UNIVERSITY OF SOUTHAMPTON

ANALYSIS OF GENES INVOLVED IN METHANOL OXIDATION IN *METHYLOBACTERIUM EXTORQUENS*

By

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A thesis submitted for the degree of
Master of Philosophy

Department of Biochemistry
September, 2001

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY

Master of Philosophy

ANALYSIS OF GENES INVOLVED IN METHANOL OXIDATION IN METHYLOBACTERIUM EXTORQUENS

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In methylotrophic bacteria such as Methylobacterium extorquens methanol is oxidised to formaldehyde by methanol dehydrogenase (MDH) located in the periplasm. From MDH the electrons are passed along an electron transport chain including 2 cytochromes (cytochrome c_L and cytochrome c_H) and finally to a terminal oxidase. The protons released from methanol oxidation form a proton motive force that can be utilised by ATP synthase to produce ATP for the energy requirements of the bacteria.

Methanol dehydrogenase has a $\alpha_2\beta_2$ configuration. The X-ray structure of this enzyme showed that the active site is found in the α-subunit and contains the prosthetic group pyrrolo-quinoline quinone (PQQ). PQQ is sandwiched between the 'ceiling' of the active site formed by a unique disulphide bridge between adjacent cysteines (Cys 103, Cys 104) and the 'floor' of the active site formed by tryptophan 243. The active site of MDH also contains a calcium atom co-ordinated to both PQQ and 2 protein groups (Glu 177, Asn 261). The mechanism by which MDH catalyses the oxidation of methanol is poorly understood but an active site base (Asp 303) has been implicated in the reaction mechanism.

There are at least 32 genes implicated in methanol oxidation. Most of these were identified in two species: Methylobacterium extorquens and Paracoccus denitrificans. Although the functions of some of these genes have been determined many have not.

This thesis describes work carried out to produce a site directed mutation in MDH changing aspartate 303 to a glutamate (D303E). Attempts to make mutations at other amino acids of MDH including active site residues (Arg331, Asn261, Glu177 and Trp243) and a residue implicated in the interaction of MDH with cytochrome c_L (Lys205) were unsuccessful probably due to the high GC content of the mxaF gene.

To determine the roles of several methanol oxidation genes found in the large operon (mxaFJGIR(S)ACKLDB) in M.extorquens characterisation of mutants strains was undertaken. This indicated that expression of the α and β subunit genes (mxaF and mxaI) is dependent upon each other. Mutations in mxaF, I, R, S and C resulted in strains that could not grow on methanol indicating that these genes have an essential role in methanol oxidation. Mutations in mxaD and in the intergenic region between mxaR and mxaS resulted in strains that could grow on methanol but the MDH activity was lower than wild-type MDH.

A double mutant MDH which lacks calcium from the active site ($mxaA^-$) and contains an active site base mutation (D303E) was produced with the aim of reconstituting this enzyme with barium to produce an enzyme with potential for studying the reaction mechanism. This enzyme could not be reconstituted with calcium or barium probably because of the altered structure of the active site.

Many of the site directed mutants of MDH have resulted in low levels of MDH expression and hence limiting characterisation. A study was carried out on the effects of growth conditions on the expression of MDH from mutant strains of M.extorquens. This work indicated that changing pH, the concentration of tetracycline, the carbon substrates and the concentration of formaldehyde could not increase the expression of MDH from these mutant strains.

CONTENTS

CHAPTER 3 - Site directed mutagenesis of methanol dehydrogenase in Methylobacterium extorquens

CHAPTER 4 - Analysis of several *mxa* genes by insertion of a kanamycin resistance gene

FIGURES

ABBREVIATIONS

ADH	alcohol dehydrogenase
Amp^r	ampicillin resistance
ATP	adenosine 5'- triphosphate
BSA	bovine serum albumen
CTP	cytosine 5'-triphosphate
dATP	deoxyadenosine 5'-triphosphate
DCPIP	2,6-dichlorophenolindophenol
dCTP	deoxycytidine 5'-triphosphate
ddATP	dideoxyadenosine 5'-triphosphate
ddCTP	dideoxycytosine 5'-triphosphate
ddGTP	dideoxyguanosine 5'-triphosphate
ddttp	dideoxythymidine 5'-triphosphate
DEAE	diethyaminoethyl
dGTP	deoxyguanosine 5'-triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleaside 5'-triphosphate
ds	double stranded
dTTP	deoxythymidine 5'-triphosphate
dUTP	deoxyuridine 5'-triphosphate
E.coli	*Escherichia coli*
EDTA	ethylene diamine tetraacetic acid
FPLC	Fast protein liquid chromatography
GDH	glucose dehydrogenase
GTP	guanosine 5'-triphosphate
HEPES	N-([2-hydroxyethyl]piperazine-N'-{2-ethanesuphonic acid])
HPLC	high performance liquid chromatography
LB	Luria-Bertani
LTQ	lysine tyrosyl quinone
M.extorquens	*Methylobacterium extorquens*
MDH	methanol dehydrogenase

NAD	nicotinamide adenine dinucleotide
P.denitrificans	*Paracoccus denitrificans*
PCR	polymerase chain reaction
PES	phenazine ethosulphate
PQQ	pyrroloquinoline quinone
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
TBS	Tris buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
Tet^r	tetracycline resistance
TPQ	6-hydroxydopa quinone
Tris	Tris (hydroxymethyl)aminoethane
TTP	thymidine 5'-triphosphate
TTQ	tryptophan tryptophylquinone
UV-VIS	ultra violet - visible
v/v	volume:volume ratio
w/v	weight:volume ratio

CHAPTER 1

INTRODUCTION

1.1 Introduction

Much of our knowledge of the composition of the system for methanol oxidation and associated electron transport chains operating in methylotrophs is based on work with *Methylobacterium extorquens* and *Paracoccus denitrificans*. The major proteins involved in methanol oxidation are located in the periplasm. The research described in this thesis is primarily concerned with phenotypic characterisation of mutant strains of *Methylobacterium extorquens* AM1 (previously *Pseudomonas* AM1).

This chapter reviews relevant aspects of methylotrophic electron transport. A definition of methylotrophy will be given, followed by an introduction to energy transduction during methanol oxidation. The focus will be on *Methylobacterium extorquens* and especially on the many genes that are thought to be involved in methanol oxidation.

1.2 Methylotrophs

Methylotrophs are defined as "able to grow at the expense of reduced carbon compounds which contain one or more carbon atoms but containing no carbon-carbon bonds" (Colby & Zatman, 1972). A wide range of substrates are utilised including methanol, methane, formate, formaldehyde and methylated amines. In nature methanol arises by the hydrolysis of methyl esters and ethers in plant lignin and pectins. It is for this reason that methylotrophs are found on leaf surfaces and in soil. Some methylotrophs are obligate whilst others, including *Methylobacterium extorquens,* are facultative and can also utilise other compounds such as pyruvate, succinate or fructose. Most methylotrophs are aerobes although exceptions include

Paracoccus denitrificans and *Hyphomicrobium X* which use nitrate as the terminal electron acceptor.

1.3 Carbon assimilation in methylotrophic bacteria

There are three major carbon assimilation pathways in methylotrophic bacteria differing in the oxidation level of the carbon units when assimilated. The ribulose biphosphate (RuBP) pathway for example in *Paracoccus denitrificans* utilises ribulose 5- phosphate kinase and ribulose 1,5- bisphosphate carboxylase to fix CO_2 as 3-phosphoglycerate and regenerates ribulose 1,5-bisphosphate to form glyceraldehyde phosphate.

The ribulose monophosphate (RuMP) pathway, for example in *Methylophilus methylotrophus,* fixes formaldehyde in a three-step process including fixation, cleavage and rearrangement. Four possible variants exist as there are two cleavage enzymes and two possible rearrangements. This pathway is more common in methylotrophs than the RuBP pathway.

The serine pathway was first described by Large in 1961 in *Pseudomonas AM1* (now *Methylobacterium extorquens AM1*) (Large *et al.*, 1961) (Fig.1.1). This pathway fixes both CO_2 and formaldehyde in approximately equal amounts (Fig.1.2). The pathway involves the condensation of glycine and methylene tetrahydrofolate by serine transhydroxymethylase. Two variants occur in methylotrophs; in the ICL^+ pathway isocitrate lyase is involved in a known route of oxidation of acetyl coA to glyoxylate. This pathway also has a measurable malate thiokinase activity. In the ICL^- pathway, for example in *Methylobacterium extorquens,* the pathway for acetyl coA oxidation is unknown.

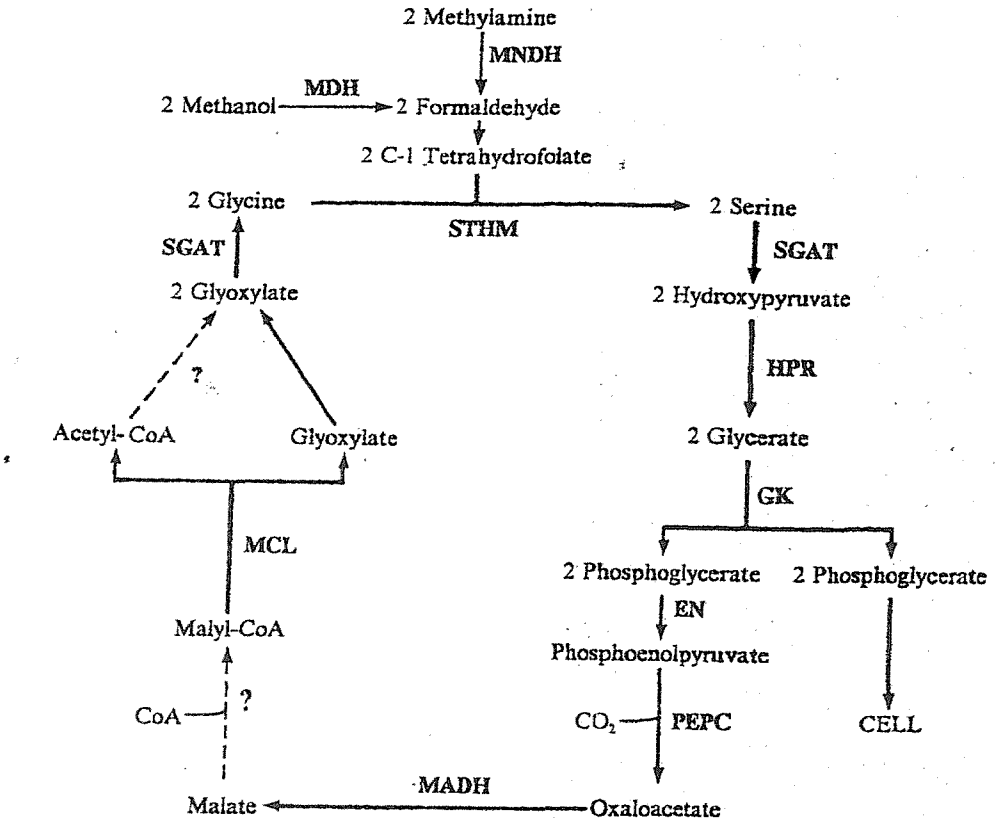

Fig. 1.1 The serine pathway for formaldehyde assimilation (icl^- variant)
This isocitrate lyase negative (icl^-) variant of the serine pathway is found in *Methylobacterium extorquens* AM1. The dashed lines indicate an unknown pathway. The icl^+ variant differs from the icl^- variant by having a measurable malate thiokinase and a known route for the oxidation of acetyl-CoA to glyoxylate utilising isocitrate lyase. MDH, methanol dehydrogenase; MNDH, methylamine dehydrogenase; STHM, serine transhydroxymethylase; SGAT, serine-glyoxylate aminotransferase; HPR, hydroxypyruvate reductase; GK, glycerate kinase; EN, enolase; PEPC, phosphenolpyruvate carboxylase; MADH, malate dehydrogenase; MCL, malyl-CoA lyase (Figure adapted from Stone and Goodwin, 1989).

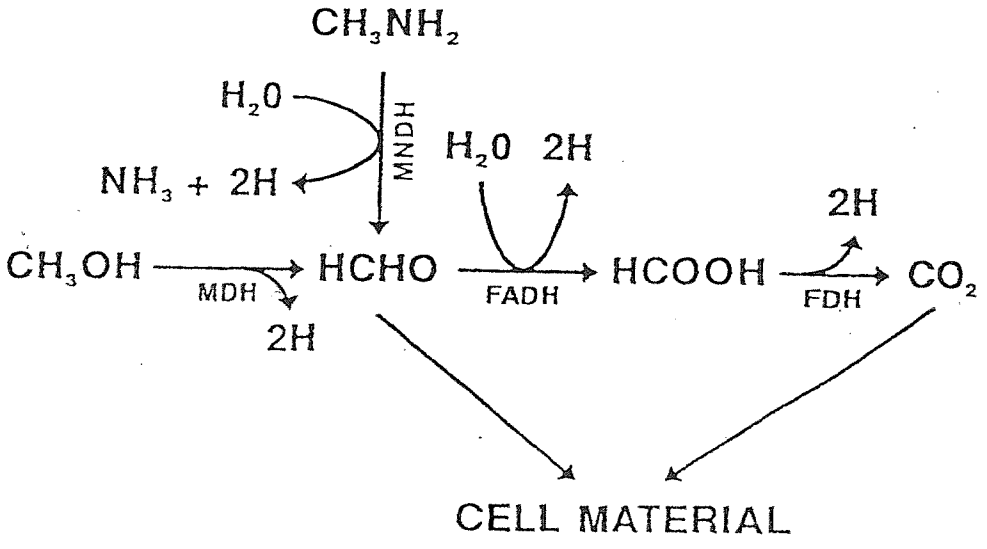

Fig. 1.2 The oxidation of methanol and methylamine in methylotrophs
The oxidation of methanol and methylamine produces formaldehyde and carbon dioxide which in *Methylobacterium extorquens* AM1 are required for carbon assimilation via the serine pathway (see Fig 1.1). The protons produced in the oxidation of these substrates allows ATP to be formed via an ATPase (see Fig 1.3). MDH, methanol dehydrogenase; MNDH, methylamine dehydrogenase; FADH, formaldehyde dehydrogenase; FDH, formate dehydrogenase.

1.4 *Methylobacterium extorquens* AM1

Methylobacterium extorquens AM1 was originally found as an airborne contaminant of liquid growth medium. This bacterium was previously called *Pseudomonas* AM1 (Green and Bousfield, 1982). It is a facultative methylotroph that can utilise methanol, methylamine, succinate and pyruvate but not methane. It is pink in colour, Gram negative, non sporing, has a 30°C optimal temperature for growth, and is rod shaped with a single polar flagellum (Anthony, 1982). It uses the serine pathway for carbon assimilation (Stone and Goodwin, 1989)(Fig 1.1).

1.5 Electron transport chains in methanol utilising bacteria

The components of electron transport chains used in methanol oxidation are found in the periplasm of bacteria with exception of the terminal oxidases which are located in the periplasmic membrane (Fig.1.3). When facultative methylotrophs are switched from multi-carbon compounds to methanol, up to 90% of electron transport switches to the periplasm.

The initial enzyme in methanol oxidation is methanol dehydrogenase (EC1.1.99.8). Methanol dehydrogenase (MDH) is an NAD^+ independent enzyme and is composed of two alpha and two beta subunits with a total mass of 149 kDa. MDH oxidises methanol to formaldehyde which is subsequently either assimilated to cell material or further oxidised to carbon dioxide for energy. The electrons from MDH are sequentially passed to a novel cytochrome c (cytochrome c_L) and then to a typical cytochrome (cytochrome c_H). From cytochrome c $_{H,}$ the electrons are passed to a terminal oxidase in the periplasmic membrane, forming water from protons and oxygen. Some methylotrophs, such as *Acetobacter methanolicus,* use cytochrome *co*, whereas others such as *Methylobacterium extorquens* use cytochrome aa_3 as the terminal oxidase (Fukumori *et al.*, 1985). The type of cytochrome oxidase used is sometimes determined by growth conditions, for

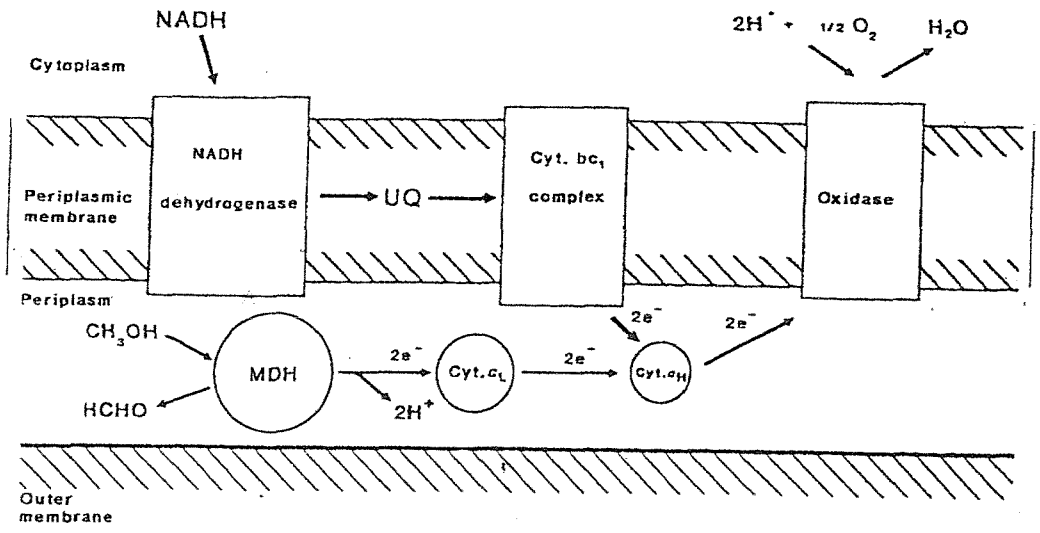

Fig. 1.3 The electron transport chain for methanol oxidation
The proteins involved in methanol oxidation are found in the periplasm except for the terminal oxidase which is located in the periplasmic membrane. MDH, methanol dehydrogenase; Cyt.c_L, cytochrome c_L; Cyt.c_H, cytochrome c_H. (Figure taken from Anthony, 1992a).

example, under carbon limitation *Methylophilus* uses cytochrome aa_3 but in carbon excess uses a cytochrome *co* (Cross and Anthony, 1980).

The passage of electrons coupled to the release of protons in the periplasm creates a proton motive force which when dissipated, via an ATPase, forms ATP in the cytoplasm. Such an electron transport chain has been confirmed *in vitro* using soluble dehydrogenases, cytochromes and oxidases from *Methylophilus methylotrophus* (Fround *et al.*, 1984) and *Methylobacterium extorquens* (Mukai *et al.*, 1990)

As the methanol oxidation system bypasses NADH dehydrogenase and the cytochrome bc_1 complex, fewer protons are pumped into the periplasm than in utilisation of most other substrates (Anthony, 1988). As a result no more than one mole of ATP is produced per mole of methanol oxidised and in this way it is analogous to the oxidation of inorganic substrates. This occurs despite the redox potential of the methanol/formaldehyde couple indicating a potential yield of two moles of ATP per mole of methanol. As methylotrophic bacteria are rarely ATP limited (they are limited by NADH), there would be little benefit of increasing the P/O ratio to two (Anthony, 1992).

1.6 Methanol dehydrogenase

Methanol dehydrogenase is found in the periplasm of methylotrophic bacteria at a concentration of 0.5 mM and accounts for 10-15% of the total soluble protein (Beardmore-Gray *et al.*, 1983; Anthony, 1986). MDH is globular in shape and could form a monolayer in the periplasm one molecule deep. The dimensions of MDH are 55Å x 55Å x 100Å and interestingly there is little room for movement in the periplasm as the periplasmic space is only 70Å in width. MDH was first purified and characterised from *Methylobacterium extorquens* subsequently the enzyme has been studied in other methylotrophs indicating very similar properties.

MDH is an NAD^+ independent enzyme but has a novel prosthetic group pyrrolo-quinoline quinone (PQQ) which gives the enzyme a characteristic absorption spectrum peak at 345 nm and 400 nm (Fig.1.4). Most MDH enzymes are basic and stable down to a pH of four. Most MDH enzymes have an isoelectric point around 8 an exception being MDH from *Paracoccus denitrificans* which has an unusually acidic enzyme with an isoelectric point of 3.8. There are several ways of assaying MDH including artificial electron acceptors such as phenazine ethosulphate (PES) which when re-oxidised by 2,6-dichlorophenol-indophenol (DCPIP) can be followed at 600 nm (Bamforth and Quayle, 1978). Other assays include a cytochrome linked assay or measuring oxygen consumption with an oxygen electrode (Beardmore-Gray *et al.*, 1983).

1.7 Pyrrolo-quinoline quinone

In the 1960s a novel prosthetic group was discovered in methanol dehydrogenase and glucose dehydrogenase and was originally thought based on fluorescence properties to be a pteridine. In the 1970s the prosthetic group was shown to be a quinone structure with two nitrogen atoms. X ray analysis of crystalline acetonyl derivatives indicated that the structure was an orthoquinone derivative of a fused quinoline and pyrrole rings (Salisbury *et al.*, 1979). The structure was later identified as 2,7,9 tricarboxypyrrolo-quinoline quinone or PQQ as it is more commonly known.

PQQ is reddish brown in colour, highly polar, acidic and has a low molecular weight. It has a characteristic absorption spectrum between 300 and 420 nm but is sensitive to the environment (Anthony, 1986). The quantity of PQQ can be determined by the absorption at 249 nm. PQQ also has a characteristic green

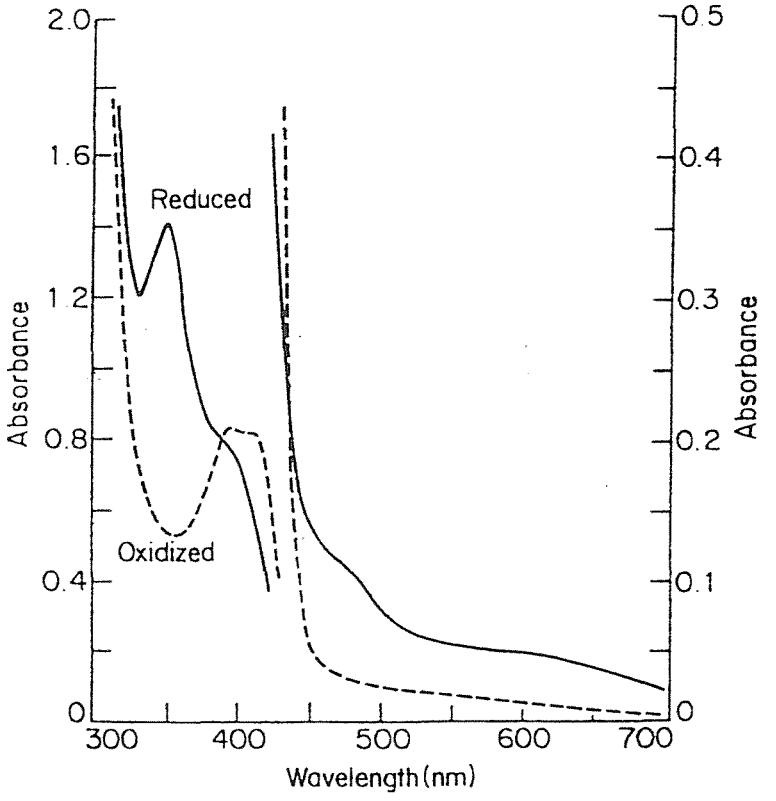

Fig. 1.4 The absorption spectra of methanol dehydrogenase
The dashed line indicates the oxidised form of the enzyme (PQQ). The solid line indicates the reduced form of the enzyme ($PQQH_2$). The semiquinone form (PQQH*) has a spectrum like the oxidised form. (Figure taken from Duine *et al.*, 1981).

fluorescence at low pH with an excitation maximum at 255 nm and a maximum emmision at 460 nm. PQQ is formed from a tyrosine and a glutamate residue as determined by using labelled substrates (Fig. 1.5) (Houck *et al.*, 1988). A model for the synthesis of PQQ is shown in Figure1.6. The genes and gene regulation involved in PQQ synthesis are discussed in Section 1.20 and 1.21b.

PQQ remains non covalently bound to the enzyme throughout the reaction cycle and therefore is not a coenzyme (Anthony, 1993). It cannot be removed from MDH without denaturation of the enzyme. Unlike other PQQ containing enzymes, glucose dehydrogenase and alcohol dehydrogenase, PQQ from MDH cannot be dissociated and subsequently reconstituted (Anthony and Zatman, 1967).

As well as a prosthetic group in enzymes, PQQ has been shown to have various other physiological roles. For example in *Escherichia coli* PQQ has been shown to be a chemoattractant (de Jonge *et al.*, 1996). Many bacteria under certain conditions excrete PQQ to act as a growth signal (Ameyama *et al.*, 1988). Interestingly some bacteria, including *E.coli*, make PQQ requiring apoenzymes despite not being able to make PQQ. These bacteria take PQQ up from the media and save energy as PQQ is energetically expensive to produce (Groen *et al.*, 1996); it would also allow extra substrates to be used.

1.8a The c type cytochromes in methylotrophs

All methylotrophs have at least two soluble cytochromes in the periplasm (Anthony, 1986). There are two main cytochromes found at high concentration in *Methylobacterium extorquens*, cytochrome c_L and cytochrome c_H. The absorbance spectra of the two cytochromes are very similar but they can be distinguished by their isoelectric points. Whereas cytochrome c_H has a PI of 8.5, cytochrome c_L has a very low PI of 3.5. Under certain growth conditions or in mutants lacking wild-type cytochromes, other c type cytochromes can be detected (Day *et al.*,

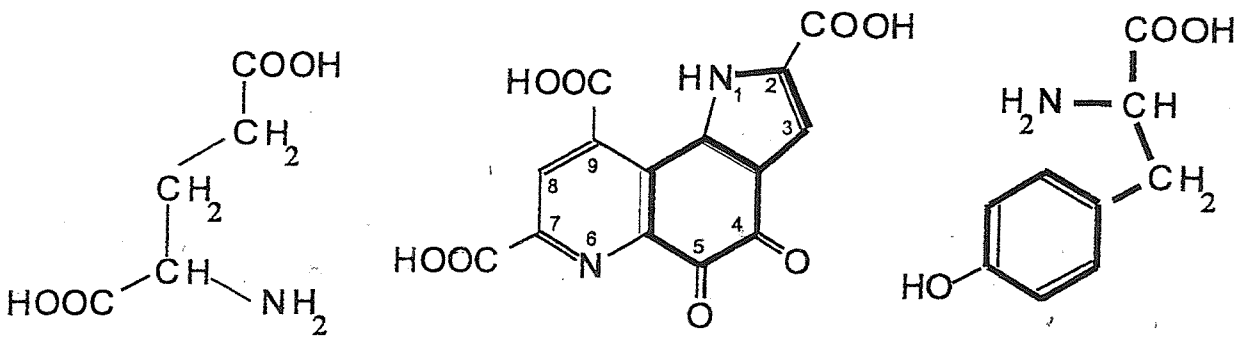

Fig. 1.5 The structure of pyrrolo-quinoline quinone and the amino acids from which the carbon backbone is derived. (Taken from Goodwin and Anthony, 1998)

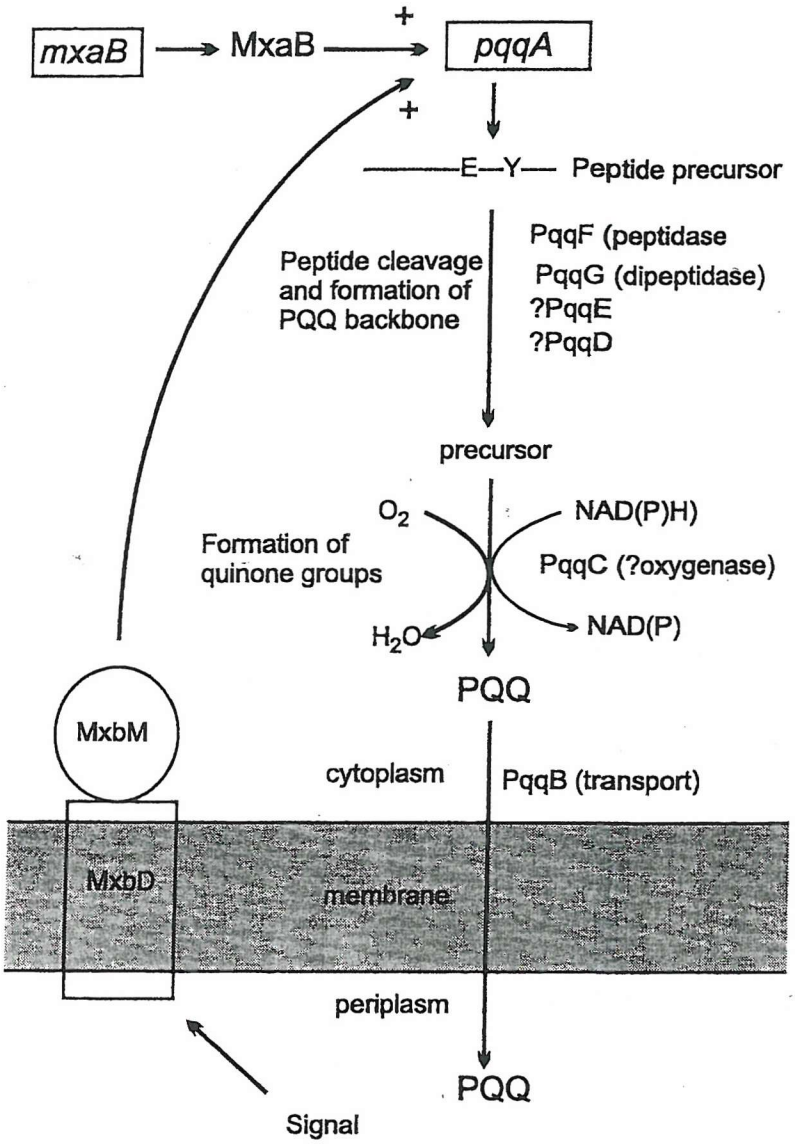

Fig. 1.6 A model for PQQ synthesis in *Methylobacterium extorquens* including proposed gene functions.

A signal, which could be formaldehyde, is detected by *mxbD* which in turn stimulates *mxbM* to act on the *pqqA* gene promoter. The precursor of PQQ is processed in the cytoplasm forming PQQ which is then transported, by the product of the *pqqB* gene product, into the periplasm. The product of the *mxaB* gene also stimulates *pqqA* gene transcription (Figure taken from Goodwin and Anthony 1998)

1990). As well as the soluble cytochromes there are also membrane bound cytochrome c molecules. These will include those in the bc_1 complex and the o-type oxidases of some methylotrophs (Anthony, 1992).

1.8b Cytochrome c_L

Cytochrome c_L is only found in methylotrophs (Anthony, 1988). Mutants of the gene encoding cytochrome c_L (*mxaG*), fail to grow on methanol but do grow on methylamine (Nunn and Lidstrom, 1986). Experiments using MDH, cytochrome c_L and horse heart cytochrome c as a second electron acceptor, indicated that the reduction of cytochrome c_L was methanol dependent and happened concurrently with formaldehyde production (Beardmore-Gray *et al.*, 1983). Cytochrome c_L is larger than a typical class 1 cytochrome c being 17-21kDa. It has a low pI of 3.5 to 4.5 (O'Keefe and Anthony., 1980a).

Cytochrome c_L lacks some lysine residues that are found in cytochrome c_H which are important for binding to the terminal oxidase (see Section 1.8c) (Anthony, 1988).

Cytochrome c_L has five alpha helices with three of them enveloping the haem group. It is this region around the haem group that shows the only homology to normal cytochromes. The haem in cytochrome c_L is covalently bound to a cysteine (Nunn and Anthony, 1988; Anthony, 1989) with the fifth and sixth ligands being a histidine and the sulphur atom of a methionine respectively giving the cytochrome a normal absorption spectrum (Pettigrew and Moore, 1987; Moore and Pettigrew, 1990). An interesting feature of cytochrome c_L is when the pH is raised, the iron of the heam shows autoreduction with a pK value of 10 and a mechanism has been proposed (Anthony, 1992).

1.8c The interaction of MDH and cytochrome c_L

An interaction between MDH and cytochrome c_L is required as the first step in the passage of electrons from methanol to the terminal oxidase. Electrostatic interactions have long been suggested as having a role in the interaction process as 10 mM salt causes 50% inhibition of electron transfer (Chan and Anthony, 1991; Cox *et al.*, 1992).

Much focus of the interaction process has been on 15 lysine residues in the beta subunit. They form a well-defined patch of positive charge on one side of an alpha helix (Nunn *et al.*, 1989; Anthony 1990). However it was subsequently shown by modification experiments that the beta subunit was not involved. Instead it is now thought that lysines on the alpha subunit are important for the docking process (Chan and Anthony, 1991; Cox *et al.*, 1992). Modification of these residues to a neutral charge inhibited the passage of electrons to cytochrome c_L but not to PES. When the same residues were modified to retain the net positive charge, the electron flow was virtually unaffected. When modification of cytochrome c_L lysine residues was carried out, little effect was seen, but when modification of carboxyl groups was carried out, the activity was lost. Interestingly the docking process does not appear to by stopped by 50 μm EDTA but it does stop the electron transfer. It has been proposed that despite being docked together the two proteins have to undergo a second form of interaction to allow electron transfer but which is inhibited by the binding of EDTA to the lysines (Dales, 1995).

Experiments with *Paracoccus denitrificans* (Harris and Davidson, 1993) indicated that a hydrophobic region on MDH is important for the docking of cytochrome c_L with MDH. This has been backed up by deducing the position of this region by x-ray analysis to be in suitable region to influence the interaction . It is now thought that a hydrophobic interaction is not important for the initial docking event, but is important for a second interaction allowing electron transfer to occur.

Cross linking studies have confirmed that MDH interacts with cytochrome c_L separates and then interacts with cytochrome c_H rather than forming a three-protein complex (Chan and Anthony, 1991; Cox *et al.*, 1992).

1.8d Cytochrome c_H

Cytochrome c_H is a typical class 1 c type cytochrome as seen for example in mitochondria (O'Keefe *et al.*, 1980). It is small and basic and its role is to transfer electrons between cytochrome c_L and the terminal oxidase. It also functions as the electron carrier between the cytochrome bc1 complex and the terminal oxidase (Anthony 1986, 1992). The x-ray struture of cytochrome c_H has recently been solved and is shown in Figure 1.7. The x-ray structure has shown that a water residue suggested as part of the electron transfer pathway is absent and a tryptophan residue replaces an invariant tyrosine in other cytochrome c molecules.

1.9 The substrates of methanol dehydrogenase and M protein

Methanol dehydrogenases, with the exception of the enzyme from *Rhodopseudomonas acidophila,* only oxidise primary alcohols. It is the steric configuration, rather than the substituent group on the substrate, that determines if oxidation takes place. Examples of primary alcohols that are not oxidised due to steric hindrance include glycerol, 1,2 propandiol and methylpropanol. Substrates that are oxidised include ethanol, allyl alcohol, crotyl alcohol and cinnamyl alcohol but the rate of oxidation is approximately 30% of the rate of methanol. Generally speaking, as the size of the substrate increases, the affinity for the substrate decreases. The km for methanol is approximately 10 to 20 μM (Anthony, 1986; Anthony, 1988).

Interestingly when MDH is assayed in the absence of an added substrate, a rate is still recorded. Although it was originally thought that some molecules of

methanol may be bound to the enzyme, it was subsequently shown that indeed two molecules of methanol are present but there are also 90 unidentified substrate molecules present on the enzyme (Ghosh and Quayle 1981).

In *M.extorquens, M.methylotrophus* and *P.denitrificans,* a protein involved in regulation of MDH activity called M protein has been described. M protein is thought to be a soluble trimeric protein with a subunit size of 45 kDa. Little is known about the protein as research has been hampered by small 3 kDa peptides that seem to mimic the action of M protein. One of the main actions of M protein is to decrease the affinity of formaldehyde for MDH (Long and Anthony, 1991). This is useful, as the oxidation of formaldehyde is energetically very wasteful because it is needed for carbon assimilation via the serine pathway (Figure 1.1). In *M.extorquens,* where the affinity of MDH for methanol and formaldehyde is the same, the presence of M protein decreases the affinity of formaldehyde for MDH by up to 64 times and the V_{max} is halved. This has the effect of greatly increasing the affinity of MDH for methanol and other substrates such as 1,2 propandiol (Page *et al.*, 1986).

1.10 Activators of methanol dehydrogenase

Aerobically prepared methanol dehydrogenase assayed at pH 9 in the dye linked assay has an absolute requirement for ammonium or methylamine salts. At low pH a higher concentration of salt is required indicating that the free base is the active species (Anthony and Zatman, 1964). Esters of glycine and beta alanine can also act as activators. Lysine, long chain alkyamines, diamines or triamines do not act as an activator (Anthony, 1986). It has been shown that ammonia is not essential for PQQ reduction but is essential for reoxidation although the exact role is uncertain. Interestingly, anaerobically prepared crude extract from *Hyphobacterium X* does not have a

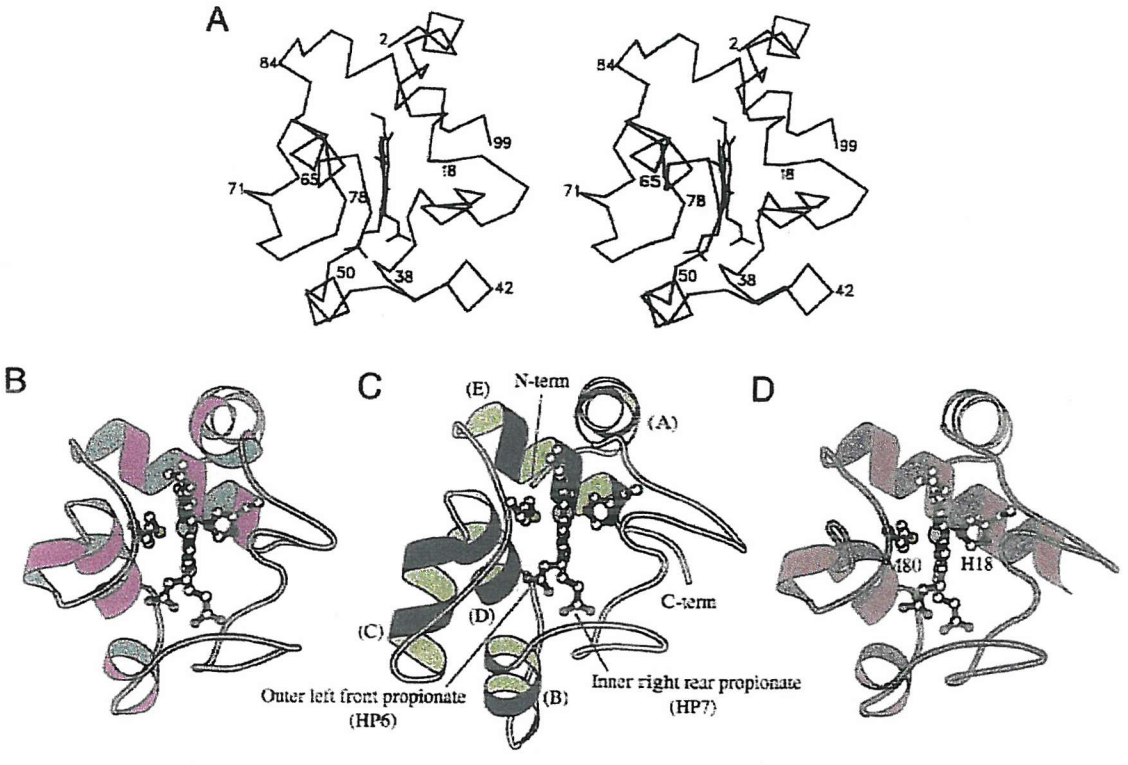

Fig. 1.7 The overall folds of cytochrome c_H, cytochrome c and cytochrome c_2. A: Stereo view of the C_α trace of cytochrome c_H . B: Cytochrome c_H. C: Cytochrome c_2 from *R. rubrum*. D: Tuna cytochrome c. The coloured structures are presented in the same view as that presented in the stereo view of cytochrome c_H. (Figure taken from Read *et al.*, 1999)

requirement for ammonia but on exposure to the air it becomes dependent. It is possible that *in vivo* another compound acts as an activator but is destroyed by oxygen during the aerobic preparation of the enzyme.

1.11 Inhibitors of methanol dehydrogenase

Phosphate and other salts have been shown to inhibit the interaction of MDH and cytochrome c_L by shielding the charges on the proteins. In *M.methylotrophus* EDTA was shown to inhibit electron transfer by affecting the interaction of MDH and cytochrome c_L (Beardmoore-Gray and Anthony, 1984) but it is now thought that it stops electron transfer rather than interaction of the two proteins (Dales and Anthony, 1995). EGTA is a very strong reversible inhibitor of electron transfer but inhibition can be relieved after gel filtration in a calcium free buffer (Chan and Anthony, 1992a). Phenazine methyl sulphate and phenazine ethyl sulphate irreversibly inhibit MDH in seconds but it can be protected from occurring by initially adding methanol or cyanide. Cyclopropanol irreversibly inhibits MDH by forming a covalent adduct with the C5 carbonyl group of PQQ (Mincey *et al.*, 1981).

1.12a The general structure of methanol dehydrogenase

MDH was originally thought to be a homodimer totalling 112-158 kDa. However a previously considered contaminant was shown, after separation from the large subunit by SDS gel filtration, to have an N terminal sequence identical to a gene (*mxaI*) in a large methanol oxidation operon. The *mxaI* gene product is a 74 amino acid polypeptide of 8.5 kDa and is now known to be the beta subunit. The alpha subunit, encoded by the *mxaF* gene, is 599 amino acids in length and is 66kd. Two of each of the subunits come together to form a tetramer of 149 kd (Figure 1.8).

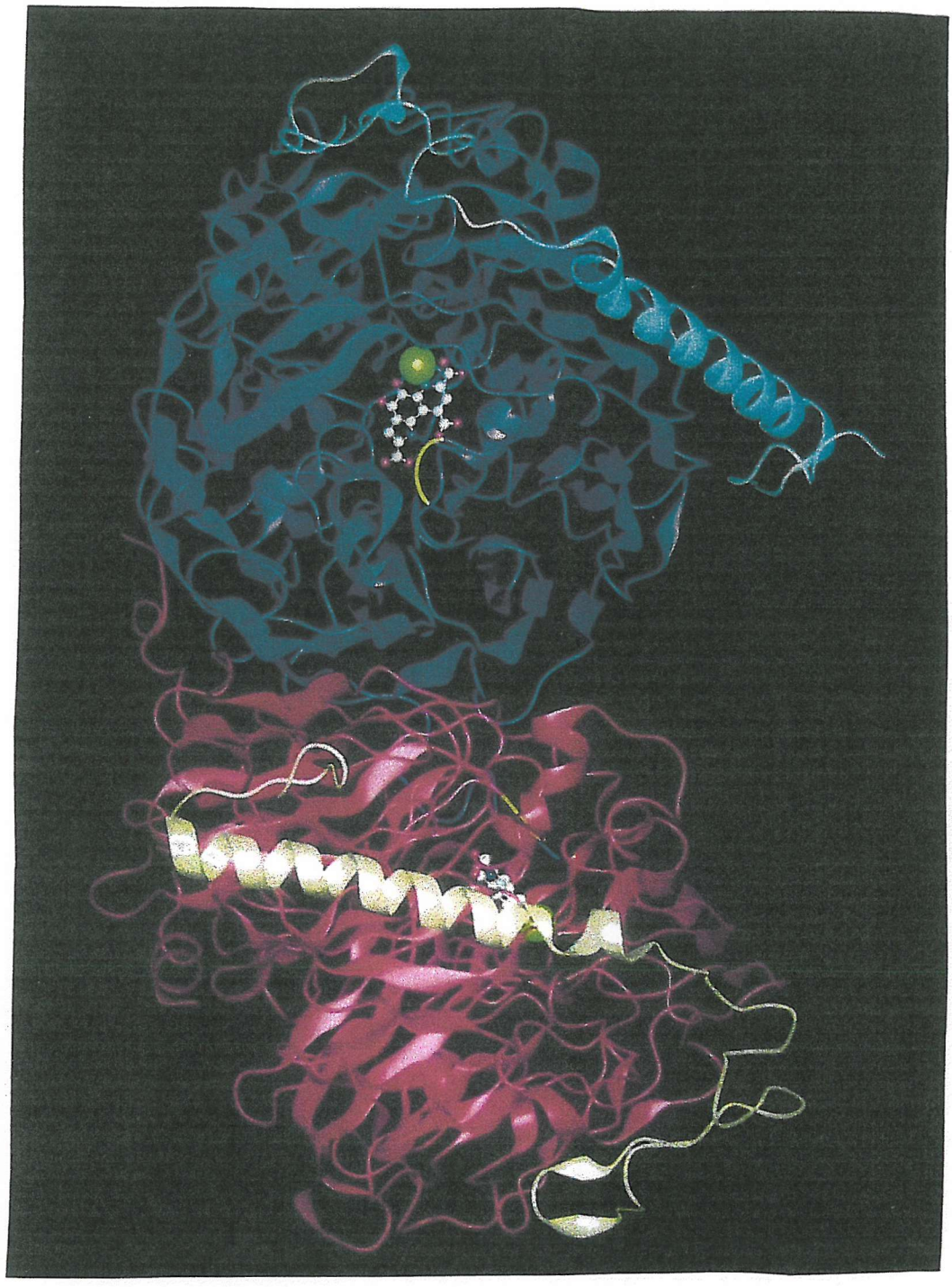

Fig. 1.8 Ribbon diagram of the methanol dehydrogenase tetramer from *Methylobacterium extorquens*. The alpha subunits are shown in red and dark blue. The "J" shaped beta subunits are shown in light blue and yellow. PQQ is shown in white and the calcium ions in green. (Taken from Goodwin and Anthony, 1995)

(Nunn and Anthony, 1988; Nunn *et al.*, 1989; Anderson *et al.*, 1990; Ghosh *et al.*, 1994). Each alpha subunit has been shown to contain one molecule of PQQ and one calcium atom (Adachi *et al.*, 1990; Richardson and Anthony, 1992). The alpha and beta subunits cannot be separated without denaturation and loss of the prosthetic group. Denaturation cannot be achieved with high salt concentrations but can be achieved by using 2% SDS, 8M urea or a pH of 12. Such findings indicate that PQQ is tightly bound and that some hydrophobic interactions exist between the subunits.

1.12b X-ray structure of methanol dehydrogenase

The x-ray structure of methanol dehydrogenase has been solved for enzymes from various strains including *Methylophilus methylotrophus* (White *et al.*, 1993) *Methylobacterium extorquens* (Ghosh *et al.*, 1995), *Methylophilus methylotrophus* W3A1 (Xia *et al.*, 1996) and *Paracoccus denitrificans* (Chen *et al.*, 1992). They show remarkable similarity, with a tetrameric structure where the beta subunits lie on the surface of the alpha subunits making hydrophobic and ionic interactions along its length (Fig. 1.9). The two beta subunits do not contact each other but the two alpha subunits contact each other over a region of 40 amino acids (Ghosh *et al.*, 1995).

The alpha subunit is an eight bladed superbarrel structure and is described as a propeller motif (Fig. 1.10). Each propeller blade or "W" motif is made up of four antiparallel beta strands named A to D with the D strands on the outside of the structure. The eight blades are arranged around a pseudo eight fold axis of symmetry (Anthony *et al.*, 1994; Ghosh *et al.*, 1995). This propeller motif with twisted beta sheets allows a particularly compact structure to be adopted. Studies by Murzin predicted that such an eight bladed motif would not occur (Murzin, 1992). Although several seven bladed superbarrels have been seen for example in

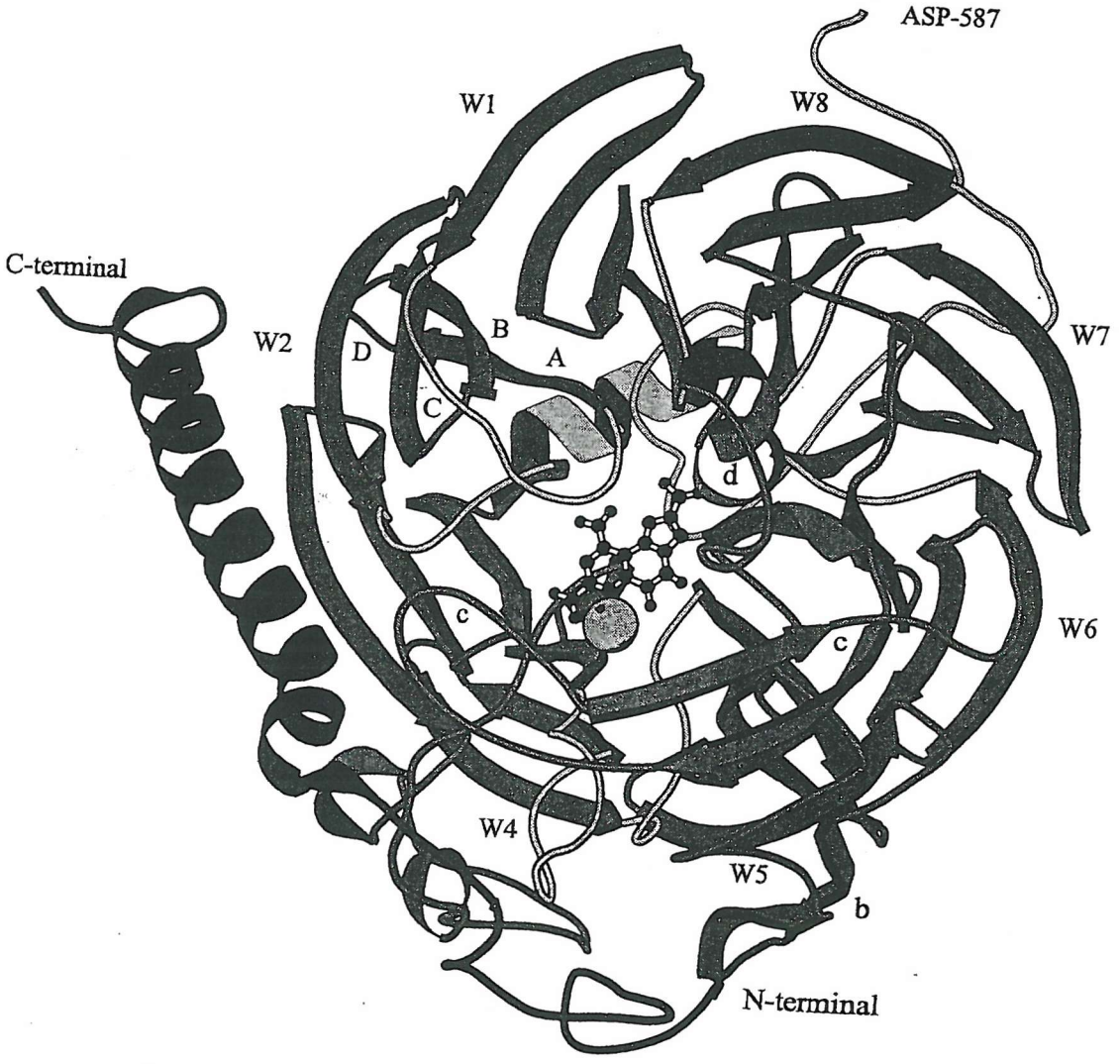

Fig. 1.9 A drawing of one half of the MDH tetramer based on the X-ray structure.

This diagram is looking down the pseudo 8-fold axis and simplified to show the 'W' motifs of the alpha chain and the long apha helix of the beta subunit. The centre of the alpha subunit shows the calcium atom represented as a sphere and PQQ (Figure taken from Goodwin and Anthony 1998 based on the structure in Ghosh *et al.*, 1995).

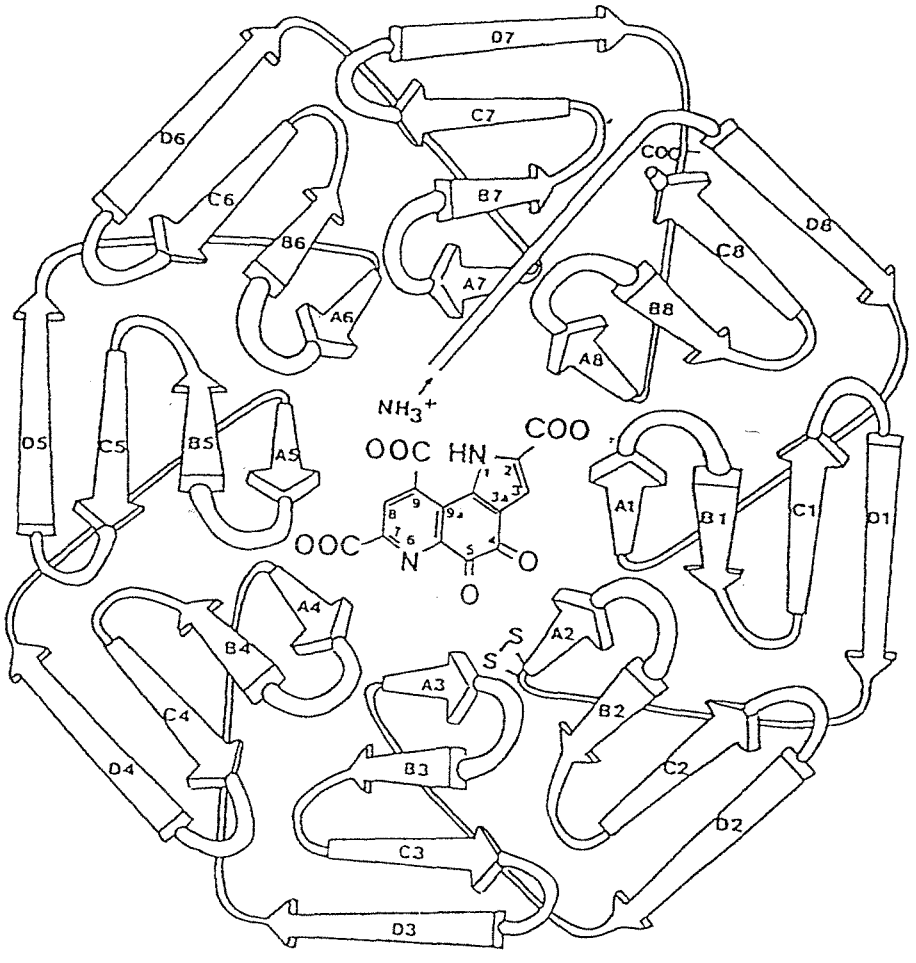

Fig. 1.10 Schematic diagram of the alpha subunit of methanol dehydrogenase. The alpha subunit is made up of eight so-called propeller blades (1-8) each consisting of four antiparallel beta strands (A-D) indicated by arrows. The A strands are found the centre of the structure and the D strands on the outside. The final propeller structure has pseudo eight fold symmetry. There are many loops between the beta strands and at the N and C terminals that have been omitted for clarity. (Figure taken from Anthony *et* al., 1994).

methylamine dehydrogenase it was considered that the extra blade would not be accommodated.

That an eight bladed structure is found is due to some special packing arrangements and especially by a 11-residue consensus sequence called the tryptophan docking motif (TDM) (Anthony *et al.*, 1994; Ghosh *et al* 1995). This motif is located around the C and D regions of each blade with exception of the eighth blade (Figure 1.11). The most important interaction is that from the peptide bond between residues six and seven of the motif and the indole ring of a tryptophan at position 11 of the motif in the next blade. Other interactions include a hydrogen bond between the main chain carbonyl of residue four to the tryptophan at position 11. The motif is held in place by interactions between the residue at position one and the tryptophan with exception of blades one and seven. In the eighth blade the C/D region is made up of the N and C terminal regions.

The TDM has been described as a stabilising girdle for the superbarrel and both glucose and alcohol dehydrogenases have similar arrangements. Other interesting points include the particularly hydrophilic A strands and the presence of glycine in five out of eight points of contact between the A strands also allowing tight packing. A long loop between strands B and C on blade six forms an antiparallel beta sheets which lies on top of the superbarrel (Fig. 1.12).

The superbarrel structure is also seen in many other proteins (Bork and Doolittle, 1994) (Fig. 1.13, Fig.1.14). Like MDH, nitrite reductase has eight blades (Fulop, *et al.*, 1995). Galactose oxidase, methylamine dehydrogenase and the beta subunit of transducin have seven blades (Ito *et al.*, 1994; Vellieux *et al.*, 1989; Sondek *et al* ., 1996). Viral neuraminidase has six blades (Varghese *et al.*, 1982) and both haemopexin and sinovial collagenase have four blades (Faber *et al.*, 1995; Li *et al.*, 1995).

Position / Motif	1	2	3	4	5	-	6	7	8	9	10	11
W1	Ala 77	Leu	Gly	Leu	Asp	Asp	Pro	Gly	Thr	Ile	Leu	Trp 88
W2	Ala 135	Leu	Asn	Ala	Glu	-	Thr	Gly	Glu	Thr	Val	Trp 145
W3	Ala 186	Tyr	Asp	Val	Lys	-	Thr	Gly	Glu	Gln	Val	Trp 196
W4	Gly 282	Arg	Asp	Ala	Asp	-	Thr	Gly	Glu	Ala	Lys	Phe 292
W5	Thr 337	Leu	Asp	Arg	Thr	-	Asp	Gly	Ala	Gln	Val	Ser 347
W6	Ala 457	Tyr	Asn	Ala	Ile	-	Thr	Gly	Asp	Tyr	Lys	Trp 467
W7	Ala 498	Arg	Asp	Ser	Asp	-	Thr	Gly	Asp	Leu	Leu	Trp 508
W8	Val 577	Phe	Ser	Leu	Asp 581	-	Gln 39	Leu	Arg	Pro	Ala	Trp 44
MDH Consensus	Ala	X	Asp/ Asn	X	X	-	Thr	Gly	Asp/ Glu	X	X	Trp
ADH Consensus	Ala	X	Asp	Ala	X	-	Thr	Gly	Lys/ Glu	X	Leu/ Val	Trp

Fig. 1.11 The tryptophan docking motif of the alpha subunit of MDH
This shows the sequence of the tryptophan docking motif of MDH from *Methylobacterium extorquens* and the consensus sequences for MDH and ADH. The docking motif occurs at the end of the C-strands and beginning of the D-strands and consists of a stacking interaction between alanine in position 1, tryptophan in position 11 and the peptide bond between positions 6 and 7 in the previous motif (Taken from Ghosh *et al.*, 1995).

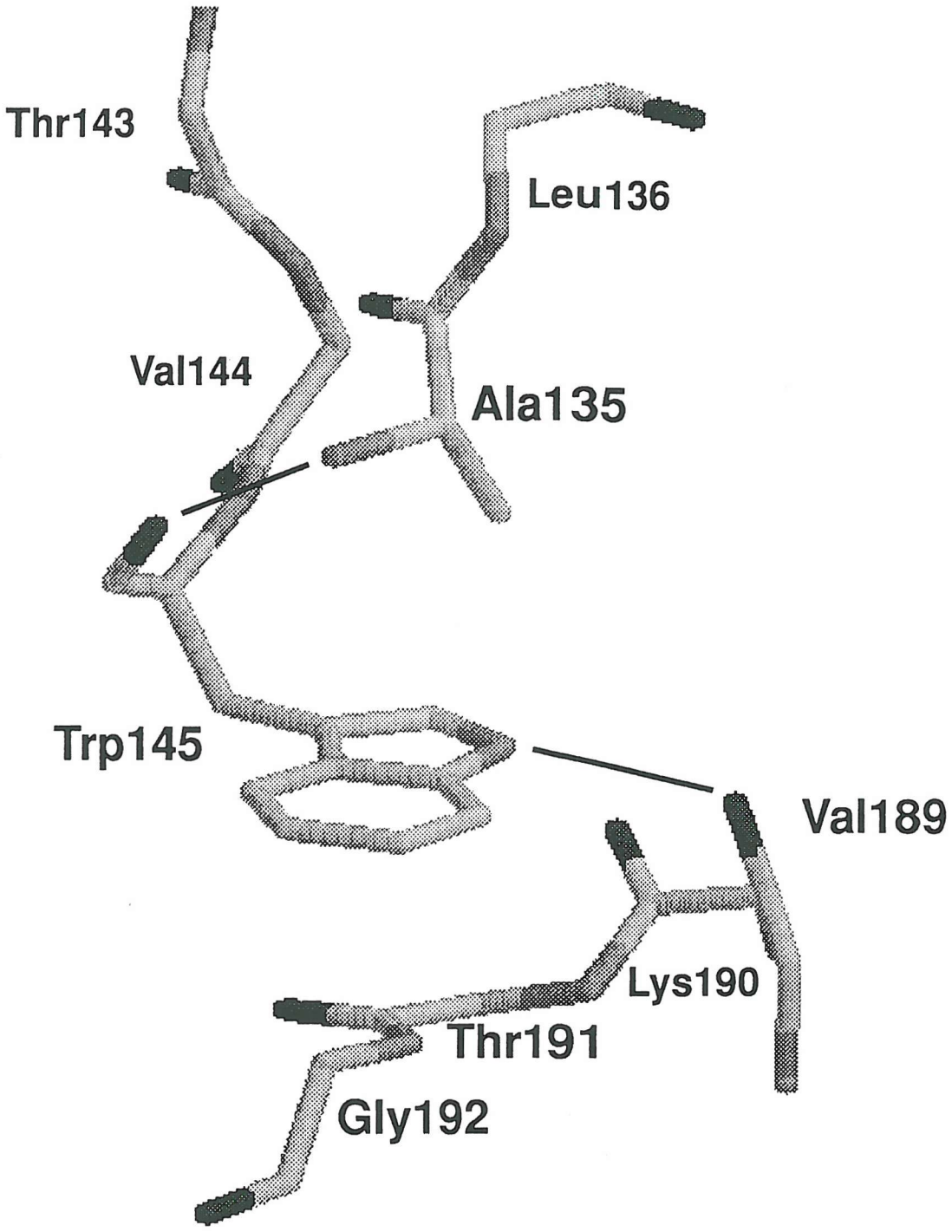

Fig. 1.12 Part of a typical tryptophan-docking motif.
This shows the interactions of Trp145 (in W2) with Ala135 (also in W2), and the interaction with the plane of the peptide bond between Thr191 and Gly192 (in W3). For clarity the remaining residues of the two motifs are omitted, and the side chains are omitted from all residues except for Trp145 and Ala135. (Figure taken from Anthony and Ghosh 1998)

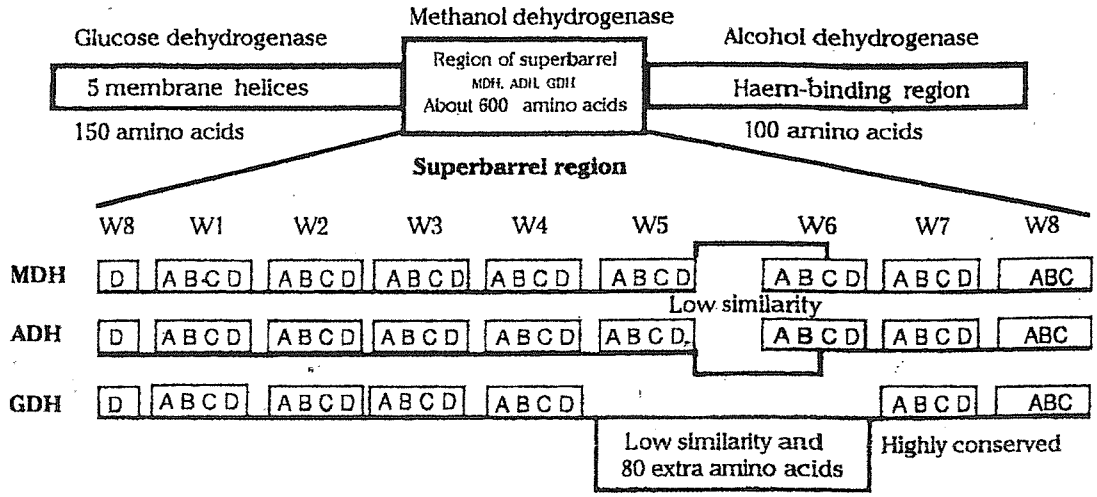

Fig. 1.13 Sequence alignment of the superbarrel regions of MDH, GDH and ADH.

Each 'W' represents a four stranded beta sheet composed of four beta strands A-D. There are many loops between, and within each of the beta sheets.

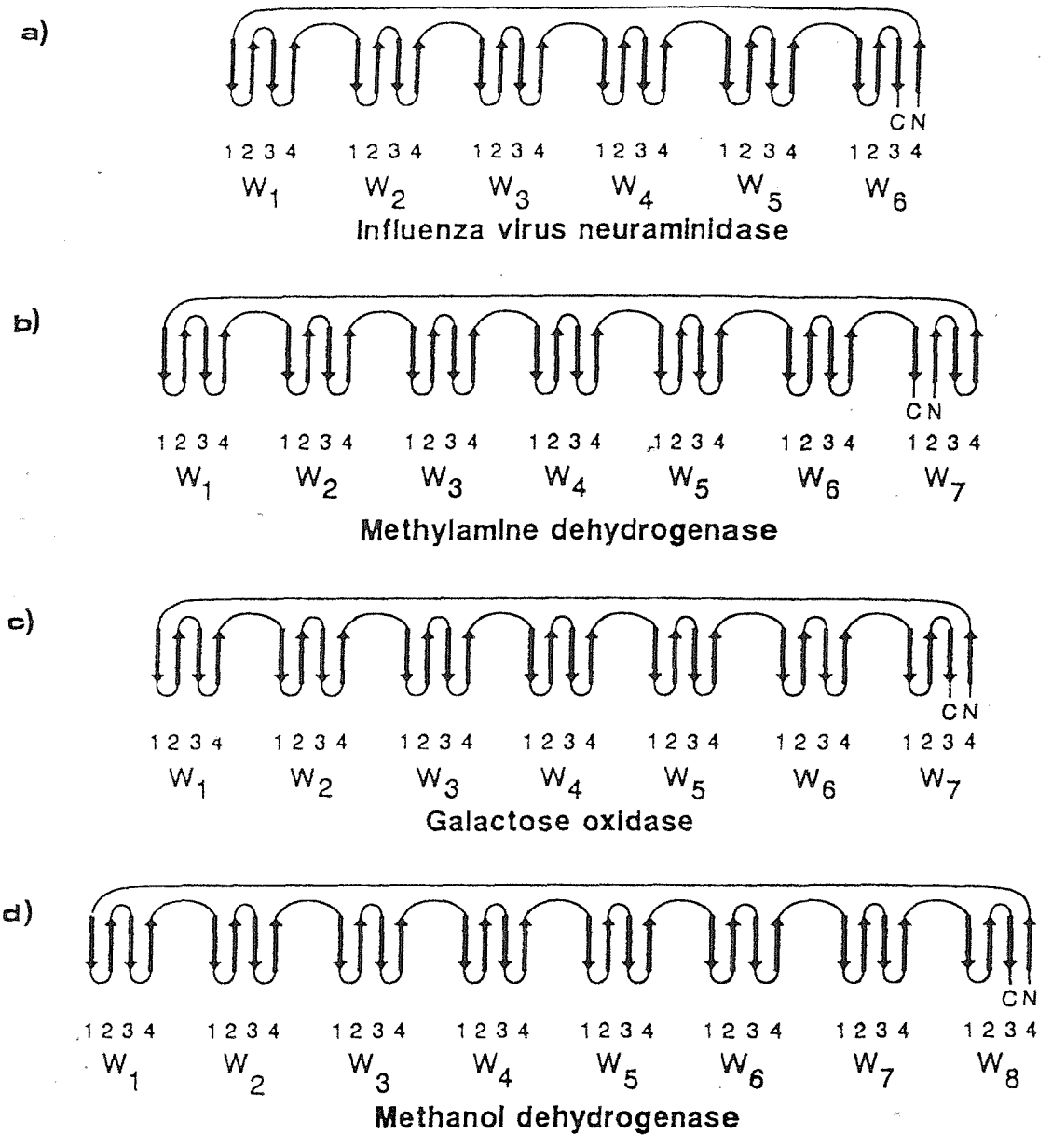

Fig. 1.14 Topological diagrams of four proteins known to have similar four-strand, beta-sheet superbarrel structures

a) Influenza neuraminidase headpiece, one of 4 identical subunits; b) Methylamine dehydrogenase heavy subunit; c) Galactose oxidase second domain; d) Methanol dehydrogenase alpha subunit (Figure taken from Xia *et al.*, 1992).

1.12c The beta subunit of methanol dehydrogenase

The beta subunit of MDH is 74 amino acids in length and has a molecular weight of 8.5 kDa. In total 39% of the amino acids in the beta subunit are charged and it is especially rich in lysines. The lysines were once thought to be involved in the binding of MDH to cytochrome c_L but this has now been dismissed because of a lack of conservation and because cytochrome c_L only cross links with the alpha subunit (Nunn *et al.*, 1989; Cox *et al.*, 1992).

The beta subunit can be seen in the crystal structure of the protein (Fig. 1.15). It forms an elongated j shape lying on the surface of an alpha subunit with no contact with the other beta subunit of the tetramer. The C terminal takes the form of an alpha helix whilst the N terminal region is more random but does contain a disulphide bridge between cysteines six and 12 (Xia *et al.*, 1992; Ghosh *et al.*, 1994). The role of the beta subunit is still not known but a role in maintaining the integrity of the alpha subunit has been proposed. No deletion mutants of the gene encoding the beta subunit are available to date. The gene, *mxaI*, is 291 base pairs in length and has a has a signal sequence indicating a periplasmic location.

1.12d The active site of methanol dehydrogenase

The active site of methanol dehydrogenase is located in the alpha subunit. It is found deep within the enzyme and contains both the prosthetic group, pyrrolo-quinoline quinone in the semiquinone form, (Duine *et al.*, 1987) and a calcium atom (Adachi *et al.*, 1990; Richardson *et al.*, 1992; Ghosh *et al.*, 1995; Goodwin and Anthony, 1996) (Figure 1.16). The x-ray structure clearly indicated that PQQ was located at the active site and not in a highly conserved region elsewhere in the protein (Figure 1.17). The active site is reached via a funnel leading from a surface depression and despite being surface accessible, many of the amino acids around the entrance are hydrophobic (Anthony *et al.*, 1994; Ghosh *et al.*, 1995) (Fig. 1.18).

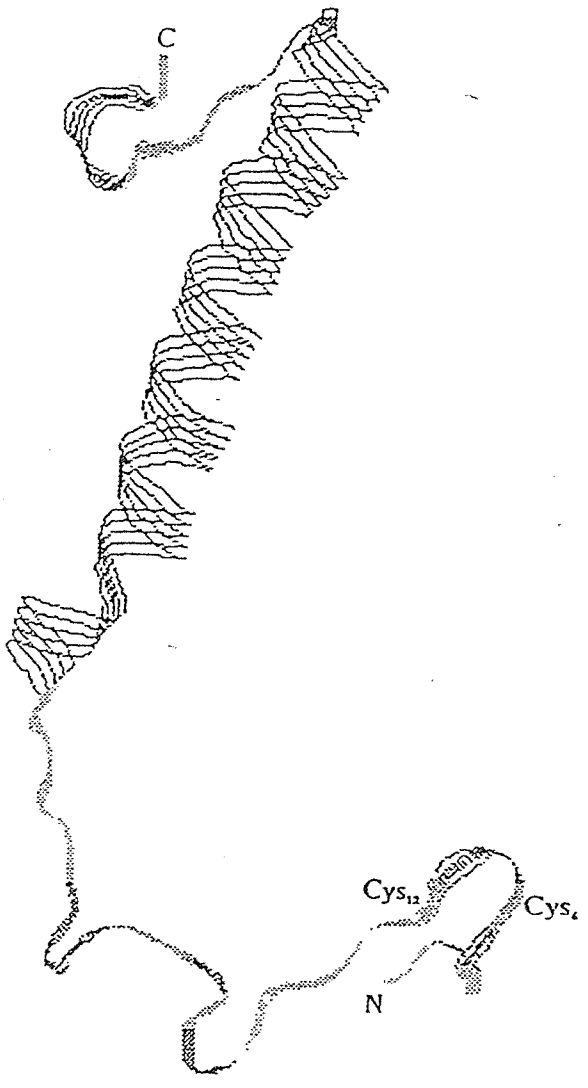

Fig. 1.15 The beta subunit of methanol dehydrogenase
The beta subunit is 74 amino acids in length. The N-terminal region is irregular and contains a disulphide bridge between Cys 6 and Cys 12. The C-terminal region forms an alpha helix. (Figure taken from Avezoux, 1995).

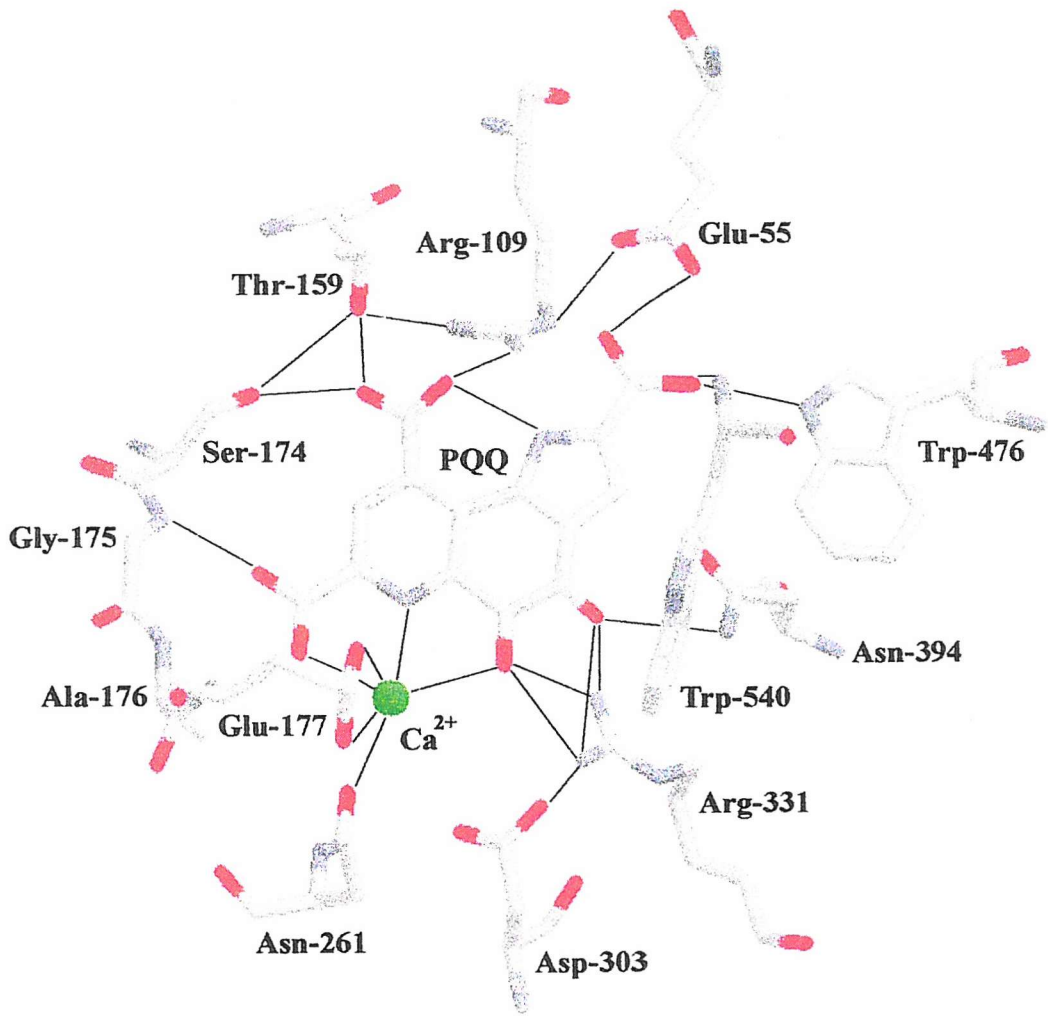

Fig. 1.16 The active site of methanol dehydrogenase
The active site of MDH contains PQQ, calcium and many amino acids including the proposed catalytic base aspartate 303. There are many interactions between PQQ, calcium and the active site amino acids of MDH.

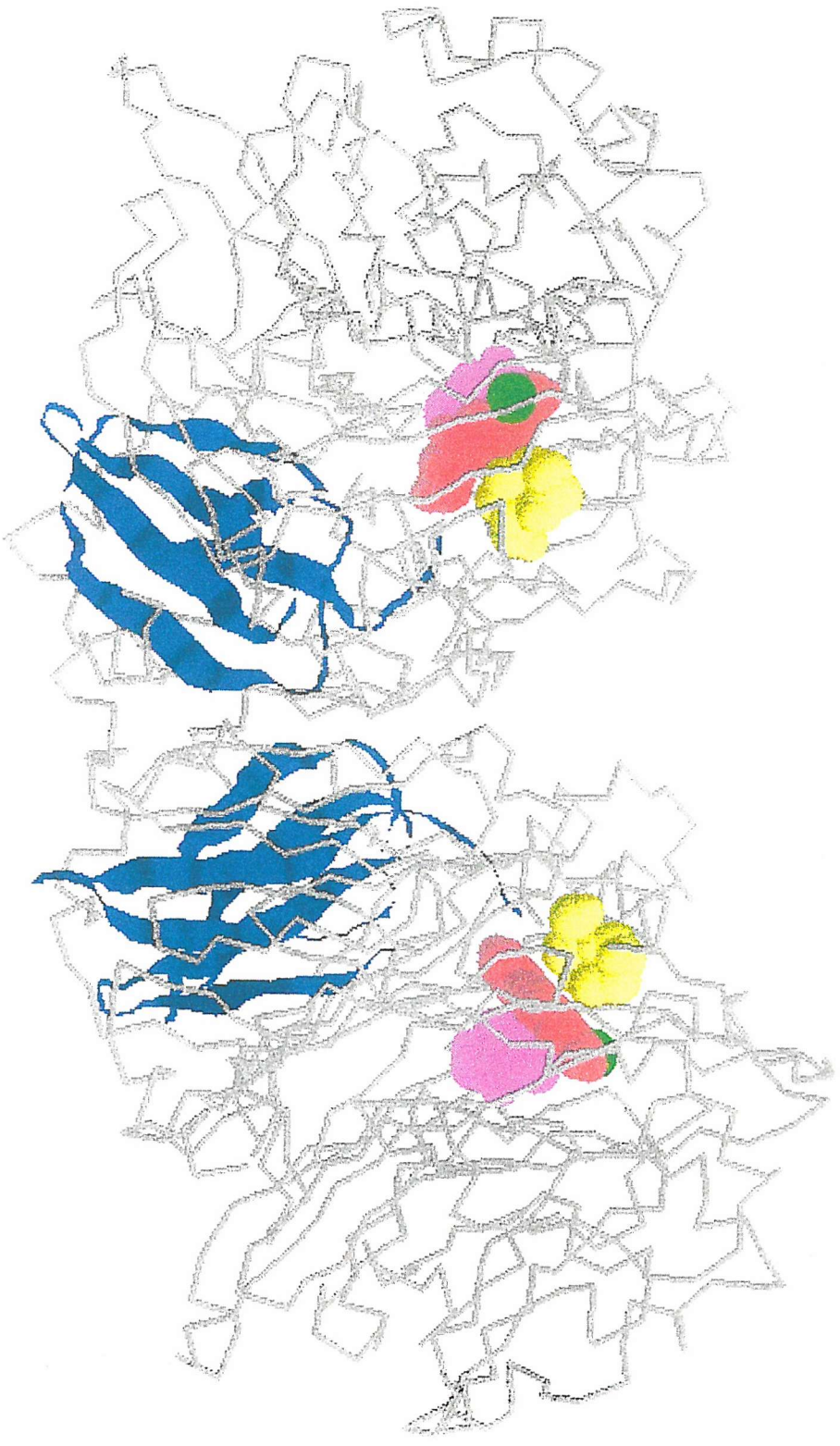

Fig. 1.17 The alpha subunit dimer showing the highly conserved region
The highly conserved region, which was once proposed to be the PQQ binding domain, is indicated by the blue ribbon. PQQ (in orange) is shown sandwiched between Tryptophan 243 (in magenta) below and a disulphide bridge between Cysteines 103 and 104 above (in yellow). (Figure taken from Anthony *et al.*, 1994).

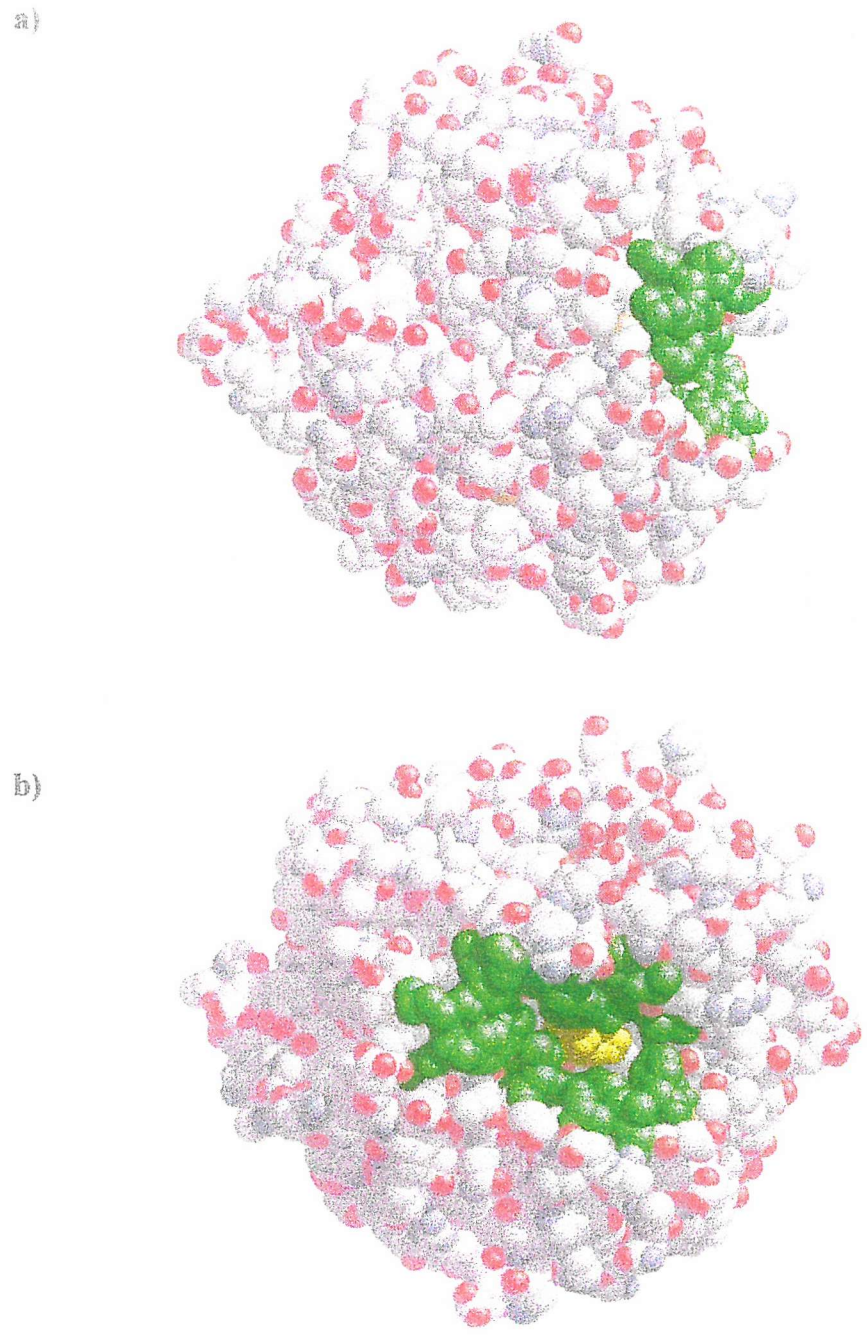

Fig. 1.18 The hydrophobic funnel on the surface of the alpha subunit of MDH. The active site of MDH is located at the bottom of a hydrophobic funnel. The atoms of the surface accessible hydrophobic residues are indicated in green and the disulphide bridge in yellow. A) side view; b) looking into the active site funnel (Anthony *et al.*, 1994).

ENDOR experiments indicate that the environment of PQQ is a hydrophobic chamber (Duine *et al.*, 1984).

The ceiling of the chamber is formed by a novel eight membered ring formed by two adjacent cysteine residues (Anthony *et al.*, 1994; Ghosh *et al.*, 1995) (Fig. 1.19). Both the sulphur atoms of the cysteines are within four Å of PQQ and would thus interact by van der Waals forces. Predictions by Ramachandran indicated that two adjacent cysteines forming a disulphide bridge would form a distorted structure with a *cis* peptide bond and x ray analysis with a dipeptide model seemed to confirm this arrangement (Ramachandran *et al.*, 1968; Chandrasekaren *et al.*, 1969; Mez, 1974; Capasso *et al.*, 1977; Blake *et al.*, 1994). Subsequently the disulphide bond was shown to be 1.7 Å, not 2 Å, with a 35° deviation in the peptide bond and in fact formed an unusual, non planar, *trans* peptide bond (Ghosh *et al.*, 1995). Sevaral roles have been proposed for the disulphide bridge including involvment in electron transfer and MDH assembly (Avezoux *et al.*, 1995)

Adjacent cysteines forming disulphide bridges have also been seen in the C terminal of mercuric ion reductase but only in the inactive form and reduction of the thiols is required for the correct configuration of the murcuric ion and subsequent activity (Miller *et al.*, 1989; Schiering *et al.*, 1991). The agonist binding site of the acetyl choline receptor also has adjacent cysteines that are reducable but there are no x ray data (Kao *et al.*, 1986).

The floor of the active site is formed by tryptophan 243 which is planar and in contact with PQQ. Many of the residues surrounding PQQ are conserved between species. These include (numbers relate to positions in *M. extorquens*) Asp 105, Arg 109, Glu 55, Ser 174, Thr 159, Asn 394 and Arg 331 and interact with PQQ via hydrogen bonds and ionic interactions.

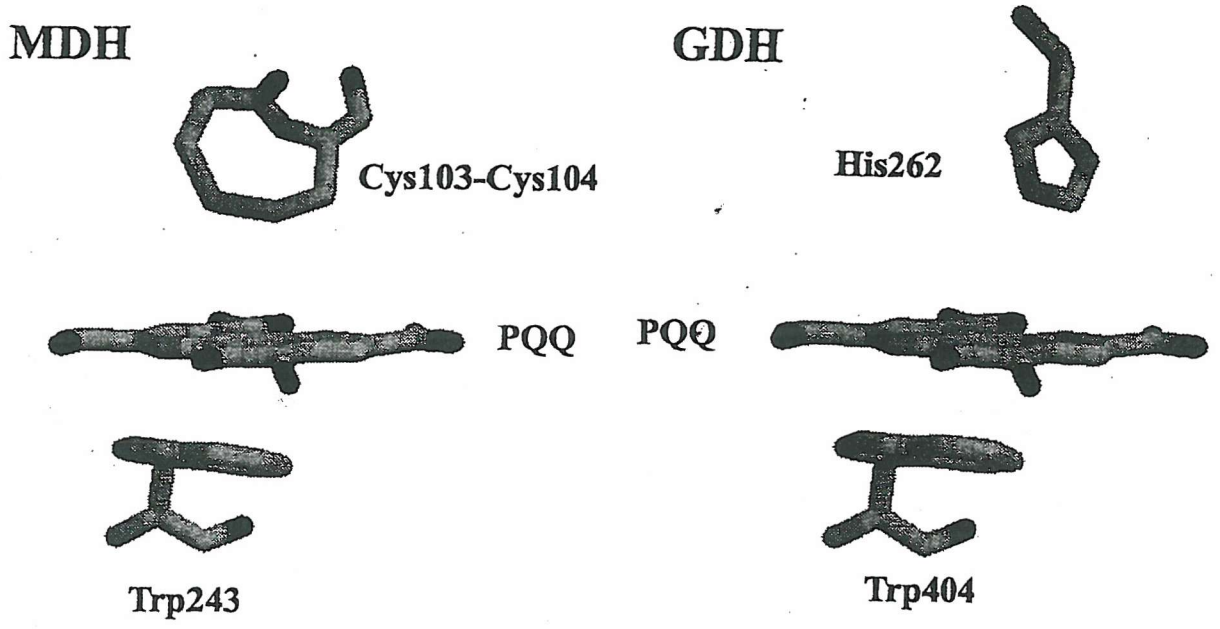

Fig. 1.19 The ceiling and floor of the active site chamber of methanol dehydrogenase

The ceiling of the active site chamber of MDH is formed from a novel disulphide bridge between residues Cys103 and Cys 104 that forms a nine membered ring. The floor of the active site is formed by Trp 243 which is found in the same plan as PQQ. For comparison the equivalent residues in GDH are shown: the ceiling is formed by His-262 and the floor by Trp-404.

1.12e Calcium at the active site of MDH

Using radio labelled calcium it was determined that there are 2 calcium atoms per MDH tetramer (Richardson and Anthony, 1992). When the crystal structure of MDH was solved, an isolated electron density at the active site surrounded by oxygen atoms from amino acids, glutamate 177 and asparagine 261, and PQQ it was interpreted as a calcium atom (Ghosh *et al.*, 1995).

The study of the roles of calcium has been greatly assisted by the production of three mutant strains of *M. extorquens* that lack the ability to insert calcium into the active site (see Section 1.19g). These strains, mutated in the genes *mxaA, K* and *L,* had enzymes of the same molecular weight, tetrameric structure, N terminal sequence and had PQQ present but differed from the wild type enzyme in the lack of activity and in the absorption spectrum. On incubation with calcium salts however, there was a time dependent and irreversible return to the wild type absorption spectrum and activity (Richardson and Anthony, 1992). An exception to the irreversibility of reconstitution is in a strain called *Methylophaga marina* which grows in high salt concentrations (Chan *et al.*, 1992).

The calcium has several roles in methanol dehydrogenase. Firstly it is thought to act as a Lewis acid in the reaction mechanism, polarising the C5 carbonyl, allowing the attack by an oxyanion or hydride (Anthony *et al.*, 1994) (see Section 1.18). This role however could potentially be achieved by arginine 331. The calcium also has the role of maintaining PQQ in the correct configuration at the active site. The duel role of calcium ie catalytic and structural has been seen in other enzymes such as phospholipase A_2 (Scott *et al.*, 1990).

1.12f Methanol dehydrogenase containing strontium or barium

The production of methanol dehydrogenase without the presence of the calcium atom in the active site allowed not only reconstitution with calcium, but also reconstitution with other ions (Goodwin and Anthony, 1996; Goodwin *et al.*, 1996; Richardson and Anthony, 1992). Many ions were tried including potassium, cesium, magnesium, and lithium but active enzymes were not produced. Incubation with calcium to these inactive enzymes produced active enzymes indicating that these other ions had not produced an inactive holoenzyme . In fact, in the case of potassium, this ion was found to be a potent inhibitor of reconstitution.

Two ions that did produce active enzymes on incubation with the apoenzyme were strontium and barium (Figure 1.20). The first interesting point that was noticed with the barium enzyme was that it did not have an endogenous rate that is seen in the wild type enzyme. It was deduced that the reason for this is because the barium enzyme has a km value for methanol approximately 1000 times higher than that for the wild type enzyme (20 μM). It was also found that this decreased affinity was for all alcohols, not just methanol. The barium enzyme oxidised formaldehyde but did not oxidise butane 1,3 diol. The V_{max} value for the strontium and the barium enzymes were higher than measured with the wild type enzyme. In the case of the barium enzyme the V_{max} was double that of the wild type enzyme because of a 50% reduction in the activation energy for the reaction.

The barium enzyme also allowed studies on the activation of the MDH catalysed reaction by ammonia. This was possible because the main problem in studying the activation process, the endogenous rate (see Section 1.9), is absent in the barium enzyme. It was deduced that there are two probable ammonia binding sites; one site results in activation of the reaction whilst the other site results in inhibition of the reaction but only at high ammonia concentrations. The effect that low concentration of ammonia has on the reaction is to increase the v_{max} by

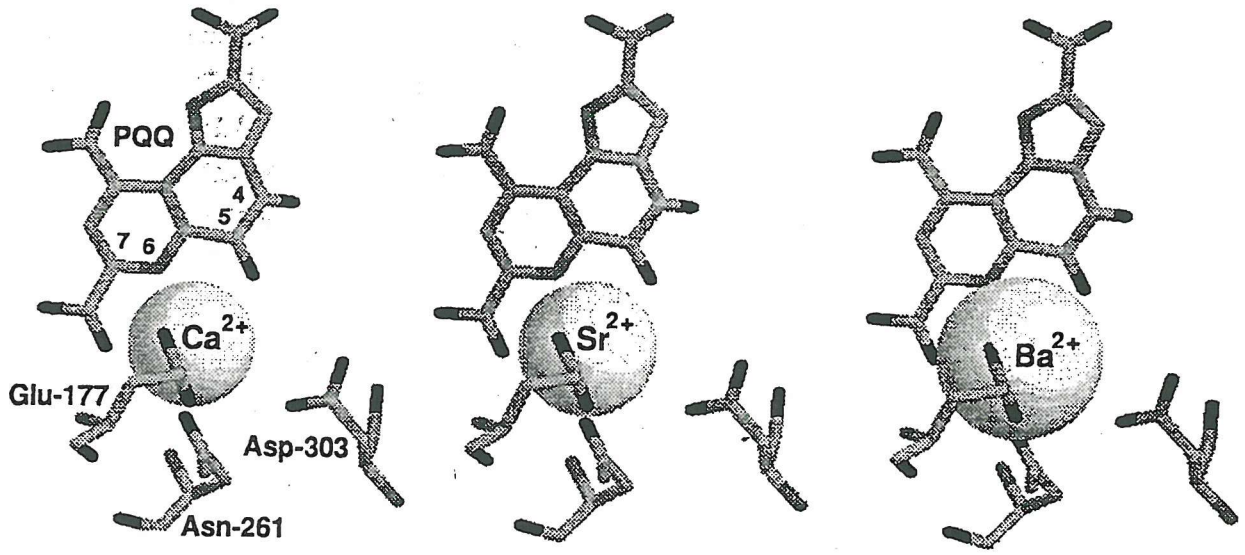

Fig 1.20 Representations of the active site of MDH containing calcium, strontium and barium. (Figure taken from Goodwin and Anthony, 1996)

increasing the rate of reduction of PQQ by the substrate but at high concentrations the affinity for methanol is decreased probably due to the methanol and activator sites being close together. The steady state kinetic data indicate that methanol and ammonia more strongly in the absence of the other, also indicating close binding sites. For a product to be formed, both substrate and the activator are required. The amount of activator needed in the barium enzyme is 26 times that needed in the wild-type enzyme to reach the same level of activity. Another activator of MDH, glycine ethyl ester gave similar results but did not show the inhibition seen with ammonia.

The barium enzyme also allowed the production of the oxidised form of PQQ which was not easily possible before because it was always purified in the semiquinone state unless oxidised by Wursters blue and protected by cyanide. The reduction of the oxidised form of PQQ was slow but speeded up 3.5 times with addition of methanol, 33 times with the addition of ammonia and gave an exceptionally fast rate with methanol plus ammonia.

Although it was hoped that a covalent intermediate may have been seen in the greatly decreased reduction process in all experiments an isosbestic point was observed, indicating the presence of only two species. As the spectrum of MDH changed concurently to the reduction process, the two species are thought to be the oxidised and reduced states. This does not rule out the possibility that a methanol or ammonia adduct was formed but gave a spectrum just like the reduced or oxidised species or that the production and conversion of such a species were so transient as not to be seen.

1.13a Glucose dehydrogenase

Glucose dehydrogenase (GDH) was first discovered by Hauge in 1964 (Hauge, 1964). There are two types of glucose dehydrogenase (Duine *et al.*, 1982 Duine *et*

al., 1987). A membrane bound enzyme with the active site in the periplasm and a soluble peripasmic enzyme only found in *Acinetobacter calcoaceticus*. The membrane bound enzyme is a monomer of 87 kDa. There is one mole of PQQ per mole of the enzyme and five predicted membrane spaning helices formed by residues 1-154 at the N terminal. The enzyme catalyses the conversion of glucose to gluconic acid. Protein sequencing from various sources indicates that the membrane bound enzyme has a superbarrel structure like MDH; for example, the periplasmic region of *E. coli* membrane bound enzyme at residues 155-796 shows 26% sequence identity to the alpha subunit of MDH of *M. extorquens* (Figure 1.13)(Anthony, 1992a). Such similarity may indicate a common origin. Molecular modelling has indicated that like MDH, GDH has an eight bladed superbarrel structure and possesses the tryptophan docking motif. Unlike MDH, GDH has no beta subunit. Especially interesting are the similarities in the active site regions of MDH and GDH (see Figure 1.23). In MDH, aspartate 303 is the proposed active site base and a similarly positioned aspartate (Asp 466) is present in GDH. Arginine 331 in MDH is replaced by lysine 493. The roof of the active site in GDH is formed by histidine 262 rather than 2 cysteines as found in MDH (see Figure1.19). The apparent fewer interactions between PQQ and the surrounding amino acids when compared to MDH may explain why PQQ can be dissociated and then reconstituted back into GDH but not MDH. A more open active site in GDH may allow access for the larger substrates used in GDH when compared to MDH. The electrons from glucose are passed to ubiquinone in the periplasmic membrane. From ubiquinone electrons are passed either to ubiquinone oxidase or to the bc_1 complex (Yamanda *et al.*, 1993).

The enzyme is found in aerobic bacteria for example *Pseudomonas* or acetic acid bacteria that do not have the phosphotransferase or glycolytic pathway for glucose uptake. Some bacteria, for example *A.calcoaceticus,* cannot oxidise

gluconic acid further and is released into the growth medium. Unlike the soluble GDH enzyme the membrane bound enzyme cannot oxidise disaccharides although remarkably a single amino acid mutation in the enzyme from *G. suboxydans* resulted in confering the ability of the enzyme to oxidise maltose (Cleton-Jansen *et al.*, 1989; Cozier and Anthony, 1995).

The soluble form of GDH is a dimer of 50kDa and has 1 PQQ per monomer (Cleton-Jansen *et al.* 1989; Duine *et al.*, 1983) and the crystal structure has recently been solved (Dewanti *et al* 1999). In *A. calcoaceticus* both the soluble and the membrane bound forms of the enzyme exist. Unlike the membrane bound enzyme, the soluble form can oxidise disaccharides and lacks the membrane spanning regions. The soluble form does not react with ubiquinone and no definite links to an electron transport chain have been shown.

1.13b Metal ions in GDH

The presence of a calcium ion in GDH was first shown in the soluble enzyme from *A.calcoaceticus* (Geiger and Gorish, 1989). Calcium can be removed from soluble GDH by subjecting the enzyme to low pH, high salt, high temperatures. It was shown that the reconstitution requires PQQ plus Cd^{2+}, Ca^{2+} or Mn^{2+}. This study has been extended by using a mutant strain of *A. calcoaceticus* that is unable to synthesis PQQ (Matsushita *et al.*, 1995). In this study an active enzyme could only be formed by incubation with Cd^{2+}, Ca^{2+}, Sr^{2+} or Mn^{2+} but not Mg^{2+} is. Using a monomer of soluble GDH apoenzyme expressed and purified from *E. coli* calcium was shown to be required dimerisation and incorporation of PQQ (Olsthoorn and Duine, 1996). The membrane bound enzyme can also be reconstituted with PQQ plus metal ions including Mg^{2+}, Ca^{2+} and Zn^{2+} (Matsushita *et al.*, 1995).

1.13c The mechanism of GDH

Molecular modelling and chemical modification have indicated that the mechanism of GDH may be very similar to that of MDH (see Sections 1.13a and 1.17)(Cozier and Anthony, 1995; Imanaga, 1989). Especially interesting is the presence of an active site aspartate in both GDH and MDH and probably both act as active site bases in the reaction mechansm. There is also a residue, lysine 494, which could be the functional equivelent to arginine 331 in MDH which may act as a Lewis acid in the reaction mechanism. An important difference between GDH and MDH is the lack of a semiquinone intermediate throughout the reaction cycle in GDH which is mirrored in the absence of the disulphide bridge which is thought to stabilise the intermediate.

1.14 Alcohol dehydrogenase

There are three types of alcohol dehydrogenase (ADH). Type I ADH is a soluble enzyme with an absorption spectrum like MDH (Groen *et al.*, 1984; Grorisch and Rupp 1989; Mutzel and Gorisch 1991; Toyama *et al.*, 1995). It has a wide range of substrates including secondary alcohols. The k_m for ethanol is 15 μM but has a 1000 times lower affinity for methanol. The optimal pH for the enzyme is nine. The electron acceptor from the enzyme is a specific c type cytochrome . In the phenazine ethyl sulphate linked assay system, ammonia is required but the reaction is inhibited by EDTA by forming inactive monomers deficient in PQQ. In *P. aeruginosa* ADH is an alpha 2, beta 2, tetramer with 2 molecules of PQQ (Schrover *et al.*, 1993) but in *P. putida* no beta subunit has been found (Toyama *et al.*, 1995).

Type II ADH is found for example in *Comamonas testosteroni*. It is a soluble monomer of 71 kDa and found in the periplasm (Groen *et al.*, 1986; deJong *et al.*, 1995). It was first isolated as an apoenzyme lacking PQQ. Reconstitution with PQQ and calcium is possible and results in a conformational change. The enzyme oxidises

primary and secondary alcohols except methanol . The optimal pH is 7.7 in the dye linked assay and it does not require ammonia. The gene sequence of ADH indicates amino acid sequences like MDH and like MDH, ADH has the tryptophan docking motif (see Section 1.17, Figures 1.22 and 1.13). The active site of this type II ADH , like MDH, contains PQQ and a calcium atom (Figure 1.23). The ceiling of the active site is formed by a disulphide bridge as in MDH.

Type III ADH has been described from *Acetobacter* and *Gluconobacter* (Beppu, 1993; Matsushita and Adachi, 1993; Matsushita *et al.*, 1994). The enzyme oxidises primary alcohols from ethanol to hexanol and there is some activity with formaldehyde. The optimal pH is between 4 and 6 and doesn't require ammonia as an activator. Type III ADH is composed of three subunits. Subunit one is 72- 80 kDa and, like type II ADH, contains PQQ and haem c. Residues 1-590 of ADH from *Acetobacter aceti* show 31% identity to the alpha subunit of MDH (Cozier *et al.*, 1995). Subunit two contains three haem c molecules, is 48-53 kDa and is firmly attached to the membrane allowing electron transfer to ubiquinone. Subunit three is firmly attached to subunit one and is 14-17kDa.

1.15 Aldehyde dehydrogenase

Aldehyde dehydrogenases catalyse the oxidation of aldehydes from two to four carbons in length. Such aldehydes are produced from the oxidation of alcohols by alcohol dehydrogenase. Most of the studies on these enzymes have been from enzymes released by detergent from the membranes of acetic acid bacteria (Matsushita and Adachi, 1993. The enzyme has a pH optimum of four. An electron acceptor has not been determined although it is probably ubiquinone.

1.16 Other quinoproteins

After the identification of PQQ in methanol and glucose dehydrogenases, many other enzymes were shown to contain PQQ including aldehyde, alcohol and quinate dehydrogenases and lupanine hydroxylase (Duine, 1991; Anthony *et al.*, 1994). Lupanine hydroxylase is a soluble enzyme found in *pseudomonas* and required for growth on alkaloids. This enzyme and Type II and III alcohol dehydrogenases contain haem and are thus called haemoquinoproteins (Matsushita *et al.*, 1994; Groen *et al.*, 1986). A number of PQQ containing dehydrogenases exist in acetic acid bacteria and may include glycerol and fructose dehydrogenases. Unfortunately a number of PQQ containing enzymes were incorrectly identified. This was because a number of PQQ like structures exist (Fig. 1.21). Unlike PQQ, these other structures are derived from the amino acids within the enzyme. One PQQ like structure is tryptophan tryptophylquinone (TTQ). It is formed from two tryptophan molecules and is found in bacterial amine dehydrogenase which catalyses the oxidative deamination of primary amines using a blue copper protein as an electron acceptor (McIntire *et al.*, 1991; Eady and large, 1971). Another PQQ like structure is 6-hydroxydopaquinone (TPQ) and is formed from tyrosine (Klinman, 1995; Janes *et al.*, 1990). It is found in copper containing amine oxidase and catalyses the oxidative deamination of primary amines to aldehydes and hydrogen peroxide. It is involved in bacterial growth, secondary metabolism in plants and oxidation of neurotransmitters in animals. Lysine tyrosyl quinone (LTQ) is formed from tyrosine and lysine (Klinman, 1995). It is found in lysyl oxidase, a copper containing amine oxidase and has a major role in development and repair of collagen and elastin connective tissues. Galactose oxidase which catalyses oxidation of primary alcohols was once thought to be a quinoprotein but has been shown to have a tyrosine covalently bonded to a cysteine (Ito *et al.*, 1991). This arrangement stabilises a free radical on the tyrosine which is coordinated to a copper ion.

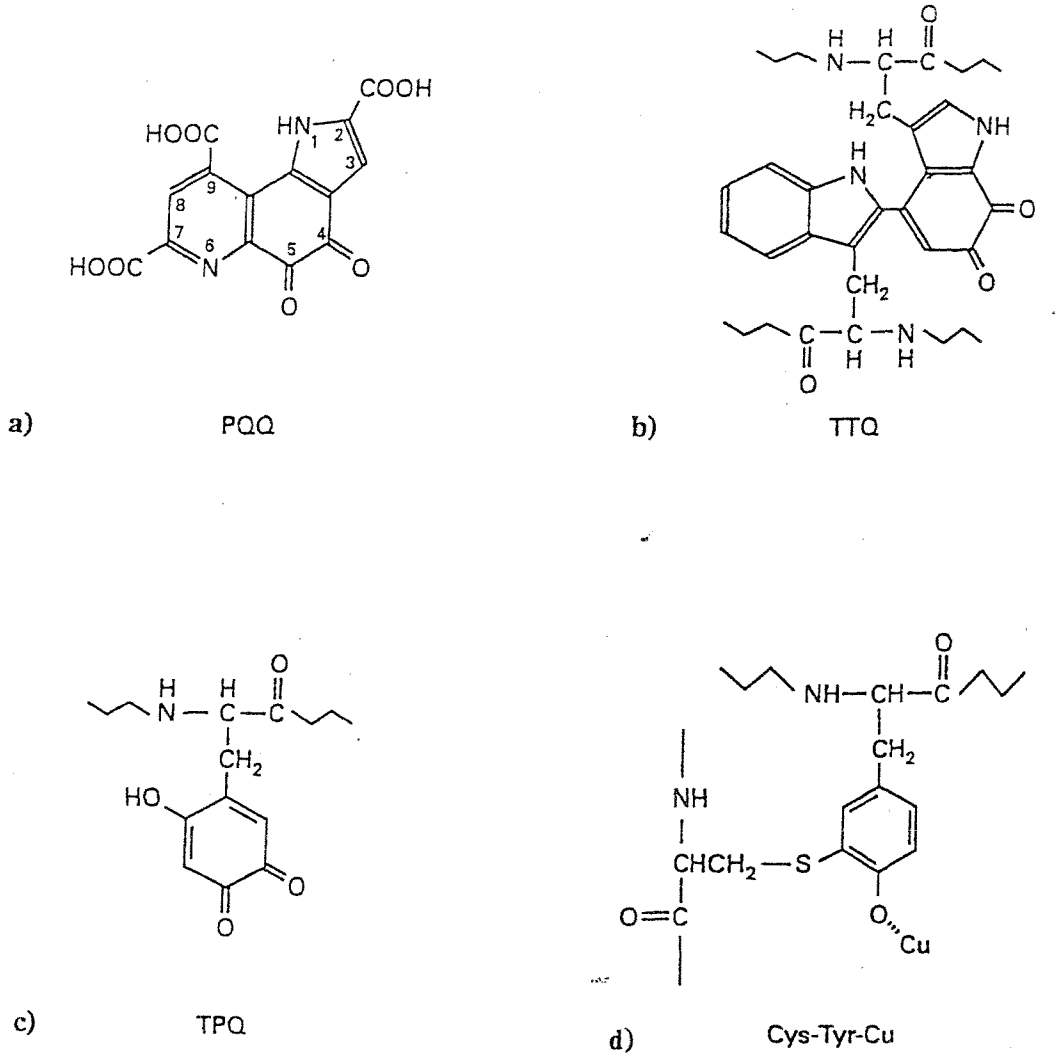

Fig. 1.21 PQQ and related prosthetic groups

Pyrrolo-quinoline quinone (PQQ) is found in a variety of different dehydrogenases including: methanol, higher alcohols, aldose sugar, quinate, lupanine, aldehydes and polyvinyl alcohol. Tryptophan tryptophylquinone (TTQ) is found in bacterial dehydrogenases for methylamine and aromatic amines. 6-hydroxydopa quinone (TPQ) is found in copper-containing amine oxidases. Although not strictly a quinoprotein, galactose oxidase contains the prosthetic group Cys-Tyr-Cu. (Figure taken from Anthony *et al.*, 1994).

1.17 Homology between PQQ containing enzymes

Gene sequencing of enzymes from various sources indicates that several dehydrogenases have a superbarrel structure like MDH and perhaps indicates a common origin (Anthony *et al.*, 1994). Examples of strong similarity include a 31% identity between MDH and residues 1 to 590 in ADH from *A. aceti* and a 26% identity between MDH and residues 155-796 of GDH from *E. coli*. Molecular modelling by Cozier based on the structure of MDH has indicated that both ADH and GDH have an eight bladed superbarrel structure with the tryptophan docking motif (Cozier *et al.*, 1995; Cozier and Anthony, 1995) (Figure 1.22). Unlike MDH, GDH and ADH do not have a beta subunit (Anthony *et* al. , 1994).

Whereas MDH and ADH have a disulphide bridge forming the roof of the active site, GDH has only one cysteine in the equivalent position. Another interesting similarity among the three proteins is a conservered region of 65 amino acids that was considered at one time to be the PQQ binding site (Inoue *et al.*, 1990; Cleton-Janson *et al.*, 1990; Anthony, 1992) (Fig. 1.17). Subsequently the X-ray structure of MDH has revealed that this region corresponds to a beta sheet in the W7 and W8 blades. Although no other function has been described, such conservation would probably suggest an important role. The similarity in the active site region of MDH and subunit one of Type III ADH with the disulphide bridge, tryptophan at the bottom of the active site, an active site base and the coordination of the calcium atom has lead to the conclusion that the mechanisms of the enzymes are very similar (Fig. 1.23).

1.18 The mechanism of methanol dehydrogenase

Using stopped flow spectrophotometric studies, methanol dehydrogenase has been shown to have a ping-pong mechanism. This involves reduction of PQQ, product release and 2 sequential electron donations to cytochrome c_L reforming the oxidised

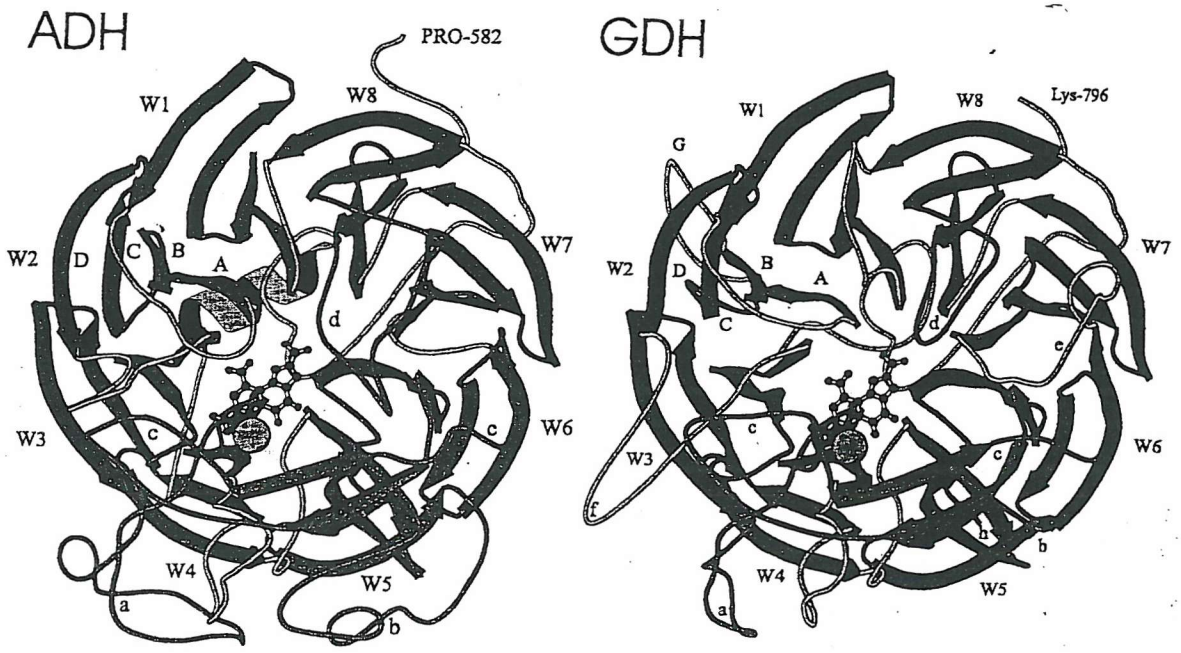

Fig. 1.22 Schematic representation of alcohol dehydrogenase (ADH) and glucose dehydrogenase (GDH).
These model structures of GDH and ADH are based on methanol dehydrogenase for which the crystal structure has been solved. The prosthetic group, PQQ, is shown as a ball and stick structure and the calcium as a Van der Waal's sphere. Figures taken from Cozier *et al.*, 1995 (ADH) and Cozier and Anthony, 1995 (GDH).

form of the enzyme (Dijkstra *et al.*, 1989) (Fig. 1.24 and Fig 1.25). Much of the focus on the mechanism has been centred on the C5 carbonyl group. This has been because of its reactivity to nucleophilic reagents with adducts forming with methanol, cyanide, aldehydes, ketones, urea, ammonia and amines (Dekker *et al.*, 1982; Duine *et al.*, 1987; Itoh *et al.* 1993) (Fig.1. 26). Of particular interest is the reactivity of the carbonyl to cyclopropranol (Frank *et al.*, 1989). The reaction leads to a covalent adduct and inactivation of the enzyme. It is thought that an active site base removes a proton from cyclopropranol allowing ring opening and adduct formation. The active site base is proposed to be aspartate 303 although another prospective residue is glutamate 177 (Blake *et al.*, 1994). Studies of the X-ray structure indicate a close proximity of an arginine (Arg 331) to aspartate 303 which could diminish the ability to act as an active site base however on further inspection it is thought that other interactions to the arginine may allow the aspartate to have a sufficiently low pK for proton abstraction. The role of the calcium atom is thought to be as a Lewis acid by polarising the C5 carbonyl allowing the attack by an oxyanion or hydride (Anthony *et al.*, 1994). This role however potentially could be achieved by arginine 331. The calcium also may bind the oxygen atom of the substrate. The duel role of calcium ie catalytic and structural has been seen in other enzymes such as phosholipase A_2 (Scott *et al.*, 1990).

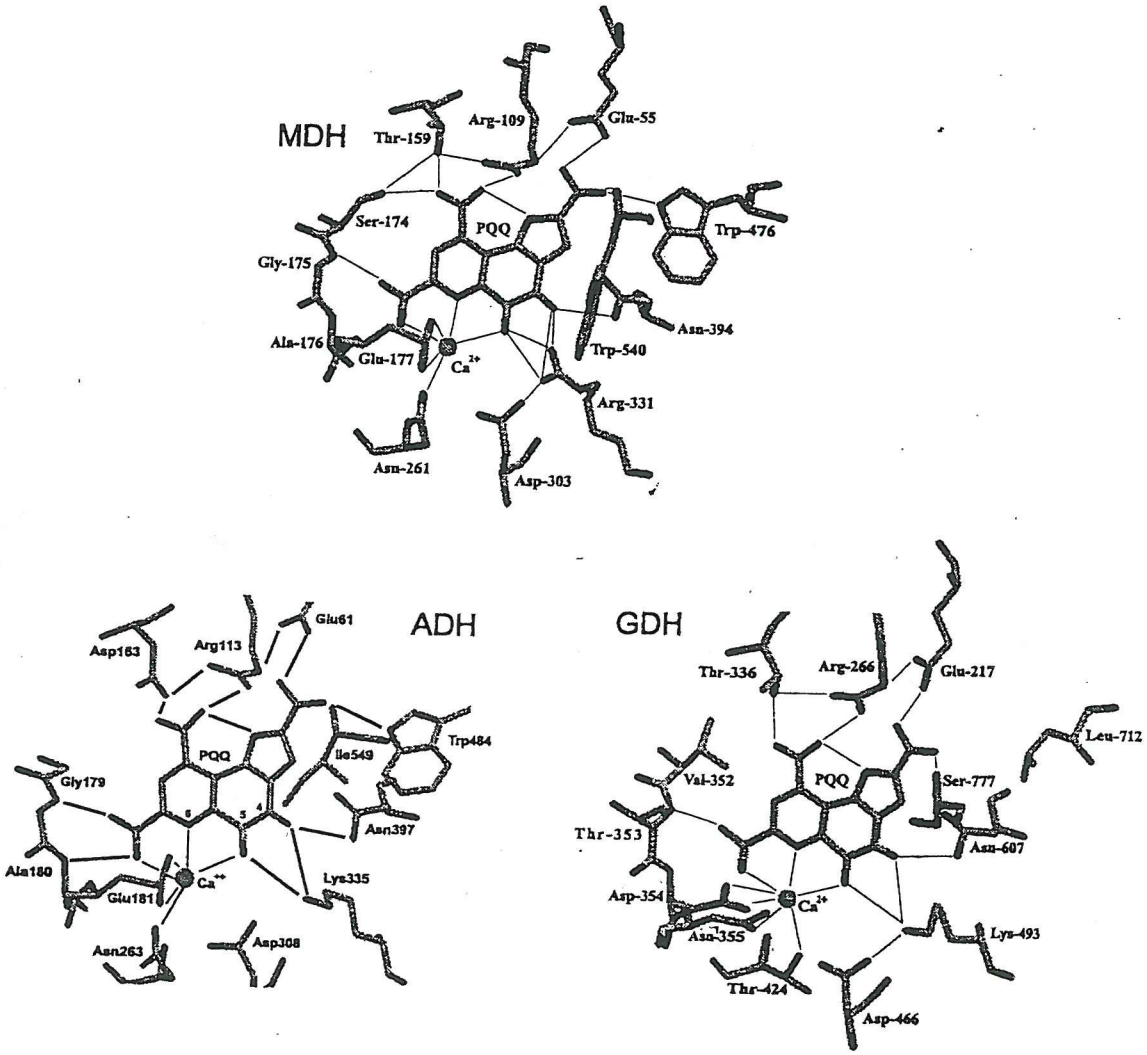

Fig. 1.23 Comparison of the active sites of MDH, ADH and GDH.
The active sites of MDH, ADH and GDH show remarkable similarity. The active site of MDH was determined by x-ray crystallography and that of ADH (type III) and GDH modelled on the MDH structure (Figure taken from Goodwin and Anthony, 1998).

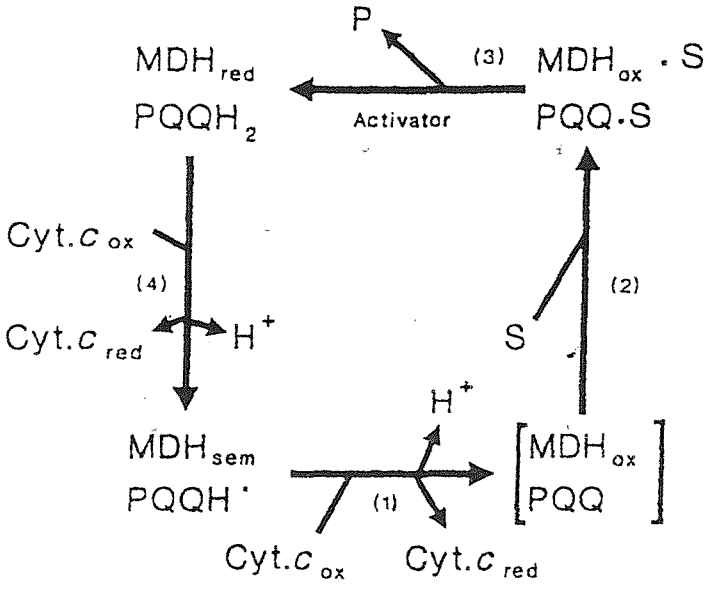

Fig. 1.24 The reaction cycle of MDH

MDH in the oxidised from (PQQ) binds with the substrate (S) forming a complex (step 2). In the presence of an activator the reduced form of MDH ($PQQH_2$) is produced and the product (P), formaldehyde, is released (step 3). There then follows two sequential interactions with cytochrome c_L ($Cyt.c_L$). In the first interaction (step 4) the cytochrome is reduced producing the semiquinone form of MDH ($PQQH^*$) and in the second interaction (step1) another cytochrome is produced leaving the oxidised form of MDH ready for another reaction cycle. In each of the two steps involving cytochrome reduction, a single proton is released into the periplasm creating a proton motive force. In the dye linked assay system phenazine ethosulphate (PES) replaces cytochrome c_L (Figure taken from Anthony, 1992 based on work by Frank *et al* 1988).

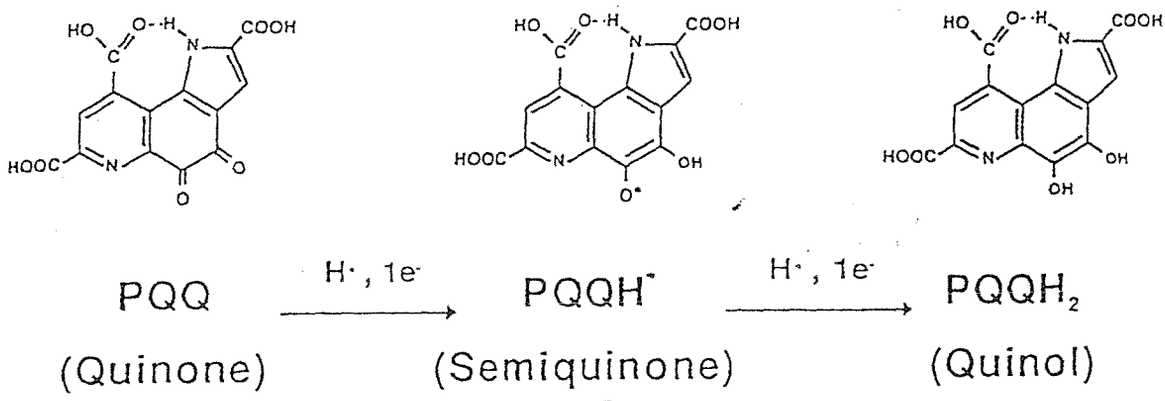

Fig. 1.25 The three oxidation forms of PQQ
PQQ can exist in three forms: the quinone form (PQQ), the semiquinone form ($PQQH^{\cdot}$) and the quinol form ($PQQH_2$). When MDH is isolated it is found in the semiquinone form.

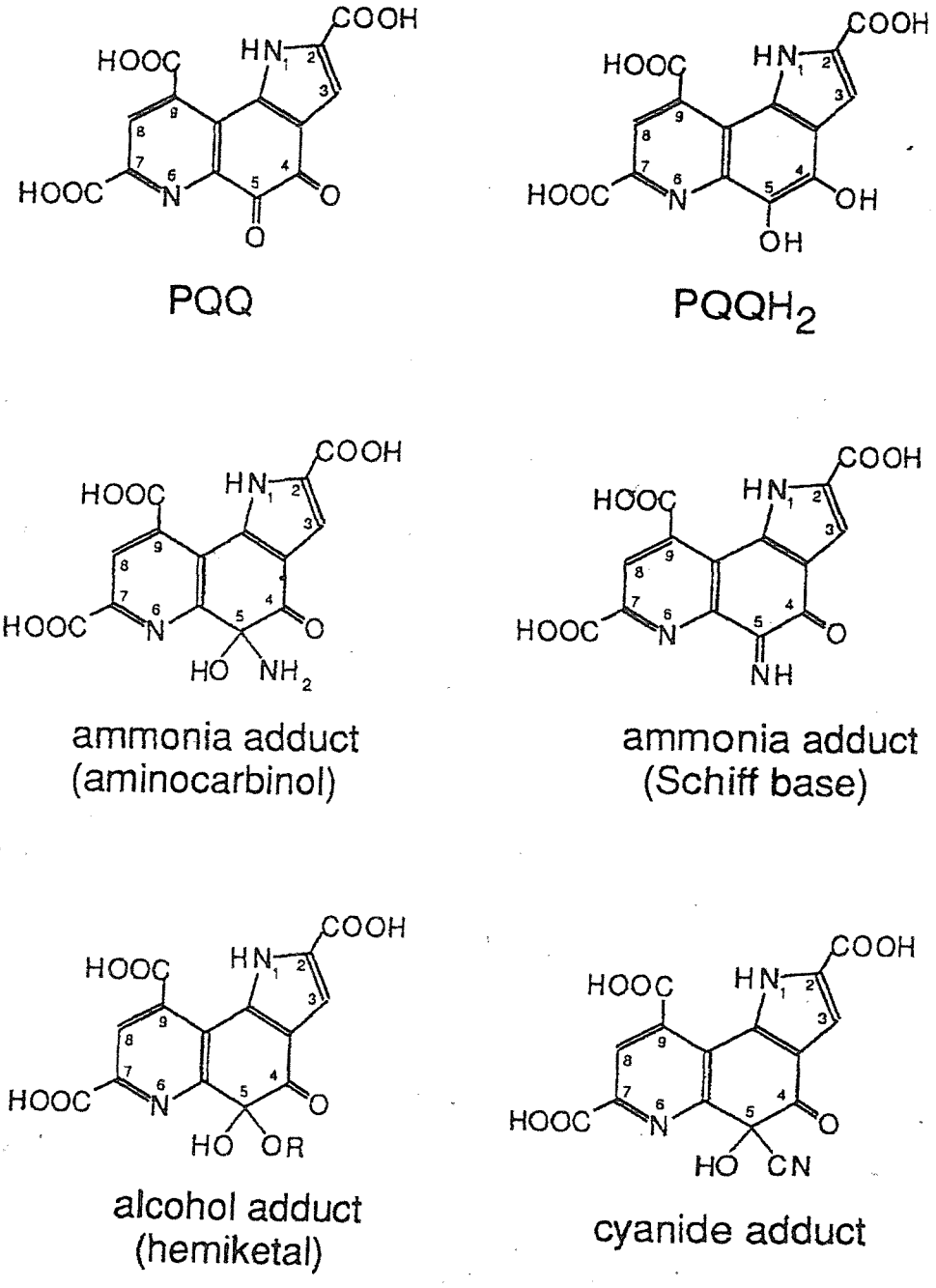

Fig. 1.26 PQQ and the adducts that it forms with ammonia, alcohol and cyanide.

Two catalytic mechanisms for MDH have been proposed. In the first, an oxyanion is produced by proton extraction which then attacks the C5 carbonyl forming a hemiketal intermediate. The pyrrole nitrogen allows the C4 ionisation and facilitates the extraction (Figure 1.27). The second proposed method is a classic acid/base hydride transfer with aspartate 303 and the calcium atom fulfilling catalytic and Lewis acid roles (Figure 1.28). What evidence there is indicates that the hemiketal mechanism is more likely. This includes a slight change in the absorbance spectrum during the reaction with deuterated methanol. Deuterated benzyl alcohols have indicated that the pro-s hydrogen is removed from the substrate and the pro-r hydrogen is retained in the aldehyde product (Frank *et al* 1988).

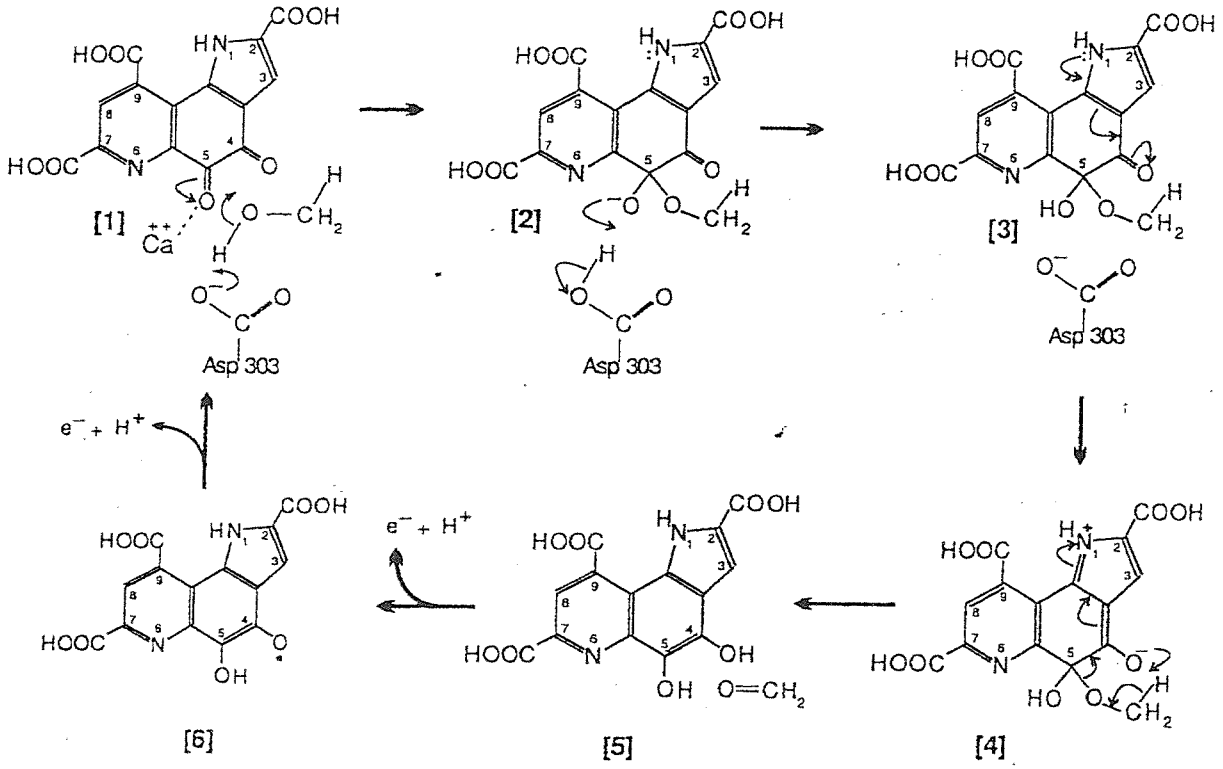

Fig. 1.27 The catalytic mechanism of MDH involving a hemiketal intermediate and a role for the pyrrole nitrogen.

At stage 1 PQQ is in the oxidised form. The catalytic base Aspartate 303 removes a proton from methanol and due to the polarisation of the C5 carbonyl by calcium the methanol oxygen reacts with this carbonyl (stage 2). The free oxygen on the C5 carbonyl removes the proton from Aspartate 303 forming the hemiketal intermediate (Stage 3). The C4 carboxyl group is activated by an arrangement involving the pyrrole nitrogen and the C4 carbonyl removes a proton from the C5 adduct leading to release of the reaction product (stages 3, 4 and 5). The reduced form of PQQ is then oxidised in two subsequent reactions with cytochrome c_L firstly forming a semiquinone intermediate (stage 6) and then the fully oxidised form (stage 1)(Figure taken from Anthony and Ghosh, 1998).

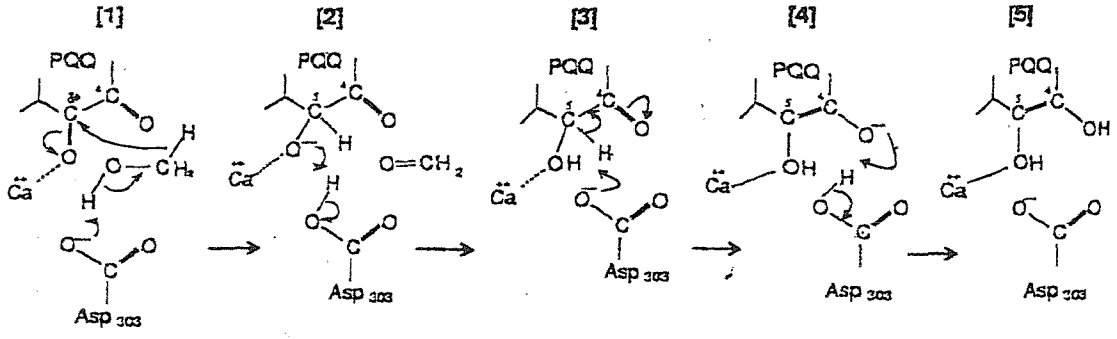

Fig. 1.28 Reaction mechanism of MDH involving acid/base catalysed hydride transfer.

In this mechanism Aspartate 303 and the C5 carbonyl remove a proton from methanol producing the reaction product, formaldehyde. The free oxygen at the C5 position then removes the proton from the catalytic base. A rearrangement occurs involving reprotonation of Aspartate 303 and so that the C4 oxygen can remove the proton from Aspartate 303. In this mechanism, as in the hemiketal mechanism, the calcium atom acts as a Lewis acid polarizing the C5 carbonyl. (Figure taken from Anthony and Ghosh, 1998)

1.19 The genetics of methanol oxidation

1.19a Introduction

Methanol oxidation requires upwards of 30 genes (Fig. 1.29)(Lidstrom *et al.*, 1994; Goodwin and Anthony, 1998). Most of the studies on methanol oxidation genetics have been from *Paracoccus denitrificans* and *Methylobacterium extorquens*. Particularly useful have been mutant strains of these bacteria although many gene functions are still unknown. Originally the genes involved in methanol oxidation were termed mox genes but are now given the prefix *mx*. The methanol oxidation genes are located in four chromosomal linkage groups named (*mxa, mxb, mxc* and *mxd*) (Fig. 1.30). Those involved in PQQ biosynthesis are given the prefix *pqq*.

1.19b The *mxa* gene cluster

The *mxa* operon is composed of 11 genes :- *mxaFJGIR(S)ACKLD* (Lidstrom *et al.*, 1994; Goodwin and Anthony, 1998). The operon has been sequenced and this has given several clues to the funtions of the genes within the operon and also shed light on the operons regulation (Figures 1.29,1.31 and 1.32). The operon is thought to be controlled by a single promoter upstream of *mxaF*, the first gene in the operon. The regulation of the *mxa* operon is discused in Section 1.21a. Unless stated otherwise the genes in the *mxa* operon from *M. extorquens* are discused in turn below.

1.19c The *mxaF* gene

MxaF is 1881 base pairs in length and encodes the alpha subunit of MDH (Figure 1.10). The gene from *P. denitrificans* has 76% homology to that from *M. extorquens*. Like all genes that produce proteins destined for the periplasm a gene region encoding a 22-27 amino acid signal peptide is required. The signal

	Proposed function and location of gene product	Organism		
		M. extorquens	*M. organophilum*	*P. dentrificans*
Structural genes	α subunit of MDH (P)	*mxaF*	*mxaF*	*mxaF*
	cytochrome C_L (P)	*mxaG*	*mxaG*	*mxaG*
	β subunit of MDH (P)	*mxaI*	*mxaI*	*mxaI*
Regulatory genes	sensor kinase – response regulator (M/C)	*mxcQE* *mxbDM*	*mxcQE* *mxbDM*	*mxaYX*
	(C)	*mxaB*	*mxaB*	
	(M)			*mxaZ*
		mxbN	*mxbN*	
Insertion of Ca^{2+} into MDH	? Ca^{2+} binding (P)	*mxaA*		
	(C)	*mxaK*		
	(M)	*mxaL*		
Other	Third subunit of MDH or molecular chaperone (P)	*mxaJ*	*mxaJ*	
	Unknown (C)	*mxaR*		*mxaR*
	Unknown	*mxaS*		*mxaS*
	Unknown		*mxaW*	
	Unknown (C)	*mxaC*		
	Unknown (P)	*mxaD*		
	Unknown		*mxcU*	
	Unknown	*mxdR*		
	Unknown	*mxdS*		

Fig. 1.29 The genes involved in methanol oxidation in *Methylobacterium* and *Paracoccus denitrificans.*

There are many genes involved in methanol oxidation. Some genes have a regulatory function, some have roles in processing and others are structural genes. Many of the gene functions have not been determined yet. Some of the genes are found in the periplasm (P), some in the cytoplasm c_L and others in the membrane (M) Figure taken from Anthony and Goodwin, 1998.

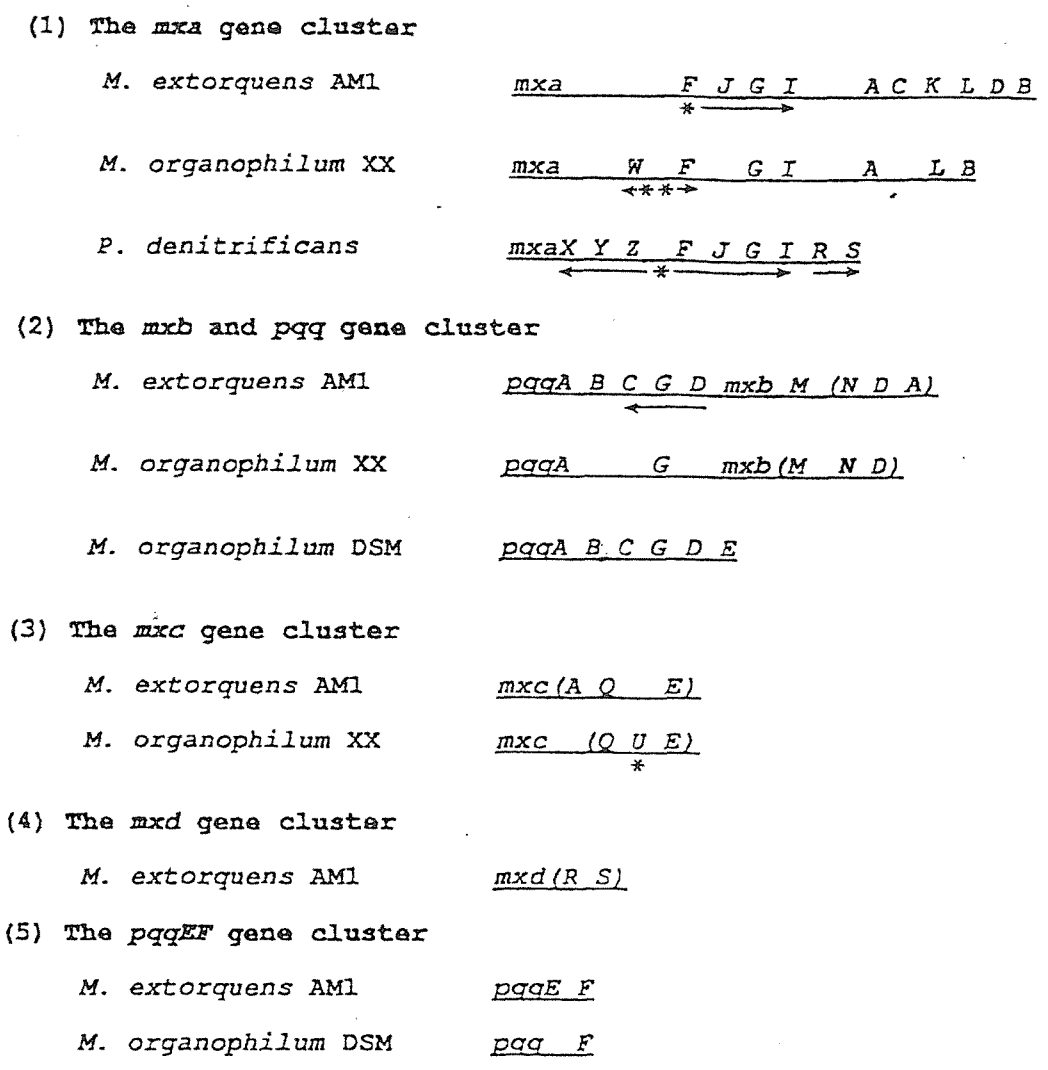

Fig. 1.30 Organisation of the methanol oxidation (*mxa*) genes and the PQQ biosynthetic (*pqq*) genes.

The *mxa* genes are located in four operons and the pqq genes in two operons. The asterisk indicates a known methanol regulated promoter. Arrows indicate the direction of transcription where known. (Figure taken and adapted from Goodwin and Anthony, 1995)

Sequence information for *mxa* genes	Reference
FJ	Anderson *et al* 1990
G	Nunn and Anthony 1988
I	Nunn *et al* 1989
RS	Amaratunga *et al* 1997
ACKLD	Morris *et al* 1995

Fig. 1.31 References to the nucleotide sequences of the genes in the *mxa* gene cluster

peptide will however be cleaved off by a signal peptidase in the membrane as the protein is translocated (Goodwin and Anthony, 1995).

1.19d The *mxaJ* gene

MxaJ encodes a 30kDa protein of unknown function (Anderson and Lidstrom, 1988; Harms *et al.*, 1987; Nunn and Anthony, 1988; Nunn *et al.*, 1989; Anderson *et al.*, 1990; van Spanning *et al.*, 1991).). The gene is 903 base pairs in length. A mutant of *mxaJ* in *M. extorquens* could not grow on or oxidise methanol. The mutant could however oxidise ethanol, pyruvate and methylamine. No alpha or beta subunits of MDH were detected but wild-type levels of cytochrome c_L were seen. In *P. denitrificans* however, the same mutation resulted in inactive MDH being made. It has therefore been suggested that the *Paracoccus* enzyme is more stable or that another protein in this organism promotes stability.

When a 32kDa protein was co-purified with MDH in *A. methanolicus,* it was proposed as an extra γ subunit. The first 29 amino acids of this protein were shown to have 41% and 48% homology to *mxaJ* in *M. extorquens* and *P. denitrificans* respectively (Matsushita *et al.*, 1993). When a 30kDa protein co-purified with MDH from *M. extorquens,* N terminal sequencing revealed that this protein had no homology to *mxaJ* and was considered a contaminant (Dales *et al.*, 1992). Data base analysis has given no clues as to the function of *mxaJ*

1.19e The *mxaG* gene

The *mxaG* gene is 594 base pairs in length has been sequenced (Nunn and Anthony, 1988). This gene encodes cytochrome c_L which has a signal sequence targeting the polypeptide to the periplasm. Cytochrome c_L acts as the electron acceptor from methanol dehydrogenase (Sections 1.8b and 1.8c).

1.19f The *mxaI* gene

The *mxaI* gene is 291 base pairs in length and has been sequenced (Nunn *et al.*, 1989). It is the fourth gene in the *mxa* gene operon and codes for the 8.5kDa beta subunit of methanol dehydrogenase (Section 1.12c). The gene encodes a signal sequence at the N terminus of the protein allowing the transportation of the polypeptide into the periplasm.

1.19g The *MxaR* gene

Sequencing has revealed that the 1023 base pair *mxaR* gene is potentially a protein of 38.6kDa. The gene has 60% homology to the equivalent gene in *Paracoccus denitrificans* (van Spanning *et al.*, 1991). The gene has revealed no potential signal peptide or membrane spanning regions and the protein is therefore thought to be cytoplasmic. The protein is thought to have two ATP binding sites. One of these sites is a glycine rich P loop (Walker A sequence) capable of binding ATP or GTP, the other is characteristic of a motif that only binds ATP. An insertion mutant in the *mxaR* gene in *Paracoccus denitrificans* resulted in a strain that produced inactive MDH and cytochrome c_L (van Spanning *et al.*, 1991; Harms, 1993). It was shown that the MDH produced was inactive and therefore a role in modifying the alpha subunit directly or via acting on other regulatory factors is postulated. No promoter has been located near the *mxaR* gene and it is thought that it is controlled by the promoter upstream of *mxaF*.

1.19h The *MxaS* gene

MxaS is a gene of 591 base pairs potentially encoding a protein of 22.3kDa (Harms, 1993). There is doubt over whether it is a true open reading frame. This is because the first 228 base pairs of the potential ORF show atypical codon usage and predict the N terminal to have an unlikely amino acid sequence containing many

arginines and prolines but very few glutamates or lysines (Amaratunga *et al.*, 1997a and b). This region of unusual amino acid composition is thought to be an N terminal extension of the protein from *Paracoccus denitrificans*. It is uncertain what the role of such an extension might be. Overall there is 20% homology between the genes of *Methylobacterium extorquens* and *Paracoccus denitrificans.*

1.19I The *mxaA, K* and *L* genes

DNA sequencing indicates that *mxaA* is 921 base pairs in length and has a periplasmic location, *mxaK* is 624 base pairs in length and is cytoplasmic, *mxaL* is 1008 base pairs in length and is an integral membrane protein (Fig 1.31). Bacteria with a mutation in either *mxa A, K* or *L* (Richardson and Anthony, 1992) have an MDH enzyme much like the wild-type including having no free thiols, the same proportion of alpha helix and beta sheet, the same N terminal sequence, the same denaturation properties and interacting with cytochrome c_L in the same way . There were however differences to the wild-type enzyme in the absorption spectrum and the oxidation state of the prosthetic group. The absorption spectrum had a lower peak at 345 nm (the region associated with PQQ) and an extra band at 520. This is due to PQQ being in the oxidised rather than the semiquinone state as found in wild-type enzyme. The PQQ levels were like that of the wild-type enzyme. The MDH from these mutant strains was also different to the wild-type enzyme as it did not react with cyclopropanol this could be because the ring opening of cyclopropanol is no longer catalysed by the active site of MDH. When MDH from the mutant strains was studied by atomic absorption spectroscopy, the calcium atoms shown to be present in wild-type MDH were absent. When MDH from these mutant strains were incubated with calcium, reconstitution occurred in a concentration and time dependent fashion. The pH optimum for reconstitution is 10.5 and reconstitution is irreversible (Goodwin and Anthony, 1995). Associated

with the reconstitution was an increase in the 345nm peak and a decrease in the 520 nm peak, giving a spectrum like the wild type enzyme. It was concluded that in the absence of calcium the PQQ is in an altered configuration in the active site but when reconstituted the configuration and enzyme activity are regained. The newly reconstituted enzyme was also found to react with cyclopropanol and produced the same spectrum as the reaction of cyclopropanol with wild-type enzyme.

Although it was originally thought that a single calcium atom had a role in binding the tetramer together, subsequent experiments using radioactive calcium showed that there are two moles of calcium for every mole of the enzyme and the x-ray structure indicated that both are in the alpha subunits. Further analysis by Arrhenius plots indicated that reconstitution is associated with a conformational change of MDH occuring at 15°C. Although a report of *Methylophaga marina* producing a calcium deficient enzyme which was red in colour the enzyme produced in the *mxa A, K* and *L* mutants were typically wild-type (green) in colour (Chan *et al.*, 1992).

Several suggestions as to the possible roles of the *mxa A, K* and *L* genes have been proposed. When bacteria containing mutations in either of these genes were grown in normal calcium conditions the MDH activity was 25% of the wild type bacteria. It has been suggested that these genes encode proteins that create a localised high concentration of calcium and thereby facilitating the entry into the apoenzyme. It is also possible the products of these genes are involved in stabilising the MDH apoenzyme in a recipient state for calcium insertion. Such theories have now been supported by the location of a possible calcium binding site in the predicted sequence for the *mxaA* gene (Morris *et al.*, 1995).

1.19j The *mxaD* gene

The *mxaD* is 528 base pairs in length (Morris *et al* 1995). There is contradicting

evidence for the role of *mxaD*. The original findings of a *mxaD* mutation strain indicated that the mutation led to production of an altered form of cytochrome c_L (cytochrome c-553), probably composed of cytochrome c_L with its signal peptide still attached (Nunn and Lidstrom, 1986). Subsequently it has been shown that the altered form has no resemblance to cytochrome c_L but is a novel cytochrome that is always present in wild-type bacteria (Day *et al.*, 1990). The exact role of the *mxaD* gene is still to be discovered. Interestingly the genome of *Methylobacter albus* BG8 (a methane utilising bacterium) contains *mxaD* but in a different place to *M. extorquens*. The order in *Methylobacter albus* BG8 is *mxaA, C, K, D* and then *L* as opposed to the order in *M. extorquens* which is *mxaA, C, K, L* and then *D* (Arps *et al.*, 1995).

1.19k The *mxaC* gene

The mxaC gene has been sequenced and is 1065 base pairs in length (Morris *et al.*, 1995). There is no signal sequence in the polypeptide and therefore is a cytoplasmic protein. No current evidence to a role of the *mxaC* gene product.

1.19L The other methanol oxidation gene clusters

The *mxb* genes are adjacent to the *PQQ* gene cluster (Figure 1.30). The *mxbD* gene encodes a membrane bound sensor kinase which is activated by an unknown signal but results in the soluble DNA binding *mxbM* gene product being activated and stimulating the promoters of *pqq* and *mxa* genes. *MxbN* also stimulates the *mxa* promoter but separately to the other *mxb* genes. The *mxc* genes include *mxcQ* and *mxcE* which are analogous to *mxbD* and *mxbM* except that these genes are required for the transcription of the latter genes and thus are higher in the hierarchy of regulation (see Section 1.21a). The *mxd* genes include *mxdR* and *mxdS* but their functions are unknown.

1.20 The genes involved in pyrrolo-quinoline quinone (PQQ) biosynthesis

PQQ is synthesised from a residue of glutamate and tyrosine (Houck *et al.,* 1991) (Figure 1.5). The processing and the transport of PQQ into the partially folded MDH requires at least seven genes (Biville *et al.*, 1989; Morris *et al.*, 1994; Toyama *et al.*, 1997). Most of the information about the *pqq* genes has been deduced by using complementation of mutant strains. The *pqq* genes in *M. extorquens* are found in two operons (Figure 1.30). The first includes *pqqA, B, C/D* and *E* and the second includes *pqqF* and *G. PqqA* was considered to be a putative polypeptide precursor for the formation of PQQ. Evidence from deletion mutants of *pqqA* in *M. extorquens* found that these cells grow normally on methanol and produced PQQ, although at a reduced level (Toyama *et al.*, 1998). *PqqB* is thought to be involved in transport of PQQ into the periplasm (Velterop *et al.,* 1995). The *pqqC/D* gene is so called as it shows similarity to the N terminal of *pqqC* and the C terminal of *pqqD* from *K. pneumoniae* and in *M. extorquens* a single protein of 42kDa can do the same function (Velterop *et al.*, 1995). *PqqC/D* catalyses the last step in PQQ biosynthesis. In the absence of *pqqC/D* a PQQ intermediate accumulates and can be converted to PQQ by the addition of *pqqC/D* protein extract *in vitro*. The *pqqE* gene product contains a motif that is also found in the MoaA, a protein involved in donating metal ions to cofactors in *E.coli* (Toyama *et al.*, 1997). *PqqF* and *G* show characteristics of divalent cation containing endopeptidases (Mealenberg *et al.*, 1992). Another gene (*mxbM*) has also been implicated in the methanol induced induction of PQQ biosynthesis (Toyama *et al.*, 1998).

1.21a The regulation of methanol oxidation genes.

Growth of cells on multi-carbon substrates gives a low level of methanol dehydrogenase expression (Dunstan *et al.*, 1972; O'Connor and Hanson, 1977). On

transfer to methanol the same cells have approximately six times higher level of MDH activity and a 6 to 10 times higher level of MDH RNA transcripts suggesting a methanol-dependent regulation of the methanol oxidation genes (Morris and Lidstrom, 1992). Using promoter probe vectors in *M. organophilum* seven regulatory genes were located: *mxaB, mxaW, mxbD, mxbM, mxcE* and *mxcQ* (Xu *et al.*, 1993). It was deduced that at least some of these gene products activate the promoters of methanol oxidation genes via a signal transduction pathway. Using transcriptional fusion analysis with the *lacZ*, it was deduced that *mxaB* (found 11kb downstream of *mxaF*) positively regulates the *mxaF* promoter in a methanol dependant and orientation specific manner (Morris *et* al. , 1992). *MxaB* mutants were found to contain no immunoprecipitatable MDH or cytochrome c_L. It was also deduced that *mxaB* has an adjacent promoter (Springer *et al.*, 1998).

MxaW and *mxcU* were also regulated in a methanol dependant manner but not by *mxaB* (Morris and Lidstrom, 1992; Xu *et al.*, 1993). *MxaW* mutations have no effect on bacterial growth and gene databases indicate that no similar gene sequences exist (Springer *et al.*, 1998). Two pairs of genes are particularly responsible for the genetic regulation of methanol oxidation genes. These are *mxbD* and *mxbM*.and *mxcE* and *mxcQ*. *MxbD* and *mxcQ* show sequence homology to the histidine kinase superfamily but are slightly different to each other in their periplasmic loops maybe indicating slightly different roles. They are membrane bound proteins with two membrane spanning domains. *MxbM* and *mxcE* have been shown to be DNA-binding proteins. It has therefore been suggested that signals from the environment (probably including formaldehyde) are detected by the membrane bound kinases leading to autophosphorylation (deVries *et al.*, 1988). This then activates the response-regulated DNA-binding proteins that then increase the rate of transcription. It is thought that there is a hierarchy of methanol oxidation regulation as mutants in *mxcQ* and *mxcE* showed a decrease in the levels of *mxbD*.

Therefore it is suggested that the *mxbD* gene is switched on by one signal and activated by another. As *mxbD* and *mxbM* but not *mxcQ* and *E* are essential for *mxaW, mxcU* and *pqqA* expression a basal level of *mxbD* and *mxbM* expression must exist. This was shown by making a fusion of the *mxbD* promoter with the *xylE* gene. It was also discovered that a region (229 to 129) upstream of the *mxbD* start signal was essential for activity (Springer *et al.*, 1997). Another regulatory gene (*mxbN*) has been identified as essential for methanol oxidation and it is probable that many more are involved in the fine tuning of the system. A summary of the genetic regulation and biosynthesis of MDH is shown in Figure 1.32.

Studies of the regulatory system in *Paracoccus denitrificans* indicate that it has signal sensors and response regulators much the same as in *M. organophilum* (Harms *et al.*, 1993; Stock *et al.*, 1989; Ninfa, 1991). *MxaY* has significant homology to a membrane bound histidine kinase which on activation autophosphorylates and passes the phosphate to *mxaX* which in turn is activated and increases the expression of *mxaF* (Harms *et al.*, 1993). Another gene, *mxaZ,* may also be a membrane spanning regulatory protein. Like *mxaY, mxaZ* stimulates *mxaX* (Yang *et al.*, 1995) although mutants do grow slowly on methanol with a decreased *mxaF* expression. These three regulatory genes are found upstream of the *mxaF* gene and transcribed in the opposite direction (Harms *et* al. , 1993).

RNA polymerase has been purified from *M. extorquens* during growth on methanol and on succinate indicating that the enzymes have the same complement of sigma factors, although run off assays showed a greater specificity for methanol oxidation genes (Davagnino *et al.*, 1998). *M. extorquens* is unusual in that it doesn't have a typical δ70 consensus promoter sequence, however another sequence some 180 base pairs upstream of *mxaF* has been identified (Morris and Lidstrom, 1992).

Although formaldehyde is thought to be a signal to increase the rate of expression of the methanol oxidation genes, in *Methylophilus methylotrophus* (an

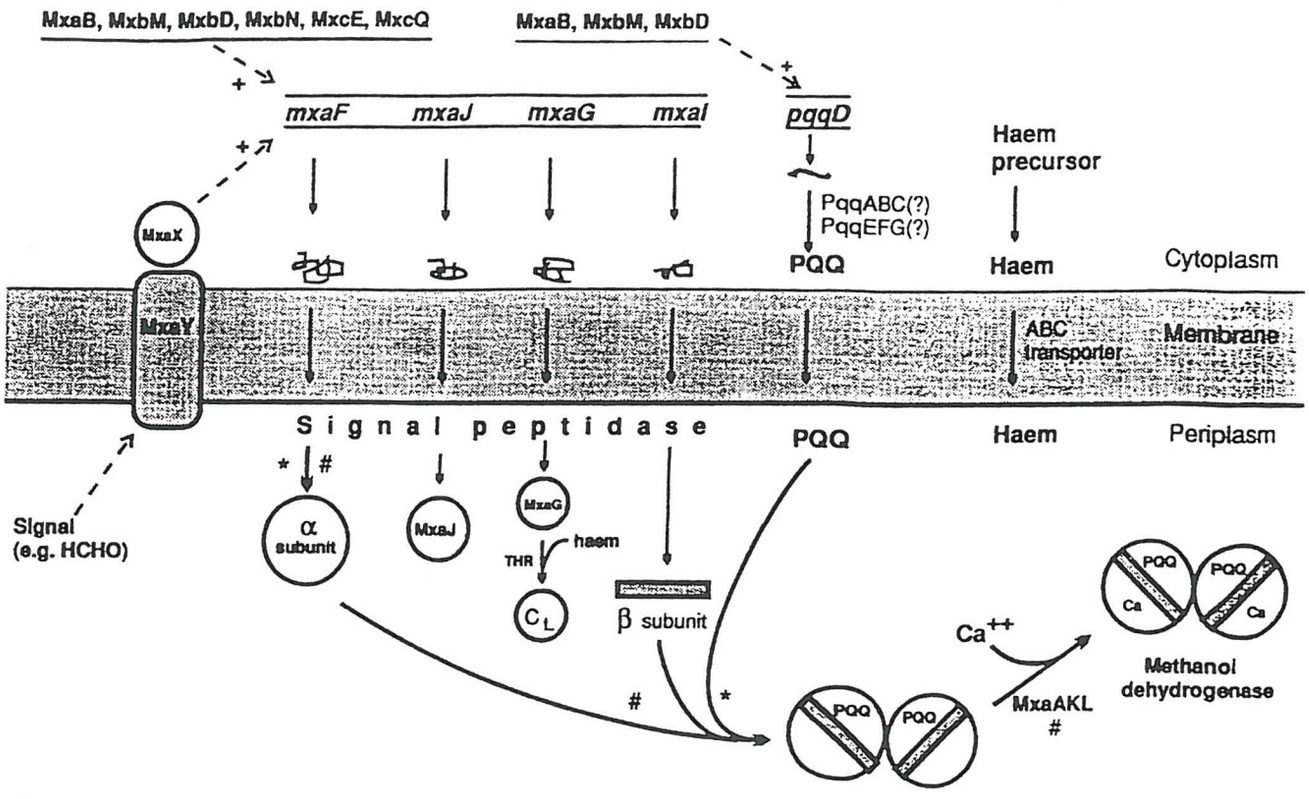

Fig. 1.32 The regulation of the *mxa* gene operon and the proposed pathway of assembly of MDH.

MxcQ is stimulated by an unknown signal and activates *MxcE*. *MxcE* increases the production of *MxbM, D* and *N*. A second unknown signal stimulates *MxaD* and results in *MxbM* activation which along with *MxbN* and *MxaB* (which shows positive feedback) increases the transcription of the mxa operon. The gene products from the mxa operon, with the exception of *MxaL* which remains in the membrane, are transported into the periplasm. In the periplasm MDH is assembled with PQQ and lastly calcium insertion which requires the *MxaA, K* and *L* proteins. The point of PQQ insertion is unknown and could be at either of two stages indicated by the asterix. It is in the periplasm also that the heam group is inserted into cytochrome c_L. (Figure taken from Goodwin and Anthony, 1995)

obligate methylotroph) grown during high methanol conditions the MDH activity decreased, probably to diminish the excess formation of the toxic formaldehyde product. MDH activity is also greatly reduced during periods of oxygen limitation. In *P. denitrificans* the methanol oxidation genes are thought to be controlled by formaldehyde and MDH activity is low under carbon limitation and in low growth rates. In general the methanol oxidation system is only implemented when there is no other preferred system.

1.21b Regulation of pyrrolo-quinoline quinone synthesis

A summary of PQQ synthesis regulation is shown in Figure 1.6. PQQ is only made when the apoenzyme of methanol dehydrogenase (MDH) is present (van Kleef and Duine, 1989). MDH however can be present without the availability of PQQ and in these conditions PQQ can be added to the growth medium to give a fully active enzymes. The promoter for *pqqA* is different from the promoter before the *mxaF* gene promoter (Barta and Hanson, 1993). There is however a promoter similar to that before *mxaF* upstream of *pqqA* in *M. organophilum* suggesting a common regulatory mechanism (Xu *et al.* , 1993). DNA sequence analysis indicates that *pqqA* and *B* are co-transcribed. Two different transcripts are found; either *pqqA* on its own or *pqqA* with *pqqB* (Ramamoorthi and Lidstrom, 1995). *pqqA* encodes a possible precursor of PQQ and *pqqB* produces a product involved in polypeptide translocation (Velterop *et al.*, 1995). *mxbD, M* and *mxaB* increase *pqqA* expression. As the transcription rates of the *pqq* genes and the levels of PQQ are not directly proportional another level of regulation is predicted. In *K. pneumoniae* the *pqqC* and *D* genes encode 29kDa and 10.4kDa proteins respectively . In *M. extorquens* it appears that these two genes have fused and produce a single protein of 42kDa which carries out the functions of both genes. Using a reporter gene *(xylE)* with the *pqqD* promoter it was deduced that *mxbM, mxbD* and *mxaB* are

required for transcription of *pqqD* and *mxbM, mxbD* are particularly responsible for the final PQQ concentration in cells (Ramamoorthi *et al* 1995).

A promoter has been located upstream of *pqqF* and is regulated by the availability of single carbon compounds. It is not however regulated by the same regulatory genes as the MDH genes (Springer *et al.*, 1996).

1.22 The processing and assembly of methanol dehydrogenase and cytochrome c_L

Methanol dehydrogenase is composed of two types of subunit, calcium atoms and the prosthetic group, PQQ. The genes involved in producing an active MDH are expressed in the cytoplasm but the resulting enzyme is found in the periplasm. The polypeptides therefore are transported into the periplasm probably either by the sec or the signal recognition pathway (Goodwin and Anthony, 1995) (Fig. 1.32). For this to occur the polypeptides, are expressed as a preprotein with an extension at the N terminal of approximately 25 amino acids which acts as a signal sequence targeting the protein to the periplasm. The extension is however cleaved off by a signal peptidase in the membrane after translocation. The way in which MDH comes together is poorly understood. It is unclear when PQQ is added as it will not bind to the folded or fully unfolded enzyme. This is in contrast to GDH where PQQ can be added after the enzyme is fully folded, and it is thought that this is because the active site is more accessible in GDH when compared to MDH.

Cytochrome c_L requires several genes for inserting the haem group after translocation into the periplasm (Page and Ferguson, 1989, 1990; Poole *et al.*, 1994). The haem is attached at the reduced haem binding site (CXXCH) and it is possible that this is achieved by an equivalent of a mitochondrial haem c lyase (Beckman and Kranz, 1993; Thonymeyer *et al.*, 1994; Sambongi and Ferguson, 1994).

1.23 Aims of the work presented in this thesis

The work presented in this thesis includes:

1) the production and characterisation of MDH from *Methylobacterium extorquens* with site directed mutations to deduce the roles of specific amino acids
2) analysis of several genes from the *mxa* operon of *Methylobacterium extorquens* which have been mutated
3) the production and characterisation of a unique MDH from *Methylobacterium extorquens* which has both an active site mutation and lacks calcium ions in the active site
4) a study of the effects of growth conditions on the expression of MDH in *Methylobacterium extorquens*

Chapter 2

MATERIALS AND METHODS

2.1 Materials and chemicals

All chemicals were of an analytical grade and obtained from the Sigma Chemical Company Ltd. , Poole, Dorset with the exceptions of those listed below:

Amersham International plc (USB), Aylesbury, Bucks, UK:

Thermo Sequenase ™ florescent labelled primer cycle sequencing kit with 7-deaza-dGTP

AT Biochem Malvern, PA, USA:

Long Ranger ™ gel solution for DNA sequencing (50% stock)

Bio-Rad Laboratories Ltd, Hemel Hempstead, Herts, UK:

SDS-PAGE Low molecular weight standards

Difco Laboratories, Detroit, Michigan, USA:

Bacto-Agar

Bacto® Nutrient agar

Bacto® Tryptone

Bacto® Yeast extract

Nutrient broth

Fisons plc, Loughborough, UK :

AnalaR grade water

Methanol

Life Technologies Ltd, Renfrewshire, Scotland, UK:

1kb DNA ladder

Millipore Corp, Bedford, MA, USA:

Ultrafree-MC 10,000 MW cut-off filtration units

National Diagnostics, New Jersey, USA:

Protogel (30% w/v acrylamide and 0.8% w/v bisacrylamide stock solution protein and sequencing electrophoresis grade)

Phamacia Biotech, Uppsala, Sweden:

Agarose NA

Pharmacia LKB, Milton Keynes, UK

DEAE- Sepharose fast flow ; Hiload superdex 200 ; Hiload Superdex 75; Mono-Q HR 5/5 prepacked anion exchange column; Sephadex G-25 HR 10/10 prepacked fast desalting column; S-Sepharose fast flow

Promega Corporation, Southampton , UK:

Wizard™ minipreps DNA purification system

QIAGEN Ltd, Dorking, Surrey, UK:

QIAEX II gel extracton kit

Satorius GmBH, Gottingen, Germany:

0.2 um Cellulose nitrate filters

Stratagene Cloning Systems , La Jolla, California, USA:

Native *Pfu* DNA polymerase

Cloned *Pfu* DNA polymerase

United States Biochemicals, Cleveland, Ohio, USA:

Sequenase version 2.0 DNA sequencing kit

2.2 Bacterial strains and plasmids

The bacterial strains used are shown in Tables 2.1 and 2.2. Plasmids used are shown in Table 2.3.

Table 2.1 *E. coli* strains and genotypes

Strain	Genotype	Source/ Description
HB101	F^-, *leu*B6, *pro*A2, *rec*A13, *thi*-1, *ara*-14, *lac*Y1, *gal*K2, *xyl*-5, *mtl*-1*rps* L20(Str^r), *sup*E44, hs*d*S20 (r_B^-,n_B^-)	Highly transformable cloning host for growth/purification of plasmid DNA.
CRS603		Carries conjugation-proficient plasmid pRK2013
XL1-Blue	supE44, hsdR17, recA1, endA1, gyrA96, thi1, relA1, lac, [F':proAB$^+$, laci^{0}ZΔ M15, Tn10 (Tet^r)]	Permits blue/white selection;permits superinfection by helper phage for rescue of single stranded phagemid DNA

Table 2.2 ***Methylobacterium extorquens*** **strains and genotypes**

Strain	Genotype	Reference
M. extorquens Wild-type	Rif^r	Nunn & Lidstrom 1986
M. extorquens *mxaF*::kan^r	*mxaF* deletion/substitution mutant, Kan^r	Amaratunga, K 1995 (Southampton University thesis)

Table 2.3 Plasmids

Plasmid	Characteristics	Source/Reference
pBluescriptR II KS+	Amp^r	Stratagene Cloning Sysetems
pTM1	Amp^r, pBluescript II KS+ (2.83kb a *mxa*FJ Sal-X ba I fragment)	Majekodunmi, T Southampton University
pTM2	Amp^r, pTM1 (D303E *mxa*FJ)	Majekodunmi, T Southampton University
pTM3	Amp^r, pTM1 (R331K *mxa*FJ)	Majekodunmi, T Southampton University
pTM4	Amp^r, pTM1 (R331K *mxa*FJ)	Majekodunmi, T Southampton University
pBR322	Amp^r, Tet^r	Promega
pKA1	Amp^r, (Tet^s), pBR322 lacking Sal I site (6.8kb *mxa*FJGI HindIII-BamHI fragment).	Amaratunga, K Southampton University
pRK310	Tet^r, lacZ, IncP1 plasmid	Ditta *et al.*, 1985
pRK310(*mxa*FJGI)	Tet^r, pRK310 with 6.8kb HindIII-Bam HI *mxa*FJGI fragment.	Nunn & Lidstrom, 1986

2.3a Media and growth conditions for *E. coli*

All *E. coli* strains grown for plasmid minipreps and transformations were grown in 10ml volumes of Luria-Bertani (LB) medium. All *E. coli* strains were grown overnight at 37°C in liquid culture with vigourous shaking. All 10 ml cultures were inoculated with a single bacterial colony. Any antibiotics that were added were prepared as described by Sambrook *et al.*, (1989).

2.3b Media and growth conditions for *M. extorquens*

M. extorquens was grown in basal medium (minimal medium) as described by Day and Anthony (1990), by mixing 100ml of stock 1, 0.5ml of stock 3 (see Table 2.4) and any autoclavable carbon sources at pH7.0, for every litre of culture medium required. The volume was made up with deionised water and autoclaved at 121°C (15 pounds per square inch) for 20 minutes. Once below 45°C, 2ml of autoclaved stock 2 (see Table 2.4), any filter sterilised carbon sources and antibiotics were added. Methylamine and succinate were added at a concentration of 0.4% (w/v) before autoclaving. Other carbon sources were sterilized and filtered through a 0.22μm filter and added to autoclaved media at a concentration of 0.2% (w/v) except methanol which was added to a final concentration of 0.5% (v/v). Stock solutions were made up to 100x final concentration, and adjusted to pH 7.0. Cultures were left for one day without shaking at 30°C followed by vigorous shaking for between 3 and 14 days. Baffled flasks were used with a 10% inoculum.

Table 2.4 Stock solutions for minimal media for growing *Methylobacterium extorquens*

Stock solution	Component	g per litre
Stock 1	$(NH_4)_2SO_4$	$30.0gl^{-1}$
	$NaH_2PO_4.2H_2O$	$5.7gl^{-1}$
	K_2HPO_4	$15.3gl^{-1}$
Stock 2	$MgSO_4.7H_2O$	$100.0gl^{-1}$
Stock 3	$CaCl_2.2H_2O$	$5.3gl^{-1}$
	$FeSO_4.7H_2O$	$2.0gl^{-1}$
	$MnSO_4.4H_2O$	$0.2gl^{-1}$
	$ZnSO_4.7H_2O$	$0.2gl^{-1}$
	$CuSO_4.5H_2O$	$0.04gl^{-1}$
	$CoCL_2.6H_2O$	$0.04gl^{-1}$
	Na_2MoO_4	$0.04gl^{-1}$
	H_3BO_3	$0.03gl^{-1}$
	1M HCL	$10.0ml^{-1}$

2.4 Maintainance of bacterial strains

M. extorquens was grown for five days and *E. coli* for eight hours to reach stationary phase and 1ml of culture was mixed with 0.5 ml of sterile glycerol. The sample was than placed at -20°C in a plastic cryogenic tube for long term storage. Cells were retrieved by plating a loopful of glycerol stock onto the

appropriate growth media.

2.5 Small scale extraction of plasmid DNA (miniprep)

From 10 ml overnight cultures of *E.coli* DNA was extracted using the Wizard™ miniprep DNA purification system (Promega) according to the manufacturers instructions.

2.6 Cloning in plasmid vectors

Using an appropriate restriction enzyme, insert DNA of the clone of interest was prepared. This was achieved by adding 1μl of the restriction enzyme with 1μl of the enzyme buffer and making the volume up to 10μl and left for 4 hours at 37°C. Digested DNA was then run on an agarose gel and then purified with the QIAEX II gel extraction kit (QIAGEN) according to the manufacturers instructions. Vector DNA was digested and purified in the same way.

2.7 Ligation of vector and insert DNA

Vector and insert DNA were mixed with DNA ligase buffer (2μl of 10x concentration, i.e. 300mM Tris-HCl, pH 7.8,100mM $MgCl_2$,100mM DTT, 10mM ATP) and 1μl T4 DNA ligase. The mixture was left overnight suspended in 25°C water which was then cooled to 4°C.

2.8 Transformation of *E. coli* by the $CaCl_2$ / heat shock method

A 10ml volume of sterile LB broth was inoculated with 200μl *E. coli* XL1-Blue grown to stationary phase. The culture was placed on ice 10 minutes prior to the end of exponential phase and then spun for 10 minutes in a microfuge. Cells were resuspended in 5 ml of sterile ice cold 50 mM $CaCl_2$ and 10 mM Tris-HCl (pH8.0). After being chilled on ice for 15 minutes the cells were pelleted and resuspended in

850 μl of the calcium solution. Aliquots of 300 μl were made and placed on ice for 1 hour. 15 μl of DNA was added to the competent cells and left for 1 hour on ice. Cells were then placed in a water bath for 2 minutes at 42 °C and again chilled on ice for 5 minutes. 1 ml of LB broth was added to the transformed cells and left to incubate at 37°C. The cells were then pelleted and resuspended in 100 μl of LB broth and plated onto LB broth and incubated overnight at 37°C.

2.9 Confirmation of transformation with the desired DNA

Colonies growing on LB broth with the appropriate antibiotics after transformation were tested for their uptake of the desired plasmid by extraction of the plasmid by the miniprep procedure and analysis by restriction enzyme digestion and agarose electrophoresis.

2.10 Agarose gel electrophoresis

Restriction digests were analysed on a 0.6 % (w/v) agarose gel (nucleic acid grade, Pharmacia) made up in TAE buffer (0.04 M Tris-Acetate, 1 mM EDTA) containing 0.6 μg ml^{-1} of ethidium bromide. The DNA was mixed with 0.1 volume of gel loading buffer (30% v/v glycerol, 0.3% w/v bromphenol blue made up in TE buffer; 10 mM Tris-HCl, pH 8.0; 1 mM EDTA) and loaded on to a gel. A lane with DNA size markers was added made up from 1μl DNA ladder (GIBCO BRL), 8 μl of sterile water and 1μl of gel loading buffer. Electrophoresis was carried out at 70 V for 90 mins in TAE buffer containing 0.6μg ml^{-1} ethidium bromide. The gel was viewed under UV light.

2.11a Automated DNA sequencing

Sequencing of double stranded DNA was done on a LICOR model 4000 automated DNA sequencer. Sequencing reactions were carried out using the solutions provided

in the Thermo Sequenase ™ fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham, UK) listed below:

Table 2.5 The components of the solutions provided in the Thermo Sequenase ™ fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP

Component	Contents
Sequenase ® buffer (5x concentrate)	200 mM Tris-HCl, pH 7.5, 250mM NaCl, 100mM $MgCl_2$
Dithiothreitol	0.1 M.
Labelling mix (dGTP)(5x concentrate)	7.5 μM dGTP, 7.5 μM dCTP, 7.5 μM dTTP.
Termination mix (ddG)	80 μM dGTP, 80 μM dATP, 80 μM dCTP, 80 μM dTTP, 8 μM ddGTP, 50mM NaCl.
ddA, ddT and ddC Termination mixes	As above with appropriate dideoxynucleotide analog (ddA, ddT and ddC)
Sequenase ® Version 2.0 enzyme	13 units/μM (diluted 1:8 in enzyme dilution buffer prior to use).
Enzyme dilution buffer	10 mM Tris-HCl pH 7.5, 5 mM DTT, 0.5 mg ml^{-1} BSA.
Stop solution	95% formamide, 20mM EDTA, 0.05% Bromophenol blue 0.05 % Xylene cyanol FF.

Double stranded DNA (0.4 pmol) was added to 2μl of the appropriate primer labelled with an IRD41 infra red tag (MWG-BIOTECH, Germany) and made up to 25 μl with water. 6 μl of this mixture was added to 2 μl of the appropriate termination mixture containing one of the dideoxynucleotides and 5 μl of sterile light mineral oil added. The mixtures were then added to a thermal cycler (Omnigene, Hybaid) and heated as follows:

Step 1 - 95°C for 5 minutes; Step 2- 95°C for 30 seconds; Step 3 -annealing temperature for 30 seconds; Step 4; - 70°C for 1 minute and steps 2 through 4 repeated for 20 cycles. Formaldehyde loading dye (4 μl) was added to each tube to stop the reaction. 2 μl of each sample was denatured by heating to 95°C for 3 minutes prior to loading on the sequencing gel. Samples not loaded immediately were stored at -20°C.

2.11b Sequencing gel electrophoresis on 5 % Long Ranger ™ gels

The gel solution was prepared by dissolving the following in deionized water and making up to a final volume of 75ml: 31.5g urea; 7.5ml Long Ranger ™ gel solution (50% stock solution); 9 ml Tris/Borate /EDTA (TBE, 10x stock). TEMED (37.5 μl) and 150 μl ammonium persulphate (25% stock, freshly made), were added to 10 ml of the gel solution to make a plug at the bottom of the gel. Once this plug had set, the same volumes of TEMED and ammonium persulphate were added to the remaining 65 °ml of gel solution. The solution was poured between the glass plates of a Sequi-Gen gel nucleic acid sequencing cell (Bio-Rad), kept at an angle of around 10°C , using a clean 50 ml syringe. Any bubbles were excluded with the aid of a "bubble getter" (Promega). The flat edge of a sharks tooth comb was inserted into the top of the gel and left to set overnight. The top of the gel apparatus was covered with clingfilm to prevent the gel from drying out and

shrinking. All gels were run in 0.6x TBE buffer (60ml 10x TBE in 1L), at 1300-1600V. The temperature of gels was maintained at around 40-45 °C. To maximize the number of bases read with a single primer, a second set of the same sequencing reactions was loaded once the bromophenol blue dyefront of the frist set was about 3 cm from the bottom of the gel. Once the bromophenol blue dye front of the second set reached the bottom of the gel, the apparatus was disassembled and the glass plates prised apart, keeping the unsiliconized plate underneath. Two sheets of Whatman 3MM ™ filter paper were laid on top of the gel excluding any air bubbles, and tne gel gently peeled off the glass plate. After laying the paper side down, clingfilm was laid on top of the gel, once again excluding any air bubbles. Once the clingfilm and filter paper were cut to size, the gel was dryed on a model 583 gel dryer (Bio-Rad) at 80°C for 1-2 hours. Once dry, the cling was removed and the gel exposed to x-ray film for autoradiography (Hyperfilm MP, Amersham) for 2-10 days, depending on the radioactivity detected on the dry gel with a Geiger-Muller counter prior to autoradiography.

2.12 Development of X-ray film

Autoradiography cassettes were opened in a dark room and the X-ray film was washed in the following solutions in succession:

Developer	**5 minutes**
Stop solution	**2 minutes**
Fixer	**5 minutes**

After fixing, the film was washed thoroughly with water and left to drip dry.

2.13 QuikChange ™ site directed mutagenesis

Site directed mutagenesis was carried out using the QuikChange™ site directed mutagenesis system (Stratagene Cloning Systems, CA USA) according to the manufacturer's instructions. To a range of pBluescript DNA concentrations (5-50 ng), 5 μl of reaction buffer was added with 250 ng of complementary oligonucleotide primers containing the desired mutation. Deoxyribonucleotides (2.5 mM) were added and overlayed with 30 μl of light mineral oil and placed in the thermal cycler (Omnigene, Hybaid). The reactions were incubated at 95°C for 5 minutes and 1 μl of native or cloned *Pfu* DNA polymerase (2.5 U/ml) and the reactions cycled as followed: Step1- 95°C for 30 seconds; Step2- 95°C for 30 seconds; Step3- 45°C for 1 minute; Step 4- 63°C for 2 minutes per kb of plasmid length. Steps 2 through 4 were repeated for 16 cycles. Following temperature cycling, reactions were cooled on ice for 5 minutes and *Dpn1* (1 μl of 10 U/μl) was added and left to incubate at 37°C for 2 hours. A summary of this mutagenesis system is shown in Figure 2.1.

Following cleavage of the original template DNA by *Dpn1*, 30μl of new, potentially mutated, DNA mixture was added to competent *E. coli* cells and transformed using the $CaCl_2$ heat shock method. Transformed cells were plated onto LB agar supplemented with ampicillin (100 mg/ml) and incubated overnight at 37°C. Individual colonies were added to 10 mlof liquid LB with ampicillin and grown up to stationary phase. Glycerol stocks were made and automated sequencing of the plasmid was used to confirm the presence of the desired mutation

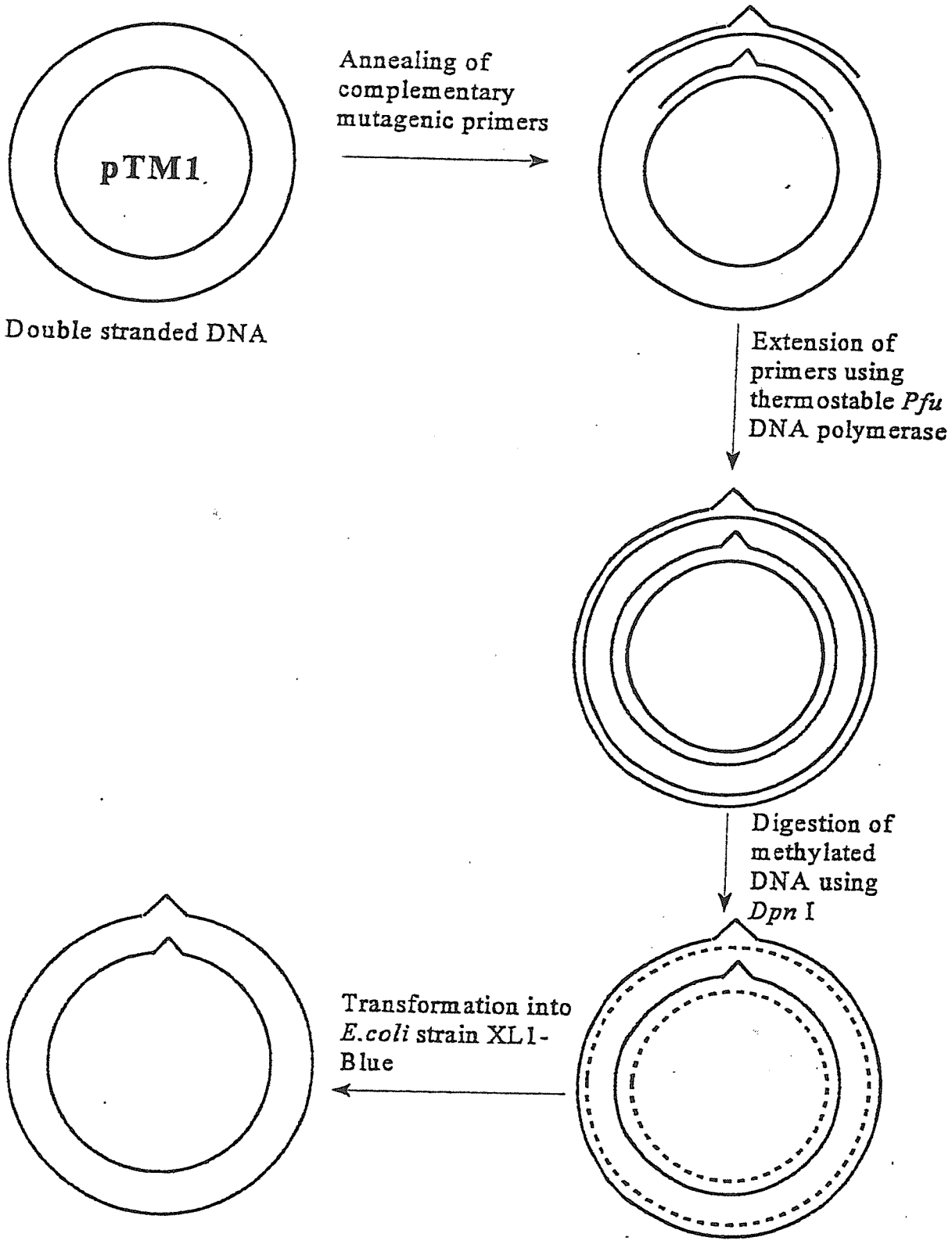

Fig.2.1 The Stratagene QuikChange™ mutagenesis system.

2.14 Synthesis of oligonucleotides

Oligonucleotides used in automated sequencing were synthesised by MWG-BIOTECH (Hamburg, Germany) and primers used in mutagenesis reactions or manual sequencing were synthesised by Oswel (Southampton , UK).

2.15 Harvesting *Methylobacterium extorquens* cells from culture

Cells were harvested at 10,000 rpm for 15 minutes at 4°C. Harvested cells were washed twice and resuspended in 20 mM Tris-HCl pH8 and stored at -20°C if necessary. Cells used for whole cell experiments were washed and resuspended in 25 mM Hepes buffer pH7 and stored at 4°C if necessary.

2.16 Estimation of dry weight of cells in suspension.

Estimation of the dry weight of cells was achieved by measuring the OD of cell suspensions at 650 nm in a PYE Unicam spectrophotometer and calculated by reading from the standard curve shown in Figure 2.2. The standard curve was obtained by suspending washed cells in buffer and measuring the absorbance at various cell dilutions; dry weight was determined by evaporating samples of the original suspension to dryness at 104°C (Dunstan, 1972).

2.17 Preparation of cell free extracts of *Methylobacterium extorquens*.

Equal amounts of cells and 20 mM Tris buffer pH8 were mixed and sonicated using an MSE Soniprep 150 ultrasonic disintegrator for 20 cycles of 30 seconds on 30 seconds off keeping the sample on ice at all times. The sonicated cells were then centrifuged at 10,000 g for 15 minutes at 4°C to remove cell debris followed by membrane removal by centrifugation at 130,000 g for 1 hour at 4°C.

2.18 Purification of methanol dehydrogenase

From the cell free extract as described above, MDH was prepared using a modified method of that described by Day and Anthony (Day and Anthony,1990). The cell free extract was applied to an anion exchange column (25x250 mm) of DEAE Sepharose (Pharmacia), previously equilibrated in 20 mM Tris HCl pH 8.0 and the eluate, including MDH, collected using a fraction collector. All fractions were tested for MDH activity using a micro titre plate assay and also analysed by SDS polyacylamide gel electrophoresis and any MDH containing fractions were pooled together and concentrated to 10 ml using a YM-30 30 kDa cut-off membrane (Amicon). The sample was then applied to a gel filtration column (20x600mm) of Superdex -200 (Pharmacia) equilibrated in 20 mM Tris-HCl pH8.0 containing 200 mM NaCl at a flow rate of 1.0 ml per minute $^{-1}$. Fractions containing MDH were pooled and concentrated to 10 ml using a YM-30 30 kDa cut-off membrane (Amicon). The final step was to dialyse the sample against 20 mM Tris HCl pH 8.0. The final sample was then divided into 1ml samples and stored at -20°C.

2.19 Measurement of methanol dehydrogenase activity

2.19a Dye linked assy system

The dye linked assay system was based on the method used by Anthony and Zatman (1967). To a 1 ml solution containing 100 mM Tris-HCl buffer pH 8, 15 mM ammonium chloride, 5 mM methanol, 86 mM dichlorophenolindophenol (DCPIP) and the enzyme at room temperature, phenazine ethosulphate (PES) was added to start the reaction. The rate of reduction of DCPIP was estimated by following the absorbance at 600 nm (ϵ= 21,500 $M^{-1}cm^{-1}$).

2.19b Microtitre dye linked assay

The dye linked assay described above was adapted for use in micro titre plates. Using the concentrations stated above, a mixture was made with all the assay components except the sample to a total volume of 10 mls. Enzyme samples of approximately 10 μl were added to 200 μl of the assay mix and left at room temperature for at least 5 minutes. A colour change from green to yellow is characteristic of DCPIP reduction.

2.20 Measurement of protein concentration

Protein concentrations were estimated using the bicinchoninic acid (BCA) adapted for micro titre plates (Redinbaugh and Turley, 1986) and read on a Dynatech MR580 micro-ELISA auto reader. Standards were made using bovine serum albumin fraction V at concentrations between 0 and 1.0 mg ml^{-1}.

2.21 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a Mini-Protean ^{R} II electrophoresis cell (Bio-Rad) using the Laemmli buffer system (Laemmli, 1970). The composition of the gels, running buffer, and loading buffer are as follows:

COMPONENT	CONTENTS
12 % resolving gel	4.0 acrylamide\bisacrylamide (37.5:1) 30 % stock 2.5 ml 1.5 M Tris-HCl, pH 8.8, 0.3 % SDS 3.5 ml AnalaR water 10 µl TEMED 40 µl Ammonium persulfate (25% freshly prepared stock)
4.5 % stacking gel	1.5 ml acrylamide\bisacrylamide (37.5:1) 30% stock 2.5 ml 0.5 M Tris-HCl, pH 6.8, 0.4% SDS 6.0 ml AnalaR water 10 µl TEMED 40 µl Ammonium persulphate (25% freshly prepared stock)
Running buffer (5x stock)	15 gl^{-1} Tris base 72 gl^{-1} Glycine 5 gl^{-1} SDS
Loading buffer (5x stock)	1.0 ml 0.5M Tris-HCl, pH 6.8 4.0 ml AnalaR water 1.6 ml 10 % w/v SDS 0.8 ml Glycerol 0.4 ml B- mercaptoethanol 0.2 ml 0.04 % w/v bromophenol blue

Samples (15 μl) were loaded on assembled gel and run at 30 mA for 1 hour. Proteins were detected by staining with Coomassie brilliant blue R250 as described by Weber and Osborn (1975). Low range molecular weight standards were loaded (5 μl) to determine the size of the proteins in the protein sample.

2.22 Western blotting

After separating protein samples by SDS-PAGE proteins were then electrophoretically transferred to nitrocellulose (0.2 mm, Satorius, Goettingen, Germany) using the mini trans-blot system (Bio-Rad) according to the manufacturers instructions. Blotting was carried out at 100 V, 230 mA for 30 minutes in a buffer containing 25 mM Tris, 192 mM glycine and 20% v/v methanol pH 8.3. The nitrocellulose was soaked in blocking solution containing Tris buffered saline (TBS; 50 mM Tris HCl and 150 NaCl pH 7.5), 5 % w/v bovine serum albumin and 0.05 % w/v sodium azide. This solution was replaced with blocking solution containing the primary (rabbit anti MDH alpha subunit, anti beta subunit or anti cytochrome c_L immunoglobulins) at a 1:1000 dilution and left gently shaking overnight. This solution was removed and the nitrocellulose washed in 50 mls of TBS three times and soaked in this solution for 20 minutes. Next the secondary antibody was added (sheep anti rabbit IgG, A and M conjugated to horseradish peroxidase; Serotec and Amersham) in blocking solution at a 1;1000 dilution and was gently shaken for 3 hours. The nitrocellulose was then washed in TBS 4 times and left to soak in TBS for 15 minutes. Finally the horseradish peroxidase substrate was added (10 mg 4-chloro-1 naphthol dissolved in 2 mls of methanol and made up to 20 mls with TBS and 10 μl of 30% hydrogen peroxide) to develop the blot which takes up to 30 minutes. The blot can then be washed and photographed

2.23 Absorption Spectroscopy

All absorption spectra were recorded using either a SLM-AMINCO DW-2000 UV-VIS spectrophotometer or a Shimadzu UV-3000 duel wavelength/ double beam spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Spectra were recorded at a scan speed of 100 nm min^{-1} with a 2 nm spectral band width and a 10 mm light path in 1 ml Hellma Quartz curettes.

Chapter-3

Site directed mutagenesis of methanol dehydrogenase in *Methylobacterium extorquens.*

3.1 Introduction

Site directed mutagenesis is an exceptionally accurate way of determining the function of a specific amino acid in an enzyme. The protein with the mutation may have properties like that of the wild-type protein but in many cases the mutation results in changes in the protein's ability to function correctly and thereby indicating that the residue that was mutated had an important role. Such changes in the protein due to the mutation could result, for example, in the loss of ability of the protein to interact with another protein correctly, the loss of catalysis by the enzyme or the inability of the protein to fold correctly. In some cases the best site directed mutation will be where all function of the protein is removed but in other examples, especially with amino acids which are involved in catalysis, much more information can be deduced from an active protein with altered characteristics.

There are several features of MDH that make it an ideal target for site directed mutagenesis. Firstly the DNA sequence of the *mxaF* (alpha subunit) and *mxaI* (beta subunit) is known. Secondly the x-ray structure has been determined and therefore potentially interesting residues can be located. Thirdly a wealth of knowledge of the enzyme has allowed specific aspects of the enzyme to be studied. Fourthly a fragment of the *mxaF* gene including the active site residues has been isolated and inserted into a plasmid which is ideal for site directed mutagenesis techniques. Previous attempts at site directed mutagenesis in methanol dehydrogenase have proved to be very problematic at several different levels.

Probably the most common way of creating site directed mutants is the

Kunkel method (Kunkel *et al.*, 1987). This method involves production of a DNA template, including the site of interest, in a strain that incorporates uracils. To this DNA, a mutagenic primer to the codon of interest is added and extended in vitro. This DNA is then incorporated into a strain that will not incorporate uracils leaving only the mutated DNA to be replicated. Several attempts to use this method concluded (Majekodunmi, 1997) that the *E. coli* strain used for incorporating uracils was not functioning correctly and in another study (Amaratunga, 1995), the region of interest was shown to be unsuitable for cloning into M13 (a step needed for the Kunkel method). Using helper phage, single stranded DNA was isolated but no mutagenic primers were able to bind as indicated by the inability to prime DNA sequencing. Another method of site directed mutagenesis, a unique site elimination method devised by Deng and Nickloff.(Deng and Nickloff, 1992), has also been attempted (Majekodunmi, 1997). This method involves using two mutagenic primers, one incorporating the mutation of interest and the other removing a unique restriction site, thus providing a means of selection. This technique was attempted using pBR322, a plasmid that includes the gene of interest, as a template. This method however failed to produce any transformants probably due to the size and highly supercoiled nature of the plasmid.

These problems have been overcome by the development of two other mutagenesis techniques. Initially the mutagenesis problem was overcome by synthesising a small sequence of DNA and then cloning it into M13. This method is time consuming but did lead to the production of three active site mutations (see Table 3.1). These mutations were in the roof of the active site chamber and included a switch of cysteines 103 and 104 to serines and a double mutation, with both cysteines being replaced by serine in the same protein (Amaratunga. K,

1995). Because of the problems associated with this method, another method, the Quik™Change method, was developed (Majekodunmi, 1997) (see Figure 3.1 and Section 3.2). Using this method, three mutants were obtained: a mutation at aspartate 303 to a glutamate (D303E) and to an asparagine (D303N), and a mutation of arginine 331 to a lysine (R331K) (Majekodunmi, 1997) (see Table 3.1).

The second problem in creating and analysing site directed mutations in methanol dehydrogenase is achieving sufficient expression of the protein from the mutated DNA to enable a detailed characterisation program (this is discussed more fully in Chapter 6). This has been a problem in five out of six site directed mutants of methanol dehydrogenase produced including D303N, R331K (Majekodunmi, 1997), C103S, C104S and the double mutant C103S/C104S (Amaratunga, 1995). In addition the C103S, C104S and the double mutant C103S/C104S (Amaratunga, 1995) was also prone to loss during purification attempts. As a general rule it was concluded that if a site directed mutant strain does not grow on methanol then the expression of the protein will be poor.

A third problem associated with methanol dehydrogenase site directed mutants is that many of the mutants that have been produced do not yield active enzymes thus restricting characterisation of the mutant protein. This has proved to be the case in D303N, R331K (Majekodunmi, 1997), C103S, C104S and the double mutant C103S/C104S (Amaratunga, 1995).

Despite a prediction that any new mutants of MDH would be hard to create it was decided that the Quik™Change method of site directed mutagenesis would

Table 3.1 Summary of site directed mutations that have been produced in methanol dehydrogenase from *Methylobacterium extorquens*

Residue	Mutation	Growth on methanol?	Growth on methylamine?	Growth on succinate?	MDH produced?	Activity in dye linked assay?
Aspartate 303	Glutamate	yes	yes	yes	yes, near wild-type levels	yes
Aspartate 303	Asparagine	no	yes	yes	yes, but at a very low level	no
Arginine 331	Lysine	no	yes	yes	yes, but at a very low level	no
Cysteine 103	Serine	no	yes	yes	yes, but at a low level	no
Cysteine 104	Serine	no	yes	yes	yes, but at a low level	no
Cysteines 103+104	Serine	no	yes	yes	yes, but only the alpha subunit and at a low level	no

be used to analyse specific MDH residues. This was perhaps more promising in the light of the findings that a bacterial strain where aspartate 303 of MDH was mutated to a glutamate produced large amounts of an active enzyme and has resulted in some very interesting results (Afolabi, 1999).

3.2 The Quik-Change ™ mutagenesis system and the cloning system for expression of MDH in *Methylobacterium extorquens AM1*

To enable the use of this mutagenesis system to be used on methanol dehydrogenase from *Methylobacterium extorquens AM1,* a suitable substrate for the mutagenesis process was produced avoiding the problems discussed in section 3.1. This work was carried out by Tosin Majekodunmi in our laboratory:

Firstly a small region of 2.82kb containing the region of interest, was cloned into pBluscript (a small plasmid which is not supercoiled). This new construct is 5.79kb in total and was named pTM1. The construct has the ampicillin resistance gene so it can be used for screening. This plasmid was suitable for QuikChange™ site directed mutagenesis (see Figure 3.1).

Site directed mutagenesis was carried out using the QuikChange™ site directed mutagenesis system (Stratagene Cloning Systems, CA USA) according to the manufacturer's instructions. To a range of pTM1(discussed above) clone DNA concentrations (5-50 ng) 5 μl of reaction buffer was added with 250 ng of complementary oligonucleotide primers containing the desired mutation. Deoxyribonucleotides (2.5 mM) were added and overlayed with 30 μl of light mineral oil and placed in the thermal cycler (Omnigene, Hybaid). The reactions were incubated at 95°C for 5 minutes and 1 μl of native or cloned *Pfu* DNA polymerase (2.5 U/ml) and the reactions cycled as followed:

Step1- 95°C for 30 seconds;

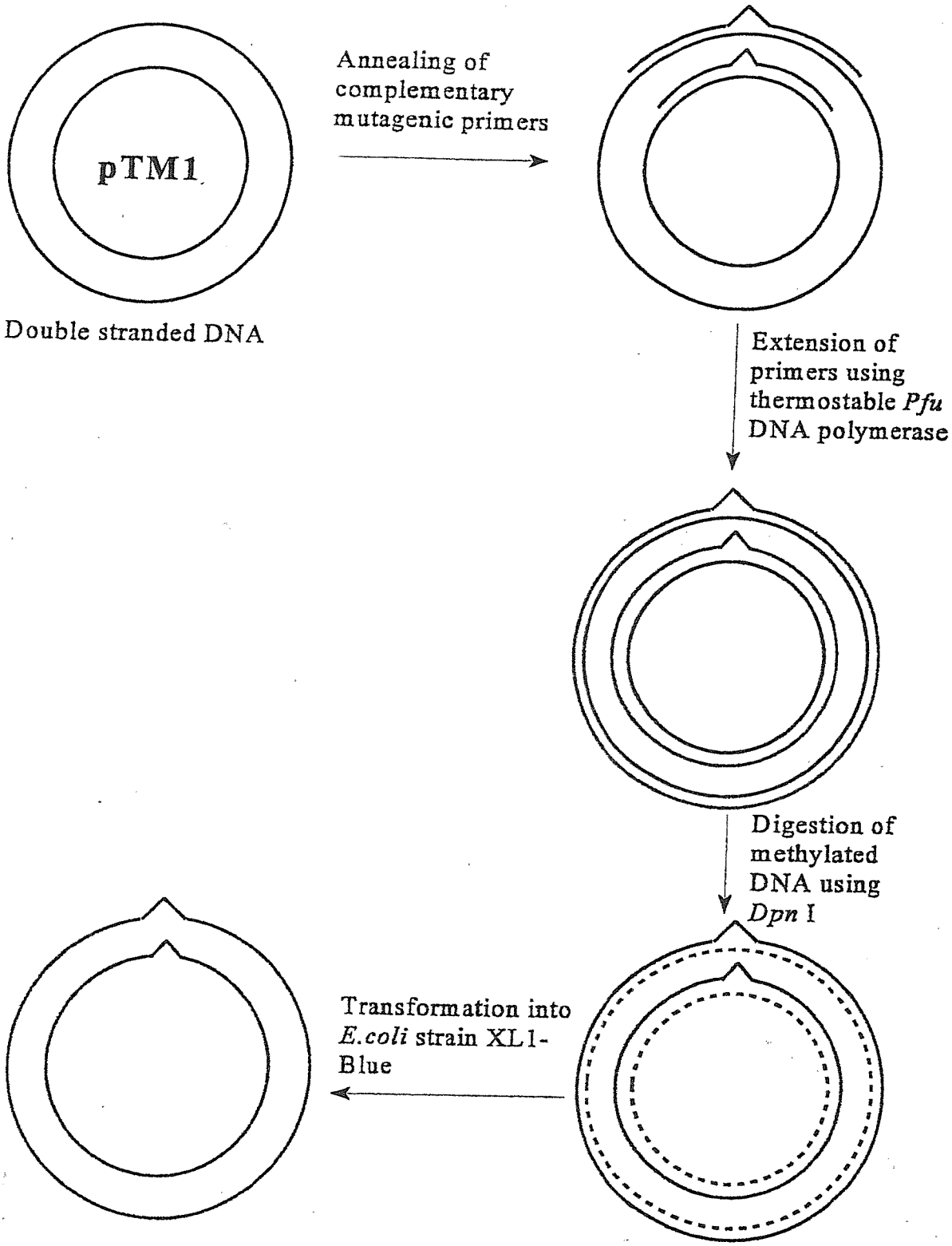

Fig.3.1 The Stratagene QuikChange™ mutagenesis system.

Step2- 95°C for 30 seconds;

Step3- 45°C for 1 minute;

Step 4- 63°C for 2 minutes per kb of plasmid length.

(Steps 2 through 4 were repeated for 16 cycles).

Following temperature cycling, reactions were cooled on ice for 5 minutes and Dpn1 (1 μl of 10 U/μl) was added and left to incubate at 37°C for 2 hours. Dpn1 does not cleave DNA that has not been methylated, for example, the newly synthesised mutant DNA in this experiment. (The template DNA, which was produced in *E. coli* (XL-1 blue) which contains dam methylase, is methylated and is removed by Dpn1). 30μl of this mixture was added to competent *E. coli* (XL1 blue) cells and transformed using the $CaCl_2$ heat shock method. Potentially transformed cells were plated onto LB agar supplemented with ampicillin (100 mg/ml) and incubated overnight at 37°C. In these cells, which will only grow on ampicillin if transformed, the nick left in the DNA, between the end of the extended DNA and the beginning of the primer, is repaired by DNA ligase. Individual colonies were added to 10 ml of liquid LB with ampicillin and grown up to stationary phase and glycerol stocks were made.

Plasmid purification from these cells and DNA sequencing, as described in the methods section, is used to ensure that the desired mutation has been achieved. The mutated section of DNA is excised and inserted into a construct to complete the *mxaF* gene. This complete, mutated, *mxaF* gene is then placed into another plasmid (pRK310) that is capable of replication in *M. extorquens* (Ditta *et al.*, 1985) and has the tetracycline resistance gene thus making it suitable for selection. This plasmid is transformed into *M. extorquens* using triparental mating.

Triparental mating uses three different strains of bacteria with different genetic properties. One strain has the gene of interest with the required site

directed mutation and is referred to as the donor strain. Another strain has genes that allow mobilisation of plasmids from one strain to another and this strain is referred to as the mobilising strain. The final strain is the recipient strain that will receive the mutated plasmid and express the mutant protein. The donor strain was created by transforming the plasmid (pRK310) containing the gene for the alpha subunit of methanol dehydrogenase (*mxaF*) with a point mutation at aspartate 303 into *E. coli* (Hb101) using the calcium chloride/heat shock method. As pRK310 has a tetracycline resistance gene selection of *E. coli* cells that were successfully transformed was achieved by growing cells on LB agar in the presence of the appropriate concentration of tetracycline.

The mobilising strain was created by transforming *E. coli* (CSR600) with a plasmid (pRK2013) containing the *tra* genes that allow mobilisation of plasmids form one strain to another. The *M. extorquens* recipient strain has no *mxaF* gene (Amaratunga *et al.*, 1996). A mixture of all three of the above strains was made by adding a sterilised loop full of each strain onto minimal medium agar containing methanol, methylamine, tetracycline and kanamycin then left at 30°C for the recipient strain to grow. The theory behind this procedure is that the mobilising plasmid will enter the donor strain. This will allow both the mobilising plasmid and the *mxaF* mutant plasmid, in the donor strain to enter the media and transform the recipient strain conferring tetracycline resistance. Thus, only a successfully transformed recipient strain will have the tetracycline resistant phenotype. Because the mobilising plasmid doesn't contain an origin of replication, it will be lost from the recipient strain in subsequent cell replication.

3.3 Design of mutagenic primers

Critical to the success of the mutagenesis system is the design of the mutagenic primers. Several factors must be considered in their design. These include: that the primers are between 25 and 45 bases in length, the melting temperature is approximately 10°C higher than the extension temperature of 68°C, the mutation codon is in or near to the middle of the primer and the primer should have a minimum GC content of 40%.

3.4Analysis of the residues that coordinate to the calcium ion in methanol dehydrogenase by site directed mutagenesis

3.4a Introduction

Two amino acid residues in the active site of methanol dehydrogenase, glutamate 177 and asparagine 261, coordinate to the calcium atom (see Figure 3.2). A programme of site directed mutagenesis of these two residues may produce interesting findings they are probably critical to the catalytic efficiency and structural maintenance of the enzyme due to their association with the calcium atom.

The calcium atom is predicted to have a role as a Lewis acid (although another nearby residue arginine 331 may fulfill this role) in the catalytic mechanism polarising the C5 carbonyl of pyrrolo-quinoline quinone preparing it for attack by the substrate oxygen (see Figures 1.27b and 1.28 and Section 1.12e). The calcium binding residues coordinate the calcium maintaining, it in the correct

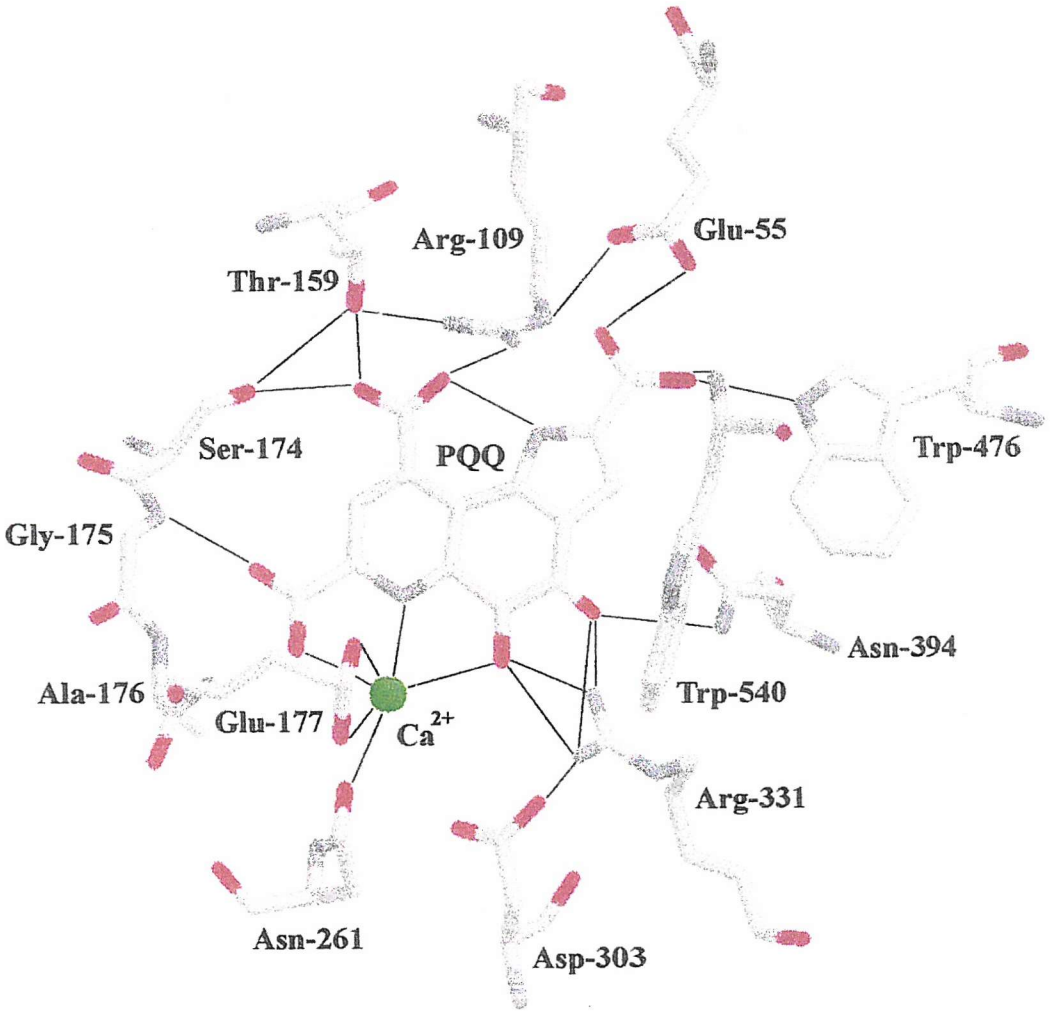

Fig. 3.2 The active site of methanol dehydrogenase
The active site of MDH contains PQQ, calcium and many amino acids including the proposed catalytic base aspartate 303. There are many interactions between PQQ, calcium and the active site amino acids of MDH.

position. Site directed mutagenesis of these residues may give further in sites into the mechanism of MDH.

The calcium atom has also been implicated, by experiments involving the reconstitution of the enzyme lacking metal ions, in maintaining the prosthetic group in the correct orientation (see Section 1.12e). Such conclusions were based on the change in absorbance spectra and concurrent return of activity upon reconstitution. The correct coordination of the calcium would probably be important for this structural role. Site directed mutagenesis of the calcium binding residues may result in a better understanding of the structural roles of calcium and the reconstitution process. Another reason to study these residues is that they could be involved in binding the substrate. Would site directed mutagenesis of these residues alter the affinity for the substrate or change the position of the bound substrate so as to alter the catalytic efficiency?

Glutamate 177 has also been suggested to be an active site base in the mechanism of MDH (see section 1.18). Site directed mutagenesis of this residue could possibly deduce if this residue acts as an active site base or not despite recent findings that this role is probably carried out by aspartate 303 (Anthony *et al.*, 1994; Ghosh *et al.*, 1995).

3.4b Site directed mutagenesis of asparagine 261 and glutamate 177, the calcium binding residues at the active site of methanol dehydrogenase, using the QuikChange™ mutagensis system.

Like all site directed mutagenesis experiments, the choice of what change to make to the target residue is a critical one. In the case of glutamate 177 several exchanges were considered. A change to glutamine would retain a similarly sized

amino acid in this position but removes the charge. Another possible change could be to change the glutamate to an alanine thus removing the charge but also reducing the size of the residue which could result in allowing easier access for the substrate but probably greatly affecting the position and therefore the role of the calcium atom. One other change that cannot be overlooked is a change from a glutamate to an aspartate which would retain the negative charge at the position but slightly decrease the size of the residue at this position. Possibly this last mutation could be the most rewarding as the retention of charge could permit the enzyme to function normally. This has been backed up by the finding that the reverse mutation, ie from aspartate to glutamate, produced an active enzyme in the active site residue aspartate 303 (Afolabi, 1999).

In the case of asparagine 261 the same three changes as described above for glutamate 177 (ie to glutamine, aspartate and alanine) are probably the best candidates for revealing new information as to the roles of this residue.

The QuikChange™ mutagenesis system is described in Section 3.2. The complementary mutagenic primers used are shown below along with the wild type DNA sequence at the site of interest. The amino acid of interest highlighted in bold type. Also shown below is the sequencing primer used for determining if the mutagenesis process has been successful (see Chapter 2).

Glutamate 177 (Wild-type) Sequence
5'-GGC TCC TCG GGC GCC **GAG** CTC GGC GTG CGC GGC TAC CTG-3'
3'-CCG AGG AGC CCG CGG **CAC** GAG CCG CAC GCG CCG ATG GAC-5'

Complementary primersused for the substitution of Glutamate 177 to Aspartate
Primer 1 5'- CG GGC GCC **GAC** CTC G-3'
Primer 2 3'- GC CCG CGG **CTG** GAG C-5'

Complementary primers used for the substitution of Glutamate 177 to Alanine
Primer 1 5'- CG GGC GCC **GCG** CTC G-3'
Primer 2 3'- GC CCG CGG **CGC** GAG C-5'

Complementary primers used for the substitution of Glutamate 177 to Glutamine
Primer 1 5'- CG GGC GCC **CAG** CTC G-3'
Primer 2 3'- GC CCG CGG **GTC** GAG C-5'

Wild-type sequence around Asparagine 261
5'-ATC TAC TTC GGC ACC GGC **AAC** CCG GCG CCG TGG AAC GAG-3'
3'-TAG ATG AAG CCG TGG CCG **TTG** GGC CGC GGC ACC TTG CTC-5'

Complementary mutagenic primers for the replacement of Asparagine 261 with Aspartate
5'-AC TTC GGC ACC GGC **GAC** CCG G-3'
3'-TG AAG CCG TGG CCG **CTG** GGC C-5'

Complementary mutagenic primers for the replacement of Asparagine 261 with Alanine
5'-AC TTC GGC ACC GGC **GCC** CCG G-3'
3'-TG AAG CCG TGG CCG **CGG** GGC C-5'

Complementary mutagenic primers for the replacement of Asparagine 261 with Glutamine
5'-AC TTC GGC ACC GGC **CAG** CCG G-3'
3'-TG AAG CCG TGG CCG **GTC** GGC C-5'

Sequencing primer for sequencing mutations at amino acid positions 243, 261, 303 and 331
5'-AGA ACC CCC ATT ACG GT-3'

Sequencing primer for sequencing mutations at amino acid positions 177 and 205
5'-G CTC ACG ATC GCC CCC TA-3'

3.4c Results of mutagenesis experiments

The purification of the plasmid used for the mutagenesis (pBluescript), proved to be successful approximately 95% of the time as indicated by the intensity of bands of the appropriate size on an agarose gel.

When the potentially mutated plasmid was transformed into *E. coli*, very few successful transformants were produced, indicating that either the plasmid DNA had been lost during the mutagenesis process or the strain was not receptive to transformation by the plasmid. In addition to this, when the few colonies that did grow on ampicillin plates were selected and grown up to 10 ml and the mutated plasmid extracted, no DNA could be detected. To overcome this problem the ampicillin concentration in the growth media was increased from 100mg/ml to 200mg/ml so that the chances of any non transformed bacteria growing on the selection plates was decreased. These results were intriguing as the mutagenesis control sample where mutagenesis was not attempted virtually always seemed to transform bacteria efficiently. To test if the PCR may have affected the template DNA, a sample of DNA was removed before and after the mutagenesis reactions and analysed on an agarose gel. To counteract this problem a change from *E. coli* strain XL1 to HB101 was carried out. This strain however did not appear any more receptive to transformation. Also another method of transformation was attempted (Goodwin, M. Personnel communication). This method is different from the usual $CaCl_2$/heat shock method (see Chapter 2); instead of leaving the recipient cells on ice for one hour prior to the addition of the mutated plasmid they were now left on

ice for just five minutes. The cells were then spread directly onto preheated (37°C) LB agar plates containing ampicillin and incubated at this temperature overnight. This method proved to make no difference initially but later when transformation was achieved by either of the above methods the modified method proved more efficient.

It was also considered that the mutagenic primers may not be permitting the PCR process. To test this theory that the primers may not be binding to the template DNA effectively the same primers were used to sequence wild-type DNA. In all cases the results were inconclusive. For this reason many of the mutagenic primers were redesigned to decrease the melting point and to decrease the chances of secondary structure formation. Initially this did not result in any change of fortune but eventually more successful transformations which did contain DNA of the appropriate size resulted.

To test that the plasmid DNA from the transformed bacteria contained the appropriate mutation, automated DNA sequencing was carried out. This led to further problems. Initially the sequencing results were of very poor quality, probably due to excessive secondary structure in the DNA caused by it being GC rich, and it was not possible to determine if the desired mutation had been produced. To overcome this problem a change to diazodideoxynucleotides from dideoxynucleotides in the sequencing reactions was undertaken and was shown to be beneficial. In addition to this alteration, on the advice of Simon Baker (personal communication), it was decided to add 3% dimethylsulphoxide (DMSO) to the mutagenesis and sequencing reactions. This organic solvent also restricts secondary structure formation and was also shown to be beneficial. When the sequence of the plasmid DNA was able to be determined, it was always of wild-type. To help overcome this problem, the length of time that the original wild-

type template (methylated) was digested by the action of Dpn1 was increased to three hours thus increasing the chances of removal. This did not result in less wild-type transformants being produced. At this point it was decided to target other residues for site directed mutagenesis. The first of these was aspartate 303, the proposed active site base in the mechanism of methanol dehydrogenase.

3.5 Analysis of aspartate 303 in the active site of methanol dehydrogenase from *Methylobacterium extorquens.*

3.5a Introduction

Despite more than 30 years of study the catalytic mechanism of methanol dehydrogenase is poorly understood. There several reasons why the study of the catalytic mechanism of methanol dehydrogenase has been difficult. Firstly, when the enzyme is purified it is always found in the semiquinone form (deBeer *et al.*, 1983; Frank *et al.*, 1988; Dijkstra *et al.*, 1989). The inability to purify the enzyme in the oxidised form prevents experiments following the reduction of the enzyme. Attempts to produce the oxidised form using cyanide with Wursters Blue or PES have produced inactive enzymes with altered absorbance spectra (Frank *et al.*, 1988).

A second problem is that in the absence of any added substrate the enzyme, at high pH and in the presence of ammonia, catalyses a measurable rate termed "the endogenous rate" (Anthony & Zatman, 1964; Duine *et* al., 1978, 1980). The endogenous reductant is thought to be a low concentration of contaminating alcohols and aldehydes found, for example, in buffers and other reagents. Studies indicate that there are approximately 90 moles of endogenous reductant for every mole of methanol dehydrogenase. The mechanism of oxidation of the endogenous reductant is the same as that for added methanol Beardmore-Gray *et al.*, 1983;

Anthony, 1986).

Linked to the endogenous rate problem another difficulty in determining the mechanism and kinetics of methanol oxidation is the very high affinity of methanol dehydrogenase for methanol (km 20 μM) ; therefore at very low concentrations of methanol there will be a very high catalytic rate.

Using stopped flow spectrophotometry methanol dehydrogenase has been shown to have a ping-pong mechanism. This involves reduction of PQQ, product release and 2 sequential electron donations to cytochrome c_L leaving the oxidised form (Dijkstra *et al.*, 1989) (Fig. 1.24; 1.25). The rate-limiting step for the reaction is the conversion of the oxidised form containing the substrate to the reduced form of the enzyme with the product. It is this step that requires an enzyme activator such as ammonia or glycine ethyl ester. Much of the focus on the mechanism has been centred on the C5 carbonyl group. This has been because of its reactivity to nucleophilic reagents, with adducts forming with methanol, cyanide, aldehydes, ketones, urea, ammonia and amines (Itoh *et al.*, 1993; Duine *et al.*, 1987; Dekker *et al.*, 1982) (Fig.1. 26). Of particular interest is the reaction of the carbonyl with cyclopropranol (Frank *et al.*, 1989).The reaction leads to a covalent adduct and inactivation of the enzyme. It is thought that an active site base removes a proton from cyclopropranol allowing ring opening and adduct formation.

The role of the calcium atom is thought to be to act as a Lewis acid by polarising the C5 carbonyl allowing the attack by an oxyanion or hydride (Anthony *et al.*, 1994)(Fig 1.27 and 1.28). This role however potentially could be achieved by arginine 331. Evidence for this role of the calcium atom has come from studies of methanol dehydrogenase reconstituted with Strontium or Barium (Harris & Davidson, 1994; Goodwin & Anthony, 1996)(see Section 1.12f). The calcium also may bind the oxygen atom of the substrate although this seems

unlikely on analysis of the x ray structure (Xia *et al.*, 1999). The duel role of calcium, ie catalytic and structural has been seen in other enzymes such as phosholipase A_2 (Scott *et al.*, 1990).

Two catalytic mechanisms have been proposed In the first an oxyanion is produced by proton extraction and then attacks the C5 carbonyl forming a hemiketal intermediate (Figure 1.27). The pyrrole nitrogen allows the C4 ionisation and facilitates the extraction (Itoh *et al.*, 1998). The second proposed method is a classic acid/base hydride transfer (Figure 1.28). What evidence there is indicates that the hemiketal mechanism is more likely. This includes a slight change in the absorbance spectrum during the reaction with deuterated methanol possibly indicating an intermediate species (Frank *et al.*, 1988; Dijkstra *et al.*, 1989). No evidence for the formation of an intermediate could be obtained from the barium containing enzyme. Deuterated benzyl alcohols have indicated that the pro-s hydrogen is removed from the substrate and the pro-r hydrogen is retained in the aldehyde product.

The active site base is proposed to be aspartate 303. Studies of the X-ray structure indicate that although there is a close proximity of an arginine to aspartate 303 (which could diminish the ability to act as an active site base) other interactions with the arginine may allow the aspartate to have a sufficiently low pK for proton abstraction.

3.5b Site directed mutation of aspartate 303 in the active site of methanol dehydrogenase from *Methylobacterium extorquens*.

Probably the best way of analysing the role of a specific amino acid in a protein is to mutate that residue to another amino acid and compare the mutant enzyme to the wild-type protein. Such a mutation was attempted at the proposed active site

base residue aspartate 303. Like all site directed mutagenesis experiments, the choice of what amino acid exchange to make is an extremely important one. In the case of aspartate 303 it was decided to change this residue to a glutamate. This would have the effect of retaining the negative charge at this position but would make the carboxylate side chain closer to PQQ.

The QuikChange™ mutagenesis system is described in Section 3.2. The complementary mutagenic primers used are shown below along with the wild type DNA sequence at the site of interest. The amino acid of interest is highlighted in bold type. Also shown below is the sequencing primer used for determining if the mutagenesis process has been successful (see Chapter 2).

Aspartate 303 (Wild-type) Sequence
5'-G CAC GAC GAG TGG **GAC** TAT GCC GGA-3'
3'-C GTG CTG CTC ACC **CTG** ATA CGG CGG-5'

Complementary mutagenic primers for the replacement of Aspartate 303 with glutamate
Primer 1 5'-G CAC GAC GAG TGG **GAG** TAT GCC GGA-3'
Primer 2 3'-C GTG CTG CTC ACC **CTC** ATA CGG CGG-5'

Complementary mutagenic primers for the replacement of Aspartate 303 with Asparagine
5'-G CAC GAC GAG TGG **AAC** TAT GCC GGA-3'
3'-C GTG CTG CTC ACC **TTG** ATA CGG CGG-5'

Sequencing primer for sequencing mutations at amino acid positions 243, 261, 303 and 331
5'-AGA ACC CCC ATT ACG GT-3'

3.5c Results of mutagenesis experiments

Using exactly the same protocol as described above for the attempts to create site

directed mutants with the calcium binding residues, two D303E mutant strains were produced as indicated by DNA sequencing of the purified plasmids from bacteria transformed with the products from the PCR mutagenesis system. This mutant strain will be discussed in more detail in Chapter 5.

3.6 Analysis of tryptophan 243 at the active site of methanol dehydrogenase in *Methylobacterium extorquens* .

3.6a Introduction

The active site of methanol dehydrogenase is found in the alpha subunit. It contains PQQ and a calcium atom in a hydrophobic chamber which is reached via a hydrophobic funnel (see Figure 1.18). PQQ has many interactions with the surrounding amino acids and also some to the calcium atom (see Figure 3.2). In *M. extorquens,* tryptophan 243 forms the floor of the active site (see Figure 3.3). It is in the same plane as PQQ. To understand the roles of this residue, a program of site directed mutagenesis using the QuikChange™ system described in Section 3.2 was undertaken.

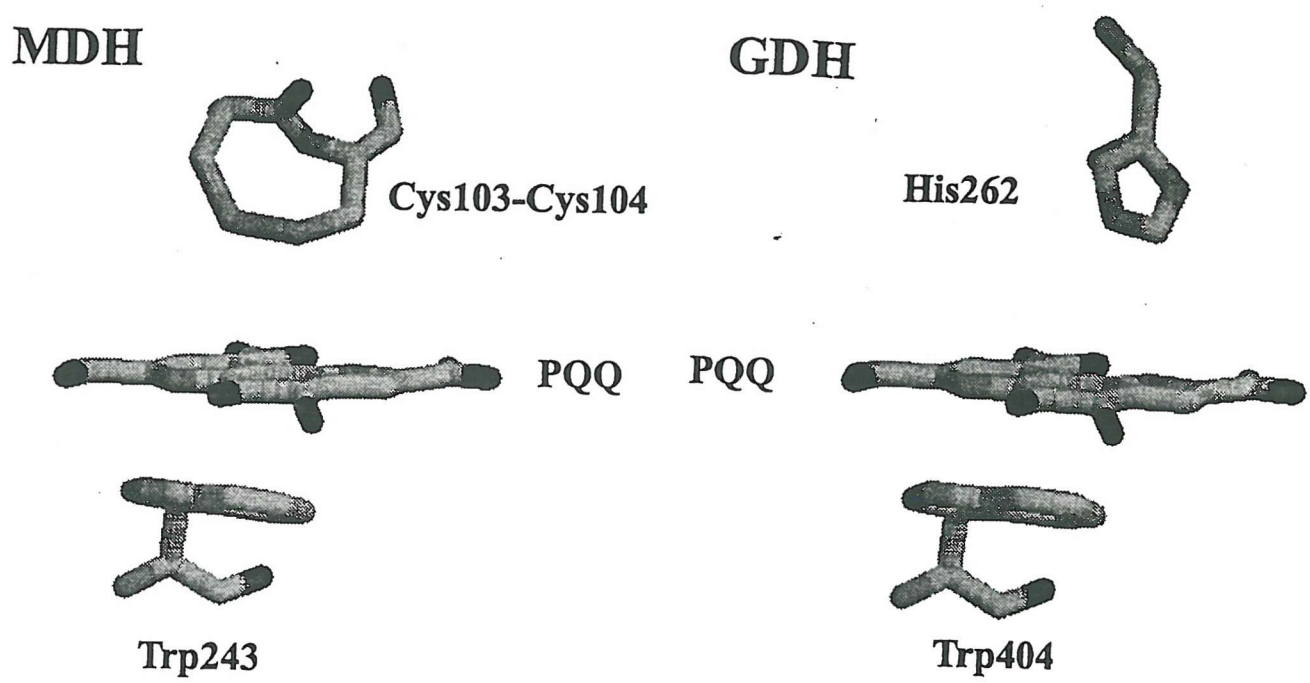

Fig. 3.3 The ceiling and floor of the active site chamber of methanol dehydrogenase. The ceiling of the active site chamber of MDH is formed from a novel disulphide bridge between residues Cys 103 and Cys 104 that form a nine membered ring. The floor of the active site is formed by Typ 243 which is found in the same plane as PQQ. For comparison the equivalent residues in GDH are shown: the ceiling is formed by His 262 and the floor by Typ 404. (Figure taken from Goodwin and Anthony, 1998)

3.6b Site directed mutagenesis of tryptophan 243.

In an attempt to create an enzyme with a mutation at residue 243 that would retain some of the characteristics of the wild type enzyme it was decided that a mutation from a tryptophan to a tyrosine would be a suitable exchange. This would retain a polar, aromatic residue at this point and hopefully only have minor a effect on the active site arrangement.

The complementary mutagenic primers used are shown below along with the wild type DNA sequence at the site of interest. The amino acid of interest is highlighted in bold type. Also shown below is the sequencing primer used for determining if the mutagenesis process has been successful (see Chapter 2).

Wild-type sequence around Tryptophan 243
5'-GGC GGC ACC AAC **TGG** GGC TGG TAC GCC TAC-3'
3'-CCG CCG TGG TTG **ACC** CCG ACC ATG CGG ATG-5'

Complementary primers for the substitution of tryptophan 243 with tyrosine
5'- GGC ACC AAC **TAC** GGC TGG TAC-3'
3'- CCG TGG TTG **GTA** CCG ACC ATG -5'

Sequencing primer for sequencing mutations at amino acid positions 243, 261, 303 and 331
5'-AGA ACC CCC ATT ACG GT-3'

3.6c Results of mutagenesis experiments

Despite several attempts to produce this site directed mutation, no mutant strain was produced. The possible reasons for this are probably the same as the problems encountered in producing site directed mutants of the calcium binding residues discuses in Section 3.4c.

3.7 Analysis of Lysine 205 near the active site funnel of methanol dehydrogenase in *Methylobacterium extorquens.*

3.7a Introduction

The electron transport chain for the oxidation of methanol involves the interaction and electron transfer between several proteins (see Section 1.5). The interaction between MDH and cytochrome c_L, the first step in the chain, has been widely studied and research has concentrated on the many lysines that are found in the alpha and beta subunits (see Section 1.8c). Analysis using TNBS, a compound that reacts with lysines, indicated three critical lysine residues namely the residues at positions 30, 205, and 583 (Majekodunmi, 1997). Interestingly residues 30 and 583 are found at the opposite side of the enzyme to the active site.

Lysine 205 on the other hand is located quite near to the active site funnel of MDH (see Figure 3.4). Other residues have also been implicated using cross linking reagents and dual labelling with TNBS and AMCA. To understand the importance of these residues site directed mutagenesis could be used.

3.7b Site directed mutagenesis of lysine 205.

Because of the position of lysine 205 on the surface of MDH it was decided that the best replacement for this amino acid would be a threonine. This would abolish any effects that the positively charged lysine could have in the interaction between MDH and cytochrome c_L. Would such a mutation reduce or abolish any interaction between MDH and cytochrome c_L or would it have no effect at all ?.

The QuikChange™ mutagenesis system is described in Section 3.2. The

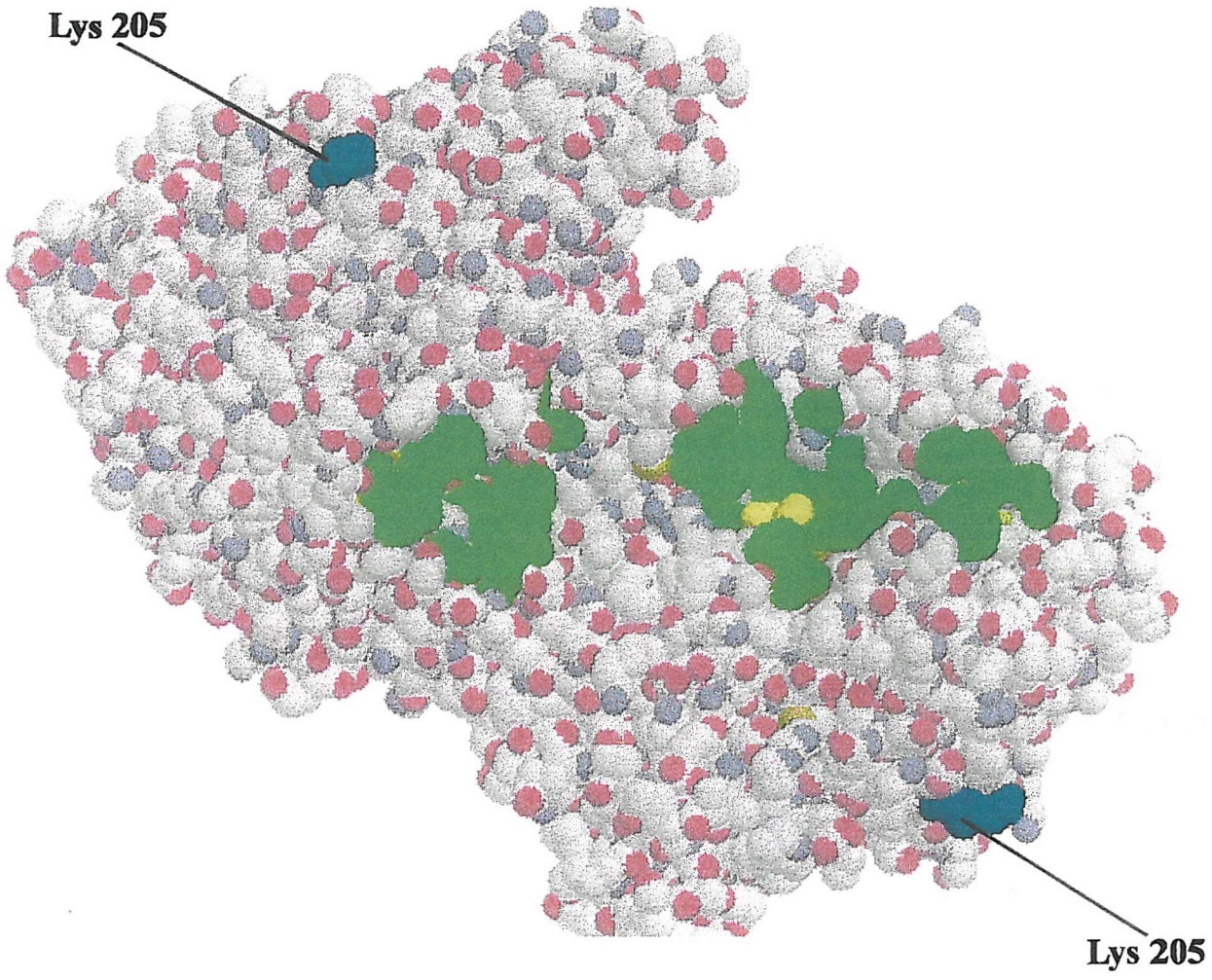

Fig. 3.4 The tetrameric structure of MDH showing lysine 205. Lysine 205 is highlighted in blue. The hydrophobic funnel leading to the active site is shown in green. Cys-103, in the roof of the active site is shown in yellow (Figure taken from Majekodunmi, 1997)

complementary mutagenic primers used are shown below along with the wild type DNA sequence at the site of interest. The amino acid of interest highlighted in bold type. Also shown below is the sequencing primer used for determining if the mutagenesis process has been successful (see Chapter 2).

Wild-type sequence around lysine 205
5'-GGT CCG GAC AAG GAC CTG CTG CTG CTG-3'
3'-CCA GGC CTG TTC CTG GAC GAC GAC GAC-5'

Complementary primers for the substitution of lysine 205 with threonine
5'- GGT CCG GAC ACG GAC CTG CTG-3'
3'- CCA GGC CTG TGC CTG GAC GAC-5'

Sequencing primer for sequencing mutations at amino acid positions 177 and 205
5'-G CTC ACG ATC GCC CCC TA-3'

3.7cResults of mutagenesis experiments

Despite several attempts to produce this site directed mutation, no mutant strain was produced. The possible reasons for this are probably the same as the problems encountered in producing site directed mutants of the calcium binding residues discuses in Section 3.4c.

3.8 Analysis of arginine 331 in the active site of methanol dehydrogenase in *Methylobacterium extorquens* .

3.8a Introduction

Arginine 331 is located at the active site of methanol dehydrogenase in *M. extorquens (*see Figure 3.2). An arginine is also found in the equivalent position in MDH from other strains (Xia *et al.*, 1992; White *et al.*, 1993; Anthony *et al.*, 1994; Ghosh *et al.*, 1995; Xia *et al.*, 1999). By contrast, models of ADH and

GDH indicate that in these enzymes the arginine is replaced by a lysine (Cozier *et al.*, 1995; Cozier and Anthony, 1995) (see Figure 1.23). The position in the active site and the conservation of the positively charged residue at this site indicates that the residue may have an important role. In *M. extorquens* the arginine is coordinated to PQQ and Aspartate 303, the enzyme's active site base.

Although it is generally considered that the calcium atom at the active site of MDH has the role of a Lewis acid polarising the C5 carbonyl it is also a possible that this role may be done by arginine 331 (Xia *et al.*, 1996) (see Figures 1.27 and 1.28). This residue is also very close to the substrate binding site and it is possible that the amino group may interact with the substrate's alcohol group and if so may have a role in substrate binding or release of the product.

3.8b Site directed mutation of arginine 331 by the QuikChange mutagenesis system

It was decided that probably the best residue for the arginine to be changed to would be a lysine as it would retain the charge at this position of the active site. This mutation would however increase the size of the residue at this point bringing the ϵ-amino group of lysine closer to the C4 carbonyl and forming new hydrogen bonds with the C-5 carbonyl. This could have effects on the substrate binding as the substrate binding site will be smaller probably restricting only smaller alcohols to act as substrates. This mutation may decrease the interaction of the residue at position 331 with the calcium ion which could have interesting implications if the calcium ion acts as a Lewis acid in the reaction mechanism. The mutation would also change the interaction of the residue at position 331 and the active site base (Aspartate 303) which would probably have implications in the reaction mechanisms. A change to an alanine was also attempted.

The QuikChange™ mutagenesis system is described in Section 3.2. The complementary mutagenic primers used are shown below along with the wild type DNA sequence at the site of interest. The amino acid of interest highlighted in bold type. Also shown below is the sequencing primer used for determining if the mutagenesis process has been successful (see Chapter 2).

Wild-type sequence around arginine 331
5'-C CAC CCG GAC CGC AAC GGC ATC GTC-3'
3'-G GTG GGC CTG GCG TTG CCG TAG CAG-5'

Complementary primers for substitution of Arginine 331 with lysine
5'-C CAC CCG GAC AAG AAC GGC ATC GTC-3'
3'-G GTG GGC CTG TTC TTG CCG TAG CAG-5'

Complementary primers for the substitution of Arginine 331 with alanine
5'-C CAC CCG GAC GCC AAC GGC ATC GTC-3'
3'-G GTG GGC CTG CGG TTG CCG TAG CAG-5'

3.8c Results of mutagenesis experiments

Despite several attempts to produce this site directed mutation, no mutant strain was produced. The possible reasons for this are probably the same as the problems encountered in producing site directed mutants of the calcium binding residues discused in Section 3.4c.

3.9 Conclusions

Using the QuikChange™ mutagenesis system the codon mutation for changing aspartate 303 to a glutamate in the *mxaF* gene has been achieved. Attempts to make site directed mutations at amino acid positions 261, 177, 331, 205 and 243 in the *mxaF* gene using the QuikChange™ mutagenesis system failed in all cases It appears that the production of MDH mutants in *M. extorquens* using mutagenic

primers is exceptionally difficult and success is limited by the high degree of secondary structure primarily caused by the GC rich nature of the MDH DNA, restricting both the production of suitable mutagenic primers and DNA sequencing. As described above, some of the problems encountered here are similar to those encountered by other attempts to produce site directed mutants of MDH (Majekodunmi, 1997; Amaratunga, 1995) and although other methods of creating some of these mutants are potentially suitable such as the Kunkel method as used to produce mutations in the *mxaG* gene (Dales, 1995) or *in vitro* synthesis (Amaratunga, 1995), it has been shown that they are probably less productive or more time consuming than the methods tried above when producing *mxaF* mutations. Due to time restraints, attempts to produce site directed mutants of MDH were aborted.

Chapter 4

Analysis of several *mxa* genes by insertion of a kanamycin resistance gene

4.1 Introduction

Methanol oxidation requires upwards of 30 genes (Section 1.19 and Figure 1.29). Research has mainly focussed on methanol oxidation genetics of *Paracoccus denitrificans* and *Methylobacterium extorquens*. Particularly useful have been mutant strains of the bacteria, although many gene functions are still unknown. Originally the genes involved in methanol oxidation were termed *mox* genes but are now given the prefix *mx*. The methanol oxidation genes are located in four chromosomal linkage groups named (*mxa, mxb, mxc* and *mxd*) (Fig. 1.30). Those involved in PQQ biosynthesis are given the prefix *pqq* (Lidstrom *et al.*, 1994)

The *mxa* operon is composed of 11 genes and is thought to be controlled by a single promoter upstream of *mxaF*, the first gene in the operon. *MxaF* is 1881 base pairs in length and encodes the alpha subunit of MDH. The gene from *P. denitrificans* has 76% homology to that from *M. extorquens*. Like all genes that are targeted to the periplasm a 22-27 amino acid signal peptide is required (Goodwin and Anthony, 1995). The signal peptide will however be cleaved off by a signal peptidase in the membrane as the protein is translocated. The next gene in the operon is *mxaJ*. It is 903 base pairs and potentially encodes a 30 kDa protein but its function is unknown. The *mxa G* gene would produce a protein of 20 kDa but has no known function in *M. extorquens* but in *P. denitrificans* the *mxa G* gene product is a protein homologous to cytochrome c_L call ed cytochrome c_{551i}. The *mxaI* gene codes for the beta subunit which is 74 amino acids in length. There is uncertainty over whether *mxaS* is an open reading frame. Three of the genes *mxaA, K* and *L* have been implicated in the insertion of calcium into the alpha subunit. The other genes *mxaR, C* and *D* have no known function.

4.2 Production of insertion and deletion mutants in *Methylobacterium extorquens* by electroporation and homologous recombination

The best way to study the function of a specific gene is to delete or alter the gene and study the phenotype of the mutant strain. A classic way to do this is to insert an antibiotic resistance gene into the gene of interest *in vitro* and then insert this DNA into the cell's genome by homologous recombination. This results in some cells having the native gene of interest disrupted by the drug resistance gene thus deleting the gene's function. Any cell in which the recombination has occurred can be selected for by growing the cells in the presence of the appropriate antibiotic.

In *Methylobacterium extorquens* there has been no simple way of introducing DNA into the bacteria and a complex series of cloning and mating has to be undertaken. This difficulty has been overcome by the finding that electroporation techniques give the same number of tranformants as compared to the $CaCl_2$/heat shock method (10^5- 10^6 cells/μg DNA). This new technique was used to produce a series of mutants that are potentially very useful in deducing both gene functions in the *mxa* operon and also mechanistic aspects of MDH catalysis (Toyama *et al.*, 1998) (see Figure 4.1, Table 4.1 and Chapter 5).

4.3a Characterisation of a mutant strain of *M. extorquens* with a kanamycin resistance gene inserted into the *mxaF* gene (MDH alpha subunit)

The mxa F gene is 1881 base pairs in length and encodes the alpha subunit of MDH. The gene from *P. denitrificans* has 76% homology to that from *M. extorquens.* Like all genes that are targeted to the periplasm a 22-27 amino acid signal peptide is required. The signal peptide is however be cleaved off by a signal peptidase in the membrane as the protein is translocated (Goodwin and Anthony, 1995).

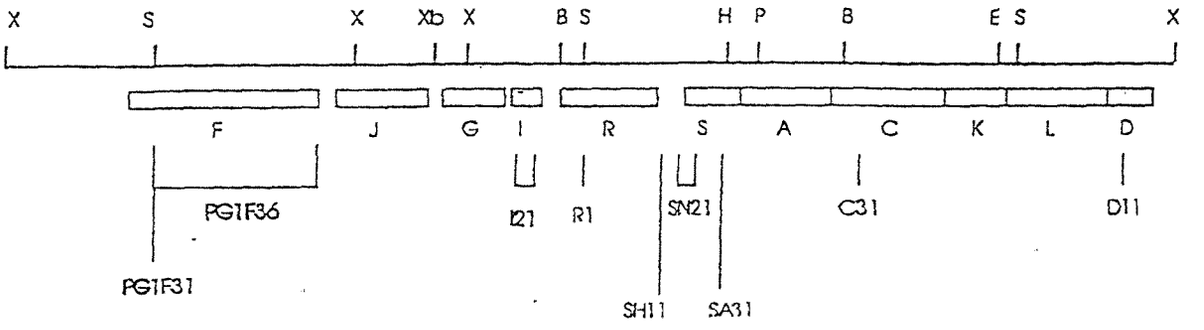

Fig. 4.1 The positions of insertion-deletion mutants within the *mxa* gene cluster.

A series of mutations in the *mxa* operon were made by insertion of the kanamycin resistance gene. A single line indicates that the mutation is an insertion in that position and two lines indicates a gene deletion with the resistance gene replacing that part of the gene. The genes are not shown to scale. (Toyama, 1997)

Mutant	Insertion strain	Plasmid used for mutation	Restriction site for pUCK4 Kan^r gene insertion	Interrupted gene	Mutation type
PG1F31	PG1		*Sal1*	*mxaF*	Insertion
PG1F36	PG1			*mxaF*	Deletion
I21	AM1			*mxaI*	Deletion
R1	AM1	pHT9XbHKan	*Bgl2*	*mxaR*	Insertion
SN21	AM1	pHT9BgHKanN	*Nru1 sites*	*mxaS*	Deletion
SA31	AM1	pHT9BgHKanA	*Asc1*	*mxaS*	Insertion
C31	AM1	pCMPN5Kan2	*Bgl2*	*mxaC*	Insertion
D11	AM1	pUC19A3S1Kan	*BsaB1*	*mxaD*	Insertion
SH11	AM1	pHT9BgHKanH	*Hinc2*	*none*	Insertion

Table 4.1 Summary of the insertion/deletion mutants produced in the *mxa* operon of *M. extorquens* (Toyama *et al.*, 1997)

Two different *mxaF* mutation strains were made (Toyama *et al.*, 1998): a kanamycin resistance gene insertion mutant near the start of the gene (strain PG1F31) and a mutant with a large portion of the gene removed and replaced with a kanamycin resistance gene (strain PG1F36) (see Figure 4.1).

Although it is well documented that the *mxaF* gene encodes the alpha subunit of MDH, such a mutation would answer several questions; do such mutations stop the production of MDH? Do the mutations have implications in the expression of genes downstream in the operon? Do other genes implicated in methanol oxidation get down regulated in the absence of MDH alpha subunit, for example the PQQ genes? In the absence of MDH does methanol still get oxidised? What are the secondary effects on cell metabolism, for example the decreased levels of formaldehyde? Probably the most useful experiments that could be undertaken with such a mutant would be based on the fact that these *mxaF* mutants were created in a strain that also lacks the *mxaA* gene, a gene that is essential for calcium insertion into MDH. This has lead to the possibility of creating a unique "double" mutant in which a site directed mutant could be expressed and then reconstituted with an ion different from that in the wild-type enzyme. This could be achieved by transforming the combined *mxaF* and *mxaA* deletion mutant with a site directed mutant in the *mxaF* gene, growing this strain in a suitable selection media, purifying the MDH produced in this strain and then reconstituting with a suitable ion. In the light of some very interesting findings from MDH reconstituted with barium (see Section 1.12f)(Goodwin and Anthony, 1996; Goodwin *et al.*, 1996) and recent evidence of the site directed mutant at aspartate 303 having some similarities to the barium enzyme (Majekodunmi, 1997), the combination of these two alterations in the same enzyme would be both a unique and potentially a highly rewarding construction. The creation and the characterisation of such a double mutant is discussed in Chapter 5.

4.3b Results

My results showed that both the mutation strains in the *mxaF* gene (insertion mutant PG1F31 and deletion mutant PG1F36)(produced by Toyama *et al.*, 1998) resulted, not surprisingly, in the absence of MDH alpha subunits from crude extracts, as detected by Western blot analysis. A more surprising result was the absence of the beta subunit from the crude extract also determined by Western blot analysis. This indicates that either the alpha subunit is needed to stop the beta subunit from being broken down or the absence of the alpha subunit in some way leads to a decrease in the expression of the *mxaI* gene encoding the beta subunit. It is interesting to note that cytochrome c_L was detected at normal levels in the mutant strains possibly indicating that the mutation did not have direct polar effects on the transcription of the *mxa* gene cluster (*mxaF,J,G,I,R,(S),A,C,K,L,D,B*).

The finding that a crude extract of this strain was inactive in the dye linked assay is not surprising because of the absence of the alpha and beta subunits. The observation that both mutant strains grew well on succinate, methylamine or either of these two supplemented with methanol indicates that there are no fatal physiological effects produced by the mutations. A summary of the results obtained from these mutant strains is given in Tables 4.2 and 4.3,

4.4a Characterisation of a mutant strain of *M. extorquens* in which a kanamycin resistance gene has been inserted mutation of the *mxaI* gene (MDH beta subunit)

The beta subunit of MDH is 74 amino acids in length and has a molecular weight of 8.5 kDa (see Sections 1.8c, 1.12a, 1.12b, 1.12c and 1.19f). It forms an elongated j shape lying on the surface of an alpha subunit with no contact with the other beta subunit of the tetramer. The *mxaI* gene (291 base pairs) in the large methanol oxidation operon in *Methylobacterium extorquens* encodes the beta subunit. The

role of the beta subunit is still not known but a role in maintaining the integrity of the alpha subunit has been proposed. A single mutation in this gene was made (Toyama *et al.*, 1997) by deleting a large segment in the middle of the gene and replacing the segment with the kanamycin resistance gene (see Figure 4.1 and Table 4.1).

4.4b Results

The incorporation of the kanamycin resistance gene replacing part of the *mxaI* gene resulted in a strain that did not produce any MDH beta subunit as detected by Western blot analysis. More surprising was the absence of the alpha subunit from this strain as determined by Western blotting. This indicates that either the beta subunit is needed to stop the alpha subunit from being broken down or the absence of the beta subunit in some way leads to a decrease in the expression of the *mxaF* gene encoding the alpha subunit. It is interesting to note that cytochrome c_L was detected at normal levels in this mutant strain possibly indicating that the insertion mutant did not have direct polar effects on the transcription of the *mxa* gene cluster (*mxaF,J,G,I,R,(S),A,C,K,L,D,B*). The finding that the crude extract of this strain was inactive in the dye linked assay is not surprising because of the absence of the alpha and beta subunits. A summary of the results obtained from this mutant is shown in Tables 4.2 and 4.3.

4.5a Characterisation of a mutant strain of *M. extorquens* in which a kanamycin resistance gene has been inserted into the *MxaR* gene.

An insertion mutation strain was made (strain R1) (Toyama *et al.*, 1998) by inserting a kanamycin resistance gene near the start of *mxaR* (see Figure 4.1 and Table 4.1). Such a mutation could indicate the role of the *mxaR* gene maybe giving similar effects to the mutation in *Paracocccus*. The mutation could for example

have effects on the expression of other genes or maybe effecting the processing of MDH.

The *mxaR* gene (1023 base pairs) potentially encodes a protein of 38.6 kDa. Sequencing revealed no potential signal peptide or transmembrene domains so a cytoplasmic location is predicted and no promoter sequences were located. Sequencing did however reveal two ATP binding sites. One of these sites is a glycine rich P loop (Walker A sequence) capable of binding both ATP and GTP and the other solely binding ATP but the function of the gene product is still unknown. The protein from *Methylobacterium extorquens* has 60% homology to the equivalent protein in *Paracoccus denitrificans*. A mutation in the gene from *Paracoccus* resulted in the strain not being able to grow on methanol, producing cytochrome c_L but producing MDH but in an inactive state (van Spanning *et al.*, 1991; Harms, 1993). Such evidence has suggested that the gene product may have a role in MDH processing. No promoter has been located around the *mxaR* gene and it is thought that it is controlled by the promoter upstream of *mxaF*. A homologue of this gene has been found in *B. subtilis* and in *A. fulgidus* and therefore it maybe part of a large gene family.

4.5b Results

The insertion of the kanamycin resistance gene replacing part of the *mxaR* gene resulted in a strain that produced both the alpha and beta subunits of MDH as deduced by Western blot analysis. This indicates that the mutation has no effects on the expression of adjacent genes *mxaF* or *mxaI*. Interestingly no MDH activity in the dye linked assay could be detected with this strain. This finding could be due to the enzyme existing in an inactive holoenzyme state or perhaps existing as an apoenzyme lacking a critical part of the active site such as PQQ or calcium. This could be due to the mutation somehow affecting one or more of the genes or

proteins involved in processing and assembling MDH. It is also possible that the kanamycin gene product has an inhibitory effect on the dye linked assay system but no examples of this situation in the literature have been found. Western blot analysis revealed that cytochrome c_L was present indicating that the mutation did not affect the transcription at least of upstream genes. This mutant was unable to grow on methanol which is not surprising considering the lack of active MDH. The mutant could however grow on methylamine and succinate indicating that the mutation has had no fatal effect on cell physiology. A summary of the results obtained from this mutant is shown in Tables 4.2 and 4.3.

4.6a Characterisation of mutant strains of *M. extorquens* in which a kanamycin resistance gene has been inserted into, or next to, the *mxaS* gene

MxaS is a gene of 591 base pairs potentially encoding a protein of 22.3kDa (Harms *et al.*, 1993). There is doubt over whether it is a true open reading frame. This is because the first 228 base pairs of the potential ORF show atypical codon usage and predict the N terminal to have an unlikely amino acid sequence containing many arginines and prolines but very few glutamates or lysines (Amaratunga *et al.*, 1997a and b). This region of unusual amino acid composition is thought to be an N terminal extension of the protein from *Paracoccus denitrificans*. It is uncertain what the role of such an extension might be. Overall there is 20% homology between the genes of *Methylobacterium extorquens* and *Paracoccus denitrificans.*

Three new strains of *M. extorquens* were made with mutations in or near the *mxaS* gene (Toyama *et al.*, 1998) (see Figure 4.1 and Table 4.1). The strain with a mutation in the intergenic region (between *mxaR* and *mxaS*) (SH11) was made by inserting a kanamycin resistance gene in a site 20 base pairs downstream of the termination sequence of the *mxaR* gene. Such a mutation could have effects on the

expression of other genes in the operon especially if a promoter is located in this region.

The deletion mutant (SN21) of 202 base pairs in total, includes the later half of the intergenic region and the first 23 amino acids of the perspective protein. This mutation would certainly remove any specific promoters for the *mxaS* gene (usually within 60 base pairs of the start of the gene), maybe having effects on the expression of downstream genes and possibly deduce a role of the extension in the protein when compared to the protein from *Paracoccus denitrificans*.

The mutation in the *mxaS* gene (S31) is a kanamycin insertion mutation located in the region with homology to the *mxaS* homologous gene from *Paracoccus denitrificans*. This mutation could reveal the function of the *mxaS* gene in *Methylobacterium extorquens*. The mutation could, for example, have effects on the expression of other genes or in the processing of MDH.

4.6b Characterisation of the mutant strain of *M. extorquens* in which a kanamycin resistance gene has been inserted into the intergenic region between *mxaR* and *mxaS* (SH11)

The insertion of the kanamycin resistance gene 20 base pairs downstream of *mxaR* termination codon in a probably intergenic region resulted in a mutant strain that could grow on methanol, succinate and methylamine. This indicates that the mutation had no major effects on the methanol oxidation system and that the intergenic region is a definite non-coding region. Western blot analysis revealed the presence of MDH (alpha and beta subunits) and cytochrome c_L. When assayed by the dye linked assay system this mutant was shown to have a similar activity to wild type. This finding indicates that the mutation does not have effects on the expression or function of any of the adjacent genes in the *mxa* gene cluster. A previous hypothesis that there could be a gene promoter in the intergenic region can probably

be ruled out or, if present, classed as non-essential. Evidence from gene sequencing also indicates that there is not a promoter (Amaratunga *et al.*, 1997a and b).

A large batch culture (15 L) of this mutant was grown to enable further studies of whole cell physiology and also to allow purification of MDH. The purification procedure was carried out the same as for the wild type strain of *M. extorquens*. This yielded a total of 120 mg of pure protein. A summary of results from this mutant strain and protein is given in Tables 4.2, 4.3, 4.4, 4.5 and 4.6.

4.6c Characterisation of the mutant strain of *M. extorquens* with a kanamycin resistance gene inserted into the *mxaS* gene replacing a 202 base pair region including half of the intergenic region between *mxaR* and *mxaS* and the first 23 amino acids of the putative *mxaS* gene (SN21)

This mutation resulted in a strain that produced both the alpha and beta subunits of MDH as deduced by Western blot analysis. This indicates that the mutation has no effect on the expression of adjacent genes *mxaF* and *mxaI*. Interestingly, no MDH activity in the dye linked assay could be detected with this strain. This finding could be due to the enzyme existing in an inactive holoenzyme state or perhaps existing as an apoenzyme lacking a critical part of the active site such as PQQ or calcium. This could be due to the mutation somehow affecting one or more of the genes or proteins involved in processing and assembling of MDH. It is also possible that the kanamycin gene product has an inhibitory effect on the dye linked assay system but no examples of this situation in the literature have been found. Western blot analysis revealed that cytochrome c_L was present indicating that the mutation did not affect the transcription of upstream genes. This mutant was unable to grow on methanol which is not surprising considering the lack of active MDH. The mutant could however grow on methylamine and succinate indicating that the mutation has

had no fatal effect on cell physiology. A summary of the results obtained from this mutant is shown in Tables 4.2 and 4.3.

4.6d Characterisation of the mutant strain of *M. extorquens* in which a kanamycin resistance gene has been inserted into the *mxaS* gene in a region homologous to the equivalent gene in *Paracoccus denitrificans* (SA31).

This mutation resulted in a strain that produced both the alpha and beta subunits of MDH as deduced by Western blot analysis. This indicates that the mutation has no polar effects on the expression of adjacent genes *mxaF* or *mxaI*. Interestingly no MDH activity in the dye linked assay could be detected with this strain. This finding could be due to the enzyme existing in an inactive holoenzyme state or perhaps existing as an apoenzyme lacking a critical part of the active site such as PQQ or calcium. This could be due to the mutation somehow affecting one or more of the genes or proteins involved in processing and assembling MDH. It is also possible that the kanamycin gene product has an inhibitory effect on the dye linked assay system but no examples of this situation in the literature have been found. Western blot analysis revealed that cytochrome c_L was present indicating that the mutation did not affect the transcription at least of upstream genes. This mutant was unable to grow on methanol which is not surprising considering the lack of active MDH. The mutant could however grow on methylamine and succinate indicating that the mutation has had no fatal effect on cell physiology. A summary of the results obtained from this mutant is given in Tables 4.2 and 4.3.

4.7 Characterisation of a mutant strain of *M. extorquens* in which a kanamycin resistance gene has been inserted into the *mxaC* gene.

The *mxaC* gene in *Methylobacterium extorquens* is 1065 base pairs in size. No function has been found for the gene product. An insertion mutant was created

(StrainC31) (Toyama *et al.*, 1998) by introducing a kanamycin resistance gene into roughly the middle of the *mxaC* gene (see Figure 4.1 and Table 4.1). Such a mutant could reveal information into the role of the *mxaC* gene product in methanol oxidation perhaps indicating a role in gene regulation or MDH processing.

4.7b Results

This mutation resulted in a strain that produces both subunits of MDH as indicated by Western blotting. The enzyme was however inactive in the dye linked assay system. The strain also produces cytochrome c_L as indicated by Western blotting. These results are the same as those recorded from mutant strain SA31 discussed in section 4.6d.

Because this gene has not been studied before in any great detail and previous hypotheses that this gene maybe involved in calcium insertion into MDH have not been confirmed a large batch culture (15 L) of this mutant was grown to enable further studies of whole cell physiology and also to allow purification of MDH. The purification procedure was carried out the same as for the wild type strain of *M.extorquens*. This yielded a total of 81 mg of pure protein. During the preparation of the mutant enzyme it was observed that the protein had an unusual brown colouration compared to wild-type (pink-green). The absorbance spectrum of the purified enzyme did not reveal any clues as to the reason behind the unusual colour. A summary of the results obtained from this mutant is shown in Tables 4.2, 4.3, 4.4, 4.5 and 4.6.

4.8a Characterisation of a mutant strain of *M. extorquens* in which a kanamycin resistance gene has been inserted in the *mxaD* gene

There is contradicting evidence for the role of *mxaD*. The original findings for a *mxaD* mutation strain indicated that the mutation lead to production of an altered form of cytochrome c_L (cytochrome c-553), probably composed of cytochrome c_L with its signal peptide still attached (Nunn and Lidstrom, 1986). Subsequently it has been shown that the altered form has no resemblance to cytochrome c_L but is a novel cytochrome that is always present in wild-type bacteria (Day *et al.*, 1990). The exact role of the *mxaD* gene is still to be discovered.

The predicted *mxaD* gene in *Methylobacterium extorquens* is 528 base pairs in length. No function has been deduced for the *mxaD* gene product but a gene homologous to the *mxaD* gene has been located at another site in the genome perhaps as a result of gene duplication. An insertion mutant was made (D11) by introducing a kanamycin resistance gene into roughly the middle of the *mxaD* gene (see Figure 4.1 and Table 4.1). Such a mutation could reveal a role for the *mxaD* gene in MDH processing or in gene regulation. It could also indicate if the *mxaD* gene and its homologue are functionally equivalent.

4.8b Results

This mutation resulted in a strain that could grow on methanol, succinate and methylamine. This indicates that the mutation had no major effects on the methanol oxidation system. Western blot analysis revealed the presence of MDH (alpha and beta subunits) and cytochrome c_L. When assayed by the dye linked assay system this mutant was shown to have a similar activity to wild type. This finding indicates that the mutation had not had effects on the expression or function of any of the adjacent genes in the *mxa* gene cluster. A large batch culture (15 L) of this mutant was grown to enable further studies of whole cell physiology and also to allow purification of

MDH. The purification procedure was carried out the same as for the wild type strain of *M. extorquens*. This yielded a total of 103 mg of pure protein. A summary of the results obtained from this mutant is shown in Tables 4.2, 4.3, 4.4, 4.5 and 4.6.

4.9 Conclusions

Characterisation of the mutant strains of *M. extorquens* produced by Dr Toyama (Toyama *et al.*, 1998) has given a valuable insight into the role of genes in the *mxa* operon. The first interesting finding was that the mutant strains with a mutation in either the *mxaF* and *mxaI* genes lead to the absence of both of these gene products. This indicates that either the gene products depend on each other for stability or that when one of the genes is not being expressed it leads to a down-regulation in the transcription or translation of the other. The finding that cytochrome c_L was present in these mutant strains at wild-type levels indicates the lack of expression is due to instability in the proteins rather than a lack of transcription (the cytochrome c_L gene is in the same gene operon as *mxaF* and *I*). In all other mutant strains the alpha and beta subunits were produced.

All of the mutant strains grew on methylamine and succinate but mutants in the *mxa F, I, R, S* and *C* genes could not grow on methanol indicating that they have an important role in the methanol oxidation system. In the case of the genes encoding the alpha and beta subunits (*mxa F* and *I*) this is not surprising but the roles of the other genes is still not known. A mutation in the intergenic region between *mxaR* and *mxaS* produced a strain (SH11) that could grow on methanol indicating that this region (which is part of the *mxaS* ORF in *P. denitrificans*) is not essential in *M. extoquens*.

MDH was purified from mutant strains in the *mxaC* (C31), *D* (D11) genes and from a strain with a mutation in the intergenic region between *mxaR* and *mxaS*

(SH11). The specific activity of the purified enzymes was measured and indicated that the mutations in the *mxaD* gene and in the intergenic region produced strains with MDH that have reduced activity compared to wild-type. This indicates that these genes have an important but non-essential role in methanol oxidation. These strains also have a reduced methanol dependant oxygen consumption compared to wild-type. The strain (C31) with a mutation in the *mxaC* gene produced inactive MDH indicating that this gene has an essential role in methanol oxidation.

Mutant	Interupted gene	Kan^R gene mutation type	Alpha subunit detected?	Beta subunit detected?	Cytochrome c_L detected?
PG1F31	*mxaF*	Insertion	-	-	+
PG1F36	*mxaF*	Deletion	-	-	+
I21	*mxaI*	Deletion	-	-	+
R1	*mxaR*	Insertion	+	+	+
SH11	*none*	Insertion	+	+	+
SN21	*mxaS*	Deletion	+	+	+
SA31	*mxaS*	Insertion	+	+	+
C31	*mxaC*	Insertion	+	+	+
D11	*mxaD*	Insertion	+	+	+

Table 4.2 The presence of the alpha and beta subunits of MDH and cytochrome c_L as detected by Western blotting in Kanamycin resistance gene insertion/ deletion mutants of genes in the *mxa* gene operon

Mutant	Gene	Kanamycin mutation type	Growth on methanol	Growth on methylamine	Growth on succinate
PG1F31	*mxaF*	Insertion	-	++	++
PG1F36	*mxaF*	Deletion	-	++	++
I21	*mxaI*	Deletion	-	++	++
R1	*mxaR*	Insertion	-	++	++
SH11	*none*	Insertion	++	++	++
SN21	*mxaS*	Deletion	-	++	++
SA31	*mxaS*	Insertion	-	++	++
C31	*mxaC*	Insertion	-	++	++
D11	*mxaD*	Insertion	++	++	++

Table 4.3 The growth of various insertion/deletion mutants strains of *M. extorquens* on various growth substrates.

Strain	Gene mutated	Mutation type	Proposed gene function	Concentration of purified enzyme
Wild-type	None	None	None	17.5mg/ml
SH11	Intergenic region between *mxaR* and *mxaS*	Kanamycin insertion	None	13.22 mg/ml
C31	*mxaC*	Kanamycin insertion	Calcium insertion into MDH alpha subunit?	8.1mg/ml
D11	*mxaD*	Kanamycin insertion	Calcium insertion into MDH alpha subunit?	10.31 mg/ml

Table 4.4 The concentrations of MDH purified from various insertion/deletion *mxa* gene mutants in *M. extorquens*

Methylobacterium extorquens strain	Mutation	Specific activity, expressed as a percentage of wild-type (6.1μmol/min/mg)
Wild-type	None	100
SH11	Kanamycin resistance gene inserted between *mxaR* and *mxaS*	33.3
C31	Kanamycin resistance gene inserted into *mxaC*	0
D11	Kanamycin resistance gene inserted into *mxaD*	74.9

The specific activity was measured using the dye linked assay system described in Section 2.

Table 4.5 The specific activity of MDH purified from several insertion/deletion mutant strains of *M. extorquens*

Methylobacterium extorquens strain	Mutation (kanamycin resistance gene)	Methanol dependant oxygen uptake in μmol O_2/mg dry cell weight	Average rates of methanol dependant O_2 uptake expressed as a percentage of wild-type (6.8μl/mg dry weight/hr)
Wild-type	None	4.0	100
SH11	inserted between *mxaR* and *mxaS*	0.6	15.7
C31	inserted into *mxaC*	0	0
D11	inserted into *mxaD*	1.1	27.6

Table 4.6 The oxygen uptake with wild-type and mutant strains of *M. extorquens*. Wild-type, SH11 and D11 were grown in minimal salts media supplemented with methanol. C31 was grown in minimal salts media supplemented with methanol and methylamine. SH11, C31 and D11 were grown in the presence of kanamycin. Oxygen uptake was measured using a Rank oxygen electrode. The incubation vessel contained 25mM HEPES buffer (pH7) and 2mg dry weight equivelent of washed cells in 3ml volume. Endogenous uptake was measured for 5 mins and 15 μmoles of methanol added. Oxygen uptake was measured for 10 mins. The ammount of oxygen consumed was calculated by assuming 0.601μg of O_2 are dissolved in 2.7ml of buffer at 30 °C.

Chapter 5

Production of a site directed mutant (D303E) lacking metal ions in the active site

5.1 Introduction

5.1a Aspartate 303 in the active site of methanol dehydrogenase from *Methylobacterium extorquens*

Despite more than 30 years of study the catalytic mechanism of methanol dehydrogenase is poorly understood (Section 1.18). Based on evidence from the reaction of MDH with cyclopropanol, it has been concluded that the catalytic mechanism of MDH involves an active site base. This role is probably carried out by aspartate 303.

To understand the function of aspartate 303 in more detail a site directed mutant of this residue was created using the Stratagene QuikChange™ system (see Chapter 3). The aspartate was changed to a glutamate which would retain an acidic residue at the active site without changing the size of the amino acid greatly and thus potentially the enzyme may retain some catalytic activity.

Characterisation of this D303E mutant gave some very interesting results (Majekodunmi, 1997; Afolabi, 1999). When the enzyme was purified and assayed by the dye linked assay system (Section 2.19a) in the absence of a substrate no activity could be detected. By contrast, in the wild-type enzyme in the absence of substrate usually a rate is detected because of the presence of approximately 90 moles of unidentified endogenous reductant. Even more surprising was that no activity could be detected with 5 mM methanol, the standard assay concentration. Subsequent experiments with much higher concentrations of methanol have now indicated that the mutant is active but has a greatly increased k_M (250 mM)

approximately 10,000 times that of the wild-type enzyme. This finding would explain why no activity was detected in the presence of an endogenous substrate alone. This mutant protein is therefore ideal for the mechanistic studies that had previously been hampered by the endogenous rate. Further studies have indicated that: the V_{max} of the enzyme is unchanged compared to wild-type, ammonia is needed for maximal activity and that methanol and ammonia (the enzyme activator) bind more weakly in the presence of each other (Afolabi, 1999).

5.1b Methanol dehydrogenase containing strontium or barium

The production of methanol dehydrogenase without the presence of the calcium ion in the active site allowed not only reconstitution with calcium but also reconstitution with other ions (Section 1.12f)(Goodwin and Anthony, 1996; Goodwin *et al*., 1996; Richardson and Anthony, 1992). Two ions that did produce active enzymes on incubation with the apoenzyme were strontium and barium. The first interesting point that was noticed with the barium enzyme was that it did not have an endogenous rate that is seen in the wild-type enzyme. It was deduced that the reason for this is because the barium enzyme has a K_M value for methanol approximately 1000 times higher than that of the wild-type enzyme (20 μM). The barium enzyme has a V_{max} double that of the wild-type enzyme and is thought to be because of a 50% reduction of the activation energy for the reaction.

The barium enzyme also allowed studies on the activation of the MDH catalysed reaction by ammonia (Afolabi, 1999). This was possible because the main problem in studying the activation was the endogenous rate which as discussed above is absent in the barium enzyme.

As both the aspartate to glutamate mutation at position 303 mutant of MDH and the insertion of a barium atom at the active site of MDH have produced some

very revealing information about MDH it was decided that a mutant containing both the site directed mutation and the presence of barium at the active site at the same time could be rewarding. It would also be the first example of such a double mutation. To produce such a mutant a strain of bacteria with the site directed mutation but without any metal at the active site must be produced.

5.2 Creation of a metal deficient site directed mutant of methanol dehydrogenase in *Methylobacterium extorquens* using triparental mating.

To produce a metal deficient site directed mutant of methanol dehydrogenase in *Methylobacterium extorquens*, triparental mating using three different strains of bacteria with different genetic properties were needed. One strain has the gene of interest with the required site directed mutation and is referred to as the donor strain. Another strain has genes that allow mobilisation of plasmids from one strain to another and is referred to as the mobilising strain. The final strain is the recipient strain that will receive the site directed plasmid and express the mutant protein. This recipient strain has a mutation in the *mxaA* gene that is critical for calcium insertion in the alpha subunit. Throughout the formation of the mutant it was important that suitable selection procedures are available.

The donor strain was created by transforming the plasmid (pRK310) containing the gene for the alpha subunit of methanol dehydrogenase (*mxaF*) with a point mutation at the active site residue aspartate 303 into *E. coli* (HB101) using the calcium chloride/heat shock method. Initially the purification of pRK310 proved difficult but was achieved when the lysis stage of the plasmid preparation technique was left slightly longer. As pRK310 has a tetracycline resistance gene, selection of *E. coli* cells that were successfully transformed was achieved by growing cells on LB agar in the presence of the appropriate concentration of tetracycline.

The mobilising strain was created by transforming *E. coli* (CSR600) with a

plasmid (pRK2013) containing the *tra* genes that allow mobilisation of plasmids form one strain to another. The *M. extorquens* recipient strain was a gift from Dr Hirohide Toyama. This strain (PG1F31) has no gene encoding the alpha subunit (*mxaF)* or an essential gene for calcium insertion (*mxaA*) (see Chapter 4). This recipient strain was made using a strain with a deletion in (PG1) and by inserting a kanamycin resistance gene into the *mxaF* gene. A mixture of all three of the above strains was made by adding a sterilised loop full of each strain onto minimal media agar containing methanol, methylamine, tetracycline and kanamycin then left at 30°C for the recipient strain to grow. The theory behind this procedure is that the mobilising plasmid will move into the media and enter the donor strain. This will allow both the mobilising plasmid and the *mxaF* mutant plasmid, in the donor strain, to enter the media and transform the recipient strain conferring tetracycline resistance. Thus only successfully transformed recipient strain will have the tetracycline and kanamycin resistant phenotype. Because the mobilising plasmid doesn't contain an origin of replication, it will be lost from the recipient strain in subsequent cell replication. The addition of methylamine is essential for the recipient strain to grow as the methanol oxidation system will be not functioning without the *mxaF* (alpha subunit) gene. The addition of methanol is to stimulate any possible methanol dependant regulatory systems so that expression of the mutant MDH is at the optimal level (see Section 1.21a).

In parallel to the triparental mating experiments, diparental mating was also attempted. This method uses the *E.coli* strain S17-1 which has the plasmid (RP4) which has the genes for allowing transfer of plasmids to *M.extorquens*. The pRK310 plasmid with the D303E mutation was transformed into S17-1 using the $CaCl_2$/heatshock method. Cells that were transformed with the pRK310 plasmid became tetracycline resistant as this plasmid contains the tetracycline resistance gene and therefore the cells could be selected for by growing in LB media

containing tetracycline. These cells were then mixed with the recipient strain (PG1F31) by adding a sterilised loop full of each strain onto minimal media agar containing methanol, methylamine, tetracycline and kanamycin then left at 30°C for the recipient strain to grow. As for the triparental mating technique this should result eventually with only the recipient strain with the pRK310 plasmid growing successfully.

Eventually the triparental technique yielded bacterial colonies that grew on growth media containing the appropriate antibiotics. These were selected and grown in liquid minimal media containing the same supplements as the selection plates.

A cell free extract of this strain did not show any methanol-dependent activity in either the dye linked assay system or in the oxygen electrode assay. The strain does however produce alpha and beta subunits of MDH (see Figure 5.1). MDH was purified from this strain at a concentration of 3.1mg/ml. Reconstitution of this enzyme using a range of pH values (7 to 11) at various calcium and barium concentrations (0-100mM) failed to produce any active MDH as indicated by the dye linked assay. This has prevented a full characterisation of the double mutant. MDH lacking calcium ions from the active site can normally be reconstituted using 10mM calcium at pH9 in less than 1 hour to produce fully active enzyme (Goodwin *et al.*, 1996). The reason for the lack of reconstitution could be because calcium cannot enter into the active site because access is blocked by the unusual glutamate at the active site.

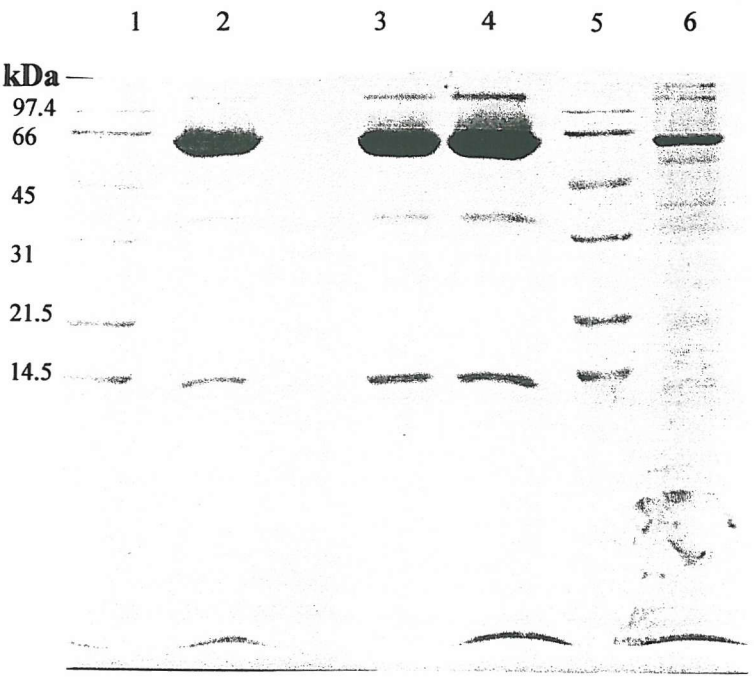

Figure 5.1 Coomassie-stained MDH separated by SDS-PAGE (12%).
Lanes 1and 5, molecular weight standards; lanes 2, 3 and 4, purified MDH from a strain of *M. extorquens* which lacks the *mxa A* gene and contains a site directed mutation (D303E); lane 6, wild-type MDH.

CHAPTER 6

The effects of growth conditions on methanol dehydrogenase expression.

6.1 The regulation of methanol oxidation genes.

The growth of methylotrophic bacteria on methanol requires upwards of 30 genes. Some of these genes have structural roles, some are involved in the regulation of methanol oxidation and others are involved in the biosynthesis of active MDH and cytochrome c_L (see Sections 1.19, 1.20, 1.21a, 1.21b and 1.22).The regulation of all of these genes is needed for the bacteria to function efficiently. The regulation of the methanol oxidation system is discussed in detail in Section 1.21a and the regulation of PQQ synthesis is discussed in Section 1.21b.

6.2 Analysis of potential factors that could affect the expression of methanol dehydrogenase in *Methylobacterium extorquens* AM1

As discussed in Chapter 3, many of the site directed mutants of methanol dehydrogenase have shown insufficient expression of an active protein from the mutated DNA to permit a detailed characterisation program. This has been a problem in five out of six site directed mutants of methanol dehydrogenase including D303N, R331K (Majekodunmi, 1997), C103S, C104S and the double mutant C103S/C104S (Amaratunga, 1995). In addition the C103S, C104S and the double mutant C103S/C104S (Amaratunga,1995) was also prone to loss during purification attempts.

In an attempt to increase the protein expression of these site directed mutants, and also possibly to increase the enzyme's activity, a study of some of the factors that could be implicated in affecting protein expression was undertaken.

6.3 Loss of the plasmid containing the mutated MDH gene from the expression strain.

For mutated MDH to be expressed in *M. extorquens*, the bacteria must contain the mutated *mxaF* gene. Because of the methods of cloning that have been used to create the site directed mutants that are available, the *mxaF* gene is located on the pRK310 plasmid in the cells cytoplasm. One potential reason that expression could be reduced in an MDH site directed mutant when compared with wild-type *M. extorquens* is the loss of the expression plasmid. This should not happen in these mutants because pRK310 also contains the tetracycline resistance gene which applies selection pressure to maintain the plasmid.

To check that the plasmid was still in the expression strain it was extracted using the plasmid purification technique as described in Chapter 2 and analysed on an agarose gel. The purification of pRK310 containing a site directed mutation from *M. extorquens* was poor but it was no different to the purification of the same plasmid without a mutation indicating that loss of the mutated plasmid from the bacteria is not the cause of the limited expression of MDH.

6.4 Formaldehyde

Formaldehyde is thought to be one of the signals that increases the expression of the *mxa* operon and thus stimulate the cell's ability to oxidise methanol. The activation of the *mxa* genes therefore is an example of positive feedback as more oxidation of methanol to formaldehyde increases the presence of MDH and thus leads to more methanol oxidation. Although formaldehyde is thought to be a signal to increase the rate of expression of the methanol oxidation genes, it was also shown in *Methylophilus methylotrophus*, an obligate methylotroph, that in high methanol conditions that MDH activity was shown to decrease probably to

reduce the excess formation of the toxic formaldehyde product. Activity is also greatly reduced during periods of oxygen limitation or low methanol concentrations. In *P. denitrificans* the regulation of the *mxa* operon also appears to be controlled by formaldehyde and MDH activity is low under carbon limitation and in low growth rates. Formaldehyde is also the product of methylamine oxidation by methylamine dehydrogenase. Although this could stimulate the *mxa* operon, the other product of methylamine oxidation is ammonia which has been recorded as an inhibitory signal for methanol oxidation.

To analyse the effects of formaldehyde on the expression of MDH it was added to 10 ml cell cultures of the various *M. extorquens* mutants, and the wild type bacteria, at various concentrations at various times throughout the growth period. This would deduce show what, if any, concentrations of formaldehyde stimulate the *mxa* operon and if the time of addition is important. To test if the formaldehyde had any effect on expression, the 10 ml samples of bacteria were sonicated and the cell free extract analysed for the presence of MDH by SDS polyacrylamide gel electrophoresis and Western blotting and the enzyme activity assayed by the dye linked assay. To determine if the *mxa* gene operon was being transcribed Western blots detecting cytochrome c_L were also carried out. It is assumed that as the gene for cytochrome c_L (*mxaG*) is downstream of the gene for the MDH alpha subunit (*mxaF*), and there are no promoters between the two genes, if cytochrome c_L is present then the MDH alpha subunit gene must be undergoing transcription and it is at another level of production that expression of the protein is lost.

In all cases it was not possible to increase the expression of MDH from the various site directed mutants in the *mxaF* gene. The presence of cytochrome c_L in all of the Western blots implied that the MDH alpha subunit gene must be undergoing transcription and it is at another level of production that expression of

the protein is lost.

6.5 Tetracycline

The antibiotic, tetracycline, added to the growth media of several of the methanol dehydrogenase mutants of *M. extorquens*. The reason for this is that the plasmid constructs (pRK310) used for expressing the mutant enzymes contain the tetracycline resistance gene and the addition of tetracycline will maintain the plasmid. This gene was also very beneficial in the selection procedures needed for creating the mutants. Tetracycline is known to reduce cell growth and is light and heat sensitive.

To study the effect of tetracyline on cell growth and MDH expression various concentrations of tetracycline, was added at various times throughout the growth period and in the light or the dark. (It was noted that the effects of the dark may not be beneficial as it is known that *M. extorquens* grows better in the light). 10 ml samples of bacteria were sonicated and the cell free extract analysed for the presence of MDH by SDS polyacrylamide gel electrophoresis and Western blotting and the enzyme activity assayed by the dye linked assay. The results indicated that all changes in the concentration of tetracycline do not increases the expression of MDH .

6.6 Carbon growth substrates

Wild-type *M. extorquens* can grow solely on methanol as its carbon substrate. In several of the active site MDH site directed mutants including D303N, R331K, C103S, C104S and the C103S/C104S double mutation, the ability to grow on methanol has been lost. In these mutants another carbon source is required and

either nutrient broth, methylamine or succinate is chosen. Despite the mutant bacteria not being able to grow on methanol it is still added to the growth media as it is required as a stimulatory growth signal for the methanol oxidation genes. Of special interest would be a switch from succinate to methylamine which has been reported to increase expression of MDH.

To study the effects of various combinations of carbon sources and switching from one growth media to another in the growth media on the growth of bacteria and the expression of MDH, 10 ml samples of bacteria were grown on the various carbon sources, sonicated and the cell free extract analysed for the presence of MDH by SDS polyacrylamide gel electrophoresis and Western blotting and the enzyme activity assayed by the dye linked assay. The results indicated that all of the changes in the carbon substrate used to grow *M. extorquens* made do not increases the expression of MDH .

6.7 pH

M. extorquens grows optimally at a pH of 7. *In vitro,* however, the optimal pH for MDH activity using the dye linked assay (Section 2.19a) is 9. To study the effects of pH on the growth of *M. extorquens* and the expression of MDH a series of experiments was conducted. This involved growing 10 ml samples of bacteria at various pH conditions. Cells were then sonicated and the cell free extract analysed for the presence of MDH by SDS polyacrylamide gel electrophoresis and Western blotting and the enzyme activity assayed by the dye linked assay. The results indicated that all changes in the pH of growth media do not increase the expression of MDH.

6.8 Results

It was concluded that none of the factors experimented with increased the

expression of MDH in *M. extorquens*. Although this was an exceptionally disappointing result, it was a study that potentially could have opened up a whole range of new experiments on any new or existing mutant strains of *M. extorquens*.

CHAPTER 7

GENERAL SUMMARY AND DISCUSSION

7.1 Introduction

Chapter 3 described the production of a site directed mutation at the active site of MDH in *M. extorquens*. Chapter 4 described the characterisation of several strains of *M. extorquens* with mutations in the *mxa* gene operon. Chapter 5 described the production of a strain of *M. extorquens* that produces MDH with an active site mutation and lacking metal ions from the active site. Chapter 6 described a study of the effects of growth conditions on the expression of MDH from *M. extorquens*.

7.2 Production of site directed mutations in MDH of *M. extorquens*

The production of site directed mutations in MDH from *M. extorquens* has proved very difficult. A method developed by Dr Majekodunmi was used to try and make various mutations including active site residues and a residue involved in the docking of MDH and cytochrome c_L. This work resulted in the production of a mutant strain of *M. extorquens* producing MDH with aspartate 303 (the active site base) mutated to a glutamate. All other mutations attempted failed probably due to the high GC content of the DNA.

7.3 Characterisation of several strains of *M. extorquens* with mutations in the *mxa* gene operon.

Several strains of *M. extorquens* with mutations in the *mxa* gene operon were given as a kind gift from Dr. H. Toyama. Strains with mutations in the *mxa F* or *I* genes produced a phenotype lacking both alpha and beta subunits indicating that the two gene products are dependant on each other. All of the mutant strains grew on

methylamine and succinate but mutants in the *mxa F, I, R, S* and *C* genes could not grow on methanol indicating that they have an important role in the methanol oxidation system. In the case of the genes encoding the alpha and beta subunits (*mxa F* and *I*) this is not surprising but the roles of the other genes is still not known. A mutation in the intergenic region between *mxaR* and *mxaS* produced a strain (SH11) that could grow on methanol indicating that this region (which is part of the *mxaS* ORF in *P. denitrificans*) is not essential in *M. extorquens*.

MDH was purified from mutant strains in the *mxaC* (C31) and *D* (D11) genes and from a strain with a mutation in the intergenic region between *mxaR* and *mxaS* (SH11). The specific activity of the purified enzymes was measured and indicated that the mutations in the *mxaD* gene and in the intergenic region produced strains with MDH that have reduced activity compared to wild-type. This indicates that these genes have an important but non-essential role in methanol oxidation. These strains also have a reduced methanol dependant oxygen consumption compared to wild-type. The strain (C31) with a mutation in the *mxaC* gene produced inactive MDH indicating that this gene has an essential role in methanol oxidation.

7.3 The production of a strain of *M. extorquens* that produces MDH with an active site mutation and lacking metal ions from the active site.

Characterisation of D303E by T. Majekodunmi and P. Afolabi had indicated that aspartate 303 to be the active site base of MDH. The D303E enzyme has a K_M for methanol approximately 10,000 times that of the wild-type enzyme. An increase in K_M was also seen in a mutant strain of *M. extorquens* where the calcium at the active site of MDH was replaced by barium (Goodwin *et al.*, 1997). A mutant strain of *M. extorquens* was made that contained both the site directed mutation and lacked ions from the active site of MDH. MDH from this strain could not be reconstituted with barium and the characterisation of this "double mutant" could

therefore not be achieved.

7.4 The effects of growth conditions on MDH expression in *M. extorquens*

Most of the mutant strains of *M. extorquens* with site directed mutations in MDH have shown to produce very little or inactive protein. In an attempt to increase the yield of enzyme various parameters in the growth media of the mutant strains was changed. This included changing the pH, the concentration of tetracycline, the carbon substrates and the concentration of formaldehyde. These experiments did not result in an increased expression or activity of MDH.

7.5 Future work

Another assay system that could be used to study mutant proteins is the cytochrome linked assay. This assay could reveal information about the enzyme that is not shown by the dye linked assay system. This would be due to the difference in the electron acceptor in the assay system. Whereas in the dye-linked assay system the electron acceptor is artificial, the use of cytochromes would more likely mimic the situation *in vivo*.

Due to the recurrent problem of MDH site directed mutants showing instability, analysis of protein stability could be useful. This could involve determining the limits of MDH stability purely by using enzyme activity as a measure or detailed unfolding experiments using the protein's florescence properties. Studies with circular dichroism could be useful in determining if a particular site directed mutation leads to an incorrectly folded configuration of the enzyme.

For several of the mutants that do not show any enzyme activity it would be useful to determine if PQQ or calcium were present. In the case of PQQ if it were present, it would also be useful to determine its redox state. This can be analysed

by producing an absorbence spectrum. The absorbence spectra could also reveal information about the conformation of PQQ in the active site. In addition the reaction of PQQ with cyclopropanol could be studied. If metal ions were shown to be absent from the enzyme then reconstitution could be carried out. This could involve adding various different ions such as barium or strontium or studying the effects of pH on the reconstitution process. Another useful experiment could be to grow mutant strains that show reduced or no activity in the presence of PQQ. In the case of the kanamycin insertion mutant in *mxaC* this could restore MDH activity as previous results indicate that the MDH from this mutant doesn't have PQQ in the active site.

A potentially useful study for any enzyme that is active is to study the activation process. As well as determining a Km value for ammonia and for example studying the effects of pH on the activation process, there are other activators of MDH that could be analysed.

To confirm any site directed mutation has in fact been achieved Both X-ray crystallography and mass spectroscopy could be carried out.

To determine if the lack of expression of MDH in a mutant is due to the lack of transcription of the *mxa* operon, a simple test is to produce a western blot testing for the product, cytochrome c_L, of the downstream gene *mxaG.* If present then as there are no promoters between the two genes then it can be assumed that MDH is being transcribed but is lost at a further processing point. A more accurate way of determining the presence of cytochrome c_L would be to use a complementation assay. This would involve producing a strain of bacteria with no *mxaG* (the gene for cytochrome c_L) and then adding the plasmid used for expressing MDH (which also contains *mxaG*) and testing for the presence of cytochrome c_L by Western blotting.

Another possibility for the lack of expression from a plasmid that has been

transformed into a bacterium is that it could have incorporated by homologous recombination into the host DNA. To test this theory the plasmid could be extracted from the expression bacteria and tested for its presence by agarose gel electrophoresis. The plasmid could be transformed into a bacteria such as *E.coli* Hb101 that allows easy purification of plasmids and also would confer the antibiotic resistance trait and then the plasmid extracted again and sequenced to determine that the mutation is still intact.

Another way of analysing mutant strains is to run the cell contents on a two dimensional gel. This technique separates proteins by size and by their isoelectric point. This produces a detailed map of the proteins present and sometimes particular proteins can be shown to be absent in a mutant strain when compared to the wild type. This technique could be particularly useful for example in the kanamycin resistance gene insertion mutants of *mxaC* and *mxaD* as the gene products of these genes have so far not been identified.

As described in Section 1.19h, the *mxaS* gene in *P.dentrificans* is shorter than the putative gene in *M.extorquens*. It is uncertain of the roles of extension. On intriguing experiment would be to express the *mxaS* gene of *P.denitrificans* in *M. extorquens* to deduce if the gene has the same role.

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