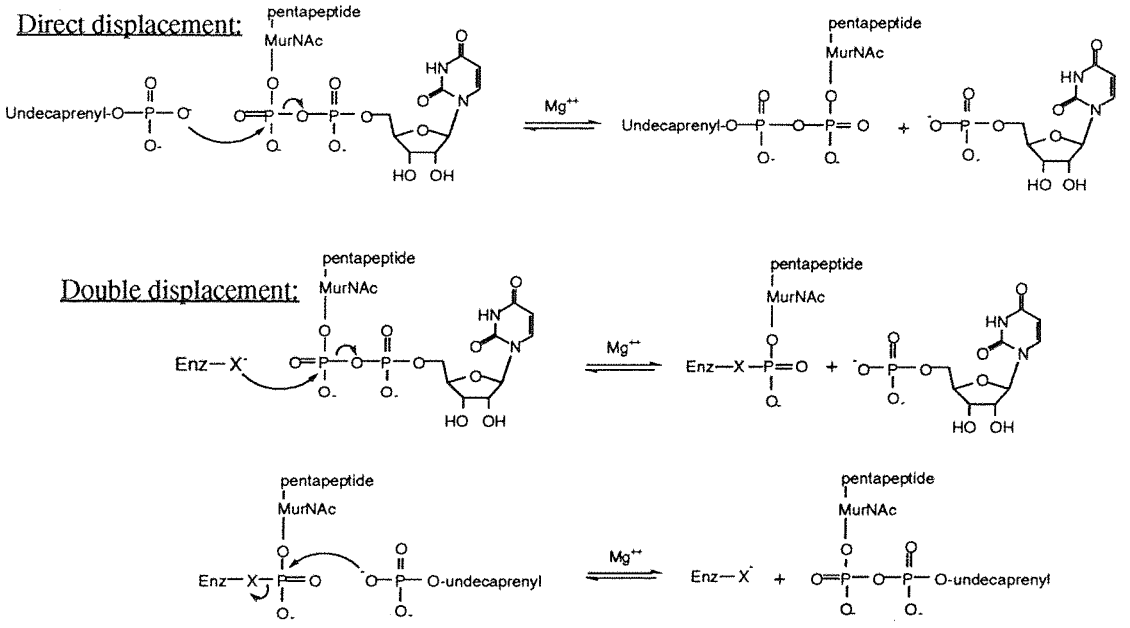


Figure 5.1. Direct and double displacement mechanisms for the reaction catalysed by translocase I.

Results obtained with the *S. aureus* enzyme by Neuhaus lend support to the double displacement mechanism, but do not prove it. The enzyme catalyses the exchange of the UMP moiety of UDPMurNAc-pentapeptide with free UMP. This arises through the reversibility of the enzyme catalysed reaction, not by virtue of its mechanistic course. With *S. aureus* particulate enzyme, the rate of exchange is 6 - 24 times the rate of transfer.[107] As discussed in 1.5.3, the observation can only be reconciled with the direct displacement mechanism if dissociation of undecaprenyl diphospho-MurNAc-pentapeptide from the enzyme is the rate-limiting step. If it could be demonstrated that the enzyme can catalyse the exchange reaction (fig. 5.2) in the

absence of lipid acceptor (undecaprenyl phosphate), this would be convincing evidence in favour of the double displacement mechanism.

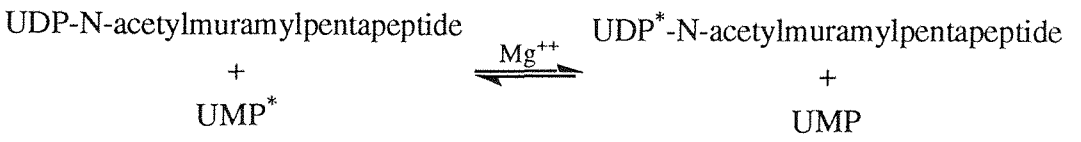


Figure 5.2. Isotope exchange reaction catalysed by translocase I.

Heydanek and Neuhaus attempted just this by extracting lyophilised membranes with organic solvent at -15 °C.[107] When resuspended in 5 mM Tris buffer at pH 7.8, this lipid-depleted preparation could catalyse both the exchange reaction and the release of UMP (called transfer assay B) from UDPMurNAc-pentapeptide. However, undecaprenyl phosphate need only be present in catalytic quantities to allow the reaction to proceed back and forth. Therefore, to use this isotope exchange data as evidence for the double displacement mechanism, one would also have to demonstrate the absence of undecaprenyl phospahte from the preparation, not a trivial undertaking.

A further complication, reported in the same paper is that the authors demonstrate the ability of the particulate enzyme preparation (be it translocase I or another protein) to hydrolyse UDPMurNAc-pentapeptide to phospho-MurNAc-pentapeptide and UMP. Therefore the assertion by the authors that release of UMP from UDPMurNAc-pentapeptide by the lipid-depleted enzyme preparation indicates the presence of undecaprenyl phosphate is not valid.

Our research has taken a direct approach to the problem. If this intermedaite could be detected rather than inferred, then we would have conclusive evidence for the double displacement mechanism. If no intermediate could be detected, the matter would remain unresolved. Since UDPMurNAc-pentapeptide carrying a carbon-14 radiolabel in the peptide part of the molecule was available, the strategy was to attempt detection of the proposed intermediate enzyme-phospho-MurNAc-L-Ala-γ-D-Glu-*m*-DAP-[^{14}C]-D-Ala-[^{14}C]-D-Ala.

5.2. Precipitation of radiolabelled species.

If the translocase I catalysed reaction proceeds via the double displacement mechanism as the evidence discussed above suggests, then short incubation of solubilised enzyme with UDPMurNAc-L-Ala-γ-D-Glu-*m*-DAP-[^{14}C]-D-Ala-[^{14}C]-D-Ala should result in the following radiolabelled species:

1. UDPMurNAc-[^{14}C]-pentapeptide
2. phospho-MurNAc-[^{14}C]-pentapeptide

3. lipid-PP-MurNAc-[^{14}C]-pentapeptide
4. enz-P-MurNAc-[^{14}C]-pentapeptide

Rapid addition of acetone to the incubation mixture will precipitate the protein and some or all of the lipid-linked product with it. It should be possible to extract the latter from the pellet with a suitable organic solvent. This is essentially the experiment which was carried out (described fully in the experimental chapter).

Enzyme solubilised in 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol, 1 % Triton X-100 was incubated with $MgCl_2$, KCl and radiolabelled substrate at 25 °C for 1 min. The protein was precipitated with an equal volume of acetone (chilled to -20 °C) and the pellet washed twice with 50/50 100 mM Tris pH 7.0/acetone. The pellet was then extracted three times with 2:1 $CHCl_3$/MeOH. Radioactivity was measured in the pellet and each of the supernatants. A control contained 6 mg/mL BSA in place of solubilised translocase I preparation. The results are presented in table 5.1.

	Radioactivity, cpm	
Fraction	**Control**	**Experiment**
Incubation supernatant	159678	137268
Acetone wash supernatant - 1	4300	7958
Acetone wash supernatant - 2	610	6924
$CHCl_3$/MeOH extraction - 1	42	1924
$CHCl_3$/MeOH extraction - 2	26	794
$CHCl_3$/MeOH extraction - 3	33	426
Final pellet	186	5280

Table 5.1. Radioactivity and pellets and supernatant fractions from labelling experiment with [^{14}C]-UDPMurNAc-pentapeptide.

These results implied that radioactivity is present in the pellet which is not readily extracted with 2:1 $CHCl_3$/MeOH. 5280 cpm/min equates to 29.3 pmoles of radiolabelled species. Supposing this were all enzyme-linked intermediate, this would imply the presence of 1.2 µg of stoichiometrically labelled protein. If 1 % of the enzyme population was labelled this would imply 160 µg of translocase I per liter of culture (since enzyme from approximately 0.75 L of culture was used in the experiment). In order to characterise further the pellet-bound radioactivity, the next step was to carry out gel filtration column chromatography which would separate the radiolabelled species according to molecular size.

5.3. Resolution of radiolabelled species by gel filtration chromatography.

For these experiments the detergents used were 3.0 % CHAPS during the labelling experiment and 2 % SDS (to ensure complete solubilisation of the acetone precipitate) during chromatography. 3.0 % CHAPS was used in place of Triton X-100 because the CMC of CHAPS is much higher than that of Triton X-100, 0.49 % *vs.* 0.02 % respectively. Consequently, the two washing steps after precipitation of radiolabelled species would be sure to bring the detergent concentration below its CMC. It was hoped that this would ensure separation of any radiolabelled translocase I and lipid-linked product before the chromatography step.

5.3.1. Chromatography of [^{14}C]-UDPMurNAc-pentapeptide-treated translocase I.

Particulate enzyme (1 mL, 8 mg of protein) was solubilised by addition of an equal volume of Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol, 6 % CHAPS and stirred at 4 °C for 1 hour. Unsolubilised material was removed by centrifugation at 100000 *g* for 30 min at 4 °C. $MgCl_2$ and KCl were added to 25 mM and 50 mM respectively to the solubilised preparation and the mixture brought to 25 °C in a water bath. This was incubated with [^{14}C]-UDPMurNAc-pentapeptide (1.3 µCi, final concentration 4 µM) for 1 min before addition of an equal volume of acetone to precipitate protein. The precipitate was collected by centrifugation and washed twice with 50/50 100 mM Tris pH 7.0/acetone as described in the experimental chapter. The final pellet was dissolved in 50 mM sodium phosphate pH 7.0, 0.2 M NaCl, 2 % SDS with the aid of sonication. A small amount of blue dextran (2 mg) was added as a void volume marker for chromatography. The sample (0.5 mL) was then chromatographed on a column of sephadex G75 (73 x 1 cm, 57 mL bed volume) equilibrated with 50 mM sodium phosphate pH 7.0, 0.2 M NaCl, 2 % SDS at a flow-rate of 10.7 mL/hr. Fractions (1 mL) were collected and radioactivity measured. The void volume was identified by the presence (by eye) of blue dextran in fractions (fig. 5.3).

Two high molecular weight species are present in addition to a low molecular weight species (presumably [^{14}C]-UDPMurNAc-pentapeptide). The exclusion limit of sephadex G75 (fine grade) is 80 kDa for proteins. Enzyme-linked intermedite would have a molecular weight of about 40 kDa and lipid-linked product has a molecular weight of 1731. However both species are extremely hydrophobic and will be associated with SDS micelles. The aggregation number (the number of monomers per micelle) is 60. The M_r of SDS is 288. Therefore the M_r of an SDS micelle is about 17 kDa. Thus if the two species are enzyme-linked intermediate and lipid-

linked product (smaller and larger peaks respectively), they have anomalously high molecular weights, even taking into account association with detergent micelles. The remainder of the experiments in this chapter are concerned with the characterisation of these two peaks.

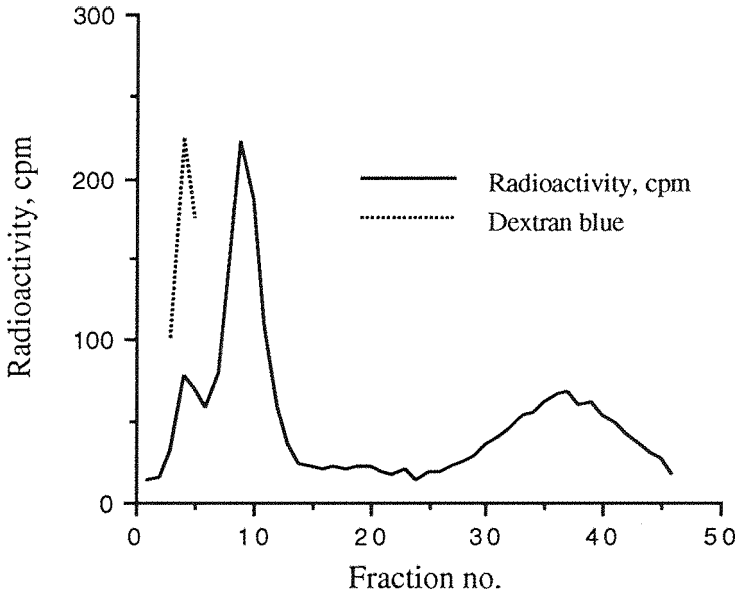


Figure 5.3. Chromatography of radiolabelled species on sephadex G75.

5.3.2. Extraction of the radioactive pellet with 2:1 $CHCl_3$/MeOH.

Translocase I solubilised with 3 % CHAPS was treated with [^{14}C]-UDPMurNAc-pentapeptide exactly as in 5.2.1. The final pellet was extracted three times with 2:1 $CHCl_3$/MeOH with the aid of sonication prior to gel filtration chromatography on the same column of sephadex G75 as in 5.2.1. The elution of radioactivity from the column is presented in fig. 5.4.

The larger peak, suggested above to be lipid-linked product has been reduced 2 - 3-fold, whilst the smaller peak, suggested above to be enzyme-linked intermediate, appears unchanged. This behaviour is consistent with their proposed identities.

5.3.3. Treatment of radiolabelled species with UMP.

A new batch of membranes was used in the remainder of the experiments in this chapter. Two lots of translocase I solubilised in 3 % CHAPS were treated with [^{14}C]-UDPMurNAc-pentapeptide as in 5.2.1. One was treated with 10 mM UMP for 1 min at 25 °C directly after the 1 min incubation with [^{14}C]-UDPMurNAc-pentapeptide as described in the experimental chapter. The rationale here is that the vast excess of UMP should have chased all the radiolabel present as intermediate or product back to starting material. The pellets were dissolved in 50 mM sodium

phosphate pH 7.0, 0.2 M NaCl, 2 % SDS and chromatographed on sephadex G75 as in 5.2.1. The results are compared in fig. 5.5.

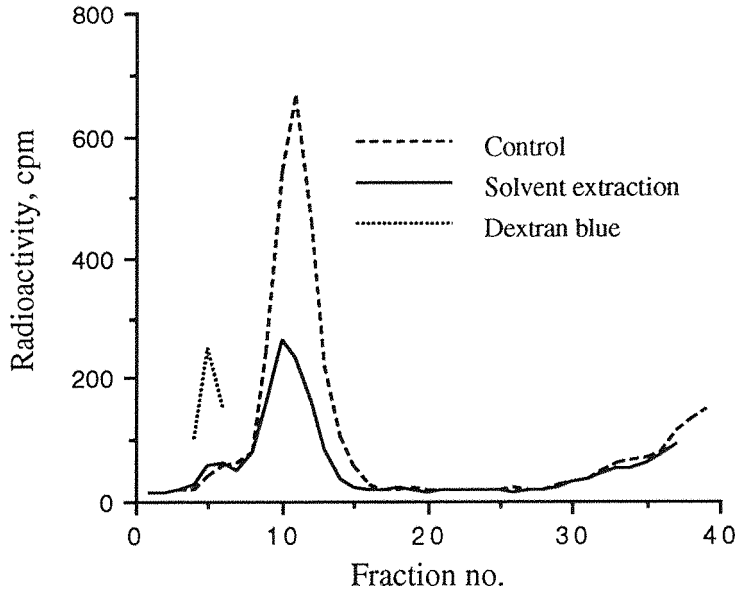


Figure 5.4. Elution of radioactivity from sephadex G75 with or without extraction with 2:1 $CHCl_3$/MeOH prior to chromatography.

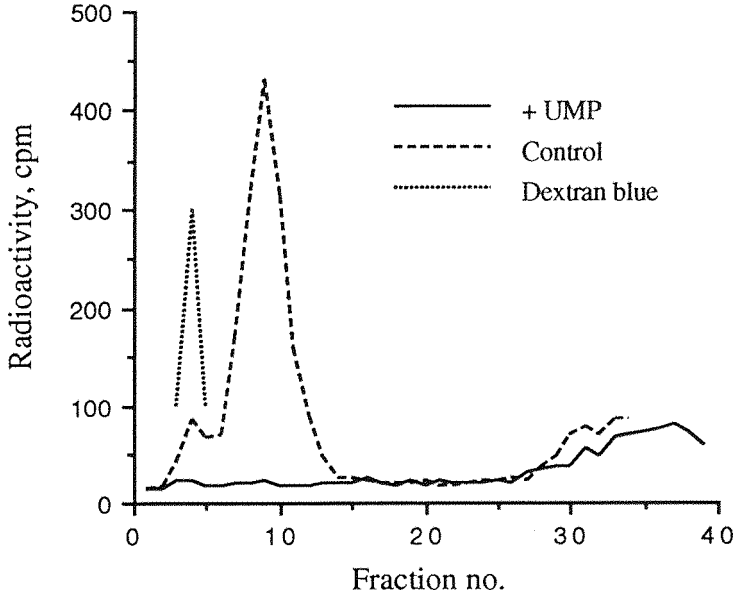


Figure 5.5. Elution of radioactivity from sephadex G75 with or without incubation with 10 mM UMP.

The two high molecular weight species were not present after incubation with 10 mM UMP. This shows that these two peaks have not arisen through some association of [^{14}C]-UDPMurNAc-pentapeptide with a high molecular weight complex of perhaps protein/lipid/detergent. The absence of the two peaks following treatment with UMP is consistent with their proposed identities.

5.3.4. Treatment of native translocase I with hydroxylamine after radiolabelling.

Hydroxylamine is a carbonyl-specific reagent in the context of protein chemistry which has previously been used in the investigation of proposed acyl-phosphate intermediates.[151] The rationale behind the present experiment is that we suspect that the proposed enzyme-linked intermediate arises through attack of an active-site carboxylate on the phosphodiester bond of the substrate, UDPMurNAc-pentapeptide. This would generate an acyl-phosphate. This idea is discussed at length in chapter 6 as is the supporting evidence. Reaction of hydroxylamine with an acyl-phosphate will generate a hydroxamic acid (fig. 5.6) in the translocase I active-site resulting in loss of radiolabel and inactive enzyme.

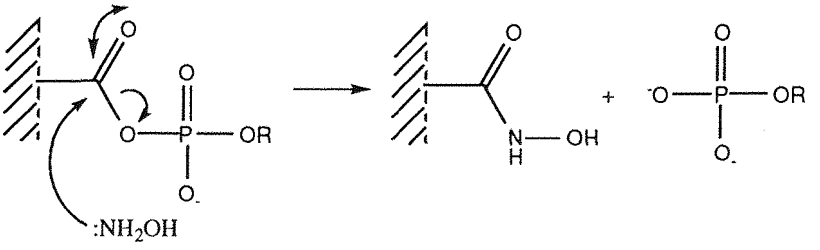


Figure 5.6. Reaction of hydroxylamine with the proposed acyl-phosphate intermediate.

In this experiment membranes from the same batch as in 5.2.3 were solubilised as in 5.2.1. The 100000 *g* supernatant was adjusted to pH 9.5 with 3 M NaOH and incubated with $MgCl_2$, KCl and [^{14}C]-UDPMurNAc-pentapeptide for 15 s before addition of 1.54 M hydroxylamine pH 9.5 to 100 mM as described in the experimental chapter. The mixture was incubated at 25 °C for a further 105 s before precipitation and column chromatography as in 5.2.1. The column data are presented in fig. 5.7.

The elution profile was essentially unchanged by treatment with hydroxylamine. It had been expected that this treatment with hydroxylamine would selectively remove the smaller peak, radioactive lipid-linked product being generate in the first 15 s before addition of hydroxylamine. It might be that an acyl-phosphate was present, but that it was not accessible to the hydroxylamine by virtue of its being

in the active-site. If that is the case, then the acyl-phosphate should become accessible to the hydroxylamine upon denaturation/unfolding of the enzyme.

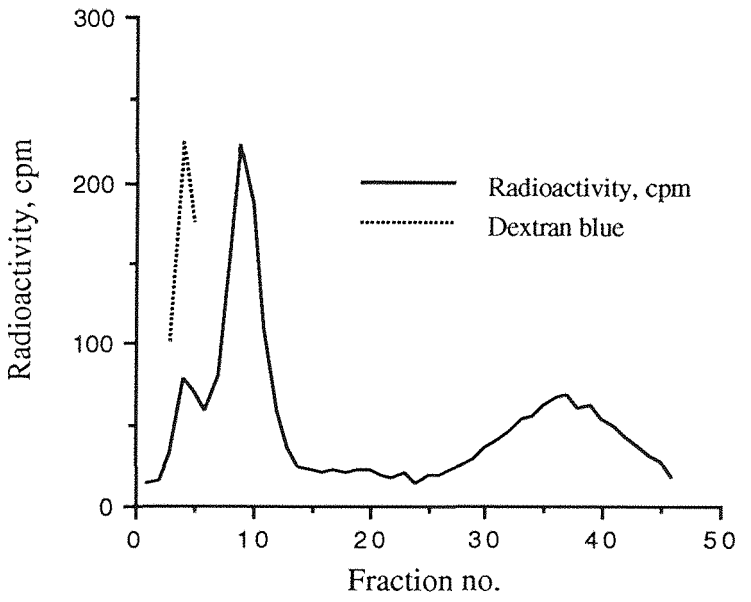


Figure 5.7. Elution of radioactivity from sephadex G75 after treatment of native translocase I with 100 mM hydroxylamine at pH 9.5.

5.3.5 Treatment of denatured translocase I with hydroxylamine after radiolabelling.

Membranes from the same batch as in 5.3.3 were solubilised, treated with [^{14}C]-UDPMurNAc-pentapeptide, precipitated and the final pellet dissolved in 50 mM sodium phosphate, 0.2 M NaCl, 2 % SDS exactly as in 5.3.1. At this point the pH was adjusted to pH 9.5 with 3 M NaOH and 1.54 M hydroxylamine pH 9.5 was added to 100 mM. The mixture was allowed to stand at room temperature for 15 min before chromatography on the same column of sephadex G75 as was used for all the experiments in 5.3 as in 5.3.1. The column data from this experiment are compared with that from the control in 5.3.3 (same batch of membranes) in fig. 5.8.

The larger peak (proposed to be lipid-linked product) was unaffected by the hydroxylamine treatment whereas the smaller peak (proposed to be enzyme-linked intermediate) is reduced to the point where it cannot be distinguished from the larger peak. It is not known whether it was the hydroxylamine or the basic conditions which caused the disappearance of this peak. In either case, the behaviour of the two peaks under these conditions is consistent with their proposed identities since pyrophosphates are not labile to hydroxylamine or mild base, whereas acyl-phosphates are.[151]

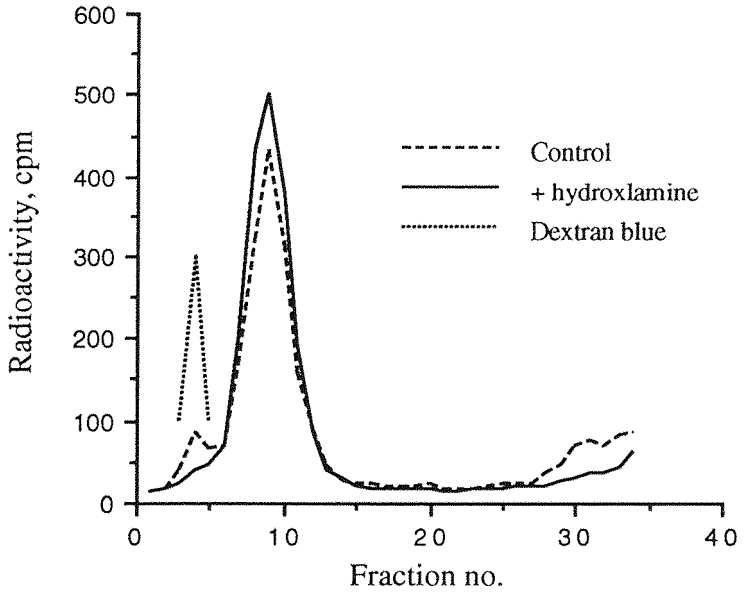


Figure 5.8. Elution of radioactivity from sephadex G75 with or without treatment with 100 mM hydroxylamine after dissolution of the final pellet in 50 mM sodium phosphate, 0.2 M NaCl, 2 % SDS.

5.4. Summary.

Interpretation of the above data requires caution on account of the low levels of radioactivity being considered. However, the results are reproducible and consistent with the proposed identities of the peaks. Having completed these experiments it was later realised that a useful control experiment would have been to exercise the protocol on a solubilised preparation of *E. coli* JM109 (pTrc99A), the empty expression vector. If the above assertions as to the identities of the peaks are correct, this experiment would give the same result, but with a 30-fold reduction (the level of overexpression) in radioactivity incorporated. This would make the small peak indistinguishable from the baseline and would place the larger peak on the borderline of detectability. By this time, room temperature ranged from 16 - 22 °C and it was discovered that SDS was not soluble in 50 mM sodium phosphate pH 7.0, 0.2 M NaCl at these temperatures. In fact, the critical micellar temperature (CMT), the lowest temperature at which a given detergent can form miceles, for SDS in 50 mM sodium phosphate pH 7.4, 0.1 M NaCl is 23.5 °C.[152] Since warm room facilities were not available, this control experiment could not be performed.

An alternative approach towards detection of the enzyme-linked intermediate would be to use UDPMurNAc-pentapeptide carrying a ^{32}P radiolabel (fig. 5.9).

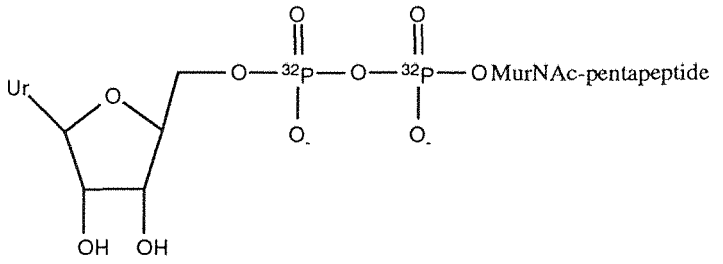


Figure 5.9. [^{32}P]-UDPMurNAc-pentapeptide.

Methodology for the preparation of this material has been described by Anderson and co-workers.[116]. The enzyme-linked intermediate would then be labelled with ^{32}P instead of ^{14}C. Since the specific activity of ^{32}P is, mole for mole, about 10000 times greater than that of ^{14}C, the intermediate generated should contain enough radoactivity to permit SDS-PAGE followed by autoradiography. If the hypothesis is sound, this would give a band around 40 kDa. The main disadvantage of this experiment is the large (1 - 5 mCi) quantities of radioactivity required in the generation of the radiolabelled substrate.

In summary, the results obtained in this series of experiments are consistent with the existence of a covalent enzyme-linked intermediate, implying that the translocase I catalysed reaction follows the double displacement mechanism. It is hoped that this methodology can be utilised in the future in conjunction with purified enzyme to fully elucidate the mechanism of the reaction.

Chapter 6 - Evidence for Active-site Carboxylates.

6.1. Introduction.

The advent of molecular biology has allowed the rapid cloning and sequencing of genes which code for proteins of interest to biological chemists. Knowing the DNA sequence of a gene allows inference of the amino acid sequence of the protein encoded by that gene. Over time a large amount of data relating primary amino acid sequence to the structure and function of proteins has been collected such that it is now possible to make educated predictions about the nature of the protein from its amino acid sequence and alignments with amino acid sequences of functionally related proteins.

In terms of structure these include predictions as to whether the protein is likely to be soluble or membrane bound and which parts are likely to form recognised secondary structural features such as α-helices and ß-sheets.

In terms of function, sequence similarity to known proteins can give an indication as to what the protein does. For example, finding a sequence motif characteristic of an NADH-binding domain would suggest involvement in a redox reaction. It is becoming increasingly common that the sequence of a new protein of unknown function bears sufficient similarity to a previously characterised protein from another organism that it is possible to assign the function of the new protein with a high degree of confidence. Several such examples appear in the following section.

When the sequences of several functionally related proteins are compared at once it rapidly becomes apparent that certain individual amino acid residues are constant. If an amino acid residue which is critical to structure or function is mutated during the course of evolution, this will result in an inactive protein and possibly the demise of the organism. Consequently, catalytic residues and residues of key structural importance are conserved. Hence conserved residues revealed by multiple alignments often have important functions.

The presence of known consensus sequences for protein export/targetting such as a leader sequence (e.g. KDEL) or a glycosylation site or an isoprenylation site can hint at sub/extracellular localisation. In the case of membrane proteins it is reasonable to make topological assignments according to the 'positive inside' rule. This rule was originally proposed by von Heijne based on the empirical observation that in a topological database of bacterial inner membrane proteins "basic residues Arg and Lys are four times less prevalent in periplasmic as compared to cytosolic loops,

whereas no comparable effect is observed for the acidic residues Asp and Glu".[153] This predicted rule has been borne out almost without exception.[154] A likely molecular basis for the 'positive inside' rule arises from the observation that only comparatively short loops (≤ 60 residues) are subject to the rule and that short loops are translocated independently of the cell's secretory system (*sec*).[155] The *sec*-independently translocated loops are influenced by the membrane electrochemical potential (which in *E. coli* gives the cytoplasm negative potential with respect to the periplasm).[156] Thus *sec*-independent translocation of positively charged loops is reduced due to the potential gradient.

Programs are available to perform phylogenetic analyses. Where an evolutionary relationship between several sequences from different sources are suspected it is possible to calculate relative similarities and construct an evolutionary tree. Qualitatively this is very useful: it shows evolutionary relationships and points of divergence; but quantitatively it can be misleading since rates of spontaneous mutation vary from one organism to the next.

In 1991 Ikeda *et al;* cloned and identified the structural gene *mraY* which codes for translocase I in *E. coli*.[27] A hydropathy analysis was conducted: this involves assigning a score to the average hydrophobicity at each amino acid position and a given number of amino acids (usually ten) flanking it on either side. This analysis showed that translocase I was likely to be a membrane protein with between seven and ten membrane-spanning domains.

In this chapter, predictions are made about the structure and topology of translocase I and its being part of an evolutionary superfamily of phospho-aminosugar transferases. On the basis of amino acid sequence alignments and mechanistic insight a series of three conserved residues in conserved domains have been investigated by site-directed mutagenesis.

6.2. Homology searches.

The basic local alignment search tool (BLAST) facility made freely available through an N.I.H. public server utility was used to search the major protein sequence databases for sequences having signifcant homology with the derived amino acid sequence of translocase I. The algorithm searches for high scoring segment pairs (HSP's) between the query sequence and the database sequences. It reports 'hits' above a certain quality which is set at a default value unless defined by the user. The programs and parameters used in this case are recorded in the experimental section. The algorithm returns sequences ordered by the probability of such a match occurring by chance. The results of the most recent search (made Nov 1995) are summarised in table 6.1.

Sequence	Function	Accession no.	Probability[a]
E. coli	translocase I	sp P15876	7.9×10^{-260}
B. subtilis	translocase I	sp Q03521	1.3×10^{-102}
C. griseus	GPT (hamster)	sp P24140	0.032
H. influenzae	translocase I	gp U32793	7.3×10^{-208}
L.mexicana	GPT	pir A44495	0.87
M. leprae	rfe?	gp U15186	0.58
P.aeruginosa	LPS biosynthesis	gp U17293	5.4×10^{-11}
S. acidocaldarius	sugar transferase?	sp P39465	2.6×10^{-8}
S. aureus	methicillin resistance	gp D21131	6.7×10^{-12}
S. cerevisiae	GPT?	sp P07286	0.049
S. pombe	GPT?	gp U09454	2.1×10^{-5}
Y. enterocolitica	LPS biosynthesis	gp Z47767	1.6×10^{-10}
C. longicaudatus	GPT (hamster)	sp P23338	0.032
M. musculus	GPT (mouse)	sp P42867	0.0013
R. meliloti	translocase I?	gp L25875	5.9×10^{-28}
*B. subtilis yvh*1	GlcNAc-1P transferase	-	-

Table 6.1. Results of a pan-database search for sequences homologous to that of translocase I using the BLAST algorithm. [a]Probability of the match occuring by chance. [?]Function is putative and was assigned on the basis of similarity to a protein of known function with ≤40% identity.

The significance of each identified sequence will be discussed in turn. The inferred *Haemophilus influenzae* protein sequence is 77 % identical to that of *E. coli* translocase I. The corresponding gene was identified as the *H. influenzae mraY* gene based on this sequence similarity this following sequencing of its entire genome.[157] Like the *H. influenzae* sequence the *Mycobacterium leprae* sequence is derived from a whole genome sequencing programme.

The *Staphylococcus aureus Llm* gene (low level methicillin resistance) was identified as a determinant in methicillin resistance.[105] Transposon mutagenesis[†] of a high-level methicillin resistant *S. aureus* strain gave a low-level resistant mutant. It was shown that the altered phenotype was not due to a reduction in production of PBP2' (also called PBP2α, see section 1.4.3) encoded by the *mecA* gene, but to disruption of a novel gene which the authors designated *Llm*. It was postulated that

[†] Transposon mutagenesis is a non-specific method for insertional inactivation of genes. The transposon inserts into the host genomic DNA and the desired mutant is identified by its altered phenotype.

Llm was involved in cell wall metabolism since the mutant phenotype was also characterised by an increased rate of Triton X-100-induced autolysis. A pairwise sequence alignment between the inferred amino acid sequence of the Llm protein and translocase I performed by Dr. M. Black of SB Pharmaceuticals revealed 26 % identitiy and 56 % similarity. The sequence similarity suggests that *Llm* may be the structural gene for the *S. aureus* translocase I. A request was made for the requisite strains in order to test the hypothesis but was denied. Work is underway at Shionogi & Co. Ltd., Japan to determine the phsiological role of *Llm* (H. Maki, personal communication). Two reports from Zhu *et al;* and Scocca *et al;* appeared in the literature at about the same time both reporting the cloning and functional identification of dolichol phosphate: N-acetylglucosamine-1-phosphate transferase (GPT) from hamster.[158, 159] This gene confers tunicamycin resistance to chinese hamster ovary cells when overexpressed.The GPT gene has also been cloned from mouse and is 96 % identical to the hamster GPT.[160]

In tunicamycin resistant strains of *Leishmania amazonensis mexicana*, an increase in the copy number of a 63 kb circular amplicon from which the above gene was cloned was accompanied by an increase in N-acetylglucosamine transferase activity. The sequence conferred tunicamycin resistance when expressed from an expression vector in *Leishmania.* Thus the *Le. amazoniensis* sequence is almost certainly a dolichol phosphate: N-acetylglucosamine-1-phosphate transferase (GPT). N-acetylglucosamine[161] This is included in the global sequence alignment in the next section on account of its biochemical characterisation despite its low actual homology with translocase I. The *Pseudomonas aeruginosa rfbA* gene is involved in B-band lipopolysaccharide biosynthesis; no positive biochemical identification has been made.[162]

The *Sulfolobus acidocaldarius* gene is an open reading frame which was accidentally cloned along with a functional geranylgeranyl pyrophosphate synthase gene.[163] The physiological function is not known, but a pairwise sequence alignment between the inferred amino acid sequence of the *S. acidocaldarius* protein and translocase I performed by Dr. M. Black of SB Pharmaceuticals revealed 22.7 % identitiy and 54.2 % similarity. Another example is the accidental cloning of a partial open reading frame from *Rhizobium meliloti* which has 45% sequence identity with *E. coli* translocase I.[164] The *Yersinia enterocolitica* amino acid sequence is derived from a gene required for lipopolysaccharide biosynthesis. This sequence has not yet been reported in the literature.

The *Bacillus subtilis* gene was sequenced as part of the genome sequencing project for that organism and is 41% identical with *E.coli* translocase I.[165] Thus it is highly likely that this is the *B. subtilis* translocase I.

The *Saccharomyces cerevisiae* (baker's yeast) gene was cloned by its ability to confer tunicamycin resistance and was tentatively designated a GPT since this is the target for tunicamycin.[121] Another yeast tunicamycin resistance gene was cloned from *Schizosaccharomyces pombe* (fission yeast) which showed 50% identity to the *S. cerevisiae* gene.[166]

We propose that these proteins shown or postulated to be N-acetylglucosamine-1-phosphate transferases either in prokaryotic or eukaryotic systems exhibit sequence similarity with translocase I because they have an analogous reaction mechanism and are related in evolution.

Karamata and co-workers have cloned a gene designated *yvhJ* from *B. subtilis* 168 whose product catalyses the first step in teichuronic acid biosynthesis, namely transfer of GlcNAc-1-phosphate from UMP to a polyprenyl phosphate carrier (D. Karamata, personal communication). This sequence has not been deposited in the databases yet but we predict that it will be similar to that of translocase I.

6.3. Sequence alignments.

To try to identify possible key residues within the translocase I sequence multiple alignments have been made at various times. The most recent global alignment is reported in appendix 6.

Three sequences among those discussed above have been omitted from this analysis. The hamster GPT sequence of Zhu *et al;* and the mouse GPT sequence of Rajput *et al;* have not been included on account of their near identity to the hamster GPT sequence of Scocca *et al;* since they would not improve the data. Also, the *R. meliloti* sequence of Leach *et al;* has not been included since this is not a complete sequence.

It is immediately apparent that there are three domains of similarity and these are summarised and labelled one through three in fig. 6.1. Domain 1 contains two conserved aspartate (D) residues. In the *S. cerevisiae, S. pombe, S.aureus* and *L. mexicana* sequences this DD pair is followed three residues downstream by another aspartate. The DDXXD motif has been characterised as a Mg^{2+}-pyrophosphate-binding domain in enzymes whose substrates contain an allylic prenyl pyrophosphate. The conserved motif was recognised in prenyl pyrophosphate synthase enzymes and was summarised by Ashby *et al;*.[167] Caratolli *et al;* studying caratenoid biosynthesis in *Neurospora crassa* suggested that conserved arginine (R) residues downstream of the DDXXD motif might also be involved in pyrophosphate-binding.[168] Farnesyl pyrophosphate (FPP) synthase condenses geranyl pyrophosphate (GPP) and isopentenyl pyrophosphate (IPP) to form FPP. As such it contains two aspartate-rich domains conforming to the consensus. Marrero *et al;* mutated either the first or third aspartate in the second domain to glutamate (DDXXD → EDXXD or DDXXE).[169]

The EDXXD mutant had a V_{max} decreased by 90-fold and a $K_{m(IPP)}$ increased 25-fold compared to the wild-type enzyme. The K_m for GPP was unchanged. The DDXXE mutant had kinetic parameters close to that of the wild-type enzyme.

Sequence	Domain 1	Domain 2	Domain 3
E. coli	-GFVDDYRK-	--NAVNLTDGLDG--	-NTYPAQVFMGD-
B.subtilis	-GFLDDYIK-	--NAVNLTDGLDG--	-NRDPAKVFMGD-
C. griseus	-GFADDVLN-	--NAINILAGING--	-NWYPSQVFVGD-
H. influenzae	-GFVDDFRK-	--NAVNLTDGLDG--	-NTYPAQVFMGD-
L. mexicana	-GFVDDVLD-	--NSINILAGVNG--	-NRYPARVFVGD-
M. leprae	-GLIDDRWG-	--NAINFVDGLDG--	-NFHRAKIFMGD-
P. aeruginosa	-GFLDDHGH-	--NLYNFMDGIDG--	-NFPPARIFMGD-
S. acidocaldarius	-GLLDDIFN-	--NAFNMLEGLNG--	-NFYPAKTFPGN-
S. aureus	-GLVDDIYD-	--NAINLIDGLDG--	-NFHPAKIFLGD-
S. cerevisiae	-GIADDLFD-	--NSINILAGVNG--	-NRWPATVFVGD-
S. pombe	-GILDDLFD-	--NSINIIAGVNG--	-NWWPSRVFVGD-
Y. enterocolitica	-GFWDDHGH-	--NLYNFMDGIDG--	-NFPPAKIFMGD-
Consensus sequence	-GXXDDXXX-	--NXXNXXXGXXG--	-NXXP$^{A}/_{S}$XXFXGD-

Figure 6.1. Domains 1-3 extracted from the global alignment. Conserved residues are emphasised as a consensus sequence.

A parallel study also conducted on the rat FPP synthase carried out by Joly and Edwards found that in the first aspartate-rich domain changing the first aspartate to glutamate (DDXXD → EDXXD) did not affect the enzyme.[170] On the other hand changing the second or third aspartate to glutamate or changing either of the two conserved asparagine residues downstream of the motif to lysine (R → K) reduced V_{max} 1000-fold. They also mutated the domain 2 second aspartate (not investigated by Marrero *et al;*) to glutamate and found that it was not critical for enzyme activity. Interestingly, none of the mutations made in this study affected the K_m value for either substrate. A subsequent study if the yeast FPP synthase confirmed the essemtial role of these aspartate and arginine residues. The three aspartates from each domain and the asparagines from the first domain were individually mutated to alanine and glutamine respectively. This resulted in almost completely inactive enzyme in every case. Tarshis *et al;* have recently reported the crystal structure at 2.6 A° resolution of recombinant avian FPP synthase.[171] The enzyme has ten core α-helices arranged around a large central cavity, presumably the active-site. The two aspartate-rich domains are on opposite walls of this cavity and each bind one samarium atom (a

derivative for X-ray crystallography purposes), supporting their proposed role in substrate-binding.

Based on this precedent we suggest that this conserved domain among polyprenyl phosphate: phospho-aminosugar transferases may be involved in substrate-binding. Note also that in sequences not having the third aspartate, the corresponding amino acid is lysine, asparagine or histidine, all capable of hydrogen-bonding interactions. The exception is the *M. leprae* sequence; this will be discussed later.

As to the significance of the second domain of similarity, the NXXNXXXGXXG sequence is not part of any recognised motif that I am aware of. Suffice to say that there is a conserved element and in that respect it is likely to be important to the enzyme in some way.

The third domain of similarity contains the largest number of conserved residues. The consensus sequence is $NXXP^{A}/_{S}XXFXGDX_6G$. There are two exceptions to this. First is again the *M. leprae rfe* sequence which has arginine (R) in place of proline (P). Conservation of a proline residue is especially interesting because as a conformationally restricted amino acid it has a unique effect on protein local structure. The *rfe* gene of *E. coli* has been studied in detail and is almost certainly the structural gene for the first enzyme in the biosynthetic pathway for O-specific lipopolysaccharide.[172] This first step is the transfer of GlcNAc-1-phosphate from UMP to undecaprenyl phosphate. Translation of the DNA sequence gives a protein smaller than translocase I (39.5 kDa and 29 kDa for translocase I and rfe respectively) with no domain 1 or domain 3 consensus. It does have the NXXNXXXGXXG consensus motif of domain 2. From its function (which has been demonstrated biochemically[172]) it would be expected in the light of the discussion so far to be homologous with translocase I and the other proteins in the alignment. This serves as a reminder of the intrinsically speculative nature of deriving functional relationships from sequence alignments. It is of course possible that there is more than one class of polyprenyl phosphate: phospho-aminosugar transferase! The second exception is the *S. acidocaldarius* sequence which has asparagine (N) in place of aspartate (D). This anomaly becomes relevant later.

These exceptions aside, this degree of local conservation through proteins from such a variety of sources (gram positive, gram negative, archaebacterial, protozoan, yeast and mammalian) is remarkable indeed.

Two possible mechanisms of catalysis by translocase I were discussed in chapter one. One involves direct nucleophilic attack by undecaprenyl phosphate on the phosphodiester bond of UDPMurNAc-pentapeptide to give products in one step. The other involves a covalent enzyme-linked species. A nucleophilic group, such as a carboxylate, in the enzyme active-site attacks UDPMurNAc-pentapeptide yielding a

reactive species which is in turn attacked by undecaprenyl phosphate to give product. If the reaction proceeds via this mechanism as is the conclusion of chapter five, translocase I should contain a highly conserved amino acid residue capable of this chemistry.

Early alignments revealed the three aspartates already mentioned, but no conserved glutamate, serine, cysteine or histidine. One tyrosine residue (W132) was conserved, but there is no precedent for tyrosine being involved in phosphate transfer reactions in bacteria, only in eukaryotes. In any case, that tyrosine is not conserved in the global alignment. Given that the DD pair (D115 and D116) may be involved in substrate-binding through a magnesium bridge, our hypothesis was that the other aspartate, D267, might be the active-site nucleophile.

6.4. Secondary structural predictions.

A hydropathy analysis performed by Ikeda *et al;* indicated between seven and ten membrane spanning domains within translocase I.[27] The method of Eisenberg was used to predict possible membrane-associated helices and their classification for translocase I.[173] The program measures the average hydrophobicity of 21 residue segments and assigns them as globular, transmembrane of surface associated based on a plot of hydrophobic moment against average hydrophobicity. The program predicted ten membrane associated helices as summarised in table 6.2.

Helix	Segment	Sequence	Classification
1	26 - 46	AIVSLLTALFISLWMGPRMIA	Globular
2	72 - 92	PTMGGIMILTAIVISVLLWAY	Transmembrane
3	94 - 114	SNPYVWCVLVVLVGYGVIGFV	Transmembrane
4	132 - 152	WKYFWMSVIALGVAFALYLAG	Transmembrane
5	168 - 188	VMPQLGLFYILLAYFVIVGTG	Transmembrane
6	200 - 220	LAIMPTVFVAGGFALVAWATG	Transmembrane
7	236 - 256	AGELVIVCTAIVGAGLGFLWF	Transmembrane
8	263 - 283	VFMGDVGSLALGGALGIIAVL	Globular
9	288 - 308	FLLVIMGGVFVVETLSVILQV	Surface
10	338 - 358	VIVRFWIISLMLVLIGLATLK	Transmembrane

Table 6.2. Results of secondary structural predictions using the method of Eisenberg.

Using this data a model was constructed of translocase I embedded in the membrane bilayer.

While it must be remembered that such a model as this is highly speculative, it is nevertheless interesting to consider it in the light of other evidence. The number of

charged residues on either side and within the membrane was counted. There are none in the regions predicted to be deep inside the membrane (because this is one of the criteria upon which the predictions are made). On one side of the bilayer there are 2 negative and 5 positive charges. On the other side there are 12 negative and 22 positive charges. Thus there is a marked difference in the distribution of charged residues. This is not a feature of the predictive program. The positive inside rule proposed by von Heijne can indicate which side is likely to be the cytoplasmic face and which side is likely to be exposed to the periplasm. Significantly, all the residues implicated by the global sequence alignment are located in predicted cytoplasm-exposed regions rather than within the membrane or on the periplasmic side which they would have to be if they are involved in substrate-binding/catalysis. These extra pieces of information are summarised in fig. 6.2.

6.5. Site-directed mutagenesis.

An alternative to protein chemical methods for the investigation of possible key active-site residues in the study of an enzyme is site-directed mutagenesis. Using what are now standard genetic techniques (in *E. coli* at least) it is possible to specifically change an amino acid residue of a protein to a different one. Differences between mutant and wildtype proteins can yield information concerning the function of that residue.

In June of 1994 the offer of facilities to do the necessary molecular biology was made by our collaborators, SmithKline Beecham Pharmaceuticals. Although the protein had not been purified the opportunity to investigate the three conserved aspartate residues was taken. It was decided to change each of them individually to asparagine since this is a conservative mutation in that asparagine has the ability to hydrogen bond and is sterically similar to aspartate. In addition the putative active-site nucleophile, D267, would be changed to alanine. Also, since the DD pair was postulated to chelate Mg^{2+} in substrate-binding, a double mutant, D115/116N would be made.

The remainder of this chapter is devoted to this work and the characterisation of the mutant proteins. At this time, the *S. acidocaldarius* protein bearing an asparagine in place of aspartate in domain 3 had not been reported.

The method of Kunkel was chosen over the phosphorothioate method of Eckstein.[174, 175] Although the frequency of mutation is generally higher with Eckstein's method (routinely >90 %), this method has more steps and requires larger quantities of DNA than Kunkel's method. So with only five mutations to make Kunkel's was the method of choice.

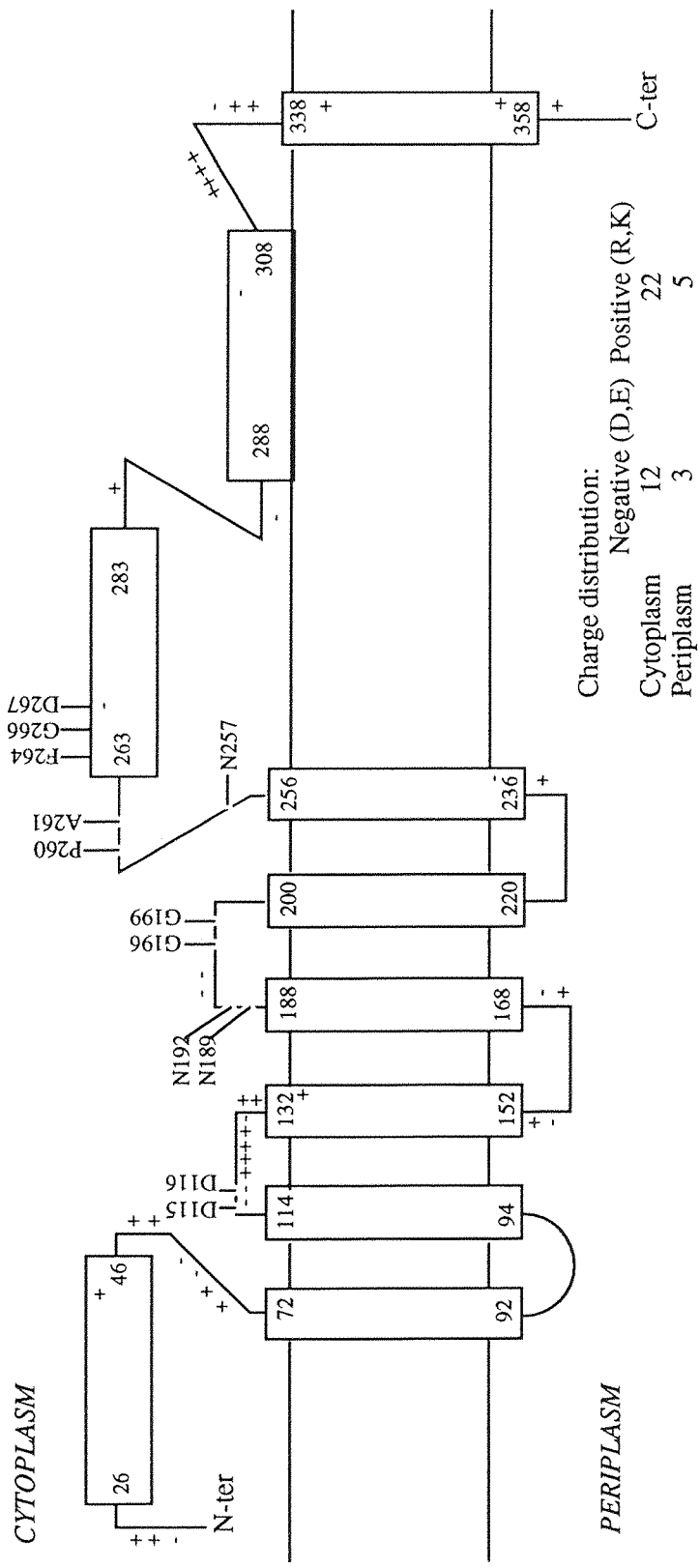


Figure 6.2. Topological model of translocase I including distribution of charged residues, conserved residues and membrane orientation.

Mutagenesis was carried out using the Bio-Rad M13 Mutagene kit as recommended by the manufacturer. Mutagenic oligonucleotide sequences are provided in the experimental section as are full details of methods. An 841bp *SspI*/*Eco*RI fragment from pBROC525 (appendix 1) was cloned into the *Hinc*II/*Eco*RI site of M13mp18 giving M525.

Ligated DNA was transformed into *E. coli* MV1190; insert positive recombinants were identified by the blue/white plaque assay. Five plaques were picked at random and used to infect cultures of *E.coli* MV1190. Double stranded M525 was prepared from each culture and digested with *StyI*/*Eco*RI and fragments assessed by gel electrophoresis to confirm insertion of the partial *mraY* fragment. Phage M525 contained in the culture supernatant of one insert positive clone was used to prepare uracil-containing single-stranded M525 by growth through *E. coli* CJ236, a *dut*$^-$, *ung*$^-$strain, infecting at a phage particle:cell ratio = 0.15. Uracil content was verified by titering the phage-containing culture supernatant on both *E. coli* MV1190 and *E. coli* CJ236. This DNA served throughout as a template for mutagenesis reactions.

Phosphorylated mutagenic oligonucleotides were used as primers for complementary strand synthesis with uracil-containing template M525 as described in the experimental section. Synthesis of the complementary strand was judged by gel electrophoresis of the reaction mixture. Double-stranded M525 DNA generated by the mutagenesis reaction was transformed into *E. coli* MV1190. Plaques were picked at random and used to infect cultures of *E. coli* MV1190. Single-stranded DNA was prepared from phage particles and mutant clones were identified by sequencing. The percentage of mutant clones in plaques screened was 20 %, 9 %, 75 %, 75 % and 100 % for D267A, D267N, D115N, D116N and D115/116N respectively. Since annealing and reaction conditions were the same for each mutagenesis reaction, it is reasonable to assume that the differences in the rate of mutation is attributable to differences in priming. Clones were checked for secondary mutations by sequencing the entire *StyI*/*Eco*RI fragment. The requisite mutant fragments were cloned back into the expression vector, pBROC525. In each case two insert positive recombinants were sequenced across the mutation site to ensure selection of mutant and not wild-type clones

In the course of screening for mutants at D267, one deletion/mutation clone was found which resulted in a frame-shift and introductin of a stop codon. The natural sequence is ...QVFMGDVGSLAG..., the new sequence is ...QVFMGMWVRWR(stop). This C-terminal truncated mutant could be of interest in the future so a sample of phage-containing culture supernatant was retained.

It should be brought to the reader's attention at this point that because the mutant proteins are expressed in *E. coli* there will be a certain amount of

contamination by chromosomally-derived translocase I which will of course be wild-type. A strategy sometimes adopted to avoid this is to express the mutants in a host whose chromosomal copy has been inactiviated by insertion or deletion mutagenesis. This is not possible in this case since translocase I is an essential protein. In an effort to allow differential purification of plasmid-derived translocase I, an attempt was made to express a fusion protein where a 3 kDa peptide containing a Ni^{2+}-binding hexahistidine motif and an enterokinase cleavage site was fused to the N-terminus of translocase I. This was however unsuccessful as is described fully in chapter 3.

6.6. Characterisation of mutant translocase I preparations.

All of the mutant strains along with the wildtype strain and a control strain carrying the empty expression vector were grown in LB media and induced with 0.2 mM IPTG at O.D. = 1.7 as described in chapter 3. Care was taken to ensure that growth times and conditions were the same for each culture. Cells were harvested and membranes prepared as described in chapter 3.

In the first instance membranes were extracted with 0.5 % Triton X-100 at 2 mg protein/mL and assayed using the standard 0.3 mL fluorescence enhancement assay (FEA) method described in the experimental chapter. Table 6.3 lists the results of this experiment.

Strain	Specific activity, u/min/mg		% wt
E.coli JM109(pTrc99A)	0.42	0.71	-
E.coli JM109(pBROC525)	11.90	15.00	100
E.coli JM109(pBROC525, D115N)		0.79	0.5
E.coli JM109(pBROC525, D116N)		0.50	0.0
E.coli JM109(pBROC525, D115/116N)		nd	nd
E.coli JM109(pBROC525, D267A)	1.11		6.0
E.coli JM109(pBROC525, D267N)	1.23		7.0

Table 6.3. Relative activities of mutant translocase I preparations in the fluorescence enhancement assay. Two sets of data correspond to two sets of experiments.

The background activity arises from chromosomally derived translocase I, which obviously was fully active. Note that the mutant preparations have residual activity when it would be expected that the proposed active-site mutants would not be active at all. It is impossible to say whether the residual activity is there because of an altered level of background compared to the control strain, *E. coli* JM109 (pTrc99A), or because the mutation has merely perturbed the structure of the protein resulting in reduced activity.

An attempt was made to increase the level of expression of functional translocase I from pBROC525. *E. coli* JM109(pBROC525) was grown to OD_{600nm} = 0.6 in 500 mL LB/amp and cooled to room temperature by running under a cold tap. IPTG was added to 0.2 mM and the culture shaken overnight on the bench. The following morning cells were harvested and enzyme prepared and assayed. However, yield of activity was only comparable to that obtained by the established protocol.

The next step was to dissect any remaining activity of mutant enzyme from that of the contaminating wildtype activity. If a preparation of enzyme activity contains two enzyme isoforms whose K_m and/or V_{max} values differ, these activities can sometimes be distinguished by a Michaelis-Menten analysis using the Eadie-Hofstee plot. When two isoforms are present, so are two corresponding sets of data as shown in fig. 6.3 with an imaginary data set where the isoform with higher specific activity and lower K_m is present as a contaminant.

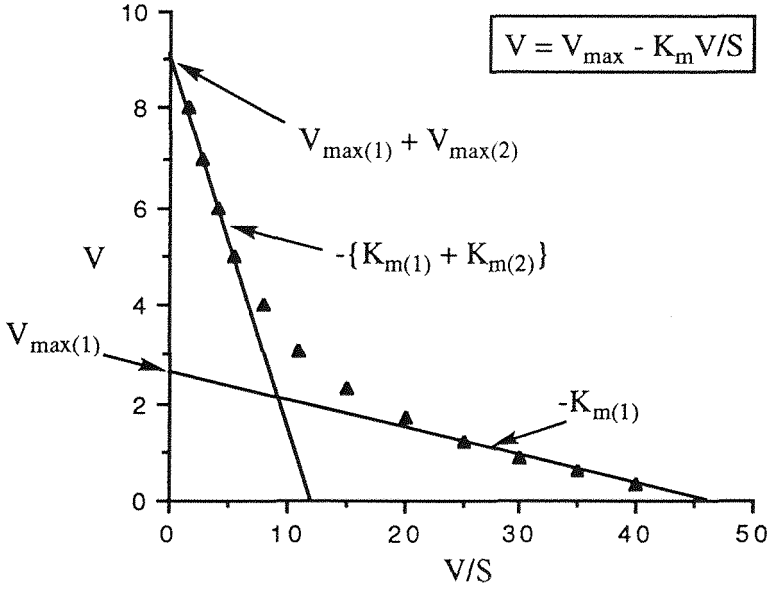


Figure 6.3. Eadie-Hofstee plot when two enzyme isoforms are present. Isoform 2 is present in excess over isoform 1 which has higher specific activity and lower K_m.

This analysis requires measurement of the activity of the mutant preparations at low substrate concentrations. The sensitivity of the FEA is not sufficient for this requirement. Translocase I catalyses an isotope exchange reaction by virtue of the reversibility of the overall reaction (fig. 6.4). A protocol was developed based on literature methodology[109] to measure this activity in particulate preparations as described in chapter 8. Briefly, enzyme was incubated with UDPMurNAc-pentapeptide and [^{14}C]-UMP. Reaction was terminated by boiling and UMP hydrolysed with alkaline phosphatase.

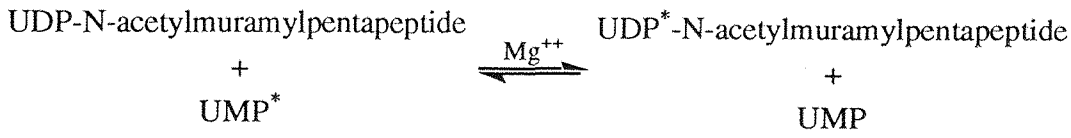


Figure 6.4. Isotope exchange catalysed by translocase I.

UDPMurNAc-pentapeptide was separated from uridine by ion exchange chromatography on Dowex-1 chloride and [^{14}C]-UDPMurNAc-pentapepitde quantified by scintillation counting. The kinetic equations associated with this isotope exchange reaction are analagous to those for the full reaction with two assumptions. In this protocol, enzyme is pre-incubated with UDPMurNAc-pentapeptide for 2 min to allow attainment of the equilibrium in fig. 6.5, if this is the actual mechanism.

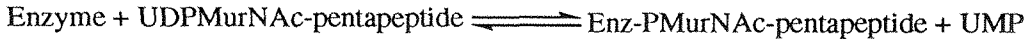


Figure 6.5. An equilibrium set up prior to the addition of [^{14}C]-UDPMurNAc-pentapeptide in the isotope exchange assay of translocase I.

So the first assumption is that the concentration of covalent enzyme-linked species and hence the concentration of UMP is negligable. The second is that endogenous undecaprenyl phosphate and undecaprenyl pyrophosphoryl-MurNAc-pentapeptide in the membranes does not participate to such an extent that it becomes significant in the kinetic analysis. Equation [6.1] is the initial exchange rate equation which rearranges to equation [6.2], the Eadie-Hofstee representation. Kinetic constants are *apparent*because [^{14}C]-UMP is not saturating.

$$\frac{R}{R_{\max(app)}} = \frac{[UDPMurNAc-pentapeptide]}{(K_{m(app)} + [UDPMurNAc-pentapeptide])} \qquad .....[6.1]$$

$$R = R_{\max(app)} - K_{m(app)} \frac{R}{[UDPMurNAc-pentapeptide]} \qquad .....[6.2]$$

In performing these experiments it rapidly became apparent that substrate inhibition was taking place above about 500 μM UDPMurNAc-pentapeptide as shown in fig. 6.6.

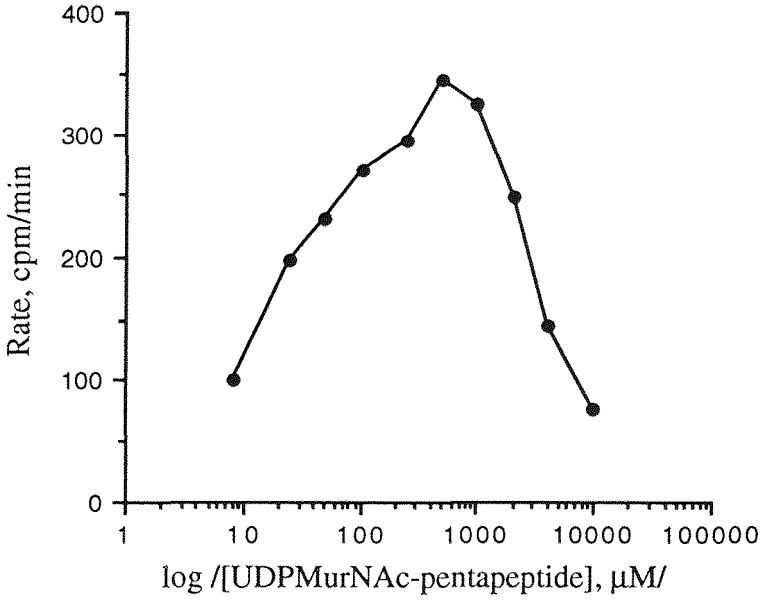


Figure 6.6. Isotope exchange rate, R, versus [UDPMurNAc-pentapeptide]. The assays contained 10 μL of 6.4 mg/mL *E. coli* JM109 (pTrc99A) membrane preparation and was conducted as described in the experimental chapter.

The presence of substrate inhibition in this system has implications for the catalytic mechanism of translocase I, a question which was addressed in chapter 5. Substrate inhibition is characteristic of ping pong mechanisms and arises through the substrate in question binding to enzyme or an enzyme complex to give a catalytically non-productive complex (fig. 6.7).

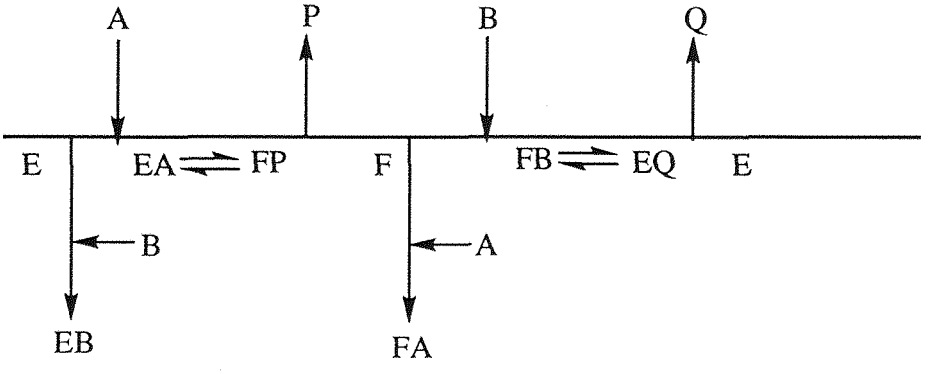


Figure 6.7. Substrate inhibition by one or both substrates in a ping pong system.

A credible scenario is that free translocase I reacts with UDPMurNAc-pentapeptide to form enzyme-P-MurNAc-pentapeptide.UMP, UMP dissociates from this 'FP' complex and then a second molecule of UDPMurNAc-pentapeptide binds at

the UMP site resulting in a dead-end complex. Whilst interesting, the practical implication is imposition of an upper limit on assay UDPMurNAc-pentapeptide concentration in the current series of experiments.

For each of the wildtype, control and five mutant preparations, the rate of appearance of radioactivity in UDP-MurNAc-pentapeptide was measured with 10, 20, 30, 40, 50, 75, 100, 125, 150, 200, 250, 300, 350 or 400 μM UDPMurNAc-pentapeptide. [^{14}C]-UMP concentration was constant at 2.2 μM (~6000 cpm/50 μL assay). In every case one isoform only was apparent. Plots of rate of incorporation against substrate showed substrate-dependent approach to a maximal rate which could not be exceeded by increasing substrate concentration. Replots using the Eadie-Hofstee rearrangement (equation [6.2]) gave the apparent kinetic parameters K_m and R_{max} (table 6.4).

Plasmid	**$K_{m(app)}$** **μM**	**$V_{max(app)}$** **cpm/min**	**Specific activity** **cpm/min/mg**
pTrc99A	17	310	4840
pBROC525	14	105	40540
pBROC525 (D115N)	84	310	3180
pBROC525 (D116N)	52	231	2370
pBROC525 (D115/116N)	35	129	1690
pBROC525 (D267A)	30	286	3110
pBROC525 (D267N)	43	209	2140

Table 6.4. $K_{m(app)}$ and $R_{max(app)}$ and specific activities for wildtype, control and mutant preparations in the isotope exchange assay. pTrc99A is the empty expression vector.

As expected the control strain, *E. coli* JM109 pTrc99A, and the overexpressing strain, *E. coli* JM109 pBROC525, have the same apparent K_m value. The mutant strains however gave consistently elevated K_m values, each of them being at least double that determined for the wildtype enzyme preparations. This indicates that the models and assumptions which have been applied are probably less than accurate. Only further study of the enzyme will resolve these inaccuracies.

Nevertheless, it is still clear from these results that either the mutant enzymes are completely inactive, or that they are active, but that the present methods are not sufficient to measure the activity, or that the mutations have interfered with expression or folding/targetting resulting in incomplete or misfolded polypeptides, although this is unlikely given the conservative nature of the Asp to Asn mutation. In the absence of a purification protocol it is not possible to distinguish between these.

6.7. Labelling experiments with mutant preparations.

In chapter 5 methodology was established for the reproducible detection of the putative enzyme-linked intermediate proposed and identified in that chapter. The radiolabelled enzyme species is proposed to be formed by attack of an active-site carboxylate (maybe D267) on the phosphodiester bond of UDPMurNAc-pentapeptide containing [^{14}C]-D-Ala-[^{14}C]-D-Ala. If the roles proposed for D115, D116 and D267 are correct, then the mutants D267A and D267N will not form this radiolabelled species. The mutants D115N and D116N, whilst not catalytically efficient, should form the radiolabelled species since this requires one partial turnover only.

With this in mind, the mutant preparations D115N, D116N and D267N were each subjected to the protocol established in chapter 5. The exact procedure in these cases is detailed in the experimental chapter. For comparison, the elution profile of overexpressed wild-type and mutant enzyme preparations treated in this way are shown in fig. 6.8.

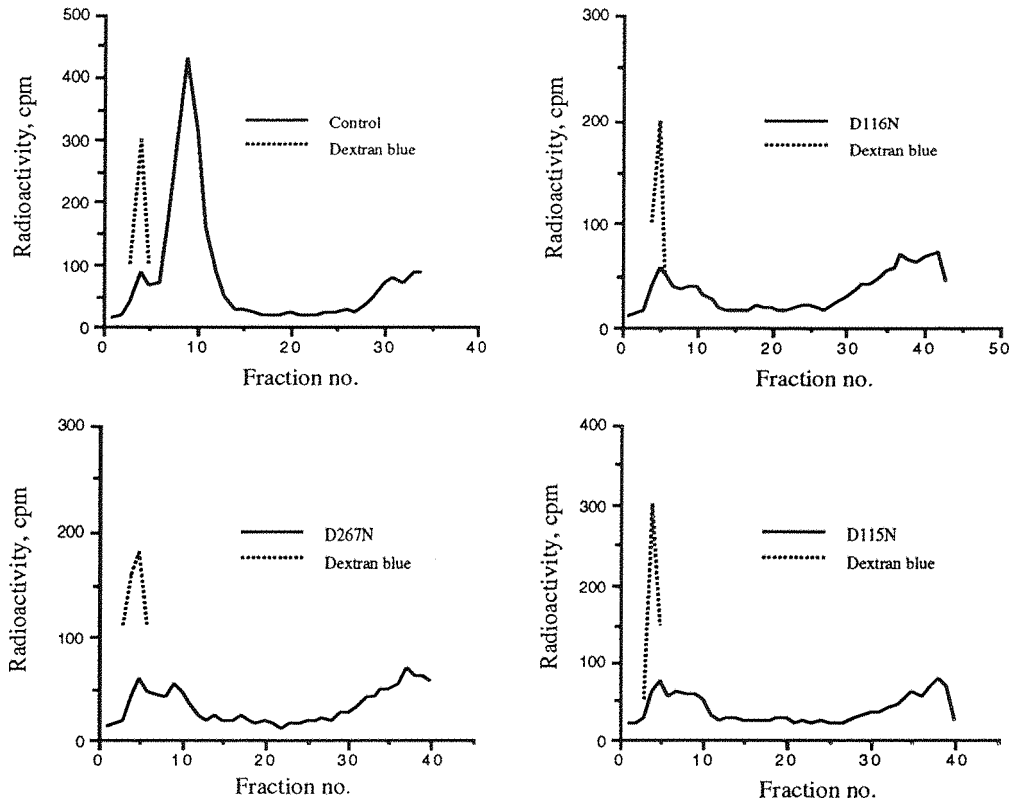


Figure 6.8. Elution profile from sephadex G75 of wildtype and mutant enzyme preparations after treatment with UDP-MurNAc-L-Ala-γ-D-Glu-*m*-DAP-[^{14}C]-D-Ala-[^{14}C]-D-Ala.

The high molecular weight species tentatively assigned as enzyme-linked species in chapter 5 is clearly present in each of the mutant preparation experiments. It was not known what contribution, if any, the chromosomally derived translocase I could make to this peak. If no significant contribution is made, then this would seem to imply that D267 is not the active-site nucleophile (and neither is D115 or D116). This conclusion is supported by the presence of asparagine in domain three of the *S. acidocaldarius* protein where all the others have aspartate.

Chapter 7. Discussion and Future Directions.

The object of this chapter is to consider the results, their implications and where they might lead as a whole rather than as individual sections. Many of the ideas discussed in this chapter are speculative and cannot be tested by experiment using the current methodologies. I include them as hints and starting points for future workers.

The biggest obstacle in this project has been the heterogeneity and low levels of specific activity of the translocase I preparations used. We have increased the specific activity of translocase I preparations approximately 30-fold using a multicopy plasmid carrying the *mraY*gene under the control of the strong *trc* promoter. The amount of translocase I protein present is still small since SDS-PAGE of whole cells from induced and uninduced cultures did not reveal any new bands in the 20 - 80 kDa region. It is unlikely that the membranes have become saturated with protein. It seems more likely that *mraY* is a poorly expressed gene, even with an optimal promoter like *trc*, perhaps due to some form of natural down-regulation. Also, the protein has to be correctly inserted into the membrane. There are two extreme models for insertion of membrane proteins.[176] In one, the co-translational model, the nascent strand is 'stitched' into the membrane as it is synthesised by the ribosome. In the other, the post-translational model, protein synthesis is completed before insertion begins. Pairs of helices insert as 'helical hairpins'. It is not knownprecisely which model occurs with *E. coli* inner membrane integral proteins, or if the true mechanism lies somewhere between the two extremes. Either model is a complex process which might reduce the efficiency of expression.

At first consideration, it might be expected that overexpression of a membrane protein would reach a point where so much extra protein was being inserted into the membrane that the membrane would no longer function correctly and the cell would die as a consequence. However, there is evidence to suggest that synthesis of phospholipids is coupled to membrane protein synthesis Weiner*et al;* found that when fumarate reductase (*E. coli*) was overexpressed 40-fold in *E. coli* this enzyme now represented 50 % of all membrane bound protein.[177] Despite this, the ratio of membrane protein to phospholipid was unchanged. Examination of overproducing cells under the electron microscope revealed membrane structures extending into the interior of the cell. A similar phenomenon appeared to be occuring in cells of *E. coli* K12 overproducing F_0F_1 ATPase.[178] It would be interesting to see if overproduction of translocase I resulted in any morphological changes.

Despite the apparent low levels of protein, the activity is easily detected and is sufficient for kinetic inhibition experiments and accurate detection in fractions following column chromatography, even when greater than 90 % of activity is lost as was the case with DEAE-sepharose. A typical 4 L preparation of *E. coli* JM109 (pBROC525) induced with IPTG gives enough enzyme for 500 standard fluorescence enhancement assays. Thus it is worth continuing experiments directed towards purification and kinetic characterisation using the present expression system. However, it is my opinion that it would be difficult to characterise translocase I and the inhibitors mureidomycin A, tunicamycin and liposidomycin B further than has already been done in the absence of at least a partially purified preparation. The presence of undecaprenyl phosphate in the crude detergent extract (albeit in contaminating quantities) introduces uncertainties into any isotope exchange experiments directed towards mechanistic analysis. The presence of significant quantities of phospholipids means that the precise detergent/lipid environment of the enzyme is unknown. It seems unlikely that contaminating activities are a problem in the case of translocase I, but it could only increase confidence in results if the preparation were pure. There are also direct advantages of purification. It was generally observed that when translocase I was desalted by gel filtration on sephadex G75, the recovery of activity was 150 - 200 % of that applied to the column. It was also noted that this treatment, or indeed dialysis against the solubilising buffer (but with 0.2 % rather than 0.5 % Triton X-100) increased the stability of the preparation. However, enzyme treated this way had been assayed in the presence of phospholipids so it might have been these or even the substrates/products which had the stabilising effect.

It should be pointed out that the optimisation of stability of solubilised activity experiment reported in chapter 2 was carried out using the activity of the solubilised preparation prior to reconstitution with phospholipids. The activity of translocase I as observed in a standard fluorescence enhancement assay is somewhat less stable, losing >50 % of activity over 24 hr on ice (no phospholipids added). This was not a problem in practice because solubilised preparation could be stored at -70 °C without loss of activity.

Much of the 'groundwork' towards purification of translocase I has been done in this project. Firstly, the development of a rapid, sensitive and cost effective standard protocol for the detection of activity will facilitate evaluation of purification steps. Resins such as DEAE-sepharose, polyprenyl-agarose (a custom prepared matrix) and reactive dye-linked resins have been shown to adsorb translocase I, with apparent high specificity in the case of polyprenyl-agarose. It was also found that phenyl-agarose afforded a degree of purification, with translocase I eluting at the end

of the salt gradient. In that case, a less hydrophobic HIC resin, such as pentyl-agarose would be worthy of investigation in the future.

The key problem at present is that activity is lost following any form of adsorptive chromatography. The two possibilities are that translocase I is separated from an essential factor or phospholipid, or that upon binding structural integrity is lost resulting in irreversible loss of activity. In the first case it ought to be possible to locate the factor in eluted fractions or include an essential phospholipid in the column buffer, although experiments to this end have been unsuccessful so date. If binding to a matrix results in unfolding or aggregation, this is most probably a function of the detergent. In this case a different choice of detergent is in order. Consideration of general precedent suggest CHAPS, octyl glucoside and the MEGA series, possibly with additives such as lauryl sarcosinate, deoxycholate or lubrol PX. CHAPS and octyl glucoside are generally favoured detergents for many good reasons, but they are expensive, prohibitively so if used routinely for preparative scale column chromatography. The MEGA series are less widely used, but have been documented to possess most of the beneficial properties of CHAPS and octyl glucoside.[126, 179] These are also expensive to buy, but unlike CHAPS and octyl glucoside they can easily be prepared on a large scale from cheap starting materials. Assays would still have to be carried out in Triton X-100 since the assay relies on the difference between the micellar and the bulk aqueous compartment for the change in fluorescence.

Aside from the mode of action of the antibiotics, the central concept of this project has been formation of a covalent enzyme-linked intermediate on the translocase I-catalysed reaction pathway involving an active-site nucleophile (fig. 7.1).

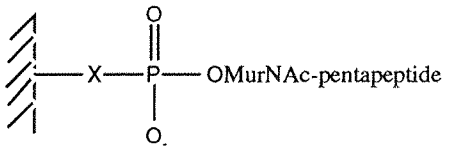


Figure 7.1. Putative covalent enzyme-linked intermediate formed by attack by an active-site nucleophile on the substrate phosphodiester bond.

This was the basic hypothesis which has directed our research, and which we have built upon. Results from amino acid sequence alignments experiments described in chapter 6 have revealed a new superfamily of enzymes. Knowing the primary amino acid sequence of translocase I as inferred from the gene sequence allowed comparison with other protein sequences in the databases. Three domains of

similarity persist through the sequences recovered. Outside of these domains there is no significant overall homology. This is hardly surprising given the evolutionary diversity of the source species. The superfamily is one of phosphoamino-sugar transferases. These enzymes catalyse the transfer of a phosphoamino-sugar from a nucleoside carrier, UMP, to a membrane bound polyprenyl phosphate acceptor molecule. The type of chemical reaction catalysed by these enzymes is the same. Whilst the primary amino acid sequences vary enormously, we suggest that the mechanism is also the same for these enzymes.

The first domain of similarity contains an adjacent pair of aspartate residues which are completely conserved. It seems very likely from literature precedent (including X-ray crystal structure data on FPP synthase[171]) that these are involved in binding a Mg^{2+}-UDPMurNAc-pentapeptide adduct. The third domain contains an aspartate residue which is conserved in all but one of the sequences and we have suggested that this may be the active-site nucleophile. This is a bold assertion given that the mechanism of catalysis has not been proven, but the topological model constructed for translocase I places all three of the conserved domains in cytoplasm-exposed regions of the protein, which they would presumably need to be if they were involved in catalysis/substrate-binding.

Results from chapter 5 were consistent with the presence of a covalent enzyme-linked intermediate. Neuhaus and co-workers observed that the *S. aureus* enzyme catalyses the isotope exchange reaction and that it proceeds 6 - 24 times faster than the transfer reaction. These facts make a direct displacement mechanism not involving an enzyme-linked intermediate improbable. Neuhaus' evidence is a valid argument for the double displacement (ping pong) mechanism even though the specific activities for the exchange and transfer reactions were not measured under conditions of saturating substrates (for any of the substrates). This is because if the mechanism was a direct displacement, the isotope exchange reaction would have to proceed through formation of the lipid-linked product. The presence of the lipid acceptor at a non-saturating concentration would then affect the exchange and transfer rates equally. In the exchange assays, UMP was present at concentrations far below K_m. This would reduce the exchange rate from the maximum rate by at least 10-fold. Despite this factor, the exchange reaction still proceeded faster than the transfer reaction. I think that this is a critical point in the argument and it is one that has not been made in the literature.

So the question facing us was 'Is the nucleophilic residue the carboxylate, aspartate-267?'. The sequence alignments showed no other conserved nucleophilic residues capable of the chemistry that was required (Glu, Ser, His, Tyr, Cys, Thr). Literature precedent indicates that candidate residues are aspartate, serine, histidine

and cysteine (table 7.1). To date there are no examples of a tyrosyl-phosphate species in a bacterial system.

Protein/Process	Phosphorylated residue	Reference
Chemotaxis response regulator, CheY	Asp	Sanders, 1989[180]
Chemotaxis response regulator, CheA	His	Bourret, 1991[181]
E. coli alkaline phosphatase	Ser	Walsh, 1979[182]
E. coli acid phosphatase	His	Ostanin, 1992[183]
Signal transduction in *Rhodobacter*, RegA	Asp	Inoue, 1995[184]
Signal transduction in *Rhodobacter*, RegB	His	Inoue, 1995[184]
Nitrogen assimilation regulation in *Enterococcus*, NTRC	Ser	Keener, 1988[185]
Nitrogen assimilation regulation in *E. coli*, NR_I	Asp	Weiss, 1988[186]
Nitrogen assimilation regulation in *E. coli*, NR_{II}	His	Weiss, 1988[186]
Agronacterium virulence factor, VirG	Asp	Jin, 1990b[187]
Agronacterium virulence factor, VirA	His	Jin, 1990a[188]
E. coli mannitol specific membrane bound enzyme II	Cys + His	Pas, 1988a[189] Pas, 1988b[190]

Table 7.1. Examples of amino acid residues forming covalent phosphoenzyme linkages in bacterial phosphate transfer reactions.

The preliminary data presented in chapter 5 provide further clues. Treatment with hydroxylamine appeared to have no effect on the native enzyme. This could mean one of two things; either the intermediate is not an acyl phosphate (hence no reaction with NH_2OH) or that the acyl-phosphate species was not accessible to the hydroxylamine. Treatment of SDS-denatured enzyme with hydroxylamine at pH 9.5 apparently led to loss of label. With hindsight this experiment should have been performed at neutral pH to avoid the possibility of basic hydrolysis. Even so, a phosphoserine species (fig. 7.2) would not be hydrolysed under such conditions; phosphohistidine (fig. 7.2) intermediates (in NR_{II} and CheA) are also stable to basic conditions (2 M NaOH) and are relatively stable to hydroxylamine ($t_{1/2}$ for CheA in 04 M NH_2OH pH 7.6 was 50 min).

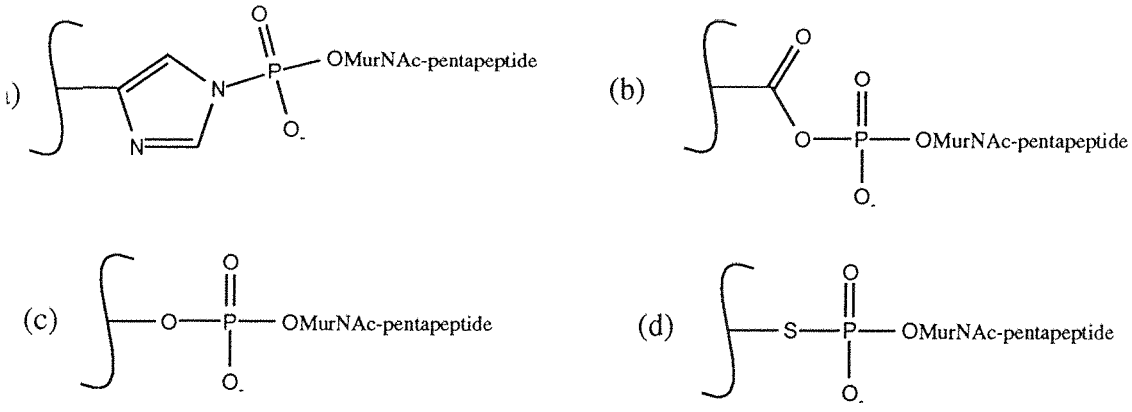


Figure 7.2. Structures of possible intermediates, (a) phosphohistidine, (b) acyl-phosphate, (c) phosphoserine and (d) phosphocysteine.

E. coli translocase I contains only two cysteines (C100 and C242), both of which the topological model places within the membrane bilayer, and neither of which is well conserved, even amongst the bacteria considered in the global sequence alignment. Thus the evidence at hand, albeit tenuous, points towards an acyl-phosphate intermediate, with D267 being the best candidate.

But, we have two pieces of evidence which are not consistent with this hypothesis. These are that the *S. acidocaldarius* sequence (not a functionally characterised protein, but highly homologous with translocase I) has asparagine (N) where all the other sequences have aspartate (D267 in *E. coli* translocase I). Also, the translocase I mutant, D267N, was able to form the radiolabelled species designated as the enzyme-linked intermediate. Clearly, if D267 is the catalytic residue, the D267 mutant should be completely inactive and should not be labelled in this way.

The final word therefore is that the double displacement, ping pong mechanism is very probably the mechanism by which the reaction proceeds, but that we cannot say at present which is the catalytic residue. The definitive experiment would be to generate the intermediate on a large scale, denature the protein and reduce it with sodium borotritide. If it was an acyl-phosphate the product would contain tritiated homoserine (fig. 7.3).

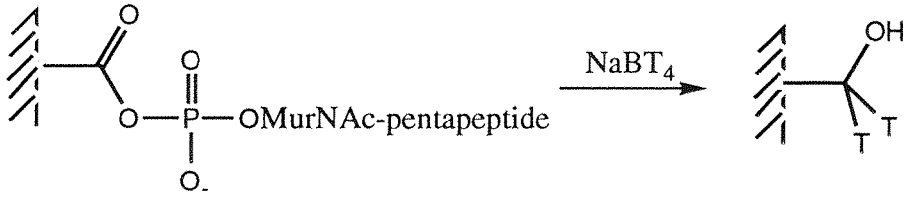


Figure 7.3. Reduction of an acyl-phosphate intermediate with sodium borotritide.

Tryptic digestion and sequencing of fragments would identify the active-site carboxylate in this case. This rationale was successfully used to identify aspartate-57 as the phosphorylated aspartate in CheY by Sanders *et al;*[180] and D52 in VirG.[187]

The actual mechanism and the residue which is the active-site nucleophile have bearing on the possible identity of the EI* complex formed during slow-binding inhibition of translocase I by mureidomycin A (and liposidomycin B since this also appears to be a slow-binding inhibitor). The tightly associated EI* complex arises through an isomerisation of EI, a rapid equilibrium reversible complex of enzyme and inhibitor. The isomerisation can either be a reversible covalent step, or it can be a conformational change of the complex. If we were to suppose that the enzyme really does follow a ping pong mechanism and that the catalytic residue is an aspartate, then we can speculate on possible chemical reactions between translocase I and bound mureidomycin A. Mureidomycin A contains an enolamine ether function which might react with a nucleophilic active-site residue (fig. 7.4).

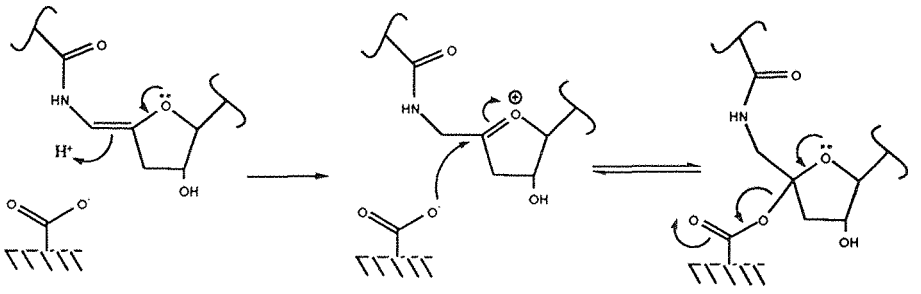


Figure 7.4. Reversible reaction of the enol ether of mureidomycin A with a translocase I active-site nucleophile.

The species generated is labile and would easily decompose back to mureidomycin A and free translocase I, i.e. the EI complex. The problem with this scheme is that in the reaction of translocase I with substrate, the translocase I active-site nucleophile would attack a phosphorus centre five atoms away from the ribose ring. Another scenario is that the carbonyl of the amide is activated to nucleophilic attack by the presence of the alkene or by conformational strain induced by binding to translocase I (fig. 7.5).

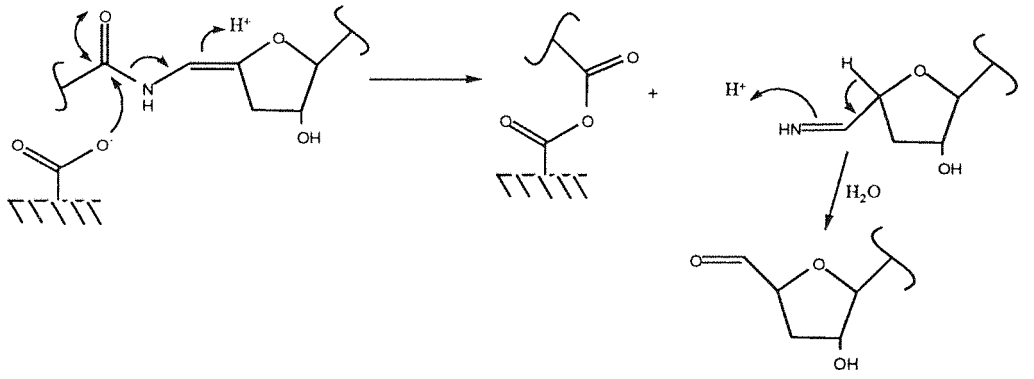


Figure 7.5. Nucleophilic attack of a translocase I active-site residue on carbonyl-activated mureidomycin A.

The anhydride generated could be hydrolysed to give free, active translocase I. This scenario is compatible with the finding that mureidomycin A did not lose potency when incubated with translocase I because this experiment (4.6.6) involved only a 3 min incubation and the process in fig. 7.5 would be stoichiometric with enzyme ($k_{off} < 1 min^{-1}$). The inhibitor would not then be significantly depleted during the assay.

Unlike mureidomycin A, liposidomycin B does not contain any obviously reactive functionality, except for its primary amine and possibly the sulfate group. It also has two ester functions, which are possible electrophilic centres.

To illustrate the transformation from EI to EI*, two examples are taken from a review by Morrison and Walsh.[148] The first is inhibition of acetoacetate decarboxylase by acetoacetone. In this case, a covalent adduct is formed with a lysine side-chain and subsequent enolisation yields a conjugated eneamine (fig. 7.6).

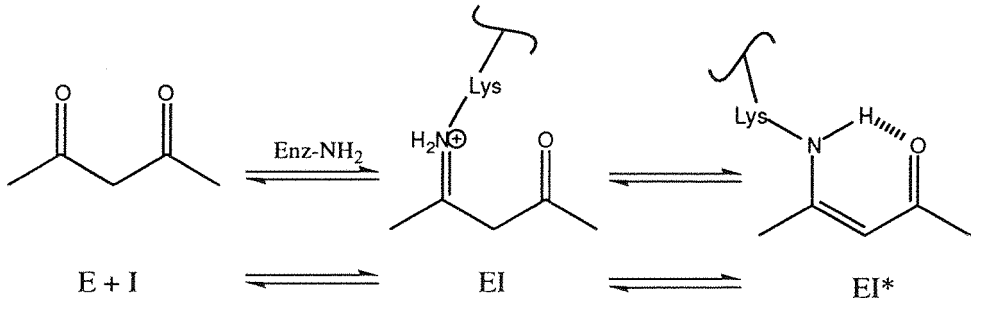


Figure 7.6. Slow-binding inhibition of acetoacetate decarboxylase by acetoacetone.

The second example is inhibition of isopentenyl pyrophosphate isomerase by an aza-analogue of the proposed transition state (fig. 7.7).

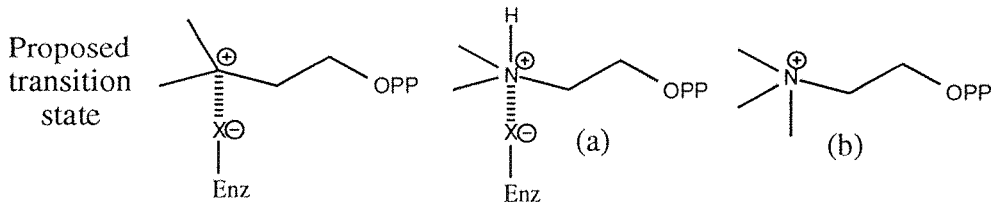


Figure 7.7. Slow-binding inhibition of isopentenyl pyrophosphate isomerase.

Inhibition by the aza-analogue (a) was time-dependent and reversible with a dissociation half-time of >10 hr. The corresponding trimethylammonium analogue (b) was also an inhibitor, but was not a slow-binding inhibitor.

This leads to consideration of a non-covalent isomerisation of the EI complex to EI* for mureidomycin A/liposidomycin B with translocase I. If as seems to be the case, the reaction catalysed by translocase I follows the double displacement mechanism, it may be that the enzyme active-site can adopt two conformations, one for each half of the overall reaction (fig. 7.8).

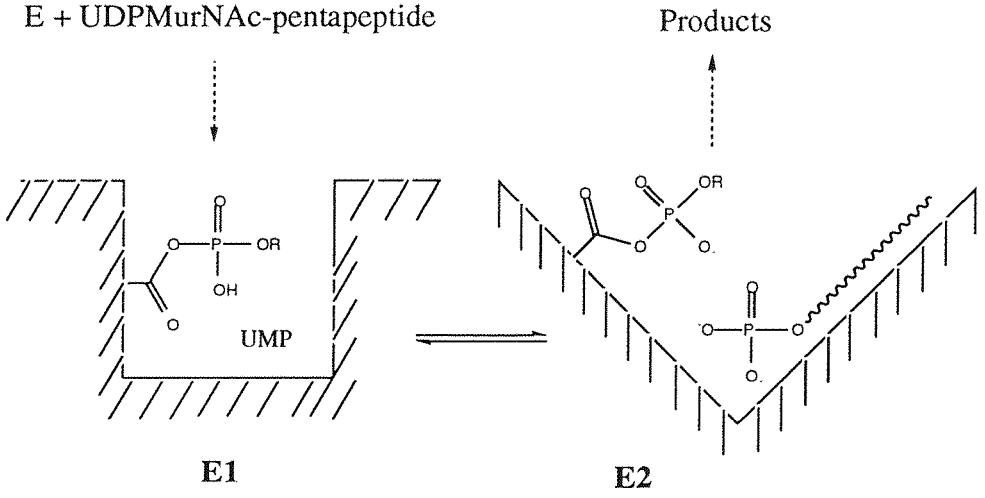


Figure 7.8. Possible conformational change during catalysis by translocase I.

If this were the case, mureidomycin A/liposidomycin B might bind to one conformation more tightly than the other, e.g. fig. 7.9.

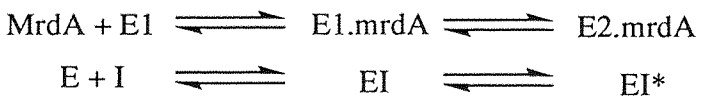


Figure 7.9. Possible mechanism for slow-binding inhibition of translocase I by mureidomycin A.

To reiterate, the ultimate goal of the research is rational design of therapeutic antibiotics. Target molecules might be UMP analogues designed to be active-site directed irreversible inhibitors (fig. 7.10).

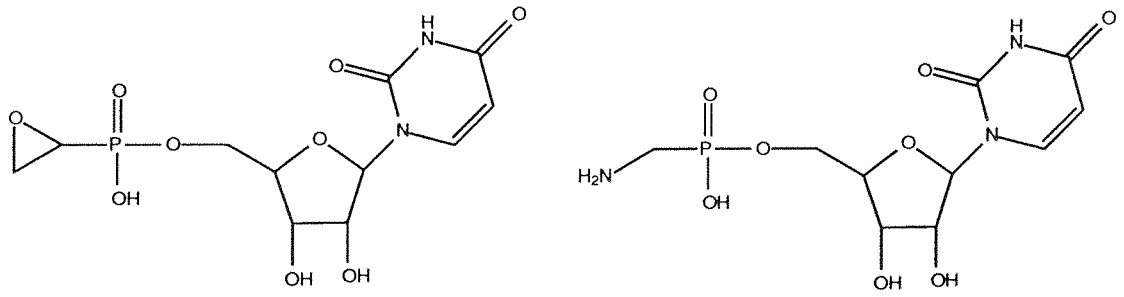


Figure 7.10. Potential translocase I inhibitors.

The rationale for the epoxide compound is that this may react with an active-site nucleophile resulting in a stable adduct. The amine compound might attack an acyl-phosphate intermediate resulting in an amide bond in the active-site, again a stable adduct.

However, a major problem with such compounds would be one of selectivity. If the GlcNAc-1-phosphate transferase of mammalian glycoprotein biosynthesis follows the same reaction mechanism as translocase I as we have proposed, these compounds would be toxic to humans as well as bacteria. Mureidomycin A did not inhibit the GlcNAc-1-phosphate transferase involved in lipid-linked GlcNAc formation from UDPGlcNAc in a membrane preparation of *B. subtilis* [72] and by analogy would be expected not to inhibit the mammalian GlcNAc-1-phosphate transferase.

It is this selectivity which makes mureidomycin A such a remarkable compound. For this reason I believe that future research should be directed toward development of this class of compounds as therapeutic antibiotics.

Chapter 8. Experimental.

8.1. General Methods.

8.1.1. Biconchininic acid (BCA) protein assay.

The method of Smith *et al.* was used to determine protein over the range 10 - 50 μg/mL (0.5 - 2.5 mg/mL in a 20 μL sample volume).[191]

Reagent A: 1 g sodium biconchininate
2 g sodium carbonate
0.16 g sodium tartrate
0.4 g sodium hydroxide
0.95 g sodium hydrogen carbonate

Make to 100 mL with H_20 and adjust to pH 11.25 with 10 M NaOH.

Reagent B: 0.4 g copper sulphate pentahydrate in 10 mL H_20.

Standard working reagent (SWR): 100 vols A + 2 vols B
(stable for 1 week).

Procedure: Add 1 mL SWR to 20 μL sample and mix. Incubate at 37 °C for 30 min. Cool to room temperature. Read absorbance at 562 nm (colour stable for 1 hr). Standards (0.5, 1.0, 1.5, 2.0, 2.5 mg/mL BSA) in duplicate, unknowns in triplicate.

Buffers often contained ß-mercaptoethanol which gives a slight positive reaction with the BCA reagent. When ß-mercaptoethanol was present in buffers, buffer only was included in the protein assay as a control and the small positive reaction subtracted from the unknowns.

8.1.2. Bradford protein assay.

In early experiments the method of Bradford was used to determine protein concentration relative to bovine serum albumin standards.[192] Brilliant blue G250 (20 mg) was dissolved in ethanol and added to 80 % *ortho*-phosphoric acid (20 mL) and made to 200 mL with water. 20 μL of sample containing between 5 μg and 20 μg of protein was mixed with 1 mL of the above prepare reagent and the absorbance at 595 nm measured. Standards were carried out in duplicate, unknowns in triplicate.

8.1.3. Sterilisation.

Media and equipment were sterilised by autoclaving at 121 °C for 25 min. Where solutions could not be autoclaved for stability reasons, sterilisation was effected by passage through an 0.22 μm sterile filter.

8.1.4. Buffer exchange/desalting columns.

Columns of sephadex-G10 (3 mL bed volume) were used to change buffers (but not detergents) or to desalt enzyme samples after chromatography. The column was equilibrated with the new buffer by flowing 10 mL of that buffer through the column (columns stored in 0.1 M NaCl, 0.02 % NaN_3 at 4 °C). Sample (1 mL) was applied and eluted with 1.5 mL of the equilibration buffer. Recovery of enzyme activity was routinely greater than 90 %.

8.1.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

SDS-PAGE was carried out using the BioRad Mini Protean II system as recommended by the manufacturer. The acrylamide stock contained acrylamide and bis-acrylamide (39:1) and was a 30 % or 40 % stock solution in water stored at 4 °C in the dark. Gel slabs were 1 mm thick and were 5 - 6 cm high (12 % acrylamide final concentration) with 1 cm of stacking gel (4.5 % acrylamide final concentration). Gel recipes were for two gels: (APS = ammonium persulfate)

<u>Running gel recipe</u>

Water	5.25 mL	6.75 mL (for 40 % acrylamide)
1.5 M Tris pH 8.8	3.75 mL	
10 % SDS	150 μl	
Acrylamide	6.0 mL	4.5 mL (for 40 % acrylamide)
	---	
10 % APS	75 μL	
TEMED	7.5 μL	

<u>Stacking gel recipe</u>

Water	5.9 mL	6.275 mL (for 40 % acrylamide)
0.5 M Tris pH 6.8	2.5 mL	
10 % SDS	100 μL	
Acrylamide	1.5 mL	1.125 mL (for 40% acrylamide)
	---	
10 % APS	50 μL	

TEMED	10 µL

Wells were made in the stacking gel with combs supplied with the aparatus to hold up to 25 µL of sample. Protein samples contained 5 - 20 µg protein in 10 µL. Sample concentration was achieved where necessary by precipitation of protein with 5 % trichloroacetic acid (on ice, 20 min) and centrifugation (13500 rpm, 10 min). Pellets were dissolved in 10 µL 100 mM NaOH to ensure neutralisation of the acid. 'Loading buffer' was prepared according to the recipe:

0.75 mL	80 % glycerol
2.0 mL	10 % SDS
1.0 mL	0.5 M Tris pH 6.8
0.2 mL	0.05 % bromophenol blue
5.55 mL	H_2O

1 volume of ß-mercaptoethanol was added to 19 volumes of 'loading buffer' just before use. 1 part of protein sample was added to two parts 'loading buffer' + ß-mercaptoethanol and boiled for 2 min. When whole cells were the sample, chromosomal DNA was sheared by sonication where necessary to allow loading of the sample onto the gel.

Molecular weight markers were chicken egg white lysozyme (14.3 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (29 kDa), chicken egg ovalbumin (43 kDa) and bovine serum albumin (66 kDa).

Gels were run at 200 V in 25 mM Tris-glycine pH 8.3, 0.1 % SDS (which was stored as a 5x concentrated stock solution) for 30 - 45 min, stained with Coomassie blue (0.25 % Coomassie brilliant blue R250 in 10 % acetic acid, 45 % methanol) and destained with 10 % acetic acid, 10 % methanol.

Gels were dried between sheets of Promega gel drying film as recommended by the manufacturer.

8.2. Chapter 2 experimental.

8.2.2.1. Radiochemical assay - general method.

The exact assay constituents and order of addition in any given experiment are listed in the relevant section. The general methodology used is as follows. Lipids to be included in an assay were taken from a stock solution in organic solvent and placed/mixed in a microfuge tube. Solvent was removed with a stream of nitrogen. The residue was dispersed either by an aliquot of detergent, in the case of particulate enzyme, or by addition of the enzyme sample itself in the case of detergent solubilised enzyme. Where detergent was included in the assay of particulate enzyme, the final assay concentration never exceeded the CMC of that detergent. In assays of solubilised enzyme, detergent concentration was constant, adding extra detergent if necessary. Assays were in a final volume of 50 µL and were started either by addition of a preprepared stock of 4:1 (mole/mole) UDPMurNAc-pentapeptide:[^{14}C]-UDPMurNAc-pentapeptide (specific activity 78 µCi/µmol) or by addition of enzyme (plus any lipids). In either case, all assay constituents were equilibrated to the assay temperature before beginning the assay. Assays were stopped by addition of an equal volume (50 µL) of 6 M pyridinium acetate, pH 4.2, or in the case of time-course assays, a 50 µL aliquot was removed and added to 50 µL of 6 M pyridinium acetate, pH 4.2. The two were mixed by pipetting, then vortexed for 2 s and held on ice until the series of assays was complete. n-Butanol (100 µL) was added to the stopped reaction mixtures and mixed by vortexing (5 s). The phases were separated by microcentrifugation (pulse, 13500 rpm). The upper organic phase was generally larger than the lower aqueous phase, but the respective volumes never varied within any given batch of assays. The variance is due to variance of the assay mixture. The volume of the upper phase was noted and a proportion, usually 100 µL of 120 µL, removed (avoiding any precipitate at the interface) for scintillation counting. Optiphase Hi-Safe 3 (4.5 mL) was added to the butanol extracts in disposable plastic inserts. Radioactivity was counted using a Packard Tri-CARB 300CD liquid scintillation counter monitoring the 4 - 156 keV window for 5 or 10 min. Single time-point assays were carried out in duplicate and the average result quoted. Controls contained buffer only and were measured in triplicate. Knowing the molar concentration of the stock radiolabelled substrate, and knowing the number of counts/min given by a 5 nCi aliquot of [^{14}C]-UDPMurNAc-pentapeptide, it was possible to convert data obtained as counts/min to pmoles.

8.2.3. Comparison of translocase I activity in particulate preparations from *E. coli* JM105 and *E. coli* JM109 (pBROC512).

Escherichia coli JM109 (pBROC 512) provided by SKB was grown to high biomass at 37 °C in 5 mL of Luria broth (LB, bacto-tryptone, 10 g/L, yeast extract, 5 g/L, sodium chloride, 10 g/L) containing kanamycin (50 μg/mL) and a sample stored in 7 % DMSO at -70 °C for future use. This culture was also used to innoculate 500 mL of the same medium and a plate of LB-kanamycin agar which once grown was kept at 4 °C. Growth at 37 °C was monitored by absorbance at 600 nm and a growth curve constructed. A 500 mL culture was grown in the same way from the plate; at the start of log phase growth (Abs_{600nm} = 0.6) isopropylthiogalactoside (IPTG, 12 mg, final concentration 0.1 mM) was added. At the end of log phase growth cells were harvested by centrifugation (4400 *g*, 10 min), washed in 50 mM potassium phosphate, 2 mM ß-mercaptoethanol (140 μL/L), pH 7.0 (200 mL) and collected by centrifugation (4400*g*, 10 min). Cells were resuspended in the above buffer (~1 mL/g cells) to give a thin paste and incubated in a sonication tube with lysozyme (10 mg, 30 min, rt) prior to thorough sonication on ice. Whole cells and debris were removed by centrifugation (12000 *g*, 30 min). Cell envelopes were collected from the supernatant by ultracentrifugation (10^5g, 30 min). The membranes were washed by resuspension in 10 mM potassium phosphate, 2 mM ß-mercaptoethanol, pH 7.0 and pelleted as above. Protein concentration was determined by the method of Bradford. The membrane suspension was stored at -20 °C in small aliquots. *E. coli* JM105 membranes were prepared in the same way, but without kanamycin or IPTG-induction.

Membranes were assayed for translocase I activity as described above. Assays contained 100 mM Tris, pH 7.5, 10 % DMSO, 25 mM $MgCl_2$, 1.4 μM [^{14}C]-UDPMurNAc-pentapeptide (5.0 nCi/50 μL aliquot) and membranes (0.38 mg protein/mL) and were carried out at room temperature (≈25 °C). 5.4 nCi of [^{14}C]-UDPMurNAc-pentapeptide gave 5865 cpm. The specific activity was 78 μCi/μmole, hence 1000 cpm = 11.9 pmol.

8.2.4.1. Preparation of farnesyl phosphate.

The method described is based on that of Danilov *et. al;*.[193] 3,7,11-trimethylpentadeca-2,6,10-trien-1-ol (farnesol, 0.9 mL, 3.9 mmol) was stirred with tetra-N-butylammonium dihydrogen phosphate (6.6 g, 19.5 mmol) in refluxing chloroform (20 mL) until the salt had dissolved before addition of trichloroacetonitrile (2.5 mL, 25.0 mmol). After 90 min a strong streak was visible in tlc (R_f. 0.10 - 0.30). The reaction was stopped by cooling and rotary evaporation of solvent under reduced pressure (starting material remained but refluxing for longer gave a coloured solution). The resulting sludge was taken up in upper phase

equilibrium water-butanol (50 mL) and washed with lower phase equilibrium water-butanol (4 x 25 mL). Solvent was removed from the organic phase by rotary evaporation and co-evaporated with toluene-methanol (2:1, 2 x 40 mL). The residue was dissolved in chloroform-methanol (2:1, 30 mL) and stirred with dowex 50W resin (ammonium form, 3.0 g) overnight. The resin was filtered and washed with chloroform-methanol (2:1, 3 x 15 mL). The combined filtrate and washings were concentrated to 10 mL by rotary evaporation and applied to a column of DEAE-sephadex in methanol (chloride form, 23 x 2.5 cm initially). The column was washed with methanol (300 mL) followed by 30 mM ammonium acetate in methanol (400 mL). Two spots (r.f. 0.08 and 0.39) eluted together. Alkaline phosphatase digestion (The sample (200 µl) was evaporated to dryness and taken up in 50mM Tris-NaOH pH 10.4, 100 mM glycine, 1mM magnesium chloride, 1mM zinc chloride (200 µl) before addition of the enzyme. The mixture was incubated at 25 °C for 15 min) removed the lower spot and yielded farnesol (as judged by tlc) whilst the upper spot was unchanged. The compounds were resolved by two passes through a column of G10-sephadex. In each case the pooled fractions were evaporated to dryness, dissolved in the minimum of methanol and applied to the G10-sephadex column (53 x 1.6 cm) in methanol. The column was washed with methanol and the fractions examined by tlc. Evaporation of solvent afforded nearly pure white solid of ammonium farnesyl phosphate (48 mg, 4 % yield). ^{1}H-NMR ($CDCl_3$) 1.6-1.7 (s, 12H, CH_3), 2.05 (m, 8H, CH_2), 4.4 (t, 2H, CH_2-O-P), 5.1 (t, 2H, $(=CH_2)_2$), and 5.4 (t, 1H, =CH) ppm. ^{31}P-NMR ($CDCl_3$) 4.24 (s, phosphate) ppm.

8.2.4.2. Assay of particulate enzyme in the presence of farnesyl phosphate.

Where farnesyl phosphate (FP) was included in the assay, membrane stocks were pre-incubated with the prenyl phosphate. Stock solutions of FP in 1:1 $CHCl_3$/MeOH were prepared at 35, 17.5, 7, 3.5 and 1.75 mM. For each of these 1 µL of stock solution was added to 50 µL membrane suspension and vortexed. These preincubations gave farnesyl phosphate concentrations in the assay of 140, 70, 28, 14 and 7 µM respectively. The concentration of [^{14}C]-UDP-MurNAc-pentapeptide in the assay is 1.4 µM. The mixture was left on ice for 30 min vortexing once in this time and once again immediately before addition to the reaction mixture. Suitable controls were included to allow for any inhibition of the assay by the solvents included (0.2 % each in the final assay mixture). Assay mixtures were as in 8.2.3 with *E. coli* JM109 (pBROC512) membranes at 1 mg protein/mL.

8.2.4.3. Assay of particulate enzyme in the presence of snake venom nucleotidase.

Assay mixtures were as in 8.2.3 with *E. coli* JM109 (pBROC512) membranes at 1 mg protein/mL and 20 munits of fresh partially purified snake venom nucleotidase (Sigma, 20 munits/50 µL assay).

8.2.4.4. Effect of ATP on particulate translocase I activity.

Assay mixtures were as in 8.2.3 with *E. coli* JM109 (pBROC512) membranes at 1 mg protein/mL and ATP at 0.1, 1, 5, 10 or 20 mM. Reactions were at 25 °C and were stopped at 1 min or 30 min at each concentration of ATP.

8.2.5.1. Comparison of particulate enzyme activity in *E. coli* JM105, JM109 (pBROC512) and JM109 (pBROC525).

A stab culture of *E. coli* JM109 (pBROC525) was provided by SB Pharmaceuticals. This was used to innoculate LB (5 mL) containing 100 µg/mL ampicillin and grown overnight at 37 °C. Of this overnight culture, 1.5 mL was added to 1 mL of sterile 50 % aq. glycerol such that the final glycerol concentration was 20 %. 1 mL of this was stored in a Nunc cryotube at -70 °C. The overnight culture was also streaked out on LB/amp agar (100 µg/mL antibiotic).

Each of *E. coli* JM105, JM109 (pBROC512) and JM109 (pBROC525) were grown in 5 mL LB containing no antibiotic, 50 µg/mL kanamycin or 100 µg/mL ampicillin respectively overnight at 37 °C. These were used to innoculate (2 %) 500 mL each of LB in 2 L baffled flasks containing antibiotics as appropriate. At OD_{600nm} = 0.6 , solid IPTG was added to the cultures of *E. coli* JM109 (pBROC512) and JM109 (pBROC525) to final concentration 48 mg/L, 0.2 mM. Cultures were grown for a further 4 hr and cells harvested by centrifugation (4400 *g*, 10 min). At this point OD_{600nm} = 1.83, 1.60 and 1.70 for *E. coli* JM105,JM109 (pBROC512) and JM109 (pBROC525) respectively. Cells were stored overnight at -70 °C. Thawed cells were resuspended in 50 mM Tris, pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$ (buffer A), 2 mL buffer/g cells. Lysozyme (small spatula end, approximately 5 mg) was added and cells gently stirred at room temperature for 30 min, followed by sonication on ice (3 x 1.5 min at 25 kcs/s, 5 µ). Whole cells and debris were removed by centrifugation (12000 *g*, 30 min) and membranes collected from the supernatant by ultracentrifugation (10^5 *g*, 30 min). Membrane pellets were resuspended in buffer A + 1 M NaCl using a teflon homogeniser and stirred gently for 15 min and 4 °C. Salt stripped membranes were collected by ultracentrifugation (10^5 *g*, 30 min) and resuspended in 400 µL each of buffer A. Since buffer A contains ß-mercaptoethanol it was prepared freshly each time it was used. Protein concentrations were determined using the BCA assay at 5.75 mg/mL, 2.35 mg/mL and 3.75 mg/mL for *E. coli* JM105,

JM109 (pBROC512) and JM109 (pBROC525) respectively. The preparations were frozen in liquid nitrogen and stored at -20 °C.

Particulate enzyme was assayed as follows: 20 µL of 50 µg/mL heptaprenyl phosphate (1.65 nmoles per assay) in 1:1 isopropanol/toluene was placed in a microfuge tube and solvent removed with a stream of nitrogen. The lipid was dispersed with 5 µL 0.1 % Triton X-100 and 25 µL 200 mM Tris pH 9.0, 50 mM $MgCl_2$ was added. 10 µL of enzyme (diluted 10-fold with buffer A for pBROC525) was added and incubated at 37 °C for 5 min. Reaction was started by addition of 10 µL UDPMurNAc-pentapeptide (5 µL [^{14}C]-UDPMurNAc-pentapeptide, 2 nCi/µL, 28 µM and 5 µL UDPMurNAc-pentapeptide, 112 µM). Incubations were at 37 °C for 30 min for *E. coli* JM105 and 15 min for JM109 (pBROC512) and JM109 (pBROC525). Product was quantified as in 8.2.2.1.

8.2.5.2. Comparison of translocase I activity in preparations of *E. coli* JM109 (pBROC525) induced with IPTG in early or late logarithmic phase.

LB + 100 µg/mL ampicillin (LB/amp, 20 mL) was innoculated with a single colony of *E. coli* JM109 (pBROC525) and grown overnight at 37 °C. This was used to innoculate 2 x 1 L of LB/amp at 1 %. These cultures were grown to either OD_{600nm} = 0.6 or OD_{600nm} = 1.9 and induced with IPTG (final concentration, 0.2 mM). Cultures were grown for a further 3 hr when OD_{600nm} = 1.95 and 2.25 for 'early' or 'late' induced cultures respectively, and harvested by centrifugation (4400 *g*, 10 min). Pellets were washed in 50 mM Tris, pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$ (50 mL each), collected by centrifugation (4400 *g*, 10 min) and stored overnight at -70 °C. Wet weights of cell pellets were 4.33 g and 5.48 g for 'early' and 'late' induced cultures respectively. Thawed cells were resuspended in buffer A (10 mL/g cells) at 4 °C and homogenised using a teflon homogeniser. Cells were broken with two passes through a Stansted cell disrupter with a forward pressure of 50 psi and a back pressure of 2 psi. Membranes were isolated as in 8.2.5.1. The pellet from the 'early' preparation was resuspended in a final volume of 3.4 mL and the 'late' in a final volume of 2.75 mL. Protein was determined by the BCA assay to be 13.7 mg/mL for the 'early' preparation and 17.3 mg/mL for the 'late' preparation. Membranes were diluted 5-fold for enzyme assays. Assays were conducted exactly as in 8.2.5.1 except that the temperature throughout was 25 °C and time-courses (1, 2 and 3 min timepoints) were measured.

8.2.5.3. Effect of varying IPTG concentration on expression of translocase I.

Three 500 mL cultures of *E. coli* JM109 (pBROC525) were grown to OD_{600nm} = 1.7 as described in 8.2.5.2 and induced with 0.05 mM, 0.2 mM or 1.0 mM IPTG. Cultures were grown for a further 90 min and harvested and washed as in

8.2.5.2. Wet weights of cell pellets were 4.72 g, 5.19 g and 4.75 g for cultures induced with 0.05 mM, 0.2 mM and 1.0 mM IPTG respectively. Membranes were prepared and assayed as in 8.2.5.2 (table 8.1).

IPTG, mM	Membrane suspension, mL	Protein, mg/mL	Spec. act. pmol/min/mg	Yield of activity nmol/min/g cells
0.05	3.71	12.6	343	3.40
0.2	3.71	13.5	519	5.01
1.0	3.21	12.4	348	2.92

Table 8.1. Data from 'Effect of varying IPTG' experiment.

8.2.5.4. Overnight induction of *E. coli* JM109 (pBROC525).

LB/amp (500 mL) was innoculated with 2.5 mL of an overnight culture of *E. coli* JM109 (pBROC525) and grown to OD_{600nm} = 1.3 at 37 °C. The culture was cooled to 20 - 25 °C by running under a cold tap with constant swirling. IPTG was added to 0.2 mM. The culture was grown overnight on the bench with shaking (15 hr). Cells were harvested (wet weight, 5.0 g) by centrifugation (4400 *g*, 10 min). Cells were resuspended in 50 mM Tris, pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$ (50 mL) and membranes prepared as described in 8.2.5.2 except that membranes were stripped of associated proteins by stirring in buffer A + 1 M KCl for 1 hr at 4 °C rather than 1 M NaCl for 15 min. The final volume of membrane suspension was 2.4 mL, protein concentration (BCA assay) = 3.5 mg/mL. Translocase I was solubilised at 2 mg protein/mL 50 mM Tris, pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol, 0.5 % Trition X-100 with stirring for 1 hr at 4 °C. Unsolubilised material was removed by ultracentrifugation (10^5 *g*, 30 min) and enzyme assayed by the fluorescence enhancement method (8.4.6.4). The assay contained 100 mM Tris, pH 7.5, 50 mM KCl, 25 mM $MgCl_2$, 0.2 % Triton X-100, 100 µg/mL phosphatidylglycerol, 40 µM dodecaprenyl phosphate and 60 µL enzyme in a final volume of 300 µL. The reaction was started by the addition of 25 µL of 10x diluted stock dansyl-UDPMurNAc-pentapeptide (SB stock, ≈3.15 mM) giving 26 µM final assay concentration. Rates were measured at 1.7 and 2.0 units/min giving a specific activity of approximately (assuming 70 % of protein in the membrane was extracted as was consistently observed at this detergent/protein ratio) 11.0 units/min/mg protein. This is slightly less than that observed in standard preparations e.g. section 6.6 (pTrc99A/pBROC525 comp).

8.2.5.5. SDS-PAGE of IPTG-induced cells of *E. coli* JM109 (pBROC525).

A 50 mL LB/amp culture of *E. coli* JM109 (pBROC525) was grown to OD_{600nm} = 1.7 as in 8.2.5.2 and split in two. One 25 mL aliquot was induced with IPTG (0.2 mM) and one was not. After 3 hr growth at 37 °C, cells were collected from 5 mL of each culture by centrifugation (4400 *g*, 10 min). The pellet was resuspended in 1 mL 50 mM Tris, pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$ and 10 µL of this mixed with 10 µL of SDS-PAGE loading buffer. 5 µL or 20 µL of each sample was analysed on a 12 % SDS-PAGE gel using the protocol described in section 8.1.

8.2.6.1. Preparation of sodium deoxycholate.

Deoxycholic acid (1 g) was recrystallised from the minimum of hot ethanol with ether. The precipitate was filtered, washed with ether and dried in a dessicator to give a first crop of 0.75 g. The solid was resuspended in water (5 mL). An equimolar amount of 3 M NaOH was added and made to 7.5 mL to give a 10 % solution of sodium deoxycholate.

8.2.6.2. Solubilisation experiments.

Membranes were prepared from *E. coli* JM109 (pBROC512) as described in section 8.2.3. Triton X-100 and CHAPS were obtained from Pierce as part of a kit. Deoxycholic acid, sodium lauroyl sarcosinate and N,N',N'-polyoxyethylene(10)-N-tallow-1,3-diaminopropane (EDT-20) were obtained from Sigma. The latter two were made to 10% solutions in the solubilisation buffer defined below.

Membrane fragments were extracted at 4 mg protein/mL in an 0.5 mL total volume with detergent at 0.25 %, 0.5 %, 1.0 % or 3.0 % in 100 mM potassium phosphate, pH 7.5, 25 mM $MgCl_2$, 2 mM ß-mercaptoethanol. After mixing by shaking (rather than by vortexing) the mixtures were incubated for 1hr at 4 °C before centrifugation at 10^5 *g* for 1 hr. Supernatants were taken and assayed for protein and translocase I activity. Detergent concentration in assays of CHAPS solubilised enzyme was maintained at 1 % w/v. Assays contained 100 mM Tris pH 7.5, 25 mM $MgCl_2$, 10 nCi, 2.8 µM [^{14}C]-UDPMurNAc-pentapeptide, 11.2 µM UDPMurNAc-pentapeptide, extraction supernatant or 4x diluted membrane suspension, (10 µL) in a volume of 50 µL. Reactions were started by addition of enzyme and gentle vortexing. After incubation for 15 min or 30 min at 30 °C reactions were stopped by addition of 6 M pyridinium acetate pH 4.2 (50 µL) and vortexed. Product was quantified as in 8.2.2.1.

8.2.6.3. Stability of Triton X-100 solublised enzyme.

Aliquots of enzyme prepared and solubilised with 0.5 % Triton X-100 as in 8.2.6.2 (200 µL) were diluted to 250 µL with appropriate stock solutions to give the following additives at the given final concentration: 5 %, 10 %, 20 % glycerol, 20 % sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF. Activity remaining after one week on ice in the control was 60 % and for those samples with additives was 74 %, 78 %, 86 %, 81 %, 77 %, 68 % and 68 % respectively. Enzyme was assayed exactly as in 8.2.6.2.

8.2.6.4. Comparison of Tris buffer and phosphate buffer.

Membranes of *E. coli* JM109 (pBROC512) were prepared as in 8.2.3, except that the preparative buffer was 50 mM Tris, pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, instead of 100 mM potassium phosphate, pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$. Protein was measured using the BCA assay. Enzyme activity was measured exactly as in 8.2.5.1. Total activity in the preparation fresh, and after 1.5, 6 and 8 days was 148, 143, 127 and 109 pmol/min at 37 °C respectively.

8.2.6.5. pH-rate profile of Triton X-100 solubilised translocase I.

Membranes of *E. coli* JM109 (pBROC512) were prepared as in 8.2.3. Enzyme was solubilised at 5 mg protein/mL in 100 mM potassium phosphate, pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol by stirring for 1 hr at 4 °C. Unsolubilised material was removed by ultracentrifugation (10^5 *g*, 30 min). The supernatant contained 3.85 mg protein/mL (BCA assay). Enzyme was assayed at pH 7.0, 7.5, 8.0, 8.5, 9.0 and 9.5 in duplicate for 15 min at 30 °C. Each assay contained 25 µL 200 mM Tris, pH varied, 50 mM $MgCl_2$, 4.4 µL H_2O, 10 µL enzyme, 5 µL [^{14}C]-UDPMurNAc-pentapeptide (2 nCi/µL, 28 µM) and 5 µL 100 µM UDPMurNAc-pentapeptide. Reactions were started by addition of substrate. Product was quantified as in 8.2.2.1. The amount of product at pH 7.0, 7.5, 8.0, 8.5, 9.0 and 9.5 was 3.4, 6.4, 14.6, 14.2, 12.4 and 12.4 pmol respectively.

8.2.6.6. Heat stability of Triton X-100 solubilised translocase I.

Translocase I was prepared and assayed exactly as in 8.2.6.5. Aliquots of enzyme were held on ice, at 37 °C, 50 °C or 60 °C for 15 min prior to assay. The amount of product after incubation on ice, at 37 °C, 50 °C or 60 °C was 15.2, 16.0, 12.3 and zero pmol after 15 min respectively.

8.2.7.1. Assay of translocase I with 0 - 5 mM UDPMurNAc-pentapeptide over 16 hr.

Crude membranes containing translocase I were prepared from *E. coli* JM109 (pBROC512) as in 8.2.3. The protein concentration was 10 mg/mL (Bradford assay). Enzyme assays contained 100 mM Tris, pH 7.5, 25 mM $MgCl_2$, membrane fragments (0.5 mg protein/mL), 10 nCi [^{14}C]-UDPMurNAc-pentapeptide (2.8 μM final concentration) and unlabelled UDPMurNAc-pentapeptide such that the final concentration of the substrate was 22.2 μM, 47.2 μM, 72.2 μM, 97.2 μM, 250 μM, 500 μM, 750 μM, 1 mM, 2 mM or 5 mM. Assays were started by addition of UDPMurNAc-pentapeptide and were incubated at 20 °C for 16 hr. Product was quantified as in 8.2.2.1.

8.2.7.2. Malachite green assay for inorganic phosphate.

This assay based on a literature method[132] gives a linear response over the range 0.2 - 10 nmoles of inorganic phosphate in a 50 μL sample volume. All solutions were prepared with high quality deionised water.

Stock solutions:
1. 0.045 % malachite green oxalate
2. 4.2 % ammonium molybdate in 4 M HCl
3. 34 % sodium citrate
4. 5 mM potassium dihydrogen phosphate (68 mg/100 mL)

Working reagent (WR):
- mix 3 vol stock 1 + 1 vol stock 2
- stir for 20 min
- filter off any precipitate

Procedure:
- add 800 μL WR to 50 μL sample
- incubate at room temperature for 10 min
- add 100 μL stock 3, stand for 1 min
- read absorbance at 660 nm

Standard curves were prepared from a 25x diluted sample of stock 4 (i.e. 0.2 mM phosphate) at 0, 2, 4, 6, 8 and 10 nmoles of phosphate in a 50 μL sample volume. Standards were measured in duplicate, unknowns in triplicate.

8.2.7.3. Assay of 5'-nucleotidase activity.

The mixture contained 25 μL of 200 mM Tris, 50 mM $MgCl_2$ at pH 9.0, 10 μL of 2 mM uridine monophosphate, 15 μL water and 0.1 units of agarose-

immobilised snake venom nucleotide phosphatase (Sigma, 1 unit is defined as hydrolysing 1 μmole of inorganic phosphate from adenosine monophosphate per minute at 37 °C and pH 9.0). Incubation was for 1 min at 37 °C before assessment of inorganic phosphate.

8.2.7.4. Phosphate-release assay.

Membranes of *E. coli* JM109 (pBROC512) were prepared as in 8.2.3, except that the preparative buffer was 50 mM Tris, pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$. An assay mixture was prepared such that it contained enough for removal of at least 10 x 50 μL aliquots for quantitation of product by either the radiochemical assay or by the phosphate-release method.

The reaction contained 100 mM Tris, pH 9.0, 25 mM $MgCl_2$, 0.01 % Triton X-100, membrane fragments (4 mg protein/mL), 4 mM UDPMurNAc-pentapeptide and 2.8 μM [^{14}C]-UDPMurNAc-pentapeptide (total 120 nCi) in a volume of 600 μL. The assay was carried out at 37 °C, starting by addition of substrate. Aliquots (50 μL) were withdrawn at 0, 1, 2, 4 and 8 hr and quantified by either method. The radiochemical method was as in 8.2.2.1.

In the phosphate-release method, the 50 μL aliquot was boiled for 1 min and plunged to an ice-bath. After pulse microcentrifugation to return all the liquid to the bottom of the microfuge tube, the liquid was added to a pellet of agarose-immobilised 5'-nucleotidase containing 0.1 units of enzyme (1 unit is defined as hydrolysing 1 μmol ATP/min at 37 °C at pH 9.0 in saturating ATP). The 5'-nucleotidase was resuspended by vortexing and the mixture incubated for 5 min at 37 °C. 5'-nucleotidase was then pelleted by microcentrifugation. A control containing 0.4 mM UMP (20 nmoles in 50 μL) was included to check that all the UMP present was hydrolysed. Inorganic phosphate was quantified using the Malachite green assay described in 8.2.7.2.

8.2.7.5. Assay of particulate enzyme in the presence or absence of heptaprenyl phosphate.

Membranes of *E. coli* JM109 (pBROC512) were prepared as in 8.2.3, except that the preparative buffer was 50 mM Tris, pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$. Heptaprenyl phosphate (10 μL, 50 μg/mL, 8.25 nmoles) in 1:1 isopropanol/toluene was placed in a microfuge tube and solvent removed with a stream of nitrogen. The lipid was dispersed with 0.1 % Triton X-100 (5 μL). Buffer, 0.5 M Tris, pH 9.0, 125 mM $MgCl_2$ (10 μL) was added followed by membrane fragments (10 μL, 10 mg protein/mL). This was incubated for 5 min at 37 °C before addition of 5 μL 2 nCi/μL [^{14}C]-UDPMurNAc-pentapeptide (28 μM) and 5.6 μL

immobilised snake venom nucleotide phosphatase (Sigma, 1 unit is defined as hydrolysing 1 μmole of inorganic phosphate from adenosine monophosphate per minute at 37 °C and pH 9.0). Incubation was for 1 min at 37 °C before assessment of inorganic phosphate.

8.2.7.4. Phosphate-release assay.

Membranes of *E. coli* JM109 (pBROC512) were prepared as in 8.2.3, except that the preparative buffer was 50 mM Tris, pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$. An assay mixture was prepared such that it contained enough for removal of at least 10 x 50 μL aliquots for quantitation of product by either the radiochemical assay or by the phosphate-release method.

The reaction contained 100 mM Tris, pH 9.0, 25 mM $MgCl_2$, 0.01 % Triton X-100, membrane fragments (4 mg protein/mL), 4 mM UDPMurNAc-pentapeptide and 2.8 μM [^{14}C]-UDPMurNAc-pentapeptide (total 120 nCi) in a volume of 600 μL. The assay was carried out at 37 °C, starting by addition of substrate. Aliquots (50 μL) were withdrawn at 0, 1, 2, 4 and 8 hr and quantified by either method. The radiochemical method was as in 8.2.2.1.

In the phosphate-release method, the 50 μL aliquot was boiled for 1 min and plunged to an ice-bath. After pulse microcentrifugation to return all the liquid to the bottom of the microfuge tube, the liquid was added to a pellet of agarose-immobilised 5'-nucleotidase containing 0.1 units of enzyme (1 unit is defined as hydrolysing 1 μmol ATP/min at 37 °C at pH 9.0 in saturating ATP). The 5'-nucleotidase was resuspended by vortexing and the mixture incubated for 5 min at 37 °C. 5'-nucleotidase was then pelleted by microcentrifugation. A control containing 0.4 mM UMP (20 nmoles in 50 μL) was included to check that all the UMP present was hydrolysed, which it was. Inorganic phosphate was quantified using the Malachite green assay described in 8.2.7.2.

8.2.7.5. Assay of particulate enzyme in the presence or absence of heptaprenyl phosphate.

Membranes of *E. coli* JM109 (pBROC512) were prepared as in 8.2.3, except that the preparative buffer was 50 mM Tris, pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$. Heptaprenyl phosphate (10 μL, 50 μg/mL, 8.25 nmoles) in 1:1 isopropanol/toluene was placed in a microfuge tube and solvent removed with a stream of nitrogen. The lipid was dispersed with 0.1 % Triton X-100 (5 μL). Buffer, 0.5 M Tris, pH 9.0, 125 mM $MgCl_2$ (10 μL) was added followed by membrane fragments (10 μL, 10 mg protein/mL). This was incubated for 5 min at 37 °C before addition of 5 μL 2 nCi/μL [^{14}C]-UDPMurNAc-pentapeptide (28 μM) and 5.6 μL

UDPMurNAc-pentapeptide (100 μM) (premixed) to start the reaction. The same assay was performed in the absence of heptaprenyl phosphate.

8.2.7.6. Acid precipitation of ^{14}C-labelled products.

Since the butanol-extraction method used above measures both lipid-linked product and phospho-MurNAc-pentapeptide a method of determination of lipid-linked product based on that Weppner and Neuhaus[118] was employed to distinguish the associated activities of the enzyme. Membranes of *E. coli* JM109 (pBROC512) were prepared as in 8.2.3, except that the preparative buffer was 50 mM Tris, pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$. The reaction mixture contained 100 mM Tris pH 9.0, 25 mM $MgCl_2$, 60 nCi [^{14}C]-UDPMurNAc-pentapeptide (2.8 μM), 11.2 μM UDPMurNAc-pentapeptide and membranes (1.4 mg protein/mL). Reaction was at 37 °C and was started by addition of substrate. Aliquots (50 μL) were withdrawn at 0, 5, 10, 20 and 30 min and added to ice-chilled 0.3 M acetic acid (100 μL). Quenched reaction aliquots were held on ice for 15 - 45 min then centrifuged (13500 rpm, 15 min). The supernatant was discarded and the pellet washed wuth chilled 0.3M acetic acid (2 x 0.5 mL), resuspended in butanol (200 μL) and radioactivity measured as above. The zero time point gave natural background radioactivity.

8.2.8.1. Effect of phospholipids on Triton X-100 solubilised translocase I.

Membranes were prepared from IPTG-induced *E. coli* JM109 (pBROC525) using 0.2 mM IPTG as described in 8.2.5.3. Protein concentration was 20.2 mg/mL (BCA assay). Membranes were extracted at 4 mg protein/mL in 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 0.5 % Triton X-100, 20 % glycerol with stirring (1 hr, 4 °C). Unsolubilised material was removed by centrifugation (10^5 *g*, 30 min). Supernatant protein concentration was 1.65 mg/mL (BCA assay). A mixture of lipids was prepared by placing 4 μL of 5 mg/mL HP (20 μg) in 1:1 isopropanol:toluene and 10 μL of 10 mg/mL (100 μg) PE in $CHCl_3$ in an microfuge tube and solvent removed with a stream of nitrogen before addition of 10 μL of 10 mg/mL (100 μg) PG in 1.0 % Triton X-100 and 40 μL of 0.1 % Triton X-100. The lipid mix was added (5 μL per 100 μL sample) to each fraction and the fractions assayed for translocase I activity. Assays contained 100 mM Tris pH 7.5, 25 mM $MgCl_2$, 10 nCi [^{14}C]-UDPMurNAc-pentapeptide (2.8 μM), 11.2 μM UDPMurNAc-pentapeptide and enzyme (15 μL, giving final assay concentrations of 5.7 μg/mL (9.4 μM) HP, 28.5 μg/mL PG and PE) in a volume of 50 μL. Reaction was at 37 °C for 30 min and was started by addition of substrate. Product was quantified as in 8.2.2.1.

8.2.8.2. Effect of phospholipids on CHAPS-solubilised translocase I.

This experiment was carried out exactly as 8.2.8.1. except that enzyme solubilisation buffer contained 1.5 % CHAPS, not Triton X-100 and assays contained 1 % CHAPS (final concentration). The extraction supernatant contained 1.13 mg protein/mL. The assay protein concentration was then 0.34 mg/mL.

8.2.8.3. Lipid dependence in the fluorescence enhancement assay.

Membranes were prepared from IPTG-induced *E. coli* JM109 (pBROC525) using 0.2 mM IPTG as described in 8.2.5.3. Membranes were extracted at 2 mg protein/mL in 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 0.5 % Triton X-100, 20 % glycerol with stirring (1 hr, 4 °C). Unsolubilised material was removed by centrifugation (10^5 *g*, 30 min). Lipids, 16.8 µL of 0.5 mg/mL HP in 1:1 isopropanol/toluene, 6 µL of 5 mg/mL PG in $CHCl_3$ or 6 µL of 5 mg/mL PE in $CHCl_3$, or combinations thereof were placed in a microfuge tube and solvent removed with a stream of nitrogen. Solubilised enzyme in 0.5 % Triton X-100 (0.3 mL) was added and vortexed thoroughly and allowed to stand for 10 min at rt before being transferred to a fluorimeter cuvette followed by 0.45 mL 1 % Triton X-100, 0.65 mL H_2O and 1.5 mL 200 mM Tris pH 8.5, 50 mM $MgCl_2$. Reaction was started by addition of 100 µL of stock dansyl-UDPMurNAc-pentapeptide estimated at 0.3 mM. Total reaction volume was 3 mL and reaction was at rt. Change in fluorescence emission at 554 nm (excitation at 340 nm) was recorded with a BBC SE2 chart recorder.

8.3. Chapter 3 experimental.

8.3.2.1. Fractionation of Triton X-100 solubilised translocase I with DEAE-sephadex.

Membranes of *E. coli* JM109 (pBROC512) were prepared as in 8.2.3. The protein concentration was 11.5 mg/mL (BCA assay). Translocase I was solubilised in 100 mM potassium phosphate pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 0.5 % Triton X-100, 20 % glycerol with stirring (1 hr, 4 °C). Unsolubilised material was removed by centrifugation (10^5 *g*, 30 min). Solubilised enzyme (1 mL) was applied under gravity to a column (2 mL) of DEAE-sephadex in 100 mM phosphate, pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol, 0.1 % Triton X-100. After washing with the same buffer (2 mL) the column was washed with buffer + 0.5 M NaCl, then buffer + 1.0 M NaCl (2 mL each). Fractions were assayed for 15 min each at 37 °C. Each assay contained 25 µL 200 mM Tris, pH 7.5, 50 mM $MgCl_2$, 4.4 µL H_2O, 10 µL enzyme, 5 µL [^{14}C]-UDPMurNAc-pentapeptide (2 nCi/µL, 28 µM) and 5 µL 100 µM UDPMurNAc-pentapeptide. Reactions were started by addition of substrate. Product was quantified as in 8.2.2.1.

8.3.2.2. Fractionation of CHAPS-solubilised translocase I with DEAE-sephadex.

Membranes of *E. coli* JM109 (pBROC525) were prepared as in 8.2.8.1. The protein concentration was 14.3 mg/mL (BCA assay). Enzyme was solubilised at 4 mg protein/mL in 50 mM Tris pH 8.2, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 1.5 % CHAPS, 20 % glycerol. The 10^5 *g* supernatant was diluted with 50 mM Tris pH 8.2, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol such that the final concentration of CHAPS was 0.6 %. This was applied to a column of DEAE-sephadex A50 (1.5 mL) equilibrated with 50 mM Tris pH 8.2, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol, 0.6 % w/v CHAPS. The column was washed with the same buffer (3 mL) followed by buffer + 330 mM NaCl (3 mL). A mixture of lipids was prepared by placing 4 µL of 5 mg/mL HP (20 µg) in 1:1 isopropanol:toluene and 10 µL of 10 mg/mL (100 µg) PE in $CHCl_3$ in an eppendorf tube and solvent removed with a stream of nitrogen before addition of 10 µL of 10 mg/mL (100 µg) PG in 1.0 % Triton X-100 and 40 µL of 0.1 % Triton X-100. The lipid mix was added (5 µL per 100 µL sample) to each fraction and the fractions assayed for translocase I activity and protein (BCA assay). Assays contained 100 mM Tris pH 7.5, 25 mM $MgCl_2$, 0.68 % CHAPS, 10 nCi [^{14}C]-UDPMurNAc-pentapeptide (2.8 µM), 11.2 µM UDPMurNAc-pentapeptide and enzyme (5 µL, giving final assay concentrations of 1.9 µg/mL (3.1 µM) HP, 9.5 µg/mL PG and PE)

in a volume of 50 µL. Reaction was at 37 °C for 30 min and was started by addition of substrate. Product was quantified as in 8.2.2.1.

8.3.2.3. Standard protocol for preparation of *E. coli* JM109 (pBROC525) membranes.

The following experimental section is a standard protocol for the preparation of membrane fragments containing translocase I which was used in almost all experiments following optimisation of growth procedures. An overnight culture derived from a single colony of *E. coli* JM109 (pBROC525), LB + 100 µg/mL ampicillin, was used to innoculate (2 %) 4 x 1 L of LB/amp in 2 L baffled flasks. The cultures were grown at 37 °C shaking at 240 rpm to OD_{600nm} = 1.7 (zero on media), which typically took 4-5 hr. IPTG dissolved in water was added to 0.2 mM and the cultures incubated for a further 90 min before harvesting by centrifugation (4400 *g*, 10 min). Cell pellets were stored overnight at -70 °C. Cells were resuspended in freshly prepared ice-cold 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$ (buffer A, 10 mL/g) using a teflon homogeniser. Cells were broken with two passes through a Stansted cell disrupter (driven with compressed nitrogen) with a forward pressure of 50 psi and a back pressure of 2 psi. Whole cells and debris were removed by centrifugation (12000 *g*, 30 min) and membranes pelleted from the supernatant by ultracentrifugation (10^5 *g*, 30 min). Membranes were resuspended and stirred in buffer A + 1 M KCl for 1 hr at 4 °C before collection by ultracentrifugation. Membranes were resuspended in ice-cold buffer A (10 - 20 mL) and protein determined by the BCA assay. The suspension was diluted to 8 mg protein/mL and flash frozen in liquid nitrogen in 0.5 mL and 1 mL aliquots. These were stored at -70 °C. No loss of activity was observed during storage at this temperature or at -20 °C.

8.3.2.4. Fractionation of Triton X-100-solubilised translocase I with DEAE-sepharose.

Membranes (8.3.2.3) were solubilised at 2 mg protein/mL in 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 0.5 % Triton X-100, 20 % glycerol with stirring (1 hr, 4 °C). The 10^5 *g* supernatant (5 mL) was diluted to 25 mL with 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 7.5 % glycerol, 63 mM NaCl such that the applicate buffer was 50 mM Tris pH7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 10 % glycerol, 0.1 % Triton X-100, 50 mM NaCl. This was applied to a column of DEAE-sepharose fast flow (4.5 x 1.6 cm) equilibrated with 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 10 % glycerol, 0.1 % Triton X-100, 50 mM NaCl at a flow rate of 0.5 mL/min. The column was washed with the same buffer (15 mL) and enzyme eluted with a 50 - 600 mM NaCl gradient over 160 mL

collecting 5 mL fractions. Fractions were assayed for activity as below and for protein. In some cases protein concentration was too low to detect reliably.

7.3 µL of 2.5 mg/mL HP in 1:1 isopropanol:toluene and 6 µL of 5 mg/mL PG in chloroform were placed in a microfuge tube and solvent removed with a stream of nitrogen before addition of 0.3 mL of enzyme sample. This was vortexed and incubated at 30 °C for 5 min before being transferred to the fluorimeter cuvette (in the cell apparatus maintained at the same temperature). 1 % Triton X-100, water then 1.5 mL 200 mM Tris pH 8.5, 50 mM $MgCl_2$ and 16 µM dansyl-UDPMurNAc-pentapeptide solution were added such that the final assay volume was 3 mL and the final concentration of Triton X-100 was 0.2 %. Increase in fluorescence at 554 nm (excitation, 340 nm) was measured as a function of time.

8.3.2.5. Mixing of fractions after DEAE-sepharose chromatography.

Enzyme was prepared, the chromatography performed and active fractions identified as in 8.2.3.4 except that the applicate buffer contained 30 mM NaCl, not 50 mM, and the NaCl gradient was 30 - 500 mM. Fraction 4 was most active, 0.35 u/min in the assay protocol described in 8.2.3.4.

Fractions or mixtures (1:1) of fractions were assayed using the radiochemical assay. Aliquots of stock HP and PG solutions in organic solvents were placed in microfuge tubes, solvent removed with a stream of nitrogen and the residue dispersed with enzyme sample such that the concentrations in the sample were 60.8 µg/mL (100 µM) HP and 100 µg/mL PG. Assays contained 100 mM Tris pH 8.5, 25 mM $MgCl_2$, 10 nCi [^{14}C]-UDPMurNAc-pentapeptide (2.8 µM), 11.2 µM UDPMurNAc-pentapeptide and enzyme (5 µL, containing lipids) in a volume of 50 µL. Reaction was at 37 °C and were started by addition of enzyme. Product was quantified as in 8.2.2.1.

8.3.2.6. Fractionation of Triton X-100-solubilised translocase I with CM-sephadex.

Membranes of *E. coli* JM109 (pBROC512) were prepared as in 8.2.3 except that the preparative buffer contained 50 mM Tris pH 7.5 rather than 100 mM potassium phosphate pH 7.5. Membranes were solubilised at 4 mg protein/mL in 50 mM Tris pH 8.2, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 0.5 % Triton X-100, 20 % glycerol. Unsolubilised material was removed by ultracentrifugation (10^5 *g* , 30 min). Solubilised enzyme (1mL) was exchanged into 10 mM MOPS pH 6.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol, 0.1 % Triton X-100 using a 3 mL column of sephadex G10. The sample (1.5 mL) was applied to a column of carboxymethyl-sephadex (2 mL) equilibrated with the above buffer. After washing with the same buffer (2 mL) the column was washed with buffer + 0.5 M NaCl, then

buffer + 1.0 M NaCl (4 mL each). Fractions were assayed for translocase I activity. Assays contained 100 mM Tris pH 9.0, 25 mM $MgCl_2$, 10 nCi [^{14}C]-UDPMurNAc-pentapeptide (2.8 µM), 11.2 µM UDPMurNAc-pentapeptide and enzyme (30 µL) in a volume of 50 µL. Reaction was at 37 °C for 1 hr and were started by addition of enzyme. Product was quantified as in 8.2.2.1.

8.3.2.7. Fractionation of Triton X-100-solubilised translocase I with cellulose-phosphate.

The protocol for this experiment was identical to that in 8.3.2.6 except that the resin was cellulose-phosphate (2 mL).

8.3.2.8. Fractionation of CHAPS-solubilised translocase I with cellulose-phosphate.

Membranes of *E. coli* JM109 (pBROC525) were prepared as in 8.2.8.1. The protein concentration was 14.3 mg/mL (BCA assay).The membrane suspension was titrated to pH 6.5 by addition of 1 M Mes. Membranes were solubilised at 4 mg protein/mL in 50 mM Mes pH 6.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 1.5 % CHAPS, 20 % glycerol. Unsolubilised material was removed by ultracentrifugation (10^5 *g*, 30 min). The extraction supernatant was diluted with 50 mM Mes pH 6.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol such that the final detergent concentration was 0.6 %. Diluted sample (2.5 mL) was applied to a column of cellulose-phosphate (1.5 mL) equilibrated with 50 mM Mes pH 6.5, 2mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol, 0.6 % CHAPS. The column was washed with the same buffer (3 mL) followed by buffer + 330 mM NaCl (3 mL). Lipids (HP, PG and PE) were added and enzyme assayed as in 8.3.2.2.

8.3.3. Fractionation of Triton X-100-solubilised translocase I with phenyl-agarose.

Membranes of *E. coli* JM109 (pBROC 525) were prepared as in 8.2.8.1. Membranes were solubilised at 4 mg protein/mL in 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 0.5 % Triton X-100, 20 % glycerol. Unsolubilised material was removed by ultracentrifugation (10^5 *g* , 30 min). Solubilised enzyme was brought to 0.5 M $(NH_4)_2SO_4$ by portionwise addition of solid. A 1 mL column of phenyl-agarose was equilibrated with 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol, 0.1 % Triton X-100, 0.5 M $(NH_4)_2SO_4$. Solubilised enzyme (2 mL, 6.4 mg protein) was applied and the column washed with buffer + 0.5 M - 0 M $(NH_4)_2SO_4$ in 0.1 M steps (1 mL each) collecting 1 mL fractions at each step. Fractions were assayed as in 8.2.8.1 using 5 µL of sample + lipids incubating at

37 °C for 1 hr. Protein was determined by the BCA assay which is tolerant to $(NH_4)_2SO_4$ to 1.5 M.[126]

8.3.4.1. Binding of Triton X-100-solubilised translocase I to dye-ligand resins.

Membranes were prepared as in 8.3.2.3 and solubilised as in 8.3.3. Solubilised enzyme was diluted 2-fold with solubilisation buffer lacking detergent such that the final detergent concentration was 0.25 %. The same procedure was used for each of six reactive dye-linked resins, namely cibacron blue 3GA-agarose, reactive blue 4-agarose, reactive brown 10-agarose, reactive green 19-agarose, reactive red 120-agarose and reactive yellow 86-agarose (purchased as a kit from Sigma). Resin (200 mg) was washed with copious amounts of water and equilibrated with 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol, 0.1 % Triton X-100 (buffer B). 2-Fold diluted Triton X-100 solubilised enzyme (1 mL) was gently stirred with resin for 2 hr at 4 °C. The incubation mixture was poured into a Pastuer pipette plugged with cotton wool and the flow through collected. The column was washed with a further 2mL of buffer B and the combined fractions assayed for translocase I activity as follows. A mixture of lipids was prepared by placing 80 µL of 0.5 mg/mL HP (40 µg) in 1:1 isopropanol:toluene, 40 µL of 5 mg/mL (200 µg) PE in $CHCl_3$ and 40 µL of 5 mg/mL (200 µg) PG in $CHCl_3$. 10 µL of this mixture was added to a microfuge tube and solvent removed with a stream of nitrogen followed by addition of 100 µL of enzyme sample (giving final concentrations of 20, 100 and 100 µg/mL respectively). Assays were time-courses and contained 100 mM Tris pH 8.5, 25 mM $MgCl_2$, 2.8 µM [^{14}C]-UDPMurNAc-pentapeptide (10 nCi/50 µL), 11.2 µM UDPMurNAc-pentapeptide and enzyme/lipids (15 µL per 50 µL) in a volume of 50 µL. Assays were at 37 °C and were started by addition of enzyme. Product was quantified as in 8.2.2.1.

8.3.4.2. Fractionation of Triton X-100-solubilised translocase I with cibacron blue 3GA-agarose and reactive red-120-agarose.

Membranes were prepared as in 8.3.2.3 and solubilised as in 8.3.3. except that the protein concentration was 2 mg/mL. Enzyme (1 mL) was stirred gently for 1 hr with 200 mg of each of cibacron blue 3GA-agarose and reactive red 120-agarose (both swollen and washed thoroughly and equilibrated with 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol, 0.1 % Triton X-100). The resins were poured into a pasteur pipette plugged with cotton wool and the flow through collected. The resins were washed with the above buffer (2 mL) followed by buffer + 200 mM NaCl (2 mL), buffer + 400 mM NaCl (2 mL) and buffer + 10 mM UMP (2 mL). Fractions were assayed using the fluorescence enhancement method described in detail in section 8.4. Assays contained 100 mM Tris pH 8.5, 25 mM

$MgCl_2$, 0.2 % Triton X-100 (final, constant), dansyl-UDPMurNAc-pentapeptide (estimated at 10 µM), 4.6 µM HP, 10 µg/mL PG and enzyme (0.3 mL) in a final volume of 3 mL. Assays were at 30 °C and were started by addition of enzyme/lipids. Controls showed that the 40 mM NaCl or 1 mM UMP carried to the assay in eluate fractions gave 30 % and 50 % reduction of initial rate respectively.

8.3.4.3. Fractionation of Triton X-100-solubilised translocase I with UDP-agarose.

Membranes of *E. coli* JM109 (pBROC512) were prepared as in 8.2.3. The protein concentration was 11.5 mg/mL (BCA assay). Translocase I was solubilised in 100 mM potassium phosphate pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 0.5 % Triton X-100, 20 % glycerol with stirring (1 hr, 4 °C). Unsolubilised material was removed by centrifugation (10^5 *g*, 30 min). Solubilised enzyme (1 mL) was applied under gravity to a column (2 mL) of UDP-agarose (attached through ribose hydroxyls, 11 atom spacer, Sigma) in 100 mM phosphate pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol, 0.1 % Triton X-100. After washing with the same buffer (2mL) the column was washed with buffer + 0.5M NaCl, then buffer + 1.0M NaCl (2mL each). Fractions were assayed as in 8.3.2.1.

8.3.4.4. Fractionation of Triton X-100-solubilised translocase I with UDPMurNAc-pentapeptide-agarose.

Thoroughly washed (water) epoxy-activated agarose (1.0 g, 7 - 10 µmol activated sites, cross-linked 4 % beaded agarose, 1,4-bis(2:3-epoxypropoxy)butane linker, 12 atom spacing, Sigma) was resuspended in 0.25 M $NaHCO_3$, pH 9.0 (4 mL) and UDP-MurNAc-pentapeptide (12.7 mg, 45 % pure, i.e. 4.8 mg, 4.0 µmol) in 0.25 M $NaHCO_3$, pH 9.0 (200 µL) was added. After gentle shaking at room temperature for 3 hr the resin was filtered and the filtrate assessed for UDP-MurNAc-pentapeptide by measuring absorbance at 262 nm ($\varepsilon_{262nm}=10^4$ $M^{-1}cm^{-1}$). On this basis it was found that 0.915 mg, 0.77 µmol of substrate was coupled to the resin. The resin was washed with water (100 mL) and unreacted resin attachment sites were blocked by resuspending the resin in 0.1 % w/v methylamine in 0.25 M $NaHCO_3$ pH 10.0 (10 mL) and shaking at ambient temperature for 3 hr. The resin was washed thoroughly with distilled water and stored in 0.1 M NaCl, 0.02 % NaN_3 at 4 °C.

Membranes were prepared as in 8.3.2.3 and solubilised as in 8.3.3. The resin was equilibrated with 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol, 0.1 % Triton X-100. Solubilised enzyme (2 mL) was applied to the resin (2 mL in a syringe column) under gravity at room temperature. The column was washed with 2 mL of the above buffer followed by 3 mL of buffer + 1 M NaCl. The salt wash fraction was desalted as in the general methods section. Assays contained

100 mM Tris pH 8.5, 25 mM $MgCl_2$, 10 nCi [^{14}C]-UDPMurNAc-pentapeptide (2.8 μM), 11.2 μM UDPMurNAc-pentapeptide and enzyme (15 μL) in a volume of 50 μL. Reaction was at 37 °C for 1 hr and were started by addition of substrate. Product was quantified as in 8.2.2.1.

8.3.4.5. Fractionation of Triton X-100-solubilised translocase I with tunicamycin-agarose.

Epoxy-activated agarose (1 g, 7 - 13 μmoles activated sites, same resin as 8.3.4.4) was swollen in neutral 100 mM phosphate (50 mL) for 30 min with periodic swirling. The resin was filtered on a glass sinter under reduced pressure and washed with distilled water to remove stabilising lactose. The resin was resuspended in 0.25 M $NaHCO_3$ pH 10.0 (6 mL) and tunicamycin (200 μL of 10 mg/mL stock in 0.25 M $NaHCO_3$ pH 10.0) was added. After gentle stirring at ambient temperature for 5 hr the resin was filtered and washed with the above buffer (2 x 5 mL). The combined wash and filtrate was concentrated by rotary evaporation under reduced pressure (liquid N_2 cooled) to ~1 mL and examined by tlc (solvent was 5:2 chloroform/methanol). Authentic tunicamycin at 0.1 mg/mL could be detected by UV illumination at r.f. 0.42. No such spot was visible in the combined wash + filtrate. Unreacted resin attachment sites were blocked by resuspending the resin in 0.1 % w/v methylamine in 0.25 M $NaHCO_3$ pH 10.0 (10 mL) and shaking at ambient temperature for 3 hr. The resin was washed thoroughly with distilled water and stored in 0.1 M NaCl, 0.02 % NaN_3 at 4 °C.

The column experiment with tunicamycin-agarose used the same protocol as with UDPMurNAc-pentapeptide-agarose (8.3.4.4).

8.3.4.6. Fractionation of CHAPS-solubilised translocase I with polyprenyl-agarose.

A crude mixture of prenols (mainly C_{50} and C_{55} homologues, Sigma), (5 mg, ≈7 μmol) was dissolved completely in DMF (2 mL) and NaOH (0.28 mg, 7 μmol) in water (2 mL) was added with vigorous stirring to give an emulsion. After 5 min, thoroughly washed (H_2O then 50/50 H_2O/DMF) epoxy-activated agarose (1.0 g, 7 - 10 μmol activated sites, same resin as 8.3.4.4) was added.The mixture was stirred slowly in the dark at room temperature for two days. The resin was filtered, washed with 50/50 H_2O/DMF (100 mL) and resuspended in 0.8 M methylamine in 0.25 M $NaHCO_3$, pH 10.0 (10 mL) and shaken gently for 4 hr. The resin was filtered, washed with H_2O (5 x 40 mL) and stored in the dark at 4 °C as a suspension in 0.1 M NaCl, 0.02 % NaN_3.

Membranes were prepared as in 8.3.2.3. Membranes were solubilised at 4 mg protein/mL in 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 1.5 %

CHAPS, 20 % glycerol. Unsolubilised material was removed by ultracentrifugation (10^5 *g* , 30 min). Solubilised enzyme was diluted with 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol such that the final concentration of detergent was 0.6 %. Polyprenyl-agarose was equilibrated with 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol, 0.6 % CHAPS. Diluted enzyme (5 mL) was incubated with the resin (all of that prepared) overnight at 4 °C in the dark. The resin filtered by pouring into a column and was washed with the equilibration buffer (5 mL). Lipids (HP, PG, PE) were added to each of the flow through and wash fractions and to a retained sample of applied enzyme as in 8.3.2.2 and assayed as in that section using a 0 - 10 min time-course for applied enzyme and a 0 - 30 min time-course for wash fractions (table 8.2).

Fraction	Rate, pmol/min	Volume of fraction, mL	Total activity, pmol/min	Protein, mg
Applied	21.75	2.0	2900	2.8
Flow through	0.23	5.3	81	1.1
Wash	0.12	5.7	46	2.1

Table 8.2. Data from polyprenyl-agarose experiment.

The resin was washed thoroughly waith 50/50 buffer A/ethanol and equilibrated again with 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol, 0.6 % CHAPS. The column experiment was repeated except that the sample was applied to the resin columnwise under gravity. the column was washed with the equilibration buffer (5 mL) followed by buffer + 10 % ethanol (5 mL) and buffer + 5 µg/mL HP (5 mL). Again, fractions were assayed as in the first experiment.

8.3.5. Fractionation of Triton X-100-solubilised translocase I using sephadex-G75 and UDP-hexanolamine-agarose.

Membranes were prepared as in 8.3.2.3 and solubilised at 4 mg protein/mL in 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol, 1 % Triton X-100 giving 1.82 mL of extract. Of this, 150 µL was retained for activity and protein assays. The remainder was applied to a column of sephadex G75 (15 x 1.6 cm) equilibrated with 50 mM Tris pH 8.0, 1 mM $MgCl_2$, 2 mM ß-mercaptoethanol, 0.2 % Triton X-100 at 4 °C. The column was flowed with the same buffer collecting 2 mL fractions. Activity was assessed using the standardised fluorescence enhancement assay protocol described in section 8.4. The 2x assay buffer contained detergent to maintain the assay concentration at 0.2 %. Activity appeared after 11 mL of buffer after flowing the sample into the column (flow adaptor and peristaltic pump). Total

activity applied was 17.3 u/min. Total activity recovered was 33.3 u/min (193 %). The two active fractions were pooled and $MgCl_2$ added to 25 mM before application to a column of UDP-hexanolamine agarose (2 mL, attached through amine function, 1 atom spacer, Sigma) in a PolyPrep column (BioRad) equilibrated with 50 mM Tris pH 8.0, 25 mM $MgCl_2$, 2 mM ß-mercaptoethanol, 0.2 % Triton X-100 at room temperature in a columnwise fashion. The column was washed with 2 mL of the same buffer followed by 50 mM Tris pH 8.0, 5 mM UMP, 25 mM EDTA, 2 mM ß-mercaptoethanol, 0.2 % Triton X-100 (2 x 2 mL). These two fractions were dialysed against 200 volumes of 50 mM Tris pH 8.0, 5 mM $MgCl_2$, 2 mM ß-mercaptoethanol, 20 % glycerol, 0.2 % Triton X-100 at 4 °C for 3 hr before assaying for translocase I activity. It has been shown in other experiments that the enzyme is stable to this treatment. The flow through and wash fractions contained >50 % of the applied activity and no activity was seen in either UMP/EDTA wash fraction.

8.3.6.1. Construction of pPB1.

Molecular biology general methods are described in section 8.6. pBROC511 was prepared at SB by Dr.M.Burnham. An 8.5 kbp *Kpn*I fragment from Kohara phage 110^{125} was cloned into the *Sal*I site of pUC19 giving pBROC508. A 1092 bp *Mlu*I fragment from this plasmid containing the *mraY* gene was blunt-ended by filling in overhangs with Taq polymerase and cloned into the *Sma*I site of pUC19 giving pBROC511 Fortuitously, pBROC511 contains an *AflIII* site directly at the ATG start codon of the *mraY* gene. The strain *E. coli* JM109(pBROC511) was a gift from Dr.M.Burnham. Plasmid DNA was isolated from a 35 mL overnight culture of this strain and analysed by restriction analysis with *Afl*III, *Eco*RI, *Hind*III and *Afl*III/*Hind*III digests. Single digest gave one fragment only; the double digest released the required 1200 bp fragment. pBROC511 DNA (5 µg) was digested with *Afl*III/*Hind*III and the *mraY* fragment purified by preparative gel electrophoresis. The Invitrogen phagemid, pRSET-B (1 µg), was digested with *NcoI*/*Hind*III and purified with the Wizard DNA 'clean up' system, the minor fragment being only 11 bp. The *mraY* and pRSET-B fragments were ligated and the mixture used to transform cells of *E. coli* MV1190. Five transformant colonies were selected at random and plasmid DNA's prepared from 1 mL LB cultures. Three of five showed the right fragments (1.15 and 2.95 kbp) when digested with *Hinc*II/*Sca*I. The original vector contains a *Sca*I site remote from the multiple cloning site and no *Hinc*II sites. One clone was further analysed with *Sca*I, *ScaI*/*Hinc*II and *ScaI*/*Eco*RI digests giving the expected 4.1, 1.15/2.95 and 1.55/2.55 kbp fragment sizes respectively and was denoted pPB1.

8.3.6.2. Growth and transformation of *E. coli* C600 (pGP1-2).

E. coli C600 (pGP1-2) was a gift from Dr. Mark Bradley of Southampton University. The plasmid harboured in this strain carries kanamycin resistance and bacteriophage T7 gene *1* coding for T7 RNA polymerase. Expression of this gene is induced by heat shock.[141] This strain was grown in LB + 50 μg/mL kanamycin at 30 °C. Transformation with pPB1 was carried out as described in the molecular biology general methods section (8.6.5) except that heat shock was at 35 °C. Transformation plates contained ampicillin (100 μg/mL) as well as kanamycin (50 μg/mL).

8.3.6.4. Experiments with *E. coli* BL21(de3)(pLysS).

E. coli BL21(de3)(pLysS) was a gift from Dr. Mark Bradley of Southampton University. This strain was transformed with approximately 250 ng of pPB1 as described in the molecular biology general methods section (8.6.5). After transformation the growth medium was LB + 100 μg/mL ampicillin. After overnight incubation the transformation plate (LB/amp agar) at 37 °C, pinprick colonies were visible (>200 indicating efficient transformation). After a total incubation time of approximately 24 hr, colonies had reached a pickable size. Colonies were discrete and of uniform size. One colony was picked at random and used to innoculate 5 mL of LB/amp which was incubated overnight at 37 °C. The overnight culture was used to innoculate 500 mL LB/amp at 2 % innoculum. After 1.5 hr growth at 37 °C, the OD_{600nm} was 0.12 and was 0.71 after 2.75 hr growth. At this point sterile aq. IPTG was added to 1 mM. After a further 3 hr growth the OD_{600nm} was 2.26.

A second colony was picked at random from the transformation plate and grown overnight in 5 mL of LB/amp and used to innoculate (1 %) 500 mL LB/amp in a 2 L baffled flask. Growth was monitored at 600 nm (table 8.3).

Time, hr	OD_{600nm}
8.25	0.25
9	0.45
9.75	0.60*
12.25	0.60
12.5	0.61

Table 8.3. Growth of strain *E. coli* BL21(de3)(pLysS)(pPB1). *Sterile IPTG was added to 1 mM.

Cells were collected by centrifugation (2 x 250 mL, 4400 *g*, 10 min) and stored at -20 °C. The culture was also streaked out on LB/amp agar and grown overnight at 37 °C. A single colony was picked from this plate and grown overnight

in LB/amp. The overnight culture was used to innoculate (2 %) 500 mL of LB/amp in a 2 L baffled flask. Growth at 37 °C was monitored at 600 nm (table 8.4).

Time, hr	OD_{600nm}
2	0.36
2.5	0.69*
2.75	0.84
3	1.10
3.25	1.27
3.5	1.48
4	1.80
5.75	2.15

Table 8.4. Growth of strain *E. coli* BL21(de3)(pLysS)(pPB1). *Sterile IPTG was added to 1 mM.

8.3.6.5. Assay of solubilised membranes from *E. coli* BL21(de3)(pLysS)(pPB1).

Membranes were isolated from the cells of *E. coli* BL21(de3)(pLysS)(pPB1) stored in 8.3.6.4 as from *E. coli* JM109 (pBROC525) in 8.3.2.3 except that cell breakage was effected by sonication rather than by an air driven cell disrupter. These cells were fragile since lysis was observed (judged by viscosity due to release of DNA) upon resuspension of the cell pellet. Membranes were also prepared as in 8.3.2.3 from cells of *E. coli* JM109 (pTrc99A) grown as for *E. coli* JM109 (pBROC525). Membranes from *E. coli* BL21(de3)(pLysS)(pPB1) and *E.coli* JM109 (pTrc99A) were solubilised in 50 mM Tris pH 7.5, 1 mM $MgCl_2$, 2 mM ß-mercaptoethanol, 20 % glycerol, 0.5 % Triton X-100 at a protein concentration of 1 mg/mL using the standard protocol as described previously. *E. coli* JM109 (pTrc99A) was used as a control in the absence of membranes from a more suitable control strain. These extracts were assayed for translocase I activity using the standardised fluorescence enhancement assay protocol described in section 8.4.6.4 with the chart recorder set at 5min/cm. The rates for *E. coli* JM109 (pTrc99A) and *E. coli* BL21(de3)(pLysS)(pPB1) extracts were 0.06 and 0.03 u/min respectively.

8.3.6.6. [^{35}S]-Methionine labelling of pPB1-derived protein.

E. coli BL21(de3)(pLysS) was transformed with either pRSET-B or pRSETA/CAT (contains a gene coding for his^6-chloramphenicol acetyl transferase, 29 kDa) to create negative and positive control strains for this expression experiment. The growth media used were Luria-Berkhardt broth (10 g/L tryptic soy broth, 5g/L yeast extract and 10 g/L NaCl) and M9 minimal medium + glucose + 18 amino acids.

5x M9 salts solution was prepared as in Maniatis.[194] The growth medium contained 1x M9 salts, 0.5 % glucose and 0.01 % of each of L-alanine, L-aspartic acid, L-glutamic acid, L-phenylalanine, L-glycine, L-histidine, L-isoleucine, L-lysine, L-leucine, L-aspargine, L-proline, L-arginine, L-serine, L-threonine, L-valine, L-tryptophan and L-tyrosine.

Single colonies were picked from fresh transformation plates of each of *E. coli* BL21(de3)(pLysS)(pRSET-B), *E. coli* BL21(de3)(pLysS)(pRSET/CAT) and *E. coli* BL21(de3)(pLysS)(pPB1) and grown overnight at 37 °C in 5 mL of LB/amp. Overnight cultures were used to innoculate (2.5 %) 20 mL each of LB/amp. These cultures were grown to OD_{600nm} = 0.6. Cells were collected by centrifugation (13500 rpm, 30 s) from 1 mL of each culture in sterile microfuge tubes. Cells were washed with 1 mL of prewarmed M9/glucose/18 aa's medium, resuspended in 1 mL of that medium and grown at 37 °C for 1 hr. Sterile aq. IPTG was added to 1 mM (5 μL of 200 mM stock) and cells grown for 20 min. Rifampicin was added to 200 μg/mL (10 μL of 20 mg/mL stock solution in methanol) and 0.5 mL cells grwon for 30 min in fresh, sterile, prewarmed microfuge tubes. [^{35}S]-Methionine (10 μCi in 5 μL) was added and cells grown for a further 5 min. Cells were collected by centrifugation (13500 rpm, 30 s) and stored overnight at -20 °C. The cell pellet was resuspended in 100 μL SDS-PAGE loading buffer and analysed on a 12 % SDS-PAGE gel along with appropriate mlecular weight markers as described in the general methods section. The gel was dried and exposed to a sheet of Amersham Hyperfilm ECL for 5 days. The orientation of the gel was noted with two pieces of luminescent tape. The film was developed as recommended by the manufacturers.

8.4. Chapter 4 experimental.

8.4.2.1. Preparation of UDPMurNAc-pentapeptide.

UDPMurNAc-pentapeptide was isolated from cells of *Bacillus subtilis* W23 on several occassions. The report below is typical of the protocol used.

PYP medium:	Bacteriological peptone	20 g/L	
	Yeast extract	1.5 g/L	pH adjusted to 7.2
	K_2HPO_4	4.5 g/L	

Cell wall synthesis medium (CWSM):

Na_2HPO_4	0.26 g/L	
NH_4Cl	2.0 g/L	
KCl	4.0 g/L	
$MgCl_2$	4.0 g/L	
Na_2SO_4	0.15 g/L	
$FeSO_4$	0.1 g/L	pH adjusted to 7.4
Glucose	2.0 g/L	
Uracil	40m g/L	
L-glutamic acid	120 mg/L	
L-lysine	500 mg/L	
meso-diaminopimelic acid	120 mg/L	
L-alanine	50 mg/L	
Chloramphenicol	50 mg/L	
Ampicillin	50 mg/L	
Vancomycin	12.5 mg/L	

CWSM was sterilised by autoclaving minus $FeSO_4$ and antibiotics. Solutions containing 1. $FeSO_4$ and 2. antibiotics were added through an 0.22 μM sterile filter immediately before innoculation.

Uridinediphospho-N-acetylmuramyl-L-Ala-γ-D-Glu-*m*-DAP-D-Ala-D-Ala was isolated from cultures of *Bacillus subtilis* W23 (NCIMB 11824) based on protocols described by Lugtenberg *et al;* and Fluoret *et al;*.[47, 115] *B. subtilis* W23 was grown to stationary phase in 50 mL of PYP medium in a baffled flask at 37 °C with shaking at 240 rpm. This was used to innoculate at 2 % 4 x 500 mL PYP in 2 L baffled flasks and grown to Abs_{600nm} = 1.5. The cultures were chilled on an ice-bath and cells pelleted by centrifugation (4400*g*, 10 min). Pellets were quickly

resuspended in 50 mL of CWSM. This was added (25 mL each) to 2 x 225 mL of prewarmed CWSM in 2 L baffled flasks and incubated at 37 °C shaking at 240 rpm for 45 min before chilling on an ice-bath. Cells were collected by centrifugation (4400*g*, 10 min) and stored at -20 °C (6.5 g, wet weight). Thawed cells were resuspended in ice-cold 5 % TCA (5 mL/g cells) and left on ice for 30 min. Precipitated material was removed by centrifugation (4400*g*, 10 min). The pellet was extracted twice with half volumes of ice-cold 5 % TCA. The supernatants were pooled and extracted with three equal volumes of ether. The aqueous phase was neutralised with 3 M NaOH and concentrated to ~1.5 mL by rotary evaporation under reduced pressure. This was clarified by microcentrifugation and applied to a column of sephadex G15 (74 x 1 cm, void volume ~22 mL by blue dextran calibration) in water. UDPMurNAc-pentapeptide was eluted from the column with water at a flow rate of 0.95 mL/min collecting 1 mL fractions. Fractions containing UDPMurNAc-pentapeptide were identified by nucleotide absorbance at 262 nm. Fractions were assessed by reversed phase HPLC on an analytical C-18 column eluting with 0.1 M ammonium phosphate pH 4.6 at a flow rate of 0.8 mL/min. UDPMurNAc-pentapeptide was positively identified by co-injection with an authentic standard (a gift from SB Pharmaceuticals). UDPMurNAc-pentapeptide has λ_{max} = 262 nm. Concentration was determined using the molar extinction coefficient for uridine, $\varepsilon_{262nm} = 10^4\ M^{-1}cm^{-1}$. The yield was 33 mg white solid, 67 % pure.

UDPMurNAc-pentapeptide was prepared on a larger scale in two halves starting from 8 L PYP in each half. This protocol was identical to the one above except for the chromatography step and gave 55 g cells, wet weight. The extraction procedure afforded 12 mL of clear yellow liquid which was chromatographed 6 mL at a time on a 225 mL column of Biogel P2 (46 x 2.5 cm) eluting with water collecting 2.5 mL fracions at a flow rate of 0.5 mL/min. The yield was 192 mg of material, 44 % pure by nucleotide absorbance.

8.4.2.2. 'One pot' preparation of UDPMurNAc-pentapeptide.

Cells of *B. subtilis* were grown to OD_{600nm} = 1.3 in 4 x 500 mL PYP medium in 2 L baffled flasks. Sterile chloramphenicol, vancomycin and ampicillin were added to 50 μg/mL, 12.5 μg/mL and 50 μg/mL respectively. After a further 5 min growth, 50 mL sterile solution containing uracil, L-glutamic acid, L-lysine, *meso*-diaminopimelic acid and L-alanine was added to each of the flasks such that the final concentrations were 40 mg/L, 120 mg/L, 500 mg/L, 120 mg/L and 50 mg/L as in the CWSM in 8.4.2.1. Cells were grown for a further 40 min to a final OD_{600nm} = 1.1. UDPMurNAc-pentapeptide was isolated as in 8.4.2.1 with chromatography on the same Biogel P2 column as described in that section.

8.4.2.3. Preparation of UDP-N-acetylmuramyl-L-Ala-γ-D-Glu-(N$^{\varepsilon}$-dansyl)-*m*-DAP-D-Ala-D-Ala.

The title compound was prepared by a method based on that of Weppner and Neuhaus.[118] UDPMurNAc-pentapeptide (2.5 μmol, 3 mg) was dissolved in 0.25 M sodium carbonate pH 10.0, 50 % aq. acetone (6 mL). Four additions of dansyl chloride (60 μmol, 16 mg) were made at 20 min intervals, the reaction mixture being stirred at rt in the dark 20 min. After the last addition acetone was removed by rotary evaporation and the aqueous sample concentrated to ~1 mL. Yellow solid was removed by microcentrifugation. The dansyl-UDPMurNAc-pentapeptide was separated from dansic acid by passage down a column of sephadex G15 (74 x 1 cm) eluting with water and detecting product by absorbance at 262 nm. The pooled fractions showed UV absorbance maxima at 249 nm and 327 nm (ratio 249/327 = 5.1), a fluorescence excitation maximum at 340 nm and fluorescence emission maximum at 565 nm. The solution was stored at -20 °C.

The material was characterised by MALDI-TOF MS and reverse-phase HPLC. MALDI-TOF mass spectra were recorded on a Vestec benchtop model in negative ion mode with an accelerating voltage, 28000 V. Samples were prepared by mixing 10 μL of each of a range of dilutions of aqueous dansyl-UDPMurNAc-pentapeptide with 10 μL of a saturated solution of matrix (sinapinic acid, dihydroxybutyric acid or a-cyano-4-hydroxycinnamic acid) in 70:30 water:acetonitrile. 2 μL aliquots were applied to sample wells and allowed to air dry. The time of flight data were approximately mass calibrated against a mixture of gramicidin S and aprotinin in a sinapinic acid matrix measured in positive ion mode accelerating with a potential of 28000 V.

HPLC analysis was performed using a Vydac Protein and Peptide analytical C-18 column eluting with water at a flow rate of 0.8 mL/min monitoring effluent at 250 nm. 30 μL of a 10-fold diluted stock dansyl-UDPMurNAc-pentapeptide gave the trace in fig. 8.1.

Dansyl-UDPMurNAc-pentapeptide was prepared on a larger scale at SB Pharmaceuticals. The reaction was a direct scale-up of that above using 192 mg of 44 % pure (i.e. 84 mg) UDPMurNAc-pentapeptide. The product was purified on a column of Biogel P2 (46 x 2.5 cm) eluting with water collecting 5 mL fractions (15 mL applied). Fractions whose absorbance spectra showed maxima at 249 and 327 nm with an absorbance ratio of 5:1 were pooled and lyophilised to give 74 mg of yellow solid. This was dissolved in 15 mL of water and stored in 1 mL aliquots at -70 °C.

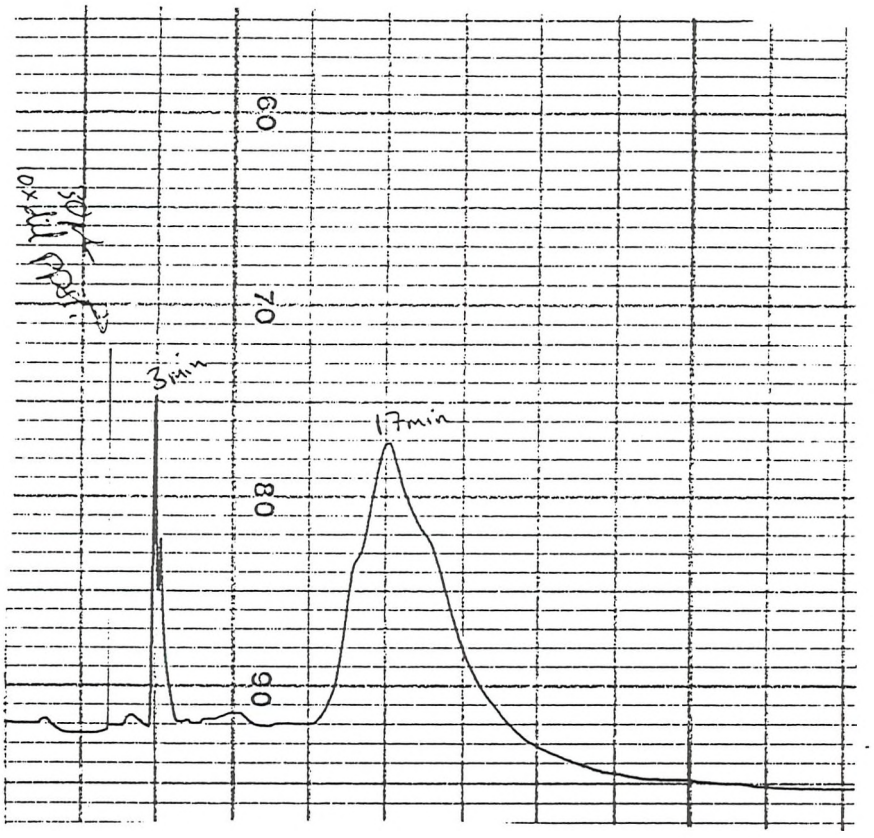


Figure 8.1. HPLC analysis of dansyl-UDPMurNAc-pentapeptide. The peak at 3 min is UDPMurNAc-pentapeptide as determined from the retention time of an authentic standard under the same column conditions.

8.4.3.1. Incubation of dansyl-UDPMurNAc-pentapeptide with translocase I.

Membranes of *E. coli* JM109 (pBROC525) were prepared as in 8.3.2.3. Membranes were solubilised at 4 mg protin/mL in 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol, 0.5 % Triton X-100 with stirring for 1 hr at 4 °C. Unsolubilised material was removed by ultracentrifugation (10^5 *g*, 30 min). Heptaprenyl phosphate (7.3 µL, 5 mg/mL) was placed in a microfuge tube, solvent removed with a stream of nitrogen and solubilised in 1 % Triton X-100 (0.45 mL). This, as well as 200 mM Tris pH 8.5, 50 mM $MgCl_2$ (1.5 mL) and detergent solubilised enzyme (0.3 mL) were incubated together in a plastic cuvette at 25 °C for 10 min. In this time the fluorescence emission spectrum in the absence of dansyl-UDPMurNAc-pentapeptide (dPP) was recorded exciting at 340 nm. There was no fluorescence emission between 400 nm and 600 nm. Reaction was started by addition of dPP (0.75 mL) to an 70 µM. The mixture was mixed thoroughly with a plastic paddle.

8.4.3.2. Time-course assay of fluorescence enhancement upon incubation of dansyl-UDPMurNAc-pentapeptide with translocase I.

Detergent solubilised enzyme was prepared as in 8.4.3.1. Reaction mixtures contained 100 mM Tris pH 8.5, 25 mM $MgCl_2$, 0.2 % Triton X-100, enzyme (0.3 mL or 0.6 mL) and 14 µM dPP. Change in fluorescence at 554 nm (ex. 340 nm) with time was recorded on a BBC SE2 chart recorder set at 200 mV and 12 cm/hr chart speed.

8.4.3.3. Time-course assay of translocase I in the presence of phospholipids.

Detergent solubilised enzyme was prepared as in 8.4.3.1. Stock solutions of lipids were mixed: 40 µL 5 mg/mL PE, 40 µL 5 mg/mL PG, 11.2 µL 5 mg/mL HP, 8.8 µL 1:1 isopropanol/toluene (100 µL total). Of this, 30 µL was taken to a microfuge tube and solvent removed with a stream of nitrogen. The residue was solubilised in enzyme (0.3 mL). Reaction mixtures were as in 8.4.3.2 except that dPP was present at 9 µM or 18 µM. Enzyme was diluted with 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol as required.

8.4.3.4. Lipid dependence in the fluorescence enhancement assay.

See 8.2.8.3.

8.4.3.5. Effect of temperature on relative fluorescence of an equilibrium reaction mixture.

The final assay performed in section 8.4.3.4 was allowed to go to equilibrium (30 min) and the fluorescence measured at 25 °C, 31 °C and 38 °C by adjusting the water-beth. The water bath maintains the temperature of the cuvette holding assembly. Temperature was measured directly in the cuvettte.

8.4.3.6 Radiochemical and fluorescence enhancement assays of translocase I under comparable conditions.

Membranes of *E. coli* JM109 (pBROC525) were prepared as in 8.3.2.3. Membranes were solubilised at 2 mg protein/mL in 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol, 0.5 % Triton X-100 with stirring for 1 hr at 4 °C. Unsolubilised material was removed by ultracentrifugation (10^5 *g*, 30 min). Assays were performed at 25 °C. The fluorescence enhancement assay contained 100 mM Tris pH 8.5, 25 mM $MgCl_2$, 0.2 % Triton X-100, 4.6 µM HP, 10 µg/mL each of PE and PG, enzyme (0.3 mL) and 9 µM dPP in a volume of 3 mL. Lipids were solubilised in the enzyme preparation and reaction started by addition of dPP monitoring ΔF at 554 nm (ex. 340 nm). The radiochemical assay was a three-point time-course assay at 3, 6 and 12 min. The reaction mixture contained 100 mM Tris pH 8.5, 25 mM $MgCl_2$, 0.2 % Triton X-100, 4.6 µM HP, 10 µg/mL each of PE

and PG, enzyme (20 µL), 40 nCi [^{14}C]-UDPMurNAc-pentapeptide and UDPMurNAc-pentapeptide (total 14 µM) in a volume of 200 µL. Aliquots were withdrawn at 3, 6 and 12 min and product quantified as in 8.2.2.1. The protein concentration in the solubilised enzyme preparation was 1.38 mg/mL (BCA assay).

8.4.3.7. Comparison of CHAPS and Triton X-100 as detergents in the fluorescence enhancement assay.

Membranes of *E. coli* JM109 (pBROC525) were prepared as in 8.3.2.3. Membranes were solubilised at 2 mg protein/mL in 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol with either 1.5 % CHAPS or 0.5 % Triton X-100 with stirring for 1 hr at 4 °C. Unsolubilised material was removed by ultracentrifugation (10^5 *g*, 30 min). The protein concentrations in the supernatants were 1.03 mg/mL and 1.28 mg/mL for CHAPS and Triton X-100 respectively (BCA assay). Assays were conducted at 30 °C. Radiochemical assay contained 100 mM Tris pH 8.5, 25 mM $MgCl_2$, 0.2 % Triton X-100 or 0.6 % CHAPS, 10 µM HP, 10 µg/mL of PG, enzyme (20 µL), 40 nCi [^{14}C]-UDPMurNAc-pentapeptide and UDPMurNAc-pentapeptide (total 14 µM) in a volume of 200 µL. Aliquots (50 µL) were withdrawn at 2, 5 and 10 min and product quantified as in 8.2.2.1. Fluorescence enhancement assays contained 100 mM Tris pH 8.5, 25 mM $MgCl_2$, 0.2 % Triton X-100 or 0.6 % CHAPS, 10 µM HP, 10 µg/mL PG, enzyme (0.3 mL) and 20 µM dPP in a volume of 3 mL. The assay protocol was as in 8.4.3.6.

8.4.3.8. Extraction of fluorescent lipid-linked product into butanol.

Translocase I was solubilised in 0.5 % Triton X-100 as in 8.4.3.7. The assay contained 100 mM Tris pH 8.5, 25 mM $MgCl_2$, 0.2 % Triton X-100, 10 µM HP, 10 µg/mL PG, enzyme (0.3 mL) and 17.5 µM dPP in a volume of 3 mL at 30 °C. A control contained 17.5 µM dPP in water. Samples were extracted with n-butanol (3 mL) and the organic layer taken to a plastic fluorimeter cuvette. Fluorescence emission was recorded at 535 nm (ex. 340 nm) at 30 °C.

8.4.4.1. Characterisation of dansyl-UDPMurNAc-pentapeptide prepared at SB Pharmaceuticals as a substrate for translocase I.

Translocase I was solubilised in 0.5 % Triton X-100 as in 8.4.3.7. The absorbance spectrum of this batch of dPP contained maxima at 249 nm and 327 nm (ratio of absorbances 249/327 = 5.1, 280/260 = 0.29, 250/260 = 1.56). 200 mM Tris pH8.5, 50 mM $MgCl_2$, (1.5 mL), 1 % Triton X-100 (0.45 mL), H_2O (0.65 mL) and 3-fold diluted stock dPP (1.05 mM, 0.1 mL) were mixed in a 3 mL plastic cuvette and the fluorescence excitation and emission spectra recorded. HP (7.3 µL, 2.5 mg/mL) and PG (6.0 µL, 5 mg/mL) were mixed in a microfuge tube and solvent removed with

a stream of nitrogen. The residue was solubilised in enzyme (above, 3 mL). This was added to the cuvette to begin the assay. After 30 min at 30 °C, fluorescence spectra were recorded again.

8.4.4.2. Comparison of heptaprenyl phosphate and dodecaprenyl phosphate as a lipid acceptor substrate for translocase I.

Translocase I was solubilised in 0.5 % Triton X-100 as in 8.4.3.7. Assays conatained 100 mM Tris pH 8.5, 25 mM $MgCl_2$, 0.2 % Triton X-100, 10 μg/mL PG, 10 μM HP or DP, 16 μM dPP and enzyme (0.3 mL) in 3 mL at 30 °C. Fluorescence emission was monitored at 535 nm (ex. 340 nm).

8.4.4.3. Effect of KCl on translocase I activity.

Translocase I was solubilised in 0.5 % Triton X-100 as in 8.4.3.7. Assays contained 100 mM Tris pH 8.5, 25 mM $MgCl_2$, 0.2 % Triton X-100, 10 μM DP, 100 μg/mL PG, enzyme (0.3 mL), 16 μM dPP and 0, 25, 50, 100 or 200 mM KCl in 3 mL at 30 °C. Fluorescence emission was monitored at 535 nm (ex. 340 nm).

8.4.4.4. Translocase I activity at pH 7.5 or 8.5.

Assay were carried out as in 8.4.4.3 with 50 mM KCl with the stock buffer solution at either pH 7.5 or 8.5.

8.4.4.5. Effect of phosphatidylglycerol on translocase I activity in the presence of dodecaprenyl phosphate.

Assays were carried out as in 8.4.4.2 with 10 μM DP and 0, 20 or 100 μg/mL (final assay concentration) PG.

8.4.4.6. Effect on translocase I activity of divalent metal ions.

Assays were carried out using the standard FEA protocol (8.4.6.1) with 40 μM DP and 26 μM dPP except that the 2x assay buffer did not contain $MgCl_2$. Salts $MgCl_2$, $MnCl_2$, $CaCl_2$, $ZnCl_2$ and $NiCl_2$ were added from 200 mM stock solutions in water.

8.4.4.7. Effect of varying Triton X-100 concentration on translocase I activity.

Assays were carried out using the standard FEA protocol (8.4.6.1) with 40 μM DP and 26 μM dPP except that membranes were solubilised at 2 mg protein/mL in buffer containing 0.5 % Triton X-100 so that using 60 μL of enzyme per assay would give 0.1 % Triton X-100 in the assay. Triton X-100 concentration was adjusted as required with additions from 1 % or 10 % stock solutions as appropriate. Assays were carried out in duplicate.

8.4.5. Determination of apparent K_m for UMP.

Assay were carried out using the standard FEA protocol (8.4.6.1) with 40 μM DP and 26 μM dPP. UMP was added when equilibrium had been reached (7min) in a 5 μL aliquot of an appropriate stock solution. The 'forward' part of the assay was identical in every case.

8.4.6.1. Verification of the antibiotic potency of mureidomycin A.

Pseudomonas putida ATCC 33015 was grown overnight in benzoate medium (0.5 g/L yeast extract, 3.0 g/L $(NH_4)_2HPO_4$, 1.2 g/L KH_2PO_4, 5.0 g/L NaCl, 0.35 g/L $MgCl_2.5H_2O$ and 3.0 g/L sodium benzoate) at 30 °C with shaking. Aliquots of the overnight culture (50 μL) of a stationary phase culture were taken to 5 mL of prewarmed medium containing 0, 1, 10, 25, 50 or 100 μg/mL of mureidomycin A. Growth was followed by monitoring absorbance at 600 nm.

8.4.6.2. Inhibition of translocase I activity by mureidomycin A in the radiochemical assay.

Membranes of *E. coli* JM109 (pBROC525) were prepared as in 8.3.2.3 and solubilised at 4 mg protein/mL in 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol, 0.5 % Triton X-100 with stirring for 1 hr at 4 °C. Unsolubilised material was removed by ultracentrifugation (10^5 *g*, 30 min). Solubilised enzyme was diluted 3-fold with 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol, 0.5 % Triton X-100. A mixture of lipids (HP, PE and PG) was prepared as in 8.2.8.1. 15 μL of this was added to 200 μL diluted enzyme of which 10 μL was used per assay giving 9.4 μM HP, and 28.5 μg/mL PE and PG in the assay. Assays were time-courses and contained 100 mM Tris pH 8.5, 25 mM $MgCl_2$, 0.1 % Triton X-100, 40 nCi (2.8 μM) [^{14}C]-UDPMurNAc-pentapeptide, 11.2 μM UDPMurNAc-pentapeptide, enzyme/lipids (40 μL) and 0, 0.1, 1.0 or 10 μg/mL mrdA in 200 μL at 37 °C. Aliquots were withdrawn at 2, 5 and 10 min and product quantified as in 8.2.2.1.

8.4.6.3. Dilution of inhibitor experiment for mureidomycin A.

Membranes of *E. coli* JM109 (pBROC525) were prepared as in 8.3.2.3 and solubilised at 4 mg protein/mL in 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol, 1 % Triton X-100 with stirring for 1 hr at 4 °C. Unsolubilised material was removed by ultracentrifugation (10^5 *g*, 30 min). Assays contained 100 mM Tris pH 7.5, 25 mM $MgCl_2$, 50 mM KCl, 0.2 % Triton X-100, 20 μM HP, 100 μg/mL PG, enzyme (0.3 mL) and 16 μM dPP at 30 °C in 3.0 mL. Assays were started by addition of substrate or substrate + inhibitor. Assays with 10, 25 or 50

nM mrdA gave initial rates 41 %, 29 % or 15 % of the no inhibitor control respectively. With 50 nM mrdA activity was completely abolished after 5 min, so this concentration of inhibitor was used for the main experiment. Enzyme only assayed as above gave initial rate 3.2 u/min. After 5 min, 0.6 mL of this reaction mixture was transferred to a new cuvette containing assay constituents (Tris, $MgCl_2$, KCl, Triton X-100, HP, PG, dPP) in 2.4 mL such that the final concentrations would be the same as in the first reaction excepting the material transferred. MrdA was also added such that the final concentration would be 10 nM. The initial rate in this assay was 0.7 u/min. The experiment was repeated except that the first assay contained 50 nM mrdA (added with the dPP). The initial rate in the first assay was 1.7 u/min, activity being completely abolished after 5 min when 0.6 mL was transferred to the second assay (no added mrdA). The initial rate was zero and the rate after 30 min was also zero.

8.4.6.4. Standard fluorescence enhancement assay (FEA) protocol.

This methodology is referred to in other sections as the standard FEA protocol and any deviations are listed in those sections.

1. Enzyme was premixed with lipids and held on ice before use in assays. Membranes of *E. coli* JM109 (pBROC525) were prepared as in 8.3.2.3. Enzyme was extracted from membranes at a concentration of 4 mg/mL with 50 mM Tris pH 7.5, 1 mM $MgCl_2$, 2 mM ß-mercaptoethanol, 20 % glycerol, 1 % Triton X-100 by stirring in the ultracentrifuge tube at 4 °C for 1 hr. Unsolubilised material was removed by centrifugation (10^5g, 30min) and supernatant held on ice. Enzyme was flash frozen (liquid nitrogen) and stored at -70 °C for 2 - 3 days without significant loss of activity. Longer storage times have not been investigated. Enzyme mixed with lipids was no less stable than enzyme not mixed with lipids. For each assay required, 2.3 µL (or multiples thereof) of 5 mg/mL dodecaprenyl phosphate (DP, Sigma supplied in 1:1 toluene/isopropanol) and 6.0 µL (or multiples thereof) of 5 mg/mL phosphatidylglycerol (PG) in chloroform were placed in a microfuge tube and solvent removed with a stream of nitrogen. Care was taken to keep the lipids near the bottom of the tube. Enzyme, 60 µL (or multiples thereof) was added and lipids taken up into solution by careful pipetting followed by gentle vortexing for 10 s. Enzyme (60 µL) was then transferred to a fresh microfuge tube and equilibrated to 34 °C in the water bath used to maintain buffers and the fluorimeter itself. This gave 30 °C inside the quartz cuvette.

2. Whilst enzyme was being warmed (typically 1 min), either the chart recorder was set up (settings sensitivity, 0.2 V, chart speed, 1 cm/min) or the cuvette was rinsed out after the preceeding assay. The cuvette was rinsed out whilst still in the fluorimeter cuvette holder with water held at 34 °C.

3. The assay was set up and started as follows: 150 µL 2x assay buffer (200 mM Tris pH 7.5, 100 mM KCl, 50 mM $MgCl_2$, pH adjusted at room temperature), 80 µL water and 60 µL enzyme/lipids (final concentrations 40 µM and 100 µg/mL for DP and PG respectively) are added from the 34 °C water bath in that order. At this point the mixture was pipetted up and down five times to effect thorough mixing.
4. Immediately, 10 µL of stock fluorescent substrate (giving 105 µM assay concentration) was added and the reaction mixture vigorously pipetted up and down several times. The fluorimeter unit was closed and the chart recorder started.

8.4.6.5. Substrate-dependence of inhibition of translocase I by mureidomycin A with respect to dansyl-UDPMurNAc-pentapeptide.

The standard FEA protocol was used (8.4.6.4) except that dPP was used at 26 µM.

8.4.6.6. Substrate-dependence of inhibition of translocase I by mureidomycin A with respect to dodecaprenyl phosphate.

The standard FEA protocol was used with the following adjustments. DP was added solubilised in 60 µL of 1 % Triton X-100, at the same time as dPP, after and separately from the enzyme. The final assay detergent concentration was therefore 0.4 %, but was constant at this concentration. The concentration of mrdA used was 200 nM (in the final 300 µL assay volume). Enzyme assayed this way gave initial rate 2.0 u/min. Enzyme was also assayed:
1. in the presence of mrdA.
2. in the absence of mrdA but with a 3 min incubation of enzyme in the assay mixture in the cuvette at 30 °C before addition of substrates DP and dPP.
3. as in 2. but with mrdA present in the 3 min pre-incubation.

8.4.6.7. Time-dependence of inhibition by mureidomycin A in the reverse reaction.

The standard FEA protocol (8.4.6.4) was used in both experiments, except where mrdA was present in the forward part of the reaction the concentration of dPP was 26 µM.

8.4.6.8. Loss of potency of mureidomycin A upon incubation with translocase I.

The standard FEA protocol (8.4.6.4) was used. Where enzyme was diluted, the diluting buffer was 50 mM Tris pH 7.5, 1 mM $MgCl_2$, 2 mM ß-mercaptoethanol, 20 % glycerol, 1 % Triton X-100. By trial and error over several assays, a concentration of mrdA (300 nM) was found which gave almost, but not quite, complete inhibition at the steady-state final rate. Enzyme was assayed in the presence

of 300 nM mrdA. After 3 min a 100 µL aliquot of this reaction mixture was boiled in a closed, pierced microfuge tube for 1 min. This was centrifuged (13500 rpm, 1min) at 4 °C. Enzyme was diluted 5-fold and assayed, the assay containing 80 µL of the centrifugation supernatnt. Three control experiments were conducted. 1. No mrdA in the first assay. 2. No mrdA in the first assay, but mrdA (80 nM) added to the second assay. 3. MrdA (300 nM) in the first assay + HP/PG solubilised in 1 % Triton X-100, but no enzyme.

Control 1.

1st assay: 150 µL 2x buffer
80 µL H_2O
60 µL enzyme + HP/PG
10 µL dPP

2nd assay: 150 µL 2x buffer
60 µL dil. enzyme + HP/PG
10 µL dPP
80 µL 1st assay mixture

Control 2.

1st assay: 150 µL 2x buffer
80 µL H_2O
60 µL enzyme + HP/PG
10 µL dPP

2nd assay: 150 µL 2x buffer
60 µL dil. enzyme + HP/PG
10 µL dPP
80 µL 1st assay mixture
2.4 µL 10 µM mrdA

Control 3.

1st assay: 150 µL 2x buffer
71 µL H_2O
60 µL HP/PG in 1 % TX100
10 µL dPP
9 µL 10 µM mrdA

2nd assay: 150 µL 2x buffer
60 µL dil. enzyme + HP/PG
10 µL dPP
80 µL 1st assay mixture

Experiment

1st assay: 150 µL 2x buffer
71 µL H_2O
60 µL enzyme + HP/PG
10 µL dPP
9 µL 10 µM mrdA

2nd assay: 150 µL 2x buffer
60 µL dil. enzyme + HP/PG
10 µL dPP
80 µL 1st assay mixture

Constituents appearing below the line in each assay list were added simultaneously to begin the assay. 2x buffer = 200 mM Tris pH 7.5, 50 mM $MgCl_2$, 100 mM KCl. dPP stock concentration was 3.15 mM.

8.4.6.9. Measurement of K_i for mureidomycin A.

The standard FEA protocol (8.4.6.4) was used for both competition experiments, determination of K_m/V_{max} values and for the determination of K_i for mrdA, excepting that substrate concentrations were varied as described in the relevant results section. Straight lines were fitted to data using the least squares method.

8.4.6.10. Measurement of k_{on} and K_i* for mureidomycin A.

The standard FEA protocol (8.4.6.4) was used except that the dPP concentration used was 53 μM. To measure k_{obs} at lower inhibitor concentrations enzyme was diluted with 50 mM Tris pH 7.5, 1 mM $MgCl_2$, 2 mM ß-mercaptoethanol, 20 % glycerol, 1 % Triton X-100. The data set 275 nM - 425 nM was collected using neat enzyme. The data set 100 - 180 nM mrdA was collected using 2- fold diluted enzyme. The data set 50 - 100 nM mrdA was collected using 5-fold diluted enzyme. The effect of diluting the enzyme was that the time-course was linear for a greater time allowing lower concentrations of inhibitor (with correspondingly greater $t_{1/2}$ times for the onset of slow-binding inhibition) to be analysed.

8.4.6.11. Dialysis of mureidomycin A-inactivated translocase I.

Assays were carried out as in 8.4.6.3. Dialysis tubing was size 1-8/32" from Medicell International Ltd., U.K. This prepared by boiling in 1 mM EDTA, washed thoroughly with water and was stored in ethanol. It was washed thoroughly with distilled water prior to use. After dialysis, detergent was added to the assays to maintain its concentration at 0.2 %. Enzyme was inactivated in an assay with 50 nM mrdA and 2 mL of the assay reaction mixture dialysed against 100 volumes of 50 mM Tris pH 7.5, 1 mM $MgCl_2$, 2 mM ß-mercaptoethanol, 20 % glycerol, 0.2 % Triton X-100 at 4 °C for 3 hr. It was generally found that the sample was concentrated by dialysis, presumably due to the presence of glycerol in the dialysis buffer. A control was treated similarly. The dialysed samples were assayed at times after removal from the dialysis tubing as shown in table 8.5.

Time, hr	Control activity, u/min	MA-treated activity,u/min
0.5	1.15	-
0.75	-	0.06
4.75	1.50	0.05
20.5	1.20	0.07
72	1.30	0.06

Table 8.5. Activity after dialysis of mureidomycin A-treated enzyme.

8.4.6.12. Gel-filtration of mureidomycin A-inactivated translocase I.

Assays were carried out as in 8.4.6.3 except that where the assay mixture was to be gel filtered, 0.6 mL of enzyme was used to make detection of activity after gel filtration more reliable. The column used was one of sephadex G75 (15 x 1.6 cm). All experiments were at 4 °C. The column buffer was 50 mM Tris pH 7.5, 1 mM $MgCl_2$, 2 mM ß-mercaptoethanol, 0.2 % Triton X-100. The column was calibrated and tested by chromatography of a mixture of blue dextran (average M_r $2x10^6$) and bromophenol blue (M_r 670) dissolved in 6 mL of running buffer. The dyes were separated. The flow rate was ~1 mL/min. The void volume was ~12 mL (bed volume, ~30 mL). The bromophenol blue appeared after ~22 mL.

Enzyme was assayed with or without 100 nM mrd A. The assay mixtures were then gel filtered, control first, collecting 2.5 mL fractions starting after the sample had been flowed into the column. Fraction 4 was identified as containing the most activity in the control and so the corresponding fraction in the positive experiment was used for assays. The active fraction was held on ice and assayed at times after gel filtration. Table 8.6 summarises the results of this experiment.

Time, hr	Control activity, %	Time, hr	Activity, %
0	35	0.3	8.5
2	32	0.75	10
4	43	3.3	10
24	38	21	8.0

Table 8.6. Activity of gel filtered translocase I after treatment with mureidomycin A. Activities are expressed as a percentage of the original activity taken to the assay mixture before chromatography.

The activity of the enzyme sample used for the plus mureidomycin A had an activity of 3.5 u/min in the absence and 1.6 u/min (initial rate) in the presence of mureidomycin A. When this sample of enzyme was taken to the column, the steady-state final rate was 0.15 u/min.

8.4.6.13. Desalting of mureidomycin A-inactivated translocase I using Penefsky spin-columns.

Enzyme was prepared and assayed acccording to the standard FEA protocol except that dPP concentration was 26 μM. A rapid method for desalting of protein samples on a small scale has been described by Penefsky.[150] Sephadex G50 (fine) was equilibrated with 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 0.2 % Triton X-100. Plastipak 1 mL syringes (Becton Dickinson, Dublin, Ireland) were plugged with a disc of Whatman no.1 filter paper cut with a hole punch. This was wetted with buffer and the end of the syringe fitted with a luer cap. Resin was poured into the syringe and allowed to settle such that the final bed volume was 1 mL. The minicolumn was then allowed to drain under gravity (now ~0.95 mL). The column was then placed in a pyrex test-tube (8 mm diameter) and spun in an MSE minor benchtop centrifuge fitted with swing out buckets with the speed setting at 4.5 (~100*g*) for 1 min to give partially dessicated resin (~0.75 mL). 100 μL of sample was applied, the column was moved to a fresh test-tube and centrifuged for a further 30 s.

This method was tested with a mixture of dextran blue (average M_r $2x10^6$, dark blue) and cobalt (II) acetate (pink). When dissolved in 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 0.2 % Triton X-100 these two compounds have non-overlapping UV absorbances at 10 and 30 mg/mL respectively. UV analysis of the spin-column effluent after application of a 1:1 mixture of the two solutions showed that it was completely free of cobalt (II) acetate. Indeed, the cobalt salt could clearly be seen to have been sequestered in the upper third of the column resin. The method was also tested for recovery of enzyme activity. This was found to be quantitative in every case.

In these experiments it was important to assay the column effluent without delay. For this reason it was demonstrated that the FEA was equally viable when lipids were added directly to the reaction mixture and the assay started by addition of enzyme. Again, detergent was added to the assay to maintain its concentration at 0.2 %. Enzyme was incubated in an assay mixture containing 400 nM mrd A and was seen to be completely inactivated after 5 min. The reaction mixture was cooled in an ice bath before desalting in the above protocol. This part of the experiment was carried out at 4 °C and desalted samples were held on ice.

8.4.7.1. Kinetics of inhibition of translocase I by tunicamycin.

All experiments with tunicamycin were carried out using solubilised translocase I prepared and assayed using the standard FEA protocol as in 8.4.6.4. Tunicamycin was prepared as a stock solution in methanol. The volume of tunicamycin stock solution added was always 3 μL. Methanol at 1 % v/v had no effect on the assay.

8.4.7.2. IC_{50} for tunicamycin.

Translocase I was assayed (8.4.7.1) in the presence of 0, 2, 5 or 10 μM tunicamycin. Initial rates were 3.7, 1.6, 0.7 and 0.4 u/min and time-courses were linear for 30 s, 1, 3 and 5 min repsectively.

8.4.7.3. 'Dilution of inhibitor' experiment for tunicamycin.

The protocol for this experiment was the same as that used for mureidmycin A (8.4.6.3) except that 30 μL, not 60 μL, of incubation mixture was carried through to the second assay. The experiment was performed twice with the same result both times.

8.4.8.1. Kinetics of inhibition of translocase I by liposidomycin B.

All experiments with liposidomycin B were carried out using solubilised translocase I prepared and assayed using the standard FEA protocol as in 8.4.6.4. Liposidomycin B (lipB, 1.14 mg, M_r 1009) was dissolved in 113 μL ultrapure water giving a 10 mM stock solution which was stored at -20 °C.

8.4.8.2. IC_{50} for liposidomycin B.

Translocase I was assayed (8.4.8.1) in the presence of 0, 100, 200 or 500 nM lipB. Initial rates were 3.7, 3.1, 1.5 and 0.65 u/min respectively.

8.4.8.3. 'Dilution of inhibitor' experiment for liposidomycin B.

The protocol for this experiment was the same as that used for mureidmycin A (8.4.6.3) except that 30 μL, not 60 μL, of incubation mixture was carried through to the second assay. The concentration of lipB use in the first assay was 250 nM and the incubation was for 2 min before transfer of the 30 μL aliquot to the second assay. As with mureidomycin A, lipB was added to the control second assay for consistency. The experiment was performed twice with the same result both times.

8.4.8.3. Dialysis of liposidomycin B-treated translocase I.

The dialysis tubing used was the same as that in 8.4.6.11. Dialysed samples were held on ice and 60 µL aliquots assayed according to the standard FEA protocol (table 8.7).

Time	Initial rate, u/min	
	Control	+ 500 nM lipB
25 min	1.0	-
30 min	-	0
3 hr 45 min	0.55	0
18 hr	0.40	0

Table 8.7. Dialysis of liposidomycin B-inactivated translocase I.

8.4.8.4. Loss of potency of liposidomycin B upon incubation with translocase I.

The protocol used in this experiment was the same as that used for mureidomycin A in 8.4.6.8 except that the first incubation time was 5 min. The concentration of lipB used in the first incubation was 300 nM.

8.4.8.5. Time-dependence of inhibition by liposidomycin B in the reverse reaction.

A standard enzyme assay was allowed to proceed to equilibrium (3 min). LipB was added in 6 µL to 200 nM and at times after that UMP was added in 6 µL to 1 mM. The initial reverse rates were measured. A control did not contain lipB.

8.5. Chapter 5 experimental.

8.5.2.1. Precipitation of radiolabelled species.

Membranes were prepared as in 8.3.2.3. The protein concentration was 8 mg/mL (BCA assay). Membranes (3.5 mL) were solubilised by addition of an equal volume of 50 mM Tris pH 7.5, 2 mM ß-mercaptoethanol, 1 mM $MgCl_2$, 40 % glycerol, 1 % Triton X-100. Unsolubilised material was removed by ultracentrifugation (10^5 *g*, 30 min). 2 M $MgCl_2$ (75 µL) and 2 M KCl (150 µL) were added to 6 mL of solubilised preparation to give approximately 25 mM $MgCl_2$ and 50 mM KCl in keeping with observed activity in the fluorescence enhancement assay. Of this 6 mL was equilibrated to 25 °C with stirring in a 38 mL centrifuge tube in a water bath. UDPMurNAc-L-Ala-γ-D-Glu-*m*-DAP-[^{14}C]-D-Ala-[^{14}C]-D-Ala (4 µL, 1.3 µCi, 1.15 mM) was added. The mixture was vortexed and incubated at 25 °C for 1 min. Acetone (-20 °C, 6 mL) was added, the mixture held at -20 °C for 10 min and centrifuged (12000 *g*, 10 min). The supernatant was decanted and set aside. The pellet was resuspended in 100 mM Tris pH 7.0 (6 mL). A second aliquot of acetone (-20 °C, 6 mL) was added and the process repeated except that this time the pellet was washed with 50/50 100 mM Tris pH7.0/acetone (10 mL). This was immediately centrifuged (13500 rpm, 5 min). The pellet was extracted three times with 2:1 $CHCl_3$/MeOH (3 x 1 mL), keeping the supernatant each time. The final pellet was resuspended in 100 mM Tris pH 7.0 (0.5 mL). This and 0.5 mL of each supernatant fraction was mixed with Optiphase Hi-Safe 3 in a disposable plastic vial and radioactivity determined as in 8.2.2.1. At the same time, a control was treated the same way, starting with 6 mL of 6 mg/mL BSA in 100 mM Tris pH 7.0.

8.5.3.1. Column chromatography of radiolabelled species.

Membranes were prepared as in 8.3.2.3. The protein concentration was 8 mg/mL (BCA assay). Membranes (1 mL) were solubilised by addition of an equal volume of 50 mM Tris pH 7.5, 2mM ß-mercaptoethanol, 1 mM $MgCl_2$, 20 % glycerol, 6 % CHAPS with stirring for 1 hr at 4 °C. Unsolubilised material was removed by centrifugation (10^5 *g*, 30 min). The supernatant was collected and 2 M $MgCl_2$ (25 µL) and 2 M KCl (50 µL) added as in 8.5.3.1. This was equilibrated to 25 °C in a glass tube in a water bath. [^{14}C]-UDPMurNAc-pentapeptide (8.7 µL, 1.3 µCi, 0.94 mM) was added giving a final concentration of approximately 4 µM. Incubation was for 1 min before addition of acetone (-20 °C, 2 mL). This was mixed by pipetting and held at -20 °C for 10 min. Precipitate was collected by centrifugation (12000 *g*, 10 min) and resuspended in 100 mM Tris pH 7.0 (1 mL). This was divided equally into two microfuge tubes and acetone (-20°C, 0.5 mL) added to each. These were

thoroughly vortexed and held at -20 °C for 10 min. Precipitate was collected by centrifugation (13500 rpm, 5 min) and washed with 50/50 100 mM Tris pH 7.0/acetone (1 mL), again with thorough vortexing to remove as much [^{14}C]-UDPMurNAc-pentapeptide as possible. The final pellet was dissolved completely in 50 mM sodium phosphate pH 7.0, 0.2 M NaCl, 2 % SDS (450 µL). This was sonicated at rt (3 min, 5 µ, 21 kc/s). Blue dextran (2 mg) dissolved in the same buffer (50 µL) was added to the sample as a calibrant. This was applied to a column of sephadex G75 fine grade (73 x 1 cm, 57 mL bed volume) equilibrated with 50 mM sodium phosphate pH 7.0, 0.2 M NaCl, 2 % SDS. At the time when these experiments were performed, rt ranged from 25 °C to 32 °C depending on the time of the day. Columns were run late afternoon/early evening when rt was approximately 30 °C. Sample was flowed into the column under gravity and followed into the column bed with buffer (0.5 mL). The column was then flowed at 10.7 mL/hr using a peristaltic pump. Fractions were collected using a Pharmacia fraction collector beginning shortly before emergence of the blue dextran marker. Fractions were collected at 5.6 min intervals (1 mL each) and mixed with Optiphase Hi-Safe 3 (3.5 mL). Radioactivity was measured as in 8.2.2.1. Distribution of dextran blue was noted by eye and assigned arbitrary values for convenience in analysis of the data.

8.5.3.2. Extraction with 2:1 $CHCl_3$/MeOH prior to column chromatography of radiolabelled species.

Enzyme from the same batch as that in 8.5.3.1 was solubilised and treated with [^{14}C]-UDPMurNAc-pentapeptide exactly as in that section. The final pellet was extracted three times with 2:1 $CHCl_3$/MeOH (3 x 1 mL). Extraction was promoted by sonication (3 min, 5 µ, 21 kc/s) on ice which rapidly gave a very fine suspension. The first organic solvent supernatant was visibly yellow and the final pellet when dissolved in column buffer (8.5.3.1) gave an almost colourless solution as opposed to a straw yellow solution in 8.5.3.1. The final pellet was dried with a stream of nitrogen, dissolved in 50 mM sodium phosphate, 0.2 M NaCl, 2 % SDS and chromatographed exactly as in 8.5.3.1.

8.5.3.3. Treatment of radiolabelled species with UMP.

This experiment used a new batch of membrane preparation. Therefore the experiment described in 8.5.3.1 was repeated as a control for this and subsequent experiments. The protocol was identical to that in 8.5.3.1 for the control experiment. For the UMP experiment, the protocol was again identical except that after the incubation for 1 min with [^{14}C]-UDPMurNAc-pentapeptide, UMP (7.4 mg dissolved in 50 µL water) was added to a final concentration of approximately 10 mM and the mixture incubated for a further 1 min before precipitation, chromatography, etc;.

8.5.3.4. Treatment of native translocase I with hydroxylamine.

CHAPS-solubilised translocase I was prepared as in 8.5.3.1 from the same batch of membranes as in 8.5.3.3. The pH of the preparation was adjusted to 9.5 with 3 M NaOH (as judged by comparison with 200 mM Tris pH 9.5 using universal indicator paper). $MgCl_2$ and KCl were added as in 8.5.3.1. [^{14}C]-UDPMurNAc-pentapeptide (8.7 µL, 1.3 µCi, 0.94 mM) was added and the mixture incubated for 15 s before addition of 1.54 M hydroxylamine pH 9.5 (139 µL) to give a final concentration of approximately 100 mM. The mixture was incubated for a further 105 s before precipitation, chromatography, etc; as in 8.5.3.1.

8.5.3.5. Treatment of denatured translocase I with hydroxylamine.

CHAPS-solubilised translocase I was prepared as in 8.5.3.1 from the same batch of membranes as in 8.5.3.3. Labelling with [^{14}C]-UDPMurNAc-pentapeptide, precipitation, washing and dissolution in column buffer (8.5.3.1) was carried out exactly as in 8.5.3.1. At this point, pH was adjusted to 9.5 and hydroxylamine added (from a 1.54 M stock at pH 9.5) to 100 mM as in 8.5.3.4. This was incubated at rt for 15 min before chromatography as in 8.5.3.1.

8.6. Chapter 6 experimental.

8.6.1. Homology searches using the BLAST algorithm.

The protein and translated sequence databases were searched for primary sequences having significant local homology to the *E. coli* translocase I amino-acid sequence using the BLAST algorithm. Aligning sequences were selected by sending a standard program message to the 'blast' server, email address <blast@ncbi.nlm.nih.gov> as per instructions in the 'blast email manual'. Complete records were retrieved from databases using the accession numbers again by sending a standard program message to the 'retrieve' server, email address <retrieve@ncbi.nlm.nih.gov>.

Searching example:
PROGRAM blastp
DATALIB nr
EXPECT 10
BEGIN
>E. coli mraY
<primary amino acid sequence in Fasts-Pearson format>
<blank line>

Retrieve example:
DATALIB swissprot
BEGIN
<accession no.1> [acc]
<accession no.2> [acc]
<blank line>

A detailed description of how to use BLAST and the email server are contained in the BLAST manuals which can be obtained by sending the message 'help' to the above sites. Databases can now also be accessed through a facility based in Cambridge, England. The facility is accessed through the Internet using the Netscape program by first contacting mol.biol.@soton. The appropriate links are provided at this site.

8.6.2. Sequence alignments.

The global sequence alignment was performed by Dr. Ian Giles, Dept. of Biochemistry, Southampton Univ. using the clustalw program with default parameter settings using the blosum30 scoring matrix. [195]

8.6.3. Secondary structural predictions.

Prediction of membrane associated helices was performed by Dr. Ian Giles, Dept. of Biochemistry, Southampton Univ. using the method of Eisenberg.[173]

8.6.4. Molecular biology general methods.

Oligonucleotides were obtained from R&D Systems Europe Ltd. Reagents were obtained from Sigma Chemical Co. unless otherwise stated and were of the highest grade available. All materials were sterilised by autoclaving or filtration. Restriction enzymes were obtained from Promega unless otherwise stated and were used with the 4-core buffer system.

DNA Preparations. Double-stranded DNA was extracted from cells using either the Promega 'Wizard Midipreps DNA purification system', the Rapid Pure Miniprep^{TM} system as recommended by the manufacturer (except that once cells were lysed pipetting and vortexing was avoided so as not to contaminate samples with sheared chromosomal DNA as this interferes with sequencing) or by phenol-chloroform/ ethanol precipitation as described in Maniatis.[194] Single-stranded DNA was prepared from phage particles using the Promega 'Magic M13 miniprep system, or by phenol-ether/ ethanol precipitation, also as described in Maniatis.[194] Quality of DNA was judged by gel electrophoresis and quantity by UV absorbance. Where necessary DNA was purified using the Promega Wizard DNA Clean-up kit.

Agarose gel electrophoresis. DNA was analysed by electrophoresis through gel slabs containing 0.7 % agarose (or 1.0 % agarose for preparative scale) in 0.5x TBE (45 mM Tris-borate pH 8.0, 1 mM EDTA), 5 μg/mL ethidium bromide with a potential difference of 10 V/cm. Samples were prepared by addition of a 1/5th volume of 0.25 % bromophenol blue, 0.25 % xylene cyanol FF, 15 % ficoll. DNA intercalated with ethidium bromide was visualised with an ultraviolet illuminator. Molecular weight markers were phage lamda DNA digested with either *Hind*III or *Hind*III/*Eco*RI.

Restriction digest analysis. Plasmid, phage and recombinant DNA's were analysed by digestion with given restriction enzymes and the resulting fragments visualised by gel electrophoresis to test for desired restriction sites and DNA inserts. For analytical digests, typically 100 ng DNA in 8 μL TE (10 mM Tris pH 8.0, 1 mM EDTA) or water (12.5 μg/mL) was placed in an eppendorf tube. To this was added 1 μL of an appropriate buffer (Promega '4-core' system) and 0.5 μL of Promega standard concentration enzyme (one or two) was pipetted onto the side of the tube. The stock restriction enzymes were diluted at least tenfold to avoid 'star' activity. Reactions were started by centrifugation and incubated at 37 °C for 45 min. When DNA's were

digested with the intention of fragment purification, 1 - 2 μg of DNA was incubated with 5 μL of each restriction enzyme and 10 μL of buffer in a final volume of 100 μL. Incubation was for 1 hr. Before preparative scale gel electrophoresis, 5 μL of reaction mixture was run out to check that digestion was complete.

Electroelution. DNA fragments purified by preparative scale gel electrophoresis were eluted from chunks of agarose gel using the BioTrap BT1000 system (Schleicher and Schuell, Dassel, Germany) as recommended by the manufacturer with 0.5x TBE as buffer. Fragments were eluted with a potential of 200 V for 20 min. Gel chunks were retrieved from the apparatus and examined under UV illumination to check that DNA had been completely eluted. The potential was reversed for 15 s to detach any DNA from the impervious membrane and ~200 μL final DNA solution collected. By this method, recovery of DNA was routinely >90 % by UV absorbance.

Preparation of competent cells. Typically, 100 mL of LB was innoculated with 1 mL of an overnight culture and grown to OD_{600nm} = 0.8. Cells were collected by centrifugation (5000 *g*, 5 min) and resuspended in 50mL ice-cold sterile 0.1 M $CaCl_2$. Cells were held on ice for 30 min, collected by centrifugation then resuspended in 2.5 mL ice-cold sterile 0.1 M $CaCl_2$. The suspension was mixed with 1.25 mL 50 % sterile glycerol and 200 μL aliquots stored at -70 °C.

Transformation. Competent cells (200 μL) were thawed on ice, 0.1 - 1 μg plasmid or phage DNA was added and the mixture allowed to stand on ice for 30min. Cells were incubated at 42 °C for 30 s then returned to ice. In the case of plasmid DNA heat-shocked cells (200 μL) were incubated with 1 mL LB at 37 °C for 1 hr and 100 μL spread out on appropriate agar plates prewarmed to 37 °C and incubated overnight at 37 °C. With phage DNA, heat-shocked cells were mixed with 100 μL of a mid logarithmic phase culture of the same strain. This mixture was added to 0.3 mL top agar (0.7 % w/v agar) maintained at 42 °C, poured onto agar plates pre-warmed to 37 °C and incubated overnight at 37 °C.

Ligation. DNA fragments were ligated using the Amersham T4 DNA ligase system as recommended by the manufacturer. Relative concentrations of DNA fragments were routinely estimated by gel electrophoresis analysis and ligated at an approximate 1:1 molar ratio. Competent cells were transformed with recombinant DNA using the entire rection volume (typically <20 μL). In evey case controls were cut vector fragment only and cut vector fragment + ligase.

Preparation of phage-containing supernatants. Phage were grown through *E. coli* MV1190 or *E. coli* CJ236 where uracil-containing phage DNA's were required. An aliquot of an overnight culture was used to innoculate LB media and the culture grown to an OD_{600nm} of 0.3. In every case cells were grown at 37 °C since below 35 °C the sex pilus necessary for phage infection does not form. Baffled flasks were avoided as vigorous agitation can result in shearing of the pilus from the cell surface. The culture was infected with either a plug of agar containing a single phage plaque or with an aliquot of an existing phage stock. After 4.5 - 5 hr cells were pelleted by centrifugation (17000 *g*, 15 min) and the supernatant carefully removed avoiding the cell pellet. The supernatant was recentrifuged (17000 *g*, 15 min) and stored at 4 °C.

Titering of phage stocks. For a given phage-containing supernatant the number of plaque forming units per mL (pfu/mL) was determined by a standard method described in Maniatis.[194] Serial dilutions of phage stock ranging from 10^6 to 10^{12} were made with LB. Diluted phage stock (100 µL) was mixed with 200 µL of a mid-logarithmic phase culture of appropriate cells and allowed to stand for 5 min at room temperature to allow for phage adhesion. This was then added to 0.3 mL 'top' agar (0.7 % w/v) maintained at 42 °C, mixed by turning, poured onto prewarmed agar plates, allowed to set and incubated overnight at 37 °C.

Blue/white plaque assay. To identify insert positive recombinants after cloning of a fragment into the multiple cloning site of M13mp18, the blue/white plaque assay[194] was used. The procedure was as for transformation of cells with phage DNA except that 40 µL of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), 20 mg/mL in DMF and 4 µL IPTG (200 mg/mL) were added to the 'top' agar before addition of transformed cells. Generally, religated phage give blue plaques whilst those carrying insert give white (clear) plaques.

Phosphorylation of oligonucleotides. Before use in mutagenesis reactions, supplied oligonucleotides were first phosphorylated. Stock solutions were diluted to 40 pmol/µL and 5 µL added to 6 µL of 5x kinase buffer and 13 µL sterile water. Reaction was started by addition of 6 µL of 2 mM ATP and the mixture incubated at 37 °C for 45 min before stopping by heating to 65 °C for 10 min. The mixture was diluted with 10 µL water (oligonucleotide concentration 5 pmol/mL) and stored at 4 °C.

Complementary strand synthesis using mutagenic oligonucleotides. This was carried out as recommended in the BioRad M13 Mutagene kit manual. Template DNA (0.1 pmol) was mixed with phosphorylated primer (2.5 pmol) and 1 µL of 10x

annealing buffer (kit supplied) in a total volume of 10 μL and heated to 70 °C followed by cooling to 30 °C over ~45 min (beaker of water standing on the bench) and then moved onto ice. Synthesis buffer, ligase and diluted T7 DNA polymerase were added in that order (kit supplied). The mixture was incubated on ice for 5 min, at room temperature for 5 min and then 37 °C for 30 min before termination with 'synthesis stop' buffer (sterile 10 mM Tris pH 8.0, 10 mM EDTA, 90 μL). Synthesis of the second strand was judged by running a 10 μL aliquot out on an agarose gel using the single-stranded template as a standard. A control reaction contained no primer (i.e. no second strand synthesis).

DNA sequencing Sequencing of single-stranded and double-stranded DNA's was carried out using either the Pharmacia T7 sequencing kit or the Amersham USB Sequenase™ Version 2.0 kit with α-^{35}S-dATP. Double-stranded DNA was denatured prior to primer annealing by the following method: 10 μg DNA was resuspended in 60 μL sterile TE, 1 M NaOH (15 μL) was added and the mixture incubated at room temperature for 5 min before addition of 7 μL 5 M ammonium acetate pH 4.6. DNA was precipitated with two volumes of ethanol held at -20 °C by holding at -20 °C for 15 min and spinning at full speed in a benchtop microfuge for 10 min. The DNA pellet was washed by resuspension in 75 % ethanol held at -20 °C and spinning for 10 min again. Typically, for one set of sequencing reactions 1 μg of single-stranded or 2 - 5 μg of double-stranded DNA was used. Primer sequences are provided in the appendix. Sequencing reactions were carried out exactly as recommended by the kit suppliers. Where compression artefacts were suspected, the true sequence was resolved by sequencing with dITP rather than dGTP exactly as provided for in the Amershem kit.

Sequencing plates were prepared each time by washing with dilute SDS solution, water, then ethanol, silanised with a portion of dimethyldichlorosilane in 1,1,1-trichloroethane and again wiped with ethanol. Appropriate volumes of ammonium persulphate and TEMED were added to gel solutions. Where a gradient was required 5 mL of 'bottom' gel containing bromophenol blue prepared exactly as described by Maniatis[194] was poured into the plates (using a 10 mL pipette and automatic pipettor), 5 mL of 'top' gel (supplied premixed by Gibco. BRL: 5.7 % w/v acrylamide, 0.3 % w/v N,N'- methylenebisacrylamide, 7.0 M urea, 100 mM Tris-borate, pH8.3, 1mM EDTA, 3.0mM N,N,N',N'-tetramethylethylenediamine) was drawn into the pipette followed by 5 mL of 'bottom' gel. Three bubbles were pulled through to effect partial mixing and this poured into the plates. The remiander was filled with 'top' gel. Sharks tooth combs were inserted upside down to form the top of the gel and the plates clamped (not on the spacers) with bulldog clips below the combs whilst polymerising. The gel was pre-run for ~45 min to warm it prior to

loading samples. For gradient gels the top reservoir of the rig (Bethesda Research Laboratories, Life Technologies Inc., model 52) contained 1x TBE and the bottom 2.5x TBE. For non gradient gels both reservoirs contained 1x TBE. The gel was run for 1.5 - 2 hr at 2000 V. The gel was adsorbed onto blotting paper, fixed with 10 % AcOH, 10 % MeOH (1 L) and rinsed with water (1 L) before drying under Saran-wrap using a BioRad model 583 gel dryer. The dried gel was monitored for radioactivity and exposed (Amersham HyperFilm-MP) overnight or longer if required. Autorads were developed as recommended by the supplier. Gels were loaded in every case in the lane order TCGA.

8.6.5. Construction of mutant pBROC525's.

pBROC525 DNA (appendix 1) was isolated from cells of *E. coli* JM109 (pBROC525) and verified by restriction analysis: *Sty*I, *Eco*RI, *Bam*HI, *Ssp*I, *Sty*I/*Eco*RI and *Ssp*I/*Eco*RI. All digests gave the expected fragments. pBROC525 was digested on a large scale with *Ssp*I/*Eco*RI and the required fragment (825 bp) purified by preparative scale gel electrophoresis and electroelution. The partial *mraY* fragment was ligated with *Hinc*II/*Eco*RI digested M13mp18 purified with the Wizard DNA 'clean up' system. The ligation reaction mixture was used to transform *E. coli* MV1190; clones carrying insert were identified using the blue/white plaque assay. Five insert positive clones were picked at random and the phage grown through *E. coli* MV1190. Double-stranded, replicative form (RF) DNA was prepared from cells as for plasmid DNA and tested for the presence of the 641 bp *Sty*I/*Eco*RI partial *mraY* fragment by restriction analysis with those enzymes. One clone showing a clean 7403/641 bp fragment pair was denoted M525 (appendix 1) and carried through to further experiments.

For this clone the culture supernatant was found to contain 1.5×10^{11} pfu/mL. This was used to infect a culture of *E. coli* CJ236 at a phage particle:cell ratio of ~0.15 to generate uracil-containing phage M525. This was titered on *E. coli* MV1190 and *E. coli* CJ236; the titers were 4.7×10^{6} and 3.5×10^{11} pfu/mL respectively thus verifying the uracil content of the phage DNA. A stock of uracil-containing single-stranded M525 DNA was prepared as recommended in the BioRad M13 Mutagene kit manual for use as a template in mutagenesis reactions. No problems were encountered in synthesising the complementary strand using any of the five primers carrying the required mutations. The primer for the D115/116N mutation was 35 bp long compared to 25 bp for the other four. This was to compensate for expected reduced primer binding in this AT-rich region of the gene.

Mutagenic primers:

D115N	5'-ggC TTT gTT AAT gAT TAT CgC AAA g-3'
D116N	5'-ggC TTT gTT gAT AAT TAT CgC AAA g-3'
D115/116N	5'-ggT gTT ATT ggC TT gTT AAT AAT TAT CgC AAA g-3'

D267A 5'-gTC TTA ATg ggC gCT gTA ggT TCg C-3'
D267N 5'-gTC TTA ATg ggC AAT gTA ggT TCg C-3'
g appears in lower case to avoid confusion with C

In each case 15 μL of stopped mutagenesis reaction was used to transform cells of *E. coli* MV1190. For each mutagenesis reaction five or more plaques were picked at random and used to infect 1.5 mL cultures of *E. coli* MV1190. Single-stranded DNA was purified from the culture supernatant and sequenced using an appropriate primer to screen for the required mutations.

Sequencing primers:

mraY160 5'-AAACTTTCCTTTggTCAggT-3'
mraY340 5'-gTTATTggCTTTgTTgATgA-3'
mraY510 5'-AgATgTgATgCCgCAgCTgg-3'
mraY680 5'-ACTTTgCCAgCTACTTgCAT-3'

In the case of D115N, D116N and D115/116N one clone was sequenced in all four lanes, TCGA, whilst the rest were sequenced in the A-lane only. Following identification of mutants, remaining culture supernatant was used to prepare single-stranded ΔM525 DNA's and the entire *StyI/Eco*RI fragment sequenced using the primers listed in the appendix to check for secondary mutations. None were found. Screening for mutants at the D267 codon revealed one clone with a deletion here and a mutation several bases downstream. The new sequence (5'-TTTATgggCgATgTAggTTCgCTggCg-3' to 5'-TTTATgggC-ATgTgggTTCgCTggCg-3') codes for a C-terminal truncated translocase I; phage-containing supernatant was retained.

For each mutant 10 mL of OD_{600nm} = 0.3 *E. coli* MV1190 culture was infected with phage stock and grown for several hours. Cells were collected and RF-DNA isolated. These DNAs were digested on a 3 μg scale with *StyI/Eco*RI and the 641 bp partial *mraY* fragments purified. The original vector, pBROC525 (5 μg), was digested with the same enzymes and the larger 4.7 kb fragment purified. Care was taken to ensure that no other DNA had co-run with this fragment by running the preparative gel for 1 hr before cutting out the band. Mutant fragments were ligated with the vector fragment; one colony was found on each of the 'no ligase' and 'religated vector' control plates and 20 - 30 colonies on each mutant ligation plate. For each mutant three transformant colonies were selected at random and used to innoculate 1mL of LB/amp and grown for 4 hr. At this point each clone was plated to LB/amp. Plasmid DNA was prepared from each and digested with *StyI/Hind*III. Every clone contained the expected 950 bp fragment by comparison with similarly digested wild-type pBROC525 and molecular size markers. Two clones were selected for each mutant and grown in 3 mL overnight culture. These were used to prepare sequencing template. Sequencing across the mutations showed that all ten clones were mutant

rather than wild-type. One clone was used to prepare stab cultures for transport and glycerol stocks. The mutants were also stored as purified DNA.

8.6.6. Overnight induction of *E. coli* JM109 (pBROC525).

See 8.2.5.4.

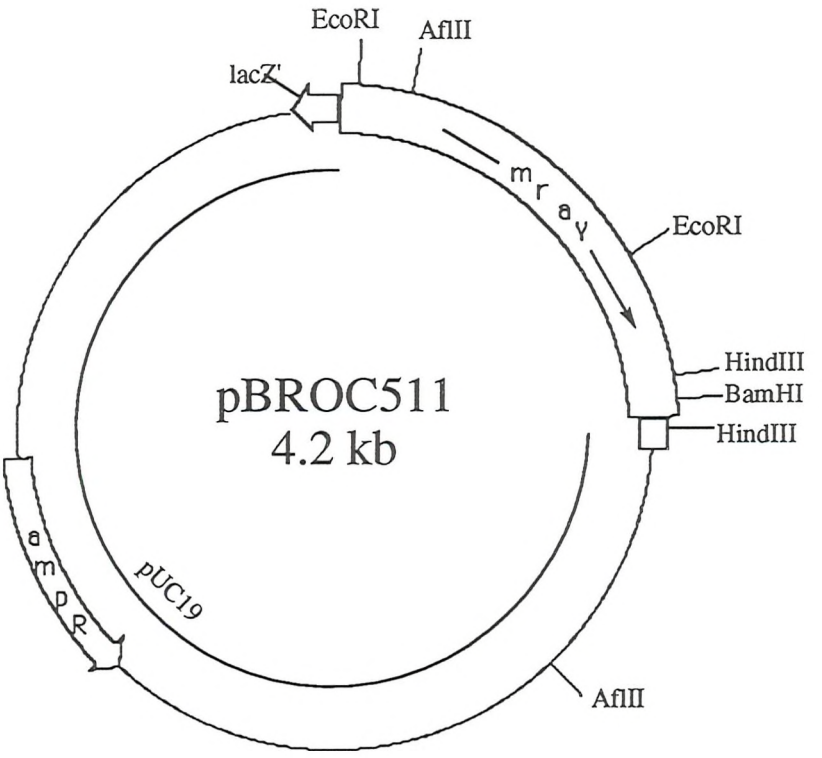
8.6.7. Isotope exchange methods.

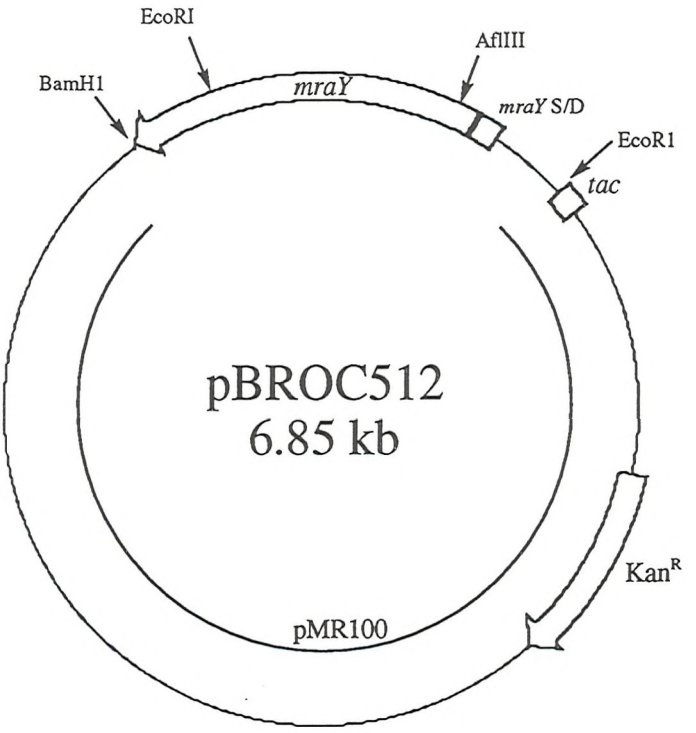
This methodology is based on that of Hammes and Neuhaus.[109] Assays were carried out at ambient temperature using particulate membrane preparations. Assays contained in a volume of 50 µL, 200 mM Tris pH 7.5, 100 mM KCl, 50 mM $MgCl_2$, 5 µL UDPMurNAc-pentapeptide, 10 µL enzyme, 5 µL 1 nCi/µL [^{14}C]-UMP. The reaction mixture was incubated for 2 min before beginning the exchange reaction by addition of radiolabelled UMP. Reaction was terminated by boiling for 2 min. Controls contained enzyme which had already been boiled. Samples were pulse centrifuged to return the liquid to the bottom of the microfuge tube. Alkaline phosphatase (2 units; type VIII-S from bovine intestinal mucosa; 1 unit hydrolyses 1 µmole of ATP/min at 37 °C at pH 9.0 in saturating ATP) was added to the mixture and incubated at room temperature for 10 min to hydrolyse UMP. The mixture was diluted with 5 mM Tris pH 7.5, 10 mM KCl (0.5 mL). This was applied to a column of Dowex-1 anion exchange resin (0.4 mL) equilibrated with 5 mM Tris pH 7.5, 10 mM KCl. The resin was prepared or regenerated by successive washes with 20 vols 0.5 M NaOH, 20 vols 5 mM Tris pH 7.5, 10 mM KCl, 20 vols 0.5 M HCl, 20 vols 5 mM Tris pH 7.5, 10 mM KCl, and 5 x 20 vols 5 mM Tris pH 7.5, 10 mM KCl. The pH of the last wash supernatant was checked. The columns were 1 mL PlastiPak syringes plugged with hole-punch cut filter paper. The column was washed with 5 x 1 mL of 5 mM Tris pH 7.5, 10 mM KCl. UDPMurNAc-pentapeptide was eluted with 5 mM Tris pH 7.5, 0.5 M KCl (1.5 mL). The eluate was transferred to a disposable plastic insert, 3 mL of Optiphase Hi-Safe 3 added and radioactivity determined as in 8.2.2.1. The specific activity of the [^{14}C]-UMP was 46.1 mCi/mmol, 0.2 mCi/mL and was diluted for use with water.

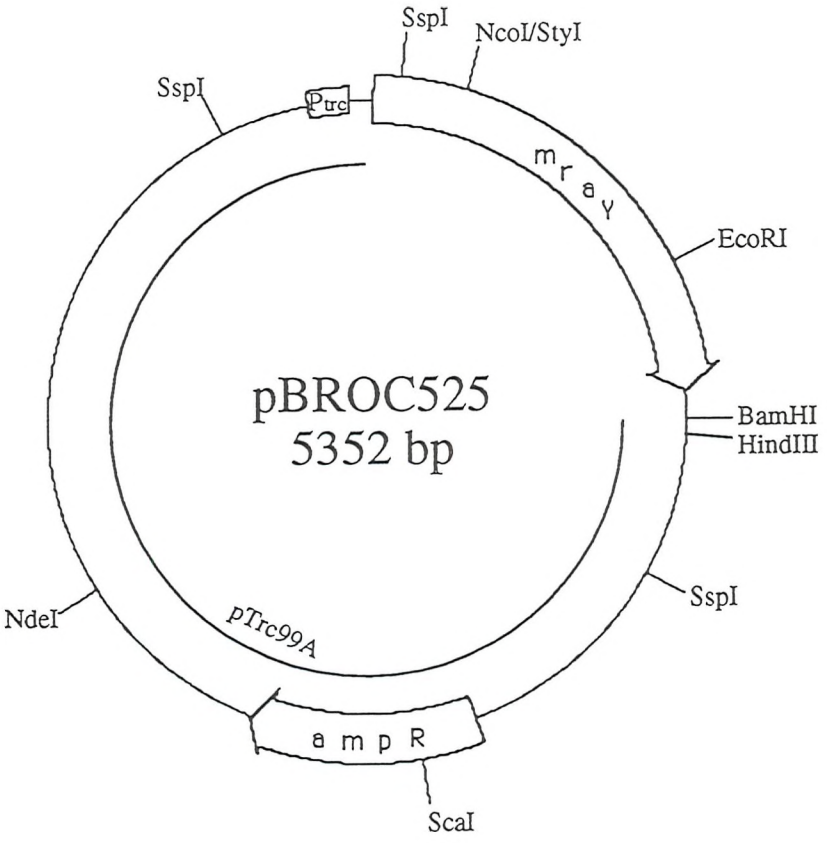
8.6.8. Labelling experiments.

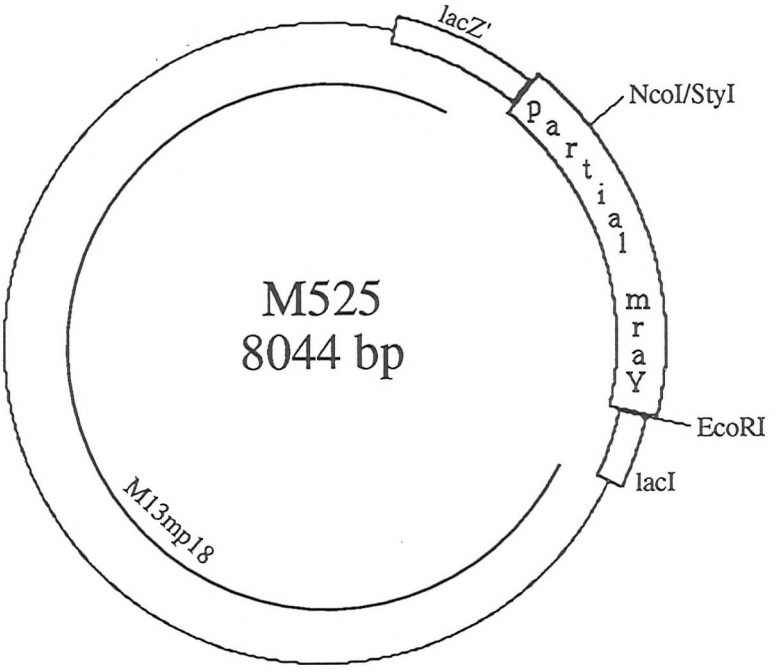
For labelling with UDP-MurNAc-L-Ala-γ-D-Glu-*m*-DAP-[^{14}C]-D-Ala-[^{14}C]-D-Ala, mutant membrane preparations (D115N, D116N and D267N) were diluted to 8 mg protein/mL and 1 mL (8 mg protein) used for each experiment. Otherwise the protocol was exactly the same as that described for the control experiment with *E. coli* JM109 pBROC525 membranes in the chapter 5 experimental section.

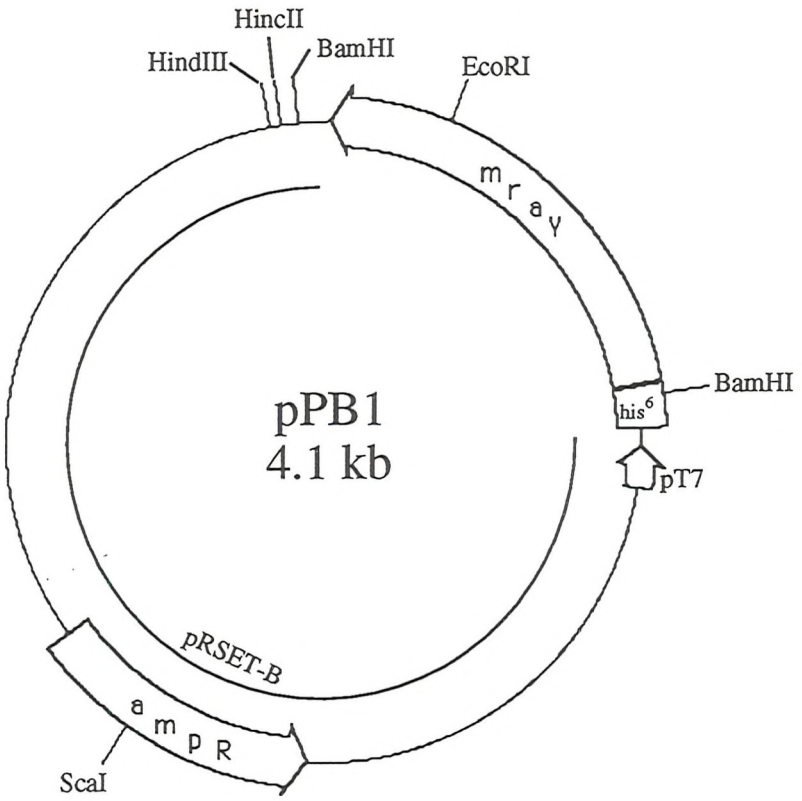
Appendix 1. Plasmid maps.




EcoRI
AflIII
BamH1
mraY
mraY S/D
EcoR1
tac
pBROC512
6.85 kb
Kan^R
pMR100


SspI
NcoI/StyI
SspI
Ptrc
mray
EcoRI
pBROC525
5352 bp
BamHI
HindIII
SspI
NdeI
pTrc99A
ampR
ScaI


lacZ'
NcoI/StyI
partial mraY
EcoRI
lacI
M525
8044 bp
M13mp18

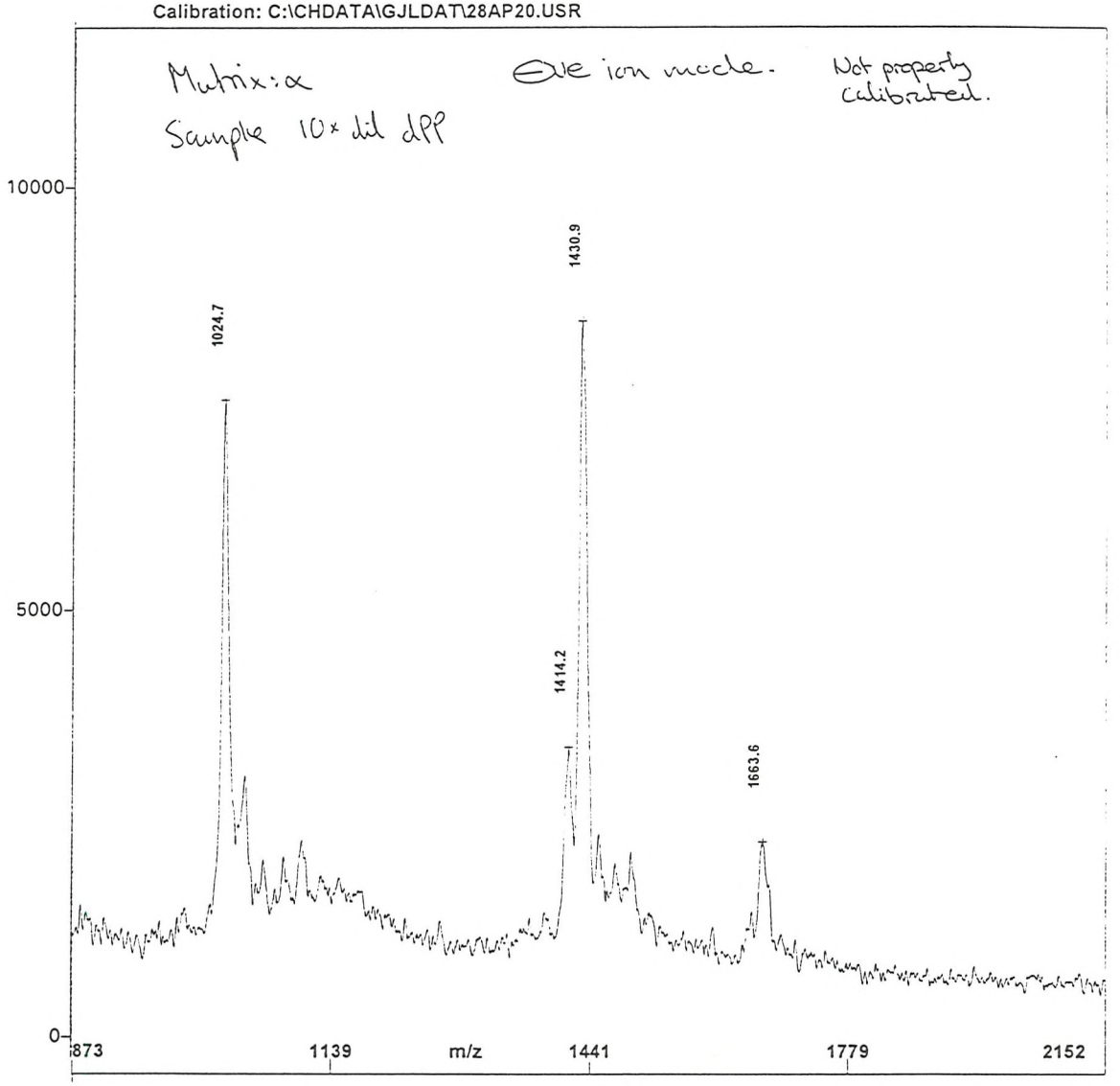

HincII
HindIII
BamHI
EcoRI
mray
pPB1
4.1 kb
BamHI
his6
pT7
pRSET-B
ampR
ScaI

Appendix 2. MALDI-TOF MS data for dansyl-UDPMurNAc-pentapeptide.

LaserTec Time-of-Flight Mass Spectrometry 4/24/94 4:45 PM

C:\CHDATA\PHILDAT\24AP008.SPC Collected: 4/24/94 4:39 PM.

Calibration: C:\CHDATA\GJLDAT\28AP20.USR

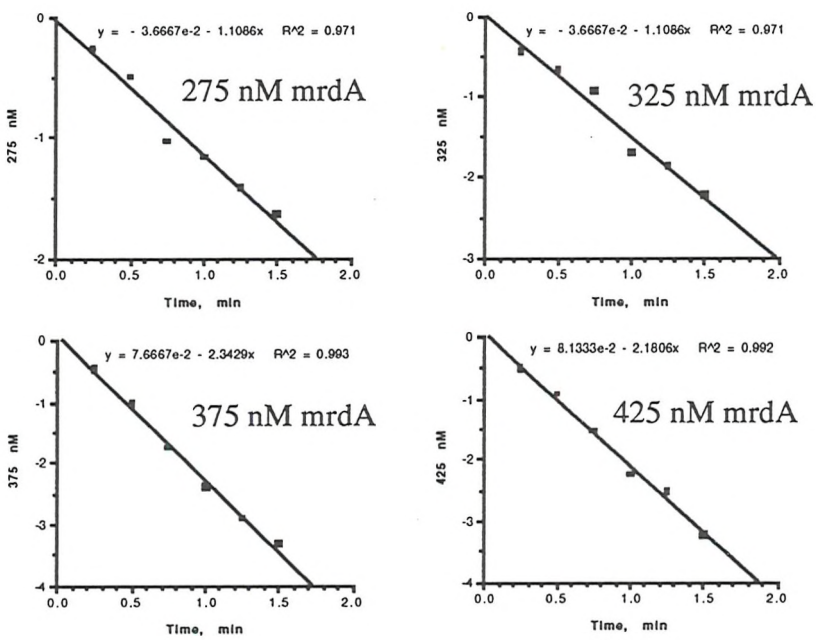


File: 24ap008 Accelerating Voltage: 28000 Laser: 540 Pressure: 5.85e-07 Torr

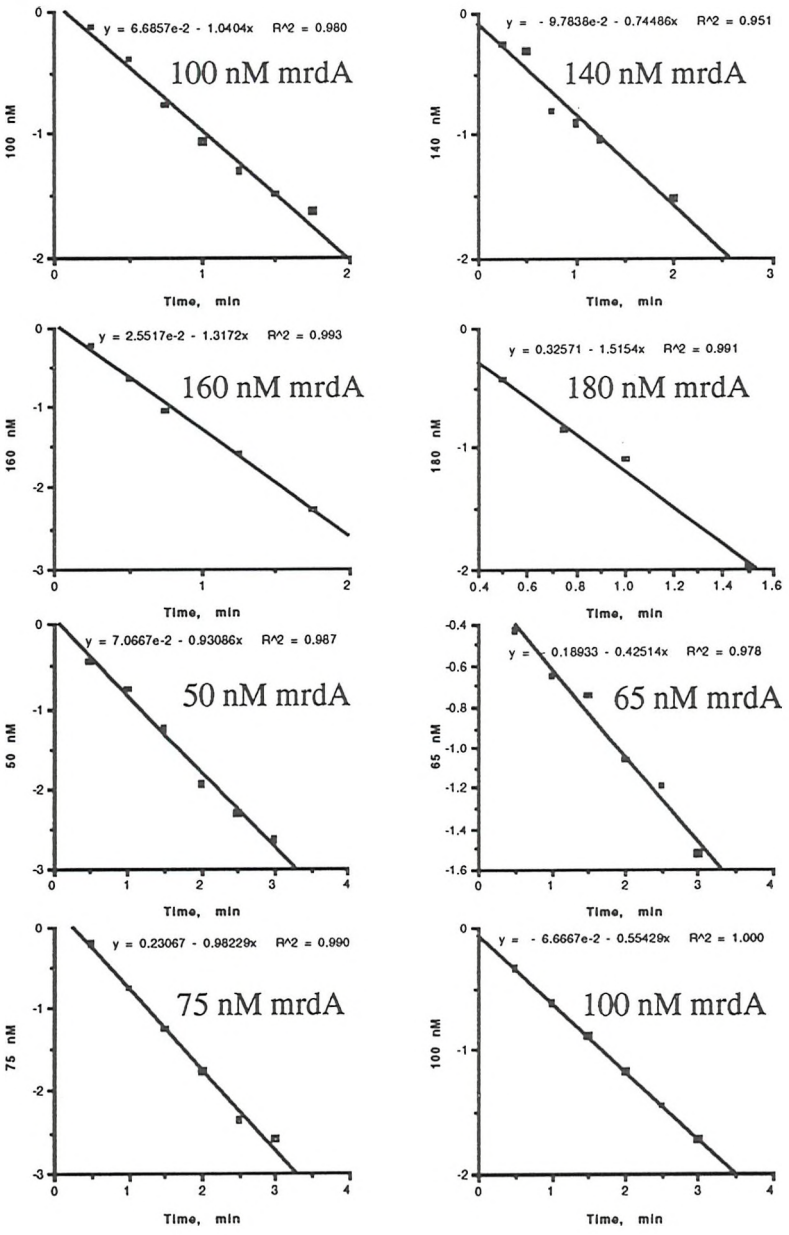
Sample: 15 Operator: Scans Averaged: 4

Method: chemlm.mnu

Appendix 3. Slow-binding data for the determination of kobs values for inhibition of translocase I activity by mureidomycin A.



P.T.O.


y = 6.6857e-2 - 1.0404x R^2 = 0.980
100 nM mrdA
100 nM
Time, min
y = - 9.7838e-2 - 0.74486x R^2 = 0.951
140 nM mrdA
140 nM
Time, min
y = 2.5517e-2 - 1.3172x R^2 = 0.993
160 nM mrdA
160 nM
Time, min
y = 0.32571 - 1.5154x R^2 = 0.991
180 nM mrdA
180 nM
Time, min
y = 7.0667e-2 - 0.93086x R^2 = 0.987
50 nM mrdA
50 nM
Time, min
y = - 0.18933 - 0.42514x R^2 = 0.978
65 nM mrdA
65 nM
Time, min
y = 0.23067 - 0.98229x R^2 = 0.990
75 nM mrdA
75 nM
Time, min
y = - 6.6667e-2 - 0.55429x R^2 = 1.000
100 nM mrdA
100 nM
Time, min

Appendix 4. Determination of K_i values.

Competitive inhibition:

Initial velocity equation for a two substrate ping pong mechanism in the absence of products:

$$\frac{V}{V_{max}} = \frac{[A][B] + K_{m(A)}}{K_{m(B)}[A] + K_{m(A)}[B] + [A][B]}$$

$$\frac{1}{V} = \frac{1}{V_{max}}\left(1 + \frac{K_{m(B)}}{[B]}\right) + \frac{K_{m(A)}}{V_{max}} \cdot \frac{1}{[A]}$$

Add in the competitive inhibition with respect to substrate A term:

$$\frac{1}{V} = \frac{1}{V_{max}}\left(1 + \frac{K_{m(B)}}{[B]}\right) + \frac{K_{m(A)}}{V_{max}}\left(1 + \frac{[I]}{K_i}\right) \cdot \frac{1}{[A]}$$

To determine K_i, plot the slopes of the double reciprocal plots against [I]:

$$\frac{K_{m(app)}}{V_{max}} = \frac{K_m}{V_{max}}\left(1 + \frac{[I]}{K_i}\right) = \frac{K_m}{V_{max}} + \frac{K_m}{V_{max} K_i} \cdot [I]$$

When the slope, $\frac{K_{m(app)}}{V_{max}} = 0$, [I] = $-K_i$.

Non-competitive inhibition:

Initial velocity equation for a two substrate ping pong mechanism in the absence of products:

$$\frac{V}{V_{max}} = \frac{[A][B] + K_{m(A)}}{K_{m(B)}[A] + K_{m(A)}[B] + [A][B]}$$

Add in the non-competitive with respect to substrate B term:

$$\frac{1}{V} = \frac{1 + {}^{[I]}/_{K_i}}{V_{max}}\left(1 + \frac{K_{m(A)}}{[A]}\right) + \frac{K_{m(A)}\left(1 + {}^{[I]}/_{K_i}\right)}{V_{max}} \cdot \frac{1}{[B]}$$

To determine K_i, plot the abscissal intercepts of the double reciprocal plots against [I]:

$$\frac{1}{V_{max(app)}} = \frac{1+{}^{[I]}/_{K_i}}{V_{max}}\left(1+\frac{K_{m(A)}}{[A]}\right) = \frac{1+{}^{[I]}/_{K_i}}{V_{max}} + \frac{K_{m(A)}\left(1+{}^{[I]}/_{K_i}\right)}{[A]V_{max}}$$

$$= \frac{1}{V_{max}} + \frac{[I]}{K_i V_{max}} + \frac{K_{m(A)}}{[A]V_{max}} + \frac{K_{m(A)}[I]}{[A]K_i V_{max}}$$

$$= \left(\frac{1}{V_{max}} + \frac{K_{m(A)}}{[A]V_{max}}\right) + \left(\frac{1}{K_i V_{max}} + \frac{K_{m(A)}}{[A]K_i V_{max}}\right).[I]$$

$$\frac{1}{V_{max(app)}} = \frac{[A]+K_{m(A)}}{[A]V_{max}} + \frac{[A]+K_{m(A)}}{[A]V_{max}K_i}.[I]$$

When $\frac{1}{V_{max(app)}} = 0$, [I] = $-K_i$.

Appendix 5. List of strains of bacteria.

E. coli JM105	*thi, rps* L (Str^r), *end* A, *sbc*B15, *hsd*R4, *sup*E, Δ(*lac-pro* AB), F'[*tra*D36, *pro* AB^+ ,*lac* I^q, *lac* ZΔM15]
E. coli JM109	*rec* A1, *sup*E44, *end*A1, *hsd*R17, *gyr*A96, *rel*A1, *thi*Δ(*lac-pro* AB), F'[*tra*D36, *pro* AB^+ ,*lac* I^q, *lac* ZΔM15]
E. coli MV1190	Δ(*lac-pro* AB), *thi, sup* E, Δ(*srl-rec* A)306::Tn10(tet^r) [F': *tra* D36, *pro* AB, *lac* I^qZΔM15]
E. coli CJ236	*dut ung thi rel* ApCJ105(Cm^r)
E. coli C600	*thr- leu- lacY- thi- supE tonA-*
E. coli JM109(de3)	*rec* A1, *de3, sup*E44, *end*A1, *hsd*R17, *gyr*A96, *rel*A1, *thi*Δ(*lac-pro* AB), F'[*tra*D36, *pro* AB^+ ,*lac* I^q, *lac* ZΔM15]
E. coli BL21(de3)	F^- *hsdS gal de3*
B. subtilis W23	
P. putida (ATCC33015)	

Appendix 6. Global sequence alignment.

```
E.coli          ---MLVWLAEHLVKYYSGFNVFSYLTFRAIVSLLTALFISLWMGPRMIAHLQKLSF-GQVVRNDG----------------------PESHFSKRGTPTMGGIMIL
B.subtilis      ----------------------MLEQVILFTILMGFLISVLLSPILIPFLRRLKF-GQSIREEG----------------------PKSHQKKSGTPTMGGVMII
C.griseus       ---------------MWAFPELPLPLLVNLFGSLLGFVATVTLIPAFRSHFIAARLCGQDLNKLS----------------------RQQIPESQGVICGAVFLII
H.influenz      ---MLVWLAEYLVRYETAFNAISYITVRANLALLTALFISLWIGPKVIKRLQILKF-GQEVRNDG----------------------PESHFAKKGTPTMGGVMIL
L.mexicana      ---MTLGLVESSRNAAFAVAAHAPVLGLILLGSIVAYVGTLRYIPNVARTLLDRNIFGIDINKSTEEQRQKFAAKRRAGQTEEKEFQKQAIPESLGILVGAMYLSV
M.leprae        -MQYVREMSSDLATFASGLLALFERSAGVPLRELALVGLTAAIITYFATGLVGVLANRLEAVAYPRE----------------------RDVHVTP--TPRMGGLAMY
P.aerugino      --------------------------MMIWMIACLVVLLFSFVATWGLRRYALATK-LMDVPNA----------------------RSSHSQP--TPRGGGVAIV
S.acidocal      ----------------------MLVSLLGILLSVIVGFVVTLISTKWVIGLCKKRGFTGKDINKLT----------------------KDDVPVLGGIGIVAGFVAG
S.aureus        --------------------------MVTLLLVAVTMIVSLTITPIVIAISKRLNL--VDKPNF----------------------RKVHTKP--ISVMGGTVIL
S.cerevisa      -MLRLFSLALITCLIYYSKNQGPSALVAAVGFGIAGYLATDMLIPRVGKSFIKIGLFGKDLSKPG----------------------RPVLPETIGAIPAAVYLFV
S.pombe         MIESCFNVGIWATGLALLMNQGQSPLLSNVGLSVLAYKATAMFIPRVGPSFIKRGFSGKDMNKVE----------------------KYVIPETMGAVSALVYFMC
Y.enteroco      --------------------------MPTFFFLLTIFFLLSVGLTYLLRLYALKNN-IIDTPNS----------------------RSSHVTP--TPRGGGVAIV

E.coli          TAIVISVLLWAYPS----------------------------NPYVWCVLVVLVGYGVIGFVDDYRKVVRKDTKGLIARWKYFWMSVIALGVAFALYLAG------
B.subtilis      LSIIVTTIVMTQKFSEI-------------------------SPEMVLLLFVTLGYGLLGFLDDYIKVVMKRNLGLTSKQKLIGQIIIAVVFYAVYHYY-------
C.griseus       LFCFIPFPFLNCFVEEQCK-----------------AFPHHEFVALIGALLAICCMIFLGFADDVLNLR--------WRHKLLLPTAASLPLLMVYFTN-------
H.influenz      FSIGVSTLLWANLA----------------------------NPYIWVCLFVLFGYGAIGFVDDFRKITRKNTDGLIARWKYFWMSVVALVAILWLYWLG------
L.mexicana      VVVLTVCLRFLGAAGEG--------------------LDNPYASLPGPLMTITVMLLLGFVDDVLDVK--------WRHKIILTALGSLPLIMTYDGSLSVLMPC
M.leprae        LGVLAAVFLASQLPALTRG------------------FVYSSGMPAVLVAGAVITGIGLIDDRWGLD---------ALTKFAGQITAASVLVTMGVA-------
P.aerugino      LVFLAALVWMLSAGSIS-----------------------GGWGGAMLGAGSGVALLGFLDDHGHIA--------ARWRLLGHFSAAIWILLWTGGF-------
S.acidocal      SFTFLLTSYNLSPGIEN-----------------------VVVSILLSSLIIGFLGLLDDIFNIS--------QATRAFLPIFASIPLILYSVGH-------
S.aureus        FSFLIGIWIGHPIET----------------------------EIKPLIIGAIIMYVLGLVDDIYDLK---------PYIKLAGQIAAALVVAFYGVT-------
S.cerevisa      MFIYIPFIFYKYMVITTSGGGHRDVSVVEDNGMNSNIFPHDKLSEYLSAILCLESTVLLGIADDLFDLR--------WRHKFFLPAIAAIPLLMVYYVD-------
S.pombe         MIIFIPVLFYKYLVPNHNPNLPSDGSVAE---VAKSQFPHDLLGAYLSALLSILSVSLLGILDDLFDIR--------WRHKFFLPAIAAIPLLVVYYVD-------
Y.enteroco      ISFLIGIILFYFLGYLP------------------------ILSVVGLIVSGGVIALVGFWDDHGHIA--------ARWRLLAHFSAAAFLLFCFGGF-------
                                                                .*  **
```

```
E.coli          --KDTPATQLVVPFFKD----------------------------------------VMPQLGLFYILLAYFVIVGT-GNAVNLTDGLDGLAIMPTVFVAGGFALV
B.subtilis      ----NFATDIRIPG-TD----------------------------------------LSFDLGWAYFILVLFMLVGG-SNAVNLTDGLDGLLSGTAAIAFGAFAIL
C.griseus       ----FGNTTIVVPKPFR-WILG-----------------------------------LHLDLGILYYVYMGLLAVFC-TNAINILAGINGLEAGQSLVISASIIVF
H.influenz      --HDTDATRLVIPFFKD----------------------------------------IMPQLGLFYIVLSYFVIVGT-GNAVNLTDGLDGLAIMPTALVAGAFALI
L.mexicana      AFGRFGLSTMNVMKEWRLGLAAPQGEPTTTFRATAPSTWFSFTVNHRSYVKVTESGAALIYLGPVYLVYLSMLCIFC-TNSINILAGVNGVEVGQSIVIAVASVVY
M.leprae        ------WSVLYIPLGGVG----------------------------------------TIVLDQTSSILLTLALTVSI-VNAINFVDGLDGLAAGLGLITAMAICIF
P.aerugino      ------PPLDVVGH--------------------------------------------AVDLGWLGHVLAVFYLVWV-LNLYNFMDGIDGIASVEAIGVCVGGALI
S.acidocal      -------TIISIPFLG------------------------------------------KVNFGILFYIIILPATLTITANAFNMLEGLNGLGAGMGLIMALALAYI
S.aureus        ------IDFISLPMG-------------------------------------------TTIHFGFLSIPITVIWIVAI-TNAINLIDGLDGLASGVSAIGLITIGFI
S.cerevisa      ----FGVTHVLIPGFMERWLKK-----------------------------------TSVDLGLWYYVYMASMAIFC-PNSINILAGVNGLEVGQCIVLAILALLN
S.pombe         ----YGVTYVSVPSIVRPFLKR-----------------------------------SLINLGFLYYFYMAAVAIFC-PNSINIIAGVNGVEAGQSLVLALVIACN
Y.enteroco      ------PVLNVSGF--------------------------------------------IIELGIFGSLFGLLFLVWM-LNLYNFMDGIDGLASAEAVTACIGMIAI
                                                                          .   *  *   *. *.

E.coli          AWATGNMNFASYLHIP------------YLRHAGELVIVCTAIVGAGLGFLWFNTYPAQVFMGDVGSLALGGALGIIAVLL------------------RQEFLLV
B.subtilis      AWNQS----------------------------QYDVAIFSVAVVGAVLGFLVFNRDPAKVFMGDTGSLALGGAIVTIAILT------------------KLEILLV
C.griseus       NLVELEGD--------------------YRDDHVFSLYFMIPFFFTTLGLLYHNWYPSQVFVGDTFCYFAGMTFAVVGILGH------------FSKTMLLFFIPQ
H.influenz      AWATGNVNFAEYLHIP------------YIKYSSEVVVFCTAIVGASLGFLWFNTYPAQVFMGDVGSLALGGALGVVAILV------------------RQEFLLV
L.mexicana      NLFQMRLDRQLTPDFSSLDAAAADARDMTSDHQLRALLLLGPFIGVSLALWRYNRYPARVFVGDSYTYFAGTVLAVSSITGV------------YSKTLLLFFAPQ
M.leprae        SVGLLRDHDG-------------------DVLFYPPAVISVVLAGSCLGFLPHNFHRAKIFMGDSGSMLVGLMLAAASTTAAGPISQNAYGTRDVFALLSPFLLVV
P.aerugino      YWLTG-----------------------HVAMVG---IPLLLACAVAGFLIWNFPPARIFMGDAGSGFLGMVIGALAIQAA------------W-TAPSLFWCWL
S.acidocal      GLKSG-----------------------GTSFYAGIVSIILASVLFGFLIFNFYPAKTFPGNIGTYFIGSVIGSIGISGY------------MYTALFFLYLPY
S.aureus        AILQAN--------------------------IFITMICCVLLGSLIGFLFYNFHPAKIFLGDSGALMIGFIIGFLSLLGF------------KNITIIALFFPI
S.cerevisa      DLLYFSMG-----PLA------------TRDSHRFSAVLIIPFLGVSLALWKWNRWPATVFVGDTYCYFAGMVFAVVGILGH------------FSKTMLLLFIPQ
S.pombe         DLFYVLSPK----NKD------------ALRAHLLSLYLVLPLIGVTAGLLKYNWWPSRVFVGDTFCYFAGMVMAVVGILGH------------FSKTLMLFFIPQ
Y.enteroco      YYISG-----------------------DHIELNSFLVLWLLACTVLGFLLWNFPPAKIFMGDAGSGFLGLMIGSLAISAG------------W-IDTRFFFCWL
                                            ...  *  *.  *.*.      *   .  .
```

```
E.coli          IMGGVFVVETLSVILQVGSFKLRGQ-------------------------RIFRMAPIHHHYELKGWPEP--RVIVRFWIISLMLVLIGLATLKVR---------
B.subtilis      IIGGVFVIETLSVILQVISFKTTGK-------------------------RIFKMSPLHHHYELVGWSEW--RVVVTFWAAGLLLAVLGIYIEVWL---------
C.griseus       VFNFLYSLPQLLHAIPCPRHRIPRLNPKTGKLEMSYSKFKTKN--LSFLGTFILKVAERLQLVTVHRGESEDGAFTECNNMTLINLLLKIFGPIHERNLTLLLLLL
H.influenz      IMGGVFVVEALSVILQVGSYKLRKQ-------------------------RIFRMAPIHHHFELKGWPEP--RVIIRFWIISLMLVLMGLVTLKLR---------
L.mexicana      VFNFLISLPQLFSIVPCPRHRVP-------------------------------TWNPRTNLLSNSHN------------YTILNVILYLFGDMHEAKLTWAILKC
M.leprae        AVMFVPMLDLLLAIVRRIRAGRSAFS----------------------------PDKMHLHHRLLQIGHSHRRVVLLIYLWVGIVAFGAASTIFFNPRNTGAVMLGA
P.aerugino      ILLGVFIVDATYTLIRRIARGEK-------------------------------FYEAHRSHAYQFASR-----RYASHLRVTLGVLAINTLWLLRWH---------
S.acidocal      VIEFVLKAKTRFKGVSFGKIDDQ-------------------------------GYLHWD---SKPNS---------------LTHIVMRIGKFKEYHIVLIIWGI
S.aureus        VILAVPFIDTLFAMIRRVKKGQHIM-----------------------------QADKSHLHHKLLALGYTHR--QTVLLIYSISILFSLSSIILYVSPPLGVVLMFV
S.cerevisa      IVNFIYSCPQLFKLVPCPRHRLPKFNEKDGLMYP--SRANLKEEPPKSIFKPILKLLYCLHLIDLEFDENN--EIISTSNMTLINLTLVWFGPMREDKLCNTILKL
S.pombe         IFNFALSVPQLFGLVECPRHRLPKLNVKTGLLENSYTEFSLNEHPLPKKTLLTISIFEKLRLIRVEYDPSTG-RPLRCTNFTIINFVLYHLGPMREDHLTICIMGL
Y.enteroco      ILLGLFIVDATWTLVRRVLGGFK-------------------------------VYEAHRSHGYQIASR-----RFKRHLPVTLSAIAINIIWLFPIALLAGLNIVN
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E.coli          ------------------------------------
B.subtilis      ------------------------------------
C.griseus       QILSSAVTFSIRYQLVRLFYDV--------------
H.influenz      ------------------------------------
L.mexicana      QVIACVLGFVVRYVLSAFLYDEVR------------
M.leprae        IVITGMATVIPLLRRRDNY-----------------
P.aerugino      ------------------------------------
S.acidocal      EILFAILAVVFQTVTITI------------------
S.aureus        LIIFSIELIVEFTGLIDNNYRPILNLISRKSSHKED
S.cerevisa      QFCIGILALLGRHAIGAIIFGHDNLWTVR-------
S.pombe         QLLTGIFGLIIRHFVAPLVYPEDNI-----------
Y.enteroco      PIIALIISYIPLLYIDYKLNAGVNND----------
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