CRYSTALLOGRAPHIC STUDIES OF ENZYMES INVOLVED IN BIOSYNTHESIS OF FATTY ACIDS; β-KETOACYL ACYL CARRIER PROTEIN SYNTHASE II AND Δ9-STEAROYL-ACP DESATURASE

Martin Moche

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ABSTRACT
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In the biosynthesis of fatty acids, the β-Ketoacyl Acyl Carrier Protein Synthases (Kas) catalyse chain elongation by the addition of two-carbon units derived from malonyl-ACP, to an acyl group bound to CoA or the small protein ACP. These enzymes are potential targets for antibiotics against bacterial or parasitical infections and their mammalian counterparts could hopefully be used as drug targets against certain cancers and obesity. The crystal structure of the Kas II isoform from *E. coli* in complex with the well known natural inhibitor cerulenin has been determined to define the active site pocket and enable rational drug design with this target. Kas belongs to a family known as condensing enzymes for their ability to catalyse the Claisen condensation reaction. Different condensing enzymes have different substrate specificities and reaction mechanisms that somehow must be reflected in their 3D structure. The crystal structure of Kas II from *Synechocystis* was determined and compared to other condensing enzymes, to elucidate structural determinants for substrate specificity and reaction mechanism in condensing enzymes.

Stearoyl Acyl Carrier Protein Desaturase is a soluble enzyme that catalyses the formation of a cis-double bond between carbon-9 and carbon-10 in stearic acid. Desaturase is known to contain a bent hydrophobic cavity, where the fatty acid substrate binds in the vicinity of a di-iron centre that is used for activation of molecular oxygen. Following reduction by ferredoxin, the di-iron centre activate molecular oxygen and reactive oxygen intermediates are formed that abstract hydrogen atoms from the bound fatty acid. The crystal structures of the molecular oxygen analogues, azide and acetate, in complex with desaturase have been determined in order to elucidate how the di-iron centre activates molecular oxygen and further controls the reactive oxygen intermediates to catalyse fatty acid desaturation. Crystal structures of iron depleted desaturase species have also been determined. Soluble desaturases act as the primary determinant for the composition of unsaturated fatty acids in plant membranes and seed storage oils, and variation in regio-specificity (double bond insertion position) among desaturase isozymes in certain plants is associated with the presence of unusual fatty acids in the seeds of these plants. The crystal structure of the protein complex between desaturase from *Ricinus Communis* and stearoyl-ACP from spinach, provides a basis for rational design of regio-specificity in soluble desaturases.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Å</td>
<td>Ångström (10^{-10} m)</td>
</tr>
<tr>
<td>ACP</td>
<td>Acyl Carrier Protein</td>
</tr>
<tr>
<td>ASA</td>
<td>Accessible Surface Area</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmatic Reticulum</td>
</tr>
<tr>
<td>fāb</td>
<td>Fatty acid biosynthesis</td>
</tr>
<tr>
<td>FAS</td>
<td>Fatty Acid Synthase</td>
</tr>
<tr>
<td>Fd</td>
<td>Ferredoxin</td>
</tr>
<tr>
<td>FNR</td>
<td>Ferredoxin NADPH reductase</td>
</tr>
<tr>
<td>KAS</td>
<td>β-Ketoacyl Acyl Carrier Protein Synthase</td>
</tr>
<tr>
<td>MCD</td>
<td>Magnetic Circular Dichroism</td>
</tr>
<tr>
<td>MMOH</td>
<td>Methane mono-oxygenase hydroxylase</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>PKS</td>
<td>Polyketide Synthase</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acids</td>
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</tbody>
</table>
1 GENERAL INTRODUCTION

Fatty acids occur as building blocks of various lipids that are essential components of biological membranes, or are consumed as fuel molecules to provide energy for the cells. Fatty acids also target proteins to the biological membranes or act as precursors for signalling molecules such as hormones. Biosynthesis of fatty acids is catalysed by discrete monofunctional enzymes in the plant plastids (1) or in the bacterial cytosol (2) however in mammals there is a single large polypeptide chain with seven catalytic domains that performs all necessary reactions in the cytosol (3). The mammalian fatty acid synthase (FAS) system is sometimes referred to as Type I while the plant and bacterial systems are called Type II (4). During biosynthesis of fatty acids the pantetheine group of either Coenzyme A (CoA) or the small protein Acyl Carrier Protein (ACP) carries all the intermediates. ACP is one of the seven domains in mammalian FAS, while in plant and bacteria it is a small soluble protein, that can recognise several different target enzymes.

The first committed step in fatty acid biosynthesis is catalysed by acetyl-CoA carboxylase, which forms malonyl-CoA from acetyl-CoA. This enzyme occurs in two isoforms in humans (5,6) and is subject to feedback inhibition by acyl-ACP products in bacteria (2). During a single elongation cycle of fatty acid biosynthesis the formation of the carbon-carbon bond is catalysed by β-ketoacyl ACP synthase (Kas) that elongates a saturated fatty acid carried by ACP by a two-carbon unit derived from malonyl-ACP. After the elongation reaction the β-Ketoacyl ACP reductase reduces the carbonyl group at C3 using NAD(P)H. The subsequent dehydration catalysed by 3-hydroxyacyl ACP dehydratase generates the enoyl substrate for the second reduction catalysed by enoyl ACP reductase. The product of a single elongation cycle regenerates a saturated fatty acid that has been elongated by two carbon units. In plant and bacterial species, some of the enzymes used in the elongation cycle occur in several isoforms. In addition to the four enzymes in the elongation cycle, mammalian FAS contains a malonyl CoA:ACP transacylase domain, ACP and a thioesterase domain for release of the palmitate product. In plants, the product of fatty acid biosynthesis is stearic acid but due to thioesterases and soluble desaturases, shorter and monounsaturated fatty acids attached to CoA are exported from the plastid into the endoplasmatic reticulum (ER). In the ER these fatty acids are desaturated, hydroxylated, elongated and esterified to glycerol-3-phosphate, forming lysophosphatidic acid, phosphatidic acid, diacylglycerol and finally triacylglycerol that is located in developing seeds as well as in the human adipose tissue.

Desaturases are iron containing proteins that use molecular oxygen to catalyse the formation of double bonds in saturated or monounsaturated fatty acids. Two kinds of desaturases have been identified, one soluble form unique to plants and one membrane bound form present in the ER (7). The soluble stearoyl acyl carrier protein desaturase from plant plastids inserts the first cis double bond into stearoyl-ACP forming oleoyl-ACP which is the first step in the biosynthesis of plant linoleic and linolenic acid. These essential fatty acids act as precursors for human elongases and desaturases in the
ER where polyunsaturated fatty acids (PUFA) are produced (8). Polyunsaturated fatty acids act as precursors for prostaglandins, leukotrienes and thromboxanes involved in for instance inflammation and pain.

1.1 AIM OF THESIS

X-ray crystallography has been used to solve 3D structures of two enzymes in the fatty acid biosynthetic pathway, either in complex with various inhibitors, or as protein substrate complexes, aiming to elucidate details regarding their substrate specificity and catalytic mechanism.

For β-ketoacyl ACP synthase (Kas), a well-known inhibitor named cerulenin exists and the initial efforts were directed to solve the structure of the complex between Kas II from *E. coli* and this inhibitor. Then the objective was to solve the crystal structure of Kas II from *Synechocystis* and compare this structure to other structures of related enzymes to establish structure-function relations as above.

With stearoyl-ACP desaturase the initial objective was to study a complex with azide as an analogue for molecular oxygen to hopefully elucidate details of the oxygen activation process in this enzyme. Another goal was to determine the crystal structure of the oxidised di-iron centre in desaturase. Finally the question regarding how desaturase recognises the substrate stearoyl-ACP was addressed by the effort to determine the crystal structure of the protein complex between desaturase and its substrate stearoyl-ACP.
2 STRUCTURAL STUDIES OF $\beta$-KETOACYL ACYL CARRIER PROTEIN SYNTHASE

2.1 INTRODUCTION

$\beta$-Ketoacyl acyl carrier protein synthase (Kas) is the enzyme, or FAS domain, that catalyses the formation of carbon-carbon bonds during fatty acid biosynthesis in a reaction known as the Claisen condensation. In biology the Claisen condensation can be divided into either a non-decarboxylating reaction catalysed by thiolases, or a decarboxylating reaction catalysed by Kas enzymes (9). The decarboxylating Kas enzymes use a malonyl-unit attached to ACP while the non-decarboxylating thiolases can activate acetyl-units attached to CoA. The decarboxylating Kas enzymes also occur in polyketide synthases (PKS) involved in synthesis of natural products such as the antibiotics erythromycin A, actinorhodin and rifamycin or the immune-suppressing agent rapamycin (10).

The decarboxylating Claisen condensation occurs by a ping-pong mechanism that can be divided into three distinct steps: (i) a transacylation where the acyl group of acyl-ACP is transferred to a cysteine residue at the active site resulting in a thioester acyl intermediate, (ii) binding and decarboxylation of the second substrate, malonyl-ACP, creating a carbanion, and (iii) formation of the carbon-carbon bond by nucleophilic attack of the reactive carbanion onto the carbonyl carbon of the thioester (Fig. 1).

Figure 1. The three steps of the decarboxylating Claisen condensation catalysed by Kas enzymes.

The transacylation (i) or decarboxylation (ii) step is sometimes assayed separately and combined with site directed mutagenesis specific details about the reaction mechanism can be obtained.
*Escherichia coli* (E. coli) has served as a model system for bacterial biosynthesis of fatty acids and much knowledge about the subject is derived from this organism (2,11). In *E. coli* there are pathways for either saturated or unsaturated fatty acid biosynthesis. *E. coli* contain three different Kas enzymes named Kas I, Kas II and Kas III, or fabB, fabF and fabH after their corresponding genes (12,13). Kas III from *E. coli* is smaller than the other two Kas proteins and catalyses the initial condensation of acetyl-CoA and malonyl-ACP to yield acetoacetyl-ACP (13). The elongating enzymes Kas I and Kas II have similar size and structure and bind and elongate fatty acids attached to ACP using malonyl-ACP. In vitro Kas I and Kas II have quite similar substrate specificity profiles, elongating saturated fatty acid substrates between 4 to 16 carbons in length (14). Kas I is essential for cell growth in *E. coli* most likely due to its ability to elongate the first intermediate cis-3-decenoyl-ACP in the unsaturated fatty acid biosynthesis pathway (15). Kas II is essential for thermal regulation of fatty acids in *E. coli* membranes by its ability to elongate the final step of the unsaturated pathway namely the elongation of cis-9-palmitoleoyl-ACP into cis-11-vaccenoyl-ACP (16-18).

Cerulenin is a mycotoxin from the fungus *Cephalosporium caerulens* known since the early seventies to inhibit fatty acid biosynthesis (19-21). Cerulenin acts as a potent irreversible inhibitor of Kas enzymes and mammalian Kas domains by covalent modification of the active site cysteine (22,23). Kas I from *E. coli* is slightly more sensitive to cerulenin than Kas II (14) while Kas III is much less sensitive and was initially identified in *E. coli* extracts based on this property (13). Thiolactomycin is an antibiotic produced by the soil fungi *Nocardia* and acts as a reversible and competitive inhibitor for malonyl-ACP but only against monofunctional plant or bacterial Kas enzymes and not against mammalian Kas domains (24-26). Like cerulenin, Kas III is less sensitive to thiolactomycin than the Kas I and Kas II enzymes from *E. coli* (27).

Kas II from *E. coli* was the first crystal structure determined for a Kas enzyme (28) and belongs to a superfamily of proteins known as condensing enzymes named after the Claisen condensation reaction catalysed by most of its members. The fold of this superfamily is called the “thiolase fold” after the first crystal structure solved for a member of the superfamily (29). Today ten structures of enzymes from this superfamily are known together with several inhibitor, intermediate, substrate and product complexes. The ongoing genome sequencing efforts rapidly increase the number of protein sequences available for members of the superfamily.

Despite knowledge obtained from biochemistry, gene sequences and crystal structures of condensing enzymes, structure-function relations regarding their reaction mechanism and substrate specificity are still debated by researchers in the field.

One aim of this summary is to discuss functional properties such as substrate specificity and catalytic mechanism for Kas II based on the crystal structures of inhibitor and intermediate complexes. The Kas II structure is then compared to structures of other condensing enzymes, with focus on the active site, in order to ask questions and provide hypotheses regarding relations between structure and catalytic mechanisms in condensing enzymes. The relevance of Kas enzymes as a drug target against bacterial
or parasitical infections or the involvement of the mammalian multisubunit (FAS) counterpart in diseases such as cancer and obesity will be briefly mentioned at the end.
2.2 STRUCTURE AND FUNCTION OF KAS II

Two crystal structures of Kas II, from *E. coli* and *Synechocystis sp.*, have been solved to 2.4 and 1.54 Å resolution, respectively (28, Paper II). The two structures of Kas II are very similar except for assignment of a few $\beta_{10}$-helices in the higher resolution structure. The sequence identity between these enzymes is 50% and 407 residues can be aligned with a root mean square deviation of 0.26 Å. The Kas II subunit consists of two mixed five-stranded $\beta$-sheets surrounded by $\alpha$-helices forming a layered structure of secondary structure elements arranged as $\alpha$-$\beta$-$\alpha$-$\beta$-$\alpha$. The two $\beta$-sheets of the subunit have a similar topology and 77 Cα atoms can be aligned with a root-mean-square deviation of 1.65 Å. This structural similarity implies that the Kas subunit evolved through gene duplication although the sequence identity is only 10% for the structurally equivalent residues.

![Figure 2. The Kas II dimer from *E. coli* with cerulenin bound in the active site (Paper I).](image)

Kas II is known to be a dimer in solution and in the *Synechocystis* structure, 3175 Å$^2$ per subunit is buried in the dimer interface. The β5 strands from both subunits run antiparallel to each other across the interface making the N-terminal β-sheet forming a continuous 10 stranded β-sheet in the dimer. A cavity filled with water molecules is present in the *Synechocystis* dimer interface and a network of water mediated and direct hydrogen bonds connect the two subunits.

The two active sites are located close to the dimer interface approximately 25 Å from each other (Fig. 2). The sulphur of the catalytic nucleophile Cys163 (*E. coli* numbering) is accessible to solvent and located next to a hydrophobic pocket where the acyl chain of
the acyl-ACP substrate binds. The second subunit contributes slightly to the active site and the tails of the longer fatty acid intermediates are predicted to be in contact with the second subunit during catalysis.

Using BLAST and ClustalW it is possible to find and align 232 sequences related to Kas II from plant, bacterial and mammalian species. The lowest sequence identity among any of these 27 028 gene pairs is 22 % but is usually much higher. Among these are several Kas domains from mammalian fatty acid synthases and polyketide synthases. Many of these genes were identified in various genome-sequencing projects and remain to be biochemically characterised. Conserved residues are expected to be important to the fold and the catalytic mechanism in Kas II. In total eight residues are strictly conserved (Table 1) while another eight residues are conservatively substituted.

<table>
<thead>
<tr>
<th>Suggested function for the conserved residue</th>
<th>Residue</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys163</td>
<td>Catalytic nucleophile, binds covalently to the fatty acid intermediate</td>
<td></td>
</tr>
<tr>
<td>Gly228</td>
<td>A side chain in this position might disturb binding of pantetheine</td>
<td></td>
</tr>
<tr>
<td>Glu241</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>Ala246</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>His303</td>
<td>Important for decarboxylation</td>
<td></td>
</tr>
<tr>
<td>Thr307</td>
<td>Binding of hydroxyl and/or phosphate of the phosphopantetheine group of the substrates</td>
<td></td>
</tr>
<tr>
<td>Lys335</td>
<td>The NZ atom interacts with His303ND1 and thereby influences the position and properties of His303. Important for decarboxylation</td>
<td></td>
</tr>
<tr>
<td>His340</td>
<td>Important for decarboxylation</td>
<td></td>
</tr>
</tbody>
</table>

The functional assignments for Gly228 and Thr307 were inferred from the crystal structure and sequence alignments only and could therefore be incorrect (Paper II). The roles of the cysteine, the two histidines and the lysine have been validated by various studies combining site directed mutagenesis with functional assays and crystal structures (30,31,32,Paper I).

2.2.1 Inhibitor and intermediate complexes as probes of catalytic mechanism and substrate specificity in Kas II

Today crystal structures of several inhibitor and fatty acid intermediate complexes are available. The structures of Kas I and Kas II from E. coli are known to be closely related, and therefore structures of inhibitor and intermediate complexes will be compared between them, with the objective to point out potential determinants for differences regarding substrate specificity and cerulenin sensitivity. The first structure
of an inhibitor complex determined was the crystal structure between *E. coli* Kas II and cerulenin (Paper I). Shortly afterwards, both the cerulenin and the thiolactomycin inhibitor complex of *E. coli* Kas I (27) as well as the decanoyl (C:10) and dodecanoyl (C:12) fatty acid intermediate complexes of the same enzyme were published (32).

Contrary to previous suggestions (22) cerulenin binds in its ring open form to the active site cysteine in both Kas I (27) and Kas II (Paper I) from *E. coli*. The binding of the cerulenin tail shows that the longer fatty acid intermediates will be in contact with residues from the second subunit. The position of the cerulenin molecules are almost identical when bound to Kas I or Kas II except for the carbon atoms at the end of the cerulenin tail. Kas I and Kas II have a sequence identity of 38 % and using the program MAPS (33) it is possible to align 359 Cα atoms with a root mean square deviation of 0.37 Å. Phe400 and Ile108 change rotamer conformations to enable binding of cerulenin in Kas II, while the corresponding residues are already in an open conformation (Phe392) in absence of cerulenin, or substituted for glycine (Ile108) in Kas I. The absence of structural rearrangements in Kas I upon binding of cerulenin could be part of the explanation why Kas I is more sensitive to cerulenin than Kas II. The cerulenin tail was expected to bind like the carbon tail of the fatty acid intermediate and this assumption turned out to be correct as revealed by the structures of Kas I in complex with cerulenin (27), decanoyl (C10) and dodecanoyl (C12) (32). A superposition of the cerulenin and fatty acid intermediate complexes in Kas I show striking similarities in the position of their carbon tails. The differences in substrate specificity between the *E. coli* Kas I and Kas II are most likely caused by differences in the identity of residues lining this fatty acid binding pocket (34). In Kas II there is also a one residue “insertion” corresponding to Ala193 in the *E. coli* enzyme that could contribute to the differences in substrate specificity between Kas I and Kas II (Paper II). The cerulenin complex does not show how the longest fatty acid substrates could bind to Kas II and there is not enough space available, suggesting that structural rearrangements occur upon binding and elongation of palmitoleic acid into cis-vaccenic acid. The single mutants Ala193Met and Ile108Phe, designed from the cerulenin complex structure (Fig. 3, Paper I), have been introduced into *E. coli* Kas II and abolish cerulenin sensitivity and the ability to elongate fatty acid substrates with more than six carbons (35). The Kas II Ile108Phe mutation was also found in a strain from *B. subtilis* selected for cerulenin resistance (36).
Figure 3. Binding of cerulenin to the active site in *E. coli* Kas II

The hydroxyl group attached to the C3 carbon of cerulenin makes a hydrogen bond to the NH-group of Phe400 and is also close to the NH-group of the catalytic nucleophile Cys163 (Fig. 3, Paper I). The main chain NH-groups of Phe400 and Cys163 are expected to form a main chain oxyanion hole used for stabilising the negative charge in the transition state during the transacylation and condensation steps of the reaction (9) (Fig. 4). This function of the main chain oxyanion hole is also supported by the fatty acid intermediate structures of Kas I where the carbonyl oxygen of the bound fatty acid points towards the main chain nitrogens of Phe400 and Cys163 (32).

The carbonyl oxygen of the cerulenin amide group makes hydrogen bonds to the NεH hydrogens of the conserved side chains of His303 and His340 (Fig. 3, Paper I). These histidine side chains, form a side chain oxyanion hole expected to facilitate decarboxylation by binding to the carbonyl oxygen of the second substrate malonyl-ACP (9) (Fig. 4). In support of this model is the recent crystal structure of a complex with the inhibitor thiolactomycin. This is a competitive inhibitor of malonyl-ACP and binds with one of its oxygen atoms in this side chain oxyanion hole (27). Recent mutational studies reveal that the side chain oxyanion hole is indispensable for the decarboxylation step and replacement of either of the conserved histidines by alanine in *E. coli* Kas I (30,31) or the Kas domain of animal fatty acid synthase abolishes decarboxylation (37). The conserved Lys335 makes a hydrogen bond to His303Nε thereby influencing the position and the electrostatic properties of this histidine and the side chain oxyanion hole. The lysine corresponding to Lys335 in *E.
coli Kas II has been mutated into alanine in E. coli Kas I and rat FAS thereby eliminating decarboxylase activity in the enzymes examined (30,31,37).

Figure 4. The Claisen condensation as catalysed by Kas II adapted from (9).

2.3 COMPARING STRUCTURES AND MECHANISMS OF CONDENSING ENZYMES

In April 2003, ten crystal structures of condensing enzymes from different species are available in the protein data bank. The structures are: Kas I from E. coli (1dd8, pdb-entry name) (34), Kas II from E. coli (1kas) (28) and Synechocystis (1e5m, Paper II), Kas III from E. coli (1eb1) (38), M. tuberculosis (1hxp) (39) and Streptomyces (1mzj) (40), thiolases from Z. ramigera (1qfl) (41) and S. cerevisiae (1afw) (42) and the unimodular polyketide synthases chalcone synthase from M. sativa (1chw) (43) and 2-pyrone synthase from G. hybrida (1ce0) (44). Kas I, Kas II and Kas III from E. coli
have already been introduced. Kas III from the R1128 synthesis pathway in Streptomyces has the same primer unit (acetyl-CoA) as the E. coli enzyme (40) while Kas III from M. tuberculosis accepts and elongates much longer primer units having the highest activity with lauroyl-CoA (C12:0) (45). The biosynthetic thiolase from Z. ramigera catalyses the formation of acetooacetyl-CoA from two units of acetyl-CoA while thiolase from S. cerevisiae is present in the peroxisomal catabolic pathway using 3-ketoacyl-CoA and CoA to form acyl-CoA and acetyl-CoA (41). Chalcone synthase uses p-coumaroyl-CoA as its primer unit and catalyses three successive condensations with malonyl-CoA before releasing chalcone from the active site (43). 2-Pyrone synthase uses acetyl-CoA as its primer and like chalcone synthase catalyse three successive condensations using malonyl-CoA before releasing its product 2-pyrone (44).

The 3D structures of condensing enzymes were compared with the objective to determine structural determinants for substrate specificity and catalytic mechanism (Paper II). Three new structures of condensing enzymes have appeared since then and in this updated comparison focus will be on residues in the active site and the two oxyanion holes. The oxyanion holes are critical for stabilising the transition states during transacylation and condensation and are also involved in the activation of the second substrate. Most of these enzymes catalyse decarboxylation of the second substrate malonyl-CoA or malonyl-ACP but biosynthetic thiolase can activate the acetyl-unit of acetyl-CoA instead. All enzymes are present as dimers except the tetrameric biosynthetic thiolase from Z. ramigera (41).

To relate the subunit structures to each other, the program MAPS (33) was used to make a multiple structural alignment based on the Cα and CB atom coordinates. The result is displayed as a diagram made with PHYLIP (46) (Fig. 5).

Figure 5. Structure relations between condensing enzymes. The different structures are represented by their entry names in the protein data bank. The ten structures of condensing enzymes divide into four groups named thiolase, Kas I and Kas II, Kas III and PKS.
From the diagram in figure 4 it appears that the Kas I and Kas II enzymes are more similar in structure to the thiolases than to the Kas III or the PKS enzymes. In the same way Kas III and PKS enzymes are structurally more related to each other than to the thiolases or the Kas I and Kas II group. In total 83 residues divided into 13 fragments appear as structurally equivalent between all these enzymes, representing 26% of the full length of E. coli Kas III, the smallest protein used in the comparison. The structurally equivalent residues belong to regions of the β-strands (β1, β4, β7, β9, β13 and β14) within the core part of the two β-sheets, part of the α-helices (α3 and α7) and the 3_10-helix γ7. The nucleophilic cysteine is located in the β5-α7 loop being structurally equivalent and identical between all condensing enzymes. The two histidines, His307 and His344 (Synechocystis numbering) in the active site of Kas II (one at the C-terminal end of β9 and the other at the C-terminal end of α12) have structural equivalents but are not invariant among all condensing enzymes.

When structures of condensing enzymes are aligned and their active sites are displayed it is clear that the thiolases and the Kas I and Kas II structures form one closely related set of structures and that the Kas III and the PKS enzymes form another closely related set of structures with respect to the identity of the residues present in the active site (Fig. 6) i.e. similar to how MAPS divides the structures of condensing enzymes based on the Cα and Cβ coordinates (Fig. 5). The catalytic nucleophile is the single feature that is conserved for all the ten condensing enzyme structures.

Figure 6. Superpositions of representatives from each of the four structural classes of condensing enzymes. Kas II from Synechocystis in yellow represents the Kas I and Kas II group and thiolase from Z. ramigera in cyan represents the thiolase group (left). Kas III from E. coli in magenta represents the Kas III group and chalcone synthase from M. sativa in green represents the PKS group (right).
In the active sites of Kas II and thiolase, the position of the cysteine carrying the fatty acid intermediates and one of the Kas II histidines (His344) is a conserved feature between these subfamilies (Fig. 6, left). The main chain oxygen hole exists in both enzymes consisting of the main chain NH-groups of Cys167 and Phe403 in *Synechocystis* Kas II and the main chain NH-groups of Cys89 and Gly380 in thiolase. The side chain oxygen hole is built up by the Ne hydrogens of His307 and His344 in *Synechocystis* Kas II and by a water molecule and His348 in thiolase (Fig. 6, left). As mentioned (Table 1) the conserved Lys339 in *Synechocystis* Kas II influences the position and electrostatic properties of His307 and in a similar way the conserved Asn316 in thiolase coordinates wat88. Glu353 in Kas II compensates the positive charge of Lys339 and this glutamate is present in 229 of the 232 available Kas II sequences.

Comparison between Kas III and chalcone synthase reveals that the nucleophilic cysteine and an active site asparagine is a conserved feature between these subfamilies. In chalcone synthase the main chain oxygen hole is disrupted since the residue corresponding to Gly306 in Kas III is a proline in chalcone synthase. Gly306 in *E. coli* Kas III and Pro375 in chalcone synthase are both flanked by glycine residues, adding flexibility to this region for these enzymes. Immediately after the three glycines in Kas III, Phe308 is situated, having its main chain in an unusual cis-conformation (47). This main chain cis-peptide is found in all Kas III and PKS enzymes except in the Kas III enzyme from *M. tuberculosis*, which has a regular trans peptide of the main chain at this position. The *M. tuberculosis* Kas III trans peptide however does not change the position of the main chain NH-group of the nearby glycine contributing to the oxygen hole.

The imidazole ring of the histidine in the active site appears to be flipped by 180 degrees between Kas III from *E. coli* and chalcone synthase (Fig. 6, right). Kas III from *Streptomyces* has its histidine orientated as in Kas III from *E. coli* while the *M. tuberculosis* enzyme has its histidine positioned as in chalcone synthase. How to model the orientation of the active site histidine in Kas III (or chalcone synthase) is not obvious. In Kas III, the side chain carbonyl oxygen of Asn274 makes a hydrogen bond to the main chain NH-group of Ser276 and the side chain NH2 group of Asn274 could be expected to form an oxygen hole together with the active site His244 (Fig. 6). Assuming correct orientation of Asn274 and formation of a side chain oxygen hole similar to Kas II, suggests that the histidine imidazole ring in Kas III from *Streptomyces* and *E. coli* should actually be oriented like in chalcone synthase and *M. tuberculosis* Kas III.

What do these structural relations imply for the reaction mechanisms in these enzymes?
2.3.1 Cysteine deprotonation and transacylation

In order for the cysteine in the active site to act as a nucleophile it must be deprotonated. The way deprotonation occurs could be suggested in part by comparing structures of condensing enzymes. Since the cysteine is located at the N-terminus of an α-helix in all condensing enzymes it is expected that its pKa is lowered by this position to the extent that a histidine residue can assist in its deprotonation (38).

The participation of a histidine residue in cysteine deprotonation has been studied biochemically in Kas III from \textit{C. wrightii} and \textit{E. coli}, chalcone synthase and thiolase (38,48-50). The condensation activity as a function of pH shifted from bell shaped, in wt Kas III from \textit{C. wrightii}, into linear when the active site histidine was mutated into arginine suggesting the active site histidine to act in cysteine deprotonation (48). In Kas III from \textit{E. coli} the rate of transacylation at pH 7.0 was six times higher in the His244Ala mutant than in the wt enzyme but at pH 6.0 the wt performs better than the mutant (38). From this study it was then suggested that the helix-dipole could be enough for deprotonation under physiological conditions (38). The pKa of the cysteine nucleophile in wt chalcone synthase, examined using iodoacetamide, is 5.5 but shifts to 6.6 or 7.6 when the active site His303 is mutated into glutamine or alanine respectively indicating deprotonation by the histidine to occur (49). In contrast, steady state kinetic measurements performed at pH 7 of the His303Gln mutant of chalcone synthase indicate that the cysteine nucleophile must already be deprotonated at pH 7 (51) probably by the helix-dipole. The His303Ala mutant is unable to perform decarboxylation and therefore inactive (51). In thiolase the His348Asn mutant retained 7% of the wt activity and His348 was therefore not interpreted as a catalytic base by the authors (50). Labelling of thiolase His348 with diethyl pyrocarbonate inactivates the enzyme (50) but not necessarily due to loss in ability of His348 to deprotonate Cys89.

In Kas I each histidine in the active site has been mutated into alanine and assayed for the rate of transacylation of myristic acid from ACP to the enzyme (31). The result of these assays conducted at pH 6.8 conclude that neither of the histidines are important to the rate of transacylation and thereby the nucleophilicity of the active site cysteine (31). Note that removal of either histidine will make the active site cysteine more accessible to solvent and myristoyl-ACP substrates and at pH 6.8 a population of Kas I molecules might already have deprotonated cysteine residues caused by the helix-dipole effect. The transacylation from acyl-ACP to Kas I as a function of pH was monitored and found to be bell-shaped for the wt enzyme but neither of the histidine to alanine mutants were analysed in this way (31).

The identity of the potentially deprotonating histidine is not an issue in Kas III, PKS or thiolase which have a single histidine in their active sites but in Kas I and Kas II there are two histidines present in the active site. Structural relations between condensing enzymes suggest His344 to be the histidine assisting in deprotonation of the nucleophile based on the structural similarity to thiolase. His344 is also closer (3.3Å) to the cysteine than His307 (4.4Å) and since the conserved Lys339 makes a hydrogen bond to the Nδ1 atom of His307 this histidine would be an unfavourable proton
acceptor (Paper II). Others suggest the other histidine to be the deprotonating histidine in Kas I and Kas II based on the observation of a potential hydroxide ion 2.5 Å from the Nδ1 atom of His298 (Synechocystis His307) in Kas I determined to 1.9 Å resolution (32). A hydroxide in this position would allow for protonation of His298 to occur but the presence of this hydroxide has not been observed in other high-resolution crystal structures of Kas I or Kas II and this way of deprotonation is therefore considered less likely. A remote possibility is that deprotonation by the active site cysteine occurs by a different mechanism in Kas I and Kas II enzymes.

The transition state of transacylation is most likely stabilised by the main chain oxanion hole i.e. independent of the histidine side chains in Kas II (Fig. 4). Despite the close structural relation between thiolase and Kas II transfer of the first acetyl group from acetyl-CoA is assumed to be stabilised by His348 and wat88 in thiolase i.e. stabilised by the motif corresponding to the side chain oxanion hole in Kas II (52).

The transacylation step is likewise suggested to be different between Kas III and chalcone synthase despite their close structural relationship in the active site (Fig. 6, right). In Kas III, transacylation is assumed to be facilitated by the main chain oxanion hole (38,53). Since the main chain oxanion hole is defective in PKS enzymes the transition state of transacylation when loading the starter unit (p-coumaroyl-CoA in chalcone synthase or acetyl-CoA in 2-pyrones synthase) is expected to be stabilised by the side chain oxanion hole like in thiolase (52,54).

2.3.2 Carbanion formation by malonyl decarboxylation vs. acetyl deprotonation

All condensing enzymes except thiolase, catalyse decarboxylation of a malonyl group attached to CoA or ACP, while biosynthetic thiolase activates the acetyl group of acetyl-CoA, to create the reactive carbanion necessary for condensation. The side chain oxanion hole created by the active site histidine residues is crucial for decarboxylation in Kas I and Kas II (Fig. 4), supported by the thiolactomycin structure and site directed mutagenesis (27,30,31). In a study using mammalian FAS from rat, the active site cysteine was mutated into glutamine and found to significantly increase the rate of decarboxylation (55). The introduced glutamine could block the main chain oxanion hole just like the fatty acid intermediates appear to do. The bound fatty acid intermediates blocking the main chain oxanion hole somehow enhance the ability of the side chain oxanion hole to facilitate decarboxylation. This makes sense since an empty condensing enzyme being an effective malonyl decarboxylase would quickly deplete the amount of malonyl-ACP substrates available for elongation.

When acetyl-CoA binds to thiolase the thioester carbonyl oxygen binds in the oxanion hole created by His348 and wat88 just like the corresponding oxygen of malonyl-ACP is expected to bind to Kas II (52,56) (Fig. 4). In thiolase the thioester oxygen of the acetylated intermediate is located in between the main and the side chain oxanion holes (41). Binding of the second acetyl-CoA molecule appear to drive the thioester oxygen of the intermediate away from the side chain oxanion hole into the main chain
oxyanion hole (52). In contrast, the fatty acid intermediates in Kas II readily bind with their thioester oxygen inside the main chain oxyanion hole leaving the side chain oxyanion hole free to facilitate decarboxylation (32). The partial blocking of the side chain oxyanion hole in thiolase might therefore contribute to the inability of thiolase to decarboxylate malonyl-CoA. Once the second molecule of acetyl-CoA is bound activation of the acetyl group is facilitated by the second cysteine (Cys378) present in thiolase (41,57,58).

The side chain oxyanion holes of Kas III and the two PKS enzymes are known to facilitate decarboxylation in a manner similar to the Kas I and Kas II enzymes (38,51,54).

2.3.3 The condensation step
During the condensation step, the reactive carbanion attacks the enzyme bound intermediate and the new carbon-carbon bond is formed. The main chain oxyanion hole is expected to stabilise the transition state of condensation in Kas I, Kas II, Kas III enzymes and the thiolases (28,52,53).

The main chain oxyanion hole is not complete in chalcone synthase and pyrone synthase, where only the main chain NH-group from the active site cysteine is left (Fig. 6, right) and therefore the side chain oxyanion hole is expected to stabilise the transition state of condensation in the PKS enzymes (54). PKS enzymes differ from Kas III and the other condensing enzymes in that they do not release their substrates for each new round of elongation (54). After condensation in chalcone synthase or pyrone synthase the enzyme traps the product in the active site again and waits for the next unit of malonyl-CoA to bind. After three rounds of elongation the PKS enzymes releases their substrates since they become too large to fit into the active site pocket (44).

2.4 FATTY ACID BIOSYNTHESIS AS A DRUG TARGET

2.4.1 Antibiotics against bacteria and parasites
The fatty acid biosynthetic pathway is known to contain useful targets for bacterial antibiotics e.g. enoyl-ACP reductase, which is the target for isoniazid used against tuberculosis and triclosan commonly found in toothpaste (59,60). *Mycobacterium tuberculosis*, the causative agent of tuberculosis, has been sequenced and shown to contain more genes for fatty acid synthesis than most other bacteria (61). Kas A from *M. tuberculosis*, a homologue to *E. coli* Kas I and Kas II, is overexpressed during isoniazid treatment but is not a direct target for isoniazid as was initially suggested (62,63). Thiolactomycin is non-toxic to mammals (24,64) and known to target Kas enzymes from *M. tuberculosis* in multidrug resistant strains of the bacteria (65).
*Plasmodium falciparum* causing malaria has recently been shown to contain a plastid (known as the apicoplast in the parasite) for fatty acid biosynthesis and the parasite is sensitive to tricosan and thirolactomycin (66,67). The parasite *Trypanosoma brucei* causing sleeping sickness has recently been shown to synthesise fatty acids using its own Kas enzymes in the bloodstream of the host to meet its strong requirement for myristate (68,69). Cerulenin inhibits one of the Kas enzymes from *T. brucei* from elongating fatty acids with more than ten carbon atoms and thirolactomycin kills the parasite in culture (68).

2.4.1.1 Cerulenin sensitivity of *M. tuberculosis* Kas III

Kas III from *M. tuberculosis* prefers to elongate lauroyl-CoA (C12:0) but can also elongate longer fatty acids such as palmitoyl-CoA (C16:0) (45). One might therefore expect Kas III from *M. tuberculosis* to be as cerulenin sensitive as Kas I or Kas II from *E. coli* but this turns out not to be the case (45). Cerulenin appears to demand a hydrophobic pocket orientated in a certain way with respect to the main and the side chain oxygen hole (27) (Paper I). Cerulenin might therefore be less effective in binding to *M. tuberculosis* Kas III since both the hydrophobic pocket that binds the acyl chain and the side chain oxygen hole have a different orientation in Kas III compared to Kas I or Kas II (Fig. 6). From mutational studies of *E. coli* Kas I it was found that the His333Asn mutant is more resistant to cerulenin than wt Kas I (27).
2.4.2 Mammalian FAS, Obesity and Cancer

Ceruleni
in has the ability to inhibit mammalian fatty acid synthases and this caused (and still causes) hesitation for derivatives of this molecule to be used as a drug in humans. C75 is a compound derived from ceruleni
tin that targets mammalian FAS and feeding this compound (C75) to mice causes a reversible loss of weight, reduction in adipose tissue and hopes for a drug against obesity (70). Mice fed with C75 are not loosing weight as a secondary effect due to illness or malaise (71). Mammalian FAS is expressed in the human brain and co-localised with neuropeptide Y (71,72) known since long to be involved in feeding (73,74).

Mammalian FAS is known to be down-regulated by dietary fatty acids in many human tissues but highly expressed in several human cancers cells such as breast, prostate, colon and ovarian cell lines in order to synthesise their own supply of fatty acids (75-77). Human FAS is therefore a putative cancer marker (77) and also an attractive target for cancer chemotherapy since selective targeting of cancer cells can occur. When tumour cells are exposed to ceruleni
tin they turn apoptotic via the mitochondrial pathway (78). This apoptosis in cancer cells appears to be induced by excess malonyl-CoA i.e. the substrate for mammalian FAS (79,80). Acetyl-CoA carboxylase, generating malonyl-CoA from acetyl-CoA, has also attracted attention in this respect (81). A small research company named FASgen Inc. associated to John Hopkins University in Baltimore is working to exploit these ideas.
2.5 CONCLUSIONS

Based on biochemical data and crystal structures of inhibitor and fatty acid intermediate complexes, the transition state of transacylation and condensation appear to be stabilised by the main chain oxyanion hole, while decarboxylation is facilitated by the side chain oxyanion hole, during Kas II catalysis.

These two oxyanion holes are present in most condensing enzymes and built up by a unique set of residues for each structural class except in the PKS enzymes that are lacking one component of the main chain oxyanion hole. The spatial location of the side chain oxyanion hole is rather similar between thiolase and the Kas I and Kas II enzymes but slightly different from the Kas III and PKS enzymes. Provided the orientation of the imidazole ring of the active site histidines are the same between Kas III and the PKS enzymes they will have the side chain oxyanion hole at identical positions in the active site. Despite the close spatial relationship in the active site the two oxyanion holes seem to be utilised quite differently for catalysis in the different condensing enzymes.

According to current knowledge the transition state of transacylation is stabilised by the main chain oxyanion hole in Kas I, Kas II and the Kas III enzymes and by the side chain oxyanion hole in the PKS and thiolase enzymes. The differences in how the side chain oxyanion hole is utilised between the Kas I and Kas II enzymes and the thiolases could depend in part of the different building blocks, a histidine versus a water molecule, that build up the motif.

The condensation step involves release of the enzyme bound intermediate and the transition state is supposed to be stabilised by the main chain oxyanion hole in Kas I, Kas II and the Kas III enzymes and the thiolases. In the PKS enzymes however where the main chain oxyanion hole is defect, the substrate stays in the active site for three rounds of elongation with malonyl-CoA, until it is expelled due to lack of space in the active site.

The helix-dipole effect is important for deprotonation of the catalytic cysteine nucleophile in all condensing enzymes, but a role for an active site histidine can not be excluded at this time.

This summary shows that it is difficult to make predictions, regarding catalytic mechanism, for a particular condensing enzyme by adapting a catalytic mechanism determined for a structurally related enzyme(s). Further studies, in particular more crystal structures of substrate, intermediate and product complexes, will be essential to unravel more details regarding the utilisation of the two oxyanion holes in condensing enzymes. The condensing enzymes in the fatty acid biosynthesis pathway are well worth studying from a medical point of view to hopefully provide drugs against cancer and obesity or new antibiotics against bacteria or parasites.
3 STRUCTURAL STUDIES OF STEAROYL ACYL CARRIER PROTEIN DESATURASE

3.1 INTRODUCTION

This summary is based on the crystal structures of the apo and one iron form of desaturase, the azide and the acetate complexes of the desaturase di-iron centre and the crystal structure of the complex between oxidised desaturase and its substrate stearoyl-ACP. The aim is to discuss structure-function relations regarding substrate specificity, activation of molecular oxygen and oxidation of the substrate, with an outline that follows the expected order of catalytic events in desaturase. Some suggestions on how to redesign regio-specificity in soluble desaturases are also presented and the concept of an ACP "recognition helix" will be addressed.

3.1.1 Molecular oxygen and iron

Molecular oxygen is a natural product of photosynthesis and a very strong oxidizing agent having the second highest electro-negativity of all elements. Despite this, it is a rather stable molecule that requires "activation" to interact with other molecules (82). Molecular oxygen is a paramagnetic species with two unpaired electrons in its triplet ground state. Most molecules are diamagnetic however and therefore have difficulties in interacting with molecular oxygen in its ground state since this would involve a change of spin state, which is "forbidden" or unlikely to occur. Molecular oxygen in its ground state therefore prefers to interact with paramagnetic species such as metals. Singlet oxygen however, which is an excited and diamagnetic state of molecular oxygen, readily interact with bio-molecules. Hydrogen peroxide is a two step reduced form of molecular oxygen that is diamagnetic and therefore interacts with most bio-molecules although not as violent as the excited singlet molecular oxygen species, due to its partial reduction.

In biological systems iron often occurs as Fe$^{2+}$ or Fe$^{3+}$ that in addition can be in the high spin or the low spin state. The high spin Fe$^{2+}$ state has the largest ion radius i.e. the most loosely bound electrons of these four states and is further paramagnetic so there is no "spin-restriction" for its interaction with molecular oxygen to occur. The low spin Fe$^{3+}$ is diamagnetic and therefore unsuitable for activation of molecular oxygen but not for reversible binding of molecular oxygen, this state is used by e.g. haemoglobin (83,84).

There exist three major classes of iron proteins that can activate molecular oxygen mononuclear, di-iron and the heme containing enzymes. "Ligand field theory" states the energy levels of the d-orbital electrons surrounding the iron ions as determined by the (protein) ligands that coordinate the metal. These proteins thereby control the spin states of their iron(s) for various purposes. Common to the di-iron and the heme enzymes is that different members of these two classes can be utilised both for
activation and reversible binding of molecular oxygen (haemoglobin and hemerythrin), while mononuclear iron enzymes apparently only activate molecular oxygen (85).

Stearoyl ACP desaturase is a di-iron enzyme that activates molecular oxygen for desaturation of fatty acids (see below). To activate molecular oxygen, the di-iron centre needs to be accessible for the oxygen molecule and should be in the reduced (ferrous) and paramagnetic state. Although each of the two iron ions in a desaturase di-iron centre is paramagnetic the di-iron centre as a whole might be diamagnetic due to anti-ferromagnetic coupling between the two irons. This is frequently occurring among di-iron enzymes especially in the oxidised state where a μ-oxo bridge can mediate the anti-ferromagnetic coupling (86-88).

3.1.2 Stearoyl acyl carrier protein desaturase

Soluble desaturases are the primary determinants for the composition of unsaturated fatty acids in plant membranes or seed storage oils (see general introduction). Stearoyl acyl carrier protein desaturase (18:0Δ9 desaturase) introduces the first cis double bond between carbon 9 and 10 into stearoyl-ACP forming oleoyl-ACP (89,90). The nomenclature 18:0Δ9 means that the double bond (Δ9) introduced into the fatty acid product are positioned between carbon 9 and 10, counting from the carboxyl end of stearic acid (18:0). 18:0Δ9 desaturases occur widely among plant species but in addition, seeds from some plants contain desaturase isozymes with different chain-length and/or regio-specificity relative to the common 18:0Δ9 desaturases e.g. 16:0Δ4 and 16:0Δ6 and are involved in biosynthesis of unusual fatty acids in their seeds (91-95).

The first crystal structure of a soluble desaturase, that of 18:0Δ9 desaturase from *Ricinus Communis* (Fig. 7), was determined in 1996 (96). Desaturase is a mainly helical protein and the structure reveals a hydrophobic "boomerang-shaped" cavity for fatty acid binding in vicinity of the di-iron centre located within a four-helix bundle (96). The ordering of catalytic events in soluble desaturases is not known, but it is assumed that binding of acyl-ACP occurs first, followed by reduction of the di-iron centre by ferredoxin and finally, activation of molecular oxygen by the di-iron centre, leading to oxidation of the bound fatty acid substrate. If the desaturase di-iron centre is reduced in the absence of fatty acid substrate, reactive oxygen species could be created. This order of events could be made in a simple way if the reducing agent, ferredoxin, would bind only to the complex between desaturase and stearoyl-ACP. Desaturase belongs to the same structural class of large four-helix bundle, di-iron carboxylate proteins as methane mono-oxygenase hydroxylase (MMOH) or the R2 subunit of ribonucleotide reductase (97) which also activate molecular oxygen for various purposes. Other smaller four-helix bundle di-iron proteins are ruberythrin that acts as an NADH peroxidase (98), bacterioferritin that mineralises and stores iron in its Fe3+ form (99) and the oxygen carrier hemerythrin (100).
Acyl Carrier Protein (ACP) is a small (~80 amino acids) acidic protein that solubilises and presents fatty acid intermediates to a large number of enzymes during type II fatty acid biosynthesis (see general introduction). Higher plants have multiple ACP isoforms that differ in expression levels depending on tissue type, developmental stage and environment (101-103). ACP is present in three distinct forms in the cell called apo-ACP, holo-ACP and acyl-ACP. Holo-ACP is formed after addition of the pantetheine cofactor to apo-ACP by the enzyme holo-acyl carrier protein synthase (AcpS) (104). Acyl-ACP is then formed after addition of the fatty acid acyl chain to the pantetheine cofactor of holo-ACP by the integral membrane enzyme acyl-acyl carrier protein synthase (Aas) (105). Both NMR (106-108) and crystal (109) structures of the helical ACP protein are available, and a recent crystal structure of E. coli ACP suggests a way for ACP to solubilise fatty acids by binding them in an interior cavity (109). A crystal structure of the complex between holo-ACP and AcpS from B. subtilis is also available (110) together with two theoretical docking models between ACP and Kas III from E. coli (111) or malonyl CoA:ACP transacylase from Streptomyces coelicolor (112). These docking models together with ACP sequence alignments suggest that ACP uses a conserved “recognition helix” to recognise several target enzymes during type II fatty acid biosynthesis (113).
3.2 CRYSTAL STRUCTURE OF THE COMPLEX BETWEEN OXIDISED DESATURASE AND ITS SUBSTRATE; STEAROYL-ACP

The structure of the complex between 18:0Δ9 desaturase from Ricinus Communis and stearoyl-ACP from spinach has been determined to 3.1 Å resolution (Fig. 8, Paper IV). Unexpectedly, there are three monomers of desaturase and only a single monomer of stearoyl-ACP present in the asymmetric unit. Before crystallisation, a 2:2 stoichiometry was observed using electrospray mass spectrometry (ESI) but in our crystals, a mixture of dimeric desaturase (2:0) and dimeric desaturase binding a single monomer of stearoyl-ACP (2:1) are present (Paper IV). In a typical in vitro assay the stoichiometry between desaturase and stearoyl-ACP is 2:2 as recently determined (114) but the in vivo situation in the plant plastid is currently unknown.

![Figure 8. The complex between desaturase and stearoyl-ACP recorded prior to crystallisation (ESI spectrum) and crystal structure.](image)

The electron density is of lower quality for the ACP part of the protein complex than for the desaturase part, especially with respect to many of the side chains. This could be due to less than full occupancy of ACP or a higher mobility of ACP or a combination of both. For some parts of our model, the electron density is not of sufficient quality to discuss details of the spinach ACP structure. However the overall root mean square deviation between spinach ACP and the structures from E. coli (109) and B. subtilis ACP (110) are 1.26 Å for 70 residues and 1.31 Å for 68 residues respectively. The corresponding sequence identities between spinach-ACP and E. coli and B. subtilis ACP are 37 % and 35 % respectively. Despite the low resolution and less well-defined electron density for ACP, the model of the complex confidently shows how the interacting proteins are arranged with respect to each other. It also gives some details about which side chains are involved in the interaction that are...
defined by the electron density, or are likely to be involved (when only the main chain is defined).

The interaction between desaturase and ACP buries 560 Å² in its interface and involves seven positively charged desaturase residues and seven negatively charged ACP residues. This electrostatic interaction occurs at a positive patch on the desaturase surface in vicinity of the hydrophobic fatty acid binding pocket. Residues contributing to the ACP interface, their interaction partners, and the definition of these side chains in the electron density has been described in detail (Paper IV). The interaction between desaturase and ACP could be summarised as consisting of at least three salt links, a few longer range electrostatic attractions but little contribution from hydrophobic interactions (Fig. 9).

![Figure 9](image)

**Figure 9.** 2fo-fe electron density map contoured at 1σ and some important residues in the interaction interface. Desaturase labels are in bold italic.

### 3.3 CRYSTAL STRUCTURES OF THE DESATURASE DI-IRON CENTRE

In recent years the desaturase di-iron centre has been observed in a number of different states (Fig. 10, Paper III and Paper IV). In the original structure determination (96), the di-iron centre appears photo-reduced by the X-ray beam, since the iron-iron distance was 4.2 Å and the μ-oxo bridge present in the oxidised state was absent (96). The di-iron centre is located within a four-helix bundle, where two pairs of anti-parallel helices provide ligands to the iron ions: α3-Glu105, α4-Glu143 and His146, α6-Glu196, α7-Glu229 and His232 (Fig. 10). The two irons are five-coordinated and they have distorted square pyramidal coordination geometry. Glu105 is a bi-dentate ligand to Fe1 and correspondingly, Glu196 is a bi-dentate ligand to Fe2. Both Glu143 and Glu229 act as bridging ligands between the iron ions. In addition to the carboxylate ligands, each iron has one nitrogen atom ligand, N81 of His146 and His232 respectively. A
The structure of the apo-form of desaturase has been determined in space group P3₁ to 3.2 Å resolution at pH 6.7, in presence of ADA buffer that acted as a metal chelator (Paper III). Electron density for the di-iron cluster was completely absent and the normally liganding residues showed some disorder. In the one-iron form of the desaturase di-iron centre, that was determined in space group P2₁2₁2₁ to 2.8 Å resolution at pH 4, Fe₁ remains in the active site, while Fe₂ is lost to the surrounding solvent. The iron depleted desaturase structures demonstrate that desaturase is able to form a stable structure in absence of iron ions and that these irons bind with different affinity to the di-iron centre, with Fe₁ as the high affinity, and Fe₂ as the low affinity site (Paper III).

**Figure 10. Crystal structures available of the desaturase di-iron centre.**

The oxidised di-iron centre has been determined from the P3₁2₁ crystals of the ACP complex that were made at 4 °C at pH 5.62 and diffracted to 3.1 Å resolution (Paper IV). The structure demonstrates that His232 is not an iron ligand in oxidised desaturase since the low affinity Fe₂ has moved 1.5 Å away from His232. The iron-iron distance
in the active site is 3.1, 3.3 and 3.4 Å in the three subunits of the asymmetric unit, consistent with previous EXAFS studies that reveal two distinct di-iron clusters with an iron-iron distance of either 3.12 or 3.41 Å (115). Mössbauer spectroscopy of desaturase as isolated, reveals that the oxidised enzyme consists of two ferric (Fe$^{3+}$) paramagnetic irons that are anti-ferromagnetically coupled to each other, making the di-iron centre into a diamagnetic species (88). The anti-ferromagnetic coupling is mediated through the μ-oxo bridge that was initially suggested to exist based on this feature and later verified by Raman spectroscopy (116). We are unable at this time to verify the presence of this μ-oxo bridge using our 3.1 Å resolution data of the oxidised di-iron centre (Paper IV), although there is an open coordination position available on each iron.

Glu229 is bridging the diferric di-iron centre as a mono-dentate ligand and should therefore contribute to the anti-ferromagnetic coupling responsible for the observed diamagnetism. The carboxylate shift observed for Glu229 must be treated with considerable care because of the low resolution but the calculated simulated annealing omit maps were of high quality (Paper IV). In fact, the side chain oxygen in Glu229, bridging the iron ions in diferric desaturase, is located where the μ-oxo bridge would be in oxidised diferric MMOH (117) and R2 (118). This means that the μ-oxo bridge of diferric desaturase could be located like the μ-oxo bridge in ruberythrin (119, 120) i.e. different from how the μ-oxo bridges are positioned in MMOH or R2. Another argument to suggest the μ-oxo bridge in desaturase to be located at the same side of the iron-iron axis as in ruberythrin, can be derived by comparing structures of the di-iron centre in complex with azide and in different redox states between desaturase (Paper III) and ruberythrin (120). When azide binds to desaturase, the μ-oxo bridge is known from spectroscopy to be either protonated or lost (121). The azide complexes of desaturase and ruberythrin are strikingly similar (see below) and in ruberythrin, azide occupies the position of the μ-oxo bridge. The Fe2 movement and loss of histidine coordination seen here in oxidised desaturase, as compared to reduced desaturase, is similar to how Fe1 in ruberythrin changes position between different redox states (120). Together, these similarities between desaturase and ruberythrin, argues for the μ-oxo bridge in desaturase to be located at the same side of the iron-iron axis as in ruberythrin.

The azide complex has been determined to 2.4 Å resolution in space group P2$_1$2$_1$2$_1$ by co-crystallising desaturase with 70mM sodium azide at pH 6.2 (Paper III). The azide ion binds the iron cluster in a μ-1,3 bridging mode, with iron-nitrogen distances of 2.5-2.6 Å thereby displacing the weakly bound water molecule present in the reduced state. There are no significant structural changes, in the geometry of the coordinating carboxylate or histidine ligands, induced by azide binding, compared to the structure of the original ferrous di-iron centre. The iron-iron distance is 4.1 Å and neither electron density nor coordination positions are available for the μ-oxo bridge. An earlier spectroscopic study demonstrated that when azide binds to oxidised desaturase, the μ-oxo bridge is either protonated or lost (121). Spectroscopy further predicted two
different and pH dependent binding models of azide, with ~90 % being in the μ-1,3 bridging mode at pH 6.2 (121), which is the pH used in our crystallisation (Paper III).

The acetate complex structure has been determined from P3₁₁₂ crystals, obtained in identical crystallisation conditions as the original P2₁₂₂₁ crystals (addition of glycerol favours the formation of the P3₁₁₂ crystals), to 2.4 Å resolution (Paper III). Acetate from the crystallisation buffer, is bridging the di-iron centre with iron-oxygen distances of 2.6 and 2.5 Å to Fe1 and Fe2 respectively. The iron-iron distance is 4.0Å and Glu196 here appears as a mono-dentate ligand to Fe2. The carboxylate shift observed was validated by calculating simulated annealing omit maps and by disturbing the structure back into its original position for re-refinement. Carboxylate shifts are expected to have an important role during di-iron enzyme catalysis (122). Acetate binding and the Glu196 carboxylate shift result in a distorted octahedral geometry of Fe1 (5 protein ligands plus one acetate ligand) and distorted trigonal bipyramidal geometry of Fe2 (four protein ligands and one acetate ligand) (Paper III).

In common between the azide and the acetate complexes are the presence of eight water molecules (four from each subunit), that form a pathway across the dimer interface connecting the high affinity iron ions (Fe1), that are 23.5 Å apart (Paper III). These water molecules form hydrogen bonds to both main chain and side chain residues of the protein and are well defined in the electron density maps. Three of the four waters were already present (96) in the original reduced desaturase structure and the distance between the azide and the closest water in this “pathway” is 3.6 Å.
3.4 THE CATALYTIC CYCLE OF SOLUBLE DESATURASE

The order of catalytic events during fatty acid desaturation by soluble desaturases remain to be fully characterised (see introduction) and the brief suggestion in figure 11 below might be incorrect on some points.

\[
\begin{align*}
&\Delta^9\text{D}_{\text{oxo}}\cdot\Delta^9\text{D}_{\text{oxo}} \to \Delta^9\text{D}_{\text{oxo}}\cdot\Delta^9\text{D}_{\text{oxo}}:18:0\text{ACP} \quad \text{(i)} \\
&\Delta^9\text{D}_{\text{oxo}}\cdot\Delta^9\text{D}_{\text{oxo}} \to 18:0\text{ACP} \quad \text{(ii)} \\
&\Delta^9\text{D}_{\text{oxo}}\cdot\Delta^9\text{D}_{\text{oxo}} \to 18:0\text{ACP} \quad \text{(iii)} \\
&\Delta^9\text{D}_{\text{oxo}}\cdot\Delta^9\text{D}_{\text{oxo}} \to 18:0\text{ACP} \quad \text{(iv)} \\
&\Delta^9\text{D}_{\text{oxo}}\cdot\Delta^9\text{D}_{\text{oxo}} \to 18:0\text{ACP} \quad \text{(v)} \\
&\Delta^9\text{D}_{\text{oxo}}\cdot\Delta^9\text{D}_{\text{oxo}} \to 18:0\text{ACP} \\
&2\text{Fd}_{\text{red}} \to \Delta^9\text{D}_{\text{oxo}}\cdot\Delta^9\text{D}_{\text{oxo}} \\
&\text{O}_2 \quad \text{to} \quad \text{H}_2\text{O} \quad \text{to} \quad 2\text{Fd}_{\text{ox}} \\
&18:1\text{ACP} \\
\end{align*}
\]

Figure 11. Catalytic cycle of soluble desaturases.

It remains to be determined whether desaturase catalysis occurs at a single subunit at a time, like in figure 11 above. It is assumed that the oxidised desaturase dimer (\(\Delta^9\text{D}_{\text{oxo}}\cdot\Delta^9\text{D}_{\text{oxo}}\)) initially binds stearoyl-ACP (18:0ACP) (i). After substrate binding desaturase binds ferredoxin (ii), and the di-iron centre is reduced in two single electron transfer steps and a water molecule is formed from the \(\mu\)-oxo bridge. After reduction of the desaturase di-iron centre (ii), ferredoxin can leave and the di-iron centre is ready to interact with molecular oxygen (iii), leading to formation of reactive oxygen intermediates e.g. a peroxy-diferric intermediate. The reactive oxygen intermediate that is able to abstract hydrogen atoms from the bound stearic acid and form oleoyl-ACP (iv), remains to be characterised. The peroxy intermediate gives rise to a \(\mu\)-oxo bridge and a water molecule and finally the product oleoyl-ACP (18:1ACP) can leave (v) from the active site, thereby completing the catalytic cycle.

In the remainder of this section the ambition is to take a closer look at some of these catalytic events.

3.4.1 Substrate recognition by desaturase

Analysis of steady-state kinetic parameters for desaturation reveals a linear increase in \(\log (k_{\text{cat}}/K_M)\) when the length of the acyl chain in acyl-ACP is varied between 15 and 18 carbons giving a \(\Delta\text{G}^{\text{binding}}\) of 3 kJ/mol per methylene group (123). By using ACP that was labelled with a fluorescent group, it was demonstrated that the acyl chain contributes significantly to the binding affinity between desaturase and stearoyl-ACP (114). This is reasonable since if holo-ACP would bind effectively in the absence of
the acyl chain, holo-ACP would be an effective inhibitor for all enzymes that use acyl-ACP for catalysis. For desaturase, holo-ACP is known to be a poor inhibitor (123). Also the release of ACP from its interaction partners could be too slow if both the acyl chain and the interacting surface gave strong contributions to the binding affinity. It is therefore not surprising that the buried surface area observed in the desaturase ACP complex is small (Paper IV), although there exists a significant electrostatic interaction between the surfaces that enables an initial recognition between these proteins to occur. Long-range electrostatic interactions are suitable and proven essential to the on-rate and thereby binding affinity and recognition between macromolecules (124). Short-range (e.g. hydrophobic) interactions influence the off-rate and for desaturase the off-rate of acyl-ACP is dependent on the length of the acyl-chain with a slower off-rate for longer acyl-chains (114).

3.4.1.1 Fatty acid chain-length specificity in desaturase

The original crystal structure (96) of the desaturase fatty acid binding pocket made a large impact in guiding mutagenesis efforts to alter desaturase chain-length specificity (94,125). In a pilot study, rational design involving site-directed mutagenesis was performed on the wt castor 18:0Δ9 desaturase making it into a 16:0Δ9 desaturase by replacement of two residues Leu118Phe/Pro179Ile (126).

![Figure 12. Stearic acid docked manually in the crystal structure of the fatty acid binding pocket of desaturase.](image)

These and other studies (91,93,95,127) have confirmed that in particular six amino acids lining the proposed fatty acid binding pocket (Met114, Thr117, Leu118, Pro179, Thr181 and Gly188) are important for chain-length specificity among soluble desaturases (Fig. 12). These six residues are invariant among 48 of the 59 available desaturase genes and all desaturases with unusual chain-length or regio-specificity have substitutions among these residues. The eleven desaturase proteins with substitutions among these six residues are listed in Table 2. A few of these desaturase gene-
products remain to be biochemically characterised but it would not be surprising to find either unusual chain-length or/and regio-specificity among these genes (Table 2).

Table 2. Desaturase genes with substitutions among the residues lining the fatty acid binding pocket known to be important to specificity among soluble desaturases.

<table>
<thead>
<tr>
<th>Residue Number</th>
<th>114</th>
<th>117</th>
<th>118</th>
<th>179</th>
<th>181</th>
<th>188</th>
</tr>
</thead>
<tbody>
<tr>
<td># genes invariant at this position (59 genes available)</td>
<td>55 Met</td>
<td>53 Thr</td>
<td>53 Leu</td>
<td>52 Pro</td>
<td>54 Thr</td>
<td>56 Gly</td>
</tr>
<tr>
<td>Castor(^a)</td>
<td>18:0(^a)</td>
<td>Met</td>
<td>Thr</td>
<td>Leu</td>
<td>Pro</td>
<td>Thr</td>
</tr>
<tr>
<td>Milkweed(^b)</td>
<td>18:0(^a)</td>
<td>Met</td>
<td>Arg</td>
<td>Leu</td>
<td>Thr</td>
<td>Thr</td>
</tr>
<tr>
<td>Geranium(^c)</td>
<td>14:0(^a)</td>
<td>Leu</td>
<td>Arg</td>
<td>Pro</td>
<td>Ile</td>
<td>Thr</td>
</tr>
<tr>
<td><em>T. alata</em>(^d)</td>
<td>16:0(^a)</td>
<td>Met</td>
<td>Thr</td>
<td>Thr</td>
<td>Pro</td>
<td>Ala</td>
</tr>
<tr>
<td>Ivy(^e)</td>
<td>16:0(^a)</td>
<td>Met</td>
<td>Arg</td>
<td>Cys</td>
<td>Ile</td>
<td>Ser</td>
</tr>
<tr>
<td>Coriander(^f)</td>
<td>16:0(^a)</td>
<td>Met</td>
<td>Arg</td>
<td>Cys</td>
<td>Thr</td>
<td>Thr</td>
</tr>
<tr>
<td>Cat Claw(^g)</td>
<td>16:0(^a)</td>
<td>Met</td>
<td>Thr</td>
<td>Trp</td>
<td>Pro</td>
<td>Thr</td>
</tr>
<tr>
<td><em>B. scoparia</em>(^h)</td>
<td>Met</td>
<td>Ile</td>
<td>Ile</td>
<td>Pro</td>
<td>Thr</td>
<td>Ala</td>
</tr>
<tr>
<td><em>A. thaliana</em>(^i)</td>
<td>Thr</td>
<td>Thr</td>
<td>Leu</td>
<td>Ser</td>
<td>Glu</td>
<td>Gly</td>
</tr>
<tr>
<td><em>A. thaliana</em>(^j)</td>
<td>Val</td>
<td>Thr</td>
<td>Leu</td>
<td>Thr</td>
<td>Tyr</td>
<td>Gly</td>
</tr>
<tr>
<td><em>A. thaliana</em>(^k)</td>
<td>Thr</td>
<td>Thr</td>
<td>Leu</td>
<td>Ser</td>
<td>Glu</td>
<td>Gly</td>
</tr>
<tr>
<td><em>L. Luteus</em>(^l)</td>
<td>Met</td>
<td>Asn</td>
<td>Leu</td>
<td>Pro</td>
<td>Thr</td>
<td>Gly</td>
</tr>
</tbody>
</table>

\(^{a}\) Genbank accession code gi|134945  
\(^{b}\) Genbank accession code gi|1762436  
\(^{c}\) Genbank accession code gi|1304409  
\(^{d}\) Genbank accession code gi|595295  
\(^{e}\) Genbank accession code not available  
\(^{f}\) Genbank accession code gi|417819  
\(^{g}\) Genbank accession code gi|2944444  
\(^{h}\) Genbank accession code gi|16588700  
\(^{i}\) Genbank accession code gi|15232941  
\(^{j}\) Genbank accession code gi|15232942  
\(^{k}\) Genbank accession code gi|15237307  
\(^{l}\) Genbank accession code gi|704824

3.4.1.2 The ACP recognition helix

Bacterial ACP can substitute for plant ACP in the reaction with plant enzymes, arguing for a conserved functionality among most plant and bacterial ACPs and after alignment of 171 plant and bacterial ACPs, it can be noticed that the most conserved residues turn up in the desaturase interface and belong to the putative ACP recognition helix. Ser39 carrying the pantetheine group of ACP is strictly conserved among all 171 plant and bacterial ACP sequences. Asp38 is the second most conserved residue and present in 167 of the plant and bacterial sequences and shown to be crucial for attachment of the pantetheine cofactor to apo-ACP by holo-ACP synthase (AcpS) in *E. coli* (128). Other residues from the recognition-helix, Leu40, Glu41, Glu44 and Glu50 are also highly conserved among plant and bacterial ACPs. Site directed mutagenesis has shown
Glu44 (corresponding to Glu41 in *Vibrio harveyi*) to be essential for productive interaction between acyl-ACP and fatty acid synthase in *Vibrio harveyi* (129).

The interface between the plant castor desaturase and spinach ACP (Paper IV) has been compared to the interface between (holo-) acyl carrier protein synthase and ACP from *Bacillus subtilis* (pdb entry 1F80) (110). From a structural alignment of the two complexes using the ACP molecule it can be seen that while the same structural motif from ACP is used for binding, there is no structural motif in common between desaturase and ACP synthase for binding of ACP. However, the contribution in % of each ACP residue to the total buried surface area has a similar distribution for these two complexes (Table 3).

<table>
<thead>
<tr>
<th>B. Subtilis ACP</th>
<th>Interface ASA (%)</th>
<th>Spinach ACP</th>
<th>Interface ASA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp35</td>
<td>2.5</td>
<td>Asp38</td>
<td>6.2</td>
</tr>
<tr>
<td>Ser36 + panetheine</td>
<td>11.8</td>
<td>Ser39 + phosphate</td>
<td>7.5</td>
</tr>
<tr>
<td>Leu37</td>
<td>16.1</td>
<td>Leu40</td>
<td>19.9</td>
</tr>
<tr>
<td>Asp38</td>
<td>1.6</td>
<td>Asp41</td>
<td>2.1</td>
</tr>
<tr>
<td>Val40</td>
<td>6.3</td>
<td>Val43</td>
<td>7.3</td>
</tr>
<tr>
<td>Glu41</td>
<td>8.2</td>
<td>Glu44</td>
<td>9.3</td>
</tr>
<tr>
<td>Val43</td>
<td>1.2</td>
<td>Val46</td>
<td>1.2</td>
</tr>
<tr>
<td>Met44</td>
<td>13.1</td>
<td>Met47</td>
<td>14.8</td>
</tr>
<tr>
<td>Glu47</td>
<td>3.5</td>
<td>Glu50</td>
<td>3.7</td>
</tr>
<tr>
<td>Asp48</td>
<td>4.7</td>
<td>Glu51</td>
<td>3.6</td>
</tr>
<tr>
<td>Asp56</td>
<td>12.4</td>
<td>Glu59</td>
<td>12.4</td>
</tr>
<tr>
<td>Glu57</td>
<td>4.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glu60</td>
<td>7.4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>% of total</td>
<td>93.6</td>
<td></td>
<td>88.0</td>
</tr>
</tbody>
</table>

The almost equivalent distributions in the interface accessible surface area (ASA), between the corresponding residues in spinach ACP and *B. subtilis* ACP are surprising since the surface of ACP synthase and desaturase have no obvious similarities, either in arrangement of secondary structure elements or in the positions of most of their side chains interacting with ACP, with two exceptions. In the structural alignment, Leu337 from desaturase is located on top of Phe25 from ACP synthase and interacts with Leu40 of spinach ACP and the corresponding Leu37 of *B. subtilis*, respectively. The alignment also puts the side chain of Arg340 from desaturase very close to Arg28 in ACP synthase, and Arg340 might thus act as a bridge between Glu50 and Glu59 in spinach ACP, similar to how Arg28 from ACP synthase is bridging Glu47 and Asp56 in *B. subtilis* ACP. In summary, desaturase and ACP synthase recognise similar parts of the ACP molecule, giving the concept of an ACP recognition helix (113) experimental validation.
3.4.1.3 Design of regio-specificity involves site-directed mutagenesis of both desaturase and ACP

The crystal structure of the protein complex between desaturase from *Ricinus Communis* (Castor Bean) and stearoyl-ACP from spinach (Paper IV) will hopefully enable rational design of regio-specificity by site-directed mutagenesis in soluble desaturases. To alter desaturase regio-specificity from Δ9 into Δ4 or Δ6, the fatty acid substrate need to get deeper down into the fatty acid binding pocket before catalysis occurs, with the di-iron centre acting as a fix-point, and one way for this to occur would be if ACP binds deeper down in the binding cavity in the Δ4 or Δ6 desaturases.

Only three of the soluble desaturases are known to have an unusual double bond insertion position, the two 16:0Δ4 desaturases from coriander (91, 92) and ivy (not published), and the 16:0Δ6 desaturase from *Thunbergia alata* (127). In desaturase, eight amino acid positions contribute ~92% of the total surface area in the ACP interface (Table 4). The identity of these amino acids in the interface varies between the 18:0Δ9 enzymes, and the 16:0Δ6 enzyme from *T. alata*, and the two 16:0Δ4 desaturases from coriander and ivy (Table 4). Note that the two 16:0Δ4 desaturases from ivy and coriander are identical in the ACP interface although their overall sequence identity is only 73%. To alter regio-specificity, residues lining the fatty acid binding cavity might need to be substituted as well (Table 2). This is realised by the simple fact that if stearic acid penetrates deeper down into desaturase due to an engineered ACP recognition surface there has to be room for the fatty acid tail at the bottom of the binding cavity.

**Table 4. Major contributors to the ACP interface representing 92% of the total surface area varies among desaturases with unusual specificity for double bond insertion position.**

<table>
<thead>
<tr>
<th>Desaturase</th>
<th>18:0Δ9</th>
<th>16:0Δ6</th>
<th>16:0Δ4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Castor</td>
<td>Arg</td>
<td>Lys</td>
<td>Lys</td>
</tr>
<tr>
<td><em>T. alata</em></td>
<td>Lys</td>
<td>Lys</td>
<td>Arg</td>
</tr>
<tr>
<td>Coriander</td>
<td>Arg</td>
<td>Lys</td>
<td>Lys</td>
</tr>
<tr>
<td>Ivy</td>
<td>Arg</td>
<td>Lys</td>
<td>Lys</td>
</tr>
</tbody>
</table>

a) Genbank accession code gi|134945
b) Genbank accession code gi|595295
c) Genbank accession code gi|417819
d) Unpublished

Among the desaturase residues present in the ACP interface the degree of conservation among the 59 available desaturase sequences suggest that residues in position 333, 337, 340 and 344 are important to modify the ACP interaction in desaturases with unusual regio-specificity as described in Paper IV. Based on the discussion in Paper IV, Δ6 or
Δ4 activity could be introduced in castor 18:0Δ9 desaturase by a quintet of site-directed mutations (D280K/R333K/L337V/R340K/R344K) were the lysine of D280K attracts the phosphate group of phospho-Ser39 in spinach ACP down into desaturase and the other four mutations alter (or weaken) the common desaturase ACP interaction surface. By using palmitoyl-ACP (16:0ACP) or myristoyl-ACP (14:0ACP) i.e. shorter substrates than stearoyl-ACP, mutations at the bottom of the fatty acid binding pocket might not be necessary.

Desaturases with unusual regio-specificity from coriander or Thunbergia alata are more efficient with acyl-ACP from the same organisms (130) and therefore these isoforms of ACP or engineered isoforms from spinach ACP might be necessary for promoting the unusual activity in the engineered desaturase mutants. How desaturases with unusual regio-specificity could discriminate between individual ACP isoforms was addressed in Paper IV. When Δ4 desaturation occurs with coriander and ivy desaturase, ACP probably binds deeper down into the enzyme, potentially involving some new residues that are currently observed in the 18:0Δ9 castor interface. The ACP gene from ivy has recently been sequenced and sequence alignments between spinach, coriander and ivy ACP shows that Asn48 and Asn56 from spinach ACP, are both glycine in coriander ACP and ivy ACP. Asn48 and Asn56 are facing the castor desaturase molecule, although at present only Asn56 contribute to the desaturase interface (Table 3) and absence of these side chains might allow for coriander and ivy ACP to slide/tilt deeper down into their Δ4 desaturases respectively. As previously described, the guanidinium head-group of Arg340 in castor desaturase appears to bridge Glu50 and Glu59 in spinach-ACP and a similar interaction occur between ACP and AcpS from B. Subtilis. It appears that this interaction is weaker, between the coriander and ivy Δ4 desaturases and their ACPs since Arg340 is a lysine in the Δ4 desaturases and in addition, Glu50 is a glycine in Ivy ACP. For spinach ACP the triple mutant E50G/N48G/N56G should make spinach-ACP more similar to coriander-ACP or ivy-ACP to promote Δ4 or Δ6 activity in the engineered castor desaturase mutants. Experiments to test these hypotheses are currently underway.

3.4.2 Reduced di-ferrous desaturase

For desaturase to activate molecular oxygen its di-iron centre need to be accessible for the oxygen molecule and further in the reduced (ferrous) and paramagnetic state (see introduction).

Ferredoxin is a small protein that reduces desaturase, potentially by two single-electron transfer steps. Ferredoxin is in turn reduced by either ferredoxin-NADPH reductase (FNR) or directly by photosystem I in the plastid (131). Desaturase is yet to be observed in a one-electron reduced form (132) and therefore transfer of the first electron seems rate limiting for reduction (7). The binding site for ferredoxin on the
desaturase (or desaturase + ACP) surface remains to be characterised, as well as the pathway taken by the electrons to reach the desaturase di-iron centre.

When spectroscopic studies are performed on reduced desaturase, the di-iron centre is usually reduced with dithionite instead of the natural reducing agent ferredoxin. The precise implications of chemical reduction with dithionite versus biological reduction with ferredoxin are currently not known. It is known that chemical reduction with dithionite reduces both subunits of the desaturase dimer in a 4e- reduction in contrast to biological 2e- reduction where one desaturase subunit is reduced at a time (132). It is further known that chemically reduced desaturase in presence of stearoyl-ACP appears more stable against re-oxidation than could be expected when compared to the rate of catalytic desaturation with ferredoxin (132). In the original crystal structure, the reduction of the desaturase di-iron centre probably occurs by photo-reduction (96), and magnetic circular dichroism (MCD) has later demonstrated chemically and photo-chemically reduced desaturase as identical species (133). The mechanism behind photo-reduction of metal centres by X-ray radiation remains to be characterised.

Recombinant desaturase purified from *E. coli* contains 81% of the protein in the oxidised state and 19% of the protein in the reduced state as determined by Mössbauer spectroscopy (88). The reduced (19%) fraction appears equivalent to desaturase reduced by dithionite (88). Mössbauer spectroscopy reveals each ferrous (Fe\(^{2+}\)) iron to be in the high spin state and that the reduced di-iron centre is a paramagnetic species consistent with the expectations for an activator of molecular oxygen (88). In the Mössbauer study, paramagnetic hyperfine interactions ruled out strong anti-ferromagnetic coupling to occur between the ferrous high spin iron of the reduced desaturase di-iron centre but is consistent with either ferromagnetic or weak anti-ferromagnetic coupling. When chemically reduced desaturase was studied using magnetic circular dichroism (MCD), the anti-ferromagnetic coupling between the high spin ferrous iron appear weak (133) like in the Mössbauer study (88), but the observed zero field splitting suggests the ground state of the reduced desaturase di-iron centre to be diamagnetic (133), which is interesting knowing that reduced desaturase is stable under molecular oxygen for hours (132). At room temperature however, low energy paramagnetic excited states can be Boltzmann populated, thereby enabling activation of molecular oxygen to occur even when the ground state of the di-iron centre is diamagnetic. This occurs in deoxy-hemerythrin, which has a diamagnetic ground state (134), but binds molecular oxygen as a peroxide species (100,135), because thermally populated paramagnetic excited states (134) activate molecular oxygen by two-electron reduction with concurrent proton donation by the hydroxo-bridge. In contrast, haemoglobin simply binds molecular oxygen by usage of a low spin Fe\(^{2+}\) state (83,84). In reduced desaturase the hydroxo-bridge used for protonation of the oxygen species in hemerythrin is absent and therefore desaturase is probably unable to act as a reversible binder of molecular oxygen.
3.4.2.1 Addition of stearoyl-ACP increases oxygen reactivity in reduced desaturase

Reduced desaturase is stable under dioxygen for hours but in presence of stearoyl-ACP the rate of re-oxidation increases (132), which suggests that the protein substrate needs to bind prior to activation of molecular oxygen by the reduced desaturase di-iron centre. This model is attractive since it would prevent the formation of reactive oxygen species in absence of stearoyl-ACP.

The coordination environment of the desaturase di-iron centre is changing upon binding of stearoyl-ACP as demonstrated by Raman spectroscopy for oxidised desaturase (136) and by MCD (133) for desaturase reduced by dithionite. The MCD study demonstrates that binding of stearoyl-ACP to reduced desaturase, opens a coordination site on Fe2 that becomes 4-coordinated and further alters the ground state of the reduced di-iron centre from diamagnetic into paramagnetic (133) thereby increasing the reactivity of the di-iron centre against molecular oxygen. Note that chemical reduction by dithionite was used in the MCD study and that the di-iron centre geometry could be different when reduced by ferredoxin.

Binding of stearoyl-ACP to reduced desaturase disturbs the CD and MCD spectra in a way that could be reproduced in part by ligand field calculations assuming that one of the bi-dentate terminal ligands shifts, from bi-dentate into mono-dentate (133) i.e. precisely the carboxylate shift in Glu196 that we are observing in the crystal structure of the acetate complex (Paper III). In this complex the reactivity against binding of acetate is indeed increased since acetate does not bind to the di-iron centre in the P2_1/2_2 crystal form but only to the di-iron centre in the P3_12 crystal form where Fe2 is coordinated as distorted trigonal bipyramidal. From ligand field theory it is known that metals coordinated in trigonal bipyramidal geometry have their d_2 orbital as their highest energy d-orbital. The z-direction in the desaturase case would be given by the axis from Fe2 down to the coordinating Nε atom of His232. A high spin ferrous iron in trigonal bipyramidal coordination is therefore a suitable geometry for delivering an electron to molecular oxygen if molecular oxygen approaches Fe2 from the direction opposite to His232.
3.4.3 The desaturase peroxo intermediate(s)

The first peroxo intermediate identified by spectroscopy in any di-iron enzyme was in MMOH (137). Since MMOH can catalyse desaturase reactions with some substrates (138) it has for long been assumed that MMOH will have oxygen intermediates in common with desaturase.

3.4.3.1 The stable μ-1,2 peroxo diferric intermediate in desaturase

In desaturase a stable peroxo intermediate can be obtained, by mixing chemically reduced enzyme with stearoyl-ACP under anaerobic conditions and then expose the sample to 1 atm O₂ (132). The desaturase peroxo-diferric intermediate is diamagnetic (139), and more stable than similar peroxo intermediates in either MMOH (140) or in mutants of R2 (141,142). The azide ion binds with μ-1,3 coordination to the ferrous ions (Paper III) and is assumed to mimic the stable μ-1,2 peroxo intermediate in desaturase (132,139). The μ-1,2 peroxo intermediate decays without formation of the product oleoyl-ACP or hydrogen peroxide but through an oxidase reaction forming water (132). The formation of water instead of product during decay of the stable μ-1,2 peroxo intermediate is probably caused by surplus electrons due to chemical reduction (132). The extra electrons delivered from surplus dithionite or from the second reduced subunit, can intercept formation of the reactive intermediate that follows the peroxo-intermediate preventing it from abstracting hydrogens from stearic acid. To reduce the stable μ-1,2 peroxo intermediate into water and diferric desaturase, two electrons and two protons need to be delivered and one possibility is that the water pathway, connecting the di-iron centres of the two subunits, performs this function (Paper III).

Crystal structures of azide complexes are available for desaturase (Paper III), ruberythrin (120) and the two double mutants F208A/Y122F (143) and E238A/Y122F (144) of the reduced R2 subunit of ribonucleotide reductase (Fig. 13). The azide ion is close to μ-1,1 bridging in both R2 mutants, occupying the position of the μ-oxo bridge (E238A/Y122F), or of a second solvent molecule (F208A/Y122F) that was coordinated by Fe₂ in the oxidised di-iron centre (118). In both mutants of R2, the azide ion extends away from the di-iron centre and occupies the available space introduced by the mutations. In contrast to these R2 mutant complexes, the azide complex in desaturase is strikingly similar to the azide complex of reduced ruberythrin, demonstrating almost identical iron coordination (Fig. 13). Like in desaturase, binding of azide to the reduced state of ruberythrin, introduces no carboxylate shifts in the surrounding ligands.
Figure 13. Crystal structures of azide complexes in di-iron enzymes

The stable μ-1,2 peroxo intermediate in desaturase and the ruberythrin peroxidase reaction, terminate with the same product namely water, suggesting that reduced desaturase can bind hydrogen peroxide and act as a peroxidase (Paper III). The rate of the potential peroxidation reaction catalysed by desaturase is expected to be low since Glu97, crucial to peroxidation in ruberythrin (120,145), is absent in desaturase (Fig. 13). Hydrogen peroxide has not been properly evaluated as a potential substrate for the reduced desaturase di-iron centre, but it is known that addition of catalase in the desaturase assay speeds up the rate of desaturation (146,147), and therefore hydrogen peroxide appears to inhibit desaturase. The hydrogen peroxide consumed by catalase has been suggested to originate from molecular oxygen reduced by ferredoxin and ferredoxin reductase used in the desaturase assay (146). It is proposed that hydrogen peroxide binds to the reduced desaturase di-iron centre, in the same way as the azide ion in the azide complex, thereby inhibiting the enzyme (Paper III). If desaturases would have a low rate of peroxidase activity, they might have a secondary role contributing to protection against oxidative stress. It has been shown that a Δ12 desaturase from C. elegans introduced and expressed in yeast (S. cerevisiae) increased the tolerance against hydrogen peroxide in the yeast (148) although this was attributed to changes in membrane fluidity and not on peroxidase activity of the introduced Δ12 desaturase gene.
3.4.3.2 Desaturase catalysis involves a transient peroxo-diferric intermediate

During desaturase catalysis, the peroxo intermediate is not the stable μ-1,2 peroxo intermediate described above. Instead it is a transient species, which forms when molecular oxygen is activated by the di-iron centre after binding of stearoyl-ACP has occurred, and possibly acts as a precursor to a highly reactive intermediate, suggested in analogy to the MMOH mechanism, that is able to abstract hydrogen atoms from the bound fatty acid. The structure of the acetate complex is expected to be a better model for this transient intermediate than the azide complex (Paper III), since the observed carboxylate shift in the acetate structure apparently mimics the distortion of the di-iron centre ligands that increase the reactivity against molecular oxygen (132) upon binding of stearoyl-ACP (133).

3.4.4 Oxidation of the fatty acid substrate in desaturase

The highly reactive intermediate, corresponding to "Q" in MMOH (149) or "X" in R2 (150), that would abstract hydrogen atoms from the saturated fatty acid bound in vicinity of the desaturase di-iron centre, remains to be characterised. The initial hydrogen abstraction occurs at the 10th carbon as suggested by experiments allowing desaturase to interact with modified fatty acid substrates where either the 9th or the 10th carbon atom was exchanged for sulphur (151) or oxygen (152) respectively. Using deuterated fatty acid substrates, it was demonstrated that the intermolecular deuterium kinetic isotope effect (KIE) was ~1 (151) but this is expected if the slowest step in the overall desaturase reaction is substrate binding or product release. By using fluorinated fatty acid substrates it was recently shown that hydrogen removal occurs in syn fashion with pro-R enantioselectivity (153), meaning that the two hydrogen atoms point in the same direction prior to desaturation.

A recent crystal structure determination of the mononuclear iron enzyme naphthalene dioxygenase (NDO) in complex with molecular oxygen and its substrates, naphthalene and indole, shows that molecular oxygen can be aligned side on to the iron (154). In addition, the oxygen molecule appears to be aligned in parallel with the double bond that is going to be di-hydroxylated in a cis-stereospecific manner by the enzyme (154). One might speculate that when stearoyl-ACP binds to oxidised desaturase, it positions the C9 and C10 carbon bond of the fatty acid in parallel to the Fe1 and Fe2 axis with the hydrogens to be removed pointing towards the iron. After reduction by ferredoxin, the di-iron centre activates molecular oxygen and aligns the peroxide intermediate in parallel to the C9-C10 carbon bond, to facilitate abstraction of the two hydrogen atoms from the bound fatty acid, that already points towards the iron. Molecular oxygen has then obtained two electrons from the di-iron centre and two electrons plus two protons from the fatty acid substrate that can be converted into a water molecule and a μ-oxo bridge. Aligning the O-O axis of the peroxide intermediate in parallel to the particular C-C carbon bond that is going to be desaturated in the bent fatty acid substrate would
then be the key for successful catalysis. If this assumption is true there is no need for a highly reactive intermediate that is similar to "Q" of MMOH (149) or "X" of the R2 subunit of ribonucleotide reductase (150) during desaturase catalysis.
3.5 CONCLUSIONS

The ambition within this project has been to study binding of the substrate stearoyl-ACP and the activation of molecular oxygen used for oxidising fatty acids in a soluble desaturase. By solving the 3D structure of desaturase from *Ricinus Communis* in complex with stearoyl-ACP from spinach, residues present in the interaction interface have been identified. The variation in identity of the side chains in the ACP interface among desaturases with unusual regio-specificity suggests that these interfacial residues contribute to regio-specificity among soluble desaturases. The structural characterisation regarding identity and interactions between residues in the desaturase-ACP interface should enable rational design of regio-specificity among soluble desaturases. The fact that desaturase and ACP synthase, being structurally unrelated in their ACP interfaces, recognise the same surface of spinach ACP and *B. subtilis* ACP, gives the concept of an ACP recognition helix experimental support.

The di-iron ligand geometry determines the spin state, accessibility and thereby the reactivity of the di-iron centre against molecular oxygen. Information of desaturase di-iron centre geometry includes; iron depleted, oxidised, reduced and oxygen analogue complexes.

A stable diferric peroxo-intermediate is detected in desaturase using spectroscopy but decays with the formation of water instead of the product oleoyl-ACP. The crystal structure of the azide complex, is assumed to mimic this stable peroxo-intermediate and is also strikingly similar to the azide complex of ruberythrin, which catalyses the formation of water from hydrogen peroxide, suggesting that reduced desaturase might have some peroxidase activity.

During desaturase catalysis the peroxo intermediate is a transient species that forms after binding of stearoyl-ACP when the di-iron centre activates molecular oxygen. The di-iron centre geometry in the crystal structure of the acetate complex could mimic this unstable diferric peroxo-intermediate, since the observed carboxylate shift of Glu196 in the crystal structure of the acetate complex, apparently mimics the distortion of the di-iron centre ligands upon binding of stearoyl-ACP, which is known to increase the reactivity against molecular oxygen.

Desaturase hydrogen abstraction from the saturated fatty acid occurs in syn fashion with pro-\(R\) enantioselectivity meaning that the removed hydrogen atoms point in the same direction prior to desaturation. Based on recent discoveries in the mononuclear iron enzyme naphthalene dioxygenase, speculations regarding the desaturation mechanism can be formulated that propose alignment of the saturated fatty acid bond that is going to be desaturated, parallel to the Fe-Fe axis and parallel to the transient desaturase peroxo-intermediate as the key step in catalysis. This abolishes the need for a highly reactive intermediate similar to “Q” in MMOH for soluble desaturases.
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5 REFERENCES
