STRUCTURAL STUDIES ON THE ENZYMATIC UNITS OF THE PEROXISOMAL MULTIFUNCTIONAL ENZYME TYPE 2 (MFE-2)

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OU LU 2004
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Academic Dissertation to be presented with the assent of the Faculty of Science, University of Oulu, for public discussion in Raahensali (Auditorium L10), Linnanmaa, on November 5th, 2004, at 12 noon.
Multifunctional enzyme type 2 (MFE-2) is a peroxisomal enzyme participating in the breakdown of fatty acids in eukaryotes. Depending on the organism, MFE-2 is composed of two to four functional units, out of which the two enzymatic ones, 2-enoyl-coenzyme A (CoA) hydratase 2 and (3R)-hydroxyacyl-CoA dehydrogenase, are found in the all MFE-2s. These units are responsible for the catalysis of the second and third steps of the peroxisomal β-oxidation of various CoA thioesters of fatty acids and fatty acyl derivatives. Their (R)-stereospecificity and ability to accept a broad range of fatty acid CoA esters as substrates, in addition to the fact that they do not share any sequence similarity with the classical mitochondrial counterparts, make the enzymatic units of MFE-2 structurally very interesting. In this study, the three-dimensional structures of the (3R)-hydroxyacyl-CoA dehydrogenase and 2-enoyl-CoA hydratase 2 units were solved by crystallographic methods.

The crystal structure of the (3R)-hydroxyacyl-CoA dehydrogenase unit of rat MFE-2 reveals a dimeric enzyme with an α/β short-chain alcohol dehydrogenase/reductase (SDR) fold. A unique feature of (3R)-hydroxyacyl-CoA dehydrogenase, however, is the separate C-terminal domain, which completes the active site cavity of the adjacent monomer and extends the dimeric interactions. The 2-enoyl-CoA hydratase 2 unit is a dimer with a unique two-domain structure proposed to evolve via gene duplication. The fold consists of two side-by-side arranged repeats of the hot-dog fold motif, thus being highly reminiscent of the tertiary structures of the (R)-specific 2-enoyl-CoA hydratase of the polyhydroxyalkanoate synthesis pathway and the β-hydroxydecanoyl thiol ester dehydrase of fatty acid synthesis type II, both from prokaryotic sources. The importance of the N-domain in the binding of bulky substrates was shown by the enzyme-product complex structure, which also indicates the active site. For the first time, it was shown that the eukaryotic hydratase 2 uses an Asp/His catalytic dyad in catalysis. Moreover, a novel catalytic mechanism was proposed for (R)-specific hydration/dehydration.

The solved structures also provide a molecular basis for understanding the effects of the patient mutations of MFE-2. They also allow discussion of the possible organisation of the three units in full-length MFE-2 of mammals.

**Keywords:** β-oxidation, hot-dog fold, SDR, X-ray crystallography
To Henna and Iiro
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Oulu, August, 2004

Kristian Koski
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACO</td>
<td>acyl-CoA oxidase</td>
</tr>
<tr>
<td>ACD</td>
<td>acyl-CoA dehydrogenase</td>
</tr>
<tr>
<td>CoA</td>
<td>coenzyme A</td>
</tr>
<tr>
<td>CH2</td>
<td>2-enoyl-CoA hydratase 2 unit of <em>Candida tropicalis</em> MFE-2</td>
</tr>
<tr>
<td>FabA</td>
<td><em>Escherichia coli</em> β-hydroxydecanoyl thiol ester dehydrase</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>HADI</td>
<td>(3S)-hydroxyacyl-CoA dehydrogenase</td>
</tr>
<tr>
<td>HADII/ABAD</td>
<td>short-chain 3-hydroxyacyl-CoA dehydrogenase type II/amyloid-β-peptide-binding alcohol dehydrogenase</td>
</tr>
<tr>
<td>HsH2</td>
<td>2-enoyl-CoA hydratase 2 unit of human MFE-2</td>
</tr>
<tr>
<td>$K_{cat}$</td>
<td>catalytic activity</td>
</tr>
<tr>
<td>MAD</td>
<td>multi-wavelength anomalous dispersion</td>
</tr>
<tr>
<td>MCD</td>
<td>medium-chain acyl-CoA dehydrogenase</td>
</tr>
<tr>
<td>MFE-1</td>
<td>peroxisomal multifunctional enzyme type 1</td>
</tr>
<tr>
<td>MFE-2</td>
<td>peroxisomal multifunctional enzyme type 2</td>
</tr>
<tr>
<td>MTP</td>
<td>mitochondrial trifunctional protein</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>PDB</td>
<td>protein data bank</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PHA</td>
<td>polyhydroxyalkanoate</td>
</tr>
<tr>
<td>pK_a</td>
<td>acid-base ionisation constant</td>
</tr>
<tr>
<td>RdDH</td>
<td>(3R)-hydroxyacyl-CoA dehydrogenase unit of rat MFE-2</td>
</tr>
<tr>
<td>SeMet</td>
<td>selenomethionine</td>
</tr>
<tr>
<td>SCD</td>
<td>short-chain acyl-CoA dehydrogenase</td>
</tr>
<tr>
<td>SCPx</td>
<td>sterol carrier protein X</td>
</tr>
<tr>
<td>SCP-2</td>
<td>sterol carrier protein type 2</td>
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<tr>
<td>SCP-2L</td>
<td>sterol carrier protein type 2-like</td>
</tr>
<tr>
<td>SDR</td>
<td>short-chain alcohol dehydrogenase/reductase</td>
</tr>
<tr>
<td>D/THCA</td>
<td>di/trihydroxycoprostanolic acid</td>
</tr>
<tr>
<td>VLCCh</td>
<td>very-long-chain acyl-CoA dehydrogenase</td>
</tr>
<tr>
<td>VLCFA</td>
<td>very-long-chain fatty acid</td>
</tr>
</tbody>
</table>
List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:


IV Koski MK, Haapalainen AM, Hiltunen JK & Glumoff T (200X) Crystal structure of 2-enoyl-CoA hydratase 2 from human peroxisomal multifunctional enzyme type 2. (Under revision)
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References
1 Introduction

Fatty acids, which are hydrophobic compounds composed of carbon, oxygen and hydrogen atoms, are crucial biological molecules participating in the formation of biological membranes and acting as precursors for hormones or intracellular messengers. Because fatty acids are highly reduced, they store much more energy for their weight than carbohydrates and therefore also provide highly efficient energy storage. Fatty acids are stored as triacylglycerols in adipose tissue and utilised for energy-metabolic purposes during periods of fasting and starvation. The energy from fatty acids is released via oxidative degradation. Oxidation may occur at the position of C2 (α-carbon) or C3 (β-carbon) or at the end of the fatty acyl tail (ϖ-end). Therefore, the three pathways involved in fatty acid oxidation are called α-oxidation, β-oxidation and ϖ-oxidation, respectively. α-oxidation and ϖ-oxidation are minor routes of fatty acid breakdown, contributing to the oxidation of phytanic acid (α-oxidation) and prostanoids (ϖ-oxidation), whereas the main route of fatty acid degradation is the β-oxidation cycle. β-oxidation is an important source of generating reducing equivalents for ATP production in mitochondria through the entry of acetyl-coenzyme A (acetyl-CoA) into the Krebs cycle.

Mammalian peroxisomes, which is where the chain-shortening of a set of bulky fatty acids and fatty acyl derivatives takes place, contain two multifunctional enzymes involved in β-oxidation, namely the multifunctional enzyme types 1 (MFE-1) and 2 (MFE-2). These two enzymes, which are responsible for catalysing the second and third steps of β-oxidation, are sequentially unrelated and thus suggested to evolve from non-homologous sources. In contrast to the monofunctional enzymes of β-oxidation, which are well characterised structurally, little is known about the structure-function relationship of peroxisomal multifunctional enzymes.

MFE-2 is a novel enzyme characterised among all those derived from yeast (Hiltunen et al. 1992) and mammals (Leenders et al. 1996). It is a key enzyme in the lipid metabolism of very-long-chain fatty acids (VLCFA), 2-methyl-branched-chain fatty acids and bile acid precursors. In humans, defects in the gene encoding MFE-2 lead to the severe phenotype, normally ending in death during the first year of life (Suzuki et al. 1997, van Grunsven et al. 1998). The present work focused on studying the structural basis on MFE-2 by solving the three-dimensional structures of its enzymatic units, (3R)-hydroxyacyl-CoA dehydrogenase and 2-enoyl-CoA hydratase 2, using X-ray crystallography.
2 Review of the literature

2.1 Fatty acid β-oxidation

2.1.1 Overview of β-oxidation

Fatty acid β-oxidation is one of the key metabolic pathways seen in a variety of living organisms from primitive bacteria to higher eukaryotes (for a review, see Kunau et al. 1995). β-Oxidation is known to occur in cytosol (bacteria), mitochondria (mammals), glyoxysomes (plants) and peroxisomes (mammals, plants, fungi). In all known cases, β-oxidation includes four reactions; flavin adenine dinucleotide (FAD)-dependent dehydrogenation, hydration, nicotinamide adenine dinucleotide (NAD)-dependent dehydrogenation and thiolatic cleavage. These reactions occur in repeated cycles with each fatty acid molecule (Fig. 1). In each cycle, a fatty acid is shortened and one two-carbon unit is released. To be able to act as substrates for β-oxidation, free fatty acids need to be first activated and linked to a carrier molecule, coenzyme A (CoA, Fig. 2).

In mammals, mitochondrial β-oxidation is the major route for degradation of fatty acids and a central metabolic process providing electrons to the respiratory chain. The acetyl-CoA generated in mitochondrial β-oxidation enters the Krebs cycle, where it is further oxidised to CO₂ or used for the formation of ketone bodies (acetoacetate, β-hydroxybutyrate). These ketone bodies are transported to such tissues as heart and brain, where they are converted back to acetyl-CoA to serve as an energy source. While mitochondria catalyse the β-oxidation of the bulk of fatty acids, a distinct set of fatty acids and fatty acyl derivatives are oxidised in peroxisomes. They include CoA esters of very-long-chain fatty acids (VLCFA), such as hexacosanoic acids (C₂₆) and tetracosanoic acid (C₂₄), long-chain dicarboxylic acids and eicosanoids (Reddy & Hashimoto 2001). In addition, pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) and CoA esters of di- and trihydroxyprostanolic acids (D/THCA) are exclusively oxidised in peroxisomes (for reviews, see Verhoeven & Jakobs 2001, Russell 2003). Both pristanic acid and D/THCA have a methyl group at the C₂ position counted from the carboxyl group, and they are thus referred to as 2-methyl-branched fatty acids (see Fig. 8). Pristanic acid is derived directly from dietary sources or indirectly via α-oxidative decarboxylation of phytanic acid, whereas D/THCA are produced from cholesterol via a complex series of enzymatic
reactions and are the immediate precursors of the primary bile acids chenodeoxycholic acid and cholic acid, respectively. Unlike the mitochondria cycle, peroxisomal β-oxidation only involves chain shortening of the fatty acids and their derivatives. For example it has been demonstrated that pristanic acid undergoes three cycles of β-oxidation in peroxisomes, after which further oxidation occurs in mitochondria (Verhoeven et al. 1998).

Fig. 1. The four-reaction β-oxidation cycle of an acyl-CoA molecule. During the cycle, the fatty acyl tail is shortened by two carbons leading to the formation of one acetyl-CoA molecule and an acyl-CoA(C_{2n-2}), which can re-enter the cycle for further oxidation.

Because of the vast diversity of the fatty acids present in nature, a set of isoenzymes with different chain-length specificities are usually needed at each step of β-oxidation for efficient degradation of fatty acids. The enzymes can be soluble, membrane-bound or part of the multifunctional enzymes evolved via gene fusion of monofunctional proteins. To date, most of the β-oxidation enzymes are well-characterised and the crystal structures of at least one of the family members of each β-oxidation step have been determined. These
structures reveal their mechanism of actions and their substrate-binding modes. Interestingly, the enzymes involved in the different steps of β-oxidation are structurally diverse. For example, no common CoA-binding motif can be detected among the β-oxidation enzymes.

2.1.2 Acyl-CoA dehydrogenation

2.1.2.1 Acyl-CoA dehydrogenases (ACDs)

The first, rate-limiting step of mitochondrial fatty acid β-oxidation, the FAD-dependent dehydrogenation of acyl-CoA thioesters to corresponding trans-2-enoyl-CoA (Fig. 1), is catalyzed by acyl-CoA dehydrogenases (ACDs), which are flavoproteins containing one noncovalently bound FAD per subunit. In the oxidative half-reaction two reducing equivalents are generated that are transferred to the electron-transferring flavoprotein and from this to the respiratory chain via electron-transferring flavoprotein dehydrogenase.

Mitochondria contain five isoforms of ACDs involved in β-oxidation, namely short-, medium-, long- and very-long-chain acyl-CoA dehydrogenases, and ACD-9 (Tanaka et al. 1990, Izai et al. 1992, Zhang et al. 2002b). As the enzyme names suggest, each of the enzymes has a characteristic pattern of substrate chain length preferences. The optimal chain lengths of saturated acyl-CoAs for short-, medium-, long- and very-long-chain ACDs are C₄, C₆–C₈, C₁₀–C₁₂ and C₁₆, respectively, although long-chain ACD may actually be more important in the β-oxidation of branched-chain fatty acids (Wanders et al. 1998) and fatty acids unsaturated at the C₄,C₅ or C₅,C₆ position (e.g., oleic acid) (Lea et al. 2000). ACD-9 is a novel ACD family member that has properties of the very-long-chain ACD (VLCD) (Zhang et al. 2002b). The ACD family members are homotetramers with a subunit size of ~45 kDa, with the exception of VLCD and ACD-9, which have bigger subunit structure (~70 kDa) and are homodimers (Izai et al. 1992, Zhang et al. 2002b). Moreover, VLCD is associated with the inner mitochondrial membrane, whereas
the other ACDs are soluble. The extra C-terminal tail in VLCD has been shown to be responsible for membrane binding as well as dimerisation of VLCD (Souri et al. 1998).

The crystal structures of short-chain ACD (SCD) from rat (Battaile et al. 2002) and medium-chain ACD (MCD) from pig (Kim et al. 1993) and human (Lee et al. 1996) have been determined. As expected based on the amino acid sequence similarities, the overall polypeptide folds of these ACDs are very similar. They are both homotetrameric enzymes with the molecular mass of one subunit being 43 kDa. The overall polypeptide fold of a monomer of MCD is shown in Figure 3. It is composed of three domains of approximately equal size: the N-terminal and C-terminal domains, which consist mainly of α-helices (domain I and III), and the middle domain comprising two orthogonal β-sheets (domain II). The α-helices form the core of the tetrameric molecule, whereas the β-sheets lie on the surface of the molecule. The complex structure with the bound FAD and fatty acyl substrate molecule (Kim et al. 1993) shows that the extended FAD is bound at the crevice between the two α-helical domains and the β-sheet domain within one monomer. The substrate-binding site locates between the α-helices αE and αG and the loop between αJ and αK. The CoA portion is partially exposed to solvent at the interface of the two monomers. In SCD, the binding cavity for the fatty acyl moiety of the substrate is shallower than the cavity in MCD. Two major reasons, namely the extra asparagine residue (Asn96 in rat SCD) found in the middle of αE and the absence of a proline residue (Pro257 in human MCD) in αG, make the substrate-binding pocket narrower in SCD compared to that in MCD. The proline residue slightly alters the helix trajectory and deepens the bottom of the binding cavity in MCD. In the uncomplexed MCD, the cavity is filled with a string of ordered water molecules (Kim et al. 1993). The presence of several water molecules in the active site cavity in the catalysis of such substrates as C₄ probably creates an entropically unfavourable state, which explains the low activity towards short-chain fatty acyl-CoAs.

The C₂-C₃ bond of the substrate is sandwiched between the carboxyl group of Glu376 (catalytic base in mammalian MCD) and the isoalloxazine ring of FAD. The mechanism of α,β-dehydrogenation is viewed as a process in which the substrates C₂-H and C₃-H are ruptured in concert. This means that the C₂-H hydrogen is abstracted as H⁺ by Glu376, whereas the C₃-H hydrogen is transferred as a hydride to the position N₅ of flavin (Ghisla et al. 1984, Pohl et al. 1986). Thioester is polarised via two tight hydrogen bonds between the carbonyl oxygen of the thioester substrate and the 2'-OH of the ribityl chain of FAD and the amide nitrogen of Glu376. The acidified thioester would be a facile proton donor to the adjacent carboxyl group of Glu376.

2.1.2.2 Acyl-CoA oxidases (ACOs)

Acyl-CoA oxidases (ACOs) are the peroxisomal equivalent of mitochondrial ACDs. However, unlike ACDs, which are reoxidised by transferring electrons to another flavoprotein, ACOs are reoxidised by molecular oxygen. As a consequence, hydrogen peroxide is formed, which is subsequently decomposed by catalase to produce water and oxygen. Two different ACOs have been identified in human and three in rat peroxisomes. The saturated straight-chain acyl-CoAs are substrates for palmitoyl-CoA oxidase, which
shows substrate preference towards medium-, long- and very-long-chain acyl-CoAs (Osumi et al. 1980). Instead, the 2-methyl branched-chain acyl-CoAs, such as pristanoyl-CoA and bile acid intermediates, are oxidised by branched-chain acyl-CoA oxidase in human or pristanoyl-CoA oxidase and trihydroxycoprostanoyl-CoA oxidase, respectively, in rat peroxisomes (Casteels et al. 1990, Schepers et al. 1990, Van Veldhoven et al. 1991). The human branched-chain acyl-CoA oxidase shares maximum (74%) overall amino acid identity with the rat trihydroxycoprostanoyl-CoA oxidase (Baumgart et al. 1996). With the exception of the homo-octameric pristanoyl-CoA oxidase from rat (Van Veldhoven et al. 1994), ACOs appear to be homodimers.

Two mRNAs for palmitoyl-CoA oxidase have been described, which are derived from a common gene via different splicing (Miyazawa et al. 1987, Osumi et al. 1987). The two palmitoyl-CoA oxidase isoforms, abbreviated as ACO I and ACO II, differ slightly in their amino acid sequences and show differences in substrate specificity (Setoyama et al. 1995). ACO I shows optimal activity towards C₁₀ acyl-CoA compared to ACO II with maximal activity on C₁₄. The crystal structure of rat ACO II complexed with FAD (Fig. 3) has been solved recently (Nakajima et al. 2002). The first 440 N-terminal residues of the 75 kDa subunit of ACO II fold very similarly to the subunit of MCD consisting of three domains. C-terminal region (approximately 220 residues), however, forms an additional α-helical domain, domain IV. This extra domain prevents the tetramer formation of ACO II by contacting with the region responsible for dimer-dimer organisation in MCD. Furthermore, the domain IV of ACO II (especially α-helix α₅) blocks the putative interaction site of electron-transferring flavoprotein (Roberts et al. 1996), preventing the access of electron-transferring flavoprotein to the FAD of the ACO molecule.

The binding cavity for the fatty acyl moieties of the substrates in ACO II is larger than in MCD, being about 28 Å long and 6 Å wide, and it can thus accommodate the acyl-chain length of C₂₃, which is in agreement with the substrate specificities previously determined (Setoyama et al. 1995). The wider and deeper active site cavity in ACO is due to the fact that the domains I and II together are rotated by 13° with respect to the domain III compared to those in MCD. The wider active site might also facilitate the access of molecular oxygen to the flavin during the oxidative half-reaction. This is a major difference compared to the mitochondrial ACDs, in which the tightly packed active-site cleft prevents oxygen from approaching the flavin.

Finally, the basic architecture of the active site cavity is remarkably similar in ACO II and MCD (the Glu421 in ACO corresponds to the catalytic Glu376 in MCD), suggesting a common mechanism for the reductive reaction. The similar structures and amino acid sequences also strongly suggest that these equivalent enzymes originated from a common ancestral gene. The reason why ACO II has very low, if any, physiologically significant activity towards short-chain substrates (≤ C₈) cannot be explained by the crystal structure. That feature, however, explains at least partially why the chain shortening of fatty acids in mammalian peroxisomes does not proceed to completion. In yeast peroxisomes, in contrast, the equivalent for acyl-CoA oxidase, Fox1p, has broad substrate specificity including short-chain substrates, and it thereby allows complete degradation of fatty acids to acetyl-CoA units (Dmochowska et al. 1990).
Fig. 3. Structural comparison of mitochondrial ACD (left) and peroxisomal ACO (right). The structural drawings were generated with Swiss-Pdb Viewer (Guex & Peitsch 1997) using the coordinates of MCD complexed with FAD (black sticks) and octanoyl-CoA (grey sticks) and ACO II complexed with FAD (black sticks). The corresponding pdb entry codes are 3MDE and 1IS2, respectively. The domains and the α-helices mentioned in the text are labelled.

2.1.3 2-enoyl-CoA hydration

After the introduction of the double bond to the fatty acyl substrate thioester by ACD or ACO at the second step of the β-oxidation cycle, a hydroxyl group is added at the C₃ of the substrate by 2-enoyl-CoA hydratase (Fig. 1). In mammalian mitochondria, two isoforms of 2-enoyl-CoA hydratases exist; a soluble 2-enoyl-CoA hydratase, also known as crotonase (hereafter referred to as hydratase 1), and a long-chain specific 2-enoyl-CoA hydratase, which is the N-terminal half of the α-subunit in the inner membrane-associated mitochondrial trifunctional protein (MTP; see chapter 2.1.6.). In mammalian peroxisomes, the corresponding step can also be catalysed by two hydratases, both of which are parts of multifunctional proteins (see chapters 2.2.2 and 2.2.3).

Hydratase 1 hydrates a very broad variety of substrates with respect to chain length (C₄-C₂₀) (Fong & Schulz 1977). The optimal substrate, crotonoyl-CoA (C₄), has a reaction rate close to that of diffusion-controlled reactions, while the rate for hexadecenoyl-CoA (C₁₆) is 150 times less (Waterson & Hill 1972). Hydratase 1 from rat mitochondria has a molecular mass of 161 kDa, with six subunits containing 261 amino acid residues (Furuta et al. 1980), and the crystal structure has been determined in binary complexes with acetoacetyl-CoA, octanoyl-CoA, hexadienoyl-CoA or cinnamoyl-CoA derivative (Engel et al. 1996, Engel et al. 1998, Bahnsen et al. 2002, Bell et al. 2002). The enzyme is built of two trimeric discs (dimer of trimers), with one substrate-binding site per subunit (Fig. 4). The monomer has an N-terminal domain, which is folded into a right-handed spiral of four turns, each turn consisting of two β-strands and an α-helix (ββα-
spiral), and a C-terminal domain comprising three α-helices in trimer formation (Fig. 4). The active site is primarily contained within one subunit, but some amino acid interactions in the binding pocket are contributed to by a neighbouring subunit in the hexamer. The fatty acyl and the β-mercaptoethylamine portions of the substrate are deeply buried inside the monomer, whereas the 3′-phosphate-ADP and pantothenic acid parts are solvent-exposed lying in the hydrophobic pocket on the surface of the molecule. Three lysine residues are salt-bridged to the phosphate groups of the 3′-phosphate-ADP moiety, namely Lys101 and Lys282 (of an adjacent subunit) are salt-bridged to the 3′-phosphate group and Lys92 to the pyrophosphate group (Engel et al. 1996). The bound CoA substrate adopts the bent state, which is stabilised via a hydrogen bond formed between the O10 of the pantetheine moiety and the N7 of the adenine unit of the substrate. In the hydratase 1/acetoacetyl-CoA complex, the active site pocket just fits around the acetyl-CoA moiety, shielded from the intertrimer space by the flexible loop (residues 113 to 120). In order to allow binding of the longer-chain substrates, the flexible loop moves, enhancing the entrance of the fatty enoyl tail into the intertrimer space (Engel et al. 1998). This mode of binding is in contrast to MCD, in which the active site locates in a cleft filled with water molecules that are successively displaced as the longer substrate binds (Kim et al. 1993).

Fig. 4. Ribbon representation of the overall fold of hydratase 1 complexed with octanoyl-CoA (pdb entry code 2DUP). On the left the two-domain structure of hydratase 1 monomer is shown. The N-terminal domain, which has the ββα-spiral fold, carries the bound octanoyl-CoA (black sticks). On the right, the trimeric disc of hydratase 1 molecule is shown. The images were generated with Swiss-Pdb Viewer (Guex & Peitsch 1997).

Hydratase 1 catalyses the reversible syn-hydration (Willadsen & Eggerer 1975) of trans-2-enoyl-CoA thioesters to the corresponding (S)-isomer (also called L-isomer) of 3-hydroxyacyl-CoA thioesters. The initial hypothesis regarding the catalytic mechanism of hydratase 1 was that it proceeds stepwise through the intermediacy of an unstable carbanionic species (Waterson & Hill 1972). Based on subsequent detailed kinetic isotope effect studies, however, it has been proposed that the mechanism is concerted (Bahnson & Anderson 1989, Bahnson & Anderson 1991), with both C2-H and C3-O bonds being
formed in the same transition state. Structural studies have revealed the presence of two catalytic glutamates, Glu164 and Glu144, that are bridged by the catalytic water molecule in the unliganded structure (Engel et al. 1996). Site-directed mutagenesis and enzyme kinetics have demonstrated that the two glutamates act together to catalyze the addition of water, and the replacement of either residue with glutamine results in a dramatic reduction in $k_{\text{cat}}$ (D’Ordine et al. 1994, Hofstein et al. 1999). Generally, the reaction is thought to proceed via an acid/base reaction, where Glu144 activates a water molecule for a nucleophilic attack at C3, and Glu164 protonates the C2 of unsaturated fatty acid (Engel et al. 1996). However, recent structural analysis of the binary complex of hydratase 1 with a cinnamoyl-CoA derivative together with the kinetic and spectroscopic data strongly suggest that both Glu144 and Glu164 are ionised and carry a negative charge, and that the three atoms of a single water molecule are added to the substrate in the hydration reaction (Bahnson et al. 2002). In hydratase 1/substrate complexes, the main-chain amino groups of Ala68 and Gly141 interact with the carbonyl oxygen of the substrate (Engel et al. 1996). This structural feature, which polarises the carbonyl of the thioester and consequently stabilises a common enolate transition state, is known as oxyanion hole (Holden et al. 2001). The polarising interaction with Gly141 is enhanced due to this residue’s position at the N-terminus of an α-helix. The mutation of Gly141 to proline results in a decrease in $k_{\text{cat}}$ of $\sim 10^6$ (Bell et al. 2001), showing the importance of Gly141 and the oxyanion hole formation for the hydratase reaction.

Hydratase 1 belongs to the low-similarity hydratase/isomerase superfamily (Müller-Newen & Stoffel 1993). More than 30 members currently comprise the superfamily, all of which are thought to have evolved from a common ancestor. In addition to hydratase 1, the crystal structures of at least six hydratase/isomerase proteins have been determined. These include 4-chlorobenzoyl-CoA dehalogenase (Benning et al. 1996), rat Δ3,5-Δ2,4-dienoyl-CoA isomerase (Modis et al. 1998), methylmalonyl-CoA decarboxylase (Benning et al. 2000), Δ3-Δ2-enoyl-CoA isomerase (Mursula et al. 2001), 3-methylglutaconyl-CoA hydratase (previously known as AUH protein) (Kurimoto et al. 2001, Ly et al. 2003) and 6-oxo camphor hydrolase (Whittingham et al. 2003). Only 4-chlorobenzoyl-CoA dehalogenase forms a trimer, while the others form hexamers as dimers of trimers, like hydratase 1. Although the family displays mechanistic diversity, almost all of the members stabilise the reaction intermediate via hydrogen bond interactions with two main-chain amino groups in the conserved oxyanion hole (Holden et al. 2001). The only exception in the family is the 6-oxo camphor hydrolase, where no such oxyanion hole via main-chain amides exists.

### 2.1.4 3-hydroxyacyl-CoA dehydrogenation

#### 2.1.4.1 Classical (3S)-hydroxyacyl-CoA hydrogenase (HADI)

The oxidation of the hydroxyl group of (3S)-hydroxyacyl-CoA to a keto group is catalysed by (3S)-hydroxyacyl-CoA dehydrogenases with concomitant reduction of NAD$^+$ to NADH (Fig. 1). As in the case of hydratases, there is one soluble (3S)-hydroxyacyl-CoA dehydrogenase in mammalian mitochondria and a membrane-bound, long-chain specific
(3S)-hydroxyacyl-CoA dehydrogenase, which is the C-terminal part of the α-subunit of MTP.

The soluble isoform, the classical (3S)-hydroxyacyl-CoA dehydrogenase (HADI), is a homodimer with a subunit molecular mass of 34 kDa, which has broad substrate chain-length specificity, exhibiting optimal activity for the C₆ to C₈ substrates (Osumi & Hashimoto 1980). The structure of human heart HADI has been solved as binary complexes with NAD⁺ or 3-hydroxybutyryl-CoA and in a ternary complex with NAD⁺ and acetoacetyl-CoA (Barycki et al. 1999, Barycki et al. 2000, Barycki et al. 2001). It has a two-domain subunit structure with an N-terminal nucleotide-binding domain (N-domain) and a C-terminal dimerisation domain (C-domain) (Fig. 5). The first six β-strands of the N-domain run parallel and are flanked by five α-helices forming the motif needed for NAD(H) binding, the Rossmann fold (Rossmann et al. 1974). The parallel β-sheet is extended by two additional parallel β-strands, which run in an opposite direction relative to the first six. One of the βαβ-spirals of the Rossmann fold is elongated, forming a large helix-turn-helix loop (Fig. 5). The C-terminal domain consists primarily of α-helices.

The substrate-binding site locates at the interface of the two domains (Fig. 5), and upon substrate binding, a significant domain movement is observed, with the N-domain rotating inward towards the C-domain. The 3'-phosphate-ADP moiety of the CoA substrate is solvent-exposed and rather disordered, but a few contacts between the CoA moiety and the protein molecule are observed, such as the salt bridge between Lys68 (in the helix-turn-helix loop) and the 3'-phosphate group (Barycki et al. 2000). The complex structures of HADI with acetoacetyl-CoA or 3-hydroxybutyryl-CoA did not indicate an obvious binding site for larger substrates. Thus, it is not clear at the present how HADI accommodates medium- and long-chain fatty acyl-CoAs.

In HADI, the catalytic pair is formed by His158 and Glu170. His158 serves as a general base by abstracting a proton from the hydroxyl group of the substrate. The subsequent ketonisation of the C₃-O bond facilitates the hydride transfer from C₃ of the substrate to the C₄ of the nicotinamide ring of NAD⁺. Glu170 is proposed to neutralise the positive charge of the protonated His158, and furthermore, Glu170 appears to be required to properly orient His158 for efficient catalysis (Barycki et al. 2001). In the ternary complex (HADI + acetoacetyl-CoA + NAD⁺), in addition to the Nₑ₂ atom of His158, the side-chains of both Ser137 and Asn208 are within hydrogen-bonding distance of the 3-hydroxyl group, and their role is suggested to stabilise the negative charge on the hydroxyl group during catalysis (Barycki et al. 2000). The enolate intermediate of acetoacetyl-CoA is stabilised by a hydrogen bond between its thioester carbonyl and the backbone amide of Asn161.

2.1.4.2 Type II 3-hydroxyacyl-CoA dehydrogenase/amyloid-β binding alcohol dehydrogenase (HADII/ABAD)

Recently, a novel short-chain 3-hydroxyacyl-CoA dehydrogenase has been identified and characterised from bovine liver and human brain (Furuta et al. 1997, He et al. 1998). Since it exhibits high affinity for amyloid β-peptide (Yan et al. 2000), the enzyme is also called a short-chain 3-hydroxyacyl-CoA dehydrogenase type II/amyloid-β-peptide-
binding alcohol dehydrogenase (HADII/ABAD). Initially, HADII/ABAD was referred to as endoplasmic reticulum-associated amyloid β-binding protein (ERAB), since it was proposed to associate with the endoplasmic reticulum (Yan et al. 1997). Recently, however, it is known to locate in mitochondria (He et al. 1999, He et al. 2002). HADII/ABAD has broad substrate specificity oxidising, in addition to 3-hydroxyacyl-CoA substrates, also 17β-hydroxysteroids, such as 17β-estradiol. Therefore, the enzyme is also called 17β-hydroxysteroid dehydrogenase type 10 (He et al. 1999). It should be noted that HADII/ABAD can also utilise the (3R)-isomers of hydroxyacyl-CoAs, although (3S)-isomers are preferred (Yan et al. 2000). The physiological role of HADII/ABAD is not fully understood, but its ability to utilise the ketone body β-hydroxybutyrate as a substrate has raised a question about its role in enhancing the cellular response to metabolic stress (Yan et al. 2000). The interaction of HADII/ABAD with β-amylloid suggests that the enzyme may contribute to the pathogenicity of cell stress induced by amyloid. Thus, it has been suggested that HADII/ABAD is a direct link from the amyloid β-peptide to mitochondrial dysfunction, which is a hallmark of the amyloid-induced neuronal toxicity in Alzheimer’s disease (He et al. 1998, Lustbader et al. 2004).

The amino acid sequence of HADII/ABAD is not related to HADI, but it shares ~30% sequence identity with the hydroxysteroid short-chain dehydrogenases. It forms a homotetramer in solution with a molecular mass of 108 kDa (one subunit 27 kDa) (He et al. 1998). The crystal structure of rat HADII/ABAD (Fig. 5) has been solved as a binary complex with a NADH cofactor, as a ternary complex with NAD+ and acetooacetate and as a ternary complex with NADH and 17β-estradiol (Powell et al. 2000). It is a single-domain enzyme having the typical Rossmann fold as the core structure (Fig. 5). The C-terminal α-helix, αE, and two β-strands, βF and βG, are involved in substrate binding and subunit association, but a separate C-terminal domain, as found in HADI, is missing in HADII/ABAD. The unique loop, which locates between βD and αD (Fig. 5) and contains several positively charged residues, such as Lys99, Lys104 and Lys105, is suggested to support the binding of the CoA substrates (Powell et al. 2000).

HADII/ABAD is a typical member of a large sequentially diverged short-chain alcohol dehydrogenase/reductase (SDR) superfamily (Jörnvall et al. 1995). Presently, the family contains around 300 members, including species variants, which display a wide substrate spectrum from steroids, alcohols, sugars and aromatic compounds to xenobiotics (Oppermann et al. 2003). The crystal structures of ~30 family members have been determined and, with the exception of the monomeric porcine testicular carbonyl reductase (Ghosh et al. 2001), all the members form a dimer or a tetramer in solution. Dimerisation is accomplished via the four-helix bundle, which comprises the long parallel α-helices, αE and αF of the Rossmann fold, of two neighbouring subunits. The G-X-X-G-X-X-G finger print of the cofactor-binding motif, locating near the N-terminal end of the polypeptide, is highly conserved among the family members. The active site of SDR enzymes, including HADII/ABAD, consists of a Ser-Tyr-Lys triad, of which the tyrosine is the most conserved. The active-site tyrosine is proposed to function as the catalytic base in the dehydrogenation reaction, whereas the active-site serine either orients the alcohol substrate or stabilises the transient reaction intermediate during the catalysis or both. Active-site lysine, instead, is proposed to lower the pK_a of the hydroxyl group of active-site tyrosine to promote proton transfer and in addition, since it forms a hydrogen bond with the nicotinamide ribose moiety, it has been proposed to orientate the coenzyme to allow
only 4-pro-S-hydride transfer (Benach et al. 1999, Winberg et al. 1999). Recently, a conserved asparagine residue (in αE), whose backbone oxygen is connected through a water molecule to the active-site lysine, was also proposed to contribute to the catalytic reaction by stabilising the position of active-site lysine (Filling et al. 2002). Moreover, the conserved N-N-A-G motif locating in the N-terminus of the β-strand βD has been shown to play an important role in maintaining the discrete structure of the central β-sheet of the Rossmann fold in SDR enzymes.

Fig. 5. Structures of the two mitochondrial dehydrogenases known to accept CoA thioesters of fatty acids as substrates. On the left, the ribbon diagram of the HADI monomer (pdb entry code 1ILO) is shown. The bound NAD+ (black sticks) and acetoacetyl-CoA (grey sticks) molecules lie in between the two domains. To visualise the two-domain structure and the substrate-binding site, the view is along the central β-sheet of the Rossmann fold. On the right, the single-domain structure of the HADII/ABAD monomer complexed with NADH (black sticks) and 17β-estradiol (grey sticks) is represented (pdb entry code 1E6W). The structure reveals the typical Rossmann fold (viewed towards the central β-sheet) composed of a β-sheet of seven parallel β-strands flanked by three parallel α-helices on each side. The vertical arrow shows the general substrate-binding loop of SDR enzymes, whereas the horizontal arrow shows the unique loop in HADII/ABAD, which is suggested to participate in CoA substrate binding. The images were generated with Swiss-Pdb Viewer (Guex & Peitsch 1997).

2.1.5 Thiolytic cleavage

The last step of β-oxidation, the cleavage reaction of 3-ketoacyl-CoA to yield acetyl-CoA, and a two-carbon shortened acyl-CoA (Fig. 1) is catalysed by 3-ketoacyl-CoA thiolases. In mammalian mitochondria, two thiolases are responsible for fatty acid β-oxidation. The long-chain 3-ketoacyl-CoA thiolase is the small subunit of the MTP, and it catalyses the last step of the chain shortening of the long-chain fatty acyl substrates, whereas the medium-chain 3-ketoacyl-CoA thiolase is soluble and completes the β-oxidation of short-chain fatty acyl-CoAs (Staack et al. 1978, Uchida et al. 1992). In addition, mammalian mitochondria contain a third thiolase, a short-chain 3-ketoacyl-CoA thiolase, also known as acetoacetyl-CoA thiolase. Unlike the long- or medium-chain 3-
ketoacyl-CoA thiolases, where the equilibrium of the reaction is in favour of the cleavage direction (degradative thiolases), acetoacetyl-CoA thiolase favours the reversible reaction, namely the synthesis of acetoacetyl-CoA from two acetyl-CoA molecules via the Claisen condensation reaction (biosynthetic thiolase). Therefore, acetoacetyl-CoA thiolase participates in ketogenesis rather than fatty acid β-oxidation (Middleton & Bartlett 1983, Thompson et al. 1989, Kunau et al. 1995). However, both biosynthetic and degradative thiolases in mitochondria are tetrameric and share significant amino acid sequence identity as well as a similar catalytic mechanism.

Peroxisomes also contain their own pool of 3-ketoacyl-CoA thiolases. For the degradation of straight-chain fatty acids, only one thiolase is found in human peroxisomes, whereas two closely related thiolases, A and B (B being induced by peroxisome proliferators and A not being so induced) are found in rat (Miyazawa et al. 1980). In addition, a protein identified as sterol carrier protein X (SCPx) (Seedorf & Assmann 1991), contains an N-terminal 46 kDa thiolase domain associated with the C-terminal 13 kDa domain, which is identical to the sterol carrier protein 2 (SCP-2). Actually, SCP-2 and SCPx are encoded by the same gene, and they arise via alternate initiation of translation. SCPx has been shown to have a crucial role in the β-oxidation of 2-methyl-branched fatty acids, since it has been demonstrated that the 3-ketoacyl esters of pristanic acid and D/THCA are cleaved by SCPx, but not by short-chain 3-ketoacyl-CoA thiolases (Antonenkov et al. 1997, Seedorf et al. 1998). Unlike the tetrameric mitochondrial thiolases and the biosynthetic thiolase, the peroxisomal thiolases are active as dimers.

At present, no structural information is available for any of the mitochondrial degradative thiolases. However, the crystal structures of peroxisomal degradative 3-ketoacyl-CoA thiolase from S. cerevisiae (Mathieu et al. 1994, Mathieu et al. 1997) and biosynthetic thiolase of Zoogloea ramigera (Modis & Wierenga 1999) have been determined. The subunit structures of both thiolases are very similar, as expected from the 38% sequence identity observed between the two enzymes. The subunit consists of two core domains, N-domain and C-domain, and a loop domain formed of the intervening residues. The two core domains have the same topology: a five-stranded mixed β-sheet covered by α-helices on both sides. Together, the two domains assemble into a five-layered αβαβα structure, with two central helices covered on each side by layers of β-strands and α-helices (Fig. 6). The loop domain of Z. ramigera thiolase contains a motif involved in tetramer formation. This motif is highly conserved in biosynthetic thiolases but absent in degradative thiolases.

The crystal structure of S. cerevisiae degradative thiolase lacks the bound ligand, and it thus allows only speculation of the ligand-binding mode. However, the biosynthetic thiolase from Z. ramigera has been crystallised in the presence of all CoA substrate intermediates (Modis & Wierenga 1999, Kursula et al. 2002). Because the cleavage reaction is reversible, these two types of thiolases share a similar mechanism, and the data obtained from Z. ramigera thiolase can thus be applied to all thiolases. The CoA molecule is bound at the interface between the two core domains and the loop domain of one subunit. The 3′-phosphate-ADP moiety of the CoA substrate is solvent-exposed, and exceptionally, is hydrogen-bonded to the protein molecule only via water molecules. However, the π electrons of the adenine ring system forms a van der Waals stacking interaction with the π electrons of an arginine side-chain (Modis & Wierenga 1999). The active site locates at the bottom of the cylindrical pocket, and in S. cerevisiae thiolase it is ex-
tended beyond the catalytic residues in such a way that the binding of the fatty acid tail of variable length is possible. In contrast, in the Z. ramigera enzyme the pocket is narrow and blocked by two methionine residues, and the pocket therefore only has room for acetoadetyl-CoA or acetyl-CoA to bind (Modis & Wierenga 1999).

Fig. 6. Ribbon diagram of S. cerevisiae peroxisomal degradative 3-ketoacyl-CoA thiolase (pdb entry code 1AFW). The thiolase monomer with the αβαβαβα fold is shown on the left, whereas the biologically active homodimer is presented on the right. The black arrows show the putative CoA binding sites. The images were generated with Swiss-Pdb Viewer (Guex & Peitsch 1997).

The catalytic triad of thiolases consists of two cysteine residues (one locating at the N-domain and the other one at the C-domain) and a histidine residue, and the reaction mechanism has been proposed to follow the classical two-step ping-pong mechanism (Thompson et al. 1989, Palmer et al. 1991, Williams et al. 1992, Mathieu et al. 1994). The first step of the degradative reaction is the attack of the nucleophilic cysteine residue (The N-domain Cys) on the C₃ atom of the 3-ketoacyl-CoA substrate, leading to the formation of a covalent bond between the C₃ atom and the Sγ atom of the cysteine. At the second step, after the acetyl-CoA formed at the first step has been released, a new CoA molecule binds to the thiolase-acyl complex, leading to the formation of the acyl-CoA product. The active-site histidine has an important role in activating the N-domain cysteine for a nucleophilic attack (Mathieu et al. 1997), whereas the C-domain cysteine acts as a base at the first reaction step by donating its proton to the leaving acetyl-CoA as well as deprotonating the incoming CoA molecule at the second step. The negative charges on the thioester oxygen atoms during the two-step reaction are stabilised via two separate oxyanion holes (Kursula et al. 2002).

2.1.6 Mitochondrial trifunctional protein (MTP)

In addition to the soluble set of β-oxidation enzymes located in the matrix, mitochondria also contains a membrane-bound multifunctional enzyme complex containing 2-enoyl-
CoA hydratase 1, (3S)-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase activities (Carpenter et al. 1992, Uchida et al. 1992). This complex, associated with the mitochondrial inner membrane and referred to as the mitochondrial trifunctional protein (MTP), trifunctional β-oxidation complex or trifunctional enzyme, is a hetero-octamer consisting of four 79 kDa α-subunits and four 48 kDa β-subunits. Since MTP shows the highest catalytic efficiency with substrates from C12 to C16 (Uchida et al. 1992), the units are also referred to as long-chain enoyl-CoA hydratase, long-chain hydroxyacyl-CoA dehydrogenase and long-chain 3-ketoacyl-CoA thiolase.

The α-subunit of MTP is a multifunctional polypeptide containing an N-terminal hydratase unit and a C-terminal dehydrogenase unit (Fig. 7). These units share significant sequence similarity with the soluble hydratase 1 and HADI, respectively, indicating that the hydratase unit of the MTP has the typical hydratase/isomerase fold with a repeated ββα spiral, whereas the dehydrogenase part has a two-domain subunit structure with an N-terminal nucleotide-binding domain and a C-terminal dimerisation domain. The β-subunit, instead, consists of only 3-ketoacyl-CoA thiolase activity. It shares ~30% sequence identity with the S. cerevisiae soluble homolog, and is thus more likely to have a general thiolase fold with a five-layered αβαβα catalytic domain.

MTP is a eukaryotic equivalent to the bacterial β-oxidation complex identified from Escherichia coli (Binstock et al. 1977) and other eubacteria (Kunau et al. 1995). However, unlike MTP, the bacterial complex is an α2β2 heterotetramer and in addition to the three activities found in MTP, also comprises Δ3,Δ2-enoyl-CoA isomerase and 3-hydroxy-CoA epimerase activities (Pramanik et al. 1979, Pawar & Schulz 1981). It has been shown by electron microscopy studies of the Pseudomonas fragi β-oxidation complex that the α- and β-subunits individually form dimers, and the two dimers hardly alter their conformations upon the formation of the α2β2 complex (Ishikawa et al. 1997). The dissociated α-subunit dimer has been shown to retain the hydratase and dehydrogenase activities, whereas the uncomplexed β-subunit dimer has lost its thiolase activity (Ishikawa et al. 1997). Thus, it is suggested that the interactions between the α- and β-subunits are crucial for stabilising the β-subunit and/or completing the substrate-binding cavity. Similar stabilisation is also suggested to occur in the MTP complex based on the results obtained from MTP-deficient patients (Ushikubo et al. 1996, Spiekerkoetter et al. 2004). It is noteworthy that experimental studies have shown that long-chain acyl-CoA intermediates of mitochondrial β-oxidation are channelled between the active sites of MTP (Yao & Schulz 1996).

### 2.1.7 Auxiliary enzymes in fatty acid β-oxidation

A normal diet contains many (poly)unsaturated fatty acids (fatty acids that contain one or more carbon-carbon double bonds) or various fatty acyl derivatives, which cannot proceed directly through β-oxidation. Therefore, numerous auxiliary enzymes are required for complete oxidation of such fatty acids. Fatty acids with a double bond at an odd-numbered position require Δ3,Δ2-enoyl-CoA isomerase or Δ3,5,Δ2,4-dienoyl-CoA isomerase as auxiliary enzymes. The Δ3,Δ2-enoyl-CoA isomerase catalyses the isomerisation of both cis- and trans-3-enoyl-CoAs to the corresponding trans-2-enoyl-CoAs (Palosaari
et al. 1990, Müller-Newen & Stoffel 1991), whereas the $\Delta^{3,5,\Delta^{2,4}}$-dienoyl-CoA isomerase metabolises trans-2,cis-5-dienoyl-CoAs to trans-2,trans-4-dienoyl-CoAs (Chen et al. 1995). Fatty acids with a double bond at an even-numbered position, on the other hand, need 2,4-dienoyl-CoA reductase to catalyse the NADPH-dependent reduction of trans-2, cis/trans-4-dienoyl-CoA into trans-3-enoyl-CoA (Kunau & Dommes 1978). The fourth auxiliary enzyme, 2-methyl-CoA racemase (Schmitz et al. 1994, Schmitz et al. 1995), is needed to convert the 2-methyl group of bile acid intermediates and pristanic acid from the ($R$)-position to the ($S$)-position, since only ($S$)-diastereomers act as substrates for peroxisomal branched-chain ACOs (Pedersen et al. 1996, Van Veldhoven et al. 1996).

So far, the crystal structures of peroxisomal $\Delta^{3,\Delta^{2}}$-enoyl-CoA isomerase from S. cerevisiae (Mursula et al. 2001) and peroxisomal $\Delta^{3,5,\Delta^{2,4}}$-dienoyl-CoA isomerase from rat (Modis et al. 1998) have been determined. They both are typical members of the hydratase/isomerase superfamily, forming hexamers made up of two trimers similarly as in the case of hydratase 1. The characterised eukaryotic 2,4-dienoyl-CoA reductases are members of the SDR superfamily (Hakkola et al. 1989, Hakkola & Hiltunen 1993), but as in the case of 2-methylacyl-CoA racemase, no structural information is available at present.

### 2.2 Peroxisomal multifunctional $\beta$-oxidation enzymes

#### 2.2.1 Presence of two MFEs in mammalian peroxisomes

Peroxisomes do not have monofunctional enzymes responsible for the second and third steps of the $\beta$-oxidation cycle. Instead, these steps, i.e, the hydration of trans-2-enoyl-CoA and the subsequent dehydrogenation of 3-hydroxy intermediate to corresponding 3-ketoacyl-CoA, are catalysed by two separate multifunctional enzymes in mammals, multifunctional enzyme type 1 (MFE-1) and multifunctional enzyme type 2 (MFE-2). MFE-1 shares notable sequence similarity and organisation of the functional units with the $\alpha$-subunit of MTP, whereas no such similarity is found with MFE-2 (Fig. 7). The only multifunctional enzyme found in yeast peroxisomes (also referred to as FOX2) is homologous with MFE-2 (Hiltunen et al. 1992).
Fig. 7. Schematic organisation of various multifunctional enzymes responsible for the second and third step of the β-oxidation cycle. Abbreviations: H1, hydratase 1; H1/I, hydratase 1/Δ3,Δ2-enoyl-CoA isomerase; H2, hydratase 2; (S)Dh, (3S)-hydroxyacyl-CoA dehydrogenase; (R)Dh, (3R)-hydroxyacyl-CoA dehydrogenase; SCP-2L, sterol carrier protein type 2 –like; X, C-terminal domain with an unknown function.

### 2.2.2 Multifunctional enzyme type 1 (MFE-1)

MFE-1, also known as L-bifunctional protein, was first identified and cloned from rat (Osumi & Hashimoto 1979, Furuta et al. 1980, Osumi et al. 1985) and later also from human (Hoefler et al. 1994). It is a 78 kDa protein with (S)-specific 2-enoyl-CoA hydratase activity in its N-terminal half and (3S)-hydroxyacyl-CoA activity in the C-terminal half of the polypeptide (Ishii et al. 1987). MFE-1 also has Δ3, Δ2-enoyl-CoA isomerase activity within the hydratase unit (Palosaari & Hiltunen 1990). At present, no structural data is available for MFE-1 or its functional units but sequence analysis studies in combination with studies involving truncated variants of MFE-1 have given some hints of the structural organisation of the full-length enzyme (Kiema et al. 2002). As a result, MFE-1 can be divided into five domains; A, B, C, D and E, A and B being mainly responsible for the hydratase and isomerase activity, and the domains C and D for the dehydrogenase activity. Domain A (residues 1-190) shows clear sequence identity (~37%) to the N-terminal region of hydratase 1 and is suggested to form a typical hydratase/isomerase fold. The two glutamates, which are essential for catalysis, as well as the alanine and glycine residues contributing to the oxyanion hole (Engel et al. 1996) are also conserved in MFE-1 (Glu103, Glu123, Ala61 and Gly100 in rat MFE-1). The same active site is shown to be responsible for the isomerase activity (Palosaari et al. 1991). The domain B (residues 191-280) is proposed to be α-helical like the dimerisation domain of hydratase 1, although their sequences are not related. The dehydrogenase unit of MFE-1 (domains C and D) shares 34% amino acid sequence identity with HADI and thus has very likely a similar two-domain structure. Namely, domain C (residues 281-474), which contains the conserved G-X-G-X-X-G motif (residues Gly310-Gly315) and a catalytic Glu-His dyad (Glu443-His431), contributes to the nucleotide-binding domain, whereas domain D (residues 480-583) contributes to the dimerisation domain. The last domain E (residues 584-
722) shares 32.5% amino acid sequence similarity with the dimerisation domain of HADI and 18% similarity with the domain D of MFE-1, indicating that the E domain has evolved by gene duplication from domain D (Kiema et al. 2002). The three C-terminal residues of MFE-1, Ser-Lys-Leu, act as a peroxisomal targeting signal type 1 (Gould et al. 1989).

Although the amino acid sequences of the α-subunits of MTP and MFE-1 resemble each other (Fig. 7), notable differences exist in their biological nature. While MTP is membrane-bound and hetero-octameric, MFE-1 is soluble and monomeric. The monomeric character of MFE-1 is unexpected, since it is the only member of the hydratase/isomerase superfamily, which is not a trimer or a hexamer. It has been estimated that, in the native structure, the C-terminal extension domain (domain E) together with the domain B stabilises the hydratase/isomerase fold (Kiema et al. 2002). The domain E has also been shown be to be crucial for dehydrogenase activity, and it probably functions as a complementary part similar to the dimerisation domain of HADI (Taskinen et al. 2002).

Although a broad variety of compounds, such as straight-chain fatty acyl-CoAs from C₄ to C₁₆, bile acid intermediates and other branched-chain fatty acids, appear to act as substrates for MFE-1 (Dieuaide-Noubhani et al. 1996, Jiang et al. 1996, Xu & Cuebas 1996, Dieuaide-Noubhani et al. 1997a, Jiang et al. 1997, Kurosawa et al. 2001), the physiological function of MFE-1 in the cell is still largely unknown. The ability of mice lacking the functional MFE-1 to survive without any severe phenotype (Qi et al. 1999) suggested that it is not essential for fatty acid β-oxidation. In the case of bile acid synthesis, the inability to convert the 24,25-trans-unsaturated derivate of D/THCA to the corresponding keto-compound is due to the fact that the (24S,25S)-diastereomer of D/THCA produced by hydratase unit of MFE-1 is not a substrate for the dehydrogenase unit of the same polypeptide (Xu & Cuebas 1996, Dieuaide-Noubhani et al. 1997a, Qin et al. 1997a). However, since the dehydrogenase unit is able to catalyse the dehydrogenation of (24S,25R)-THCA (Kurosawa et al. 2001), it is suggested that MFE-1 provides an alternative route for bile acid synthesis via a combined action with 2-methylacyl-CoA racemase (Baes et al. 2000, Cuebas et al. 2002) or hydroxylases, such as CYP3A4 (Savolainen et al. 2004). These hypotheses are strengthened by the findings that MFE-1 is upregulated if racemase, or MFE-2 is knocked out (Baes et al. 2000, Savolainen et al. 2004). The presence of isomerase activity suggests that MFE-1 contributes to the metabolism of long-chain polyunsaturated fatty acids (Gurvitz et al. 2001, Zhang et al. 2002a), and a recent study has shown the participation of MFE-1, together with MFE-2, in the chain-shortening of C₁₆ dicarboxylic acids (Ferdinandusse et al. 2004).

2.2.3 Multifunctional enzyme type 2 (MFE-2)

2.2.3.1 Nomenclature and physiological function

MFE-2, 2-enoyl-CoA hydratase 2/(3R)-hydroxyacyl-CoA dehydrogenase, was first purified and cloned from Candida tropicalis as a hydratase-dehydrogenase-epimerase (Moreno de la Garza et al. 1985, Nuttley et al. 1988). The identification of a novel (R)-specific 2-enoyl-CoA hydratase (thereafter referred to as hydratase 2) revealed that the
Epimerase reaction is actually a combined process of two separate hydratases with opposite chiral specificities (Hiltunen et al. 1989, Malila et al. 1993). The characterisation of *S. cerevisiae* MFE-2 (Hiltunen et al. 1992) showed, for the first time, the physiological function of MFE-2 in fatty acid β-oxidation via a (R)-specific route. To date, MFE-2 has been cloned and characterised from various organisms, including among others pig (Leenders et al. 1994a), human (Adamski et al. 1995), rat (Dieuaide-Noubhani et al. 1996, Qin et al. 1997b), chicken (Kobayashi et al. 1997), *Neurospora crassa* (Fosså et al. 1995) and *Glomus mosseae* (Requena et al. 1999). Because the mammalian enzyme was discovered almost simultaneously by several research groups, alternative names, such as multifunctional protein type 2, D-specific multifunctional protein 2 and D-bifunctional protein, have been used for MFE-2.

Amino acid identities between MFE-2 variants from different mammals are very high, being around 80%. The human enzyme consists of 737 amino acid residues and has a native molecular mass of ~154 kDa, suggesting that the enzyme is a dimer (Jiang et al. 1996). In *vivo*, full-length MFE-2 is partially processed to 32 kDa and 45 kDa fragments (Leenders et al. 1994b, Novikov et al. 1994, Dieuaide-Noubhani et al. 1997b). The processing site has been located between residues the Ser317 and Ala318 in rat (Qin et al. 1997a), and it has been proven that the processing occurs after targeting of the protein to the peroxisomes (Leenders et al. 1994a). Although the extent of processing varies in different tissues, both fragments are present in all tissues (Adamski et al. 1997).

The importance of MFE-2 in the metabolism of pristanic acid (Fig. 8) and D/THCA, the precursors of bile acids (Fig. 8), has been demonstrated by several research groups (Dieuaide-Noubhani et al. 1996, Dieuaide-Noubhani et al. 1997a, Jiang et al. 1997, Novikov et al. 1997, Qin et al. 1997a). The accumulation of these compounds into the plasma of MFE-2-deficient mice (Baes et al. 2000) and humans (Suzuki et al. 1997, van Grunsven et al. 1998) further supports these observations. Furthermore, the elevated ratio of C26 to C22 fatty acids in the plasma of knock-out mice and MFE-2 patients indicates that VLCFAs act as substrates for MFE-2, but not for MFE-1. Recently, MFE-2 has been shown also to be a key enzyme in the biosynthesis of docosahexaenoic acid (C22:6 n-3) via retroconversion of docosapentaenoic acid (C22:5 n-3) (Ferdinandusse et al. 2001, Su et al. 2001). Docosahexaenoic acid is an important component for normal neurological development, especially in brain and retina. Finally, MFE-2 also participates in the metabolism of eicosanoids, such as inactivation of leukotrienes (Ferdinandusse et al. 2002a).
2.2.3.2 (3R)-hydroxyacyl-CoA dehydrogenase unit

The (3R)-hydroxyacyl-CoA dehydrogenase unit locates in the N-terminus of mammalian MFE-2 (residues 1-323). Although the unit catalyses NAD$^+$-dependent dehydrogenation at the third step of $\beta$-oxidation (Fig. 1), its sequence is not related to the mitochondrial HADI or the dehydrogenase unit of MFE-1. Instead, the presence of the G-X-X-G-X-G (Gly16-Gly22) motif near the N-terminus and the Tyr-X-X-X-Lys (Tyr164-Lys168) motif in the middle part of the sequence reveals a typical member of the SDR superfamily. Interestingly, the first 261 N-terminal residues of the dehydrogenase unit of MFE-2 share 28% identity with the HADII/ABAD and thus have similarity with the short-chain 17$\beta$-hydroxysteroid dehydrogenases. This supports the finding that the dehydrogenase unit of MFE-2 has 17$\beta$-hydroxysteroid dehydrogenase activity, being able to catalyse the oxidation of 17$\beta$-estradiol and androstene-3$\beta$,17$\beta$-diol at the C17 position (Adamski et al. 1992). Initially, before the physiological function of MFE-2 was understood, the enzyme was named 17$\beta$-hydroxysteroid dehydrogenase type 4 (Leenders et al. 1994a).

The dehydrogenase unit of mammalian MFE-2 is highly stereospecific, accepting only fatty acyl-CoA esters with a hydroxyl group at the (3R)-position. Although HADII/ABAD can also catalyse (3R)-dehydrogenation, (3S) isomers are clearly preferable as substrates (Yan et al. 2000). Moreover, the chain length specificities between these enzymes vary. HADII/ABAD accepts only short-chain fatty acyl-CoAs, whereas the dehydrogenase unit of MFE-2 is able to utilise short-, medium-, long-, and very-long-chain substrates, the maximal velocities increasing when the chain length of the substrates increases from C4 to C16 (Dieuaide-Noubhani et al. 1996, Qin et al. 1997b). The dehydrogenase unit of mammalian MFE-2 also shows strict stereospecificity towards branched-chain fatty-enoyl substrates accepting only methyl groups at the (R)-position (Dieuaide-Noubhani et al. 1996, Dieuaide-Noubhani et al. 1997a, Novikov et al. 1997).

Interestingly, in fungal MFE-2, the dehydrogenase unit is duplicated (Fig. 7). The two copies, referred to as dehydrogenase A and dehydrogenase B, share ~40% amino acid
identity, A being more closely related to the dehydrogenase region of mammalian MFE-2. The duplicated dehydrogenase region of *C. tropicalis* MFE-2 has broad substrate specificity from C₄ to C₁₆ showing maximum catalytic efficiency with (3R)-hydroxydecanoyl-CoA (Qin *et al*. 1999). The two dehydrogenase units, A and B, show different substrate preferences, namely unit A acts on medium- and long-chain substrates and unit B on short-chain substrates (Qin *et al*. 1999). In addition to the physiological activity towards straight-chain fatty acyl-CoAs, the duplicated dehydrogenase unit of *C. tropicalis* MFE-2 shows minor 17β-hydroxysteroid dehydrogenase activity (Qin *et al*. 1999). Moreover, in addition to being able to catalyse the (24R,25R)-stereoisomer of THCA-CoA to corresponding 24-keto-THCA-CoA, it also accepts the (24R,25S)-stereoisomer of THCA-CoA as substrate (Qin *et al*. 2000).

### 2.2.3.3 Hydratase 2 unit

The middle part of the mammalian MFE-2, the hydratase 2 unit, is responsible for the second step of peroxisomal β-oxidation together with the hydratase unit of MFE-1. The basic difference between these two hydratases is that the created end products are enantiomers, namely hydratase 2 adds the hydroxyl group at the (R)-position, whereas the hydratase unit of MFE-1 adds the hydroxyl group at the (S)-position of the trans-2-enoyl-CoA substrates. (R)-specificity makes the hydratase 2 a key enzyme in the metabolism of 2-methyl-branched fatty acids and their derivates, since only the (25R)-isomers of D/THCA and (3R)-hydroxy-(2R)-methyl-pristanoyl-CoA are known to be intermediates in the synthesis of bile acids and in the breakdown of pristanic acid, respectively. In addition, hydratase 2 has broad substrate specificity from medium-chain (C₈) to long-chain (C₁₆) fatty enoyl-CoAs, but shows only residual activity towards short-chain substrates (Malila *et al*. 1993, Jiang *et al*. 1996, Qin *et al*. 1997). The accumulation of very-long-chain substrates, such as C₂₂ and C₂₆, in the plasma of hydratase 2-deficient patients (van Grunsven *et al*. 1999a) indicates that such a VLCFA also acts as a substrate for hydratase 2.

The hydratase 2 unit of yeast MFE-2 shows ~40% sequence identity with the mammalian ortholog. Since fungi lack the mitochondrial machinery for β-oxidation and also peroxisomal MFE-1, hydratase 2 is the only hydratase acting on the second step of β-oxidation. In line with this, the hydratase 2 part of fungal MFE-2 must also accept short-chain fatty enoyl-CoAs as substrates.

Hydratase 2 does not show any amino acid sequence similarity to hydratase 1 or the hydratase/isomerase region of MFE-1. Sequence alignment of several hydratase 2s from mammals and fungi has revealed a conserved amino acid sequence region, Y-R-L-(G)-S-G-D-X-N-P-L-H-X-D-P-X-X-A, referred to as the hydratase 2 motif (Qin *et al*. 2000a). In the same study, 12 highly conserved proline residues were replaced by alanines by site-directed mutagenesis, and the resulting constructs were tested for complementation of the *fox2* null mutant yeast strain. Out of the five noncomplementing variants, two (Asp510Ala and Glu366Ala) were successfully purified. Since the purified variants showed no (Asp510Ala) or significantly decreased (Glu366Ala) hydratase activity with-
out any fold disturbances, Asp510 and Glu366 were proposed to act as catalytic residues for hydratase 2 (Qin et al. 2000a).

### 2.2.3.4 Sterol carrier protein type 2-like (SCP-2L) domain

The last functional unit of mammalian MFE-2 shows ~40% sequence identity with the human SCP-2, being thus referred to as the sterol carrier protein type 2-like domain (SCP-2L). Except for the mycorrhizal fungus *G. mossea* (Requena et al. 1999), SCP-2L is not present in fungal (such as *S. cerevisiae*, *N. crassa* and *C. tropicalis*) MFE-2 (Fig. 7).

The crystal structure of the truncated human MFE-2 containing only the SCP-2L domain has been determined in complex with a ligand analog, Triton X-100 (Haapalainen et al. 2001). The overall polypeptide fold of SCP-2L is similar to that of rabbit and human SCP-2s (Choinowski et al. 2000, García et al. 2000), comprising a five-stranded β-sheet covered on one side by five amphipathic α-helices (Fig. 9). The Triton molecule is bound to the hydrophobic tunnel, which traverses the molecule. The entrance of the tunnel is formed by the residues in the α-helices αD and αE and the β-strand β5. The C-terminal tripeptide, Ala-Lys-Leu, known as peroxisomal targeting signal type 1 (Gould et al. 1989), is solvent-exposed in the triton-complexed SCP-2L, whereas in the unliganded rabbit and human SCP-2s the targeting signal is buried. It has been shown by molecular dynamics studies with SCP-2L that the removal of the Triton molecule causes the binding pocket to collapse, which leads to the buried targeting signal (Lensink et al. 2002). These findings support the hypothesis of a ligand-assisted protein import mechanism in peroxisomes (Choinowski et al. 2000).

![Fig. 9. Ribbon diagram of the SCP-2L domain from human MFE-2 (pdb entry code 1IKT). The bound Triton X-100 molecule is shown as a ball-and-stick model and the C-terminal peroxisomal targeting signal is shown by the black arrow. The image was generated with Swiss-Pdb Viewer (Guex & Peitsch 1997).](image)

A recently published SCP-2 structure from yellow fever mosquito shows a ligand binding mode very different from that observed in SCP-2L (Dyer et al. 2003). The tail of the bound C16 fatty acid lies in the hydrophobic tunnel similarly to the Triton molecule in
SCP-2L, but the entrance of the tunnel locates on the opposite side of the molecule. This binding mode is enhanced by the replacement of the short α-helix αB, found in SCP-2L (Fig. 9) and SCP-2, by an elongated loop resulting in an open tunnel. The different binding modes of the two closely related proteins suggest that SCP-2s may exhibit multiple binding modes for their various ligands.

2.2.3.5 MFE-2 deficiency

The functional deficiency of MFE-2 (also called D-BP deficiency) is a rare genetic disorder, but one of the most frequently occurring single-peroxisomal enzyme deficiencies (for reviews, see Clayton 2001, Möller et al. 2001). It was initially described as a MFE-1 deficiency (Watkins et al. 1989), but reinvestigations located the disease-causing mutations in the gene coding for MFE-2 (Suzuki et al. 1997, van Grunsven et al. 1999c). MFE-2 deficiency is a very severe disorder, leading in most cases to death during the first year of life. In general, children with MFE-2 deficiency show severe nervous system involvement with profound hypotonia, uncontrolled seizures and failure to acquire any significant developmental milestones. They typically have large fontanelles, macrocephaly and other dysmorphic features. Patients with deficient MFE-2 often show disordered neuronal migration. The pathological mechanism underlying this MFE-2 disorder is not fully understood. However, it has been shown that MFE-2-deficient patients have impaired antioxidative defence in their plasma, which contributes to increased oxidative stress (Ferdinandusse et al. 2003). The presence of increased oxidative stress has also been observed in patients with Alzheimer’s disease and Parkinson’s disease (Foley & Riederer 2000, Smith et al. 2000).

MFE-2 disorder can be categorised into three different subtypes, depending on the nature and location of the mutation. The most severe form is type I deficiency, where the whole MFE-2 is completely lost. The mutations belonging to this subtype are frameshift mutations or larger in-frame mutations (Table 1) that result in truncated enzymes, which, if produced at all, are probably unstable and degrade rapidly (Suzuki et al. 1997, van Grunsven et al. 1999c). Naturally, the frameshift-mutated enzymes with a premature stop codon lack the C-terminally located peroxisomal targeting signal type I (Gould et al. 1989) and thus cannot enter peroxisomes. In patients with type II or type III MFE-2 deficiency, only part of the full-length enzyme is inactive. In the type III deficiency, the dehydrogenase region is unfunctional (van Grunsven et al. 1998, van Grunsven et al. 1999c), whereas in type II deficiency a disease-causing mutation exists in the region of hydratase 2 (van Grunsven et al. 1999a).

Pristanic acid and VLCFAs (C26) accumulate in all three subtypes of MFE-2 disorder, but differences are seen in the accumulation of bile acid intermediates. In patients with type III MFE-2 disorder, the accumulated isoform is logically the product of the hydratase 2 reaction, namely (24R,25R)-D/THCA-CoA, whereas the levels of (24S,25S)- D/THCA-CoA (due to the function of the hydratase unit of MFE-1) are elevated in type I deficiency (Vreken et al. 1998). In contrast, no D/THCA accumulation is observed in type II MFE-2 deficiency (van Grunsven et al. 1999a). The underlying basis for this
phenomenon is unknown, but lends support to the hypothesis of the presence of an alternative route for bile acid synthesis (Cuevas et al. 2002, Savolainen et al. 2004).

To date, several point, frameshift and in-frame mutations (Table 1) have been identified in the MFE-2 gene (Suzuki et al. 1997, van Grunsven & Wanders 1997, van Grunsven et al. 1998, van Grunsven et al. 1999a, van Grunsven et al. 1999b, van Grunsven et al. 1999c, Paton & Pollard 2000, Ferdinandusse et al. 2002), and moreover, a number of uncharacterised mutations are known (Gloorich et al. 2003). The most common mutation of MFE-2 disorder, Gly16Ser (van Grunsven et al. 1998) changes the sequence of the conserved fingerprint of the Rossmann fold and can thus be proposed to decrease drastically the affinity of the dehydrogenase unit of MFE-2 to NAD+. A similar effect can be estimated also to occur in the mutation of Leu21Phe. However, the structural basis of the loss-of-function cannot be explained for the rest of the point mutations due to the lack of three-dimensional structures of the enzymatic units of MFE-2.

Table 1. The mutations identified in MFE-2-deficient patients. (Modified from Möller et al. 2001)

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Subtype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly16Ser (46G → A)</td>
<td>III</td>
<td>van Grunsven et al. 1998, van</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Grunsven et al. 1999c</td>
</tr>
<tr>
<td>Leu21Phe (63G → T)</td>
<td>III</td>
<td>van Grunsven et al. 1999c</td>
</tr>
<tr>
<td>Ser177Phe (530C → T)</td>
<td>III</td>
<td>Paton &amp; Pollard 2000</td>
</tr>
<tr>
<td>Val218Leu (652G → T)</td>
<td>III</td>
<td>Paton &amp; Pollard 2000</td>
</tr>
<tr>
<td>Asn457Tyr (1369A → T)</td>
<td>II</td>
<td>van Grunsven et al. 1999a</td>
</tr>
<tr>
<td>Asn457Asp (1369A → G)</td>
<td>II</td>
<td>Paton &amp; Pollard 2000</td>
</tr>
<tr>
<td>aa 39-74 in-frame deletion</td>
<td>I</td>
<td>Ferdinandusse et al. 2002</td>
</tr>
<tr>
<td>aa 94-208 in-frame deletion</td>
<td>I</td>
<td>van Grunsven &amp; Wanders 1997,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>van Grunsven et al. 1999c</td>
</tr>
<tr>
<td>aa 480-501 in-frame deletion</td>
<td>II</td>
<td>van Grunsven &amp; Wanders 1997,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>van Grunsven et al. 1999c</td>
</tr>
<tr>
<td>Frameshift at aa 94 (stop at 95)</td>
<td>I</td>
<td>Paton &amp; Pollard 2000</td>
</tr>
<tr>
<td>Frameshift at aa 102 (stop at 148)</td>
<td>I</td>
<td>Paton &amp; Pollard 2000</td>
</tr>
<tr>
<td>Frameshift at aa 140 (stop at 163)</td>
<td>I</td>
<td>Watkins et al. 1989, van Grunsven et al. 1999b</td>
</tr>
<tr>
<td>Frameshift at aa 290 (stop at 294)</td>
<td>I</td>
<td>Watkins et al. 1989, van Grunsven et al. 1999c</td>
</tr>
<tr>
<td>Frameshift at aa 404 (stop at 405)</td>
<td>I</td>
<td>Suzuki et al. 1997</td>
</tr>
</tbody>
</table>

2.3 The (R)-specific 2-enoyl-CoA hydratases of the bacterial polyhydroxyalkanoate (PHA) synthesis pathway

In addition to being a component of MFE-2, hydratase 2 is also found in certain prokaryotic microorganisms such as *Aeromonas caviae* (Fukui & Doi 1997). These prokaryotic
hydratase 2s, referred to as (R)-specific 2-enoyl-CoA hydratases or (R)-hydratases, catalyse similar reaction as hydratase 2, but the products of trans-2-enoyl-CoA hydration, i.e., (3R)-hydroxyacyl-CoAs, are subsequently polymerised to polyhydroxyalkanoate (PHA) by PHA synthetase (Fukui & Doi 1997). Thus, the physiological role of (R)-hydratases differs from that of eukaryotic hydratase 2s.

PHAs are biological polyesters used as intracellular carbon and energy storage material. They have recently attracted industrial attention because of their potential use as biodegradable plastic (Madison & Huisman 1999). Because the properties of PHA are dependent on the chain-length of the fatty acyl units, (R)-hydratases, as monomer-supplying enzymes for PHA synthesis, have been studied intensively. In addition to A. caviae, the PhaJ gene encoding (R)-hydratase has been cloned from many microorganisms, such as Rhodospirillum rubrum (Reiser et al. 2000), Aeromonas hydrophila (Park et al. 2001), Pseudomonas aeruginosa (Tsuge et al. 2000, Tsuge et al. 2003), Pseudomonas putida (Fiedler et al. 2002) and Pseudomonas oleovorans (Fiedler et al. 2002). Generally, (R)-hydratases are dimers or tetramers with subunit size of ~15 kDa, utilising only short-chain enoyl-CoA substrates with chain length ranging from C4 to C6. These enzymes share sequence identity (~15%) with the C-terminal half of eukaryotic hydratase 2, and have also the conserved hydratase 2 motif. Exceptionally, P. aeruginosa has four (R)-hydratase isoforms; PhaJPA1, PhaJPA2, PhaJPA3 and PhaJPA4 (Tsuge et al. 2000, Tsuge et al. 2003). While PhaJPA1 is a typical (R)-hydratase with preference for short-chain substrates (and a molecular size of around 15 kDa), PhaJPA2, PhaJPA3 and PhaJPA4 all prefer medium-chain (C8) enoyl-CoAs. Furthermore, PhaJPA2 and PhaJPA3 have a subunit size of ~30 kDa resembling the subunit size of eukaryotic hydratase 2. Interestingly, although PhaJPA4 has a relatively conserved hydratase 2 motif, the enzyme exhibits low (R)-specificity (Tsuge et al. 2003).

The best characterised (R)-hydratase at present is the A. caviae enzyme. It is a dimeric enzyme with a subunit size of 14 kDa, and it shows maximum catalytic efficiency towards short-chain (C4 and C6) enoyl-CoA thioesters (Fukui et al. 1998). The crystal structure of A. caviae (R)-hydratase determined by Hisano and co-workers (2003) revealed that the enzyme structurally resembles the E. coli β-hydroxydecanoyl thiol ester dehydrase (FabA) (Leesong et al. 1996), a key enzyme in the bacterial fatty acid synthesis pathway type II. In both structures, the two subunits are associated with one another to form a functional homodimer with an extended antiparallel β-sheet (Fig. 10). The subunits consist of a long and hydrophobic α-helix wrapped by the β-sheet. Since the structure resembles a hot-dog with a long core α-helix and a curved β-sheet, corresponding to the sausage and the bun, respectively, the fold was named ‘hot-dog fold’ by Leesong and co-workers (1996). The additional loop structure included by an amphipathic α-helix, referred to as overhanging segment, in (R)-hydratase (Hisano et al. 2003) accounts for the major difference between the two prokaryotic enzymes. The importance of the overhang for catalytic activity is obvious, since it houses the hydratase 2 fingerprint.

Neither (R)-hydratase nor dehydrase has been crystallised in complex with natural substrates (fatty acyl/enoyl thioesters of CoA and acyl carrier protein, respectively). Instead, the crystal structure of dehydrase in complex with 3-decynoyl-N-acetylcysteamine, a suicide inhibitor of FabA, has been solved (Leesong et al. 1996). The ligands lie at the dimer interface and are covalently bound to the catalytic histidines (His70). Based on their complex structure, the dehydration reaction is proposed to occur via acid/base ca-
talysis (Leesong et al. 1996). In this proposal, His70 functions as the catalytic base accepting a proton from the C$_2$ of the fatty acyl substrate, whereas the Asp84 of the neighbouring subunit works as an acid and protonates the leaving hydroxyl group in the dehydration reaction (Leesong et al. 1996). After aligning the FabA homodimer with the A. caviae (R)-hydratase, the side-chain of Asp31 of (R)-hydratase (an equivalent residue to Asp510 in the hydratase 2 unit of human MFE-2) superimposes on the Asp84 of FabA. More interestingly, an equivalent histidine, His36, is also present in the (R)-hydratase in the same alignment. Although the aspartate and histidine in A. caviae (R)-hydratase are derived from the same subunit, a similar reaction mechanism between (R)-hydratase and FabA can be proposed. This is in contradiction to the data obtained from eukaryotic hydratase 2, where the Asp/Glu catalytic dyad was proposed (Qin et al. 2000a). It is noteworthy that A. caviae (R)-hydratase does not have a region equivalent to the N-terminal half of the hydratase 2 unit of MFE-2 and thus lacks the putative catalytic glutamate.

To date, the hot-dog fold has also been identified in tetrameric 4-hydroxybenzoyl-CoA thioesterases from Pseudomonas sp. strains CBS-3 and SU (Benning et al. 1998, Thoden et al. 2003) and a dimeric medium-chain length acyl-CoA thioesterase II (thioesterase II) from Escherichia coli (Li et al. 2000).

Fig. 10. Structural comparison of the hot-dog fold containing (R)-hydratase and FabA. The (R)-hydratase homodimer (pdb entry code 1IQ6) is represented on the left, with the two arrows pointing to the overhanging segments housing the hydratase 2 motif. On the right is the homodimer of FabA complexed with 3-decynoyl-N-acetylcysteamine (pdb entry code 1MKA). The 3-decynoyl-N-acetylcysteamines, shown as black sticks, are bound at the subunit-subunit interface of the extended β-sheet. The images were generated with Swiss-Pdb Viewer (Guex & Peitsch 1997).
3 Outlines of the present study

MFE-2 is a novel β-oxidation enzyme which is known to be the key enzyme in the metabolism of VLCFAs, pristanic acid and bile acid. At the time this study was started, MFE-2 had been cloned from different organisms, purified and characterised and produced recombinantly on bacteria and yeast. Because the enzymatic units of MFE-2 share no amino acid sequence similarities with their classical mitochondrial counterparts, the units were suggested to have different folding, CoA-binding mode and catalytic mechanism as well. In this study, which is part of a larger project focusing on the structure-function relationship of full-length MFE-2, the focus was on the enzymatic units of MFE-2, the two main aims being:

1. To determine the high-resolution crystal structure of the (3R)-hydroxyacyl-CoA dehydrogenase unit of MFE-2.
2. To determine the high-resolution crystal structure of the 2-enoyl-CoA hydratase 2 unit of MFE-2.

Once the crystal structures of (3R)-hydroxyacyl-CoA dehydrogenase and 2-enoyl-CoA hydratase 2 were solved (as part of MFE-2s from rat and C. tropicalis, respectively), further work was focused on the hydratase 2 unit. The two more specific aims were:

1. To solve the ligand-binding mode of hydratase 2.
2. To solve the crystal structure of the hydratase 2 part of human MFE-2 to provide a molecular basis for understanding the effects of the patient mutations as well as the substrate specificity differences between mammalian and fungal hydratase 2.
4 Materials and methods

The materials and methods have been described in more detail in the original articles referred to by their Roman numerals (I-IV).

4.1 The enzymatic units of MFE-2 used in this study

For structure determination studies, the enzymatic units of MFE-2 were cloned from various species with different polypeptide lengths. The most stable variants, chosen for crystallisation experiments, are summarised in Table 2. For the sake of clarity the abbreviations of the recombinant proteins are different from those used in the original articles (Table 2).

Table 2. Recombinant MFE-2 fragments used for structure determination.

<table>
<thead>
<tr>
<th>Enzymatic unit of MFE-2</th>
<th>Species</th>
<th>Amino acid region</th>
<th>Abbr. in the thesis</th>
<th>Abbr. in the original article(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(3R)-hydroxyacyl-CoA</td>
<td>Rattus norvegicus</td>
<td>1-319</td>
<td>RnDH</td>
<td>dhΔSCP-2LA (I)</td>
</tr>
<tr>
<td>dehydrogenase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-enoyl-CoA hydratase 2</td>
<td>Candida tropicalis</td>
<td>628-906</td>
<td>CtH2</td>
<td>CtMfe2p(dhΔSCP-2LA) (II, III)</td>
</tr>
<tr>
<td>2-enoyl-CoA hydratase 2</td>
<td>Homo sapiens</td>
<td>318-615</td>
<td>HsH2</td>
<td>HsMFE-2(dhΔSCP-2LA) (IV)</td>
</tr>
</tbody>
</table>

4.2 cDNA cloning

The open reading frames of rat MFE-2 (obtained by reverse transcription from RNA isolated from livers of Wistar rats of the Laboratory Animal Centre of the University of Oulu) and human MFE-2 (Haapalainen et al. 2001) were used as templates for the PCR amplification of RnDH and HsH2, respectively. The plasmid PMK/HDE50 encoding C. tropicalis MFE-2 and used as a template for CtH2 was a gift from Dr. R. A. Rachubinski.
The oligonucleotide pairs used for amplification were: \( R_{nDH} \),
\[
\text{CACTTCC ATG GCT TCG CCT AGG TTC GAC G (5'}^{\prime}\text{-primer) and}
\]
\[
\text{CATCTGGATCC TCA ATC GCA ACA TCA GCA TCT GGT CTG AGG TTC GAC G (3'}^{\prime}\text{-primer);}
\]
\( H_{sH2} \),
\[
\text{CACTTCC ATG GCA ACA TCA GCA TCT GGT CTG AGG TTC GAC G (5'}^{\prime}\text{-primer) and}
\]
\[
\text{CATCTGGATCC TCA TAA CTT AGC TGA AGT ACC AGA TG (3'}^{\prime}\text{-primer); C}_{t}H_{2},
\]
\[
\text{ATGCT CAT ATG GAA GAC GAT CCA GTC TGG AGA (5'}^{\prime}\text{-primer) and}
\]
\[
\text{ATGCT CAT ATG GAA GAC GAT CCA GTC TGG AGA (3'}^{\prime}\text{-primer) (NcoI, NdeI}
\]
\[
\text{and BamHI restriction sites are shown in bold). The extracted PCR products were sub-
\]
\[
\text{cloned to the pUC-18 vector using the Sure Clone Ligation Kit (Amersham Biosciences).
}\]
\[
\text{From the pUC-18 vector the inserts were digested using the restriction endonucleases}
\]
\[
\text{NcoI and BamHI for } R_{nDH} \text{ and } H_{sH2}, \text{ or NdeI and BamHI for } C_{t}H_{2} \text{ and subsequently}
\]
\[
\text{ligated to pET-3 expression vectors (Novagen).}
\]

4.3 **Production of recombinant proteins**

To produce the recombinant proteins, the constructed expression vectors were transformed to \( E. \text{coli BL21(DE3) pLysS} \) cells. A single colony from a Luria Bertani plate was transferred to Luria Bertani broth and grown overnight at 37°C in the presence of ampicillin (100 µg/ml). A 10 ml portion of the overnight culture was transformed to one litre of M9ZB medium supplemented with carbenicillin (50 µg/ml) and chloramphenicol (34 µg/ml), and the growing of bacterial cells was continued under aerobic conditions at 37°C. Once A_{600} of 0.6 against the water blank was reached, recombinant protein expression was initiated with 0.4 mM of isopropyl-1-thio-β-D-galactopyranoside, and the expression was performed at 30°C (CtH2) or 37°C (RnDH2 and HsH2). After three hours of induction, cells were collected by centrifugation and washed with 120 mM NaCl, 16 mM potassium phosphate, pH 7.4. The cells were then stored at -70°C until used.

4.4 **Production of selenomethionine (SeMet) derivatives (I, II)**

To generate \( R_{nDH} \) and \( C_{t}H_{2} \) labelled with selenomethionine (SeMet), the corresponding expression vectors were transformed to a methionine auxotrophic \( E. \text{coli B834(DE3)} \) strain. Bacteria were first grown overnight at 37°C in Luria Bertani broth supplemented with 50 µg/ml carbenicillin, but the effective growth was performed in SeMet Minimal Media [48 mM Na_{2}HPO_{4}, 22 mM KH_{2}PO_{4}, 9 mM NaCl, 0.4% (w/v) glucose, 2 mM MgSO_{4}, 0.1 mM CaCl_{2}, 8 mM (NH_{4})_{2}SO_{4}, 1 × BME vitamin solution (Gibco BRL), and 0.5 mM L-SeMet (Calbiochem-Novabiochem), 50 µg/ml carbenicillin]. After 5 hours of growth (OD_{600} reached the value of 0.8-0.9), expression of SeMet proteins was initiated with isopropyl-1-thio-β-D-galactopyranoside, which was added to a final concentration of 1 mM. Induction was performed at 20°C for 20-24 hours and the cells were collected and washed similarly as native proteins.
4.5 Protein purification

For purification, the cells were first treated with lysozyme. For that procedure, ~4 g of cell pellet was suspended in 40-50 ml of the buffer used at the first purification step with final lysozyme, Dnase I, Rnase A and MgCl2 concentrations of 100 µg/ml, 25 µg/ml, 25 µg/ml and 10 mM, respectively. After 15-30 minutes of incubation at room temperature, the broken cell fractions were separated from the crude protein solution by centrifugation.

Various chromatographic methods were applied to purify the enzymes. Firstly, the crude bacterial homogenates were applied to 30-60 ml ion-exchange chromatography columns using either DEAE sepharose or Q-sepharose matrixes (Amersham Biosciences). The unbound fractions of the first step of purification were pooled and dialysed against the buffer used at the second step, which was performed using commercial anion- or cation-exchange columns (Resource Q or S, respectively; Amersham Biosciences). After the two ion-exchange chromatography steps, the purity of the recombinant proteins was generally good enough for the final step, which included a size exclusion chromatography using a Superdex™ 200 HR 10/30 column (Amersham Biosciences). However, in the case of CtH2, a Resource Phenyl-Sepharose Fast-Flow hydrophobic interaction column (Amersham Biosciences) was required as a fourth column. The purification protocols are described in more detail in the original articles I, II, and IV.

4.6 Crystallisation, data collection and processing (I-IV)

The hanging-drop vapour-diffusion method was used in all crystallisation experiments. Each drop contained 2 µl of recombinant protein sample (concentrated to 3.9-17.5 mg/ml, see Table 3) and 2 µl of well solution. Ligands, such as NAD+ and acyl-CoAs, were included into the protein solution. The initial crystallisation conditions were screened using factorial screens (Jancarik & Kim 1991, Zeelen et al. 1992) and Clear strategy screens (Brzozowski & Walton 2001) at 22°C or 4°C, and the crystallisation conditions were optimised by altering pH, protein concentration and precipitant concentration as well as by using additives, which were added separately to the crystallisation drop (0.4 µl per drop).

All the crystal transfers, from drop to drop or from drop to beam, were performed with nylon loops (Hampton Research). Prior to flash-freezing at -173°C (100 K), cryoprotective compounds, such as glycerol or polyethylene glycol (PEG), were added to the reservoir solution to a concentration of 15-25%. The quality of the obtained crystals was screened using a Nonius FR591 rotating-anode X-ray generator and a MAR345 image plate detector (MarResearch) at the Department of Biochemistry, University of Oulu. Multiwavelength anomalous dispersion (MAD) data sets were collected at the beamlines BM30 at ESRF, Grenoble, France (I), and at the EMBL beamline BW7A, DESY, Hamburg, Germany (II). The other data sets were collected at the EMBL beamlines X13 and X11 (III, IV) and at the beamline I711 at MAXLab, Lund, Sweden (II). The used detectors were either MAR345 or MARCCD (MarResearch). The data sets were processed and scaled using either the programs DENZO and SCALEPACK from the HKL package (Otwinowski & Minor 1997), or the program XDS (Kabsch 1993).
4.7 Phase determination

The primary phases for SeMet-labelled \textit{RnDH} and \textit{CtH2} were determined by the MAD method using data measured at three wavelengths. The initial positions of the selenium sites were found by the program SOLVE (Terwilliger & Berendzen 1999), and in the case of \textit{CtH2}, additional selenium sites were also found by using CNS (Brünger \textit{et al.} 1998). 2-fold averaging using the solutions of the self-rotation function calculated with GLRF (Tong & Rossmann 1997) and the program FINDNCS (Lu 1999) was performed during the phase determination of \textit{RnDH}. The initial maps were improved further by solvent-flattening calculations using the program DM (Cowtan & Zhang 1999) and by the application of phase extension.

The crystal structure of \textit{HsH2} and the ligand-complexed structure of \textit{CtH2} were phased by a molecular replacement solution using CNS and the crystal structure of SeMet \textit{CtH2} as a model.

4.8 Model building, refinement and structure validation

With the exception of the initial model of \textit{RnDH}, which was built by means of the auto-building program MAID (Levitt 2001), model building was performed using the program O (Jones \textit{et al.} 1991). Structural refinement of \textit{RnDH} and \textit{HsH2} was carried out exclusively by using CNS, with noncrystallographic symmetry restraints included, whereas REFMAC (Murshudov \textit{et al.} 1997) was mainly used for the refinement of \textit{CH2} structures. Water molecules were added to the \textit{CH2} structures using the solvent-building mode of the program ARP/wARP (Perrakis \textit{et al.} 1999).

Structural integrity was monitored by WHATIF (Vriend 1990) and PROCHECK (Laskowski \textit{et al.} 1993), and the locations and types of structural motifs were defined by PROMOTIF (Hutchinson & Thornton 1996) and DSSP (Kabsch & Sander 1983).

4.9 Other methods

A QuickChange mutagenesis kit (Stratagene) was applied to introduce point mutations to the amino acid sequence of \textit{CtH2}. Oligonucleotides for the mutagenesis as well as for the cloning of the separate MFE-2 fragments were prepared with an Applied Biosystems DNA Synthesizer (Perkin-Elmer) or purchased from Amersham Biosciences.

Protein purity after each purification step was monitored by SDS-polyacrylamide gel electrophoresis using Coomassie staining and low molecular weight standards (Bio-Rad laboratories). To concentrate the protein samples, Ultrafree-4 centrifugal filter & tube Biomax-10 K concentrators (Millipore) were used. The protein concentrations of the crystallisation samples were determined by using UV-absorbance scan together with theoretical extinction coefficient at 280 nm or by using the Bradford reagent (Bio-Rad Laboratories). The measurements of \textit{(3R)-hydroxyacyl-CoA dehydrogenase and 2-enoyl-
CoA hydratase 2 activities were based on magnesium complex formation as described (Hiltunen et al. 1989, Malila et al. 1993).

To determine the degree of SeMet labelling of RnDH crystals, mass spectrometric analyses were carried out using an LCT (Micromass LTD) orthogonal time-of-flight mass spectrometer with an OpenLynx3 Data system. The Jasco JHO spectropolarimeter was used for the circular dichroism measurements to compare the secondary structure elements between the wild-type and the mutated CtH2 variants.
5 Results

5.1 Purification and crystallisation of truncated MFE-2 variants (I-IV)

The recombinant enzymatic units of MFE-2, (3R)-hydroxyacyl-CoA dehydrogenase and 2-enoyl-CoA hydratase 2, were purified to homogeneity, as determined by SDS polyacrylamide gel electrophoresis and Coomassie staining. All the variants, RnDH, CtH2 and HsH2, eluted from the size exclusion column with the retention time of dimers. However, a small fraction of CtH2 appeared to be tetrameric at a salt concentration of 150 mM, but after decreasing the NaCl concentration in the buffer used in the size exclusion experiments to 30 mM, the contaminating peak corresponding to the tetrameric form disappeared. The purified fragments of MFE-2 showed similar catalytic efficiency as obtained for full-length MFE-2. The SeMet-labelled variants of RnDH and CtH2 behaved similarly as the native forms during purification. In the case of RnDH, the degree of labelling was determined by mass-spectrometric analysis. The results showed complete labelling, where all of the five methionines of RnDH were replaced by SeMet.

Fig. 11. Crystals of the enzymatic units of MFE-2. Optimisation of the crystallisation conditions of RnDh (A), SeMet-labelled CtH2 (B), and HsH2 (C) yielded a single good-looking crystal, whereas the crystals obtained from the co-crystallisation experiments of CtH2H813Q with trans-2-decenoyl-CoA were twinned and needle-like (D). Bar represents 0.2 mm.

Initial crystallisation screening was performed with native proteins. The screening of the RnDH was carried out in the presence of NAD+, whereas no ligands were used in the case of CtH2 and HsH2. Triangle-shaped (RnDH) or plate-like (CtH2, HsH2) crystals
were obtained as results of further optimisation of the crystallisation conditions (Figs. 11A and 11C). The SeMet-labelled proteins crystallised nearly in the same conditions as the native forms, although the SeMet CtH2 crystals were more rectangular than their plate-like native counterparts (Fig. 11B). The optimised crystallisation conditions and the preliminary X-ray analysis for SeMet-labelled RnDH and CtH2 as well as those for HsH2 are presented in Table 3.

Table 3. Crystallisation and preliminary X-ray analysis of the enzymatic units of MFE-2.

<table>
<thead>
<tr>
<th>Protein conc.</th>
<th>Precipitant solution</th>
<th>Additive</th>
<th>Ligand</th>
<th>Crystallisation temperature (°C)</th>
<th>Resolution (Å)</th>
<th>Space group</th>
<th>Molecules per asymmetric unit</th>
<th>Matthews coefficient (Å³/Da)</th>
<th>Solvent content</th>
</tr>
</thead>
<tbody>
<tr>
<td>SeMet RnDH</td>
<td>3.9</td>
<td>0.12 mM Triton X-100</td>
<td>10 mM NAD⁺</td>
<td>21</td>
<td>2.38</td>
<td>P2₁</td>
<td>4</td>
<td>2.55</td>
<td>51</td>
</tr>
<tr>
<td>SeMet CtH2</td>
<td>17.2</td>
<td>0.12 mM Triton X-100</td>
<td>1.2 mM trans-2-decenoyl-CoA</td>
<td>4</td>
<td>1.95</td>
<td>C₂</td>
<td>4</td>
<td>2.82</td>
<td>56</td>
</tr>
<tr>
<td>C/H2H813Q-ligand</td>
<td>12.0</td>
<td>0.12 mM Triton X-100</td>
<td></td>
<td>4</td>
<td>2.35</td>
<td>P2₁</td>
<td>4</td>
<td>2.39</td>
<td>48</td>
</tr>
<tr>
<td>HsH2</td>
<td>6.4</td>
<td>0.12 mM Triton X-100</td>
<td></td>
<td>21</td>
<td>3.00</td>
<td>P2₁</td>
<td>12</td>
<td>2.70</td>
<td>55</td>
</tr>
</tbody>
</table>

To obtain a ligand-complexed crystal structure of hydratase 2, firstly, the two general methods were tested with CtH2. They include soaking of the physiological substrate (e.g., trans-2-decenoyl-CoA) with the crystals before flash-freezing, and crystallisation of CtH2 in the presence of substrate analogs (e.g., straight-chain acyl-CoAs). However, although well-diffracting crystals with a novel space group (P2₁2₁2₁) were obtained by co-crystallisation experiments with octanoyl-, decanoyl- and hexadecanoyl-CoAs, the solved crystal structures did not reveal any clear electron density for the ligands. Secondly, the work was continued with CtH2 variants with dramatically reduced enzyme activity. Those variants included point mutations introduced to catalytic residues, namely either to Asp808 (equivalent to the Asp510 in human MFE-2) or to His813 (found in this study based on the SeMet CtH2 structure, see chapter 5.3.3). The crystallisation experiments of
the Asp808Ala variant in the presence of trans-2-decenoyl-CoA did not yield crystals, but instead, the co-crystallisation experiments of CtlH2His813Gln with trans-2-decenoyl-CoA were successful producing needle-like crystals (Fig. 11D) with ligand molecules included. The preliminary crystallographic data of this complex structure (CtlH2H813Q-ligand) is also shown in Table 3.

5.2 Crystal structure of the (3R)-hydroxyacyl-CoA dehydrogenase unit of rat MFE-2 complexed with NAD+ (I)

The three-wavelength MAD data of the RnDH crystal were first tentatively phased by the SOLVE (Terwilliger & Berendzen 1999) with a resolution limit of 2.6 Å. Although the program was able to find 12 selenium sites out the 20 expected in the asymmetric unit, the resulting electron density maps were not interpretable (figure of merit value = 0.34). The finding that the two dimers in the asymmetric units are related via a non-crystallographic 2-fold axis \((\kappa = 180^\circ, \phi = 0^\circ, \psi = 86^\circ)\), identified using the GLRF (Tong & Rossmann 1997), FINDNCS (Lu 1999) and the 12 selenium sites, however, enabled the calculation of better maps. After phase extension and solvent flattening, the maps were suitable for the autobuilding program MAID (Levitt 2001). The detailed refinement and structural determination statistics are presented in Table 1 in the original article I.

The tertiary structures of the subunits of one RnDH homodimer were virtually identical (Fig. 5, original article I), and with the exception of the loop regions, Asp44-Gly51 and Ala196-Val210, in the subunit B, the polypeptides were continuous from Ser3 or Pro4 to Asp303. As expected from the observed sequence similarity with the SDR proteins, RnDH has the typical Rossmann fold consisting of a seven-stranded parallel \(\beta\)-sheet \((\beta A-\beta F)\) sandwiched between two arrays of three parallel \(\alpha\)-helices \((\alpha B, \alpha C, \alpha D, \alpha E, \alpha F)\) located on each side of the \(\beta\)-sheet. The parallel \(\beta\)-sheet is, furthermore, extended by a \(\beta\)-strand \(\beta G\), whereas the rest of the polypeptide chain of RnDH, namely \(\beta\)-strands \(\beta H\) and \(\beta I\), and the \(\alpha\)-helices \(\alpha CT1, \alpha CT2\) and \(\alpha H\), form an extra domain referred to as the C-terminal domain (Fig. 12). No other SDR superfamily member has a similar two-domain architecture.

In addition to the two longest \(\alpha\)-helices, \(\alpha E\) and \(\alpha F\) of the Rossmann fold, which form the interfacial four-helix bundle typical of dimeric and tetrameric SDR proteins, the dimeric interactions are extended by the C-terminal domain. The long \(\beta\)-strand, \(\beta H\), which is a linker region between the N-terminal nucleotide-binding domain and the novel C-terminal domain, is arranged antiparallel to the corresponding \(\beta\)-strand of the neighbouring subunit. The two strands intersect spatially at Trp249, whose aromatic rings are connected by stacking interactions. The \(\beta\)-strand \(\beta I\) of monomer A pairs with the N-terminus of the \(\beta\)-strand \(\beta H\) of monomer B and vice versa. The two short \(\alpha\)-helices \(\alpha CT1\) and \(\alpha CT2\) and the long \(\alpha\)-helix \(\alpha H\) lie on the surface of the N-terminal domain of the adjacent unit (Fig. 12).

The bound NAD\(^+\) is in an extended conformation and sits on a surface created by the C-terminal ends of the \(\beta\)-strands in the Rossmann fold. The nicotinamide part is buried near the putative active site, while the adenine moiety locates close to the N-terminal part of the Rossmann fold. The amino acid residues of the conserved Gly-rich motif (Gly16-
Gly22) and the N-N-A-G motif (Asn98-Gly101) important for cofactor binding and the structural stability of the Rossmann fold, respectively, are in close contact with NAD⁺. The elongated loop between the β-strands βB and the α-helix αC forms a lid over the NAD⁺-binding pocket (Fig. 12). Interestingly, this loop is missing from the dehydrogenase B of fungal MFE-2, which shows preference to short-chain acyl-CoA substrates. The hydroxyl group of Tyr164 and the ε amino group of Lys168, the amino acids of the conserved Y-X-X-X-K catalytic motif, are within hydrogen bond distances from the hydroxyls of the nicotinamide ribose (Fig. 4, original article I). Ser151 also locates in the vicinity of the active site completing the Ser-Tyr-Lys catalytic triad. The architecture of the active site allows B face 4-pro-S-hydride transfer. The close contact between the sidechain of Asp40 and the hydroxyl group of the adenine ribose regulates the specificity toward NAD(H) over NADP(H) (Fig. 4, original article I).

Fig. 12. Ribbon diagram of the RnDH subunit (on the left) and the dimer (on the right) in complex with NAD⁺. The subunits A and B in the right-hand image are shown in dark and light grey, respectively. The bound NAD⁺ molecules are shown as black sticks, and the black arrow points to the loop structure, which is absent from the short-chain specific dehydrogenase B of fungal MFE-2. The loop is not seen in the subunit B of RnDH (right image) because of the lack of interpretable electron density. The images were generated with Swiss-Pdb Viewer (Guex & Peitsch 1997).

5.3 Structural studies on hydratase 2 unit of MFE-2

5.3.1 Structure determination (III-IV)

The complete MAD data at 2.25 Å resolution were used for phase determination of the unliganded form (apoenzyme) of hydratase 2. The positions of 8 selenium atoms were found in the asymmetric unit of the C/H2 crystal form by SOLVE (Terwilliger & Berendzen 1999), and further refinement with CNS (Brünger et al. 1998) led to two additional sites. After solvent flattening and phase extension, the electron density calculated with the help of the 10 selenium sites already revealed clear secondary structure elements for CH2 (figure of merit = 0.56). The data from the absorption peak, supplemented with additional 85% complete high resolution data (at 1.95 Å), was used in refinement, resulting in a model with crystallographic R_work and R_free values of 17.7%
and 21.4%, respectively (Table 1, original article III). The solved crystal structure revealed four monomers (two dimers) in the asymmetric unit, out of which the monomer D could be most completely built, containing residues Pro631-Asp902. Therefore, the monomer D or a C-D dimer of SeMet C/H2 was used as a model in the molecular replacement phasing of the C7H2H813Q-ligand complex and HsH2 crystal structures, respectively. The detailed protocols for the structure determination as well as the structural statistics are described in the original articles III (C/H2 and C7H2H813Q-ligand) and IV (HsH2).

### 5.3.2 Overall structure of 2-enoyl-CoA hydratase 2 from C. tropicalis MFE-2 (III)

The C/H2 subunit is composed of 7 α-helices and 11 β-strands with molecular dimensions of 35 × 40 × 45 Å (Fig. 13). The β-strands form an extended antiparallel β-sheet, which covers the whole molecule. Two buried α-helices are located under the large β-sheet, namely a sixteen-residue-long α-helix α7 and a short three-residue-long α-helix, α2. These two buried α-helices are sandwiched between the large β-sheet and a pair of two antiparallel α-helices, α1 and α5, which run almost perpendicular with respect to the α-helices α2 and α7. Moreover, the subunit contains two elongated loop structures between the α-helices α1 and α2, and α5 and α7, the latter housing an additional amphipathic α-helix α6. Together with the α-helix pair α1 and α5, the two extended loops participate in the C/H2 dimer formation (Fig. 13).

After a more careful examination, the existence of a structural repeat in the subunit of C/H2 can be observed. Namely, after dividing the polypeptide into two equal fragments and rotating the N-terminal half 180° around the vertical axis perpendicular to the β-sheet layer, the N-terminal β-strands β1-β5 superimpose on the C-terminal β-strands β7-β11, as do also the α-helices α1 and α5. The core of the C-terminal half of C/H2 (C-domain) consists of the long α-helix α7 and the covering β-strands β7-β11, forming a typical hot-dog fold first identified in the crystal structure of FabA (Leesong et al. 1996). In the N-domain of C/H2, which has a β-strand architecture topologically equivalent to that observed in the C-domain, the long central α-helix of the hot-dog structure is replaced by a discontinuous helical region. The short α-helices α2 and α3 locate at the end of the region, while the central part is more stretched and forms a bend in the continuous chain. Moreover, high temperature factors and fragmented or wholly absent electron densities indicate high structural mobility for the discontinuous helical region. The poorly interpretable electron density continues up to the N-terminus of the β-strand β2, indicating that the whole loop between the α-helix α2 and the β-strand β2 is flexible and hence referred to as flexible loop I. In addition, the N-domain of C/H2 also contains two other flexible loops, namely the hairpin loop between the β-strands β3 and β4 (flexible loop II) as well as the loop beginning at the end of the β-strand β5 and ending at the C-terminus of the β-strand β6 (flexible loop III). The flexible loop III continues as a well-defined random coil structure, which traverses the extended β-sheet. This region, which is included by a short α-helix α4, functions as a linker region between the N- and C-domains.
Fig. 13. The two-domain crystal structure of one subunit of CtH2 is shown on the left. The N-domain and the C-domain are coloured as light and dark grey, respectively. The α-helix α4 in the linker region between the two domains is labelled. The topology of the β-sheet is shown underneath the image. The right image shows the quaternary fold of CtH2, where the two α-helices, α1 from the N-domain and α5 from the C-domain, form a four-helix bundle structure, where the pairwise arranged helices from one subunit are packed against their counterparts from the other one in an anti-parallel fashion. The dimeric interactions are strengthened by the two extended loops, out of which the C-domain loops housing the hydratase 2 motif are shown by the arrows on both images. The bound CoA substrates, (3R)-hydroxydecanoyl-CoAs, are shown as black sticks. The images were generated with Swiss-Pdb Viewer (Guex & Peitsch 1997).

5.3.3 Hydratase 2 motif and catalytic residues in C. tropicalis hydratase 2 (III)

The conserved sequence pattern of hydratase 2 enzymes, the hydratase 2 motif (Qin et al. 2000a), starts at the middle of the α-helix α5 and ends at the middle of the amphipathic α-helix α6, thus forming half of the C-domain solvent-exposed loop. The proposed catalytic Asp808 locates in the random-coil polypeptide in between the α-helices α5 and α6, near the domain-domain interface. Interestingly, the equivalent residue for the Glu366 of human MFE-2 (proposed to be a catalytic residue by Qin and co-workers (2000a)), Glu659, is placed in the N-domain solvent-exposed loop locating on the opposite side of the molecule. Although, in the CtH2 dimer, this loop interacts with the C-domain solvent-exposed loop, Glu659 is not in the close vicinity of Asp808. In contrast, Glu659 is salt-bridged to a one of the conserved residues in the hydratase 2 motif, Arg804 of the neighbouring subunit (salt bridge Glu366-Arg506 in human hydratase 2; Fig. 3A, original article IV). This architecture of the active site challenged the previously proposed reaction mechanism of hydratase 2.

Careful examination of the region around Asp808 revealed four other conserved protic residues, His699, Tyr803, His813 and His830, which were either directly or indirectly (via water molecules) interacting with Asp808 (Fig. 3A, original article III). Because studies with human MFE-2 have shown that the residues corresponding to His699 and
His830 (His404 and His532, respectively) can be changed to alanines without losing the hydratase 2 activity (Qin et al. 2000a), Tyr803 and His813 were the most likely candidates for the second catalytic residue for the hydratase 2 unit. Previously, these residues have been proposed to have a structural role in the full-length MFE-2, since the alanine mutations (Tyr505Ala and His515Ala) have caused the inactivation of both hydratase 2 and (3R)-hydroxyacyl-CoA dehydrogenase activities (Qin et al. 2000a). In the present study with CtH2, less drastic changes at the amino acid level were introduced to those conserved residues. Namely, Tyr803 was replaced by phenylalanine and H813 by glutamine, yielding recombinant proteins CtH2Y803F and CtH2H813Q, respectively. After successful expression of the mutated variants, hydratase 2 activities were measured from bacterial extracts. CtH2Y803F had wild-type hydratase 2 activity, indicating that Tyr803 is not needed in catalysis. Instead, the activity of CtH2H813Q in the crude homogenate was under the detection limit. Because the expressed CtH2H813Q was also stable, it was purified to homogeneity using the same protocol as in the case of native and SeMet-labelled recombinant enzymes. During purification, CtH2H813Q showed similar behaviour as the native protein, suggesting correct folding. In the concentrated crystallisation sample (12 mg/ml) its \( k_{\text{cat}} \) value of 0.026 s\(^{-1}\) with 60 \( \mu \)M \((3R)\)-trans-2-decenoyl-CoA was around 15000 times lower than that determined for the native enzyme (388 s\(^{-1}\)). These results suggest that the histidine in the hydratase 2 motif (His813 in \( C. \) tropicalis, His515 in human) has a catalytic rather than a structural role.

### 5.3.4 Crystal structure of CtH2H813Q in complex with (3R)-hydroxydecanoyl-CoA (III)

CtH2H813Q maintained the same overall folding as the SeMet CtH2, confirming that His813 has no structural function in the protein. Three CtH2H813Q subunits out of the four found in the asymmetric unit, defined electron densities for acyl-CoA molecules. Unexpectedly, a clear density for the hydroxyl group in the (R)-position at C\(_3\) (Fig. 3C, original article III) revealed that the actual ligand in the crystal structure was the hydroxylation product of \((3R)\)-trans-2-decenoyl-CoA, namely \((3R)\)-hydroxydecanoyl-CoA. Except for the four carbons at the \( \phi \)-end of the \((3R)\)-hydroxydecanoyl-CoA molecule in subunit D, the ligand molecules had well-defined electron densities in all subunits.

The CoA-binding site locates at the surface of the extended \( \beta \)-sheet at the interface of the N- and the C-domains (Fig. 13). The bound \((3R)\)-hydroxydecanoyl-CoA molecule is in the bent conformation, a state stabilised via the hydrogen bond between O\(_{10}\) of the pantotheine unit and N\(_7\) of the adenine unit. The adenine ring of the 3'-phosphate ADP moiety points towards the protein, its \( \pi \) electrons forming a van der Waals stacking interaction with the \( \pi \) electrons of the side-chain of Arg855, and the adenine amino group hydrogen-bonding to the backbone oxygen of Phe856. The \( \varepsilon \) amino group of Lys729 forms a salt bridge with the 3'-phosphate group of the ribose ring.

Part of the pantothenic acid, and the whole \( \beta \)-mercaptoethylenamine and acyl units of the \((3R)\)-hydroxydecanoyl-CoA molecule are buried into the protein interior. Numerous hydrophobic residues, such as Leu698, Phe758, Leu812, Phe824, Ile828, Phe856, Gly858 and Ile859, cover the orifice of the substrate-binding tunnel. The only hydrogen bond
formed between the β-mercaptoethylenamine unit and the protein molecule is between
the N₄ and backbone oxygen of His699. The carbonyl oxygen of the acyl unit of the (3R)-
hydroxydecanoyl-CoA molecule is hydrogen-bonded to the backbone amide of Gly831
and a water molecule, W1 (Fig. 3B, original article III), while the (3R)-hydroxyl group
makes three hydrogen bonds with the protein, namely with the O₂ of Asp808, the Nₑ₂ of
Gln813, and the Nₑ₂ of Ans810 (Fig. 3B, original article III). The substrate-binding tun-
nel continues as an open cleft accommodating the remaining acyl chain of the substrate.
This hydrophobic cleft is surrounded by the β-strands β₂ and β₅, and especially by the
flexible loop I, which contains hydrophobic residues, such as Phe676, Phe685, Phe692
and Leu697, which interact with the σ-end of the acyl group. The cleft in the C₁₀-
complex structure has enough space to engulf a fatty acyl tail up to about C₁₆ in length.

5.3.5 Crystal structure of 2-enoyl-CoA hydratase 2 from human
MFE-2 (IV)

The crystals obtained from HsH₂ had a large unit cell with relatively low symmetry, and
altogether 12 subunits were found in the asymmetric unit (Table 3). In subunit I the
amino acids from Phe324 to Ala606 could be built, but the subunits generally contained
many regions with fragmented electron densities. The basic fold of HsH₂ resembles the
overall fold of CṭH₂, having a similar two-domain subunit structure composed of a N-
domain with an incomplete hot-dog fold, a C-domain with a complete hot-dog fold, and a
connective linker region (Fig. 2B, original article IV). Dimerisation was also
accomplished equally to that observed in CṭH₂, although no two salt bridges resembling
the Asp637-Arg809 and Arg638-Asp639 in CṭH₂ are observed in the
HsH₂ dimer. Interestingly, in the crystal structure of HsH₂ two dimers are linked via β-
sheets in such a way that the β-strand β₇ from one subunit forms an antiparallel β-sheet with the β₇ of
the other subunit from the adjacent dimer (Fig. 1B, original article IV). However, since only
the dimeric form is detected in the size exclusion experiments, the tetramerisation of
HsH₂ is probably a consequence of crystal contacts.

The C-domains of HsH₂ and CṭH₂ are practically similar, the major difference being
the elongated loop region (residues Ala549-Arg555) after the hot-dog core α-helix α₇ in
HsH₂. In contrast, larger variations occur in the N-domains of the two orthologs, mainly
due to the presence of the highly mobile loop regions, the flexible loops I-III, in both
structures. In HsH₂, the flexible loops I (Met386-Ala402) and III (Val464-Thr474) were
the most mobile structures, because of the lack of electron density in most subunits in the
asymmetric unit. Compared to the corresponding loops in CṭH₂, the flexible loop I was
more oval-shaped and lacked the short α-helix α₃, while the flexible loop III did not form a β-sheet structure with the β-strand β₃, but continued towards the C-domain,
starting the domain-domain linker region (Fig. 2B, original article IV). The flexible loop
II (Leu432-Val441), which forms a hairpin loop containing the Lys435 known to form a
salt bridge with the 3'-phosphate group of the ribose ring of the enoyl-CoA molecule, in
contrast, superimposes well on the corresponding loop of CṭH₂. Smaller differences in
the N-domains between HsH₂ and CṭH₂ locate in the solvent-exposed loop after the α-
helix α₁ as well as in the hairpin loop between the β-strands β₄ and β₅. The extreme N-
terminus of \( HsH2 \), the region preceding the \( \beta \)-strand \( \beta1 \) (Phe324-Pro335), has relatively fragmented electron densities, but clearly extends \( \beta1 \) by pairing with the \( \beta \)-strand \( \beta3 \). In \( CtH2 \), in contrast, where the modelled N-terminus is 11 residues shorter, no such elongation of \( \beta1 \) is likely to occur. Finally, the intervening bridge between the N- and C-domains of \( HsH2 \) lacks the well-defined \( \alpha \)-helix \( \alpha4 \), found in \( CtH2 \) (Fig. 2B, original article IV).

The putative active site residues of \( HsH2 \), Asp510, His515 and Gly533, superimposed on the corresponding residues, Asp808, His813 and Gly831, in \( CtH2 \). However, the relatively low resolution did not allow visualisation of the individual water molecules in the catalytic centre.
6 Discussion

6.1 (3R)-hydroxyacyl-CoA dehydrogenase unit of rat MFE-2, a novel two-domain structure among SDR proteins (I)

Although the (3R)-hydroxyacyl-CoA dehydrogenase unit of rat MFE-2 is a dimer of two-domain subunits similarly to its mitochondrial counterpart, HADI (Barycki et al. 1999), these two enzymes do not share any significant structural homology, although both have a Rossmann fold for NAD⁺ binding. Instead, RnDH shares notable identity with HADII/ABD, which is also known to accept β-oxidation intermediates as substrates (Powell et al. 2000). The similarity between RnDH and HADII/ABD is expected, since both are members of the SDR protein family. In the structural alignment of RnDH with HADII/ABAD, the β-strands of the central seven-stranded β-sheet superimpose perfectly, as do the six surrounding α-helices of the Rossmann fold. In addition, the bound NAD⁺ molecules are in the same conformation and superimpose well. Nevertheless, the C-terminal domain of RnDH, starting at the β-strand βH, does not have a counterpart in either HADII/ABAD or any other SDR enzymes so far characterised structurally, making the fold of RnDH unique. The dimerisation of RnDH is accomplished equally to the other SDR family members via the long α-helices αE and αF. In the quaternary structure of HADII/ABAD, similarly as in the two other closely related short-chain hydroxysteroid dehydrogenases, 7α-hydroxysteroid dehydrogenase (Tanaka et al. 1996) and 3α,20β-hydroxysteroid dehydrogenase (Ghosh et al. 1991), the corresponding dimer is further dimerised, forming a biologically active tetramer. The two dimers in the tetramer are dimerised via the β-strands βG, which in RnDH is paired with the β-strands βH of the same subunit. Moreover, the C-domain of the neighbouring subunit in RnDh wraps the rest of the corresponding tetramerisation area in HADII/ABAD. Therefore, similar tetramerisation is prevented in RnDH.

The side-chain conformations of the catalytic triad, Tyr164, Lys168 and Ser151 in RnDH are similar to those found in other SDR enzymes, suggesting a highly similar catalytic mechanism. The conserved asparagine in the α-helix αE, known to contribute to the catalysis of many SDR enzymes, including HADII/ABAD, is not present at the active site of RnDH. Instead, in the RnDH structure the equivalent residue is His123, whose Nε2 atom is within hydrogen bond distance from the ε amino group of Lys168. It is notewor-
thy that this His123 is highly conserved among (3R)-hydroxyacyl-CoA dehydrogenases (Fig. 1, original article I), prompting the question of its functional role in catalysis.

The crystal structure of RnDH does not include a ligand molecule, leaving the ligand-binding mode of the (3R)-hydroxyacyl-CoA dehydrogenase unit of MFE-2 as an open question. In the ternary complex of HADII/ABAD with NAD\(^+\) and 17β-estradiol, the steroid ligand binds to the pocket formed between the two elongated loops equivalent to the loops between the β-strand βD and the α-helix αE and the β-strand βF α-helix αG in RnDH. In general, the loop βF-αG is known as a substrate-binding loop among SDR enzymes, and it is typically rather flexible. This feature is also seen in the crystal structure of RnDH, where the B subunit lacks a well-defined electron density for this loop. In this mode of binding, the carbonyl oxygen of the substrate is hydrogen-bonded to the hydroxyl group of the active site tyrosine and close to the C4 group of the nicotinamide ring, the site of hydride transfer. Since the (3R)-hydroxyacyl-CoA dehydrogenase unit of MFE-2 is also known to accept 17β-estradiol as a substrate, an equal binding mode of steroid compounds is very likely due to the notable similarities in the nucleotide-binding domains of these two enzymes. In the case of acyl-CoA substrates, the elongation of the second loop in HADII/ABAD, loop βD-αE, has been proposed to be important, since it serves a positive cluster for the phosphates of the CoA moiety of the acyl-CoA substrates. No such loop elongation is observed in the RnDH structure, suggesting that RnDH may have a different binding mode for CoA substrates. Instead, in RnDH, an open tunnel is formed through the novel C-terminal domain in such a way that the tunnel reaches the active site cavity. This could be the possible ligand-binding tunnel for RnDH. The suggestion is strengthened by the existence of a positive cluster between the β-strand βF and the α-helix αCT1, which might serve as a suitable binding site for the CoA moiety of the substrate.

The elongated loop βB-αC of RnDH, the one possible factor for the long-chain acyl-CoA substrate preference (Qin et al. 1999, Qin et al. 2000b), locates at the bottom of the active site cavity close to bound NAD\(^+\). In the RnDH structure there would not be space for substrates longer than C\(_{16}\) without conformation changes of the loop. The high glycine content of this loop may be one factor that enhances the ability of the loop to take various conformations upon binding of the VLCFAs and fatty acyl derivatives known to be substrates for RnDH.

The structural basis for the strict stereospecificity of the (3R)-hydroxyacyl-CoA dehydrogenase unit of MFE-2 is not revealed by the binary structure of RnDH. The active site is almost identical to that observed in HADII/ABAD, which is known to accept both enantiomers of 3-hydroxyacyl-CoAs as substrates. One possible explanation could be the differences in CoA substrate binding, which lead to the different orientation of the acyl unit of the substrate with respect to the active site residues.
6.2 Structural studies on the 2-enoyl-CoA hydratase 2
part of MFE-2 (III, IV)

6.2.1 Unique two-domain structure of hydratase 2

The crystal structure of hydratase 2 introduces a novel fold into the family of enzymes involved in fatty acid β-oxidation, namely the hot-dog fold. The structure also confirms that hydratase 2 has evolved from a totally different evolutionary origin compared to its mitochondrial equivalent, hydratase 1, which is a typical member of the hydratase/isomerase superfamily. As already expected based on sequence analysis, the C-domain of hydratase 2 folds very similarly to the subunit of (R)-hydratase solved by Hisano and co-workers (2003). Both structures carry the solvent-exposed loop housing the conserved hydratase 2 motif (referred to as overhanging segment by Hisano and co-workers (2003)), known to be important in the (R)-specific hydration of fatty acyl-CoAs (Qin et al. 2000a). However, while in (R)-hydratase two subunits each containing a hydratase 2 motif associate side-by-side to form the functional homodimer, the N-domain and C-domain of hydratase 2 pair in such a way that one subunit resembles strikingly the homodimer of (R)-hydratase (Fig. 2, original article III). The origin of the N-domain of hydratase 2 is an intriguing question. Since it shows no significant amino acid sequence similarity to the C-domain, it can be proposed that the N-domain has arisen via gene fusion of two non-related genes with subsequent structural convergence. However, 50% identity at the DNA encoding the two domains in the C. tropicalis hydratase 2, in addition to the striking similarities in the folding, suggest that gene duplication of an ancient gene followed by mutational differentiation is a more likely explanation.

The hydratase 2 dimer does not have a counterpart in the quaternary structure of (R)-hydratase. The reason why eukaryotic hydratase 2 has to be a dimer in its active form, while the A. caviae (R)-hydratase dimer corresponding to one subunit of eukaryotic hydratase 2 is active is an open question. The probable explanation for the dimeric nature of the eukaryotic hydratase 2 is the incomplete hot-dog fold in the N-domain. The disrupted hot-dog core fold most probably leads to a thermodynamically more unstable structure, which has to be stabilised by dimeric interactions. Interestingly, the R. rubrum (R)-hydratase appears to be a homotetramer in its native form (Reiser et al. 2000). It is likely that the two (R)-hydratase dimers in R. rubrum are further dimerised via the four-helix bundle forming a quaternary structure very close to that observed in eukaryotic hydratase 2. The (R)-hydratases having equivalent molecular size with hydratase 2, such as PhaJ2PA, can be suggested to have a similar two-domain subunit structure as hydratase 2.

Among the hot-dog fold enzymes, thioesterase II also has a structural repeat within the subunit resembling the subunit structure of hydratase 2 (Li et al. 2000). The subunit structure of thioesterase 2 has an extended 12-stranded β-sheet, where the β-strands β2-β6 and β8-β12 are topologically arranged equivalently to the β-strands β1-β5 and β7-β11, respectively, in hydratase 2. However, in thioesterase II, the N-domain has complete hot-dog fold with a long core α-helix, while the C-domain core α-helix is discontinuous, resembling the incomplete hot-dog fold of the N-domain of hydratase 2. More striking differences can been seen at the quaternary structure level, namely while hydratase 2 sub-
units are dimerised via a four-helix bundle, the two subunits in the thioesterase II dimer are dimerised via exposed β-sheets. Although similar gene duplication may have occurred during the evolution of thioesterase II as in the case of hydratase 2, the lack of any amino acid sequence similarity in addition to the different functional role suggested that these two hot-dog fold-containing proteins are distant relatives evolved from the same ancestor gene but via distinct gene duplications.

6.2.2 Ligand-binding mode

The bound (3R)-hydroxydecanoyl-CoA in the crystal structure of CTH2H813Q revealed the detailed ligand-binding mode of hydratase 2. The functional hydratase 2 dimer contains two substrate-binding sites, one per subunit, which locate on sites opposite to the elongated dimer (Fig. 13). The (3R)-hydroxydecanoyl-CoA molecules are bound at the domain-domain interface between the β-strands β2 and β8. An equivalent binding mode at the subunit-subunit interface is seen in all the ligand-complexed hot-dog enzymes, such as FabA in complex with 3-decynoyl-N-acetylcysteamine and 4-hydroxybenzoyl-CoA thioesterases in complex with 4-hydroxybenzoate and its derivatives (Thoden et al. 2002, Thoden et al. 2003). However, in all the three cases, the unit corresponding to the hydratase 2 subunit contains two ligand-binding sites. This is also most likely the case in (R)-hydratase, although the crystal structure with bound ligand is not available.

In the binary structure of hydratase 2, the fatty acyl and the β-mercaptoethyleneamine units are buried into the hydrophobic tunnel, whereas the 3′-phosphate ADP and half of the pantetheine moieties lie on the surface of the molecule. Thus, the basis of how hydratase 2 binds the CoA esters of fatty acids resembles other β-oxidation enzymes, although the CoA-binding fold is strikingly different. Moreover, compared to hydratase 1, notable similarities can be detected concerning the interactions between the protein molecule and the 3′-phosphate ADP moiety of the ligand as well as in the conformation of the CoA molecule itself. Namely, in both structures the CoA is in a bent state, where the adenine amine is hydrogen-bonded to the main-chain oxygen of the protein molecule, whereas the phosphates are salt-bridged to the side-chains of lysines.

Hydratase 2, at least in mammals, can accept as substrates CoA esters of bulky fatty acids and fatty acyl derivatives, such as VLCFAs, branched-chain fatty acids and bile acid intermediates. The complex structure of C. tropicalis hydratase 2 with the C10 fatty acid substrate revealed the importance of the N-domain for the binding of the long fatty acyl unit of the ligand. The highly mobile N-domain loops, especially loop I, of hydratase 2 facilitates the passage of bulkier substrates to the active site cavity. In the A. caviae (R)-hydratase dimer, the region corresponding to the flexible loop I is stiffened up by the rigid long “hot-dog helix” of an adjacent subunit, and the preference to short-chain substrate (C4-C6) can be explained in terms of these structural features. The low activity of human hydratase 2 towards the short-chain substrates, instead, is more difficult to explain by means of structural analysis. The flexible loop I in the unliganded form of human hydratase 2 is folded differently than either unliganded or liganded forms of the fungal ortholog in such a way that the side-chain of Met386 blocks the cavity for the long carbon tail (>C6) of the fatty acyl group (Fig. 4C, original article IV). Upon the binding of
long-chain substrates, it is likely that the flexible loop straightens, creating a secondary effect on the CoA-binding pocket via the two other flexible loops, so that the enzyme affinity towards the CoA unit of the substrate is substantially increased.

6.2.3 Proposed reaction mechanism for (R)-specific hydration/dehydration (III)

The present results disproved the role of N-domain glutamate as a catalytic amino acid for hydratase 2. In contrast, clear evidence was obtained by mutagenesis as well as crystallographic studies to show that the histidine in the hydratase 2 motif (His813 and His515 in C. tropicalis and human MFE-2, respectively) acts as a second catalytic residue in addition to the previously identified aspartate (Asp808 and Asp5150 in C. tropicalis and human MFE-2, respectively). The parallel study with (R)-hydratase by Hisano and co-workers (2003) led to a similar conclusion concerning the Asp/His catalytic dyad and the suggested active sites of the homologous enzymes were superimposable. However, the (3R)-hydroxydecanoyl-CoA-complexed hydratase 2 structure is the first experimental structure among (R)-specific hydratases/dehydrases, allowing a more detailed analysis of the active site as well as a better understanding of the catalytic mechanism.

The tight hydrogen bond between the acyl hydroxy group of the substrate and Asp808 confirms the active site. Although the active site histidine, His813, was replaced by glutamine in the complex structure of hydratase 2, the molecular architecture of the active site was not changed when compared to the SeMet-labelled CtH2. Thus, His813 also seems to locate in the vicinity of the (3R)-hydroxyl group in the product complex of the wild-type enzyme. The active site of CtH2 contains five water molecules, which are present in both liganded and unliganded structures (Fig. 3, original article III). When the active site regions of apo- and holoenzyme structures of C. tropicalis hydratase 2 were superimposed, a water molecule, which is hydrogen-bonded to the Oδ2 atom of Asp808, Nε2 of His813 and the Nε2 atom of Asn810, occupies the position of the (3R)-hydroxyl group of the substrate (Fig. 3, original article III). Therefore, this water molecule can be proposed to be the water added to the C3 position of the substrate and defined as catalytic water in the hydration reaction. In the optimal configuration for the hydration reaction, the electron pair of the fourth tetrahedral position of catalytic water would be directed toward the C3 of the substrate. This requirement is fulfilled only if the carboxylate oxygen of Asp808 and the Nε2 of His813 are both unprotonated. This being the case, the acid/base mechanism previously proposed for FabA and (R)-hydratase (Leesong et al. 1996, Hisano et al. 2003) is not possible, but the catalytic water is the source of both the proton added to the C2 and the (3R)-hydroxyl group added to C3 as visualised in the Figure 4 in the original article III. Initially this enoyl-CoA hydration mechanism was suggested for hydratase 1 (Bahnson et al. 2002). It is noteworthy that the water molecule that is hydrogen-bonded to the Oδ2 atom of Asp808 is replaced by the γ-oxygen of the Ser62 of the adjacent subunit at the active site of (R)-hydratase. The replacement of Ser62 by alanine in (R)-hydratase resulted in reduced hydratase 2 activity. Thus, it is proposed that Ser62 might have an additional role in the hydration reaction in (R)-hydratase (Hisano et al. 2003).
The two hydrogen bonds from the backbone amide of Gly831 and a water molecule to the carbonyl oxygen of the substrate (Fig. 3B, original article III) create the typical oxyanion hole found upon substrate binding in many CoA ester-metabolising enzymes, such as the members of the hydratase/isomerase superfamily (Holden et al. 2001). Importantly, Gly831 locates in the N-terminus of the α-helix α7, the long hot-dog α-helix, whereupon the polarising effect of Gly831 is enhanced. The equivalent glycine is also found in (R)-hydratase and in FabA structures (Leesong et al. 1996, Hisano et al. 2003), indicating its importance in β-addition/elimination reaction. Since hydratase 2 has lost the long core α-helix in the N-domain that enables utilisation of long-chain fatty enoyl-CoAs, the loss of the other catalytic site in the hydratase 2 monomer can be explained.

Although hydratase 2 and hydratase 1 are structurally unrelated, there exist similarities in their CoA binding as well as in their active-site architecture. The hydrogen bonding network of the active-site of the (3R)-hydroxyacyl-CoA complexed hydratase 2 resembles the active site geometry of hydratase 1, although in a mirror image fashion (Fig. 5, original article III). Both enzymes use an oxyanion hole for orienting the ligand and stabilising the reaction intermediate in catalysis, but the catalytic residues are positioned opposite with respect to the formed oxyanion hole. Thus, it can be concluded that the stereochemistry of the added hydroxyl group is determined by the position of the catalytic residues with respect to the hydrogen bond formed between the carboxyl group of the substrate and the amino group of the oxyanion hole glycine. Therefore, the organisation of the active site allows the hydration to occur via the same mechanistic principles, indicating the occurrence of functional convergence during evolution.

### 6.3 Structural basis of MFE-2 deficiency (I, IV)

The crystal structures of RnDH and HsH2 allow for a rational interpretation of the physiological effect of mutant enzymes characterised from MFE-2-deficient patients (see Table 1). Four mutations with single amino acid changes are identified in the region of (3R)-hydroxyacyl-CoA dehydrogenase (Table 1). Out of these, the mutation Gly16Ser and Leu21Phe can be expected to interfere with the binding of NAD⁺, because the residues locate in the conserved Gly-rich motif of SDR enzymes. The binary structure of RnDH with NAD⁺ strengthens this proposal. The serine introduced in the Gly16Ser variant enzyme removes the critical turn in the Gly-rich loop and, moreover, its side-chain will occupy the position of the adenine ribose of NAD⁺, whereas the phenylalanine in the Leu21Phe mutation will most probably occupy the space normally accepted by the nicotinamide part of the NAD⁺ molecule. The Val218 of the α-helix αG is not in close contact with NAD⁺, but rather interacts via hydrophobic interactions with Leu21. Therefore, the bulkier side-chain of leucine in the Val218Leu mutation can be suggested to move the side-chain of Leu21 towards the NAD⁺-binding pocket, leading to a phenomenon similar to the L21F mutation. The fourth amino acid mutation belonging to the type III MFE-2 deficiency is the replacement of Ser177 by phenylalanine. This serine is not conserved among the (3R)-hydroxyacyl-CoA dehydrogenases, since in most cases it is replaced by threonine, as also in RnDH (Fig. 1, original article I). In the crystal structure of RnDH, Thr177 locates in the middle section of the α-helix αF, an important α-helix in
dimerisation. Moreover, in the RdDH dimer, Thr177 is positioned against the N-terminus of the α-helix αF of the neighbouring subunit, which houses the conserved Y-X-X-X-K catalytic motif. Thus, it can be expected that the phenylalanine in the position of Ser177 in the human ortholog will, in addition to disrupting the dimerisation, also change the positions of the side-chains of Tyr164 and Lys168 such a way that catalysis cannot proceed.

In MFE-2 deficient patients, only two point mutations are identified in the region of the hydratase 2 unit, and in both cases the mutated residue is Asn457 (Table 1). The replacement of Asn457 by tyrosine leads to severe MFE-2 deficiency, where the 45kDa hydratase 2/SCP-2L fragment is totally lost from the cell homogenates of the patients and the amount of full-length MFE-2 is also reduced (van Grunsven et al. 1999a). Instead, the patient carrying the N457D mutation has relatively mild effects, indicating a milder type of deficiency. One of the reasons for the mildness of the latter mutation can be that it is found only in one allele in the patient, although it has been shown that the mutation-containing allele is predominant (Paton & Pollard 2000). In the HsH2 structure, Asn457 locates in the middle of the N-domain β-strand β5, far from the active site of hydratase 2. Instead, the hydrogen bond formed between the Nδ2 of Asn457 and the backbone oxygen of Gly378 of the short α-helix α2, is an important stabilising interaction for the incomplete hot-dog fold of the N-domain of hydratase 2. The bulkiness and the hydrophobic nature of the side-chain of tyrosine in the N457Y mutation disrupts the N-domain incomplete hot-dog fold by pushing the α-helix α2 away from the β-sheet layer. In the N457D mutation, which is disease-causing, obviously the hydrogen bond formed between the Asp457 and Gly378 is not as favourable as in the wild-type enzyme, thus creating a disruption in protein folding and/or stability.

The in-frame deletions found in MFE-2-deficient patients create severe folding errors in the full-length enzyme, leading to unstable protein. The only exception is the relatively small deletion of the residues 480-501 in the hydratase 2 region, since, although they have unfunctional hydratase 2, the patients carrying this mutations still have (3R)-acyl-CoA dehydrogenase activity left. In this particular case, part of the linker region between the N- and C-terminal domains, including the β-strand β7 is cut off. The deleted regions in the three remaining cases are: aa 39-74 in-frame deletion, one βα turn (βc and αC) of the Rossmann fold in addition to the preceding loop structure βB-αC; aa 94-208 in-frame deletion, the catalytic motif of the (3R)-hydroxyacyl-CoA dehydrogenase unit and the helices important for dimerisation; aa 325-404 in-frame deletion, a large region of the N-domain of hydratase 2, including the N-domain solvent-exposed loop housing the Glu366 and the α-helix α1, both important in the dimerisation of the hydratase 2 unit.

6.4 Proposed organisation of the functional units of mammalian MFE-2 (IV)

At present, MFE-2 is the only multifunctional enzyme involved in fatty acid β-oxidation of which structural data is available. The crystal structures of the two enzymatic units of MFE-2 determined in this study, allow us, for the first time to discuss the organisation of the separate units in the full-length multifunctional enzyme. Human MFE-2 has been
shown to be a dimeric enzyme with a native molecular size of ~154 kDa (Jiang et al. 1996). Similarly, the recombinantly produced dehydrogenase 2 and the hydratase 2 enzymes are both dimeric, and dimerisation has been found to be crucial for enzyme activity and correct folding in both cases. Because the C-termini of the (3R)-hydroxyacyl-CoA dehydrogenase dimer and the N-termini of the hydratase 2 dimer locate on the same side of the enzymes, it is likely that these enzymatic units are able to unite without a break of the dimeric interactions (Fig.14). The SCP-2L unit, on the contrary, is a monomeric protein based on the size exclusion and crystallographic studies (Haapalainen et al. 2001), and the location of the two C-termini of the hydratase 2 unit on the opposite faces of the dimeric molecule (Fig. 13) suggests that the SCP-2L unit is also “monomeric” in the full-length enzyme. Although the 15-residue long linker region between hydratase 2 and SCP-2L (residues Pro607-Lys621) does not exist in either of the crystal structures, it can be proposed that SCP-2L most probably interacts with the C-domain of hydratase 2. If this kind of aggregation exists, it is difficult to explain the possible role of SCP-2L in the formation the extra cavity for the bulky fatty acyl substrate during hydratase 2 catalysis.

Fig. 14. Proposed assembly of the functional units of mammalian MFE-2. The two polypeptides in the MFE-2 dimer are coloured dark and light gray. The arrows show the linker regions between the two enzymatic units, which are the in vivo cleavage site of the full-length enzyme. Abbreviations: DH, (3R)-hydroxyacyl-CoA dehydrogenase unit; H2, 2-enoyl-CoA hydratase 2 unit; SCP, sterol carrier protein type 2 –like unit; AKL, peroxisomal targeting signal Ala-Lys-Leu; N, N-terminus.
In the present work, the three-dimensional structures of the recombinantly produced enzymatic units of MFE-2, namely (3R)-hydroxyacyl-CoA dehydrogenase and 2-enoyl-CoA hydratase 2, were solved by crystallographic methods. The solved structures were: [1] the (3R)-hydroxyacyl-CoA dehydrogenase unit of rat MFE-2 in complex with its cofactor, NAD$^+$ (2.38 Å), [2] the 2-enoyl-CoA hydratase 2 unit of fungal (C. tropicalis) MFE-2 as an apoenzyme (1.95 Å) and [3] as a binary complex with (3R)-hydroxydecanoyl-CoA (2.35 Å), and [4] the 2-enoyl-CoA hydratase 2 unit of human MFE-2 as an unliganded form (3.0 Å).

The (3R)-hydroxyacyl-CoA dehydrogenase unit of MFE-2 has an N-terminal nucleotide-binding domain topologically equivalent to the short-chain alcohol dehydrogenase/reductase (SDR) enzymes. The core structure of this domain is the central β-sheet surrounded by two arrays of three α-helices, which is a typical Rossmann fold known to contribute to the cofactor binding. Interestingly, the C-terminal residues of (3R)-hydroxyacyl-CoA dehydrogenase constitute a separate domain with a previously uncharacterised fold, which makes the first enzymatic unit of MFE-2 unique among the SDR enzymes. In the dimeric enzyme, this extra domain is lined with the nucleotide-binding domain of the neighbouring subunit, thus extending the dimeric interactions. Moreover, it serves a putative binding site for the 3′-phosphate ADP moiety of the fatty acyl-CoA substrate molecule. The G-X-X-G-X-G motif important in cofactor binding and the three catalytic amino acids Ser-Tyr-Lys interacting with the NAD$^+$ molecule, indicating a catalytic mechanism equal to those seen in the other SDR enzymes.

The second enzymatic unit of MFE-2, the 2-enoyl-CoA hydratase 2, on the other hand, belongs to the novel family of hot-dog fold-containing enzymes. One subunit of the dimeric enzyme is composed of an N-terminal domain with an incomplete hot-dog fold and a C-terminal domain with a complete hot-dog fold. Although these two domains lack the amino acid sequence similarity, the similar folding suggests gene duplication during hydratase 2 evolution. Interestingly, the two-domain subunit structure is highly reminiscent of the homodimeric form of the prokaryotic homolog, (R)-specific 2-enoyl-CoA hydratase of the PHA-synthesis pathway. The presence of large loop structures with high flexibility as well as the replacement of the long core helix by a discontinuous helical region in the N-terminal domain have enabled eukaryotic hydratase 2 to utilise even bulky fatty enoyl-CoAs and their derivatives, substrates which are not accepted by
prokaryotic homolog because of the presence of two rigid core α-helices of the hot-dog fold in the functional unit. The active site of hydratase 2 complexed with its product, (3R)-hydroxydecanoyl-CoA, confirms the catalytic amino acid residues and the stabilisation mechanism of the reaction intermediate via the oxyanion hole. Moreover, the hydrogen-bonding network suggests a novel catalytic mechanism for (R)-specific hydration, where the atoms added across the trans-2 double bond are derived from a single water molecule.

The determined structures of the enzymatic units of mammalian MFE-2 solved the structural basis for the reported MFE-2 patient mutations. In addition, they allowed an initial discussion about the organisation of the functional units in the biologically active mammalian MFE-2 dimer. However, many open questions concerning the structure-function relationship of MFE-2, such as possible substrate-channeling between the enzymatic units and the role of SCP-2L, still exist. Therefore, more structural work with bigger MFE-2 fragments or with full-length enzymes are needed to give an explanation for the multifunctionality of the MFE-2 in fatty acid β-oxidation.
References


