Structural and functional studies on 6-methylsalicylic acid synthase from *Penicillium patulum* and *holo*-acyl carrier protein synthase from *Escherichia coli*

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Neil Ian Johnson

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The type I polyketide synthase, 6-methylsalicylic acid synthase, is a multifunctional enzyme that catalyses the formation of 6-methylsalicylic acid from one starter unit of acetyl-CoA, three extender units of malonyl-CoA and one equivalent of the reducing cofactor NADPH. In the absence of NADPH, a triketide intermediate is released from the enzyme as triacetic acid lactone.

Using succinyl-CoA transferase, purified from porcine heart, $[2^{-13}C]$-malonyl-CoA was biosynthesised and in a linked assay, $[13C]$-labelled 6-methylsalicylic acid and $[^{13}C]$-labelled triacetic acid lactone were produced in the absence of externally added acetyl-CoA. By collision induced dissociation mass spectrometric analysis, it was determined that 6-methylsalicylic acid synthase possesses a malonyl-CoA decarboxylase activity. Similar methodology was also used to show that in the absence of NADPH, 6-methylsalicylic acid synthase is able to synthesise small amounts of orsellinic acid as well as triacetic acid lactone. It is proposed that in the absence of NADPH, the triketide intermediate is a poor substrate for the β-ketosynthase but that 1-2% can react with malonyl-CoA to give orsellinic acid.

Fluoroacetyl-CoA, chloroacetyl-CoA, and bromoacetyl-CoA were synthesised and purified and their effect/action with 6-methylsalicylic acid synthase was investigated. All were shown to inactivate the enzyme although none were incorporated into halo-products. The interaction between 6-methylsalicylic acid synthase and several N-acetylcysteamine intermediates was also investigated. The enzyme appears to prefer to synthesise its own intermediates rather than accept externally added substrate analogues.

A peptide fragment of 6-methylsalicylic acid synthase containing the acyl carrier protein domain and the holo-acyl carrier protein synthase from *Escherichia coli* were purified. The ability of the latter synthase to convert the former from the apo- acyl carrier protein to holo- acyl carrier protein was attempted. Mass spectrometry and nuclear magnetic resonance techniques were used to obtain initial structural data for the holo-acyl carrier protein synthase.
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<td>6-MSAS</td>
<td>6-Methylsalicylic acid synthase</td>
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<td>AcCoA</td>
<td>Acetyl-coenzyme-A</td>
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<td>ACP</td>
<td>Acyl carrier protein</td>
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<td>ACPS</td>
<td>Holo acyl carrier protein synthase</td>
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<td>AT/MT</td>
<td>Acyl/malonyl transferase domain</td>
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<td>Bromoacetyl-CoA</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CHS</td>
<td>Chalcone synthase</td>
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<td>Curie</td>
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<td>Cysteine</td>
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<td>DEBS</td>
<td>Deoxyerythronolide B synthase</td>
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<td>DH</td>
<td>Dehydratase domain</td>
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<tr>
<td>DTNB</td>
<td>5,5′-Dithio-bis[2-nitrobenzoic acid]</td>
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<tr>
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<td>Dithiothreitol</td>
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<td>Enoyl reductase domain</td>
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<td>ES-MS</td>
<td>Electrospray mass spectrometry</td>
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<td>Fatty acid synthase</td>
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<td>Fast protein liquid chromatography</td>
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<tr>
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<td>Heteronuclear Single Quantum Coherence</td>
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<td>ß-Keto synthase domain</td>
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<td>Lys</td>
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<td>MCoA</td>
<td>Malonyl-coenzyme-A</td>
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<td>2-[N-Morpholino]ethane sulfonic acid</td>
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<tr>
<td>MOPS</td>
<td>3-[N-Morpholino]propane-sulfonic acid</td>
</tr>
<tr>
<td>Mr</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>m u</td>
<td>Milli units</td>
</tr>
<tr>
<td>NAC</td>
<td>N-Acetyl cysteamine</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>Ammonium sulphate</td>
</tr>
<tr>
<td>OA</td>
<td>Orsellinic acid</td>
</tr>
<tr>
<td>OAS</td>
<td>Orsellinic acid synthase</td>
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<td>P-pant</td>
<td>4′-Phosphopantetheine</td>
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<td>PEG</td>
<td>Polyethylene glycol</td>
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<td>PKS</td>
<td>Polyketide synthase</td>
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<td>PMSF</td>
<td>Phenylmethylsulphonyl fluoride</td>
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<td>Q-TOF</td>
<td>Quadrupole time of flight</td>
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<td>Serine</td>
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<td>SH</td>
<td>Thiol</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>-------------</td>
<td>------------------------------------------------------</td>
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<tr>
<td>TAL</td>
<td>Triacetic acid lactone</td>
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<tr>
<td>TE</td>
<td>Thioesterase domain</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-Tetramethylethylenediamine</td>
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<tr>
<td>Tricine</td>
<td>N-Tris[Hydroxymethyl]methylglycine</td>
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<td>TRIS</td>
<td>Tris[hydroxymethyl]aminomethane</td>
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<tr>
<td>TOF</td>
<td>Time-of-flight</td>
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Chapter 1

The biosynthesis of fatty acids and polyketides

1.1 Introduction

The metabolism of living cells is defined as the overall network of chemical reactions that take place so that energy may be acquired and utilised for various functions. These reactions are catalysed by enzymes and form a complex web of biochemical pathways (figure 1.1). The products of metabolism can be divided into two groups:

*Primary metabolites*, such as common sugars, amino acids, common fatty acids, nucleotides and the polymers derived from them (polysaccharides, proteins, lipids, DNA and RNA). These chemical species are present in all living systems and are involved in all essential processes including the maintenance and growth of the cell, transport across membranes and storage of energy.

*Secondary metabolites*, also known as 'natural products', are present in only plants and micro-organisms. These products tend to be restricted to one, or several, closely related species and are derived from specialised pathways leading to a wide variety of structures. Unlike primary metabolites these compounds appear to be non-essential to life but are still thought to be of key biological importance as well as providing the organism with a distinct advantage over others.

The dividing line between the two types of metabolism is thinly veiled due to the overlap of their respective synthetic pathways (figure 1.1). In fact, complex secondary metabolites are synthesised from three types/classes of primary metabolite precursors. The first of these is shikimic acid, the precursor for many aromatic compounds. Second is the family of amino acids, which leads to peptide antibiotics and alkaloids. The third is acetate, which is the precursor for polyketides, fatty acids, prostaglandins, macrocyclic antibiotics and isoprenoids. It should be noted that the same three classes of precursors can be used to synthesise primary metabolites.
D-Glucose (+ other 4,5,6 & 7 carbon sugars)

Phophoenol Pyruvate

Pyruvate

Acetyl-CoA

Mevalonate

Isoprenoids: Terpenoids Steroids Carotenoids

Figure 1.1. Relationship between primary and secondary metabolism
Historically, secondary metabolites have often been considered as nothing more than "flotsam on the metabolic beach". While there are no known functions for the majority of these compounds, it is evident that in some cases these compounds do play a key role in the survival of the organisms which produce them, e.g. as feeding deterrents, UV protectants, nitrogen reserves, pollinator attractants, and growth regulators.

The human species has always been dependent on secondary metabolites for medicinals, flavourings for food, perfumes, pigments for artwork and clothing as well as tools to achieve spiritual enlightenment. One example of the latter is absinthe, an intensely bitter anise-flavoured liqueur that was popular in Western Europe from the 18th to the early 20th Century. It is made from a distillate of wormwood, anise, fennel, and other aromatic herbs steeped in alcohol.

The prevalent use of secondary metabolites in the modern world has led to an increase in research on the genes involved in their production and the alteration of these genes has resulted in the synthesis of novel compounds. This research could be directed towards providing new, alternative antibiotics required to combat the increasing numbers of bacterial strains that have developed resistance. It is therefore important to understand the origins of secondary metabolites and to determine the structure and function of the enzymes that synthesise them. This thesis will therefore focus mainly upon fatty acid synthases and polyketide synthases.

1.2 History

The term ‘polyketide’ was first introduced into the chemical literature in 1907 in a paper presented to the Chemical Society by Collie. In it he proposed that many natural products contained the ketene, [CH$_2$CO] unit. However, his idea could not be investigated further because there were few authentic natural product structures available at the time. This idea was later revived and extended by Robinson (1948) who having been stimulated by the evidence that acetate was the building block for fatty acids (Rittenberg and Bloch, 1944; Rittenberg and Bloch, 1945) made a hypothesis that natural products could be built from acetate units. Independently Birch (1953) put forward the ‘acetate hypothesis’. Birch proposed, by analogy with known fatty acid synthesis and the discovery that acetyl coenzyme-A acts as ‘active acetate’ in
fatty acid biosynthesis (Lynen and Reichert, 1951; Lynen, Reichert and Rueff, 1951), that polyketides were formed by the head to tail linkage with C2 acetate units, followed by a variety of secondary modifications including cyclisation, oxidation, C-alkylation, methylation and dehydration. He confirmed his ideas by carrying out the first ever biosynthetic experiments on fungal polyketides (Birch and Donovan, 1953; Birch, 1967). Since this original analogy, much of our understanding of polyketide biosynthesis has come from comparisons with developments in fatty acid biosynthesis.

1.3.1 Assembly of the β-ketoacyl backbone

The structural diversity of polyketides arises from a common pattern of biosynthesis, namely, the mechanism of carbon-carbon bond formation required to synthesise the β-ketoacyl backbone. Initiation of chain elongation usually proceeds from an acetate starter unit, that covalently attaches to an enzyme called a polyketide synthase. This is followed by the decarboxylative condensation of a β-carboxyacyl thioester extender unit with the enzyme bound acyl-thioester in a reaction which resembles a Claisen condensation (figure 1.2a). The resulting β-ketoacyl backbone undergoes further rounds of elongation until the desired backbone length is achieved. The number of acetate units condensed by the polyketide synthase is used to classify the polyketide as a diketide, triketide, tetraketide etc. Subsequent processing of the β-keto groups on the carbon backbone is the key to the diversity of polyketides.

One extreme of processing, common to the fatty acid biosynthesis pathway, occurs when all the β-keto groups are completely reduced to methylene groups (path A, figure 1.2b). This involves reduction of the ketone to an alcohol followed by the elimination of water to produce an α,β-unsaturated double bond. Finally the double bond is saturated to produce the methylene functionality. The alternative extreme of processing, which reflects polyketide biosynthesis, occurs when there is no further processing of any of the β-keto groups (path D, figure 1.2b). Polyketide metabolites can then arise from unreduced poly-β-keto chains, which undergo intramolecular condensations to produce aromatic compounds such as tetracyclines.
Figure 1.2a. The unified mechanism for polyketide backbone formation; Pant = 4’-phosphopantetheine; Cys = cysteine. The bold bonds represent either a starter acetate or equivalent intermediate acting as an electrophile for condensation with malonyl-CoA. 1.2b. Paths A to D highlight the extreme degrees of possible reduction of the polyketide backbone. B represents an intermediate level of reduction at various stages. C represents the use of alternative extender units such as methyl malonyl-CoA (P) or ethyl malonyl-CoA (B) (after O’Hagan, 1991).
One of the most interesting aspects of polyketide biosynthesis lies between the two extremes in which the fate of each β-keto group is varied after chain elongation (paths C and D, figure 1.2b). The level of reduction of each acetate unit is not the only factor responsible for the structural variety of polyketides. Other variants such as the use of different starter units (i.e. propionyl-CoA, butyryl-CoA), use of different extender units (i.e. methylmalonyl-CoA), side chain variation of the α-carbon atom (path C, figure 1.2b) and R or S chirality at hydroxyl carbon atoms (path B, figure 1.2b) also contribute towards the variation in primary polyketide structure. Also the total length of the chain can vary immensely. For example, 6-methylsalicylic acid (6-MSA) has an eight carbon length, whereas brevetoxin has a 50 carbon length. The diversity of polyketide structures is augmented even further by a number of post-elongation modifications including cyclisation, reduction, oxidation, methylation and dehydration.

1.3.2 Monofunctional and multifunctional proteins

The biosynthesis of polyketides and fatty acids is directed by polyketide synthases (PKSs) and fatty acid synthases (FASs) respectively. These enzymes vary in structure from organism to organism and can therefore be defined as either monofunctional or multifunctional proteins. A number of monofunctional proteins form a non-aggregated system of individual enzymes that catalyse fatty acid or polyketide synthesis. However, a sequence of reactions can occur more rapidly in a multifunctional enzyme complex because the active centers are close to one another; the products formed by one reaction within the complex do not diffuse away from the complex but are immediately acted upon by the next component of the system. This increases the efficiency of the pathway and the overall rate of catalysis and side reactions are minimized. Reaction intermediates can be forced to complete the intended reaction sequence, instead of escaping to conversion by enzymes that compete for the same reaction intermediate.

Multifunctional proteins have similar properties to monofunctional protein complexes except that they incorporate all the enzyme activities on a single polypeptide. The folding of the polypeptide dictates the relative position of each activity within the protein structure. Usually two polypeptides (or subunits) are required to interact in order to function. Examples include 6-deoxyerythronolide-B synthase and 6-
methylsalicylic acid synthase. Multifunctional proteins have a number of possible advantages over monofunctional protein complexes, some of which can be summarised as follows:

1) Proximity of active sites can facilitate the transfer of intermediates from one enzyme to another by channelling or direct covalent transfer, thereby minimising interactions with the external environment.
2) Covalent interactions between the enzymes may stabilise the overall enzyme structure while at the same time allowing flexibility around the active site.
3) Transcription and translation of the constituent components of a multifunctional protein are co-ordinately regulated and are expressed in the correct stoichiometry for synthesis of fatty acids and polyketides.

1.3.3 Type I and II synthases
The biosynthesis of the primary polyketide structure is achieved by the congregation of a number of enzyme activities to form a large, multifunctional PKS enzyme complex. The structural diversity in the organisation of PKS enzyme activities has resulted in the following structural classification, analogous to that designated for FASs: Type I synthases possess all their catalytic activities in a single multifunctional protein. In vivo, the dimerisation of two multifunctional polypeptides is required to express full catalytic activity. The nature of the interaction between the two subunits has to adhere to the constraint that the ketosynthase (KS) and acyl carrier protein (ACP) domains of each subunit must be in close contact. For the fungal PKS 6-methylsalicylic acid synthase (6-MSAS) these domains are located at opposite ends of the polypeptide chain and therefore the subunits are likely to be arranged in a 'head to tail' fashion (Spencer and Jordan, 1992; Child et al., 1996). However for the bacterial PKS 6-deoxyerythronolide-B synthase (6-DEBS) there are multiple copies of KS and ACP domains and these are within close vicinity of each other along the polypeptide chain. A truncated version of 6-DEBS, DEBS1+TE, has also been shown to retain catalytic competency. Therefore the arrangement of subunits is thought to be in a 'head to head' manner (Staunton et al., 1996). Type II synthases in contrast have a separate polypeptide for each activity. The discrete monofunctional polypeptides form a non-aggregated system. One example is the E.coli FAS. The type I synthases are thought to have arisen from their type II counterparts by a series of gene fusion events. Whether
these events occurred before or after the divergence of evolutionary lines is discussed in section 1.9.

1.3.4 The acyl carrier protein

Acyl carrier proteins (ACP) form an essential protein activity of PKSs and FASs, although the type II PKS chalcone synthase is an exception. Multienzyme complexes and multifunctional proteins that possess an ACP require a 4'-phosphopantetheine prosthetic group derived from coenzyme-A, in order to function (figure 1.3). The active *holo-* form of the ACP uses the 4'-phosphopantetheine prosthetic group as a presumed 'flexible' arm which, during synthesis, is thought to guide the growing carbon chain to the various enzyme activities of the synthase. Conversion of the ACP from *apo-* to *holo-* form is mediated by a separate, magnesium dependant enzyme called phosphopantetheine protein transferase (PPTase) (also known as *holo*-ACP synthase). This enzyme has been shown to transfer the 4'-phosphopantetheine group from CoA onto a conserved serine residue of *E.coli* FAS *apo*-ACP (Prescott *et al.*, 1975). However, recent research performed on the type I yeast FAS using $[^3H]$-CoA labelling has shown that the ACP located on subunit $\alpha$ is able to undergo self-pantetheinylation. A distinct FAS-activating PPTase domain on the C-terminal portion of one $\alpha$ subunit of the FAS complex is thought to interact with the N-terminal residing ACP of a second $\alpha$ subunit (Fichtlscherer *et al.*, 2000).

The study of *apo*-ACPs has been aided by their isolation from a number of *Streptomyces* type II PKS systems (Shen *et al.*, 1992; Crosby *et al.*, 1995). One such study has revealed the secondary structure (Crump *et al.*, 1996) and the three-dimensional solution structure (Crump *et al.*, 1997) of the actinorhodin (*act*) *apo*-ACP, using two-dimensional NMR techniques. The *act* *apo*-ACP structure has revealed a high degree of homology when compared with the *E.coli* FAS *apo*-ACP structure (Holak *et al.*, 1989). Both proteins consist of four helices that form a hydrophobic cleft capable of accommodating both the 4'-phosphopantetheine prosthetic group as well as an acyl chain. However, a number of hydrophilic groups, buried within the core of the protein have been identified for the *act* *apo*-ACP that are not observed for the FAS ACP (figure 1.4). The presence of these groups could reflect
Figure 1.3. Structures of a. coenzyme-A; b. acetyl-CoA; c. 4′-phosphopantetheine group bound to a serine residue of the holo acyl carrier protein (ACP).
Figure 1.4. Schematic representation of act apo-ACP structure determined by NMR showing the partially buried conserved Arg72 and Asn79 in the restrained minimised average structure. One hydrogen bonding interaction is indicated from Thr7 to Arg72 and two are indicated for Leu5 to Asn79 (after Crump et al, 1997).
the need to stabilise the PKS chain, which is extremely labile compared with the fully reduced FAS chain.

The isolation of type II act ACP in an active holo-form (Cox et al., 1997) has allowed the reactivity of the ACP to be probed. The PKS ACP has been shown to self-acylate with units of malonyl-CoA and acetoacetyl-CoA, as well as with N-acetyl cysteamine derivatives (Hitchman et al., 1998), therefore providing a reason for the absence of an active acyl transferase domain in some PKS enzyme gene sequences.

1.4.1 Fatty acids
Fatty acids are a class of compounds containing a long hydrocarbon chain and a terminal carboxylate group. Fatty acids are primary metabolites and have defined functions within the cell. They are the main components of all cell membranes, except in Archaebacteria, which possess phytanyl ether lipids derived from the terpenoid pathway (O’Hagan, 1991). Fatty acids also assist in targeting proteins to membrane locations and are fuel molecules stored as triglycerides. Derivatives of fatty acids also serve as hormones and intracellular messengers.

The diversity of naturally occurring fatty acids arises primarily through variation of chain length (6-30 carbons long) and degree of saturation. The chain is usually linear and contains an even number of carbons. However it has been shown that fatty acids with an odd number of carbons can be synthesised from a propionate (C₃) starter unit (Ingram et al., 1977). Alternative starter units can lead to an array of fatty acids with different terminal ends (table 1.1).

1.4.2 Fatty acid biosynthesis
Long chain fatty acids such as palmitic acid are synthesised in vivo in two distinct steps. The first is the conversion of acetyl-CoA to malonyl-CoA, catalysed by acetyl-CoA carboxylase (1), a biotin dependant enzyme (Wakil et al., 1958) that does not associate with fatty acid synthase (FAS). This step is necessary to circumvent the thermodynamic barrier posed by the condensation of two acetyl-CoA molecules. The second is the conversion of one unit of acetyl-CoA and seven units of malonyl-CoA to palmitic acid utilizing 14 molecules of NADPH, a multistep reaction catalysed by FAS.
<table>
<thead>
<tr>
<th>Starter unit</th>
<th>Fatty acid</th>
<th>Series</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isobutyryl-CoA (from valine)</td>
<td><img src="image" alt="Isobutyryl-CoA" /></td>
<td>$C_{18}$ iso-series (even)</td>
</tr>
<tr>
<td>2-Methylbutyryl-CoA (from leucine)</td>
<td><img src="image" alt="2-Methylbutyryl-CoA" /></td>
<td>$C_{19}$ iso-series (odd)</td>
</tr>
<tr>
<td>3-Methylbutyryl-CoA (from isoleucine)</td>
<td><img src="image" alt="3-Methylbutyryl-CoA" /></td>
<td>$C_{19}$ anteiso-series (odd)</td>
</tr>
<tr>
<td>Fluoroacetyl-CoA</td>
<td><img src="image" alt="Fluoroacetyl-CoA" /></td>
<td>$\omega$-Fluoropalmitic acid</td>
</tr>
<tr>
<td>Propionyl-CoA</td>
<td><img src="image" alt="Propionyl-CoA" /></td>
<td>$C_{17}$ (odd)</td>
</tr>
<tr>
<td>Cyclohexylcarboxyl-CoA</td>
<td><img src="image" alt="Cyclohexylcarboxyl-CoA" /></td>
<td>$\omega$-Cyclohexyl-undecanoic acid</td>
</tr>
</tbody>
</table>

Table 1.1. Fatty acids with unusual starter units (after O’Hagan, 1991).
This reaction involves numerous sequential reactions and acyl intermediates, the nature of which became primarily known from studies of fatty acid synthesis in cell-free extracts of *E. coli* (Wakil, 1970; Volpe and Vagelos, 1977; Bloch and Vance, 1977). There are over 40 steps with at least 30 acyl intermediates during the synthesis of palmitic acid. The overall reaction is summarised in (2):

\[
\begin{align*}
(1) \quad & CH_3CO-SCO_2 + CO_2 + ATP \leftrightarrow HOOCCH_2COS-CoA + ADP + P_i \\
(2) \quad & CH_3CO-SCO_2 + 7 HOOCCH_2COS-CoA + 14 NADPH + 14 H^+ \rightarrow \\
& \quad CH_3CH_2(CH_2CH_2)_nCH_2COOH + 7 CO_2 + 14 NADP^+ + 8 CoA-SH + 6 H_2O
\end{align*}
\]

The acyl carrier protein (ACP) was identified as the activity that binds all acyl intermediates as thioester derivatives. The other enzyme activities involved in the synthesis of palmitic acid are the acetyl transferase (AT), malonyl transferase (MT), β-ketoacyl synthase (KS), β-ketoacyl reductase (KR), β-hydroxyacyl dehydratase (DH), enoyl reductase (ER) and thioesterase (TE).

### 1.4.3 Fatty acid synthases

The mechanism of palmitic acid synthesis for the type I mammalian fatty acid synthase is shown in figure 1.5 and can be described as follows: Firstly acetyl-CoA is delivered, by AT, to the active thiol of a conserved KS cysteine residue. Malonyl-CoA is transferred via MT, to the active thiol of the 4'-phosphopantetheine arm of the ACP. Condensation then takes place between the acetyl moiety and the β-carbon of the malonyl moiety resulting in the release of CO₂. The resulting C₄ chain, attached to the ACP, then undergoes an NADPH dependent reduction (KR) followed by dehydration (DH) and finally enoyl reduction (ER) resulting in a fully saturated chain. The C₄ chain is then transferred back to the KS. A second malonyl-CoA unit is then transferred to the vacant ACP thiol and another round of condensation and complete reduction takes place. This process is repeated five more times until the required C₁₆ chain length has been obtained. The TE mediates product release from the enzyme.

The mechanisms used by mammals and bacteria to synthesise palmitate and the mechanism used by yeast to synthesise palmitoyl-CoA are very similar. However, the
Figure 1.5. Proposed mechanism of palmitate biosynthesis by mammalian fatty acid synthase. Cys-SH represents the active cysteine of the β-ketoacyl synthase. Pant-SH represents the 4'-phosphopantetheine thiol of the acyl carrier protein.
structural organisation of the FAS systems of these organisms varies greatly (figure 1.6). The evolutionary events resulting in different FAS structures are of great interest and will be discussed at the end of this chapter.

1.4.4 Mammalian fatty acid synthase

The mammalian FAS is a type I system of native Mr 500,000 comprised of two identical multifunctional polypeptide chains, each containing the seven discrete functional domains required for fatty acid synthesis. Upon dissociation of the native enzyme complex, six of the activities were retained whilst the KS activity was lost, even though its active site cysteine was still present (Butterworth et al., 1967; Muesing et al., 1975; Yung and Hsu, 1975; Stoops et al., 1979; Stoops and Wakil, 1981). It was therefore thought that dimerisation of the synthase was required for palmitate synthesis. Cross-linking studies of FAS with dibromopropan-2-one (DBP) suggested that the two subunits were juxtaposed in a head to tail manner such that the KS of one subunit interacts with the ACP of the opposing subunit (Stoops and Wakil, 1981). However, recently the ‘head to tail’ model has been revised. Mutation of the ACP phosphopantetheine and KS active site thiols of FAS has shown that cross-linking can occur between and within subunits (Witkowski et al., 1999). It was therefore concluded that head to tail structural and functional contacts could be made between and within subunits.

Limited proteolysis of several FAS systems has revealed that each multifunctional protein is arranged as a series of globular domains connected by polypeptide bridges (Kirschner and Bisswanger, 1976). Therefore it has been possible to isolate active fragments from FAS systems including chicken liver FAS (Mattick et al., 1983). These studies have also allowed construction of a reasonably detailed map of the synthase subunit, which is proposed to consist primarily of three major domains, I, II and III with masses of 127, 107 and 33 kDa respectively. Further proteolysis of the domains has been used to isolate the various activities, to elucidate their position within the polypeptide and to identify the linker regions between them (Schweizer et al., 1989; Amy et al., 1989; Holzer et al., 1989; Witkowski et al., 1991). As a result it has been possible to propose a mammalian FAS structural model (figure 1.7).
Figure 1.6. Molecular organisation of the genes of fatty acid synthases and a comparison with 6-methylsalicylic acid synthase (Hopwood and Sherman, 1990). KS - keto synthase; AT/MT - acetyl/malonyl transferase; PT - palmitoyl transferase; DH - dehydratase; ER - enoyl reductase; KR - keto reductase; ACP - acyl carrier protein; TE - thioesterase.
Figure 1.7. Proposed “head to tail” arrangement of the subunits of chicken fatty acid synthase. The juxtapositioning of the β-ketoacyl synthase and 4’-phosphopantetheine thiol of the ACP results in the formation of two active centres for palmitate biosynthesis. AT, acetyl transferase; MT, malonyl transferase; KS, ketosynthase; DH, dehydratase; ER, enoyl reductase; KR, ketoreductase; ACP, acyl carrier protein; TE, thioesterase; AcCoA, acetyl-CoA; MalCoA, malonyl-CoA; cys, cysteine (after Wakil, 1989).
1.4.5 Yeast fatty acid synthase

The genetics and biochemistry of yeast FAS have been one of the most thoroughly investigated of all FASs. Genetic mapping techniques have revealed that there are two different and unlinked loci designated fas 2 and fas 1 which encode respectively the α and β subunits of the yeast FAS (Schweizer et al., 1973). SDS-polyacrylamide gel electrophoresis has revealed that the two subunits, designated α and β, are non-identical and have molecular weights of 212 and 203 kDa respectively (Stoops et al., 1978). The activities are distributed amongst the two subunits. Genetic analysis (Knobling et al., 1975) and protein studies (Kresze et al., 1976; Stoops et al., 1978) conclude that the α-subunit contains the ACP, KR and KS domains and that the β-subunit contains the AT, TE, ER and DH components as well as a novel malonyl/palmityl transferase (figure 1.6). The enzyme has a native molecular weight of \(2.4 \times 10^6\) Da and therefore the obvious conclusion made was that the FAS functions as an \(α_6β_6\) complex (Schweizer et al., 1975; Stoops et al., 1975, 1978; Lynen, 1980). The recent isolation and sequencing of fas 1 and fas 2 (Schweizer, M. et al., 1989; Schweizer, E. et al., 1987) has allowed the use of targeted \textit{in vitro} mutagenesis to establish that there are six active centers per \(α_6β_6\) complex (Schuster et al., 1995).

Due to its large size it has been possible to use electron microscopy to determine the three-dimensional topology of the native yeast FAS (Kolodziej et al., 1996) at 25 Å resolution (figure 1.8). The structure is barrel shaped, the ends of which are enclosed by two sets of three β-subunits. The wall of the barrel appears to consist of three N-shaped α-subunit pairs each with an over and underlying arch-shaped β-subunit. Inside the molecule there are six cavities, each of which is proposed to be an active site for fatty acid biosynthesis (figure 1.8). The extensive intermolecular interactions between the subunits contribute to the stability of the overall complex.

1.4.6 \textit{E.coli} fatty acid synthase

The nature of the FAS mechanism was primarily known from studies on the type II \textit{E.coli} FAS, in which the individual enzymes were isolated and then utilised in the reconstitution of the FAS system (Wakil, 1970; Volpe and Vagelos, 1977). The unravelling of the genes involved in \textit{E.coli} fatty acid biosynthesis has revealed that
Figure 1.8. Yeast fatty acid synthase $\alpha_6\beta_6$ complex. Stereo views of the wire frame structure and a solid shaded representation of its central cavity in red on its 3-fold (A) and 2-fold axis (B). Shown is an interpretive model of the organization of the $\alpha$-subunits (yellow) and the $\beta$-subunits (grey) in the structure viewed on the 2-fold axis (front half and back half, left to right). The zig-zag shape of the $\alpha$ subunits forms the wall of the barrel, and two arch-like $\beta$-subunits over- and underlay pairs of the $\alpha$-subunits and form the caps (after Kolodziej et al., 1996).
there are often multiple copies of each enzyme activity. One such example has arisen from the isolation and characterisation of three β-ketoacyl-ACP synthases, KSI, II and III, that arise from the fabB, fabF and fabH genes, respectively (D’Agnolo et al., 1975; Garwin et al., 1980; Kaupinnen et al., 1988; Tsay et al., 1992). The substrate specificity of each KS varies but all play an important role in fatty acid biosynthesis. KSI and KSII are specific for C4 to C14 acyl-ACP primers but KSII can also utilise a C16 acyl-ACP primer (D’Agnolo et al., 1975; Garwin et al., 1980). KSIII uses CoA thioesters instead of acyl-ACP as the primer (Jackowski and Rock, 1987). These properties can be highlighted at the chain initiation stage from which there are three possible routes (figure 1.9). In the first, KSIII catalyses the condensation of acetyl-CoA with malonyl-ACP to yield acetoacetyl-ACP. In the second pathway, acetyl-CoA is first transferred to the ACP by either KSIII or AT. The acetyl-ACP is then condensed with malonyl-ACP by KSI or KSII. The third route involves the decarboxylation of malonyl-ACP by KSI to form acetyl-ACP followed by subsequent condensation with malonyl-ACP.

Upon formation of acetoacetyl-ACP, the β-carbonyl group undergoes a series of reduction, dehydration and reduction events. This is followed by the condensation of malonyl-ACP with the reduced C4 chain, a reaction catalysed by the β-ketoacyl-ACP synthases. The chain elongation and reduction cycle continues until the desired chain length is reached. Chain release is catalysed by either thioesterase I or II.

X-Ray crystallography is the most direct approach to understanding the structure-function correlation of E.coli FAS proteins. The crystal structures of malonyl transacylase, enoyl reductase, thioesterase II, KSII, and KSIII domains have all been solved (Serre et al., 1995; Baldock et al., 1998; Li et al., 2000; Huang et al., 1998; Davies et al., 2000). The type II three-dimensional structures can provide a useful comparison with those activities that reside on type I FAS and PKS systems for which no crystal structures have been determined.
Figure 1.9. Pathways for the initiation of fatty acid biosynthesis. There are three potential pathways for the formation of acetoacetyl-ACP in *E. coli*. (1): KSIII catalyses the condensation of acetyl-CoA with malonyl-ACP. (2): Conversion of acetyl-CoA to acetyl-ACP by ATA or KSIII is followed by condensation with malonyl-ACP by KSI (or KSII). (3): Decarboxylation of malonyl-ACP to acetyl-ACP by KSI is then condensed with malonyl-ACP. Abbreviations: ACC, acetyl-CoA carboxylase; MTA, malonyl-CoA:ACP transacylase; ATA, acetyl-CoA:ACP transacylase; KS, β-ketoacyl-ACP synthase (after Jackowski and Alix, 1990).
1.5.1 Polyketides

Polyketides comprise a large family of structurally and functionally diverse complex molecules produced as secondary metabolites in a variety of fungi, plants and bacteria. Although a definitive role for polyketides has not yet been proven, many have important pharmacological properties. Examples include erythromycin and patulin (antibiotics), FK506 and rapamycin (immunosuppressants), monensin (antiparasitic) and adriamycin (anticancer) (figure 1.10).

1.5.2 Polyketide synthases

Polyketides are synthesised by polyketide synthase (PKS) enzymes, which catalyse the repeated condensation cycles between acyl thioesters, usually involving the acetyl, propionyl, malonyl or methylmalonyl moieties and function similarly to FASs. However, after each condensation, the subsequent sequence of ketoreduction, dehydration, and enoyl-reduction of the β-keto group, normally prevalent in fatty acid biosynthesis may be omitted or curtailed by a highly programmed PKS following some or even all condensation steps (figure 1.2). The highly programmed manner that is employed by PKSs is responsible for generating a most impressive variety of structures compared with FASs. The PKS continues to catalyse chain elongation until a defined chain length is obtained. The product is then set free from the PKS by thioesterification, acyl transfer or cyclisation mechanisms that are also used by FASs.

1.5.3 The ‘minimal PKS’ and the chain length factor

Studies on aromatic type II PKS gene clusters has led to the discovery of three genes that encode a so-called ‘minimal PKS’. The key components are a ketosynthase / acyltransferase domain (KS / AT), a ‘chain length determining factor’ (CLF) and an acyl carrier protein (ACP) which are necessary for in vivo biosynthesis of polycyclic aromatic products. The minimal PKS controls chain length, the degree and regiospecificity of ketoreduction and the regiospecificity of the first carbon chain cyclisation (McDaniel et al., 1993a, 1993b, 1994, 1995; Fu et al., 1994). This has been shown by the structural characterisation of polyketide products obtained by the construction and expression of gene recombinants encoding the subunits of three different aromatic PKSs; act from S.coelicolor, fren from S.roseofulvus, and tcm from
Figure 1.10. Structural diversity of polyketides
S. glaucescens (McDaniel et al., 1994). Further research has revealed that CLF does not alter the chain length as predicted and that it exerts major influence only in the presence of a homologous KS activity (Shen et al., 1995) and perhaps further downstream enzymes such as cyclases and aromatases (Kramer et al., 1997). The CLF has been shown to be a factor required for polyketide chain initiation due to the ability of a conserved glutamine to decarboxylate malonyl-CoA to acetyl-CoA (Bisang et al., 1999). This has led to the re-examination of the chain initiation mechanism of type I modular PKSs, prompting the identification of a previously unknown KSQ domain, which operates like CLF in type II systems (Bisang et al., 1999). The amino acid sequences of the ketosynthase and KSQ are similar except that the conserved cysteine found in KS is replaced by glutamine for KSQ.

Recently published research has revealed a different minimal PKS composition to that described above (Hitchman et al., 1998). The ability of type II PKS ACPs to self-acylate suggests that the β-ketosynthase, chain length factor, and the acyl carrier protein constitute a ‘truly’ minimal PKS in vivo.

1.6 The type I PKS, 6-deoxyerythronolide-B-synthase

1.6.1 Erythromycin biosynthesis

Erythromycin A is part of the family of macrolide antibiotics and was originally isolated from *Saccharopolyspora erythraea* (McGuire et al., 1952). It is an important antibiotic widely used in clinical medicine against infections caused by Gram-positive bacteria and is also the main treatment used to fight pulmonary infections such as Legionnaire's disease. The structural elucidation of erythromycin A (Harris et al., 1965; Martin and Rosenbrook, 1967) revealed a large 14-membered lactone ring called 6-deoxyerythronolide-B (6-DEB), with two glycosyl groups attached (figure 1.11b). Therefore the biosynthesis of erythromycin A is considered to proceed in two stages. Initially, 6-deoxyerythronolide-B-synthase (6DEBS) is used to generate 6-DEB from one starter unit of propionyl CoA and six extender units of methylmalonyl CoA. The second stage consists of modifying 6-DEB using ‘tailoring’ enzymes to carry out regiospecific hydroxylations, glycosylations and a sugar group methylation, leading to the synthesis of the final erythromycin A product.
Figure 1.11a. The genes and proteins of the 6-deoxyerythronolide B synthase and the structures of the biosynthetic intermediates (after Weissman et al., 1998); 1.11b. The structure of erythromycin A.
1.6.2 6-DEB biosynthesis

The structural genes responsible for the synthesis of the parent aglycone of erythromycin A, 6-DEB, consist of three contiguous open reading frames, \textit{eryAI}, \textit{eryAII} and \textit{eryAIII}, (of \(-10\) kb each) which encode three large multidomain type I proteins designated DEBS1 (370kDa), DEBS2 (380kDa) and DEBS3 (330kDa) respectively (Cortes \textit{et al.}, 1990) (figure 1.11a). Each DEBS protein contains two functional units called modules (hence the nomenclature, modular polyketide synthase), each of which contains all the active sites required for one cycle of condensation and \(\beta\)-carbonyl processing. Unlike aromatic PKSs that use the same set of activities (including the minimal PKS) for each round of chain elongation, 6DEBS synthesises 6-DEB, by using its six modules in a ‘processive’ rather than an ‘iterative’ manner. The loading domain of DEBS1 initiates chain elongation by catalysing the attachment of the propionyl-CoA starter unit. Six cycles of condensation and \(\beta\)-carbonyl processing follow before chain release by the thioesterase component of DEBS3 (figure 1.11a). It is fascinating to note that the sequence of the catalytic domains, apart from module 4, matches the sequence of activities expected for a processive mechanism.

1.6.3 Modification studies on 6-DEBS

The overexpression, isolation and purification of all three DEBS multienzymes (Caffrey \textit{et al.}, 1992) have allowed its structural and mechanistic properties to be investigated. The length of the polyketide product has been shortened by fusing the thioesterase domain of DEBS3 with the COOH-terminus of DEBS1 (Cortes \textit{et al.}, 1995; Bycroft \textit{et al.}, 1999). The resulting mutant, DEBS1+TE, is able to form a triketide lactone (figure 1.12a). A dimeric double helical arrangement for the subunits of both 6DEBS and DEBS1-TE has been proposed as a result of a number of cross-linking experiments (Staunton \textit{et al.}, 1996). The two subunits of each module are thought to exist in a ‘head to head’ ‘tail to tail’ manner that twist together to form a double helix (figure 1.12b). This contrasts with the classical ‘head to tail’ arrangement of subunits thought to exist for other non-modular FASs and PKSs.
Figure 1.12a. Domain organisation of the triketide lactone synthase, DEBS1+TE. The hatched area denotes the region derived from DEBS3; 1.12b. Proposed double helical structure for 6-deoxyerythronolide B synthase and DEBS1+TE (courtesy of P.F. Leadlay, University of Cambridge).
Further experiments on 6-DEBS have produced a number of novel erythromycin compounds. This has been achieved by grafting the avermectin loading module onto DEBS1 in place of its normal loading module (Marsden et al., 1998). The avermectin PKS loading module has a broad substrate specificity, so that expression of the resulting hybrid enzyme in *Saccharopolyspora erythraea* (Marsden et al., 1998) has been shown to produce erythromycins A, B and D, as well as six novel erythromycins. This type of experiment has given rise to the idea of creating combinatorial libraries of macrolides that could be useful in counteracting antibiotic resistant bacteria. Additional erythromycin compounds have recently been isolated from a mutant strain of 6-DEBS (Wilkinson et al., 2000). The two novel octaketides, both 16-membered macrolides, appear to be aberrant products of 6-DEBS in which module 4 has 'stuttered', that is, it has catalysed two successive cycles of chain extension. This has provided the first evidence that an extension module in a modular PKS has the potential to catalyse iterative rounds of chain elongation, like other type I FAS and PKS systems. The factors governing the extent of such 'stuttering' remain to be determined.

### 1.7 Chalcone and stilbene synthase

The closely related plant-specific type II PKSs, chalcone synthase (CHS) and stilbene synthase (STS), catalyse the synthesis of two similar precursors required for a large number of biologically important secondary products (figure 1.13). Both enzymes utilise a starter unit of coumaroyl CoA and then perform sequential condensation reactions with three malonyl CoA units to produce the same tetraketide intermediate. However, CHS and STS follow different C-8 intermediate folding paths, resulting in the products, naringenin and resveratrol, respectively. The chalcone, naringenin, is a precursor for products required for flower colour, protection from ultraviolet light, defence against pathogens, interaction with microorganisms and fertility, whereas the stilbene, resveratrol, is a precursor for the production of phytoalexins and stress indicators.

Both synthases are homodimers with a subunit molecular weight of ~45kDa (Schroppner and Kindl, 1984). The relatively small size of these enzymes is partly accounted for by the absence of an acyl carrier protein domain, a characteristic that distinguishes these enzymes from all other known polyketide synthases isolated to date.
Figure 1.13. Reactions catalysed by chalcone and stilbene synthase. Both enzymes produce the same tetraketide intermediate from one coumaroyl-CoA starter unit and three malonyl-CoA extender units. The additional decarboxylation, orchestrated by stilbene synthase, results in an alternative cyclisation pattern to that of chalcone synthase.
Instead all the reactions take place at a single active site via the thiol of cysteine 169 (for CHS). The highly conserved sequence surrounding the active site reveals no homology to any known fatty acid synthase or polyketide synthases (Lanz et al., 1991). Even so, the recently solved crystal structure of CHS with and without substrate analogues not only elucidates the enzyme mechanism but can also provide important clues to how type I PKSs and FASs function (Ferrer et al., 1999).

1.8 The type I PKS 6-methylsalicylic acid synthase

1.8.1 Overview

The biosynthesis of single aromatic ring compounds occurs by two main routes; the shikimate pathway and the polyketide pathway. The intermediates of the shikimate pathway utilised in primary metabolism to produce aromatic amino acids have been well documented and many of the enzymes involved have been isolated and studied (Dewick, 1989). In contrast, aromatic ring biosynthesis from polyketides is not so well understood. This is mostly because the intermediates are labile and are enzyme bound, up to and including, the point of aromatic ring release from the enzyme system.

6-Methylsalicylic acid (6-MSA) is one of the simplest polyketide-derived aromatic metabolites and is biosynthesised by 6-methylsalicylic acid synthase (6-MSAS). The enzyme requires a starter unit of acetyl-CoA, three extender units of malonyl-CoA and one unit of NADPH (figure 1.14). The aromatic tetraketide produced, 6-MSA, is the first stable intermediate to be released into solution during the biosynthesis of the secondary metabolite patulin (Forrester and Gaucher, 1972). Patulin is a potent antibiotic and mycotoxin produced by the fungal source of Penicillium patulum and is useful in veterinary medicine.

1.8.2 Enzyme topology

The isolation and purification of 6-MSAS from Penicillium patulum has yielded milligram quantities of homogenous enzyme (Dimroth et al., 1970; Spencer and Jordan, 1992b), thereby allowing its properties to be investigated in detail. 6-MSAS has a $M_r$ of 190,000 as shown by SDS gel filtration and exists as a homotetramer ($\alpha_4$) with a native $M_r$ of $\sim$780,000 (Spencer and Jordan, 1992b). The subunit $M_r$ agrees closely
Figure 1.14. The formation of 6-methylsalicylic acid and triacetic acid lactone by 6-methylsalicylic acid synthase (6-MSAS), and orsellinic acid by orsellinic acid synthase (OAS).
with the value of 190,731 Da predicted from the gene sequence without the 4'phosphopantetheine moiety (Beck et al., 1990). The amino acid sequence of the 6-MSAS subunit and the sequential order of its constituent activities are closely related to those of type I mammalian FAS systems. Sequence alignments of 6-MSAS (Beck et al., 1990), rat FAS (Amy et al., 1990), chicken FAS (Holzer et al., 1989) and *Streptomyces cerevisiae* FAS1 and FAS2 (Schweizer, M. et al., 1986) have been used to predict the positions of five of the enzyme components of 6-MSAS. The β-ketoacyl synthase (KS), acetyl / malonyl transferase (AT/MT), acyl carrier protein (ACP), dehydratase (DH) and keto reductase (KR) regions show high levels of amino acid conservation around the putative active site residues.

1.8.3 The condensing site of 6-MSAS

Analogies between the structures of type I FAS and 6-MSAS have allowed a mechanism to be postulated for 6-MSA production (Dimroth, 1970). The mechanism incorporates the individual activities as a multifunctional protein complex (figure 1.15). There are two thiol residues implicated: one, an active site cysteine of the KS (Cys-204) and the other from the 4'-phosphopantetheine prosthetic group of the ACP attached to Ser-1733. The elongation of the polyacyl chain by Claisen condensation with malonyl CoA is dependent upon the two thiols, both of which are present in all PKS and FAS systems.

Experiments using thiol specific inhibitors and cross-linking reagents have been used to study the condensing site of 6-MSAS, similar to those carried out on type I vertebrate and yeast FASs (Wakil and Stoops, 1983). Treatment of 6-MSAS with the cross-linking reagent 1,3-dibromopropan-2-one (DBP) resulted in the formation of dimers and inactivation of the enzyme (Spencer and Jordan, 1992b; Child et al., 1996) (figure 1.16a). This has been shown by SDS-PAGE, density gradient ultracentrifugation, and secondary modification with $^{14}$C-iodoacetamide, an inhibitor that preferentially alkylates the reactive KS cysteine thiol. The formation of dimers, achieved by the formation of a mixed disulfide between cysteine 204 and the 4'-phosphopantetheine group indicates that the two thiol residues are positioned close to each other (Spencer and Jordan 1992b; Child et al., 1996). These studies conclude that two pairs of functional dimers
Figure 1.15. The enzymic synthesis of 6-methylsalicylic acid (after Dimroth et al., 1970). The cysteine thiol of the β-keto-synthase thiol is represented by \( ^{\text{K}}\text{SH} \). The 4'-phosphopantetheine thiol of the acyl carrier protein is represented by \( ^{\text{ACP}}\text{SH} \).
Figure 1.16a. Cross-linking of 6-methylsalicylic acid synthase with 1,3-dibromopropan-2-one (X) and the possible mechanism of inhibition of the cross-linking reaction by acetyl CoA and malonyl CoA; 1.16b. Proposed mechanism for the modification of 6-MSAS by reaction of the mycotoxin, cerulenin, with the substrate-binding cysteine residue (Cys-204).
are present in the 6-MSAS tetramer and that, within each dimer, the KS and the ACP components are juxtaposed (figure 1.16a). This spatial arrangement of thiol residues is analogous to that predicted in type I vertebrate FASs and other PKSs. Both acetyl-CoA and malonyl-CoA prevent the cross-linking, although only acetyl-CoA protects the enzyme from inactivation by protecting the KS thiol (figure 1.16a).

Irreversible inactivation of 6MSAS can also be achieved by covalent modification of Cys-204 of the KS by cerulenin, a fungal mycotoxin (Ohno et al., 1975; Child and Shoolingin-Jordan, 1998). The mechanism of cerulenin inactivation of 6-MSAS is proposed to be similar to that of type I FASs. The inhibitor reacts with the nucleophilic substrate binding thiol of the KS component resulting in cleavage of the epoxide moiety and covalent bond formation (figure 1.16b). The hydrophobic acyl ‘tail’ of cerulenin is also implicated in the inhibitory mechanism and may play a part in recognition of the reagent by the cysteine thiol. Conserved glycine residues may form part of a hydrophobic cavity to channel substrates and analogues, such as cerulenin, towards the reactive KS thiol.

1.8.4 6-MSA release from enzyme

The KS, KR, AT/MT and DH domains are all required to interact with an intermediate-ACP at various points of the synthesis, however, there is no indication from the gene sequence that either an enoyl reductase or thioesterase sequence is present in 6-MSAS (Beck et al., 1990). The lack of an enoyl reductase is of no surprise since no alkene reduction is required. However, the absence of a thioesterase domain raises the possibility that following cyclisation, product release from the enzyme takes place by an alternative mechanism (Spencer and Jordan, 1992a). There are two pieces of evidence to suggest this. Firstly, treatment of 6-MSAS with the serine protease inhibitor PMSF, that reacts with the active site serine of the thioesterase of FAS, does not inactivate the enzyme (Lam et al., 1988). Secondly, studies so far have not shown the formation of a product-CoA ester as is found in yeast fatty acid synthase. The alternative mechanism may follow a carbanion (E1cb) mechanism (figure 1.17, lower path) (Casanova et al., 1967; Holmquist and Bruice, 1969). The carbanion can collapse spontaneously to form a reactive ketene, which is rapidly hydrated to the carboxylic acid. The possibility that 6-MSAS has a novel thioesterase that maybe responsible for
Figure 1.17. Release of 6-methylsalicylic acid from the enzyme by thioesterase (upper path) and ketene (lower path) mechanisms.
hydrolysis should not, however, be ruled out (figure 1.17, upper path).

1.8.5 Molecular programming of 6-MSAS
During the three reaction cycles required for 6MSA biosynthesis, only one reduction occurs (at the C₆ polyketide intermediate stage). If the requisite NADPH is not provided, the enzyme-bound C₆ triketide intermediate is converted to triacetic acid lactone (TAL), as also happens with fatty acid synthases. The molecular programming of the enzyme to perform a non-repetitive cycle of reactions in a stereospecific manner requires the individual enzyme activities to be used in a clearly defined order. This provides a fascinating challenge, made more complex when considering that 6-MSAS comprises only one set of enzyme activities.

Comparisons between 6-MSAS and orsellinic acid synthase (OAS) from Penicillium cyclopium, have been made at the structural and mechanistic levels in order to understand the factors that contribute to the molecular programming process. OAS catalyses the production of orsellinic acid using the same building units that 6-MSAS employs. The only difference between the two products is the presence of an additional hydroxyl group at the C-4 position of orsellinic acid (figure 1.14). This indicates the absence of a NADPH-dependant reduction step in OAS that would remove the hydroxyl group. OAS has been isolated as a homogenous protein (Gaucher and Shepherd, 1968; Spencer and Jordan, unpublished results) and has a lower subunit molecular weight of Mr ∼130,000, compared with a Mr ∼190,000 for 6-MSAS. This reflects the absence of the reductase and dehydratase in OAS that would be required for the removal of the hydroxyl group from the C₆ triketide intermediate.

The reduction of the C₆ triketide intermediate is a key decision in the 6-MSAS cycle because the C₆ triketide can either be released as TAL or can go on to form 6-MSA, depending on the absence or presence of NADPH, respectively. The reason that only a single reduction occurs per cycle is not apparent and provides an interesting problem of specificity. The reduction step yields a C₆ alcohol intermediate that is then processed further through the cycle. However, it is uncertain whether or not the dehydration occurs at the C₆ alcohol stage or at the point when the tetraketide intermediate cyclises to form 6-MSA. The former is the more attractive possibility because of the analogy
with the FAS reaction and the fact that a dehydratase protein domain has been postulated from the cDNA-derived protein sequence of the enzyme.

1.8.6 Stereochemical considerations for the C₆ intermediate

The absolute configuration of the C₆ hydroxy intermediate for the 6-MSAS reaction has not yet been determined although the configuration of the FAS alcohol has been determined as ‘R’. If 6-MSAS favoured the ‘R’ configuration then this would impose mechanistic constraints on the dehydration reaction to form the cis-double bond that is required for the aromatic ring formation (assuming that this occurs immediately after the reduction). Determination of the configuration of the alcohol would fill an important gap in understanding the overall stereochemical mechanism.

1.8.7 Stereochemical course and chiral control

The incorporation of ‘R’ and ‘S’ [1-¹³C; 2-²H]-malonyl-CoA into the fatty acids produced by FASs from yeast, rat liver and Penicillium patulum and into 6-MSA produced by 6-MSAS from Penicillium patulum (Abell and Staunton, 1984; Jordan and Spencer, 1990; Spencer and Jordan, 1990; Jordan and Spencer, 1991; Spencer and Jordan, 1992c), has shown that malonyl-CoA is incorporated into the acyl chain in a highly stereospecific manner. For instance, the steric course of FAS proceeds with the elimination of the H₉, originally in malonyl-CoA, from the ‘R’-hydroxy-intermediate during the dehydration reaction. However, for 6-MSA biosynthesis, the hydrogen atoms eliminated from the 2- and 4- positions of the C-6 intermediate arise from opposite orientations, being the H₉ and H₉ atoms of malonyl-CoA, respectively (figure 1.18). Therefore, the hydrogen atom eliminated from the 2-position has the opposite orientation in malonyl-CoA to that eliminated in the dehydration reaction in fatty acid biosynthesis. For 6-MSAS it is likely that the triketide intermediate forms a cis-double bond. Whether this is a result of the reductase or the dehydratase following a different steric course to those in the FAS reaction is unknown.

The Claisen condensations in PKSs, like those in FASs, occur with inversion of configuration so that the hydrogen atom eliminated from the 2-position of the C-6 intermediate is H₉, and that eliminated from the 4-position is H₉. This information is essential for determining the precise stereochemical course of the overall reaction and
Figure 1.18. Stages in the biosynthesis of 6-MSA from acetyl-CoA and malonyl-CoA including the stereo-specific incorporation of methylene hydrogen atoms from malonyl-CoA (after Spencer and Jordan, 1990b). The methylene hydrogen atoms of malonyl-CoA are indicated by ■(Hre) and ▲(Hsi). The ▲H atom and the ■H atom from the 2- and 4- positions of the C₆ triketide intermediate are eliminated.
poses several mechanistic restrictions at subsequent stages. It is likely that a similar steric course to 6-MSAS is found in OAS with \( H_2 \) also being eliminated from the 2-position and \( H_3 \) from the 4-position of the \( C_6 \) intermediate. Therefore, both PKSs are able to catalyse the manipulation of the polyketide intermediates with a high degree of steric control.

1.9 Evolutionary origin of fatty acid and polyketide synthases

An amino acid sequence comparison between PKSs and FASs has revealed that the catalytic domains of both exhibit a high degree of homology. The question of which one evolved from the other, or whether they both evolved from a common ancestor arises. Also, it is important to ask how the type I multifunctional polypeptides evolved. The FAS systems of yeast and mammals exist in multifunctional forms whereas those from bacteria and plants are found to be in monofunctional (type II) forms. Therefore the theory that the multifunctional synthases arose from the fusion of genes encoding the monofunctional activities would seem to be plausible. The discovery of many introns within the rat FAS genes supports ‘exon shuffling’ resulting in gene fusion (Amy et al., 1990).

Whether exon shuffling took place before or after the divergence of eukaryotes and prokaryotes is debatable. Most of the clearest examples of exon shuffling involve genes that are unique to mammalian systems and that presumably appeared relatively late in eukaryotic evolution (Patthy, 1991; Doolittle, 1987). Indeed the yeast (\( \alpha_6\beta_6 \)) and mammalian (\( \alpha_2 \)) FAS systems exhibit little sequence similarity and are connected structurally in completely different ways indicating that they have most likely evolved by a separate series of gene fusion events that occurred after the divergence of fungal and vertebrate lines. However the discovery that the type I multifunctional 6-DEBS required for polyketide synthesis in \( S. erythraea \) and mammalian FAS are composed of functional domains of similar size and amino acid sequence (Donadio et al., 1991; Cortes et al., 1990) directly challenges this view and raises the distinct possibility that gene fusion took place prior to the divergence of prokaryotes and eukaryotes.
The amino acid sequence and domain organisation of 6-MSAS from *P. patulum* shows greater similarity to mammalian FAS, than to its own fatty acid synthase (Weisner *et al.*, 1988; Beck *et al.*, 1990; Hopwood and Sherman, 1990). There is a 67% amino acid sequence homology between the ACP of 6-MSAS and rat FAS but only a 13% similarity with the FAS of *P. patulum*.

From the available data it would not seem likely that the PKSs evolved from the FASs by gene duplication within the same organism. A more plausible explanation would be that the gene fusion events that led to a common PKS and FAS took place before the divergence of evolutionary lines. However, the possibility that the mammalian FAS module was acquired by prokaryotes by a relatively late ‘horizontal’ gene transfer mechanism cannot be ruled out at present (Vining, 1992).

1.10 Summary

Comparative studies of 6-MSAS and type I mammalian FAS highlights the remarkable similarities between the two enzyme systems in terms of subunit arrangement, domain organisation and important conserved amino acid residues. Indeed, both systems use similar substrates and can produce triacetic acid lactone in the absence of NADPH. However, these resemblances are not so evident in their respective mechanisms. The repeated cycle employed in type I FASs is modified by 6-MSAS, that uses a cycle which performs only a single reduction step during chain elongation, namely, at the C₆ polyketide intermediate stage. A second contrast arises from the stereochemical events during the polymerisation process. 6-MSAS eliminates H₆ from the 2- position of the C₆ intermediate, whereas FASs commonly eliminate H₆ from the equivalent position. A third difference arises from the way in which their respective products are released. The thioesterase domain present in FASs is absent in 6-MSAS suggesting a novel mechanism for the release of 6-MSA. The specificity of the single reduction step to produce a cis double bond may drive the tetraketide towards a cyclic release from 6-MSAS. The complicated molecular programming of the enzyme needs to be studied further to understand the overall mechanism.
This thesis intends to further explore the properties of 6-MSAS and its relation to type I FASs. The decarboxylation of malonyl-CoA and the synthesis of orsellinic acid by 6-MSAS will be investigated. The synthesis of halogenated acetyl-CoA compounds and their reaction with the enzyme will be researched in experiments similar to those already performed on type I mammalian FASs. The interaction between 6-methylsalicylic acid synthase and several N-acetylcysteamine intermediates will also be investigated. Also the purification and partial characterisation of 6-MSAS ACP and E.coli holo ACP synthase will be presented. Initial NMR data of the latter enzyme will also be shown.
Chapter 2

Purification of 6-methylsalicylic acid synthase from *Penicillium patulum* and succinyl CoA transferase from porcine heart

2. The purification of 6-methylsalicylic acid synthase

2.1 Overview

The type I multifunctional enzyme complex 6-methylsalicylic acid synthase, has been isolated from *Penicillium patulum* by several research groups to various degrees of purity (Dimroth *et al*., 1970; Scott *et al*., 1974; Beck *et al*., 1990) but insufficient yields have prevented in-depth study of the properties and structure of the enzyme. However, 6-MSAS has been purified to homogeneity in milligram quantities using a more recent procedure (Spencer and Jordan, 1992b). This has been achieved by growth of *Penicillium patulum* in liquid culture from a spore inoculum, in which the culture is synchronous such that all cells are approximately of similar age and morphology (Grootwassink and Gaucher, 1980). This is followed by a series of purification steps based on precipitation and gel filtration, all of which have been carried out at 4°C due to the high instability of the enzyme. This protocol has been used as a template for the following purification procedure.

2.2 Materials

6-MSAS was isolated from the strain *Penicillium patulum* NRRL 2159A (also called *Penicillium urticae*) which was obtained from the Deutsche Sammlung fur Microorganismen (Göttingen). Czapek solution agar, bacto-agar and yeast extract were from Difco laboratories, U.S.A. Mono Q anion exchange columns, Sephacryl-S400, DEAE sepharose (fast flow) and PD-10 (Sephadex G-25) pre-packed gel filtration columns for use during the enzyme purification were from Pharmacia Fine Chemicals, Sweden. Acetyl-CoA, malonyl-CoA, NADPH, TEMED, ammonium persulphate and bovine serum albumin were obtained from Sigma Chemical Company. Dithiothreitol (DTT) was supplied by Melford Laboratories. Ammonium sulphate (analytical grade) was from B.D.H. SDS-PAGE molecular weight markers (broad range, 6-200 kDa), protein assay reagent and hydroxyapatite were obtained from Biorad. Acrylamide was obtained from National Diagnostics, USA. Borosilicate nanoflow probe tips were
obtained from Micromass. All other chemicals were from Sigma Chemical Company.

2.3 Purification methods

2.3.1 Growth of *Penicillium patulum*

Mycelia of *Penicillium patulum* were grown from spores on the surface of agar plates (49g Difco Czapek solution agar and 5g Difco bacto-agar per litre of distilled water). Using a dry wire loop, each plate was inoculated with spores from a previous culture. The plates were incubated at 25°C until maximum spore formation had occurred (usually 7-10 days). The plates were stored at 4°C for up to a period of 8 weeks before use.

Strain NRRL 2159A of *Penicillium patulum* is unusual in that it produces white spores due to a mutation in a pigment synthesis pathway. This phenotypic difference is useful as contaminants of stock cultures can be readily identified.

2.3.2 Submerged culture medium

The agar layers from eight plates were removed and carefully washed in 250ml of Triton X-100 (1ml/litre) in 1% (w/v) NaCl to remove the spores from the mycelia. The suspension containing the spores was used to inoculate eight 2-litre conical baffled flasks, each containing 600ml of a yeast extract-glucose-buffer (YGB) medium. To prevent mycelial aggregation on the flask walls, the interior of the flasks were coated with dimethylchlorosilane (BDH). The YGB medium, similar to that used by Grootwassink and Gaucher (1980), and Lam, Neway and Gaucher (1987) contained: yeast extract (24g), MgSO$_4$·7H$_2$O (2.4g), glucose (192g), KCl (0.4g), KH$_2$PO$_4$ (25g) and K$_2$HPO$_4$ (25g) in a final volume of 4.8 litres. The KH$_2$PO$_4$ and K$_2$HPO$_4$ were dissolved in 200ml of distilled H$_2$O and autoclaved separately at 15lb/in$^2$. Autoclaving the complete medium results in precipitation of the phosphate, so 20ml of the buffer was added to each flask immediately prior to inoculation. The flasks were incubated in a rotary shaker for 24 hours at 28°C with a 5cm stroke rate at a speed of 120 rotations per minute.
2.3.3 Cell disruption

The mycelia from the flasks were collected by suction filtration and washed with distilled H₂O, yielding approx. 170g dry wt. The mycelial mats were immediately broken up into 2cm x 2cm pieces and suspended in a 'Bead-Beater' bead mill (Biospec, USA) containing ~200ml of pre-chilled 100mM Tris/sulphate buffer, pH 7.6, containing 15% (v/v) glycerol, 1mM-EDTA, 5mM-β-mercaptoethanol, 0.34g/l of benzamidine and 2mM phenylmethysulphonylfluoride (PMSF)- dissolved in 0.5ml methanol beforehand. More buffer was added to the mill followed by careful placement of the rotor, in order to exclude all air. The homogenising chamber (350 ml) was surrounded by a water jacket containing a salt/ice/water mixture at -8°C. The suspended mycelia were homogenised using 0.5mm-diameter glass beads for 2 x 90s, with 2min intervening cooling periods. The suspension was filtered through muslin cloth to remove glass beads, unbroken cells and cell fragments. The used glass beads were washed in acetone so that they could be recycled. All subsequent stages were carried out at 4°C.

2.3.4 Ammonium sulphate fractionation

(NH₄)₂SO₄ (125.5g/l) was added to the crude extract, with stirring, to give 22% saturation. After 20 minutes, the precipitated protein was collected by centrifugation at 4°C in a Beckmann J2-21 centrifuge, 6 x 500ml rotor, for 25 min at 8500 r.p.m. The supernatant was retained and adjusted to 40% saturation by addition of further solid (NH₄)₂SO₄ (109g/l). After 20 minutes stirring time, the precipitated protein containing 6-MSAS was collected by centrifugation as before.

2.3.5 Poly(ethylene glycol) fractionation

The precipitate from the previous stage was resuspended in 200ml of 100mM-Tris/sulphate buffer, pH7.6, containing 6% (w/v) polyethylene glycol-6000, 5mM-β-mercaptoethanol, 1mM-EDTA and 0.34g/l of benzamidine. The solution was stirred for 20 min and centrifuged at 4°C in a Beckmann J2-21 centrifuge, 6 x 500ml rotor, for 25 min at 8500 r.p.m. The supernatant was retained and adjusted to 20% saturation by the gradual addition of 50% (w/v) polyethylene glycol-6000 (80ml/200ml supernatant) with slow stirring. The protein precipitate was collected after 20 minutes stirring, by
2.3.6 DEAE-Sepharose ion-exchange chromatography

After assaying for 6-MSAS the non-active supernatant fraction was discarded and the pellet was redissolved in 25ml of 50mM-potassium phosphate buffer, pH 7.6, containing 15% glycerol, 5mM-β-mercaptoethanol, 1mM-EDTA and 0.34g/l of benzamidine. The protein solution was loaded on to a DEAE-Sepharose column (6.4cm x 12cm) pre-equilibrated with the same buffer. Purification of 6-methylsalicylic acid synthase and fatty acid synthase was achieved by elution over a linear phosphate gradient (50-400mM; total volume 500ml). Each fraction contained 5ml. The fractions (45-75) containing 6-MSAS activity were pooled and concentrated by precipitation with (NH₄)₂SO₄ (243g/l).

2.3.7 Hydroxyapatite chromatography

The pellet containing 6-MSAS from the previous stage was dissolved in the smallest volume possible of 10mM-potassium phosphate buffer, pH 7.6, containing 15% glycerol, 2mM-DTT, 1mM-EDTA and 0.34g/l of benzamidine. The protein solution was desalted through a Pharmacia PD-10 gel-filtration column (2.5ml per column) which had been pre-equilibrated with the same buffer. This step ensured the removal of (NH₄)₂SO₄ which would have interfered with the binding of the protein to the column. The eluate (~15ml) was applied to a column of hydroxyapatite (Biogel HTP, Biorad) (3cm x 10cm) which had been pre-equilibrated with the same buffer. The 6-MSAS and FAS were separated by elution through a stepwise gradient of 10, 50, 100 and 200mM potassium phosphate buffers containing 15% glycerol, 2mM-DTT, 1mM-EDTA and 0.34g/l of benzamidine. Protein that had eluted from the column was detected at λ=280nm on a chart recorder. Each protein peak was collected and assayed for 6-MSAS activity. The majority of 6-MSAS eluted at 100mM although a substantial amount eluted at 50mM, whereas FAS eluted at 200mM. Those peaks containing 6-MSAS activity were pooled and concentrated by precipitation with (NH₄)₂SO₄ (243g/l).
2.3.8 Fast protein liquid chromatography (FPLC) Mono Q™ anion-exchange chromatography
The (NH₄)₂SO₄ precipitate from the previous stage was dissolved in 2ml of pre-chilled 40mM Tris/HCl buffer, pH 7.2, containing 5mM-DTT. The solution was desalted through a PD-10 column that had been pre-equilibrated with the dissolving buffer. The eluate was then loaded on to a Pharmacia Mono Q™ HR 5/5 column (1ml of resin) which had also been pre-equilibrated with the dissolving buffer. Elution of the enzyme from the column was achieved using an FPLC apparatus (Pharmacia) with a linear gradient (0-1M NaCl in a total volume of 35ml) at a flow rate of 1ml/min (figure 2.1a).

2.3.9 Buffer exchange and storage of 6-MSAS
The buffers used in the previous step did not contain glycerol, benzamidine, β-mercaptoethanol or EDTA since they are not compatible with the column material. Therefore, upon elution from the Mono Q™ column, the collected fractions were buffer exchanged through a PD-10 column into 100mM Tris/HCl, pH7.6, containing 20% glycerol, 1mM-EDTA, 1mM-DTT, 1mM-β-mercaptoethanol and 1mM benzamidine. The active fractions were pooled and then quick frozen by pipetting 30μl volume drops of enzyme solution into liquid nitrogen in a small dewar flask. The frozen enzyme droplets were collected and placed into screw top Eppendorfs which had been pre-cooled in liquid nitrogen (USA Scientific Plastics, Bradwell Abbey, Milton Keynes). The Eppendorfs were placed in a liquid nitrogen storage facility (-120°C) until enzyme was required.

2.3.10 Fluorometric assay for 6-MSAS activity
A fluorometric method has been developed to measure 6-MSAS activity by following the formation of 6-MSA. The polyketide product has maximal excitation and emission wavelengths of 308nm and 410nm respectively. However, using wavelengths of 310nm and 390nm for excitation and emission eliminates the interference caused by the change of NADPH fluorescence (λ_EX = 360nm, λ_EM = 465nm). The fluorescence intensity of 6-MSA is increased in the presence of bovine serum albumin (BSA) (Vogel and Lynen, 1975), therefore enabling small changes in fluorescence to be observed accurately. BSA has two distinct hydrophobic binding pockets that can bind a wide range of
ligands (Peters, 1985). When a ligand is transferred from a polar aqueous environment to the non-polar, hydrophobic binding pocket, there is an approximately 30 fold increase in intrinsic fluorescence emission (Epps et al., 1995).

In a final volume of 1ml the reaction mixture contains: Tris/sulphate buffer, pH 7.6, (0.1M); acetyl-CoA, (0.1mM); NADPH, (0.2mM); BSA, (2.5mg); 0.2-1.0 munits (mU) of 6-MSAS. Following the establishment of a stable baseline at 25°C, the reaction commenced by addition of malonyl-CoA (0.1mM), and the increase in fluorescence associated with 6-MSA formation was measured for five minutes. The extent of fluorescence change is related to that obtained with a standard solution of 6-MSA.

Units: One unit of activity is defined as that amount of enzyme catalysing the formation of 1μmole of 6-MSA per minute at 25°C under the assay conditions. The specific activity is defined as the catalytic activity per mg of protein (i.e. mU/mg).

2.3.11 Determination of protein concentration
Protein concentrations were measured using the BioRad Protein Assay Reagent (Biorad Laboratories), a variation of the method by Bradford (1976), using BSA as the standard.

2.3.12 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)
5% SDS-PAGE polyacrylamide gels were performed using the method of Laemmli (1970). Approximate molecular weights of experimental bands were determined by comparison to those of molecular weight markers (Biorad, broad range).

2.3.13 Gel silver staining
Gel silver staining was carried out using the method of Wilm (1996).

2.3.14 Mass spectrometric analysis of 6-methylsalicylic acid synthase
6-MSAS was removed from the −120°C liquid nitrogen storage facility and buffer exchanged into 5mM ammonium acetate buffer containing 1mM DTT and the
concentration of the enzyme was adjusted to 0.5mg/ml. An equal volume of a solution containing 25% acetonitrile, 50% formic acid and 25% analytical grade H₂O was added to the 6-MSAS solution. A volume of 5μl was loaded onto a borosilicate nanoflow probe tip and analysed using a Micromass LCT nanoflow time-of-flight mass spectrometer.

2.4 Results
2.4.1 Regulation of in vivo growth
Production of 6-MSAS, as the first enzyme involved in the biosynthesis of patulin (Martin and Demain, 1978), is subject to regulatory mechanisms. All enzymes involved in secondary metabolism including 6-MSAS, are induced at the end of exponential cell cycle growth during the productive phase known as the idiophase. This occurs at approximately 18 hours after spore inoculation when levels of nitrogen containing nutrients have been exhausted. After biosynthesis, 6-MSAS is prone to rapid degradation by proteolytic enzymes (Lam et al., 1987), as are many enzymes that take part in secondary metabolite pathways. This could be viewed as a crude form of regulating the level of enzyme in the cell. It is crucial therefore, to determine the optimum growth conditions that maximise the level of enzyme but minimise the level of proteolytic degradation. Although the most recent published purification (Spencer and Jordan, 1992b) used an incubation time of 26 hours before harvesting the cells, the purification followed in this thesis, employs a lower incubation time of 24 hours. This results in a similar yield of protein and a four-fold increase in activity (table 2.1) with a reduced level of proteolytic ‘nicking’ of 6-MSAS. The use of a new, more efficient rotary shaker could be a possible reason for the lower optimum time.

2.4.2 In vitro protection of 6-MSAS
Although 6-MSAS is refined at 4°C at a pH optimum of 7.6, it remains susceptible to proteolytic degradation throughout the purification, particularly during the later chromatographic steps where structural damage may occur. However, the enzyme can be protected by the addition of 15% (v/v) glycerol and protease inhibitors such as PMSF and benzamidine which extend the half-life of the enzyme from 2 to 6 hours (Lam et al., 1987).
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein (mg)</th>
<th>Total Activity (mU)</th>
<th>Specific Act. (mU/mg)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>7870</td>
<td>38,000</td>
<td>4.8</td>
<td>100</td>
</tr>
<tr>
<td>22-40% (NH₄)₂SO₄ pellet</td>
<td>2400</td>
<td>35,000</td>
<td>14.6</td>
<td>92</td>
</tr>
<tr>
<td>6-20% PEG-6000 pellet</td>
<td>580</td>
<td>32,500</td>
<td>56.0</td>
<td>86</td>
</tr>
<tr>
<td>DEAE-Sepharose eluate</td>
<td>186</td>
<td>26,000</td>
<td>139.8</td>
<td>68</td>
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<td>53</td>
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<tr>
<td>Mono Q™ eluate</td>
<td>17</td>
<td>7,000</td>
<td>411.8</td>
<td>18</td>
</tr>
</tbody>
</table>

**Table 2.1.** Purification table of 6-methylsalicylic acid synthase from *Penicillium patulum*. On average a total of 17mg of pure enzyme was obtained from each purification, based on an initial volume of 4.8 litres of medium.
Each subunit of 6-MSAS possesses two reactive thiols. These are located on the 4'-phosphopantetheine arm attached to serine-1733, and the amino acid cysteine-204. The thiol groups need to remain in a reduced state in order to maintain enzyme activity. The thiol protecting reagents DTT and β-mercaptoethanol are used to preserve the reduced state of the condensing site. In addition, the presence of EDTA in all buffers maintains synthase activity by chelating divalent metal ions that may promote disulphide formation between two reduced, adjacent thiols.

2.4.3 Purification of 6-MSAS
6-MSAS has been purified to homogeneity from *Penicillium patulum*. The purity is indicated by the presence of a single band at ~190kDa on a silver stained 5% SDS-PAGE gel (figure 2.1b). Approximately 17mg of pure enzyme was obtained from each purification.

A further two changes have been made to the most recent published purification (Spencer and Jordan, 1992b). Firstly, it was observed that upon precipitation of the DEAE sepharose and hydroxyapatite pooled fractions, there was always a residual amount of ammonium sulphate left undissolved. This was rectified by using analytical grade ammonium sulphate supplied by B.D.H. Secondly, unlike previous procedures, fresh hydroxyapatite (Biogel HTP) was used for each purification. This was found to be a better alternative to recycling used hydroxyapatite which had a reduced binding affinity. Both factors have contributed towards an improved yield of 6-MSAS.

2.4.4 Enzyme storage
Once purified, the enzyme responds well to storage in liquid nitrogen and loses about 1.5% activity per month. The storage method is an improvement upon that previously used (Spencer and Jordan, 1992b) which registered a 5% loss in activity per month through storage of 6-MSAS as an ammonium sulphate pellet at -70°C.

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Figure 2.1. Final stages of 6-MSAS purification from \textit{P. patulum}. a. FPLC Mono Q\textsuperscript{TM} anion exchange chromatography of 6-methylsalicylic acid synthase. Elution was carried out using a NaCl gradient (red line) over a period of 33 minutes. Detection of protein was carried out at 280nm; b. Silver stained 5\% SDS gel of pure 6-MSAS: Lanes 1 and 6, molecular weight markers (myosin, 200kDa; \(\beta\)-galactosidase, 116.3kDa; phosphorylase B, 97.4kDa; bovine serum albumin, 66.2kDa). Lanes 2, 3, 4 and 5; FPLC Mono Q\textsuperscript{TM} purified 6-methylsalicylic acid synthase, 2\(\mu\)g, 1\(\mu\)g, 0.5\(\mu\)g and 0.25\(\mu\)g respectively.
Figure 2.2a. Raw data obtained from 5μl of 6-methylsalicylic acid synthase at a concentration of 0.25 mg/ml (as described in section 2.3.14) analysed by nanoflow ES-MS-TOF; 2.2b. Deconvoluted spectrum of the multiple charged envelope.
2.4.5 Mass spectroscopic analysis of 6-MSAS

The predicted molecular mass of 6-MSAS from the amino acid sequence is 190,731 Da (Beck et al., 1990). The mass of the 4'-phosphopantetheine arm must be added onto the sequence derived weight, resulting in a predicted post-translationally modified mass of 191,078 Da. Each subunit of 6-MSAS has Mr 190,047 as determined by nanoflow TOF-MS (figure 2.2b). The deconvolution of the multiply charged envelope (figure 2.2a) results in a major species that is within 0.5% of the predicted molecular weight of the 6-MSAS subunit (figure 2.2b).

2. Purification of succinyl CoA transferase from porcine heart
2.5 Overview

The oxidation of ketone bodies within mammalian mitochondria provides the major metabolic fuel for the heart and kidney (Sharp and Edwards, 1978; Russel and Patel, 1982) and a secondary fuel source for the brain (White and Jencks, 1976a, 1976b) when insufficient glucose is available. A rise in the physiological level of ketone bodies is associated with metabolic disorders such as starvation and diabetes. Succinyl CoA transferase (SCAT) is the first enzyme to be utilised in the oxidation of ketone bodies. SCAT functions to activate acetoacetate by forming acetoacetyl CoA, by transfer of a CoA moiety from succinyl CoA in a reversible ping-pong reaction. The acetoacetyl-CoA produced is then broken down by a thiolase to two acetyl-CoA molecules capable of entering the tricarboxylic acid cycle.

SCAT from porcine heart is a homodimer with a subunit Mr of 52,197, as deduced from the cDNA sequence (Lin and Bridger, 1992). Each subunit consists of two domains of Mr 29k and 25k, linked by a hydrophilic 'hinge' region. The folding and assembly of the enzyme requires the productive interaction between the two domains of each subunit, involving a substantial conformational rearrangement (Rochet et al., 1997). A key feature of the enzyme is an active site amino acid residue, which is thioesterified by CoA during the reaction (Solomon and Jencks, 1969). The residue, identified as glutamate-344 (Rochet and Bridger, 1994), is known to be conserved in all CoA transferases and is responsible for the formation of a stable enzyme-CoA covalent intermediate (Hersh and Jencks, 1967a, 1967b).
2.6 Materials

Fresh porcine heart was supplied by H.M. Bennett Butchers, Funtley, Hampshire (now closed). DEAE Sephacel, KH$_2$PO$_4$, KCl, MgCl$_2$, Tricine, MOPS, EDTA, PMSF, (NH$_4$)$_2$SO$_4$, glycerol, succinic acid, succinic anhydride, coenzyme-A and acetoacetyl-CoA were purchased from Sigma Chemical Company. DTT was obtained from Melford Laboratories. Sepharose 4B resin was purchased from Pharmacia Fine Chemicals. Dialysis tubing (size 9) was from Medicell Int Ltd. YM 30 and PM 10 concentrating cell membranes were from Millipore Ltd. Yellow HE-4R and blue MX-2G Procion dyes were obtained from BASF.

2.7 Purification methods

2.7.1 Crude extract preparation

Pig heart tissue (~900g) was removed from storage at -70$^\circ$C and semi-thawed overnight at -20$^\circ$C. All subsequent stages were carried out at 4$^\circ$C. The following day the tissue was chopped into pieces (2cm x 2cm) and divided into two halves. Each was processed as follows: The tissue was placed in a chilled Waring blender and to it was added ~700ml of 50mM potassium phosphate buffer, pH 7.4, containing 1mM EDTA, 0.2M KCl, 1mM DTT and 0.2mM PMSF. The suspension was homogenised at full power for 10 x 30 seconds interspersed by 60 seconds cooling in an ice/salt bath. The suspension was supplemented with another ~700ml of the same buffer and homogenised at half power for 10 x 30 seconds interspersed by 60 seconds cooling. The homogenate was then centrifuged at 4$^\circ$C in a Beckmann J2-21 centrifuge, using a 6 x 500ml rotor, for 40 minutes at 10,000 r.p.m. The crude extract obtained was filtered through four layers of muslin cloth to remove any fats.

2.7.2 Ammonium sulphate fractionation

Solid (NH$_4$)$_2$SO$_4$ (243g/l) was added with stirring to give 40% saturation. After stirring the extract for 45 minutes, the precipitated protein was collected by centrifugation in a Beckmann J2-21 centrifuge, using a 6 x 500ml rotor, for 40 min at 10,000 r.p.m. The pellet was discarded. The supernatant was retained and adjusted to 65% saturation by the addition of further solid (NH$_4$)$_2$SO$_4$ (168g/l). After stirring for 45 minutes, the extract was centrifuged as before. The pellets were resuspended in 5mM potassium phosphate buffer, pH 7.0, containing 1mM EDTA, 10% glycerol, 1mM DTT and
0.2mM PMSF. The resulting enzyme extract was then dialysed overnight in 5 litres of the same buffer.

2.7.3 Ultra-centrifugation
Precipitated protein was removed from the dialysate by centrifugation in a Beckmann L7-1 ultracentrifuge, using a 6 x 94ml rotor, for 50 minutes at 35,000 r.p.m.

2.7.4 DEAE Sephacel column chromatography
The supernatant obtained was loaded onto a column of DEAE Sephacel (column size: 5cm x 40cm) that had been pre-equilibrated with 5mM potassium phosphate buffer, pH 7.0, containing 1mM EDTA, 10% glycerol, 1mM DTT and 0.2mM PMSF at a flow rate of 90ml/hr. In order to elute the protein, the same buffer was applied to the column, also at a flow rate of 90ml/hr. Fractions were collected (100 x 13ml volume) starting from when the protein front, visible by a yellow brown band, had reached 15cm from the column base.

2.7.5 Concentration and dialysis
The active fractions from the previous step were pooled and the resulting volume of ~500ml was reduced to ~200ml by concentrating under N₂ using a YM30 membrane in a 400ml Amicon ultra-filtration cell. The concentrated protein was then dialysed overnight into 20mM Tricine buffer, pH 8.2, containing 1mM EDTA, 10% glycerol, 1mm DTT and 0.2mM PMSF.

2.7.6 DEAE Sepharose 4B/ yellow HE-4R chromatography with KCl elution
Approximately 300ml of yellow dye bound resin was used to pour a 5cm x 30cm column which was then equilibrated overnight with 20mM Tricine buffer, pH 8.2, containing 1mM EDTA, 10% glycerol, 1mm DTT and 0.2mM PMSF. The dialysed protein was loaded onto the column at a flow rate of 90ml/hr. The column was then washed overnight in the same buffer at a flow rate of 180ml/hr. The next day, the flow rate was adjusted to 80ml/hr before application of a 0-0.4M KCl linear gradient (1100ml buffer and 1100ml buffer containing 0.4M KCl). Fractions were collected over 185 tubes (~12ml per tube). Active fractions were dialysed overnight into 5 litres of 20mM MOPS containing 1mM EDTA, 10% glycerol, 1mm DTT and 0.2mM
2.7.7 Preparation of succinyl-CoA

The synthesis of succinyl-CoA was achieved by following the method of Simon and Shemin (1953). Coenzyme-A (10mg) was dissolved in a 1.9ml Eppendorf containing 1.2ml of 0.2M NaHCO₃. Succinic anhydride was prepared from pellets by crushing them in a pestle and mortar. Crushed succinic anhydride (1.2mg) was added to the Eppendorf and dissolved in ~20mins with intermittent vortexing. The Eppendorf was kept on ice when possible due to the instability of succinyl-CoA in solution.

2.7.8 DEAE Sepharose 4B/ blue MX-2G affinity chromatography with succinyl-CoA elution

Approximately 150ml of blue dye-bound resin was used to pour a 5cm x 15cm column which was then equilibrated overnight with 20mM MOPS buffer, pH 7.2, containing 1mM EDTA, 10% glycerol, 1mm DTT and 0.2mM PMSF. The dialysed protein was loaded onto the column at a flow rate of 80ml/hr. The column was then washed overnight in the same buffer at a flow rate of 30ml/hr. There is a residual amount of cleavage of the succinyl-CoA transferase polypeptide chain on elution with succinyl-CoA that can be minimised by increasing the flow rate. This was pre-adjusted to 180ml/hr before applying a 400ml solution of 20mM MOPS buffer, pH 7.2, containing 1mM EDTA, 10% glycerol, 1mm DTT, 0.2mM PMSF and 0.3mM succinyl CoA. Fractions (50 x 5ml) were collected and immediately placed on ice to avoid protein precipitation. SCAT activity was found in fractions 15 to 25. These fractions were pooled and dialysed overnight into 5 litres of 20mM MOPS buffer, pH 7.2, containing 1mM EDTA, 10% glycerol, 1mm DTT and 0.2mM PMSF. Dialysis, using 5 litres of fresh buffer each day, was continued for a further two days to remove any remaining bound CoA from the active site of the enzyme.

2.7.9 DEAE Sepharose 4B/ blue MX-2G/ chromatography/ KCl elution

The final chromatographic stage was necessary to remove any contaminating cleavage product generated by succinyl-CoA. Blue resin (70ml) was used to pour a 2.5cm x 14cm column which was equilibrated overnight with 20mM MOPS buffer, pH 7.2, containing 1mM EDTA, 10% glycerol, 1mm DTT and 0.2mM PMSF. The dialysed
protein was loaded onto the column at a flow rate of 25ml/hr. The column (labelled as MX-2G/A) was then washed overnight in the same buffer at 25ml/hr. Approximately half of the enzyme did not bind to the dye resin due to retention of CoA at the active site. The non-bound enzyme was dialysed for one further night in the above MOPS buffer and then applied to a second column of blue resin, of similar volume, called MX-2G/B. SCAT was eluted from both columns by the application of a 0-0.5M KCl linear gradient (total volume of 420ml) at a flow rate was 50ml/hr. Fractions were collected over 100 tubes (~ 4.5ml per tube). Active fractions were pooled and dialysed overnight into 20mM MOPS buffer, pH 7.2, containing 1mM EDTA, 10% glycerol, 1mm DTT and 0.2mM PMSF.

2.7.10 Concentration
The dialysate from the previous stage was reduced to a 10ml volume by concentration under N₂ using a PM10 membrane in a 50ml Amicon ultra-filtration cell. The purified enzyme was stored in 1ml aliquots in a freezer at -70°C.

2.7.11 Succinyl-CoA transferase activity assay
The spectrophotometric assay used to determine SCAT activity was similar to previous methods (Stern et al., 1956; Rochet and Bridger, 1994). The assay performed at 30°C contains; Tris/HCl buffer, pH 8.5, (50mM); MgCl₂ (10mM); acetoacetyl CoA (50μM); SCAT (0.1-4.0 units). This resulted in a total volume of 975µl. Upon achieving a stable base line, succinate (50mM) was added to make a final volume of 1ml. The decrease in absorbance at 303nm, reflecting the utilisation of acetoacetyl CoA, was followed using a Kontron Uvikon 930 spectrophotometer.

The absorption peak of acetoacetyl CoA at 303nm is attributed to the formation of the enolate species. The presence of magnesium ions amplifies the enolate absorption by forming a chelation adduct. One unit of SCAT is defined as the amount of enzyme which catalyses the formation of 1 µmole of succinyl CoA per minute at 30°C, under the assay conditions.
2.7.12 Determination of protein concentration
As described in section 2.3.11

2.7.13 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)
Electrophoresis using 10% SDS-PAGE gels was performed as described in 2.3.12

2.7.14 Electrospray mass spectrometric analysis of SCAT
Purified enzyme was desalted through a PD-10 column into 20mM MOPS buffer, pH 7.2. The solution was adjusted to 0.1mg/ml protein with the same buffer and made up to 33% acetonitrile and 1% formic acid. Analysis was performed by electrospray mass spectrometry (Micromass Quattro II, triple quadrupole), in positive ion mode, at a cone voltage of 45V, capillary voltage of 3.3V and HV lens voltage of 0.35V in a solvent system of 50% acetonitrile. The raw data obtained at the above values were then subjected to maximum entropy analysis according to the Micromass Schedule.

2.8 Results
2.8.1 Purification of SCAT
The procedure used to purify succinyl-CoA transferase from porcine heart is an improved version of a previously used method that consisted of two ammonium sulphate fractionations, one heat and acid treatment stage and two DEAE cellulose chromatography steps, differing only in pH (Hersh and Jencks, 1967a). The latter of the two DEAE cellulose steps resulted in a large loss of enzyme activity. Therefore it was necessary to modify the purification procedure by replacing the problematic DEAE stage with a series of dye column steps (Lloyd and Shoolingin-Jordan, in press). The dye bound resins were generated in the laboratory by the covalent attachment of yellow HE-4R and blue MX-2G dyes (obtained from A.J. Lloyd and BASF, Manchester) to Sepharose 4B. Structural similarities between the dyes used and dinucleotides such as NAD+ allows them to ‘mimic’ a coenzyme substrate which SCAT recognises and binds to. The association between column-bound dye and enzyme is such that SCAT can only be removed by elution with either high salt or a preferred substrate such as succinyl-CoA. The yellow dye column step in particular was found to be crucial in the overall purification procedure. A large quantity of unwanted protein was removed during this step including a contaminating thiolase.
enzyme that would interfere with the following affinity column stage and subsequent studies on PKS enzymes. The last two dye column steps were also essential to the purification by increasing the specific activity from 174 mU/mg to a final value of ~225 mU/mg (table 2.2).

The above purification yielded SCAT of high purity with specific activities higher than that previously reported. Approximately 18.5 mg of enzyme was obtained from the blue MX/2G column steps A and B. The purity is indicated by a predominant band of ~52 kDa on a 10% SDS-PAGE gel (figure 2.3, lane 8). A smaller, lower band of ~48 kDa can also be observed. The lower band may represent either a nicked form of SCAT or a contamination.

The presence of two smaller peptide bands (~29 kDa and 25 kDa) can be observed when an excess of protein is loaded onto the gel (figure 2.3, lane 7). These bands, identified as digestion products resembling domains 1 and 2, reflect the susceptibility to proteolysis at the hinge region of each subunit. The fragmentation into two peptides corresponds with a loss of enzyme activity. Proteolysis is minimised by storing the enzyme at -70°C where it remains active for several months thereafter.

2.8.2 Mass spectrometric analysis of SCAT

The predicted molecular mass of SCAT from the amino acid sequence is 52,197 Da. Electrospray mass spectrometric analysis of SCAT using a Quattro II triple quadrupole spectrometer has revealed the major species to have a mass of 52,175 Da (figure 2.4b). The deconvolution of the multiply charged envelope (figure 2.4a) results in a major species that is within 22 mass units of the predicted molecular weight of the SCAT subunit (figure 2.4b).

The deconvoluted spectrum (figure 2.4b) does not show the presence of a protein species corresponding to the lower band which is observed by SDS-PAGE (figure 2.3). However, the raw data could be interpreted as containing two protein charge distribution envelopes (figure 2.4a). The identical masses of 52,175 Da resulting from the transformation of both envelopes could indicate the presence of two different conformationally folded states of SCAT. This requires further examination.
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Table 2.2. Purification table of succinyl-CoA transferase from porcine heart. An average total of 18.5mg of pure enzyme was obtained from each purification.
Figure 2.3. 10% SDS-PAGE gel of succinyl-CoA transferase purification stages. Lane 1 and 9, molecular weight markers (66.2kDa, 45kDa, 31kDa). Lane 2, crude extract. Lane 3, 40-65% saturation ammonium sulphate fractionation. Lane 4, DEAE Sephacel flow through. Lane 5, Yellow HE/4R eluate. Lane 6, Blue MX/2G succinyl-CoA elution. Lane 7, Blue MX/2G (column A); KCl gradient eluate. Lane 8, Blue MX/2G (column B); KCl gradient eluate.
Figure 2.4a. Raw data obtained from succinyl-CoA transferase at a concentration of 0.07mg/ml analysed by ES-MS using quadrupole detection in positive ion mode; 2.4b. Deconvoluted spectrum of the multiply charged envelope.
2.9 Conclusion

6-Methylsalicylic acid synthase has been isolated in a highly purified form with only minimal proteolytic degradation, from *Penicillium patulum* grown in liquid culture from a spore inoculum. An accurate mass spectrum has also been obtained for 6-methylsalicylic acid synthase and to date is the only multifunctional intact PKS to have been analysed by this method. Previously only over expressed domains of PKSs have been analysed using mass spectrometry, such as the ACP domain of *S. coelicolor* (Crosby et al., 1995). The logical progression from this point would be to obtain mass spectroscopic data for chemically modified 6-MSAS. For example, cerulenin inactivated 6-MSAS and DBP cross-linked 6-MSAS. Another alternative would be analysing 6-MSAS under native mass spectrometric conditions in an attempt to visualise the homotetrameric native structure. Recent advances in mass spectrometer technology should be useful in attaining such data. Having isolated homogenous, active 6-methylsalicylic acid synthase, its ability to decarboxylate malonyl-CoA and other properties will be investigated.

The lack of a defined *m/z* series for 6-MSAS makes analysis by manual transformation impossible, therefore Maximum Entropy was utilised. Figure 2.2a shows a very poor signal-to-noise ratio as compared to the data presented in figure 2.4a. This however, does not present a problem since the program Maximum Entropy can extract a clean spectrum from the raw data (figure 2.2b) with an insignificant amount of artefacts (Cottrell, 1994).

Succinyl-CoA transferase has been isolated from porcine heart. It does not possess an absolute substrate specificity (Menon & Stern, 1960). Therefore this property has been utilised by groups to exploit the ability of succinyl-CoA transferase to synthesise other acyl-CoAs (Jordan et al., 1986), an ability which is made use of in the following chapter for the preparation of [2-¹³C]-labelled malonyl-CoA.
Chapter 3 part I

The malonyl-CoA decarboxylase activity of 6-methylsalicylic acid synthase

3.1 Introduction

Research has shown that polyketide synthases possess a high degree of similarity to fatty acid synthases, both in the physical and chemical properties of the enzymes and the primary sequences of the active site domains. The mechanisms used to polymerise the carbon starter and extender units to form long chains are also similar with an acyl group bound to a ketosynthase condensing with a malonyl or methylmalonyl group bound to an ACP moiety. Decarboxylation of the extender unit accompanies the condensation and only normally occurs if the β-ketosynthase thiol is occupied by an acyl group. However, it has been reported that for a number of FAS systems, a low malonyl-CoA decarboxylase activity is present when the β-ketosynthase thiol is unoccupied. Recently, a decarboxylase activity has been reported for DEBS-1+TE (Pieper et al., 1996) and chalcone synthase (Eckermann et al., 1998).

3.1.1 Fatty acid synthase and malonyl-CoA decarboxylase

A malonyl-CoA decarboxylase activity has been shown to exist in FAS systems such as plaice liver (Wilson and Williamson, 1970), yeast (Kresze et al., 1977a and b), lactating rabbit mammary gland (Hansen et al., 1971) and E.coli (Toomey and Wakil, 1966). The decarboxylase activity for all of these FAS systems was reported to be no more than 5% of the synthase activity. However, FAS from pigeon liver has been reported to have a decarboxylase activity of 25-30% of its synthase activity (Katiyar et al., 1974).

The incorporation of the alkylating reagent iodoacetamide has been shown to increase the malonyl-CoA decarboxylase activity of yeast FAS (Kresze et al., 1977). The β-ketosynthase active site cysteine residue is alkylated resulting in a reduced synthase activity but an increased malonyl-CoA decarboxylase activity, which can catalyse the formation of acetyl-CoA at about 60% of the maximal rate of acetyl-CoA consumption by native FAS. However, the malonyl-CoA decarboxylase activity is blocked by N-ethylmaleimide, a reagent shown to inhibit the transfer of malonyl-CoA to the ACP.
(Oesterhelt et al., 1977). This indicated that the ketosynthase catalyses decarboxylation of malonyl-ACP and not malonyl-CoA.

The above observations have led to a model for the condensing site in which a basic residue is proposed to hydrogen bond to the KS cysteine when it is unoccupied by substrate. The pKa of the cysteine is increased thereby favouring covalent bond formation with the bound acyl-ACP substrate. Upon reaction of the KS cysteine with a natural substrate, the enzyme conformation is proposed to change from I to II so that the aforementioned basic residue is displaced into a position where it can interact favourably with the malonyl-ACP and promote decarboxylation (figure 3.1). If an alkylating agent such as iodoacetamide occupies the KS thiol site then it is possible that the decarboxylation occurs using the same mechanisms as those normally used for the condensation (figure 3.2) Alternatively, the binding of an acetyl unit and/or alkylating agent could alter the charge distribution of the amino acid residues within the active site, thereby enabling them to catalyse decarboxylation.

The sequence alignments of FASs and PKSs from different organisms have identified seven conserved amino acid residues within the KS domain, which could be implicated in the mechanism of malonyl-CoA decarboxylation (figure 3.3). Among these are three basic residues, two histidines and one lysine, all of which are candidates for a catalytic role in the decarboxylation of malonyl-ACP. The inactivation of chicken liver FAS with o-phthalaldehyde, which reacts with primary amines and thiols, has indicated that the ACP thiol and a lysine residue on the same or opposing subunit are within close proximity of each other (Stoops et al., 1983). Further evidence that implicates the lysine residue in a catalytic role has come from mutagenesis studies upon the KS-CLF heterodimer of the type II act PKS (Dreier and Khosla, 2000). These studies also concluded that the two conserved histidines are involved in malonyl-CoA decarboxylation.

3.1.2 A proposed mechanism for the observed malonyl-ACP decarboxylation in FAS and PKS systems

As previously mentioned, the decarboxylase activity of PKSs and FASs is maximal when either the substrate or an alkylating agent such as iodoacetamide occupies the
Figure 3.1. Hypothetical scheme for acetoacetate formation. A. Proton extraction from the β-ketosynthase thiol, thus increasing nucleophilicity of the sulphur; B. Acetylation of the β-ketosynthase thiol inducing a proposed conformational change within the β-ketosynthase active site; C. decarboxylative condensation of the 4'-phosphopantetheine bound acyl group and the β-ketosynthase thiol bound acetyl group; D. 4'-Phosphopantetheine bound acetoacetate moiety; I and II indicate two proposed conformational changes of the condensing active site. SACP acyl carrier protein thiol, and SKS: β-ketosynthase thiol; (modified from Kresze et al., 1977b).
Figure 3.2. Hypothetical scheme for iodoacetamide induced malonyl-ACP decarboxylation. A. Proton extraction from the β-ketosynthase thiol, thus increasing nucleophilicity of the sulphur; B. Alkylation of the β-ketosynthase thiol inducing a proposed conformational change within the β-ketosynthase active site; C. decarboxylative condensation of the 4'-phosphopantetheine bound acyl group; D. 4'-Phosphopantetheine bound acetyl moiety released in the presence of free CoA; I and II indicate two proposed conformational changes of the condensing active site. S\textsubscript{ACP} acyl carrier protein thiol; and S\textsubscript{KS}: β-ketosynthase thiol; (modified from Kresze et al., 1977b).
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**Figure 3.3.** Amino acid alignment of two portions of the β-ketoacyl synthase domain that contain invariant basic residues from a variety of polyketide and fatty acid synthase sources. The numbers indicate the position of the starting residue for each portion after the substrate binding cysteine of 6-MSAS. References for the sequences are as follows: DEBS, (Bevitt et al., 1992); 6-MSAS, (Beck et al., 1990); rat FAS, (Amy et al., 1989); *E. coli* Fab B, (Kauppinen et al., 1988); Fas2 from *P. patulum*, (Weisner et al., 1988); Fas2 from *S. cerevisiae*, (Mohamed et al., 1988); actinorhodin synthase ORF1, (Fernandez-Moreno et al., 1992); granaticin synthase ORF1, (Sherman et al., 1989); tetracenomycin synthase K, (Bibb et al., 1989).
cysteine thiol of the KS. These observations suggest that the alkylated condensing active site is able to generate acetyl-CoA, possibly through the mechanism shown in figure 3.2 which is summarised below:

\[
\text{MSAS}_{\text{alkylated}} + \text{malonyl-CoA} \rightarrow \text{Malonyl-MSAS}_{\text{alkylated}} + \text{CoA} \\
\text{Malonyl-MSAS}_{\text{alkylated}} \rightarrow \text{Acetyl-MSAS}_{\text{alkylated}} + \text{CO}_2 \\
\text{Acetyl-MSAS}_{\text{alkylated}} + \text{CoA} \rightarrow \text{MSAS}_{\text{alkylated}} + \text{acetyl-CoA}
\]

The acetyl-CoA generated is then able to act as a starter for the unmodified enzyme molecules still present. Therefore in the presence of excess iodoacetamide all the enzyme molecules will be modified by alkylation and hence no decarboxylase activity will be observed (Dimroth et al., 1976). Free CoA plays an important role in the binding of the correct substrate (Lin and Smith, 1978; Soulie et al., 1984). If the wrong substrate is bound then CoA acts as an acceptor and removes the acyl group thereby regenerating the condensing site for binding of the correct substrate (Wakil, 1989). It is possible that free CoA can act in an analogous manner during decarboxylation of malonyl-ACP by removing the newly generated acetyl-CoA to act as a starter.

3.1.3 The Decarboxylase Activity of PKS Systems

There is an increasing awareness of the ability of some PKS and FAS systems to catalyse decarboxylation of malonyl-ACP at a low background level in the absence of a starter unit. Recently a chalcone synthase like protein, 2-pyrone synthase, has been shown to decarboxylate malonyl-CoA when starved of natural starter substrate (Eckermann et al., 1998). Another example is the truncated mutant, DEBS1 + TE of the erythromycin producing PKS 6-deoxyerythronolide-B synthase, which has been shown to possess a methylmalonyl-CoA decarboxylase activity in the absence of its starter, propionyl-CoA (Pieper et al., 1996). It was observed that the product \((2S,3S,4S,5R)-2,4\text{-dimethyl-3,5-dihydroxy-}\it{n}\text{-heptanoic acid lactone, could be synthesised from three units of methylmalonyl-CoA, the first of which undergoes decarboxylation. Full experimental data was not available to support this conclusion.}
3.1.4 The decarboxylase activity of 6-methylsalicylic acid synthase

Similar to yeast FAS, a malonyl-CoA decarboxylase activity has been shown to exist for 6-MSA synthase when treated with iodoacetamide (Dimroth et al., 1976). Addition of 0.2 mM iodoacetamide to an incubation mixture containing NADPH, malonyl-CoA and 6-MSAS, immediately caused rapid consumption of NADPH at 20% of the activity of the unmodified synthase in the presence of the normal starter acetyl-CoA. Incubation of 6-MSAS with 0.5mM iodoacetamide resulted in the loss of native synthase activity but retention of the malonyl-CoA decarboxylase activity observed in the 0.2mM iodoacetamide experiment. Treatment with higher iodoacetamide concentrations (1-10mM) abolished all decarboxylase and synthase activity. Incorporation of [2-14C]malonyl-CoA indicated that one mole of 6-MSA originated from four moles of malonyl-CoA and one mole of NADPH (Dimroth et al., 1976). However, a native malonyl-CoA decarboxylase activity could not be shown.

All PKS and FAS systems require decarboxylation of the extender unit to occur in order to form the growing polyketide chain. It is therefore not unreasonable to expect there to be a low background decarboxylation of malonyl-CoA in the absence of a starter unit resulting in low levels of 6-MSA formation. From observations that 6-MSA could be synthesised in the absence of added acetyl-CoA our suspicions grew that 6-MSAS possessed a native malonyl-CoA decarboxylase activity. Substrate labelling studies using [2-13C]-malonyl-CoA were therefore designed in an attempt to label regio-specifically the methyl group at the 6-positions of 6-MSA and TAL.

3.2 Materials

6-Methylsalicylic acid synthase was purified from Penicillium patulum NRRL 2159A as described in chapter two. Succinyl-CoA transferase was purified from porcine heart as described in chapter two. Borosilicate nanoflow tips for ESMS were obtained from Micromass. Malonyl-CoA, NADPH, coenzyme-A, succinic anhydride, citrate synthase and oxaloacetic acid were obtained from Sigma. [2-13C] Malonic acid (99% atom [13C]) and acetic anhydride were obtained from Aldrich. HPLC grade H₂O and ammonium hydroxide were supplied by BDH. Formic acid was obtained from Fluka. HPLC grade acetonitrile was obtained from Fisher Scientific. Trifluoroacetic acid was obtained from Applied Biosystems Division. The C₁₈ reverse phase column (250mm x
4.6mm; 5μm) was supplied by Spherisorb. HPLC buffer A contained H₂O + 0.1% (v/v) TFA. HPLC buffer B contained acetonitrile + 0.1% TFA (v/v). A Superose-6 gel filtration column was obtained from Pharmacia. It is important to note that the malonyl-CoA used in all reactions and assays was HPLC-purified within the laboratory to remove any contaminating acetyl-CoA. Mass spectrometry was used to check [2-13C]malonic acid for any contaminating [2-13C]-acetate.

3.3 Methods

3.3.1 Gel-filtration purification of 6-methylsalicylic acid synthase

6-MSAS (1mg) purified from the Mono Q™ anion exchange column (chapter 2) was loaded onto a Superose-6 gel filtration column (30cm x 1cm) that had been pre-equilibrated with 100mM Tris-HCl buffer, pH 7.6, containing 10% glycerol and 1mM DTT.

3.3.2 Purification of malonyl-CoA

Malonyl-CoA was purified by HPLC prior to use in all experimental assays. Malonyl-CoA (2mg) was dissolved in H₂O (100μl) and then injected onto a C18 reverse phase column pre-equilibrated in 98% buffer A, 2% buffer B (section 3.2 for details of buffers) at a flow rate of 1 ml/min. Malonyl-CoA was eluted from the column by increasing buffer B to 80% over a period of 30 minutes at λ = 260nm and at a flow rate of 1 ml/min.

3.3.3 Determination of contaminating decarboxylase activity

A control assay was performed to determine if BSA (Sigma) contained any contaminating decarboxylase activity. In a volume of 50μl, the BSA control assay contained; BSA (1.25mg), potassium phosphate buffer, pH 7.6 (10mM), NADPH (0.2mM) and malonyl-CoA (0.2mM) (HPLC purified). After 30 minutes the assay was terminated by the addition of 10μl of 50% (w/v) TCA. Precipitated protein was removed by centrifugation for 5 minutes. A 10μl aliquot was analysed by HPLC and mass spectrometry to determine whether or not any acetyl-CoA had been synthesised.
3.3.4 Demonstration of malonyl-CoA decarboxylase activity catalysed by 6-methylosalicylic acid synthase
The following assay mixture was prepared to a final volume of 1ml using analytical grade water: potassium phosphate buffer, pH 7.6 (10mM), BSA (1.25mg), NADPH (0.2mM), malonyl-CoA (0.2mM) (HPLC purified) and 0.2-1.0 mU of 6-MSAS. The increase in fluorescence due to the enzymatic formation of 6-MSA was followed using a Hitachi F-2000 fluorescence spectrophotometer.

3.3.5 Pre-incubation of malonyl-CoA with citrate synthase
An assay was performed to determine whether there was any contaminating acetyl-CoA in any of the components of the fluorescence assay. In a volume of 975(1), the assay contained potassium phosphate buffer, pH 7.6 (10mM), BSA (1.25mg), NADPH (0.2mM) and malonyl-CoA (0.2mM) (HPLC purified). The above constituents were pre-incubated with oxaloacetate (0.2mM) and 2.15 units of citrate synthase for 30 minutes at 25°C, then, 0.2-1.0 mU (25μl) of 6-MSAS was added to the assay. The increase in fluorescence due to the enzymatic formation of 6-MSA was followed using a Hitachi F-2000 fluorescence spectrophotometer.

3.3.6 Synthesis of unlabelled and [2-13C]-labelled malonyl-CoA by incubation of unlabelled and [2-13C]-labelled malonate with succinyl-CoA transferase
A 1.9ml Eppendorf tube containing 10μl of 10mM succinyl-CoA, 10μl of 3M [2-13C] or unlabelled malonate, pH 7.0, 10μl of succinyl-CoA transferase (0.73 units) and 70μl of 20mM MOPS buffer, pH 7.2, containing 10% glycerol and 1mM EDTA was incubated at 25°C for 30 minutes. The reaction was terminated by the addition of 20μl of 50% (w/v) TCA. The sample was microcentrifuged for 5 minutes to remove precipitated protein and 100μl of the sample was then injected onto a C18 reverse phase column pre-equilibrated in 98% buffer A, 2% buffer B at λ = 260nm and at a flow rate of 1 ml/min. Buffer B was increased to 80% over a period of 30 minutes at a flow rate of 1 ml/min. The peak of interest was collected, freeze-dried and redissolved in 50% (v/v) acetonitrile. The sample was analysed by ES-MS.
3.3.7 Succinyl-CoA transferase controls
Controls were carried out to determine whether any acetyl-CoA was being produced by the succinyl-CoA transferase assay. A 1.9ml Eppendorf tube containing 10µl of 10mM succinyl-CoA, 10µl of succinyl-CoA transferase (0.73 units), 10µl of 3M unlabelled malonic acid, pH 7.0, and 70µl of 20mM MOPS buffer, pH 7.2, containing 10% glycerol and 1mM EDTA were incubated at 25°C for 30 minutes. A time zero assay was also carried out. The reaction was terminated by the addition of 20µl of 50% (w/v) TCA. Precipitated protein was removed by centrifugation for 5 minutes. A 10µl sample of each terminated reaction was then analysed by ES-MS.

3.3.8 Biosynthesis of [13C]-labelled 6-methylsalicylic acid and [13C]-labelled triacetic acid lactone
The production of [13C]-6-MSA was achieved as follows; a 1.9ml Eppendorf tube containing 10µl of 10mM succinyl-CoA, 10µl of succinyl-CoA transferase (0.73 units), 3.3 µU of 6-MSAS, 10µl of 20mM NADPH, 10µl of 10mM acetyl-CoA, 10µl of 3M [13C]-malonate, pH 7.0, and 100µl of 100mM potassium phosphate buffer, pH 7.6, was prepared. To investigate the labelling pattern of [13C]-TAL, NADPH was omitted from the reaction and replaced by water. The reactions (200µl) were terminated after 2 hours by the addition of 20µl of 50% (w/v) TCA. The samples were microcentrifuged for 5 minutes to remove precipitated protein. Unlabelled 6-MSA and TAL were synthesised using the same method except that [13C]-malonate was replaced with [12C]-malonate.

3.3.9 Reverse-phase HPLC purification of enzymically synthesised [13C] and unlabelled 6-MSA and TAL
The samples, prepared as in section 3.3.8, were loaded onto a C18 reverse phase Spherisorb column that had been pre-equilibrated with 98% buffer A and 2% buffer B. Labelled and unlabelled 6-MSA and TAL were eluted by increasing buffer B to 80% over a period of 30 minutes at λ = 300nm and at a flow rate of 1 ml/min. The peaks of interest were collected and freeze dried.
3.3.10 Electrospray mass spectrometric analysis of unlabelled, $^{13}$C-labelleled malonyl-CoA and succinyl-CoA transferase controls

Freeze-dried unlabelled and [2-$^{13}$C]-malonyl-CoA samples were dissolved in a 50% acetonitrile (v/v), 1% ammonium hydroxide (v/v) solution. Samples (10 μl) were introduced into the mass spectrometer using a Hewlett-Packard HPLC system and Rheodyne injection port fitted with a 10 μl sample loop at a flow rate of 0.01 ml/min. Mass spectrometry was performed using a Micromass Quattro II triple quadrupole mass spectrometer with an electrospray ionisation source. The source was operated at 80°C, with a cone voltage of 60 V. Spectra were recorded over the range 800 m/z to 900 m/z, in negative ion mode. Control of the mass spectrometer, data acquisition and mass measurement were performed using Masslynx 2.1 software. The succinyl-CoA transferase controls were dissolved in a 50% acetonitrile (v/v), 1% ammonium hydroxide (v/v) solution. The same procedure for ES-MS analysis was followed as described above. Spectra were recorded over the range 800 m/z to 900 m/z, in negative ion mode.

3.3.11 Nanospray collision induced dissociation (CID) mass spectrometry

Labelled (and unlabelled) 6-MSA and TAL samples were dissolved in a 50% acetonitrile (v/v), 5% ammonium hydroxide (v/v) solution. A sample (2 μl) of this solution was loaded into a borosilicate metal coated nanoflow needle (type B) and mass spectrometry was performed in negative ion mode using a Micromass Quattro II triple quadrupole mass spectrometer with a nanoelectrospray source and probe. The source was operated at 30°C, with a capillary voltage of 2.45 kV, HV lens of 0.20 kV and a cone voltage of 35 V. Spectra were recorded over the range m/z 130 to m/z 190 for 6-methylsalicylic acid and m/z 115 to m/z 150 for triacetic acid lactone. For CID tandem mass spectrometry, quadrupole (Q1) was operated in mass resolving mode to select the precursor ion. Q1 was adjusted to transmit a window of approximately 3 mass units wide to quadrupole 2 (Q2). The collision cell pressure was maintained at 10 mTorr to provide collisional damping of the fragment ions and the remaining precursor ions. Spectra were recorded over the mass range m/z 25 to m/z 160 for 6-MSA and m/z 25 to m/z 150 for TAL. Control of the mass spectrometer, data acquisition and mass measurement was performed using Masslynx 2.1 software.
3.4 Results and discussion

3.4.1 Gel-filtration of 6-methyIsalicylic acid synthase

It was essential that 6-MSAS was purified to homogeneity so as to avoid the possibility of co-purifying a contaminating decarboxylase enzyme. The regular purification of 6-MSAS used in chapter 2 has a final FPLC Mono Q™ step. The enzyme purified from this step appeared as a single band on a silver stained gel (figure 3.4), thereby indicating a single species. Therefore a further purification step was not needed to improve the purity of 6-MSAS. The gel-filtration step was simply used as an analytical tool to confirm the homogeneity of 6-MSAS prior to incubation with labelled substrates. Elution of 6-MSAS from a pre-packed Superose-6 gel-filtration (separation range approximately 13kDa to 800kDa) at a flow rate of 1 ml/min resulted in a single symmetrical peak at an elution time of 30 minutes. This indicated the presence of a single species of enzyme. The possibility of any other enzyme eluting from this column in the same single peak was therefore likely to be extremely remote.

3.4.2 Determination of any potential contaminating decarboxylase activity

It was important to eliminate the possible presence of any contaminating decarboxylase activity within BSA, a protein used to enhance the fluorescence emission signal of 6-MSA at 390nm. The HPLC and mass spectral analysis of the BSA control assay after 30 minutes revealed that no acetyl-CoA (Mr 808.6) had been synthesised, thereby indicating that BSA did not contain a decarboxylase activity.

3.4.3 Preincubation of 6-methyIsalicylic acid synthase with citrate synthase in the absence of externally added acetyl-CoA

Citrate synthase is a key enzyme in the Krebs cycle that catalyses the formation of citrate from acetyl-CoA and oxaloacetate. Therefore, the enzyme was used as a control measure that functioned to remove all traces of acetyl-CoA from 6-MSAS. This was essential in order to determine whether 6-MSAS itself was able to decarboxylate malonyl-CoA and utilise it as a starter unit. The rate of 6-MSA formation in the presence of externally added acetyl-CoA is demonstrated in figure 3.5a. The rate of 6-MSA formation in the absence of externally added acetyl-CoA but after pre-incubation with citrate synthase and oxaloacetate is demonstrated in figure 3.5b. The formation of
Figure 3.4. Silver stained 5% SDS gel of pure 6-MSAS from *P. patulum* after FPLC Mono Q™ stage: Lanes 1 and 6, molecular weight markers (myosin, 200kDa; β-galactosidase, 116.3kDa; phosphorylase B, 97.4kDa; bovine serum albumin, 66.2kDa). Lanes 2, 3, 4 and 5; Purified 6-methylsalicylic acid synthase, 2µg, 1µg, 0.5µg and 0.25µg respectively.
Figure 3.5. Fluorescence detection of 6-MSA formation. a. Production of 6-MSA by 6-MSAS in the presence of acetyl-CoA, malonyl-CoA and NADPH. Details of fluorometric assay are noted in chapter 2; b. Production of 6-MSA by 6-MSAS in the absence of externally added acetyl-CoA and following prior incubation of the assay components with citrate synthase and oxaloacetate.
6-MSA was followed for 20 minutes from which the initial rate was recorded at 5% of that containing externally added acetyl-CoA.

Had figure 3.5b shown an initial rapid increase in the rate, followed by a halting of 6-MSA production, this would have indicated that citrate synthase was unable to remove any small amounts of acetyl-CoA and that 6-MSAS could have utilised the trace amount of acetyl-CoA present. This would also show that 6-MSAS was unable to decarboxylate malonyl-CoA. However, figure 3.5b shows a steady increase in 6-MSA production over 20 minutes. This suggests that 6-MSAS has the ability to synthesise 6-MSA from four units of malonyl-CoA. The data therefore suggests that one unit of malonyl-CoA is decarboxylated to a starter unit of acetyl-CoA by the enzyme. The remaining three malonyl-CoA units are then utilised as extender units, in the normal way when acetyl-CoA is available.

3.4.4 Succinyl-CoA transferase controls
It was necessary to eliminate the remote possibility that acetyl-CoA could be synthesised during the transferase reaction from the newly synthesised malonyl-CoA which could then decarboxylate under the buffer and assay conditions chosen. The zero time and 30 minute incubations were terminated and the resultant solutions were analysed by ES-MS. The zero time assay indicates, as expected, the presence of only succinyl-CoA with Mr 866.3 (figure 3.6a). The 30 minute assay reveals the presence of newly synthesised malonyl-CoA with Mr 852.25 and succinyl-CoA with a mass of Mr 866.31 (figure 3.6b). For both assays there was no indication of any acetyl-CoA, Mr 808.6.

3.4.5 Synthesis of unlabelled and $[^{13}\text{C}]$-labelled malonyl-CoA by succinyl CoA transferase
Succinyl-CoA transferase was used to synthesise unlabelled malonyl-CoA which was used as a standard for comparison. The resulting mass of the biosynthesised malonyl-CoA was Mr 852.14 as shown by ES-MS (figure 3.7a). This compares favourably with the chemical standard (obtained from Sigma) with Mr 852.10. The $[^{2-^{13}}\text{C}]$-labelled biosynthesised malonyl-CoA was shown to have a mass of Mr 853.10 (figure 3.7b), approximately one mass unit greater than the unlabelled malonyl-CoA. This
Figure 3.6. ES-MS analysis of succinyl-CoA transferase controls (section 3.4.4) in negative ion mode showing the formation of unlabelled malonyl-CoA (Mr 852.25) from unlabelled malonate and succinyl-CoA. The peak at Mr 866.23 is succinyl-CoA.  

**a.** Time zero assay; **b.** Time 30 minute assay.
Figure 3.7. ES-MS analysis of malonyl-CoA in negative ion mode a. unlabelled malonyl-CoA prepared by succinyl-CoA transferase; b. [2-\textsuperscript{13}C]-labelled malonyl-CoA synthesised by incubation of [2-\textsuperscript{13}C]-labelled malonic acid with succinyl-CoA transferase and succinyl-CoA.
demonstrates the successful synthesis of [2-^{13}C]-labelled malonyl-CoA using succinyl-CoA transferase and succinyl-CoA as the CoA donor.

3.4.6 Analysis of standard and unlabelled 6-methylsalicylic acid and triacetic acid lactone by CID mass spectrometry

In order to gain insight into the labelling pattern of [^{13}C] in 6-MSAS derived products, it was necessary to perform a control study on unlabelled 6-MSA and TAL. Comparisons made between the unlabelled and [^{13}C] labelled molecules would lead to identifying the positions of the [^{13}C] labels within 6-MSA and TAL. All mass spectrometric analyses were carried out in negative ion mode which proved to be appropriate for the subsequent CID analysis. The 6-MSA parent ion was represented by a single charged species with Mr 150.79 (figure 3.8a). Fragmentation of the parent ion resulted in the production of only two daughter ions. The first daughter ion with Mr 106.79 is produced as a result of loss of the carboxyl group, Mr 44.00. The second daughter ion, Mr 91.82, is produced as a result of loss of both the carboxyl and methyl groups, Mr 58.97 (figure 3.8b). The TAL parent ion was represented by a single charged species, Mr 124.75 (figure 3.11a). Fragmentation of the parent ion resulted in the production of two daughter ions only. The first daughter ion, Mr 80.82, is produced as a result of loss of the carboxyl group. The second daughter ion, Mr 40.96, corresponds to the C-2 unit, CHCO (figure 3.11b). The remaining carbon chain was not observed.

The enzymatic synthesis of unlabelled 6-MSA and TAL produced near identical parent and daughter ion spectra to those obtained for the standards (figures 3.8 and 3.11). Having assigned the daughter ions it was possible to analyse the [^{13}C]-labelled 6-MSA and TAL using the same method and to thereby determine the positions of the [^{13}C]-label within the molecules and the biosynthetic origins of the carbon atoms.

3.4.7 Analysis of 6-methylsalicylic acid synthesised from [2-^{13}C]-malonyl-CoA and unlabelled acetyl-CoA

Succinyl-CoA transferase was used to convert [2-^{13}C]-malonate to [2-^{13}C]-malonyl-CoA. Labelled 6-MSA was obtained by coupling the succinyl-CoA transferase reaction with 6-MSAS, unlabelled acetyl-CoA and NADPH. The [^{13}C]-labelling pattern of the
Figure 3.8. Nanospray mass spectral analysis of unlabelled 6-MSA standard. a. parent ion; b. Collision induced dissociation mass spectrometric analysis of daughter ions produced upon fragmentation of unlabelled parent ion.
aromatic ring of 6-MSA originating from the three extender units can be predicted from the postulated reaction cycle (Dimroth, 1970) and previous experiments carried out using labelled substrates (Jordan and Spencer, 1991). The analysis of the parent ion of [13C3]-6-MSA, Mr 153.83 (figure 3.9a), was exactly three mass units higher than the unlabelled standard (Mr 150.79, figure 3.8a), as expected. Upon fragmentation, loss of the carboxyl group resulted in a daughter ion, Mr 109.85 and loss of the carboxyl and methyl group resulted in a daughter, Mr 94.87 (figure 3.9b). Both daughter ions are three mass units greater than the corresponding Mr values obtained from standard unlabelled 6-MSA (figure 3.8b). This indicates, as predicted, that there are three [13C]-labels within the aromatic ring.

3.4.8 Analysis of 6-methylsalicylic acid synthesised exclusively from [2-13C]-malonyl-CoA with [13C]-acetyl-CoA generated by 6-MSAS

The [13C]-labelling pattern in 6-MSA resulting from the decarboxylation of malonyl-CoA to yield the starter acetyl-CoA can be studied by using the previous 6-MSA fragmentation patterns as standards for comparison. If malonyl-CoA is decarboxylated to the starter acetyl-CoA by 6-MSAS, then the [13C] label of [2-13C] malonate will be incorporated into the methyl group of 6-MSA. As a result, the parent ion mass would be expected to be four mass units higher than the unlabelled product and one more than the sample from section 3.4.7 (Mr 153.83). The spectrum of 6-MSA derived exclusively from [2-13C]-malonyl-CoA indeed revealed a parent ion, Mr 154.84, four mass units greater than the unlabelled product (figure 3.10a). Upon fragmentation, the loss of the carboxyl group should not result in any loss of the [13C]-label from the resulting daughter ion. A Mr of 110.84 for the daughter ion confirms that all four 13C-labels have been retained (figure 3.10b). However, the daughter ion corresponding to the loss of the carboxyl and methyl groups produces a Mr of 94.83. The Mr is identical to that for 6-MSA derived from 13C-malonyl CoA and externally added acetyl-CoA. This indicates the loss of one 13C-label which must be derived from the methyl group of 6-MSA. The position of this label can only be as a result of decarboxylation of malonyl-CoA to the acetyl-CoA starter. The mass spectrometric data are summarised in table 3.1.
**Figure 3.9.** Nanospray mass spectrometric analysis of [2,4,6-$^{13}$C$_3$]-labelled 6-MSA biosynthesised by the succinyl-CoA transferase / 6-MSAS linked assay in the presence of externally added unlabelled acetyl-CoA. a. parent ion; b. collision induced dissociation mass spectrometric analysis of daughter ions produced upon fragmentation of parent ion.
Figure 3.10. The decarboxylation of malonyl-CoA by 6-MSAS results in the synthesis of 6-MSA from four malonyl-CoA units. Nanospray mass spectrometric analysis of [2,4,6,8-$^{13}$C$_4$]-labelled 6-MSA biosynthesised by the succinyl-CoA transferase 6-MSAS linked assay in the absence of externally added acetyl-CoA. a. parent ion; b. collision induced dissociation mass spectrometric analysis of daughter ions produced upon fragmentation of parent ion.
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</tr>
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<td>154.84 (+4)</td>
<td>111.13 (+4)</td>
</tr>
</tbody>
</table>

**Table 3.1.** The predicted masses and observed masses in negative ion mode of: unlabelled 6-MSA (figure 3.8); [2,4,6-$^{13}$C$_3$]-labelled 6-MSA (external addition of unlabelled acetyl-CoA) (figure 3.9); [2,4,6,8-$^{13}$C$_4$]-labelled 6-MSA produced as a result of the decarboxylase activity of 6-MSAS, generated from the succinyl-CoA transferase and 6-MSAS coupled assay (figure 3.10). The positions of the [$^{13}$C]-labels in the structures are indicated.
3.4.9 Analysis of triacetic acid lactone (TAL) synthesised from [2-\(^{13}\)C]-malonyl-CoA and unlabelled acetyl-CoA

TAL is produced by 6-MSAS from one starter unit of acetyl-CoA and two extender units of malonyl-CoA in the absence of NADPH. This is a stereochemically controlled enzymatic process and represents a decision point in the biosynthesis of not only 6-MSA but fatty acids also (Spencer and Jordan, unpublished results). The position of the \(^{13}\)C labels within the TAL structure originating from the extender units can therefore be predicted. The TAL synthesised in the presence of [2-\(^{13}\)C]-malonyl-CoA and unlabelled acetyl-CoA, but without NADPH, produced a parent ion, Mr 126.72, as expected, two mass units higher than that of the unlabelled TAL (figure 3.12a). Upon fragmentation, the loss of the carboxyl group resulted in a daughter ion of Mr 82.85 (figure 3.12b), which is two mass units higher than that of the corresponding unlabelled daughter ion (figure 3.11b). The CHCO fragment is one mass unit higher than that of the corresponding unlabelled daughter ion indicating the presence of a \([^{13}\)C]-label that has originated from one of the malonyl-CoA extender units.

3.4.10 Analysis of triacetic acid lactone produced exclusively from [2-\(^{13}\)C]-malonyl-CoA

Further evidence that confirms the ability of 6-MSAS to decarboxylate malonyl-CoA can be obtained from studying the labelling pattern of TAL derived exclusively from \(^{13}\)C-malonyl-CoA and comparing the findings with the previous fragmentation patterns. The presence of a single \([^{13}\)C]-label within the methyl group of the lactone would indicate that malonyl-CoA has undergone decarboxylation. The parent ion of TAL resulted in a peak of Mr 127.76 (figure 3.13a), which is three mass units higher than that of the unlabelled TAL (figure 3.11a). Upon CID, the loss of the carboxyl group resulted in a daughter ion, Mr 83.86, (figure 3.13b) indicating the presence of three \([^{13}\)C]-labels within the structure, one of which is derived from the decarboxylation of malonyl-CoA. The position of this label can be pinpointed to the methyl group, as shown by the loss of Mr 41.97 for the CHCO fragment. These studies reinforce the above conclusions that in the absence of acetyl-CoA, malonyl-CoA can provide the starter unit by decarboxylation to acetyl-CoA. The mass spectrometric data are summarised in table 3.2.
Figure 3.11. Nanospray mass spectrometric analysis of unlabelled triacetic acid lactone chemical standard. a. parent ion; b. collision induced dissociation mass spectrometric analysis of daughter ions produced upon fragmentation of parent ion.
Figure 3.12. Nanospray mass spectrometric analysis of [2,4-$^{13}$C$_2$]-labelled triacetic acid lactone biosynthesised by the succinyl-CoA transferase / 6-MSAS linked assay in the presence of externally added acetyl-CoA and in the absence of NADPH. a. parent ion; b. collision induced dissociation mass spectrometric analysis of daughter ions produced upon fragmentation of parent ion.
Figure 3.13. The decarboxylation of malonyl-CoA by 6-MSAS results in the synthesis of TAL from three malonyl-CoA units. Nanospray mass spectrometric analysis of [2,4,6-\(^{13}\)C\(_3\)]-labelled TAL biosynthesised by the succinyl-CoA transferase 6-MSAS linked assay in the absence of externally added acetyl-CoA and in the absence of NADPH. a. parent ion; b. collision induced dissociation mass spectrometric analysis of daughter ions produced upon fragmentation of parent ion.
| Table 3.2. | The predicted masses and observed masses in negative ion mode of: unlabelled TAL (figure 3.11); [2,4-^{13}C_2]-labelled TAL (external addition of unlabelled acetyl-CoA) (figure 3.12); [2,4,6-^{13}C_3]-labelled TAL produced as a result of the decarboxylase activity of 6-MSAS, generated from the succinyl-CoA tranferase and 6-MSAS coupled assay in the absence of NADPH (figure 3.13). The positions of the \[^{13}C\]-labels are indicated. |
3.5. Summary

A decarboxylase activity for 6-MSAS has been shown by alkylation of the cysteine thiol of the β-ketosynthase with iodoacetamide (Dimroth et al., 1976). However, the combination of mass spectrometric and fluorescence data obtained indicates that 6-MSAS possesses a slow, native malonyl-CoA decarboxylase activity even when the cysteine thiol of the KS is unoccupied. Therefore it is possible to synthesise 6-MSA in the absence of externally added acetyl-CoA, in an analogous way to that formed with yeast FAS (Kresze et al., 1977).

The biosynthesis of fatty acids requires acetyl-CoA and malonyl-CoA. Although acetyl-CoA is an intermediate in the biosynthesis of many cellular components, malonyl-CoA is only utilised in fatty acid biosynthesis. Hence it is possible that the cellular level of malonyl-CoA regulates fatty acid synthesis. Although a decarboxylase activity is unlikely to have an important physiological role when acetyl-CoA is available, it may play an important role in the regulation of fatty acid biosynthesis within the cell when acetyl-CoA is absent.

3.5.1 Structural studies upon the synthase condensing site

Recently, the three dimensional crystallographic structure of E.coli FAS KSII has been solved (figure 3.14). It is therefore possible to view the interaction between the active site cysteine and the surrounding conserved amino acid residues directly. The active site Cys-163 is located at the bottom of a mainly hydrophobic pocket which facilitates binding of the acyl chain of the acyl-ACP substrate. In close vicinity of the side chain of Cys-163 are the side chains of His-303 and His-340, with distances from the Sγ atom to the Ne atoms of 4.6 and 3.3Å respectively. His-340 is well positioned to act as a base, that can remove the proton from the Sγ atom of Cys-163, thus enhancing the nucleophilicity of this residue. This histidine is invariant in all sequences of condensing enzymes (Huang et al., 1998). The Cys-His pair at the active site of KSII is also reminiscent of the catalytic diad found in cysteine proteases (Kamphius et al., 1984).

The enzyme must also be able to stabilise a number of transition states and intermediates during polyketide synthesis. Charge stabilisation might be provided by
Figure 3.14. Stereo view of the active site of β-ketoacyl-acyl carrier protein synthase II (FAS KSII) from *E. coli* as seen from the entrance of the active site pocket. The location of the conserved active site residues discussed within the text are indicated on the diagram. The polypeptide chains of the two subunits are shown in grey and blue respectively (after Huang *et al.*, 1998).
His-303 and His-340, and possibly by the main chain NH of Phe-400. The location of the His-303 side chain suggests that it is involved in the decarboxylation of malonyl-ACP and in the stabilisation of the formed carbanion intermediate. Another highly conserved basic residue close to Cys-163 is Lys-335, the amino group of which is 7.8Å from the $\text{S}^\gamma$ atom. It is possible that this conserved residue reacts with $\alpha$-phthalaldehyde in both yeast FAS (Stoops et al., 1983) and 6-MSAS (Child, 1994).

The result of experiments with $\alpha$-phthalaldehyde (Child, 1994) and the determination of the primary sequence of 6-MSAS (Beck et al., 1990) indicates that there could be a role for the conserved basic lysine and histidine residues within the KS domain of 6-MSAS. The basic residues within the active site could participate in many of the stages of the condensation reaction (figure 3.15):

1) His-340 abstracts the proton from the thiol group of Cys-163 (figure 3.15a), thus enhancing its ability to attack the ACP 4'-phosphopantetheine bound acyl unit.

2) The acyl unit is now transferred onto the thiol of Cys-163. A postulated role for Lys-335 at this stage would be to protonate the ACP 4'-phosphopantetheine thiol, thus preventing rebinding of the acyl unit bound to Cys-163. The ACP 4'-phosphopantetheine thiol is now unoccupied and ready to accept an extender malonate unit from malonyl-CoA (figure 3.15b).

3) The His-303/Glu-314 is in a position to protonate the carboxyl group of malonyl-ACP. Lys-335 acts as a base promoting the decarboxylation of malonyl-ACP (Stoops et al., 1983) (figure 3.15c).

4) Lys-335 acts as a base by abstracting a proton from the ACP 4'-phosphopantetheine bound acyl group thus facilitating its condensation with the cysteine bound acyl unit (figure 3.15d). It is possible that the decarboxylation of malonyl-ACP (figure 3.15c) and subsequent condensation with the cysteine bound acyl unit is a concerted mechanism. Therefore the ketene intermediate shown in figure 3.15d would not be formed.
Figure 3.15. Functional model for the active site of KSII from *E. coli* (acyl units are in red). a. ACP-bound acyl unit is attacked by the β-KS thiol, which is in a deprotonated form; b. Acyl unit is transferred from the ACP to the β-KS thiol (Cys163); c. ACP-bound malonate unit (extender unit) is decarboxylated; d. Condensation of the ACP-bound malonate unit with the cysteine bound acyl unit; e. Collapse of the tetrahedral intermediate thus forming an ACP-bound acetoacetate unit; f. Free cysteine thiol is able to attack and displace the ACP-bound acetoacetate unit.
5) His-340 is well situated to abstract a proton from the tetrahedral intermediate promoting formation of the phosphopantetheine bound acetoacetate unit and thus vacating the thiol of Cys-163 (figure 3.15e).

6) The free ionised thiol residue of Cys-163 will attack the phosphopantetheine bound acetoacetate moiety, thus resulting in a new thioester bond and allowing the next extender unit to bind to the now free phosphopantetheine thiol (figure 3.15f).

It is possible that some of the basic residues within the condensing active site could stabilise the growing polyketide chain (Crump et al., 1997). His-340, His-303 and Lys-335 are in reasonable positions in relation to the polyketide chain to promote condensation of the carbonyl moieties and also promote stabilisation of the enzyme bound polyketide chain.

Recent research upon the KS-CLF heterodimer of the type II act PKS has confirmed the importance of the conserved lysine and histidine residues (Dreier and Khosla, 2000). To dissect the roles of these residues, each was independently replaced with alanine in the act PKS. All of the modified enzymes failed to catalyse polyketide formation or malonyl-ACP decarboxylation. However, the lysine mutant could be labelled in the presence of \[^{14}\text{C}]\text{-malonyl-CoA, holoACP and malonyl transferase}\) whereas the two histidine mutants could not. This research suggests that all of these conserved residues are important for the decarboxylation of malonyl-ACP and that both histidines may also function to transfer the malonyl group from ACP to KS. However, once the catalytic site is occupied it is highly likely that both histidines will not partake in the transfer of further acyl substrates. These observations compliment the proposed functional model for the active site of KSII (figure 3.15).

The KS of 6-MSAS possesses the same conserved residues as those implicated in the condensing site of KSII and since the two enzymes use similar substrates, the position within the active site of the conserved lysine and histidine residues of KSII can, with some certainty, be extrapolated to the KS of 6-MSAS. With the availability of the KSII crystal structure, catalytic activities can be assigned to the visualised residues of the KS.
of 6-MSAS, which were previously only tentatively assigned on the basis of sequence alignments and chemical modification (figure 3.16). However, it will not be possible to determine the true function of all the conserved residues of the KS of 6-MSAS until its crystal structure has been solved.

The overall structural organisation of KSII closely resembles that of KSIII (Davies et al., 2000) and chalcone synthase (Ferrer et al., 1999). Whereas KSII is specific for acyl-ACP substrates, both KSIII and chalcone synthase are specific for acyl-CoA substrates. The condensing site mechanisms of these enzymes are proposed to be similar. However, the role played by Lys-335 in the KSII active site mechanism is replaced by an asparagine residue for KSIII and CHS. For KSIII only a glycine residue replaces the role played by His-340 in the KSII mechanism. The glycine residue is thought to form a cavity (an oxyanion hole) with the active site cysteine. KSII, KSIII and CHS define a family of proteins that are closely related to the thiolase I structure (Mathieu et al., 1997). Thiolase I catalyses the final step in the β-oxidation pathway resulting in the formation of acetyl-CoA from acetoacetyl-CoA (Kunau et al., 1994). It is therefore interesting to speculate that all four proteins originated from a common ancestor. This observation once again underlines how closely related polyketide and fatty acid biosynthesis and fatty acid degradation are in evolutionary and mechanistic terms.
Figure 3.16. Functional model for the active site of the keto synthase of 6-MSAS (acyl units are in red). The ACP 4'-phosphopantetheine bound malonate unit undergoes decarboxylation prior to condensation with the acetyl unit bound to Cys204 of the keto synthase.
Chapter 3 part II

The synthesis of orsellinic acid by 6-methylsalicylic acid synthase

3.6 Introduction

6-Methylsalicylic acid synthase and orsellinic acid synthase catalyse the respective formation of 6-MSA and orsellinic acid from the same building units, namely, one starter unit of acetyl-CoA and three extender units of malonyl-CoA. The only difference between the two products formed is the absence of a hydroxyl group at the 4-position of 6-MSA. The removal of the hydroxyl group requires a NADPH dependant reduction step and a dehydration step. These are performed by the β-ketoreductase and dehydratase activities of 6-MSAS, respectively. An amino acid sequence comparison between 6-MSAS and OAS reveals that the latter does not possess the β-ketoreductase and dehydratase components. Gel electrophoresis studies upon OAS, which has been isolated as a homogenous protein (Gaucher and Shepherd, 1968; Spencer and Jordan, unpublished results), have revealed the enzyme to have a subunit molecular weight of Mr ~110,000, compared with a Mr ~190,000 for 6-MSAS. This difference presumably reflects the absence of the β-ketoreductase and dehydratase domains in OAS that would otherwise result in the removal of the keto group from the C₆ tetraketide intermediate.

During the investigation into the malonyl-CoA decarboxylase activity of 6-MSAS, unlabelled and [¹³C]-labelled 6-MSA and TAL were isolated by HPLC. It was noticed that for all reactions without NADPH, an additional small, but sharp, peak was
observed by HPLC that corresponded to a similar retention time to that of orsellinic acid. It was therefore important to identify this peak and to observe whether 6-MSAS possessed the additional capability to synthesise orsellinic acid. This would also provide an opportunity to obtain further evidence for the presence of a malonyl-CoA decarboxylase activity for 6-MSAS.

3.7 Materials
6-Methylsalicylic acid synthase was purified from *Penicillium patulum* NRRL 2159A as described in chapter 2. Succinyl-CoA transferase was purified from porcine heart as described in chapter 2. Borosilicate nanoflow tips for ESMS were obtained from Micromass. Malonyl-CoA, butyryl-CoA, propionyl-CoA, NADPH, coenzyme-A and succinic anhydride, were obtained from Sigma. Orsellinic acid for use as a chemical standard, [2-¹³C] malonic acid (99% atom [¹³C]) and acetic anhydride were obtained from Aldrich. HPLC grade H₂O and ammonium hydroxide were supplied by BDH. Formic acid was obtained from Fluka. HPLC grade acetonitrile was obtained from Fisher Scientific. Trifluoroacetic acid was obtained from Applied Biosystems Division. The C₁₈ reverse phase column (250mm x 4.6mm; 5µm) was supplied by Spherisorb. HPLC buffer A contained H₂O + 0.1% (v/v) TFA. HPLC buffer B contained acetonitrile + 0.1% TFA (v/v). The Superose-6 gel filtration column was obtained from Pharmacia. It is important to note that the malonyl-CoA used in all reactions and assays was HPLC-purified within the laboratory, prior to use, to remove any contaminating acetyl-CoA. Mass spectrometry was also used to check [2-¹³C]-malonic acid for any contaminating [2-¹³C]-acetate. Q-TOF MS-MS was performed courtesy of Micromass UK.

3.8 Methods
3.8.1 Gel-filtration purification of 6-methylsalicylic acid synthase
6-MSAS (1mg) purified from the Mono Q™ anion exchange column (chapter 2) was loaded onto a Superose-6 gel filtration column (30cm x 1cm) that had been pre-equilibrated with 100mM Tris-HCl buffer, pH 7.6, containing 10% glycerol and 1mM DTT.
3.8.2 Biosynthesis of $[^{13}C]$-labelled and unlabelled orsellinic acid
The production of $[^{13}C]$-labelled (and unlabelled) orsellinic acid (OA) was achieved as follows: a 1.9ml Eppendorf tube containing 100μl of 100mM potassium phosphate buffer, pH 7.6, 10μl of 10mM succinyl-CoA, 10μl of succinyl-CoA transferase (0.73 units), 3.3 mU of 6-MSAS, 10μl of 10mM acetyl-CoA and 10μl of 3M [2-$^{13}$C] (or unlabelled) malonate, pH 7.0 was prepared. In order to observe the labelling pattern of orsellinic acid, NADPH was omitted from the reaction. The reaction (200μl) was terminated after 2 hours by the addition of 20μl of 50% (w/v) TCA. The sample was microcentrifuged for 5 minutes to remove all traces of precipitated protein, prior to HPLC.

3.8.3 Reverse-phase HPLC purification of enzymically synthesised $[^{13}C]$ and unlabelled orsellinic acid
The samples, prepared as in section 3.8.2, were loaded onto a C$_{18}$ reverse phase Spherisorb column that had been pre-equilibrated with 98% buffer A and 2% buffer B. Labelled and unlabelled OA were eluted by increasing buffer B to 80% over a period of 30 minutes at λ = 300nm and at a flow rate of 1 ml/min. The peaks of interest were collected and freeze dried.

3.8.4 The use of alternative starter units to synthesise orsellinic acid analogues
Attempts were made to synthesise OA analogues by changing the acetyl-CoA starter unit to either propionyl-CoA or butyryl-CoA. The method used in 3.8.2 was followed using unlabelled malonate except that 10μl of 20mM propionyl-CoA (or butyryl-CoA) was used instead of 10μl of 10mM acetyl-CoA. The samples were analysed by HPLC using the conditions as for 3.8.3.

3.8.5 Nanospray collision induced dissociation (CID) mass spectrometry
Labelled and unlabelled OA samples were dissolved in a 50% acetonitrile (v/v), 5% ammonium hydroxide (v/v) solution. A sample (2μl) of this solution was loaded into a borosilicate metal coated nanoflow needle (type B) and mass spectrometry was performed in negative ion mode using a Micromass Quattro II triple quadrupole mass spectrometer fitted with a nanoelectrospray source and probe. The source was operated at 30°C, with a capillary voltage of 2.45 kV, HV lens voltage of 0.20 kV and a cone
voltage of 35 V. Spectra were recorded over the range \( m/z \) 150 to \( m/z \) 200 for orsellinic acid. For CID tandem mass spectrometry, quadrupole (Q1) was operated in mass resolving mode to select the precursor ion. Q1 was adjusted to transmit a window of approximately 3 mass units wide to quadrupole 2 (Q2). The collision cell pressure was maintained at 10 mTorr to provide collisional damping of the fragment ions and the remaining precursor ions. Spectra were recorded over the mass range \( m/z \) 25 to \( m/z \) 200 for orsellinic acid. Control of the mass spectrometer, data acquisition and mass measurement was performed using Masslynx 2.1 software.

3.8.6 Quadrupole Time of Flight (Q-TOF) mass spectrometry

\([^{13}\text{C}]\)-Labelled and unlabelled OA samples were dissolved in 100\(\mu l\) of acetonitrile followed by a 50 fold dilution with a 50% acetonitrile (v/v), 50% \( \text{H}_2\text{O} \) (v/v) solution. Mass spectrometry was performed in negative ion mode using a Micromass Q-TOF instrument fitted with an electrospray source and probe. The source was operated at 100\(^\circ\text{C}\), with a cone voltage of 30 V. Spectra were recorded over the range \( m/z \) 65 to \( m/z \) 180 for orsellinic acid at a rate of 1 spectrum / second.

3.9 Results and discussion

3.9.1 Gel-filtration of 6-methylnsalicylic acid synthase

As described in section 3.4.1.

3.9.2 Reverse phase HPLC purification of biosynthetic orsellinic acid

Both \([^{13}\text{C}]\)-labelled and unlabelled OA synthesised by 6-MSAS were detected by HPLC and eluted from the C\(_{18}\) column with a retention time of \( \sim \)19 minutes. In all cases a small, sharp peak was observed. A comparison of OA peak size with that of TAL (retention time \( \sim \)14 minutes) revealed that the rate of formation of OA was about 1-2% that of TAL. It can therefore be concluded that 6-MSAS can make orsellinic acid in small quantities.

3.9.3 The use of alternative starter units to synthesise orsellinic acid analogues

In a previous set of experiments, incubation of alternative starter units with 6-MSAS resulted in the synthesis of alternative 6-alkylsalicylic acids and triketide lactones (Campuzano et al., 1998). It was observed that there was an inverse relationship
between the carbon chain length of the starter unit and the level of incorporation into the final product. Therefore in order to see any small amounts of enzyme synthesised OA analogues, short chain starters such as propionyl-CoA and butyryl-CoA were used. Upon HPLC analysis of both incubations no peaks corresponding to OA analogues were detected. It was therefore concluded that either no OA analogues could be synthesised by 6-MSAS or that the levels of these compounds were too low to be detected by HPLC.

3.9.4 Analysis of standard and unlabelled orsellinic acid by CID mass spectrometry

In order to gain insight into the \([^{13}C]\)-labelling pattern of biosynthetic OA, it was necessary to perform a control study with unlabelled OA. Comparisons made between the unlabelled and \([^{13}C]\) labelled molecules would allow the identification of the positions of the \([^{13}C]\) labels in OA. All mass spectrometric analyses were carried out in negative ion mode, that proved to be appropriate for the subsequent CID analysis. The OA parent ion is represented by a single charged species with Mr 166.85 (figure 3.17a). Fragmentation of the parent ion resulted in the production of four daughter ions. The first daughter ion with Mr 122.49 is produced as a result of loss of the carboxyl group, Mr 44.00. The smallest daughter ion, Mr 40.91, corresponds to the C-2 unit, CHCO (figure 3.17b). The assignment of the remaining two daughter ions, Mr 80.77 and Mr 78.66, is described in 3.9.7.

The enzymatic synthesis of unlabelled OA produced near identical parent and daughter ion spectra to that obtained for the standard (figure 3.17). Having assigned the daughter ions it was then possible to analyse the \([^{13}C]\)-labelled OA using the same method and thereby to determine the positions of the \([^{13}C]\)-label within the molecule and the biosynthetic origins of the carbon atoms.

3.9.5 Analysis of orsellinic acid biosynthesised from \([2-{^{13}C}]\)-malonyl-CoA and unlabelled acetyl-CoA

The OA enzymically synthesised from 6-MSAS in the presence of \([2-{^{13}C}]\)-malonyl-CoA and unlabelled acetyl-CoA, but without NADPH, produced a parent ion, Mr 169.91, as expected, three mass units higher than that of the unlabelled OA (figure 3.18a). Upon fragmentation, the loss of the carboxyl group resulted in a daughter ion
Figure 3.17. Nanospray mass spectrometric analysis of orsellinic acid chemical standard. a. parent ion; b. collision induced dissociation mass spectrometric analysis of daughter ions produced upon fragmentation of parent ion.
Figure 3.18. Nanospray mass spectrometric analysis of [2,4,6-\textsuperscript{13}C\textsubscript{3}]-labelled orsellinic acid biosynthesised by the succinyl-CoA transferase / 6-MSAS linked assay in the presence of externally added acetyl-CoA and in the absence of NADPH. a. parent ion; b. collision induced dissociation mass spectrometric analysis of daughter ions produced upon fragmentation of parent ion.
of Mr 125.71 (figure 3.18b), which is three mass units higher than that of the corresponding unlabelled daughter ion (figure 3.17b). The CHCO fragment, Mr 41.78, is one mass unit higher than that of the corresponding unlabelled daughter ion indicating the presence of a $[^{13}\text{C}]$-label that has originated from one of the malonyl-CoA extender units. The remaining daughter ion, Mr 82.13, produced a broad peak that could be the result of two ions close together. Therefore this peak was left unassigned.

3.9.6 Analysis of orsellinic acid produced exclusively from $[2-^{13}\text{C}]$-malonyl-CoA

Further evidence that confirms the ability of 6-MSAS to decarboxylate malonyl-CoA can be obtained from studying the labelling pattern of OA derived exclusively from $[^{13}\text{C}]$-malonyl-CoA and comparing the findings with the previous fragmentation patterns. The presence of a single $[^{13}\text{C}]$-label within the methyl group of OA would indicate that malonyl-CoA has undergone decarboxylation. The parent ion of OA resulted in a peak of Mr 170.76 (figure 3.19a), which is four mass units higher than that of the unlabelled OA (figure 3.17a). Upon CID, the loss of the carboxyl group resulted in a daughter ion, Mr 126.77, (figure 3.19b) indicating the presence of four $[^{13}\text{C}]$-labels within the structure, one of which is derived from the decarboxylation of malonyl-CoA. The position of this label can be pinpointed to the methyl group, as shown by the loss of Mr 41.71 for the CHCO fragment. The remaining daughter ion, Mr 82.13, produced a broad peak that could be the result of two ions close together. Therefore this peak was left unassigned. These studies reinforce the conclusion from chapter 3 part I that, in the absence of acetyl-CoA, malonyl-CoA can provide the starter unit by decarboxylation to acetyl-CoA. The mass spectrometric data are summarised in table 3.3.

3.9.7 Analysis of orsellinic acid daughter ions by Q-TOF MSMS

The data obtained using the triple quadrupole mass spectrometer did not show conclusively that 6-MSAS could synthesise OA in small quantities. The predicted and observed masses of some of the parent and daughter ions shown in table 3.3 differ by as much as 0.64 mass units. Therefore it was important to clarify this data using a higher resolution analyser such as TOF. Also some of the daughter ions obtained from the $[^{13}\text{C}]$-labelled OA parent ions could not be identified using the triple quadrupole instrument. It was apparent for both $[^{13}\text{C}]$-labelled experiments that there were two
Figure 3.19. Nanospray mass spectrometric analysis of [2,4,6,8-\textsuperscript{13}C_4]-labelled orsellinic acid biosynthesised by the succinyl-CoA transferase / 6-MSAS linked assay in the presence of externally added acetyl-CoA and in the absence of NADPH. \textbf{a.} parent ion; \textbf{b.} collision induced dissociation mass spectrometric analysis of daughter ions produced upon fragmentation of parent ion.
<table>
<thead>
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<th>Structure</th>
<th>Parent ion</th>
<th>Carboxyl loss</th>
<th>CHCO fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Predicted</td>
<td>Observed</td>
<td>Predicted</td>
</tr>
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<td><img src="image1" alt="Structure 1" /></td>
<td>167.14</td>
<td>166.85</td>
<td>123.13</td>
</tr>
<tr>
<td><img src="image2" alt="Structure 2" /></td>
<td>170.14</td>
<td>169.91</td>
<td>126.13</td>
</tr>
<tr>
<td><img src="image3" alt="Structure 3" /></td>
<td>171.14</td>
<td>170.76</td>
<td>127.13</td>
</tr>
</tbody>
</table>

**Table 3.3.** Summary of results obtained using triple quadrupole Quattro II MSMS. The predicted masses and observed masses in negative ion mode of: unlabelled orsellinic acid (figure 3.17); [2,4,6-$^{13}$C$_3$]-labelled orsellinic acid (external addition of unlabelled acetyl-CoA) (figure 3.18); [2,4,6,8-$^{13}$C$_4$]-labelled orsellinic acid produced as a result of the decarboxylase activity of 6-MSAS, generated from the succinyl-CoA transferase and 6-MSAS coupled assay (figure 3.19). The positions of the [$^{13}$C]-labels in the structures are indicated.
daughter ion peaks very close to each other resulting in one broad peak at Mr 82.13 (figure 3.18) and Mr 83.18 (figure 3.19). Unfortunately, it was not possible to assign chemical structures to these daughter ion fragments due to the resolution limitations of the triple quadrupole instrument.

Both triple quadrupole and Q-TOF mass spectrometers possess a quadrupole analyser (MS1) and a collision cell. However, instead of having a second quadrupole analyser (MS2), the Q-TOF has a time of flight analyser. The time of flight analyser measures very accurately, the time taken for an ion to travel a set distance, which is directly related to the mass to charge ratio of the ion. Unlike quadrupole analysers, TOF is able to register all ions at the same time, thereby providing a sensitivity of a full spectrum equal to that of a single ion monitoring regime. A TOF mass spectrometer is also able to measure the exact mass of ions and was therefore used to clarify the data already obtained and to identify the daughter ions not assigned by the triple quadrupole instrument.

The mass spectrometric data obtained using the Q-TOF instrument are shown in figures 3.20, 3.21a and 3.21b and are summarised in table 3.4. The data acquired using the triple quadrupole instrument revealed that, for parent and daughter ions, there was a difference of up to 0.64 mass units between some of the predicted and observed masses (table 3.3). However the data obtained using the Q-TOF instrument minimised the predicted and observed mass differences to a maximum of 0.13 mass units (table 3.4). The Q-TOF data could also be used to resolve the daughter ion peaks that could not be assigned using the triple quadrupole data.

A C$_5$H$_5$O fragment can be assigned to the peaks of Mr 81.0344 for unlabelled OA (figure 3.20), Mr 83.0414 for [2,4,6,$^{13}$C$_3$]-OA (figure 3.21a) and Mr 84.0440 for [2,4,6,8,$^{13}$C$_4$]-OA (figure 3.21b). The origination of the C$_5$H$_5$O fragment from OA is shown in table 3.4.

A C$_6$H$_7$ fragment can be assigned to the peaks of Mr 79.0554 for unlabelled OA (figure 3.20), Mr 82.0655 for [2,4,6,$^{13}$C$_3$]-OA (figure 3.21a) and Mr 83.0684 for [2,4,6,8,$^{13}$C$_4$]-OA (figure 3.21b). However it was not possible to assign these daughter ions to any obvious chemical structures. Instead, exact mass assignments show
Figure 3.20. Q-TOF MS-MS analysis of the unlabelled orsellinic acid standard in negative ion mode. Daughter ions produced in the region between 75-180 Da are shown.
Figure 3.21. Q-TOF MS-MS analysis of $[^{13}\text{C}]-$labelled orsellinic acids synthesised by the succinyl-CoA transferase / 6-MSAS linked assay in the presence and absence of externally added acetyl-CoA.  

a. Fragmentation pattern of [2,4,6,$^{13}\text{C}_3$]-orsellinic acid parent ion (+ acetyl-CoA);

b. Fragmentation pattern of [2,4,6,8,$^{13}\text{C}_4$]-orsellinic acid parent ion (- acetyl-CoA).
Table 3.4. Summary of results obtained using Q-TOF MSMS. The predicted masses and observed masses in negative ion mode of: unlabelled orsellinic acid (figure 3.20); [2,4,6-\textsuperscript{13}C\textsubscript{3}]\textsuperscript{-}labelled orsellinic acid (external addition of unlabelled acetyl-CoA) (figure 3.21a); [2,4,6,8-\textsuperscript{13}C\textsubscript{4}]\textsuperscript{-}labelled orsellinic acid produced as a result of the decarboxylase activity of 6-MSAS, generated from the succinyl-CoA transferase and 6-MSAS coupled assay (figure 3.21b). The positions of the [\textsuperscript{13}C]-labels in the structures are indicated.
conclusively that carbon-carbon bonds must be rearranged in order for the precursor ion to lose two molecules of CO₂. Two possible rearrangements are shown in figure 3.22. The nature of the rearrangement is unknown but the mass measurements of the corresponding daughter ions suggest that the carbon atoms at the 2-, 4-, 6- and 8-positions of OA are retained in the C₆H₇ fragment (figure 3.22).

3.10 Summary
The data obtained in these experiments indicate that the [¹³C]-labels occur at the expected positions in the OA, supporting the proposition that 6-MSAS, in the absence of NADPH is able to synthesise OA. The absence of NADPH indicates that it is unlikely that 6-MSAS is involved in the biosynthesis of OA in vivo. It has also been shown that 6-MSAS can synthesise OA from four units of malonyl-CoA in the absence of acetyl-CoA and NADPH. This observation supports the conclusion made in chapter 3 part I, that 6-MSAS possesses a malonyl-CoA decarboxylase activity.

The major difference between the amino acid sequences of 6-MSAS and OAS is that the latter does not possess β-ketoreductase and dehydratase domains. Therefore for 6-MSAS to synthesise OA, it must ‘skip’ the β-ketoreductase and dehydratase steps. This can be viewed as an irregularity in the sequential set of condensation and chain modification events that are required to produce TAL, in the absence of NADPH. The observed rate of OA biosynthesis is ~1-2% that of TAL. This indicates that the unreduced triketide intermediate is recognised, albeit poorly, by the β-ketosynthase. Consequently malonyl-CoA is transferred, by malonyl transferase, to the active thiol of the 4’phosphopantetheine arm of the ACP. Then a condensation reaction takes place between malonyl-ACP and the KS bound unreduced triketide intermediate. The resulting tetraketide intermediate undergoes cyclisation to form OA.

It is possible that there are irregularities at other various steps of chain synthesis, leading to the background synthesis of other metabolites. However such metabolites, if they exist, remain to be identified.
Figure 3.22. Hypothetical structural rearrangement resulting in the unlabelled OA daughter ion, Mr 79.06. The OA parent ion undergoes loss of CO$_2$ to produce a daughter ion of observed Mr 123.04. The loss of COH + OH from this daughter can occur in many different ways (two shown) resulting in a structurally rearranged daughter ion of observed Mr 79.06. From the $^{[13]}$C-labelling experiments it can be concluded that this daughter ion must retain the carbon atoms that occur at the 2-, 4-, 6- and 8- positions of OA (shown in blue).
Chapter 4
Investigations with haloacetyl-CoA derivatives on 6-methylsalicylic acid synthase

4.1 Introduction

4.1.1 Natural occurrence of halogenated metabolites

Halogenated metabolites, once thought to be infrequent in nature, are actually quite common and are produced by many different organisms. The most abundant naturally synthesised organohalogens are bromine-containing metabolites, preferentially produced by bacterial marine organisms, because of the relatively high concentration of bromine in seawater compared to soil. Halogenated compounds are also produced by marine invertebrates, one such example being the Tyrian purple dye (figure 4.1) produced by several Murex species (molluscs). In terrestrial organisms mostly chlorometabolites are found, many of which have been isolated from fungi. Some like griseofulvin (figure 4.1), produced by several Penicillium species, have antibiotic activity. Others like chloromethane, are synthesised in huge amounts and are considered responsible for the destruction of the ozone layer or, like drosophilin A (figure 4.1), are very difficult to degrade. Iodinated compounds occur less frequently, although they too are produced by a variety of organisms. An example is the thyroid hormone thyroxine (figure 4.1), which is synthesised by mammals. Fluorinated metabolites are very rare, although the amount of fluoride available to organisms is higher than that of iodide and bromide.

The search for halometabolites from bacteria was, and is still, connected to the search for antibiotics or compounds with other biological activities. In fact, most organohalogens found in bacteria have been isolated from antibiotic-producing Actinomycetes species. Two examples are chloramphenicol from Streptomyces venezuelae and pyoluteorin from Pseudomonas fluorescens (figure 4.1). The characterisation of the gene cluster from the latter organism has revealed that a type I PKS is involved at the early stages of the biosynthetic pathway (Nowak-Thompson et al., 1999). However, the incorporation of two chlorine atoms into the final product takes place using an enzyme called a halogenase.
Figure 4.1. Examples of halometabolites produced by eukaryotic organisms.
4.1.2 Halogenating enzymes

Halogenases, also known as haloperoxidases, are enzymes that catalyse the two-electron oxidation of a halide (X) to the corresponding hypohallic acid according to equation [1]:

\[ H_2O_2 + H^+ + X^- \rightarrow H_2O + HOX \]

HOX may further react with a broad range of nucleophilic acceptors to form a number of diverse halogenated compounds. Three classes of haloperoxidases have been identified. Two of these classes are the haem-containing haloperoxidases and the vanadium-containing haloperoxidases that bind vanadate (VO\(_4^{3-}\)) as a prosthetic group. The third class consists of enzymes, detected in a number of bacteria, do not possess a prosthetic group (Wiesner et al., 1988; Bantleton et al., 1994). Bacterial haloperoxidases possess a Ser-His-Asp catalytic triad in the active centre that has been shown to be necessary for halogenating activity (Pelletier et al., 1995). The first step in halogenation is the formation of an acetate ester at the serine residue of the catalytic acid. As a strong oxidising agent, peracetic acid is then able to oxidise halogens to form halogenated compounds according to equation [2]:

[2]
4.1.3 The incorporation of halogenated starter units with fatty acid synthase

Rat liver FAS (Kumar et al., 1980), rabbit FAS (McCarthy and Hardie, 1982) and chicken liver FAS (Tian et al., 1989) have been shown to be irreversibly inactivated by chloroacetyl-CoA. Therefore these enzymes are unable to synthesise the corresponding ω-chlorinated fatty acids. Two mechanisms have been proposed for the action of chloroacetyl-CoA with rabbit FAS (figure 4.2). For both mechanisms, chloroacetyl-CoA is proposed to alkylate the ACP 4'-phosphopantetheine thiol.

The inactivation of mammalian FASs with chloroacetyl-CoA contrasts with a naturally occurring FAS identified in seed oils of *Dichapetalum toxicarium*, a plant from Sierra Leone that accumulates fluoroacetate on its leaves (Wood et al., 1964). The plant FAS is not inactivated by but is able to use fluoroacetate as a starter compound to synthesise a number of ω-fluorofatty acids that belong to a rare group of fluorinated natural products (figure 4.3). These compounds are thought to arise due to fluoroacetyl-CoA replacing acetyl-CoA at the initial stage of fatty acid synthesis (Harper et al., 1990). However, the restriction of fluorine to the ω-position implies the existence of enzyme constraints at subsequent stages of the biosynthetic pathway. Thus, either the acetyl-CoA carboxylase enzyme does not readily synthesise fluoromalonyl-CoA or the substrate specificity of the malonyl-ACP transferase of FAS does not extend to fluoromalonyl-ACP during chain elongation.

There are a number of uses for halogenated fatty acids in medicine. One example is the use of radiohalogenated (¹²⁵I) free fatty acids as tracers that can be used to assess both myocardial perfusion and metabolism (Corbett, 1999). Several protocols have been implemented using these compounds to detect abnormal fatty acid metabolism in ischemic heart disease as well as in nonischemic and hypertrophic cardiomyopathies.

4.1.4 The incorporation of haloacetyl-CoAs with 6-MSAS

So far, the effect of halogenated acetyl-CoA compounds on PKSs has not been investigated. The following research describes the synthesis, purification and identification of fluoro-, chloro-, and bromoacetyl-CoA. The incorporation of these compounds with 6-MSAS is also investigated in an attempt to show if any triacetic
Figure 4.2. Proposed mechanisms of action of chloroacetyl-CoA with rabbit FAS. Chloroacetyl-CoA could be transferred to the pantetheine thiol (pant-SH) either directly (mechanism 1) or via an O-ester intermediate bound to the acyl transferase (mechanism 2). The hydroxyl group indicated represents the serine residue of the acyl transferase (after McCarthy and Hardie, 1982).
Figure 4.3. The biosynthesis of \( \text{threo-18-fluoro-9,10-dihydroxy-10-stearic acid} \) by *Dichapetalum toxicarium*. The fluorinated fatty acyl chain, biosynthesised by fatty acid synthase, undergoes reduction by stearoyl desaturase to produce \( \omega \)-fluorooleoyl-SACP. Subsequent oxidation and hydrolysis steps are required to synthesise the final product (after Harper and O’Hagan, 1994).
compounds with 6-MSAS is also investigated in an attempt to show if any triacetic acid halolactones or 6-halomethylsalicylic acids are synthesised or, conversely, if these compounds are able to inhibit / inactivate the enzyme.

4.2 Materials

6-Methylsalicylic acid synthase was purified from Penicillium patulum NRRL 2159A. Fluoroacetate and phosphorus pentachloride were obtained from Aldrich. Chloroacetyl chloride and bromoacetyl chloride were obtained from Fluka. Acetyl-CoA, malonyl-CoA, NADPH and NaHCO₃ were purchased from Sigma. Borosilicate nanoflow type B tips came from Micromass. 1,3-Dibromopropan-2-one (DBP) was purchased from Lancaster Chemicals Ltd. Iodoacetamide was obtained from BDH. [¹⁴C]-Iodoacetamide was purchased from Amersham. HPLC grade H₂O and ammonium hydroxide were supplied by BDH. Formic acid was obtained from Fluka. HPLC grade acetonitrile was obtained from Fisher Scientific. Trifluoroacetic acid was obtained from Applied Biosystems Division. An analytical C₁₈ reverse phase column (250mm x 4.6mm; 5μM) was supplied by Spherisorb. A preparative C₁₈ reverse phase column (250mm x 22mm; 10μM) was supplied by Vydac. HPLC buffer A contained H₂O + 0.1% (v/v) TFA. HPLC buffer B contained acetonitrile + 0.1% (v/v) TFA.

4.3 Methods

4.3.1 Fluoroacetyl chloride synthesis

Phosphorus pentachloride (5g) and sodium fluoroacetate (2.18g) were placed in a round bottomed flask and mixed producing a strong exothermic reaction. The fluoroacetyl chloride was distilled by gently heating the reaction mixture up to 70°C. The acid chloride was collected and stored at 4°C. A sample was removed for analysis by ¹H-NMR.

4.3.2 ¹H-NMR analysis of fluoroacetyl chloride

Fluoroacetyl chloride (2-3mg) was dissolved in deuterated chloroform (0.6ml). The sample was analysed using a NMR spectrophotometer (Bruker) operating at 300MHz.
4.3.3 Fluoro-, chloro- and bromoacetyl-CoA synthesis

Each haloacetyl-CoA compound was synthesised from its corresponding acid chloride as follows; coenzyme A (10mg) was weighed into an Eppendorf and dissolved in 1.25ml of 0.2M NaHCO₃. A 50x excess of acid chloride was added (50μl) and the contents mixed by gently ‘flicking’ the Eppendorf. The reaction was allowed to proceed for 90 minutes on ice, during which time the Eppendorf was opened intermittently to release any HCl gas.

4.3.4 Reverse-phase HPLC purification of haloacetyl-CoAs

An aliquot (2μl) of each completed haloacetyl-CoA synthesis reaction was loaded onto an analytical C₁₈ reverse phase column (Spherisorb) that had been pre-equilibrated with 98% buffer A and 2% buffer B. Each haloacetyl-CoA was eluted from the column by increasing buffer B to 80% over a period of 30 minutes at λ = 260nm and at a flow rate of 1 ml/min. The peaks of interest were collected and freeze dried. The remaining portion of each reaction mixture was purified using a preparative C₁₈ reverse phase column (Vydac) using a similar acetonitrile / water gradient to that described above. The peaks of interest were collected and freeze dried.

4.3.5 Nanospray mass spectrometric analysis of haloacetyl-CoAs

The freeze dried samples collected from the HPLC analytical columns were redissolved in 50% acetonitrile and adjusted to a final concentration of 5% ammonium hydroxide. A 2μl sample of the stock solution was removed and loaded onto a borosilicate metal coated nanoflow capillary. Mass spectrometry was performed in negative ion mode using a Micromass Quattro II triple quadrupole mass spectrometer with a nanospray source and probe. The source was operated at 30°C, with a capillary voltage of 2.62 kV, HV lens voltage of 0.14 V and a cone voltage of 21 V. Spectra were recorded over the range m/z 450 to m/z 950 for coenzyme-A and fluoroacetyl-CoA, and m/z 650 to m/z 950 for chloroacetyl-CoA and bromoacetyl-CoA. Control of the mass spectrometer, data acquisition and mass measurement was performed using Masslynx 2.1 software.
4.3.6 Stability of haloacetyl-CoA compounds
The freeze dried haloacetyl-CoA compounds were resuspended, separately, at 25°C, in analytical grade water and 100mM potassium phosphate buffer, pH 7.6. Aliquots were removed at regular intervals over a four hour period and loaded onto an analytical reverse phase HPLC column pre-equilibrated in 98% buffer A and 2% buffer B. The extent of degradation of each haloacetyl-CoA was observed by applying a similar gradient to that used in 4.3.4.

4.3.7 FPLC purification of 6-MSAS
Homogenous 6-MSAS was obtained by elution from a Mono Q™ anion exchange FPLC column as described in chapter 2.

4.3.8 Buffer exchange of 6-MSAS by gel filtration
The stored enzyme contains glycerol, DTT, EDTA, benzamidine and β-mercaptoethanol. In order to prevent these reagents from interfering with further experiments it was necessary to apply the enzyme to a PD-10 gel filtration column that had been pre-equilibrated with 100mM potassium phosphate buffer, pH 7.6. The enzyme was eluted from the column in the same buffer and left on ice until required. It was always used, however, within two hours.

4.3.9 Cross-linking with 1,3-dibromopropan-2-one after pre-incubation of 6-MSAS with haloacetyl-CoA derivatives
6-MSAS (140μl of a 0.1mg/ml solution; 0.13μM) in 100mM potassium phosphate buffer, pH 7.6, was pre-incubated with each haloacetyl-CoA (final concentration 0.67mM) for five minutes at 25°C. A control containing 6-MSAS previously pre-incubated with acetyl-CoA (final concentration 0.67mM) and a control containing 6-MSAS without any haloacetyl-CoA were run in parallel. 1,3-Dibromopropan-2-one (DBP) (0.52μM; ~ 4 moles of DBP: 1 mole of 6-MSAS tetramer) was added to each of the reactions, which were then allowed to proceed for 15 minutes at 25°C. Each reaction was terminated by the addition of an equal volume of SDS electrophoresis disruption buffer. The extent of cross-linking within each reaction was observed by SDS-PAGE.
4.3.10 Reaction of 6-MSAS with $[^{14}\text{C}]$-iodoacetamide after preincubation with haloacetyl-CoAs

6-MSAS (100µl of a 0.76mg/ml solution; 1.0µM) in 100mM potassium phosphate buffer, pH 7.6, was pre-incubated separately with each haloacetyl-CoA (0.9mM) for five minutes at 25°C (total volume 110µl). A control containing 6-MSAS pre-incubated with acetyl-CoA (0.9mM) and a control containing 6-MSAS without any haloacetyl-CoA were run in parallel. $[^{14}\text{C}]$-Iodoacetamide (50µM, 0.074 µCi; 50 moles iodoacetamide: 1 mole 6-MSAS tetramer) was added to each of the reactions, which were then allowed to proceed for forty minutes at 25°C. Each reaction was then applied to a PD-10 column that had been pre-equilibrated with 100mM potassium phosphate buffer, pH 7.6, so as to remove unbound radiolabelled iodoacetamide. The enzyme was eluted with the same buffer. Fractions containing enzyme were pooled.

4.3.11 Liquid scintillation counting of $[^{14}\text{C}]$-iodoacetamide samples

The pooled fractions obtained from each reaction from 4.3.10 (~350µl) were diluted with 5ml of scintillation fluid (ReadyProt, Beckman) and analysed for 2 minutes using a Beckmann LS6500 counter with a background of 35.56 dpm for $[^{14}\text{C}]$. The efficiency of counting was 95.8% for counts per minute (cpm) were corrected to disintegrations per minute (dpm).

4.3.12 Incubation of haloacetyl-CoA derivatives with 6-MSAS to synthesise 6-halomethylsalicylic acids and triacetic acid halolactones

To investigate the formation of halomethylsalicylic acids, the following was carried out for each haloacetyl-CoA: In a final volume of 140µl, 6-MSAS (100µl of a 0.32mg/ml solution; 0.30 µM) in 100mM KH$_2$PO$_4$ buffer, pH 7.6, haloacetyl-CoA (3.5mM), malonyl-CoA (1.4mM) and NADPH (1.4mM) were added to an Eppendorph tube and gently mixed by gentle agitation. After 60 minutes at 25°C the reaction was terminated by the addition of 20µl of 50% (w/v) TCA. The sample was microcentrifuged for 5 minutes to remove precipitated protein and 100µl of the sample was then injected onto a C$_{18}$ reverse phase column pre-equilibrated in 98% buffer A, 2% buffer B at $\lambda = 300$nm and at a flow rate of 1 ml/min. Buffer B was increased to 80% over a period of
30 minutes at a flow rate of 1 ml/min. To investigate the formation of halo-lactones, NADPH was omitted from the reaction and replaced by water.

4.3.13 Inactivation of 6-MSAS on incubation with haloacetyl-CoA derivatives

In a final volume of 130μl, 6-MSAS (110 μl of a 0.67mg/ml solution; 0.88μM) in 100mM potassium phosphate buffer, pH 7.6, was incubated, separately, with each haloacetyl-CoA (3.0mM) for 30 minutes at 25°C. Each reaction was then applied to a PD-10 column that had been pre-equilibrated with 100mM potassium phosphate buffer, pH 7.6, to remove unbound haloacetyl-CoA. The enzyme was eluted with the same buffer. Fractions containing enzyme were pooled (~300μl, 0.25mg/ml) and assayed for 6-MSAS activity. The assay contains in a 1ml final volume: potassium phosphate buffer, pH 7.6, (0.1M); acetyl-CoA, (0.1mM); malonyl-CoA (0.2mM); NADPH, (0.2mM); BSA, (1.25mg). Following the establishment of a stable baseline at 25°C, the reaction was initiated by addition of a 50μl aliquot of the pooled fractions containing 6-MSAS, and the increase in fluorescence associated with 6-MSA formation was measured at 390nm. The extent of fluorescence change is related to that obtained with a standard solution of 6-MSA.

A control containing 6-MSAS (0.88μM) without haloacetyl-CoA was also performed and assayed under the same parameters as above.

4.3.14 Reaction of haloacetyl-CoA derivatives with 6-MSAS

Various concentrations of each haloacetyl-CoA were incubated with 6-MSAS (250μl of a 0.56mg/ml solution; 0.73μM) in 100mM potassium phosphate buffer, pH 7.6, at 25°C. At various time intervals, 60μl aliquots were removed and assayed for 6-MSAS activity under the same conditions as described in 4.3.13.

4.3.15 The effect of acetyl-CoA and malonyl-CoA on inactivation by haloacetyl-CoA compounds

To determine the effect of the enzyme substrates upon inactivation of 6-MSAS with each haloacetyl-CoA compound, similar procedures to 4.3.14 were used except that
the enzyme was pre-incubated with the acyl-CoA derivative (final concentration 0.5mM) prior to the addition of the inhibitor.

4.4 Results

4.4.1 The synthesis and purification of haloacetyl-CoA derivatives

Each haloacetyl-CoA derivative was synthesised from its corresponding haloacetyl chloride. Although chloro- and bromoacetyl chloride were readily available chemicals, fluoroacetyl chloride was not, and therefore was synthesised within the laboratory. Fluoroacetyl chloride was analysed by $^1$H-NMR to establish that the correct compound had been synthesised. The presence of a doublet at $\delta = 5.1$ ppm with $J = 47.16$ Hz arises due to the spin-spin coupling exerted by F ($I = \frac{1}{2}$) upon the two adjacent hydrogen atoms. This indicates that the correct compound had been made (figure 4.4).

The haloacetyl-CoA derivatives were purified by HPLC. A general rule in reverse phase chromatography is that the more hydrophobic the sample the greater the retention time exhibited by that sample. This appears to be true in the case of the haloacetyl-CoAs under investigation. The retention times of each derivative using a C$_{18}$ analytical column and a C$_{18}$ preparative column are shown in table 4.1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>C$_{18}$ analytical column retention time (mins)</th>
<th>C$_{18}$ preparative column retention time (mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoA</td>
<td>12.28</td>
<td>13.21</td>
</tr>
<tr>
<td>Fluoroacetyl-CoA</td>
<td>13.27</td>
<td>14.90</td>
</tr>
<tr>
<td>Chloroacetyl-CoA</td>
<td>14.10</td>
<td>15.54</td>
</tr>
<tr>
<td>Bromoacetyl-CoA</td>
<td>14.51</td>
<td>16.19</td>
</tr>
</tbody>
</table>

Table 4.1. Retention times of haloacetyl-CoA derivatives.

Similar retention times were observed using the C$_{18}$ preparative column although each compound eluted over a longer period (~ 1 min) than that observed using the analytical column (15 secs), as expected.
Figure 4.4. $^1$H-N.M.R spectrum of laboratory synthesised fluoroacetyl-chloride. The presence of a doublet at $\delta = 5.1$ ppm with $J = 47.16$ Hz arises due to the spin-spin coupling exerted by F ($I = \frac{1}{2}$) upon the two adjacent hydrogen atoms. The singlet at $\delta = 7.3$ ppm corresponds to dichloromethane (used for calibration).
4.4.2 Nanospray mass spectrometric analysis of haloacetyl-CoAs

Mass spectrometric analysis of the purified haloacetyl-CoA samples confirmed that they had been successfully made. Fluoroacetyl-CoA (FAcCoA) was shown to have a Mr of 826.24 (figure 4.5b). A smaller peak at Mr 766.24 on the same spectrum corresponded to CoA indicating that FAcCoA is unstable and undergoes hydrolysis under the mass spectrometric conditions used. However, there were the expected two mass peaks for both chloroacetyl-CoA (ClAcCoA) and bromoacetyl-CoA (BrAcCoA). For ClAcCoA, the mass peaks at Mr 842.17 and Mr 844.27 were present in approximately a 3:1 ratio (figure 4.6a). This reflects the isotopic abundance of Cl which has isotopes of Mr 35.0 and Mr 37.0 in approximately a 3:1 ratio. For BrAcCoA, the mass peaks at Mr 886.10 and Mr 888.10 were present in approximately a 1:1 ratio (figure 4.6b). This reflects the isotopic abundance of Br which has isotopes of Mr 79.0 and Mr 81.0 in approximately a 1:1 ratio. Also shown is the mass spectral data for coenzyme-A (figure 4.5a). The small envelope of peaks above Mr 766.24 indicate reflects the presence of lithium from the original CoA that was supplied by Sigma as a tri-lithium salt.

4.4.3 Stability of haloacetyl-CoA compounds

Each of the chemically synthesised haloacetyl-CoA compounds was monitored for signs of degradation over time. HPLC analysis revealed that ClAcCoA and BrAcCoA were stable under assay conditions over a 4 hour period. However, FAcCoA was found to degrade partially after ~ 3 hours in both analytical grade water and 100mM KH$_2$PO$_4$ buffer, pH 7.6. This was characterised by the emergence of a peak at a retention time of ~ 12.2 minutes which corresponded to CoA thereby indicating that FAcCoA had undergone hydrolysis. All experiments carried out using FAcCoA were performed within the time before significant degradation had occurred.

4.4.4 Cross-linking and SDS-PAGE analysis of 6-MSAS pre-incubated with haloacetyl-CoAs prior to incubation with 1,3-dibromopropan-2-one

The samples from 4.3.9 were analysed by 5% SDS-PAGE (figure 4.7). Enzyme that had only been incubated with DBP was shown to be represented by a number of bands other than the native subunit molecular weight (lane 2). The additional bands reflected
Figure 4.5a. Nanospray mass spectral analysis of coenzyme-A. b. Nanospray mass spectral analysis of fluoroacetyl-CoA.
Figure 4.6a. Nanospray mass spectral analysis of chloroacetyl-CoA. b. Nanospray mass spectral analysis of bromoacetyl-CoA.
Figure 4.7. 5% SDS PAGE gel showing the cross-linking pattern of 6-MSAS with DBP and the inhibition of this after pre-incubation with haloacetyl-CoA analogues. Lanes 1 and 7, molecular weight markers (Myosin, 200kDa; β-galactosidase, 116.3kDa; Phosphorylase B, 97.4kDa). Lane 2, 6-MSAS cross-linked with 1,3-dibromopropan-2-one in a 1:4 ratio. 6-MSAS was subjected to cross-linking with 1,3-dibromopropan-2-one in a 1:4 ratio after incubation with: acetyl-CoA (Lane 3); fluoroacetyl-CoA (Lane 4); chloroacetyl-CoA (Lane 5); bromoacetyl-CoA (Lane 6).
the presence of cross-linked subunits that have a higher molecular weight and therefore do not progress as far on the gel. Pre-incubation with acetyl-CoA was shown to protect the enzyme from being modified by DBP (lane 3). Acetyl-CoA is transferred onto the β-ketoacyl synthase (KS) thiol thereby blocking the reaction of DBP. Each of the haloacetyl-CoAs was also shown to protect the enzyme from cross-linking with DBP (lanes 4, 5 and 6). However, these results alone do not indicate to which of the two active site thiols of 6-MSAS the haloacetyl-CoAs are attached, in order to prevent cross-linking. The thiol group that the haloacetyl-CoAs are attached was determined by performing a similar protection experiment, except that the KS thiol specific alkylating reagent iodoacetamide was used instead of DBP.

4.4.5 Liquid scintillation counting of 6-MSAS samples pre-incubated with haloacetyl-CoAs prior to labelling with $^{14}$C-iodoacetamide

The 6-MSAS control that was not pre-incubated with any haloacetyl-CoA prior to incubation with $[^{14}\text{C}]$-iodoacetamide was shown to have a total $[^{14}\text{C}]$ radioactive count of 16035 dpm (table 4.2). This indicates that $[^{14}\text{C}]$-iodoacetamide has reacted with the enzyme. Iodoacetamide has been previously shown to react with the substrate binding cysteine of the KS domain of vertebrate fatty acid synthases (Oesterhelt et al., 1977) and 6-MSAS (Child et al., 1996). This reflects the high level of similarity between the condensing sites of vertebrate FASs and 6-MSAS. Further confirmation that iodoacetamide is specific for the KS Cys-204 residue is observed for the control where 6-MSAS has been pre-incubated with acetyl-CoA prior to the addition of iodoacetamide. The $[^{14}\text{C}]$ radioactive count for this reaction was measured at 1515 dpm, less than 10% of the control. This low count indicates that acetyl-CoA reacts with the KS and therefore prevents iodoacetamide from doing so. The pre-incubations containing FAcCoA, ClAcCoA and BrAcCoA gave $[^{14}\text{C}]$ count readings of 7703 dpm, 2798 dpm and 518 dpm respectively (table 4.2). Although BrAcCoA was able to react with the KS and prevent iodoacetamide from modifying the enzyme, the results obtained for ClAcCoA and FAcCoA were not so conclusive. ClAcCoA was shown to protect 6-MSAS from modification with $[^{14}\text{C}]$-iodoacetamide to a high level but not as well as AcCoA. FAcCoA was only able to protect ~52% of the 6-MSAS KS thiols
<table>
<thead>
<tr>
<th>Analogue CoA ester</th>
<th>$[^{14}C]$-iodoacetamide radioactive counts (dpm)</th>
<th>Percentage protection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no CoA ester)</td>
<td>16035</td>
<td>0</td>
</tr>
<tr>
<td>Acetyl-CoA</td>
<td>1515</td>
<td>90.56</td>
</tr>
<tr>
<td>Fluoroacetyl-CoA</td>
<td>7703</td>
<td>51.96</td>
</tr>
<tr>
<td>Chloroacetyl-CoA</td>
<td>2798</td>
<td>82.55</td>
</tr>
<tr>
<td>Bromoacetyl-CoA</td>
<td>518</td>
<td>96.77</td>
</tr>
</tbody>
</table>

**Table 4.2.** Radioactivity (dpm) incorporated into 6-MSAS samples that have been pre-incubated with acetyl-CoA and haloacetyl-CoAs prior to incubation with $[^{14}C]$-iodoacetamide. A 6-MSAS control that was not pre-incubated with a CoA ester analogue prior to incubation with $[^{14}C]$-iodoacetamide is also shown. The background radioactive count was measured at 36 dpm.
from modification with iodoacetamide. The implications of these results are discussed in section 4.5.

4.4.6 Incubation of haloacetyl-CoA derivatives with 6-MSAS to synthesise 6-halomethylsalicylic acids and triacetic acid halolactones
6-MSAS has previously been shown to have a broad starter substrate specificity leading to the biosynthesis of a number of 6-alkylsalicylic acid and triketide lactone analogues (Campuzano et al., 1998). It was therefore not unreasonable to expect the broad substrate specificity to extend to halogenated acetyl-CoA compounds. However, the data obtained from the HPLC analysis of the reactions described in 4.3.12 showed no signs of formation of any 6-halomethylsalicylic acids or triacetic acid halolactones. Therefore, the possibility of inactivation / inhibition of 6-MSAS by haloacetyl-CoAs was investigated.

4.4.7 Inactivation of 6-MSAS on incubation with haloacetyl-CoA derivatives
Each haloacetyl-CoA derivative was incubated separately with 6-MSAS as described in section 4.3.13. Excess substrate was then removed via a PD-10 gel filtration column. Those fractions containing enzyme were assayed and found to possess no 6-MSAS activity. A control containing 6-MSAS without haloacetyl-CoA was run in parallel and when assayed, produced a rate of 5096 units / min. The results therefore indicate that 6-MSAS is irreversibly inactivated by each of the haloacetyl-CoA derivatives.

4.4.8 Reaction of haloacetyl-CoA compounds with 6-MSAS
Having established that the haloacetyl-CoAs were able to inactivate 6-MSAS it was necessary to determine how different concentrations of these compounds would affect the activity of 6-MSAS. For all time course assays, inactivation of 6-MSAS occurred within twenty seconds. An analysis of the results obtained from the time course assays for FAcCoA (figure 4.8a), ClAcCoA (figure 4.9a) and BrAcCoA (figure 4.10a) reveal that ClAcCoA is the most potent inactivator. Both FAcCoA and BrAcCoA yielded similar results and are an order of magnitude less potent than ClAcCoA. For each haloacetyl-CoA derivative a secondary plot of initial rate of inactivation versus concentration of inhibitor was calculated (figures 4.8b, 4.9b and 4.10b). A line of best
Figure 4.8a. Time-course of inactivation of 6-MSAS (0.72 μM) with different concentrations of FAcCoA: 0.5 μM (--•--), 1.0 μM (---), 2.5 μM (—•—), 25 μM (—•—). A control containing 6-MSAS (0.73 μM) without FAcCoA is also shown (---). b. Secondary plot of initial rate of inactivation (enzyme units) versus concentration of FAcCoA (μM).
Figure 4.9a. Time-course of inactivation of 6-MSAS (0.73 μM) with different concentrations of ClAcCoA: 0.1 μM (■), 0.25 μM (▲), 0.5 μM (●), 2.5 μM (○). A control containing 6-MSAS (0.73 μM) without ClAcCoA is also shown (●). b. Secondary plot of initial rate of inactivation (enzyme units) versus concentration of ClAcCoA (μM).
Figure 4.10a. Time-course of inactivation of 6-MSAS (0.73 μM) with different concentrations of BrAcCoA: 1 μM (■), 2.5 μM (▲), 5.0 μM (●), 10μM (●). A control containing 6-MSAS (0.73 μM) without BrAcCoA is also shown (■). b. Secondary plot of initial rate of inactivation (enzyme units) versus concentration of BrAcCoA (μM).
fit was determined for each plot and the constant k (mU/μM) was obtained from each gradient. However, for each secondary plot the data point corresponding to the highest concentration of inhibitor was not found to reside on the line of best fit. The reasons for this could be due to the inaccuracy in calculating the very rapid initial rate of inactivation and that the highest concentrations used were not limiting such that a lower concentration would have given a similar initial rate. The k values of FAcCoA, ClAcCoA and BrAcCoA were 0.46, 5.70 and 0.58 (mU/μM) respectively. The interpretation of these results is discussed in section 4.5.

4.4.9 The effect of acetyl-CoA and malonyl-CoA on inactivation by haloacetyl-CoA compounds

From the experiments performed it would appear that malonyl-CoA and acetyl-CoA protect 6-MSAS from inactivation with haloacetyl-CoAs. For both the ClAcCoA (figure 4.12) and BrAcCoA (figure 4.13) reactions, protection of 6-MSAS by malonyl-CoA was marginally greater than that of acetyl-CoA yet for FAcCoA (figure 4.11) the opposite was observed. However, the protection experiments cannot unambiguously distinguish whether the ACP 4'-phosphopantetheine thiol or the KS thiol is being modified. This is because the decarboxylase activity of 6-MSAS (chapter 3) implies that malonyl-CoA will not just protect the pantetheine thiol, but because having undergone decarboxylation to acetyl-CoA it will also protect the KS thiol.

4.5 Discussion

The protection of 6-MSAS from alkylation with iodoacetamide, by pre-incubating the enzyme with haloacetyl-CoAs has shown that the KS reactive thiol is protected in the order Br > Cl > F. The three haloacetyl-CoA derivatives were also shown to completely protect 6-MSAS from cross-linking with DBP. Therefore, it is possible that FAcCoA, and to a lesser extent ClAcCoA, may protect a region of the condensing site other than the KS thiol, from cross-linking with DBP. Previous research has shown that ClAcCoA protects the ACP pantetheine thiol of rabbit FAS. (McCarthy and Hardie, 1982) (figure 4.2). In a similar fashion FAcCoA and ClAcCoA may protect the ACP pantetheine thiol of 6-MSAS. However, the question that needs to be answered is why is the KS thiol protected in the order Br > Cl > F. This can be explained on the
Figure 4.11. Time-course of inactivation of 6-MSAS (0.73 μM) by FAcCoA (2.5 μM) (●) and studies of substrate prevention of FAcCoA (2.5 μM) as follows: no treatment (○) and treatment with FAcCoA following incubation with acetyl-CoA (0.5 mM) (■) or malonyl-CoA (0.5 mM) (▲).
Figure 4.12. Time-course of inactivation of 6-MSAS (0.73 μM) by ClAcCoA (0.5 μM) and studies of substrate prevention of ClAcCoA (0.5 μM) as follows: no treatment (○-) and treatment with ClAcCoA following incubation with acetyl-CoA (0.5 mM) (■-) or malonyl-CoA (0.5 mM) (▲-).
Figure 4.13. Time-course of inactivation of 6-MSAS (0.73 μM) by BrAcCoA (5 μM) (-●-) and studies of substrate prevention of BrAcCoA (5 μM) as follows: no treatment (-♦-) and treatment with BrAcCoA following incubation with acetyl-CoA (0.5 mM) (-■-) or malonyl-CoA (0.5 mM) (-▲-).
assumption that, similar to acetyl-CoA, haloacetyl-CoA derivatives are first transferred to the KS thiol by the acyl transferase component and are then transferred to the ACP thiol. The transfer of the derivatives from the KS to the ACP thiol may be a sterically hindered process regulated by the size of the halogen. Therefore transfer of BrAcCoA from KS to ACP may not be possible due to the larger atomic size of bromine.

Kinetic experiments have shown that fluoro-, chloro-, and bromoacetyl-CoA inactivate 6-MSAS. The haloacetyl-CoA derivatives are thought to alkylate the enzyme, resulting in the elimination of the halogen atom. The site of alkylation could be the active site thiol of the KS (Cys-204) or the thiol of the ACP pantetheine arm. However, a primary amine group of a conserved lysine residue (Lys-374) could also be a site of alkylation. Previous experiments have shown that o-phthalaldehyde reacts with 6-MSAS to produce a thiosionisdole ring formed between the amine group of Lys-374 and a thiol group of either the KS or the ACP pantetheine arm (Child et al., 1996). Therefore it is possible that the haloacetyl-CoAs may alkylate the lysine residue whilst attached to either of the two reactive thiols (mechanism 2, figure 4.14).

A comparison of the k values obtained from 4.4.8 show that the most effective inactivating reagent is ClAcCoA and that FAcCoA and BrAcCoA are of similar potency (figure 4.15). This result was not anticipated since bromine is the best leaving group and hence BrAcCoA was expected to be the most effective reagent. An explanation for this result may be that bromine may partially obstruct the entry of BrAcCoA into the condensing site of 6-MSAS due to steric hindrance. The reason why FAcCoA is less effective as an inactivating reagent than ClAcCoA is that FAcCoA may undergo rapid hydrolysis when attached to the active serine of the acyl transferase domain and may not be loaded onto the KS thiol (mechanism 1, figure 4.14). Afterwards there is also the possibility that hydrolysis may occur after the haloacetyl group is bound to the KS thiol. Although all of the haloacetyl-CoAs were shown to be stable in potassium phosphate buffer for several hours, when bound to enzyme they may become more susceptible to hydrolysis.
Figure 4.14. Two possible pathways for the incorporation of haloacetyl-CoA derivatives with 6-MSAS. The derivatives may undergo hydrolysis when attached to the active serine of the acyl transferase domain (mechanism 1) or may inactivate the enzyme by alkylating a conserved lysine within the condensing site of 6-MSAS (mechanism 2). X = F, Cl or Br.
Figure 4.15. A comparison of the $k$ (mU/μM) values of fluoro-, chloro- and bromoacetyl-CoA as calculated from figures 4.8b, 4.9b and 4.10b.
It is also possible that in the presence of haloacetyl-CoA and malonyl-CoA, 6-MSAS may generate enzyme bound halo-intermediates that are unable to detach from the enzyme. The structures of these intermediates and a possible mode of inactivation for each are shown in figure 4.16. The C2 and C4 intermediates may inactivate 6-MSAS by alkylation of the amine group of Lys-374 whilst bound to either the KS thiol (Cys-204) or the ACP pantetheine arm thiol (figure 4.16). It is also possible that either of the condensing site thiol groups may be alkylated by the intermediates. However, if C6 intermediates were formed then they would be released as triacetic acid halolactones in the absence of NADPH. Since no triacetic acid halolactones were detected by HPLC then it is unlikely that any halo-derived C6 intermediates are produced.

The inactivation of 6-MSAS by the action of haloacetyl-CoAs further highlights the similarity with type I mammalian FASs. However, the amino acid residue(s) that is alkylated by the haloacetyl-CoA derivatives was not identified. A method to achieve this would be to synthesise $[^{14}C]$-haloacetyl-CoA, incubate the radiolabel derivative with 6-MSAS and perform a limited protease digest. The radiolabelled peptides could be separated by gel electrophoresis and subjected to N-terminal sequencing. Unfortunately attempts at synthesising $[2-{^{14}C}]$-bromoacetyl-CoA from $[2-{^{14}C}]$-bromoacetylchloride failed and the experiment described above could not be performed. It was suspected that the purchased $[2-{^{14}C}]$-bromoacetylchloride was not the correct compound or that it was highly unstable and, as a result, became hydrolysed before use.
Figure 4.16. Hypothetical enzyme-bound intermediates formed upon inactivation of 6-MSAS by haloacetyl-CoA derivatives. X = F, Cl or Br.
Chapter 5

Investigations with 6-methylsalicylic acid synthase using N-acetylcysteamine substrates and intermediates

5.1 Introduction

For an enzyme that catalyses a multistep reaction with all the intermediates enzyme-bound, as does 6-MSAS, it is important to establish the precise order of the events and the identity of intermediates from the initial binding of the substrates to the final release of product. Several methods may be used to determine the nature of the intermediates: feeding potential intermediates to the enzyme and observing the progress to product; trapping intermediates by the use of inhibitors that affect individual stages; using substrate analogues and observing the formation of any intermediate or product analogues; direct observation of enzyme bound intermediates at low temperature by NMR; observing enzyme intermediate species by mass spectroscopy etc. The choice of methods clearly depends on the nature of the reaction, the type of intermediates involved and the molecular properties of the enzyme. In the case of 6-MSAS, the trapping of thiolester intermediates is clearly not without difficulty although the possibility of carrying out reduction of any intermediates to release the equivalent alcohol intermediates is possible. The use of substrate analogues, although a useful approach, always leaves a doubt that the reaction pathway of the analogue may be different from that of the true substrate. For an enzyme of the size of 6-MSAS, direct observation by NMR is unlikely to be feasible and, similarly, the use of mass spectrometry is exceptionally challenging with the current technology available. The method used in this study, therefore, relied on the synthesis of putative enzyme intermediates as thiolesters with the expectation that the enzyme would recognise these and complete their transformation selectively into 6-MSA. Since there are substantial difficulties in making CoA-thiolesters of potential intermediates, the strategy was to prepare N-acetylcysteamine (NAC) derivatives instead. These have been shown on previous occasions to be accepted by enzymes that normally utilise CoA thiol esters as substrates and, in particular, have been exploited in fatty acid and polyketide synthases. For instance S-acetyl-NAC has been shown to protect pigeon liver fatty acid synthase against inactivation by phenylmethylsulfonyl fluoride (Kumar, 1975). Recently a number of research papers
have been published concerning the interaction of NAC substrate analogues with the modular polyketide synthase 6-deoxyerythronolide-B synthase. Methylmalonyl-NAC and various diketide NAC analogues have been shown to be effective substitutes for their CoA counterparts in incubations with DEBS1 + TE (Chuck et al., 1997; Pohl et al., 1998; Holzbaur et al., 1999). NAC substrate analogues have also been used to analyse the stereospecificity of the TE component of 6-DEBS (Gokhale et al., 1999).

5.2 Materials

6-Methylsalicylic acid synthase was purified from *Penicillium patulum* NRRL 2159A as described in chapter 2. Succinyl-CoA transferase was purified from porcine heart as described in chapter 2. Acetyl-CoA, malonyl-CoA, coenzyme-A, succinic anhydride and NADPH were purchased from Sigma. Acetyl-NAC was purchased from Aldrich. [2-13C] Malonic acid (99% atom [13C]) and acetic anhydride were obtained from Aldrich. 1,3-Dibromopropan-2-one (DBP) was purchased from Lancaster Chemicals Ltd. HPLC grade H2O and ammonium hydroxide were supplied by BDH. HPLC grade acetonitrile was obtained from Fisher Scientific. Trifluoroacetic acid was obtained from Applied Biosystems Division. An analytical C18 reverse phase column (250mm x 4.6mm; 5μM) was supplied by Spherisorb. HPLC buffer A contained H2O + 0.1% (v/v) TFA. HPLC buffer B contained acetonitrile + 0.1% (v/v) TFA. Borosilicate nanoflow tips for ESMS were obtained from Micromass. 6-MSAS intermediate NAC analogues were synthesised in the laboratory by Dr K-M. Cheung.

5.3 Methods

5.3.1 FPLC purification of 6-MSAS

Homogenous 6-MSAS was obtained by elution from a Mono Q™ anion exchange FPLC column as described in chapter 2.

5.3.2 Fluorimetric assay

The conditions used for incubations with the enzyme and other necessary substrates, such as malonyl-CoA or NADPH are described in the relevant legends. The increase in fluorescence associated with 6-MSA formation was measured at 390nm. The extent of fluorescence change is related to that obtained with a standard solution of 6-MSA.
5.3.3 Cross-linking with 1,3-dibromopropan-2-one after pre-incubation of 6-methylosalicylic acid with the 3R and 3S-hydroxytriketide-NAC

6-MSAS (140μl of a 0.1mg/ml solution; 0.13μM) in 100mM potassium phosphate buffer, pH 7.6, was pre-incubated with either the 3R or 3S-hydroxytriketide-NAC (figure 5.1c and d) (final concentration 0.67mM) for five minutes at 25°C. A control containing 6-MSAS, previously pre-incubated with acetyl-CoA (final concentration 0.67mM) and a control containing 6-MSAS without any substrate, were run in parallel. 1,3-Dibromopropan-2-one (DBF) (0.52μM; ~ 4 moles of DBF; 1 mole of 6-MSAS tetramer) was added to each of the reactions, which were then allowed to proceed for 15 minutes at 25°C. Each reaction was terminated by the addition of an equal volume of SDS electrophoresis disruption buffer. The extent of cross-linking within each reaction was observed by SDS-PAGE.

5.3.4 Use of [2-13C]-malonyl-CoA to investigate incorporation of 3R and 3S-hydroxytriketide-NAC

The production of [13C]-6-MSA from 3R and 3S-hydroxytriketide-NAC was attempted as follows; a 1.9ml Eppendorf tube containing 10μl of 10mM succinyl-CoA, 10μl of succinyl-CoA transferase (0.73 units), 3.3 mU of 6-MSAS, 10μl of 20mM NADPH, 10μl of 10mM 3R or 3S hydroxytriketide-NAC, 10μl of 3M [13C]-malonate, pH 7.0, and 100μl of 100mM potassium phosphate buffer, pH 7.6, was prepared. The reactions (200μl) were terminated after 2 hours by the addition of 20μl of 50% (w/v) TCA. The samples were microcentrifuged for 5 minutes to remove precipitated protein. ES-MS and CID-MS were performed on the samples under the same conditions as described in chapter three, part I.

5.4 Results

All chemical syntheses were performed by Dr K-M Cheung in this laboratory. The structures of the polyketide derivatives are shown in figure 5.1. The conditions used for incubations with the enzyme and other necessary substrates, such as malonyl-CoA or NADPH are described in the relevant legends.
Figure 5.1 The structures of N-acetyl cysteamine analogues that are potential intermediates in the 6-MSAS pathway.
5.4.1 Incubations with acetyl-SNAC and malonyl-SNAC

Both acetyl-SNAC and malonyl-SNAC were found to be incorporated into 6-MSA in the presence of NADPH at rates 5.8% and 1.1%, respectively, compared to rates for the natural substrates, acetyl-CoA and malonyl-CoA, establishing the feasibility of using NAC derivatives in place of the natural substrates for studies with 6-MSAS. These results also highlight the importance of coenzyme-A in catalysis. Indeed the relative contributions of binding energy made by sections of the coenzyme-A molecule to catalysis by succinyl-CoA transferase have been determined (Whitty et al., 1995). It was concluded that active site binding of the α, β, and γ carbon atoms of the pantoic acid domain of coenzyme-A significantly destabilise the enzyme-coenzyme-A thiolester, enhancing its reactivity towards acetoacetate or succinate by a factor of 10^7. Similar interactions between 6-MSAS and coenzyme-A may be required to enhance the reactivity of the synthase towards acetyl-CoA and malonyl-CoA.

5.4.2 Acetoacetyl-NAC

The incorporation of acetoacetyl-NAC (figure 5.1a) with 6-MSAS in the presence of [2-14C]-malonyl-CoA was previously shown to be successful, resulting in the synthesis of [14C]-6-MSA (Bhogal thesis, 1995). The conclusion made was that the [14C]-6-MSA had originated from the successive condensations of one acetoacetyl-NAC unit with two [2-14C]-malonyl-CoA units. However, since this experiment was performed, 6-MSAS has been shown to possess a malonyl-CoA decarboxylase activity (chapter 3). Therefore it was possible that [14C]-6-MSA had been derived solely from [2-14C]-malonyl-CoA under the experimental conditions used. Kinetic assays revealed that in the presence of acetoacetyl-NAC, malonyl-CoA and NADPH, 6-MSA was synthesised at a rate of ~3% that of the rate observed with natural substrates (Bhogal thesis, 1995). This rate was interpreted as an indication that acetoacetyl-NAC had been successfully incorporated by the enzyme. However, this rate is in fact below that observed for the malonyl-CoA decarboxylase activity which is ~5% of the native rate. Therefore it is possible that acetoacetyl-NAC inhibits the malonyl-CoA decarboxylase activity and is not an enzyme substrate as previously thought.
The kinetic experiments performed previously with acetoacetyl-NAC (Bhogal thesis, 1995) were repeated to confirm that the compound was an inhibitor of 6-MSAS (figure 5.2). Acetoacetyl-NAC (0.01mM) was incubated with 6-MSAS and then assayed immediately. The rate of 6-MSA formation (~20 fluorescence units/min) was lower than that observed for the decarboxylase activity (~80 fluorescence units/min). A higher concentration of acetoacetyl-NAC (0.1mM) was found to completely inhibit 6-MSA production. Therefore any 6-MSA produced in the presence of acetoacetyl-NAC is likely to have arisen from the decarboxylation of malonyl-CoA to give the enzyme-bound starter acetyl-moiety that reacts subsequently with 3 malonyl-CoA units in the normal way. A control assay in which acetoacetyl-NAC was replaced by acetoacetyl-CoA (0.1mM) produced a rate of 6-MSA formation of ~140 fluorescence units/min. However both acetoacetyl derivatives are poor substrates compared with the natural substrates (~800 fluorescence units/min). The inability of acetoacetyl-NAC to incorporate its diketide moiety significantly into 6-MSA was disappointing and did not bode well for the use of more advanced triketide intermediates described in the next section.

5.4.3 C6-triketide-SNAC
The synthesis of the triketide-NAC derivative was successful after many attempts, the most difficult aspect being to prevent the compound cyclising non-enzymically to TAL. Incubation of the triketide-SNAC with 6-MSA synthase, again, was disappointing, with no evidence for any enzyme catalysed formation of 6-MSA in the presence of malonyl-CoA and NADPH. The formation of TAL, however, was observed but since the control without enzyme also produced TAL there was little evidence for the involvement of the enzyme in this reaction.

5.4.4 3R and 3S-hydroxytriketide-NAC
If acetyl-CoA and malonyl-CoA are incubated together with 6-MSA synthase in the absence of NADPH, there is a release of triacetic acid lactone from the enzyme. This observation, together with other considerations, demands that the NADPH dependent reduction occurs at the triketide stage. There are two possible 3-hydroxytriketide isomers, one with a 3R and the other with a 3S configuration. Previous studies on the stereochemistry of the 6-MSAS reaction, carried out in this laboratory (Spencer and Jordan, 1991) have established that the two hydrogen atoms at the C2 position of the
Figure 5.2 The effect of acetoacetyl-CoA and acetoacetyl-NAC upon the rate of 6-MSA synthesis. In a final volume of 60μl, 6-MSAS (50 μl of a 0.53mg/ml solution; 0.70μM) in potassium phosphate buffer, pH 7.6 (0.1M), was incubated, separately, with the following: acetyl-CoA (0.1mM); acetoacetyl-CoA (0.1mM); no other substrate (decarboxylase rate); acetoacetyl-NAC (0.01mM); acetoacetyl-NAC (0.1mM). Immediately each incubation was added to the assay that in a 1ml final volume contained, BSA (1.25mg) NADPH (0.2mM) and malonyl-CoA (0.2mM). For each reaction the formation of 6-MSA was observed at 390nm over a period of five minutes.
malonyl-moiety are handled in a stereospecific manner during the formation of 6-MSA. The stereochemical argument required that in order to form a 3,4-cis-double bond and to eliminate the pro-R hydrogen atom from the C4 position of the intermediate, the preferred 3-hydroxy-intermediate would have the 3R configuration. In order to test this hypothesis, the 3R- and 3S-hydroxytriketide-NAC derivatives were synthesised separately over a period extending over nearly 10 years. They were characterised unambiguously by a combination of NMR, mass spectrometry and X-ray analysis of the chemical intermediates.

Attempts were made to incorporate either the 3R or 3S compound into 6-MSA together with malonyl-CoA, either in the presence or absence of NADPH. In the presence of NADPH and at low concentrations (2.5μM and 25μM) of either triketide derivative, 6-MSA formation was observed (figure 5.3). At a higher concentration (0.25mM) of either derivative 6-MSA synthesis was virtually zero. It should be noted that in actuality, NADPH would not be required for the reduction, since the 3-hydroxytriketide compounds are intermediates subsequent to the reductive step. The coenzymes were used however in case their presence was necessary for the conformational integrity of the enzyme. In the absence of NADPH, 6-MSA formation was not observed with either the 3R or 3S-hydroxytriketide-NAC. These results suggest that 6-MSAS did not accept the triketide derivatives as substrates but as inhibitors. Also from the results obtained in the presence of NADPH, 6-MSAS appeared to be unable to distinguish between the 3R and 3S derivatives. Furthermore, the data obtained from the HPLC and mass spectrometric analysis of the reactions described in 5.3.4 showed no signs of formation of any [13C]-labelled 6-MSA arising from the incorporation of the 3R and 3S derivatives with 6-MSAS.

The inability of the enzyme to accept either the 3R or the 3S derivative as a substrate may stem from one, or a combination, of reasons. The NAC moiety may not be well recognised by the enzyme, a possibility that is suggested from the above studies with acetyl-NAC, malonyl-NAC and acetoacetyl-NAC. Alternatively, the reduced triketide chain may not be recognised by the enzyme active site if the correct conformation of the intermediate can only be generated by synthesis of the polyketide in a sequential manner from the natural C2 and C3 substrates. Therefore the enzyme may stabilise
**Figure 5.3** The effect of 3R and 3S-hydroxytriketide-NAC upon the rate of 6-MSA synthesis. In a final volume of 60μl, 6-MSAS (50 μl of a 0.51mg/ml solution; 0.70μM) in potassium phosphate buffer, pH 7.6 (0.1M), was incubated, separately, with acetyl-CoA (0.1mM) (A) or, 3R or 3S-hydroxytriketide-NAC at concentrations of: 2.5μM (B), 25μM (C), 0.25mM (D). Immediately each incubation was added to the assay that in a 1ml final volume contained, BSA (1.25mg) NADPH (0.2mM) and malonyl-CoA (0.2mM). For each reaction the formation of 6-MSA was observed at 390nm over a period of five minutes.
the intermediate in an alternative form to that found in free solution - for instance, a specific enol or keto-form in a different conformation may only be recognised specifically by the active site. Furthermore, attempting to introduce an intermediate may not find the enzyme in the correct conformation, particularly if the exchange of thiolester bonds is necessary to drive protein conformational changes.

In an attempt to determine whether the $3R$ and $3S$ hydroxy compounds were binding to the relevant active site thiol group of the enzyme, they were independently incubated with the enzyme and the integrity of both the enzyme thiols were explored using the bifunctional cross-linking reagent 1,3-dibromopropan-2-one. DBF has been shown previously to cross-link the β-ketoacyl synthase and 4'-phosphopantetheine thiols of 6-MSAS (Child et al., 1996). In the presence of excess (0.67mM) $3R$ or $3S$-hydroxytriketide-NAC there was a partial inhibition of cross-linking, indicating that the compounds were interacting weakly with the enzyme (lanes 3 and 4, figure 5.4). However, the 6-MSAS control preincubated with 0.67mM acetyl-CoA also only partially protected the enzyme from cross-linking (lane 5, figure 5.4). This suggests that the inhibition of cross-linking by the NAC analogues may be stronger than indicated by SDS-PAGE. The cross-linking experiment was performed numerous times but it was not possible to improve on the results obtained. Furthermore, indication that one of the isomers was more specific for the enzyme was not evident. The negative results, while disappointing, do nevertheless indicate the exceptional specificity of the enzyme for its substrates and suggest further that the enzyme may prefer to synthesise the polyketide de novo rather than accept a preformed intermediate polyketide chain.

### 5.4.5 Cis and trans 2- and 3-enoyl-NAC triketide derivatives

Attempts are under way to synthesise all four of the possible enoyl intermediates, namely the 2-cis and 2-trans isomers and the 3-cis and 3-cis isomers (figure 5.1e, f, g and h). Despite the lack of success with the $3R$ and $3S$-hydroxytriketide isomers, the chemical challenges themselves are enough to justify attempts to synthesise these compounds and it is remotely possible that the reductase and “cyclase” may be able to utilise them as substrates.
Figure 5.4 5% SDS PAGE gel showing the cross-linking pattern of 6-MSAS with DBP after pre-incubation with C6 hydroxy NAC derivatives. Lanes 1 and 6, molecular weight markers (myosin, 200kDa; β-galactosidase, 116.3kDa; phosphorylase B, 97.4kDa; serum albumin, 66.2kDa). Lane 2, 6-MSAS cross-linked with 1,3, dibromopropan-2-one in a 1:4 ratio. 6-MSAS was subjected to cross-linking with 1,3, dibromopropan-2-one in a 1:4 ratio after incubation with: 3R-hydroxytriketide-NAC (lane 3); 3S-hydroxytriketide-NAC (lane 4); acetyl-CoA (lane 5).
5.4.6 C8 Tetraketide derivative

It was envisaged that the proposed tetraketide intermediate would be too unstable to investigate since the tetraketide, as the free acid, rapidly cyclises non-enzymically to orsellinic acid, a more oxidised product than 6-MSA. As the thiol ester is likely to be far less stable than the free acid, no attempts were made to make the NAC-derivative. Furthermore, judging by the properties of the unreduced C6 triketide-NAC intermediate, it would be unlikely if it would survive long enough to test rigorously.

5.5 Summary

The results obtained so far suggest that 6-MSAS recognises N-acetyl cysteamine intermediates as inhibitors and not as substrates. This contrasts with research upon fatty acid and other polyketide synthases in which NAC derivatives have been incorporated into the final product by the enzyme. However, the recent discovery that some FASs and PKSs have been shown to possess a decarboxylase activity may jeopardise previous conclusions made that NAC analogues are substrates for these enzymes. For example the discovery of a malonyl-CoA decarboxylase activity for 6-MSAS compromises the preliminary observation that acetoacetyl-NAC is a substrate for the enzyme (Bhogal thesis, 1995). DEBS1 + TE has been shown to possess a methylmalonyl-CoA decarboxylase activity (Pieper et al., 1996). However, the presence of such an activity has not compromised the extensive research that has been performed on DEBS1 + TE with various NAC derivatives (Chuck et al., 1997; Pohl et al., 1998; Holzbaur et al., 1999; Gokhale et al., 1999).

There is the possibility that 6-MSAS may not be in the correct conformation to recognise the various NAC intermediates as substrates. It is interesting to compare the tetrapolymerisation reaction catalysed by 6-MSAS with that which is catalysed by porphobilinogen deaminase (1-hydroxymethylbilane synthase), an enzyme of the haem biosynthesis pathway. The latter enzyme will only accept the monopyrrole unit, porphobilinogen as a substrate, forming a covalent link with an active site group after deamination. All attempts to incorporate a dipyrromethane (dipyrrrole) or a tripyrrane (tripyrrrole) were unsuccessful, the reason being that sequential conformational changes to the enzyme were obligatory. Interestingly porphobilinogen deaminase recognised the aminomethylbilane (tetrapyrrole) as a pseudosubstrate and catalysed a slow deamination but no further addition of substrate. It is possible that 6-MSAS,
similar to porphobilinogen deaminase, may undergo sequential conformational changes during the formation of 6-MSA and will therefore not accept intermediates as substrates.
Chapter 6

The purification of 6-MSAS apo-ACP and *E.coli* holo-ACP synthase, and the partial characterisation and initial NMR studies of *E.coli* holo-ACP synthase

6.1 Introduction

6.1.1 Acyl carrier protein

Acyl carrier proteins (ACPs) play an important role in a number of biosynthetic pathways that are dependant upon acyl group transfers. ACPs are predominantly associated with the biosynthesis of fatty acids but are also utilised in the synthesis of polyketide antibiotics, non-ribosomal peptides and intermediates used in the biosynthesis of vitamins such as biotin. The ACP in each of these pathways is composed of 80-100 residues and is either an integrated domain in a larger multifunctional protein (type I) or is a structurally independent protein that is part of a non-aggregated multienzyme system (type II). Type I ACPs are found in mammals, fungi and certain mycobacteria, whereas type II ACPs are utilised by plants and the majority of bacteria.

ACPs require post-translational modification in which the inactive apo- form is converted into the active holo- form by the transfer of the 4'-phosphopantetheine (P-pant) moiety of coenzyme-A to a conserved serine residue of the apo-ACP (figure 6.1a). The β-hydroxyl sidechain of the serine residue serves as a nucleophilic group attacking the activated pyrophosphate linkage of CoA. The newly introduced thiol of the P-pant prosthetic group now acts as a nucleophile for acylation by a substrate, which may be acyl-CoA or malonyl-CoA derivatives for FASs and PKSs, or aminoacyl-AMPs for non-ribosomal peptide synthases (figure 6.1b).

The three-dimensional NMR solution structure of a *Streptomyces* type II PKS actinorhodin apo-ACP (Crump *et al.*, 1997) has shown that four α-helices are arranged so that they form a hydrophobic cleft, large enough to accommodate the P-pant prosthetic group as well as a substrate or intermediate acyl chain. More recently the crystallographic structure of the type II FAS holo-ACP from *Bacillus subtilis* has been solved (Parris *et al.*, 2000) (figure 6.2). The *B.subtilis* ACP has five α-helices, one more than the act ACP type II PKS, but four of these are arranged in a similar
Figure 6.1a General reaction scheme for post-translational phosphopantetheinylation. 4'-phosphopantetheinyl (P-pant) transferases transfer the 4'-phosphopantethine moiety from CoA to a conserved serine residue of apo-ACP to produce holo-ACP and 3',5'-ADP. b. The terminal cysteamine thiol of the phosphopantetheine cofactor acts as a nucleophile for acyl activation. For FASs and PKSs an acetyl-group is transferred from CoA to holo-ACP (path 1) to form acetyl holo-ACP. For non-ribosomal peptide synthases an aminoacyl group is transferred from aminoacyl AMP to holo-PCP to form aminoacyl holo-PCP (peptidyl carrier protein) (path 2).
Figure 6.2. Tertiary structure of holo-ACP from *B.subtilis* with the 4’-phosphopantetheine moiety (ball and stick) bound to Ser-36 of helix α3 (after Parris *et al.*, 2000).
fashion to form a hydrophobic cleft. The attachment of the P-pant group to the conserved serine residue (Ser-36) of helix α3 within the cleft is shown (figure 6.2). However, three-dimensional structures alone do not reveal how the P-pant group is loaded onto the active serine residue of an ACP.

6.1.2 4'-Phosphopantetheinyl transferases
Evidence suggests that each enzyme dependant upon 4'-phosphopantetheine (P-pant) attachment for activation has an "accomplice" enzyme, known as a P-pant transferase, responsible for this attachment (Lambalot and Walsh, 1996). The P-pant transferase superfamily can be roughly divided into two subgroups. The first of these are the enzymes responsible for modifying the peptidyl carrier protein (PCP) subunits of non-ribosomal peptide synthetases. The crystal structure of one such enzyme, the surfactin synthetase-activating (Sfp) enzyme from *Bacillus subtilis*, has been solved recently (Reuter et al., 1999). The enzyme, which functions as a dimer, transfers the P-pant moiety from CoA to a conserved serine residue of the PCP of surfactin synthetase (figure 6.1). The second subgroup comprises the acyl carrier protein synthases (ACPSs) and other enzymes that transfer the P-pant group onto the acyl carrier protein of FASs or PKSs. Both enzyme subgroups are magnesium ion dependant.

The sequence homology between the two subgroups is rather low (between 12 and 22%) and, whereas those enzymes that modify PCPs are typically ~230 amino acids in length, ACPSs are usually composed of 120 residues. Sequence alignment has revealed that the high level of conservation between ACPSs observed in two regions, residues 5-13 and 54-65 (*E.coli* ACPS numbering) (figure 6.3b), extends to both subgroups. Also there is a high level of structural homology between the Sfp enzyme and the ACPSs from *B.subtilis* (Parris et al., 2000) (figure 6.3a) and from *Streptococcus pneumoniae* (Chirgadze et al., 2000), both of whose structures have been solved recently.

6.1.3 Structural interactions between ACPS and ACP
The crystal structures of ACPS from *B.subtilis* (Parris et al., 2000) and *Streptococcus pneumoniae* (Chirgadze et al., 2000) and of the closely related Sfp enzyme from *B.subtilis* (Reuter et al., 1999) have revealed that two subunits are required to
Figure 6.3a. Tertiary structure of one ACPS molecule. b. Sequence alignment of twelve ACPS-type 4'-phosphopantetheine transferases. The invariant residues are shown in red. The secondary structure assignments for B. subtilis ACPS are shown above the sequences: α helices are shown as rectangles and β strands as arrows. The last 56 residues of Thermotoga maritima ACPS were omitted as they have no counterpart in the other enzymes. The SWISS-PROT accession numbers are given in the second column for each protein.
dimerise in order to form one active site. However, unlike the Sfp enzyme that exists as a dimer with one active site, both of the ACPS structures incorporate a third subunit to form trimers. The interaction of the three subunits allows the formation of three active sites and also permits a large hydrophobic surface from each ACPS molecule to be buried in the trimer. This oligomeric state is retained in the crystal structure of the ACPS / type II holo-ACP complex from *B. subtilis* (figure 6.4a). The contacts between holo-ACP and ACPS are predominantly hydrophilic in nature with almost all of the interactions occurring between helix \( \alpha_1 \) of ACPS and helix \( \alpha_3 \) of ACP (highlighted in figure 6.4a). A key residue in the binding of ACP to ACPS is a conserved arginine residue (Arg14) from *B. subtilis* ACPS. Arg14 forms a salt bridge with a conserved aspartate residue (Asp35) of ACP and is involved in hydrogen bonding with Asp38 of ACP. Both of these residues are close to the reactive serine residue (Ser36) that the P-pant moiety attaches to.

The requirement for two ACPS molecules to establish one active site has been shown by constructing a model based on the superimposition of the separate structures of ACPS-CoA and ACPS-ACP (figure 6.4b). A loop consisting of residues 64-78 of ACPS\#1 enlarges the active site by shifting 2Å to accommodate helix \( \alpha_4 \) from ACP. The model also shows that the \( \alpha_2 \) helix of ACP\#1 is directed at the phosphate of the CoA that is to be transferred to ACP\#1 and that three magnesium-bound water molecules are close to Ser36 of ACP\#1. Therefore this model can be used to determine a catalytic mechanism for P-pant transfer and activation of ACP. Firstly one of the metal bound water molecules is activated by the removal of a proton, either by another water molecule or by Asp35 of ACP. The activated water molecule removes the hydroxyl hydrogen from Ser36, which then carries out the nucleophilic attack on the \( \beta \)-phosphate of CoA. This results in the transfer of the P-pant group to Ser36 of ACP, and the resulting 3'5'-ADP is stabilised by interactions with magnesium and basic residues of ACPS.

6.1.4 The type I PKS 6-MSAS ACP and *E.coli* holo-ACP synthase

The following research describes the purification of the type I ACP component of 6-MSAS and of the *E.coli* holo-ACP synthase (ACPS). The attempted conversion of 6-MSAS apo-ACP to holo-ACP using *E.coli* ACPS is mentioned. The preliminary work
Figure 6.4a. Ribbon diagram showing ACP from *B. subtilis* bound in each active site of the ACPS trimer (*B. subtilis*) and the interaction between helix α3 of ACP and helix α1 of ACPS.

b. Model of interaction between ACPS (red and yellow), ACP (light blue), CoA (dark blue), Mg^{2+} (grey circle) and three water molecules (blue circle). The conformation of the 4'-phosphopantetheine group, after attachment to the ACPS, is shown as a thin grey chain.
required to solve the NMR solution structure of the *E.coli* ACPS and the first attempts at identifying the oligomeric state of an ACPS using mass spectrometry are also described.

6.2 Materials

N-Lauroyl sarcosine (sarkosyl), 2-[N-morpholino]ethane sulfonic acid (MES), glucose, CaCl₂, coenzyme-A, TEMED, ammonium persulphate and benzyolated dialysis tubing were supplied by Sigma. Disposable filter holders (0.2μM) were supplied by Schleider and Schuell. Dithiothreitol was purchased from Melford Laboratories. SP-Sepharose Fast Flow resin was supplied by Amersham Pharmacia. Yeast and tryptone were supplied by Difco Laboratories. NaCl, KH₂PO₄ and acetonitrile were from Fisher Scientific. Tris/HCl was supplied by ICN. NH₄Cl, analytical grade water and ammonium sulphate (analytical grade) was purchased from B.D.H. ¹⁵NH₄Cl was supplied by Goss Scientific Instruments. Diethylaminoethyl cellulose (DE-52) was purchased from Whatman. Vivaspin 20ml concentrators were supplied by Sartorius. PD-10 (Sephadex G-25) pre-packed gel filtration columns for use during the enzyme purification were from Pharmacia Fine Chemicals, Sweden. SDS-PAGE molecular weight markers (broad range, 6-200 kDa) and protein assay reagent were obtained from Biorad. Acrylamide was obtained from National Diagnostics, USA. Sodium deuteroacetate, NaN₃ and DSS (2,2 dimethyl-5-silapentane sulfonate) were purchased from Aldrich. A small quantity of *apo*-ACP from *Streptomyces coelicolor* was supplied courtesy of Bristol University. All other chemicals were from Sigma Chemical Company.

6.3 Purification of 6-MSAS ACP

6.3.1 Preparation of 6-MSAS ACP gene expressed in *E.coli*

6-MSAS ACP was kindly prepared by Dr. M. Sarwar using the polymerase chain reaction. The PCR DNA was restricted with *Nde*I and *Bam*HI enzymes and ligated into a *Nde*I and *Bam*HI restricted pT7-7 vector.

6.3.2 Preparation of competent cells for transformation

A single colony of *E.coli* strain BL21 (DE3) was grown overnight at 37°C in 10ml LB medium with continuous shaking. LB medium (15ml) was inoculated with 150μl of
this overnight culture and incubation was continued until the OD$_{600} = 0.3$. Cells were harvested by centrifugation at 3000 r.p.m at 4°C for 10 minutes, then resuspended in 10ml of ice-cold 50mM calcium chloride. Cells were used immediately.

6.3.3 Transformation of competent cells with plasmid DNA
A 1μl volume of pT7-7 plasmid DNA, was added to BL-21 competent cells (200μl) and the mixture stored at 4°C for 30 minutes. The cells were heat shocked for 30 seconds at 42°C, then incubated on ice for another 5 minutes. LB medium (300μl) was added and the mixture was incubated with inversion at 37°C for 1 hour. Then the whole mixture was spread on LB agar plates supplemented with ampicillin and these were left to grow overnight at 37°C. The plasmid that contained the 6-MSAS ACP gene (156 amino acids) also held the gene for ampicillin resistance. Therefore the cells which had taken up the plasmid DNA could grow on the ampicillin plates. A control plate with untransformed wild type bacteria was also set up to check for contamination.

6.3.4 Bulk growth of transformed cells
Colonies of transformed bacteria were selected individually and placed in four universals, each containing 20ml of sterile LB media (10g/l tryptone, 5g/l yeast, 5g/l NaCl) and 20μl of a 100mg/ml ampicillin stock solution. The cultures were incubated overnight at 37°C at a shaking rate of 160rpm. Each culture was then added to one of four baffled flasks containing 600ml of sterile LB media and 0.6ml of a 100mg/ml ampicillin stock solution. The flasks were grown at 37°C at a shaking rate of 160rpm in a New Brunswick Scientific Innova 4330 incubator/shaker until the OD$_{600}$ reached 1.0 (~3 hours). Then, to each flask, 0.6ml of 1M IPTG was added to induce overexpression of 6-MSAS ACP. The flasks were incubated for a further 3 hours under the same conditions as described above.

6.3.5 Harvesting of bulk growth
The bacterial cells were then obtained from the culture (2.4 litres in total) by centrifugation, at 4°C, in a Beckmann J2-21 centrifuge, 6 x 500ml rotor, for 25 min at 10000 r.p.m. The resulting pelleted cells were stored in universal containers at −20°C until required for future use.

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6.3.6 Sonication
Once thawed, the cells (12g wet weight) were resuspended in 20ml of 50mM Tris/HCl buffer, pH 8.0, containing 1mM DTT. The cells were then sonicated using a Soniprep 150 sonicator for fifteen 15 second bursts, with 40 second intervening cooling periods. At all times during sonication, the cells were jacketed in an ice water bath to keep the sonicate cool. The sonicated cells were then centrifuged for 45 minutes at 35000 r.p.m in a Beckmann L7-1 ultracentrifuge, using a 6 x 94ml rotor.

6.3.7 Sarkosyl treatment
The supernatant from the previous step was discarded. The pellet was resuspended in 20ml of 50mM Tris/HCl buffer, pH 8.0, containing 1mM DTT and 1% (w/v) sarkosyl. The solution was stirred for ten minutes at 4°C. The suspension was then sonicated and centrifuged as described in section 6.3.6.

6.3.8 Dialysis
The supernatant obtained from 6.3.7 was placed in benzoylated cellulose dialysis tubing (molecular weight cut off = 10kDa) and dialysed overnight in 5 litres of 50mM Tris/HCl buffer, pH 8.0, containing 1mM DTT. The dialysate was then centrifuged in a Beckmann J2-21 centrifuge, 6 x 500ml rotor, for 25 min at 10000 r.p.m. The supernatant was retained.

6.3.9 Ammonium sulphate fractionation
(NH₄)₂SO₄ (390g/l) was added to the supernatant, with stirring, to give 60% saturation. After 20 minutes, the precipitated protein was collected by centrifugation at 4°C in a Beckmann J2-21 centrifuge, 6 x 500ml rotor, for 25 min at 10000 r.p.m. The pellet was retained.

6.3.10 Gel filtration
The ammonium sulphate pellet was resuspended in 2ml of 50mM Tris/HCl buffer, pH 8.0, containing 1mM DTT. The suspension was loaded onto the top of a Superdex G-200 gel filtration column (1.6cm x 60cm) that had been equilibrated in the same buffer at a flow rate of 0.1ml/min. Fractions (1.2ml) were collected after 40ml (void volume of column) of buffer had been passed through the column.
6.3.11 Concentration
The fractions containing 6-MSAS ACP from the previous stage were pooled and placed in a 20ml Vivaspin concentrator (molecular weight cut off = 10kDa). The concentrating vessel was centrifuged in an Eppendorf Hermle ZK380 centrifuge at 5500 r.p.m., at 4°C, until a volume of 2ml was obtained. The purified enzyme was stored in 0.5ml aliquots in a freezer at -70°C.

6.3.12 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)
12% SDS-PAGE polyacrylamide gels were performed using the method of Laemmli (1970). Approximate molecular weights of experimental bands were determined by comparison to those of molecular weight markers (Biorad, broad range).

6.3.13 Determination of protein concentration
Protein concentrations were measured using the BioRad Protein Assay Reagent (Biorad Laboratories), a variation of the method by Bradford (1976), using BSA as the standard.

6.3.14 Electrospray mass spectrometric analysis of 6-MSAS ACP
In order to examine 6-MSAS ACP by mass spectrometry, 0.5mg of protein was buffer exchanged into analytical grade water and freeze dried overnight. 6-MSAS ACP was then dissolved in a solution containing 50% acetonitrile, 49% analytical grade water and 1% formic acid to obtain a final protein concentration of 0.2mg/ml. A 10μl volume of this solution was analysed by electrospray mass spectrometry (Micromass Quattro II, triple quadrupole) in positive ion mode, at a cone voltage of 36 V, capillary voltage of 3.77 V and HV lens voltage of 0.23 V, in a solvent system of 50% acetonitrile. The raw data obtained at the above values was then subjected to maximum entropy analysis according to the Micromass schedule.

6.3.15 Determination of aggregation state using light scattering
The aggregation state of the purified 6-MSAS ACP was analysed using a DynaPro Dynamic Light Scattering Instrument. A 20μl volume of 6-MSAS ACP (1mg/ml) was filtered through a Whatman Anotop™ Plus Syringe filter disc of 0.02μM porosity.
The filtered solution was injected into a quartz cuvette (holding volume = 12μl) which was placed into the optics area and illuminated with a laser. The DynaPro instrument analysed the light scattered by the protein solution and calculated the results.

6.4 Results

6.4.1 Purification of 6-MSAS ACP

An ACP of the type II FASs from *Saccharopolyspora erythraea* (Revill and Leadlay, 1991; Morris et al., 1993) and several ACPs of the type II PKSs from *Streptomyces coelicolor* have been expressed and purified from *E.coli* (Crosby et al., 1995). However, until now the purification of an ACP from a type I PKS has not been attempted. Unlike the type II ACPs that exist as separate proteins, the ACP component of the type I PKS 6-MSAS resides on a single polypeptide that contains all of the other enzyme activities required for the biosynthesis of 6-MSA. The ACP domain resides at the C-terminal end of the protein sequence. Attempts had previously been made at cloning and overexpressing the 6-MSAS ACP domain in *E.coli* but unfortunately the bacterial system was not able to overexpress the construct. Therefore a different strategy was required. Previous research whereby 6-MSAS was subjected to limited proteolysis by V8 protease, which hydrolysates the carboxyl side of glutamate, resulted in the production of several soluble peptides (Child et al., 1996). One such peptide, identified by N-terminal sequencing, was found to contain the ACP domain (Mr ~9000) attached to a N-terminal extension as shown in figure 6.5. The peptide is composed of 156 amino acids and has a predicted molecular weight of Mr 16,860. The DNA encoding this peptide was successfully cloned and overexpressed in *E.coli* in an effort to obtain soluble and correctly folded ACP. This would allow the structure and function of this protein to be researched. It should be noted that attempts were made to reduce the coding region of the 6-MSAS ACP but this resulted in no expression since the *E.coli* strain failed to grow with the modified insert.

The elongated 6-MSAS ACP (referred to forthwith as 6-MSAS ACP) has been purified to near homogeneity from *E.coli*. The purity is indicated by the presence of a single band at ~17kDa on a 12% SDS-PAGE gel (lane 5, figure 6.6). From each purification (2.4 litre cell culture) approximately 20mg of pure protein was obtained. However, a number of problems were encountered during the purification. Upon
Figure 6.5. The predicted position and domain boundaries of the five activities of 6-MSAS and, as highlighted, the position and size of the peptide containing the ACP domain following a limited V8 protease digest of the native 6-MSAS enzyme (KS, β-ketoacyl synthase; AT/MT, acetyl/malonyl transferase; DH, dehydratase; KR, β-ketoacyl reductase; ACP, acyl carrier protein) (Child et al., 1996). The cleavage site of V8 protease is indicated.
centrifugation of the sonicate it was found that the 6-MSAS ACP remained in the insoluble pellet. This problem was overcome by adopting a similar method to that used for solubilising actin, a bacterial outer membrane protein (Frankel et al., 1991). Soluble, active actin had been obtained by lysing the bacterial cells from which it had been expressed, in the presence of 0.2% (w/v) sarkosyl (N-laurylsarcosine). It was found by investigating a range of concentrations that for 6-MSAS ACP, 1% (w/v) sarkosyl was required to solubilise the protein. The remaining steps in the purification, namely the dialysis, ammonium sulphate fractionation and gel filtration steps, were used to try and remove as much sarkosyl as possible from the enzyme whilst maintaining it in a soluble form.

6.4.2 Mass spectrometric analysis of 6-MSAS ACP
The predicted molecular mass of the 6-MSAS ACP from the amino acid sequence is 16,860 Da in the apo- form. If the protein is in the holo- form then the P-pant arm must be added onto the sequence derived weight, resulting in a predicted post-translationally modified mass of 17,207 Da. Electrospray mass spectrometric analysis of the protein using a Quattro II triple quadrupole spectrometer revealed the major species to have a mass of 16,843 Da in positive ion mode (figure 6.7b). The deconvolution of the multiply charged envelope (figure 6.7a) results in a major species that is within 16 mass units of the predicted molecular weight of the elongated 6-MSAS ACP in the apo- form. It is therefore most likely that the protein has been purified in the apo- form and that the conditions under which it has been expressed does not allow the conversion into the holo- form.

6.4.3 Aggregation state of 6-MSAS ACP
The DynaPro instrument uses the technique of dynamic light scattering (DLS) to determine the molecular weight of a sample. With this technique, a beam of monochromatic light is directed through the sample and the fluctuation of intensity of the scattered light by the molecules is analysed. The output from the DLS is the translational diffusion coefficient, $DT$, of the particles in solution. Under the assumption of Brownian motion, this coefficient is converted to the hydrodynamic radius, $RH$, of the particles using the Stokes-Einstein equation:

$$RH = \frac{k_BT}{6\pi\eta DT}$$
Figure 6.6. 12% SDS gel of 6-MSAS ACP purification stages. Lane 1 and 6, broad range molecular weight markers (200kDa to 6.5kDa). Lane 2, whole cell lysate. Lane 3, supernatant after sonication in sarkosyl. Lane 4, 0-60% ammonium sulfate fractionation. Lane 5, Gel filtration (G-75) eluate.
Figure 6.7a. Raw data obtained from 6-MSAS ACP at a concentration of 0.2mg/ml analysed by ES-MS using quadrupole detection in positive ion mode. b. Deconvoluted spectrum of the multiple charged envelope.
where \( k_b \) is Boltzman's constant, \( T \) is the absolute temperature in degrees Kelvin, and \( \eta \) is the solvent viscosity. Under the assumption that the protein is globular then it is possible to correlate the RH value to molecular weight. For 6-MSAS ACP a RH value of 23.67 nm was calculated which corresponded to a molecular weight of \(~8000\) kDa (2.8% error). This value indicates that there is a considerable amount of aggregation of 6-MSAS ACP.

The elongated 6-MSAS ACP was found to elute from the Superdex G-200 gel filtration column at approximately 5ml after the void volume. Protein that elutes at such a volume corresponds to a native molecular weight of \( \text{Mr} > 500,000 \). This indicated that 6-MSAS ACP (predicted \( \text{Mr} 16,843 \)) had aggregated and this finding complemented the result obtained by light scattering.

6.5 Purification and partial characterisation of \( E.\text{coli} \) wild type and C118A ACPS

6.5.1 Preparation of \( E.\text{coli} \) wild type and C118A ACPS genes
The \( E.\text{coli} \) wild type and C118A mutant genes were kindly prepared by Dr. M. Sarwar using the polymerase chain reaction. The PCR DNA was restricted with \( \text{SalI} \) and \( \text{ClaI} \) enzymes and ligated into a \( \text{SalI} \) and \( \text{ClaI} \) restricted pT7-5 vector.

6.5.2 Preparation of competent cells for transformation
Performed as described in section 6.3.2.

6.5.3 Transformation of competent cells with plasmid DNA
Performed as described in section 6.3.3 except that pT7-5 plasmid DNA was used instead of that of pT7-7.

6.5.4 Bulk growth of transformed cells
Performed as described in section 6.3.4.

6.5.5 Harvesting of bulk growth
Performed as described in section 6.3.5.
6.5.6 Sonication
Once thawed the cells (12g wet weight) were resuspended in 20ml of 50mM MES buffer, pH 7.8, containing 10mM MgCl₂. The cells were then sonicated using a Soniprep 150 sonicator for fifteen 15 second bursts, with 40 second intervening cooling periods. At all times during sonication, the cells were jacketed in an ice water bath to keep the sonicate cool. The sonicated cells were then centrifuged for 45 minutes at 35000 r.p.m in a Beckmann L7-1 ultracentrifuge, using a 6 x 94ml rotor.

6.5.7 Ultracentrifugation
The sonicate was spun in a Beckmann L7-1 ultracentrifuge, using a 6 x 94ml rotor, for 60 minutes at 40000 r.p.m.

6.5.8 SP-Sepharose KCl elution
A 3cm x 30cm (200ml volume) column of SP-Sepharose was equilibrated with 3 column volumes of 50mM MES buffer, pH 6.1, containing 10mM MgCl₂ and 1mM DTT at a flow rate of 1ml/min on a FPLC system. The supernatant (~ 30ml) from the previous step was loaded onto the top of the column using a 50ml injection “superloop”. The column was washed with 200ml of the same buffer before applying a linear salt gradient (0-1M NaCl in a total volume of 500ml) at a flow rate of 1ml/min and 5ml fractions were collected. The enzyme was usually found to elute in five fractions between fractions 65 and 75.

6.5.9 Concentration
The fractions containing ACPS from the previous stage were pooled and placed in a 20ml Vivaspin concentrator (molecular weight cut off = 10kDa). The concentrating vessel was centrifuged in a Eppendorf Hermle ZK380 centrifuge at 5500 r.p.m., at 4°C, until a volume of 2ml was obtained.

6.5.10 Buffer exchange using PD-10 gel filtration
The protein solution from 6.5.9 was loaded onto a PD-10 gel filtration column that had been pre-equilibrated with analytical grade water. Enzyme was eluted with analytical grade water and those fractions containing protein were pooled and freeze-dried overnight. Freeze-dried ACPS was stored at 4°C for future use.
6.5.11 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

12% SDS-PAGE polyacrylamide gels were performed as described in section 6.3.8.

6.5.12 Preparation of minimal M9 media

2.4 litres of minimal growth media (6g/l Na₂HPO₄, 3g/l KH₂PO₄, 0.5g/l NaCl, 1g/l
¹⁵NH₄Cl) was prepared and adjusted to pH 7.4 with 1M NaOH. 600ml was placed in
each of four baffled flasks and autoclaved. Then to each flask, 1.2ml of 1M MgSO₄,
6ml of 20% (w/v) glucose and 0.1ml of 1M CaCl₂ were added.

6.5.13 Growth in minimal M9 media

Colonies of transformed bacteria were selected individually and placed in four
universals, each containing 20ml of sterile LB media (10g/l tryptone, 5g/l yeast, 5g/l
NaCl) and 20µl of a 100mg/ml ampicillin stock solution. The cultures were incubated
overnight at 37°C at a shaking rate of 160 r.p.m. Each culture was then added to a
baffled flask containing 600ml of sterile LB media and 0.6ml of a 100mg/ml
ampicillin stock solution. The flasks were grown at 37°C at a shaking rate of 160
r.p.m in a New Brunswick scientific innova 4330 incubator/shaker until the OD₆₀₀
reached 1.0 (~3 hours). The bacterial cells were then obtained from the culture (2.4
litres in total) by centrifugation at 4°C in a Beckmann J2-21 centrifuge, 6 x 500ml
rotor, for 25 min at 10000 r.p.m.

The bacterial cell pellets obtained from growth in LB media were resuspended in
~30ml of minimal media. The suspension was equally divided amongst four baffled
flasks containing minimal media and 0.6ml of a 100mg/ml ampicillin stock solution.
The flasks were grown at 30°C at a shaking rate of 160 r.p.m for 1 hour. Then to each
flask, 0.6ml of 1M IPTG was added to induce overexpression of holo-ACP synthase.
The flasks were incubated for a further 19 hours under the same conditions as
described above. The remainder of the protocol used for the purification of ¹⁵N-
labelled holo-ACP synthase was the same as that used for ACPS grown in LB media
only.

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6.5.14 Electrospray mass spectrometric analysis of wild type, mutant, and $^{15}$N-labelled ACPS

The freeze dried ACPS samples were dissolved in a solution containing 50% acetonitrile, 49% analytical grade water and 1% formic acid to obtain a final protein concentration between 0.1 and 0.2 mg/ml. A 10 µl volume of each solution was analysed by electrospray mass spectrometry (Micromass Quattro II, triple quadrupole) in positive ion mode, at a cone voltage of 40 V, capillary voltage of 3.66 V and HV lens voltage of 0.31 V, in a solvent system of 50% acetonitrile. The raw data obtained at the above values was then subjected to maximum entropy analysis according to the Micromass schedule.

6.5.15 Activity assay for ACPS using mass spectrometry

In a final volume of 75 µl the reaction mixture contains: 10 mM MES buffer, pH 6.1, 0.15 mM apo-ACP from *S. coelicolor*, 1.3 mM MgCl$_2$, 1.3 mM CoA, and 10 µM ACPS. The reaction was incubated for 30 minutes at 37°C. Then 5 µl was removed and diluted into a solution containing 50% acetonitrile, 49% analytical grade water and 1% formic acid to obtain a final protein concentration of 0.1 mg/ml. A 10 µl volume of the solution was analysed by mass spectrometry (Micromass Quattro II, triple quadrupole) in positive ion mode, at a cone voltage of 40 V, capillary voltage of 3.66 V and HV lens voltage of 0.31 V, in a solvent system of 50% acetonitrile. The raw data obtained at the above values was then subjected to maximum entropy analysis according to the Micromass schedule. A control reaction without ACPS was also carried out.

6.5.16 NMR studies on wild type and C118A ACPS

NMR experiments were performed using a Varian 600 MHz *INOVA* spectrometer equipped with a triple resonance probe and Z-field gradients. One-dimensional spectra were recorded for the wild type ACPS at a concentration of 4.8 mg/ml (total volume = 600 µl) in 90% H$_2$O / 10% D$_2$O, 20 mM sodium deuteroacetate buffer, pH 6.5, containing 1 mM NaN$_3$ and 1 mM DSS (2,2 dimethyl-5-silapentane sulphonate) at 25°C. Spectra with adequate signal to noise ratios could be achieved using 128 scans. A one-dimensional spectrum was again collected after three days and compared to the starting spectrum. Two-dimensional $^1$H-$^{15}$N HSQC spectra (heteronuclear single
quantum coherence) were recorded for both wild type and the C118A ACPS. Spectra were recorded with 1024 complex points in the first (observe) dimension and 128 complex points in the second (indirect nitrogen) dimension and 32 scans per increment. The nitrogen spectral width was set to 2000 Hz and the $^{15}$N carrier frequency was set to 122.6 ppm.

6.5.17 Quartenary interactions between subunits of wild type ACPS by Q-TOF mass spectrometry
Freeze dried ACPS was dissolved in 25mM ammonium acetate buffer, pH 7.0 to obtain a final protein concentration of 1.0pmol/μl. The solution was analysed by nanospray MS (Q-TOF II) in positive ion mode, at a cone voltage of 45V and capillary voltage of 900V with a Z-spray source. The raw data obtained at the above values was then subjected to maximum entropy analysis according to the Micromass schedule.

6.6 Results and discussion
6.6.1 Purification of E.coli wild type and C118A ACPS
Wild type ACPS has been purified to near homogeneity from E.coli. ACPS has a predicted pI of 9.3 and therefore the final step of the purification involved separating the ACPS from impurities using cation exchange chromatography. The ACPS was found to elute from the SP-Sepharose column as a single peak at approximately 0.75M NaCl (figure 6.8a). The purity is indicated by the presence of a single band observed on a 12% SDS-PAGE gel (lane 5, figure 6.8b). From each purification (2.4 litre cell culture) approximately 35mg of pure enzyme was obtained, a yield comparable to that obtained by Lambalot and Walsh (1995). However, it was discovered that the DE-52 stage could be omitted from the purification procedure resulting in an improved yield of ACPS. This modification does not affect the elution profile from the SP-Sepharose column and the level of purity of ACPS remains the same.

The purification procedure followed for the C118A ACPS mutant was the same as that followed for the wild type. Similar yields and levels of purity were observed at each of the purification stages. The reason for purifying the mutated form of ACPS is
Figure 6.8a. Final step in the purification of *E.coli* ACPS. Elution of ACPS (peak indicated) from SP-Sepharose-4B was carried out using a 0-1M NaCl gradient (red line). b. 12% SDS gel of ACPS purification stages. Lane 1 and 5, broad range molecular weight markers. Lane 2, whole cell lysate. Lane 3, supernatant after sonication. Lane 4 SP-Sepharose-4B KCl gradient eluate.
discussed in section 6.6.5.

6.6.2 Purification of $^{15}$N-labelled *E. coli* wild type and $^{15}$N-labelled C118A ACPS

In order to collect 2D-NMR data it was necessary to purify ACPS with all of the nitrogen atoms isotopically labelled ($^{15}$N). Upon attaining an OD of 1.0 in LB media the cells were harvested and then successfully overexpressed in minimal M9 media which contained $^{15}$NH$_4$Cl. From each purification (2.4 litre cell culture) approximately 20mg of pure enzyme was obtained.

6.6.3 Mass spectrometric analysis of *E. coli* ACPS preparations

The mass spectrometric data obtained from unlabelled wild type ACPS (figure 6.9), unlabelled C118A ACPS (figure 6.10), $^{15}$N-labelled wild type ACPS (figure 6.11) and $^{15}$N-labelled C118A ACPS (figure 6.12) is summarised in table 6.1:

<table>
<thead>
<tr>
<th>ACPS</th>
<th>Predicted molecular weight (Da)</th>
<th>Observed molecular weight (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>13921</td>
<td>13925</td>
</tr>
<tr>
<td>C118A mutant</td>
<td>13889</td>
<td>13893</td>
</tr>
<tr>
<td>$^{15}$N-Labelled wild type</td>
<td>14104</td>
<td>14099</td>
</tr>
<tr>
<td>$^{15}$N-Labelled C118A mutant</td>
<td>14072</td>
<td>14084</td>
</tr>
</tbody>
</table>

*Table 6.1* Summary of mass spectrometric data obtained from the various *E. coli* ACPS preparations.

The predicted and observed masses for the *E. coli* wild type and C118A mutant ACPS are within four mass units of each other and therefore indicate that the correct proteins have been isolated. For the $^{15}$N-labelled proteins the predicted molecular weights have been calculated on the assumption that all 125 nitrogen atoms on the peptide backbone and all 58 nitrogen atoms located on the amino acid side chains have been isotopically labelled. The observed molecular weights of both $^{15}$N-labelled proteins are within 0.085% of their predicted values and therefore confirm that they have been correctly labelled.
Figure 6.9a. Raw data obtained from *E. coli* holo-ACP synthase at a concentration of 0.2mg/ml analysed by ES-MS using quadrupole detection in positive ion mode.  

b. Deconvoluted spectrum of the multiple charged envelope.
Figure 6.10a. Raw data obtained from $^{15}$N-labelled E.coli holo-ACP synthase at a concentration of 0.2 mg/ml analysed by ES-MS using quadrupole detection in positive ion mode. b. Deconvoluted spectrum of the multiple charged envelope.
Figure 6.11a. Raw data obtained from *E.coli* holo-ACP synthase C118A mutant at a concentration of 0.2 mg/ml analysed by ES-MS using quadrupole detection in positive ion mode. b. Deconvoluted spectrum of the multiple charged envelope.
Figure 6.12a. Raw data obtained from $^{15}$N-labelled *E.coli* holo-ACP synthase C118A mutant at a concentration of 0.2 mg/ml analysed by ES-MS using quadrupole detection in positive ion mode. b. Deconvoluted spectrum of the multiple charged envelope.
6.6.4 Activity assay for E.coli ACPS using mass spectrometry

In order to observe ACPS activity it was necessary to have a supply of the substrate apo-ACP. Unfortunately no apo-ACP from E.coli was available. However, previous research has shown that the E.coli ACPS has a broad substrate specificity for PKS ACPs from S.coelicolor (Crosby et al., 1995). Therefore, the FAS apo-ACP from S.coelicolor was used in the activity assay. The predicted molecular mass of the apo-ACP from the amino acid sequence is 8785 Da. The deconvolution of the multiple charged envelope (figure 6.13a) results in a major species with a mass of 8787 Da (figure 6.13b) that is within two mass units of the predicted mass. The predicted molecular mass of the holo-ACP from the amino acid sequence is 9125 Da. In the presence of CoA and Mg$^{2+}$, ACPS was able to convert the ACP from the apo- to the holo- form. Deconvolution of the multiple charged envelope (figure 6.14a) revealed a major species of 9128 Da that is within 3 mass units of the predicted mass for holo-ACP (figure 6.14b). The smaller peaks at 9150 Da and 9171 Da are likely to account for ACPS plus one and two sodium ions respectively. The control assay without ACPS resulted in a major species corresponding to apo-ACP. Therefore wild type ACPS has successfully been shown to convert FAS apo-ACP from S.coelicolor to the active holo- form.

The C118A ACPS mutant and both $^{15}$N-labelled preparations were also assayed for activity. All were found to convert apo-ACP to the holo- form completely under the conditions described above. One problem with the mass spectrometric assay was that no accurate rate could be calculated for the different protein preparations. Therefore the specific activity of the C118A mutant could not be compared to that of the wild type.

6.6.5 Initial NMR data of wild type and C118A ACPS

Before collecting NMR data for ACPS, it was necessary to establish that the enzyme can maintain its conformation for long periods of time at the higher temperatures needed for data collection. A change in conformation or denaturation would be observed by a change in the NMR spectrum. A $^1$H-NMR spectrum of wild type ACPS was monitored over a period of three days. A comparison of the profile recorded at the beginning of the experimental run (A, figure 6.16) and after three days (B, figure 6.16) were different, thereby indicating that the protein had not maintained its native
Figure 6.13a. Raw data obtained from *Streptomyces coelicolor* apo-ACP at a concentration of 0.1 mg/ml analysed by ES-MS using quadrupole detection in positive ion mode. b. Deconvoluted spectrum of the multiple charged envelope.
Figure 6.14a. Raw data obtained from *Streptomyces coelicolor* ACP at a concentration of 0.1mg/ml after incubation with *E.coli* holo-ACP synthase, MgCl$_2$ and coenzyme-A, analysed by ES-MS using quadrupole detection in positive ion mode. b. Deconvoluted spectrum of the multiple charged envelope.
conformation over this time. Further evidence for this change was observed using mass spectrometry. Aliquots (10μl) of ACPS at time zero and after three days were removed and diluted into a solution containing 50% acetonitrile, 49% analytical grade water and 1% formic acid to obtain a final protein concentration of 0.2mg/ml. The samples were examined by ES-MS using triple quadrupole detection. The multiple charged envelope of ACPS after three days (figure 6.15a) consisted of twice as many peaks as that of the time zero envelope (figure 6.9a). This indicated that two species were present. Deconvolution of the multiple charged envelope results in two major species (figure 6.9b). The first of these at Mr 13926 corresponds to an ACPS subunit. However, the second peak at Mr 27852 is close to the mass of an ACPS dimer. Therefore it is very likely that the dimerisation of ACPS accounted for the change in NMR signal that was observed over three days.

Mass spectrometry of ACPS was performed in the presence of formic acid and as a result non-covalent interactions between enzyme subunits were not observed. Therefore the dimerisation of ACPS is likely to be due to the formation of a disulphide bond between the two subunits resulting in the loss of two hydrogen atoms. ACPS possesses only one cysteine residue, located at position 118 of the 125 amino acid sequence. The location of the cysteine residue at the C-terminal end could mean that it is structurally exposed on the ACPS surface. Cys118 is a non conserved residue so it is likely that it plays no important role in enzyme function. Therefore it was hoped that the successful construction and purification of the C118A mutant would yield active protein that would not dimerise. Indeed, it was found that the ¹H-NMR spectrum of the C118A mutant did not change significantly over a period of three days and that dimer formation was not observed by ES-MS. The protein was also found to be active as described in section 6.6.4.

Having established that the C118A mutant was able to provide a stable ¹H-NMR spectrum, a two-dimensional ¹H-¹⁵N HSQC spectrum was performed (B, figure 6.17). An HSQC spectrum was also performed for wild type ACPS as a comparison (A, figure 6.17). Both spectra consist of many peaks that are widely dispersed, indicating that the protein is folded. However, the peaks of the spectrum from the mutant are considerably more defined than that of the wild type. The clearly defined data that the
Figure 6.15a. Raw data obtained from *E.coli* holo-ACP synthase after 3 days at 25°C on NMR instrument at a concentration of 0.2 mg/ml analysed by ES-MS using quadrupole detection in positive ion mode. b. Deconvoluted spectrum of the multiple charged envelope after 3 days. c. Deconvoluted spectrum of the multiple charged envelope before exposure to NMR conditions.
Figure 6.16. A one dimensional 1H-NMR spectrum of wild type E.coli ACPS at the beginning of the experimental run (A), and after 3 days (B). Spectra were recorded at a concentration of 4.8mg/ml (total volume = 600 μl) in 90% H2O / 10% D2O, 20mM sodium deuteroacetate buffer, pH 6.5, containing 1mM NaN3 and 1mM DSS (2,2 dimethyl-5-silapentane sulphonate) at 250C. Spectra with adequate signal to noise ratios could be achieved using 128 scans.
Figure 6.17. Two-dimensional $^1$H-$^{15}$N HSQC spectra (heteronuclear single quantum coherence) of wild type *E. coli* ACPS (a) and C118A ACPS (b). Spectra were recorded with 1024 complex points in the first (observe) dimension and 128 complex points in the second (indirect nitrogen) dimension and 32 scans per increment. The nitrogen spectral width was set to 2000 Hz and the $^{15}$N carrier frequency was set to 122.6 ppm.
C118A mutant provides is a good starting point from which to solve the solution structure of ACPS.

### 6.6.6 Quarternary interactions observed by Q-TOF mass spectrometry

Mass spectrometry of ACPS was performed in ammonium acetate buffer, pH 7.0, in order to preserve non-covalent interactions between enzyme subunits. The production of protein ions of high mass-to-charge ratio was expected due to the omission of formic acid. Therefore in order to observe ions above 2000 m/z units, a time of flight analyser was used. From the raw data obtained a series of peaks was observed, the largest of which occurred at 2321 m/z units (figure 6.18a). Deconvolution of the multiple charged envelope resulted in the production of two major species of equal intensity (figure 6.18b). The peak at 13921.23 corresponded to the mass expected for the ACPS subunit whereas the peak at 27480.52 corresponded to the mass expected for the ACPS dimer. There were no peaks of higher mass detected corresponding to higher order oligomeric states.

The crystallographic structures of ACPS from *B.subtilis* (Parris et al., 2000) and *S.pneumoniae* (Chirgadze et al., 2000) have shown that each enzyme assembles as a tightly packed trimer. There is significant structural homology between these enzymes and Sfp from *B.subtilis* (Reuter et al., 1999). However, Sfp has been shown to pack as a dimer. The mass spectrometry data obtained with ACPS from *E.coli* has revealed only a dimeric state, thereby complementing the Sfp model and results obtained from gel filtration with ACPS from *E.coli* (Lambalot and Walsh, 1995). It is possible therefore that the ACPS trimer observed in the two crystallographic structures may not reflect the actual oligomeric state in solution. The NMR solution structure of ACPS should reveal the true oligomeric state which, from mass spectrometry, appears to be a dimer.

### 6.7 Summary and future work

The purification of 6-MSAS ACP and of ACPS from *E.coli* have been carried out successfully. Having shown that ACPS is catalytically active with apo-ACP from *S.coelicolor*, the activity of ACPS with apo-ACP from 6-MSAS was then investigated. It was hoped that the conversion of apo-6-MSAS ACP to the holo-form could be catalysed by ACPS from *E.coli* in the presence of Mg$^{2+}$ and CoA under the
Figure 6.18a. Raw data obtained from a 1.0pmol/μl solution of wild type *E. coli* ACPS under non-covalent conditions analysed by nanoflow Q-TOF. b. Deconvoluted spectrum of the multiple charged envelope.
same conditions described in section 5.5.15. Unfortunately, no conversion was observed by mass spectrometry. Therefore it is possible that either *E. coli* ACPS is not specific for 6-MSAS ACP due to the different protein sources (i.e. fungal and bacterial) or that 6-MSAS ACP, although purified, is inactive due to its state of aggregation as observed by gel filtration and light scattering.

The need to clone, overexpress and purify the C118A mutant has been justified as a result of the improved NMR data obtained so far. The next step is to optimise the protein buffer conditions so as to obtain the best possible data. It will then be necessary to purify enzyme that has been isotopically labelled with $^{13}$C and $^{15}$N. A full set of NMR experiments will then be performed from which the data collected will be processed in order to obtain a three dimensional structure. A comparison between the NMR solution structure and the two known ACPS X-ray structures (Parris *et al.*, 2000; Chiragdze *et al.*, 2000) can then be made.
References


Cottrell, J.C., & Green, B. N. (1994) VG Organic, Tudor Road, Altrincham, Cheshire, WA14 5RZ; Application Note No. 212.


