Crystal Structure of Liganded Rat Peroxisomal Multifunctional Enzyme Type 1

A FLEXIBLE MOLECULE WITH TWO INTERCONNECTED ACTIVE SITES

Prasad Kasaragod, Rajaram Venkatesan, Tiila R. Kiema, J. Kalervo Hiltunen, and Rik K. Wierenga

From the Biocenter Oulu and Department of Biochemistry, University of Oulu, FI-90014 Oulu, Finland

The crystal structure of the full-length rat peroxisomal multifunctional enzyme, type 1 (rpMFE1), has been determined at 2.8 Å resolution. This enzyme has three catalytic activities and two active sites. The N-terminal part has the crotonase fold, which builds the active site for the Δ3,Δ3-enedooyl-CoA isomerase and the Δ2-enedooyl-CoA hydratase-1 catalytic activities, and the C-terminal part has the (3S)-hydroxacyl-CoA dehydrogenase fold and makes the (3S)-hydroxacyl-CoA dehydrogenase active site. rpMFE1 is a multidomain protein having five domains (A–E). The crystal structure of full-length rpMFE1 shows a flexible arrangement of the A-domain with respect to the B–E-domains. Because of a hinge region near the end of the A-domain, two different positions of the A-domain were observed for the two protein molecules (A and B) of the asymmetric unit. In the most closed conformation, the mode of binding of CoA is stabilized by domains A and B (helix-10), as seen in other crotonase fold members. Domain B, although functionally belonging to the N-terminal part, is found tightly associated with the C-terminal part, i.e. fixed to the E-domain. The two active sites of rpMFE1 are ~40 Å apart, separated by a tunnel, characterized by an excess of positively charged side chains. Comparison of the structures of rpMFE1 with the monofunctional crotonase and (3S)-hydroxacyl-CoA dehydrogenase superfamily enzymes, as well as with the bacterial αβ2-fatty acid oxidation multienzyme complex, reveals that this tunnel could be important for substrate channeling, as observed earlier on the basis of the kinetics of rpMFE1 purified from rat liver.

Multifunctional enzyme, type 1 (MFE1), is one of the most abundant mammalian peroxisomal proteins (1). Its catalytic activities, i.e. Δ3,Δ3-enedooyl-CoA isomerase, Δ2-enedooyl-CoA hydratase-1, and (3S)-hydroxacyl-CoA dehydrogenase (Fig. 1), place it in the β-oxidation pathway for the degradation of fatty acids (2). In the isomerization step, 3E- and 3Z-enedooyl-CoA substrate molecules are converted into (2E)-enedooyl-CoA, which subsequently is converted into (3S)-hydroxacyl-CoA in the hydration step. The latter specificity is also referred to as the hydratase-1 specificity. (3S)-Hydroxacyl-CoA is then oxidized in the second active site to 3-ketoacyl-CoA, using NAD⁺ as the cofactor. Rat peroxisomal MFE1 (rpMFE1) is a monomeric enzyme of 722 residues (1) with a large excess of basic residues, resulting in a predicted pI of 9.3 (3). MFE1 is known to have two active sites, i.e. a hydratase/isomerase (H/I) active site in the N-terminal part and a dehydrogenase active site in its C-terminal part. The catalytic activities of the N-terminal active site (isomerase and hydratase) and the C-terminal active site (dehydrogenase) are also catalyzed by corresponding homologous, well studied monofunctional enzymes, i.e. isomerases, hydratases (also referred to as crotonases), and dehydrogenases. Sequence analyses have shown that the N- and C-terminal parts belong to two different superfamilies. The N-terminal part belongs to the crotonase superfamily (4), and the C-terminal part belongs to the HAD superfamily (5).

The precise physiological role of MFE1 is not known, as it has overlapping substrate and catalytic specificity (but no structural similarity) with peroxisomal multifunctional enzyme, type 2 (MFE2) (2, 6). However, based on analysis of metabolite profiles in genetically modified mice strains, it has been suggested that MFE1 is involved in a α-methylacyl-CoA racemase-MFE2 independent synthesis pathway of bile acids from (24S)-hydroxysteroids (7). Other experiments provide evidence that MFE1 is involved in the β-oxidation of long chain dicarboxylic acids (8, 9). These suggestions are in line with a broad substrate specificity of MFE1 in vitro, as it can use as substrates CoA derivatives of both linear fatty acids as well as 2-methyl branched fatty acids, including bile acids that are characterized by having a steroid moiety at the α-end of its fatty acid tail (10, 11). The broad substrate specificity requires unique structural features in the active sites.

The catalytic properties of the N-terminal part of rpMFE1 are intriguing, as this active site catalyzes both a hydratase as well as an isomerase reaction (12). The isomerase activities of the rat MFE1 have been well characterized in vitro (13). The isomerase activity can be functional also in vivo as demonstrated by the observation that expression of rpMFE1 in the Δec1 knock-out yeast strain, deficient in peroxisomal isomerase
Crystal Structure of Rat Peroxisomal MFE1

The 1.9 Å crystal structure of the B–E-domains of the C-terminal part (the dehydrogenase part, Fig. 2) of rat peroxisomal MFE1 (rpMFE1-DH) has been described previously (1), but the structure of the A-domain, which adopts the core of the crotonase fold, has not yet been reported. The complete crotonase fold is formed by domains A and B together. Several structures are known of monofunctional crotonase enzymes with isomerase activity or hydratase activities, such as Δ3,Δ2-enoyl-CoA isomerase, Δ2-enoyl-CoA hydratase-1, and Δ3,Δ2,4-dienoyl-CoA isomerase (4, 26). The C-terminal part belongs to the two domain HAD superfamily. The HAD fold consists of an NAD-binding Rossmann-like fold (domain C) plus a dimerization domain (domain D). The founding member of this superfamily is the human mitochondrial (3S)-hydroxyacyl-CoA dehydrogenase, which is also referred to as the short chain hmHAD (27). Also, the structure of the porcine homologue is known (28). Interestingly, the crotonase fold and the HAD fold (domains C and D) are also present in the α-chain of the bacterial Pseudomonas fragi fatty acid oxidation multienzyme αβ2 complex (pfFOM), whose crystal structure has been determined by Morikawa and co-workers (25). MFE1 is a homologue of the full-length α-chain of pfFOM; the α-chain of pfFOM and MFE1 have the same domain structure (Fig. 2). The α-chains of the pfFOM complex form an α2-dimer, and the β-chains of this complex form a β2-thiolase dimer. The C-terminal part of the α-chain of pfFOM, as well as of MFE1, correspond to the HAD fold, extended by one more domain (domain E), whose fold is topologically identical to the HAD dimerization domain and domain D of MFE1. Domains D and E are tightly associated. The evolutionary relationship of the D- and E-domains has been described previously (1, 5).

In this study, the crystal structure of the full-length rpMFE1 is described. The important structural features of rpMFE1 are summarized, including the mode of binding of CoA into its binding site of the crotonase domain of the H/I part. Extensive structural comparisons with enzymes of the crotonase fold and the HAD fold as well as with pfFOM provide for the first time a few enzymes, for example dihydrofolate reductase-thymidylate synthase and the bacterial fatty acid oxidation αβ2 complex. Electrostatic interactions have been proposed to be important for the channeling in the bifunctional enzyme dihydrofolate reductase-thymidylate synthase (23, 24), whereas for the bacterial fatty acid oxidation complex, a substrate-anchored diffusion mechanism has been proposed (25).

Previous studies of MFE1 (5) have subdivided the molecule into five domains (A–E), as also visualized in Fig. 2. The nomenclature introduced in Fig. 2 reflects the highly modular assembly of this enzyme, which allows for a description at three levels as follows: (i) domains with different evolutionary histories, (ii) functional units, and (iii) structural units. There are five domains (domains A–E). The two functional units are the N-terminal part (domains A and B) and the C-terminal part (domains B–E). Domain B is a linker helix, which functionally belongs to the N-terminal part but structurally to the C-terminal part. The structure analysis reported here shows that there are three structural units, i.e. domains A, C, and B/D/E, connected via flexible linkers.

The experimental kinetic data of MFE1 indicate that a substrate channeling mechanism between the two active sites must exist such that the product of the first active site is transferred to the second active site without being released into bulk solvent (16). Apparently, the promiscuous catalytic properties of the MFE1 H/I active site have been conserved in MFE1 during its evolution, whereas in the evolution of the related monofunctional Δ2-enoyl-CoA hydratases and Δ3,Δ2-enoyl-CoA isomerases only one of these activities has been preserved.

Experimental kinetic data of MFE1 indicate that a substrate channeling mechanism between the two active sites must exist such that the product of the first active site is transferred to the second active site without being released into bulk solvent (16, 17). For some structurally well characterized multifunctional enzymes, extended channels in the interior of the protein have been described (18–20) connecting the two active sites, such that the product of one active site can diffuse toward the other active site, while being shielded from bulk solvent. Good examples of these are provided by the structures of tryptophan synthase (21) and aldolase dehydrogenase (22). In these enzymes the channeled substrates are small molecules, i.e. indole and acetaldehyde, respectively. In MFE1, the substrate is a large polar, negatively charged molecule. The mechanism that allows for substrate channeling of such extensive polar molecules is poorly understood and has been addressed only in studies of
Crystal Structure of Rat Peroxisomal MFE1

FIGURE 2. Domain and assembly structure of MFE1. The A-domain is the domain with the crotonase fold, and its active site is catalyzing the isomerase and the hydratase reaction. The C-terminal helix of the crotonase fold (helix-10) is the B-domain; it is also referred to as the linker helix (25). The C-domain is the NAD-binding domain of the dehydrogenase part. The C-domain also provides the important catalytic residues of the HAD active site. The D- and E-domains are evolutionarily related and have very similar folds. They are tightly associated with each other. The E-domain provides important stabilizing interactions with the linker helix, generating the B/D/E structural unit. The C-terminal SKL sequence is the targeting signal sequence for import into the peroxisome. The relative lengths of the domains in the scheme correspond to the respective number of residues in each of the domains.

EXPERIMENTAL PROCEDURES

Expression and Purification of Rat perMFE1—The rpMFE1 encoding cDNA was cloned into the pET15b (Novagen) vector at NdeI and BamHI sites. The cloning strategy resulted in the addition of 20 residues (Met-Gly-Ser-Ser-(His)₆-Ser-Ser-Gly-Lys-Val-Pro-Arg-Gly-Ser-His) before the N-terminal Met¹ of the protein, having a His tag, preceded by a thrombin cleavage site. The resulting pET15b-rpMFE1 construct was transferred into Escherichia coli BL21(DE3) cells, which previously had been transformed with the pGro7 plasmid (Takara Bio), encoding for the GroEL and GroES chaperones. Without coexpressing with these chaperones, rpMFE1 precipitated in the cell in inclusion bodies. A single colony was inoculated into 10 ml of LB media containing ampicillin (100 μg/ml) and chloramphenicol (20 μg/ml) and grown at 37 °C overnight. These cells were used to inoculate 1000 ml of M9ZB media containing ampicillin (100 μg/ml), chloramphenicol (20 μg/ml), and 0.5 mg/ml L-arabinose to induce the chaperones. The culture was incubated at 37 °C until A₆₀₀ reached 0.8, and expression was induced at 25 °C by the addition of isopropyl 1-thio-β-D-galactopyranoside to a final concentration of 0.5 mM, and the induction was continued for 5 h. The cells were harvested, washed with 1× phosphate-buffered saline buffer, and stored at −80 °C until further use.

The cells were suspended in lysis buffer (20 mM potassium phosphate buffer, pH 7.4, 500 mM NaCl, 50 mM imidazole, 10% glycerol, 0.1% Triton X-100, 100 μg/ml lysozyme, 25 μg/ml DNase, 25 μg/ml RNase, 1 protease inhibitor tablet (Roche Applied Science), 5 mM ATP, 5 mM MgCl₂) and incubated at 22 °C for 30 min. The cells were further lysed by brief sonication followed by a centrifugation step. The supernatant was loaded onto Ni-HiTrap chelating HP 5-ml column (GE Healthcare), pre-equilibrated with the equilibration buffer (20 mM phosphate buffer, pH 7.4, 500 mM NaCl). The protein was eluted with a 100-ml linear gradient of 0–300 mM imidazole in equilibration buffer. The peak fractions containing the pure protein were pooled and concentrated using a centrifugal filter device (Amicon Ultra15 Millipore) and applied onto a Superdex-200 size exclusion chromatography column that had been pre-equilibrated with the gel filtration buffer (10 mM PIPES, pH 6.5, and 50 mM NaCl). The peak fractions were pooled, concentrated, and immediately used for crystallization. The sample was confirmed to be pure by SDS-gel electrophoresis.

Solution Studies—The purified enzyme is a monomer as confirmed by static light scattering measurements, using the Wyatt miniDAWN-TREOS device. Using the standard enzyme assay, as described previously (16), a turnover number of ~4 s⁻¹ is measured. In this (bifunctional) assay (2E)-decenoyl-CoA is used as substrate, which is converted into 3-ketodecanoyl-CoA. In the standard assay (50 mM KCl, 50 mM Tris/HCl, pH 9, 50 μg/ml bovine serum albumin, and 1 mM NAD⁺), the substrate concentration is 60 μM, and the activity is measured by monitoring the formation of NADH at 340 nm. This observed turnover number compares well with the previously reported catalytic activity of the rpMFE1 directly purified from rat liver tissue, i.e. 3.5 s⁻¹ (16). It also is similar to the catalytic efficiency of the monofunctional rpMFE1-DH construct, residues 260–722, of which the crystal structure has been described previously (1). The turnover number of this construct was found to be 2.1 s⁻¹ (29).

Crystallization—For crystallization, 8 mg/ml protein in 10 mM PIPES, pH 6.5, and 50 mM NaCl was incubated with 2 mM CoA for 30 min at room temperature and centrifuged to remove any precipitated aggregates. 1 μl of protein was mixed with 1 μl of crystallization condition in a sitting drop plate (Greiner, Jena Bioscience) with 100 μl of reservoir solution. The crystallization condition contained 100 mM MES, pH 6.0, 150 mM ammonium sulfate, and 15% w/v PEG4000. Crystals were obtained in 1 week time at 22 °C and allowed to grow for a month. The single crystal was transferred to the mother liquor...
Crystal Structure of Rat Peroxisomal MFE1

### Table 1

| Crystallographic data |
|-----------------------|
| **Data collection**   |
| Beamline              | ID14-2 |
| Temperature           | 100 K  |
| Wavelength            | 0.933 Å |
| Space group           | P2₁,2₁,2₁ |
| Unit cell parameters  | 65.70x126.50x227.07 Å |
| Vₐ                      | 2.8 Å³/Da |
| Molecules/asymmetric unit | 2 |
| Resolution range       | 46.19–2.8 Å (2.95–2.8 Å) |
| Completeness           | 98.1% (98.0%) |
| (I/σ(I))²              | 11.5 (2.6) |
| Rpim                   | 5.9% (29.7%) |
| No. of unique reflections | 46,656 (6697) |
| Redundancy             | 6.2 (5.8) |
| Wilson B-factor        | 57.9 Å² |

| Refinement |
|------------|
| Resolution | 46.2 to 2.8 Å |
| R-factor   | 22.4% |
| R-free     | 28.2% |
| No. of reflections | 44,229 |
| No. of protein atoms | 11,105 |
| No. of waters | 229 |
| No. of active site molecules | 3 |
| No. of other molecules | 3 |
| SO₄         | 5 |
| Glycerol    | 3 |

| Geometry statistics |
|---------------------|
| r.m.s.d., Å bonds   | 0.014 Å |
| r.m.s.d., Å angles  | 1.6° |
| r.m.s.d., Å B       | 0.6 Å |
| Protein main chain  | 1.7 Å² |
| Protein side chain  | 39.7 Å² |
| Average R           | 40.2 Å² |
| Protein molecule    | |
| A                    | |
| B                    | |
| Active site ligands  | |
| CoA (A)              | 89.3 Å² |
| ADP (A)              | 47.1 Å² |
| CoA (B)              | 64.7 Å² |

| Ramachandran plot (Molprobity) |
|--------------------------------|
| Favored                        | 94.3% |
| Allowed                        | 99.9% |
| Outliers                       | 0.14% |
| PDB code                       | 2X58 |

---

containing also 15% glycerol and 2 mM CoA and immediately flash-frozen in liquid nitrogen.

**Crystal Structure Determination, Refinement, and Validation**—A 2.8 Å dataset was collected from a single rpMFE1 crystal at beamline ID14-2 (ESRF, Grenoble, France). The dataset was processed using iMOSFLM (30) and SCALA (31) of the CCP4 package (32). The data collection statistics are summarized in Table 1. The structure was solved by molecular replacement with Phaser (33), using PDB codes 1ZCJ (1) and 1WDK (25) as search models. The starting model was rebuilt iteratively using subsequent cycles of COOT (34) model building and REFMAC5 (35) refinement calculations. Initially, only the protein part was built, and subsequently the water molecules and other solvent molecules were included in the model, and only at the very end the CoA molecules bound to molecules A and B were introduced, as well as the ADP molecule bound to molecule A. Some residues of the N-terminal tag peptide could be built in the final map, and they are numbered starting from 0, −1, etc. These residues are part of the N-terminal tag; they are present because a thrombin cleavage step, to remove the N-terminal tag, was not included in the purification protocol. MolProbity (36) and COOT have been used for assessing and improving the quality of the model. The final refinement statistics are summarized in Table 1.

**Sequence and Structural Analysis**—Sequence alignments were initially made with ClustalW (37) and subsequently optimized from the structural comparisons. Separate sequence alignments were made for the N-terminal part (1–290) and the C-terminal part (266–722). For the N-terminal part sequence alignment, the following sequences were used: PDB codes 2X58 (rpMFE1), 1WDK (25), 1SG4 (38), 2F6Q, 2DUB (40), 1PJH (41), and 1DCI (42), and for the C-terminal part PDB codes 2X58 (rpMFE1), 1WDK, and 1F0Y (27) were used. For the structural comparisons, the corresponding structures have been superimposed using the SSM method (43) as implemented in COOT.

The MFE1 structure has been compared with the structure of the B–E rpMFE1-DH construct (PDB code 1ZCJ (1)). It has also been compared with the structures of the apo (PDB code 1F14 (27)) and ternary (PDB code 1F0Y (27)) complexes of hmHAD. The four available structures of pfFOM have also been analyzed, i.e. PDB codes 1WDK, 1WDL (25), 1WDM (25), and 2D3T (44); PDB code 1WDK has been used as the reference structure for the comparisons. For the comparisons of the crotonase structure of the H/I part, the structures of the following members of the crotonase superfamily have been used: human mitochondrial ∆₁,∆₂-enoyl-CoA isomerase (PDB code 1SG4), rat mitochondrial ∆₂-enoyl-CoA hydratase-1 (hydratase, PDB codes 1DUB (45) and 2DUB), yeast peroxisomal ∆₁,∆₂-enoyl-CoA isomerase (PDB code 1PH), rat mitochondrial/peroxisomal (46) ∆₁,∆₂,∆₃-enoyl-CoA isomerase (PDB code 1DCI), and human peroxisomal ∆₁,∆₂-enoyl-CoA isomerase (PDB code 2F6Q).

**RESULTS AND DISCUSSION**

**Overall Structure**—For obtaining homogeneous recombinant rpMFE1 in soluble form, it turned out to be essential to use a host *E. coli* strain in which the chaperones GroEL and GroES were also coexpressed. Activity assays confirmed that the purified recombinant rpMFE1 was equally active as rpMFE1 purified directly from rat liver, as described under “Experimental Procedures.” The purified rpMFE1 was crystallized in the presence of 2 mM CoA in a MES buffer at pH 6. In this crystal form there are two molecules (molecule A and molecule B) of rpMFE1 per asymmetric unit. The structure of rpMFE1 has been refined using REFMAC5 at 2.8 Å resolution to a final R-factor and R-free of 22.4 and 28.2%, respectively, and with good geometry (Table 1). A continuous polypeptide chain could be built for both molecules. Molecule A starts at residue 4 and ends at residue 720, whereas molecule B starts at −2 and ends at 718. For both molecules, the C-terminal peroxisomal SKL targeting sequence is disordered, as commonly seen in structures of peroxisomal proteins. Molecule A is liganded in the H/I active site (CoA) and the HAD active site (ADP). Molecule B is

---

3 A. Tumbull, P. Lukacik, N. Shaqfcat, C. Smee, G. Berridge, K. Guo, F. von Delft, J. Weigelt, M. Sundstrom, C. Arrowsmith, A. Edwards, O. Gileadi, and U. Oppermann, personal communication.
liganded only in the H/I active site (CoA) (supplemental Fig. 1); binding of ADP in the HAD active site of molecule B is blocked by crystal contacts. In the structure description, we will use molecule A as the reference molecule.

As outlined in the Introduction, the structure is built up from essentially three structural units as follows: the A-domain, the C-domain, and the B/D/E-domains (Fig. 3A). The secondary structure assignment for each of the domains is given in Fig. 5. This figure also provides the nomenclature of the secondary structure elements. The B-domain is the linker helix between the A-domain and the C-domain; it is tightly associated with the E-domain, as can be deduced from a superposition analysis of molecules A and B. Molecules A and B do not exactly superimpose, due to the flexible connections between the structural regions (between the side chains of Gln355 and Glu161). In molecule A, only one hydrogen bond is formed between these two residues Arg657–His658–Lys659. Arg657 makes a salt bridge with Glu274 of the linker helix. Interestingly, in the rpMFE1-DH structure Glu274 is salt-bridged to Arg440 of domain C; this different salt bridge interaction correlates with a small adjustment of the linker helix.

The B-, C-, and D/E-domains of the dehydrogenase parts of molecules A and B adopt the same relative conformation with respect to each other. Comparison with the (unliganded) rpMFE1-DH structure (1) reveals that the assembly of the B–E-domains of the rpMFE1 and rpMFE1-DH structures is also the same.

Further superposition analyses (see below) show that the key structural difference between the two molecules in the asymmetric unit of the rpMFE1 crystal is caused by a hinge motion rotating the A-domain of the N-terminal part, with respect to the C-terminal part. Three of the most explicit structural features of the new rpMFE1 crystal structure are as follows: (i) the protruding CH2-helix of the C-terminal part, (ii) the hinge region and hinge motion between the N- and C-terminal part, and (iii) the tunnel between the active sites of the H/I part and the dehydrogenase part (Fig. 3B).

**Protruding Helix**—The protruding CH2 helix extends from domain C of the dehydrogenase part (Fig. 3A). It contacts the H/I part of the same molecule, thereby generating a tunnel. In pfFOM and hmHAD, this extended helix is part of a helix-turn-helix motif, but in rpMFE1 this helix continues as an extended loop back into the bulk of the protein. The contact area between the protruding helix and the H/I part is formed by residues Gln352–Asn353–Asn354–Gly355–Gln356 at the tip of the protruding helix and by residues Ser158–Ala159–Asp160–Glu161 of the H/I part, between the edge strands B5 and helix H6. In molecule A, only one hydrogen bond is formed between these two regions (between the side chains of Gln355 and Glu161). In molecule B, the A-domain has rotated toward the tip of the protruding helix by ~4 Å, and at the new interface two hydrogen bonds are seen (between the side chains of Asn353 and Glu161, as well as between Gly354 and Ser158). The tip of the protruding helix has the highest B-factors of molecules A and B, but the
Crystal Structure of Rat Peroxisomal MFE1

**Comparison of Two Molecules**
The two molecules show that its position with respect to the dehydrogenase part is the same. The differences of the protein-protein interface interactions at the tip of the protruding helix in molecule A and molecule B are entirely due to differences in positioning of the A-domain of the N-terminal part with respect to the C-terminal part in the two molecules of the asymmetric unit.

**Hinge Region**—The superpositioning of the B–E domains of molecules A and B reveals that the A-domain of the N-terminal part adopts two different orientations with respect to the C-terminal part. In molecule B, the A-domain is more rotated toward the N-terminal part. In molecule B, there is a salt bridge between the pyrophosphate moiety of CoA and Lys275 of the linker helix. Also, Phe271 of the linker helix now contacts the adenine ring in the molecule B complex. These ligand-protein contacts are missing in molecule A; indeed, the CoA molecule of molecule B is somewhat better defined by its electron density map (supplemental Fig. 1) than the CoA molecule bound to molecule A. The linker helix is a functional part of the N-terminal crotonase fold, being referred to as helix-10 (Fig. 2). Helix-10 is known to have salt bridge and hydrophobic van der Waals contacts with the bound CoA molecule in the homologous monofunctional enzymes of the superfamily, as, for example, described for the structure of the human isomerase, complexed with octanoyl-CoA (38). It is of interest to note that the unliganded rpMFE1 could not be crystallized, whereas cocrytalization in the presence of CoA readily produces high quality crystals. This suggests that the binding of CoA rigidifies the molecule and implies that the apoenzyme is more flexible. The functional relevance of this flexibility is not immediately apparent.

**Tunnel**—The tunnel is shaped by the protruding helix CH2 and four regions of the H/I part, i.e. B4, B5, as well as helix H9A, and the linker helix. From the dehydrogenase part, helix EH2 and also several loop regions contribute to the shape of the tunnel, in particular the loops after CH1, CB6, CH5, DH2, and EH2. There is an excess of positively charged side chains pointing into this tunnel, eight lysines and five arginines (Fig. 3B), and in this region only four acidic residues are found (Asp149, Glu161, Glu274, and Glu346). The excess of positive charge could favor a diffusion of the substrate between the two active sites along the surface of the enzyme instead of dissociating back into the bulk solvent, before reaching the second active site.

**Comparisons with the Crotonase Fold Enzymes**—The crotonase fold is characterized by having a spiral fold constructed of four ββα-units at its N terminus, followed by a helical part. The β-strands of the crotonase fold built two β-sheets, referred to as sheet A and sheet B (Fig. 5). In the canonical crotonase fold, such as found in the structure of human mitochondrial isomerase (PDB entry 1SG4) (38), the C-terminal part consists of the following four consecutive helices: H7, H8, H9, and helix-10. These four helices wrap around the surface of the core of the protein (Fig. 6). Because of domain swapping (after helix H8), in some superfamily members the C-terminal helix, helix-10, is provided by the adjacent subunit, for example in the hydratase structure (45). Helix-10 is important for stabilizing the CoA mode of binding, providing hydrophobic and salt bridge interactions (38) with CoA. In rpMFE1, there are three large structural differences with respect to the classical crotonase fold, as found in the isomerase. First, helix H8 is an extended loop. Second, helix H9 is topologically replaced by three helices (H9A, H9B, and H9C) (Fig. 6). Helix H9A contributes to the shape of the tunnel (Fig. 3B). The superposition analysis shows that helix H9C of rpMFE1 corresponds to H9 of the canonical crotonase fold (Fig. 6). Third, helix-10 is preserved...
but, interestingly, is associated with the E-domain of the dehydrogenase part in rpMFE1. In the corresponding structures of other crotonase superfamily members, this helix usually has high B-factors and is very often incompletely defined near its C terminus by the electron density map. However, in the rpMFE1 structure the helix is well defined because of its interactions with the E-domain.

The rpMFE1 crotonase fold is a monomeric member of the crotonase superfamily. One of the important trimerization motifs is near helix H9. In MFE1 (and the α-chain of pfFOM), the corresponding helix H9C is associated with the preceding helices H9A and H9B, thereby preventing the trimerization assembly as found in the classical crotonase fold enzymes.

The sequence comparisons (supplemental Table 1) show that the H/I part has the highest sequence identity to the monofunctional rat hydratase (32%). The sequence identity with the H/I part of the pfFOM complex is 27%. rpMFE1 is both a hydratase as well as an isomerase, but the sequence identities with the latter enzymes are much less, being 21% for human mitochondrial isomerase and 14% for yeast peroxisomal isomerase.

**Fig. 6** shows a superposition of the liganded active sites of rpMFE1, isomerase and hydratase. The catalytic residues are in each case glutamate residues. The catalytic site of rpMFE1 is identified by its two catalytic protic glutamates, Glu103 and Glu123, and by its oxyanion hole peptide –NH groups of Ala61 and Gly100. Fig. 6 shows that in rpMFE1 a glycerol hydroxyl group is hydrogen-bonded to the carboxylate moieties of Glu103 and Glu123; this interaction could mimic the position of the catalytic water, required for the hydration reaction (Fig. 1). It is unclear currently how it is possible that the MFE1 active site can catalyze both the isomerization reaction as well as the hydratase reaction.

**Subtle structural differences in the mode of binding of 3-enoyl-CoA** but, interestingly, is associated with the E-domain of the dehydrogenase part in rpMFE1. In the corresponding structures of other crotonase superfamily members, this helix usually has high B-factors and is very often incompletely defined near its C terminus by the electron density map. However, in the rpMFE1 structure the helix is well defined because of its interactions with the E-domain.

The rpMFE1 crotonase fold is a monomeric member of the crotonase superfamily. One of the important trimerization motifs is near helix H9. In MFE1 (and the α-chain of pfFOM), the corresponding helix H9C is associated with the preceding helices H9A and H9B, thereby preventing the trimerization assembly as found in the classical crotonase fold enzymes.

The sequence comparisons (supplemental Table 1) show that the H/I part has the highest sequence identity to the monofunctional rat hydratase (32%). The sequence identity with the H/I part of the pfFOM complex is 27%. rpMFE1 is both a hydratase as well as an isomerase, but the sequence identities with the latter enzymes are much less, being 21% for human mitochondrial isomerase and 14% for yeast peroxisomal isomerase.

**Fig. 6** shows a superposition of the liganded active sites of rpMFE1, isomerase and hydratase. The catalytic residues are in each case glutamate residues. The catalytic site of rpMFE1 is identified by its two catalytic protic glutamates, Glu103 and Glu123, and by its oxyanion hole peptide –NH groups of Ala61 and Gly100. Fig. 6 shows that in rpMFE1 a glycerol hydroxyl group is hydrogen-bonded to the carboxylate moieties of Glu103 and Glu123; this interaction could mimic the position of the catalytic water, required for the hydration reaction (Fig. 1). It is unclear currently how it is possible that the MFE1 active site can catalyze both the isomerization reaction as well as the hydratase reaction. Subtle structural differences in the mode of binding of 3-enoyl-CoA

**Subtle structural differences in the mode of binding of 3-enoyl-CoA**
Crystal Structure of Rat Peroxisomal MFE1

Comparison with hmHAD—The C-terminal part of MFE1 adopts the HAD fold, as described for hmHAD (27). From the sequence comparisons (supplemental Table 2), it is seen that rpMFE1, hmHAD, and pfFOM share ~30% sequence identities in each of the possible pairwise sequence comparisons. The highest sequence identity of rpMFE1 is with the monofunctional hmHAD sequence (33%). The critical catalytic residues are conserved in each of these three sequences. The hmHAD enzyme is a dimeric enzyme. The dimerization domain of hmHAD provides the subunit-subunit interactions that generate the native HAD dimer. The D- and E-domains of rpMFE1 share the same fold and correspond to the dimerization domains of the hmHAD dimer (Fig. 7). The hmHAD structure is known to exist in an open (unliganded) and closed (liganded) structure. The fully closed form of the hmHAD structure has been observed only in the ternary dead-end complex with bound acetoacetyl-CoA and NAD (+) (27). The hinge region is between domains C and D. In the crystal structure of rpMFE1, the HAD parts of both molecules have adopted the open conformation. Although CoA has been present in the crystallization medium, it is not bound to the HAD part of molecules A or B. This is consistent with the properties reported for hmHAD, where it has been found that substrate binding is much stronger in the presence of NAD (+) (27). However, an ADP molecule is observed to be bound in the HAD active site of molecule A (Fig. 3A), where it adopts the same mode of binding as seen for NAD (+) bound to hmHAD (27). ADP was not added to the crystallization medium, so it seems that it has been copurified. In molecule B, ADP is not bound as this mode of binding is prevented by crystal contacts.

The highest B-factor region of the dehydrogenase part (in both molecules) is near the tip of the CH2-helix. Two other regions of both molecules also have high B-factors near residues Pro45 and His606, also in the dehydrogenase part. The former residue (after helix DH2) is in an extra long loop (not present in pfFOM and hmHAD). As discussed previously (1), helix DH2 of rpMFE1 has moved away from the pfFOM and
Comparisons with pfFOM—The positioning of the A-domain with respect to the dehydrogenase part of pfMFE1 is shown in Fig. 3A. Very similar domain assemblies are also observed for the α-chains of the various structures of the α₁β₂ pfFOM complex. Altogether, four crystal structures are known of pfFOM (25, 44). These structures show a variable degree of asymmetry, ranging from highly asymmetric (PDB code WDL), via moderate asymmetry (PDB code 2D3T) (44), to a symmetric arrangement (PDB codes 1WDK and 1WDM) (25). Small angle x-ray scattering measurements (44) suggest that in solution an ensemble of assemblies with fluctuating domain arrangements exists. This conformational flexibility and a substrate-anchored diffusion mechanism are key elements of the proposed substrate channeling mechanism between the three active sites of the pfFOM complex. The rpMFE1 tunnel is not seen in any of the available crystal structures of pfFOM, because the CH2-helix is shorter and positioned differently (Fig. 8). In the pfFOM structures, the dehydrogenase part of each of the α-chains is seen to occur in the unliganded conformation in all available structures (25, 44), as in the pfMFE1 structure.

Fig. 8A compares the folds of the pfFOM α-chain (PDB code 1WDK) and rpMFE1 (molecule A). For this superpositioning, the B-E-domains of rpMFE1 and pfFOM have been used. In pfFOM the hinge region of the A-domain is in a more open conformation than the most open conformation of the rpMFE1 complex, as observed in molecule A. The structures of the complexes of the crotonase A-domains of rpMFE1 and the pfFOM α-chain are compared in Fig. 8B, highlighting the structural differences of the substrate specificity determining regions near loop-2 and helix H2 of both structures.

Substrate Channeling—The distance between the two rpMFE1 active sites is about 40 Å. Experimental kinetic data on rpMFE1 show that substrate channeling between the two active sites occurs (16, 17). The structure of the related pfFOM suggests that for this enzyme a substrate-anchored diffusion mechanism could operate (25). In one of the pfFOM structures, an acetyl-CoA molecule has been found (molecule A, PDB code 1WDM) to be bound at a position, intermediate between the two active sites, such that the fatty acid tail reaches the active site of the HAD part. It is speculated that in this position possibly the fatty acid tail can swing from the HAD active site to the hydratase active site, although the adenine moiety remains bound at its site. The available pfFOM structures currently do not include a structure of a complex with the hydratase active site complexed with a fatty acyl-CoA molecule. The intermediate pfFOM-binding site corresponds to a region of the rpMFE1 tunnel. Key interactions of the adenine moiety of the bound acetyl-CoA with side chains in the observed pfFOM-adenine binding pocket are mediated via Lys₁⁴₂, Tyr₂⁴⁹, Leu₂⁹⁰, and Leu₆₆₆. In rpMFE1, these residues are Thr₁²⁵, Val₁₂₆, Leu₂₆⁷, and Arg₆⁵⁷, respectively. The latter Arg₆⁵⁷ is salt-bridged to Glu₂⁷⁴ of the linker helix, and this salt bridge fills the space occupied in pfFOM by the acetyl-CoA molecule. In the rpMFE1 structure, this pfFOM mode of binding of acetyl-CoA is not seen. Instead the CoA is bound to the H/1 part in the classical mode-of-binding, as seen for other members of the crotonase family (Figs. 6 and 8). These observations suggest differences for the possible substrate channeling mechanisms between rpMFE1 and pfFOM. In particular, the rpMFE1 structure suggests that positively charged side chains in the tunnel between the two active sites (Fig. 3B) might facilitate a substrate crawling mechanism. In fact, the surface area between the tunnel and the dehydrogenase active site also has an excess of basic residues (Lys⁵⁷⁳, Lys⁶₃¹, and Arg⁶⁶⁹) and no acidic residues.

Concluding Remarks—rpMFE1 is a monomeric protein, although the homologous superfAMILY members of the N-terminal part are trimers (crotonase superfAMILY) and of the C-terminal part are dimers (HAD superfAMILY). The trimerization of the N-terminal part is prevented by the presence of the unique helices H9A and H9B, whereas the dimerization of the C-terminal part is blocked by the additional unique E-domain in rpMFE1. The rpMFE1 structure shows that the A-domain is flexibly connected to the C-terminal part, with the hinge region between the domains A and B (helix-10). The open/closed movement of domain A brings its CoA-binding site closer to helix-10 allowing CoA to interact both with domain A and helix-10, and these interactions in the closed form are very similar as observed in the crotonase superFamily. It is intriguing to note that helix-10 of the H/1 part belongs structurally to the dehydrogenase part, being fixed to the E-domain. The two active sites are separated by a positively charged tunnel possibly facilitating an efficient channeling of substrate from the hydratase/isomerase catalytic site to the dehydrogenase catalytic site. Further crystallographic studies are required to establish how these active sites can bind fatty acyl CoA molecules with bulky fatty acid tails, as found in substrates derived from bile acids, for example. Further structural enzymological studies are also required to understand the promiscuous catalytic properties of the crotonase active site and the molecular mechanism causing the substrate channeling.

Acknowledgments—We gratefully acknowledge the excellent support of the beamline scientists at the European Synchrotron Radiation Facility. We thank Dr. Paivi Pirilä for synthesizing the (2E)-decenoyl-CoA. We thank Tatu Haataja for providing the pGro7 plasmid.

REFERENCES
1. Taskinen, J. P., Kiema, T. R., Hiltunen, J. K., and Wierenga, R. K. (2006) J. Mol. Biol. 355, 734–746
2. Hiltunen, J. K., and Qin, Y. (2000) Biochim. Biophys. Acta 1484, 117–128
3. Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M. R., Appel, R. D., and Bairoch, A. (2005) in The Proteomics Handbook (Walker, J. M., ed) pp. 571–607, Humana Press, Inc., Totowa, NJ
4. Holden, H. M., Benning, M. M., Haller, T., and Gerlt, J. A. (2001) Acc. Chem. Res. 34, 145–157
5. Kiema, T. R., Taskinen, J. P., Pirilä, P. L., Koivuranta, K. T., Wierenga, R. K., and Hiltunen, J. K. (2002) Biochem. J. 367, 433–441
6. Bhaumik, P., Koski, M. K., Glumoff, T., Hiltunen, J. K., and Wierenga, R. K. (2005) Curr. Opin. Struct. Biol. 15, 621–628
7. Savolainen, K., Kotti, T. J., Schmitz, W., Savolainen, T. I., Sormunen, R. T., Ilves, M., Vainio, S. J., Conzelmann, E., and Hiltunen, J. K. (2004) Hum.

Crystal Structure of Rat Peroxisomal MFE1

Crystal Structure of Rat Peroxisomal MFE1
Crystal Structure of Rat Peroxisomal MFE1

Mol. Genet. 13, 955–965

8. Nguyen, S. D., Baes, M., and Van Veldhoven, P. P. (2008) Biochim. Biophys. Acta 1781, 400–405

9. Ferdinandusse, S., Denis, S., Van Roermund, C. W., Wanders, R. J., and Dacremont, G. (2004) J. Lipid Res. 45, 1104–1111

10. Kurosawa, T., Sato, M., Nakano, H., Fujiwara, M., Murai, T., Yoshimura, T., and Hashimoto, T. (2001) Steroids 66, 107–114

11. Xu, R., and Cuebas, D. A. (1996) Biochem. Biophys. Res. Commun. 221, 271–278

12. Palosaari, P. M., Vihinen, M., Mäntsälä, P. I., Alexson, S. E., Pihlajaniemi, T., and Hiltunen, J. K. (1999) J. Biol. Chem. 266, 10750–10753

13. Zhang, D., Yu, W., Geisbrecht, B. V., Gould, S. I., Sprecher, H., and Schulz, H. (2002) J. Biol. Chem. 277, 9127–9132

14. Filppula, S. A., Yagi, A. I., Kilpeläinen, S. H., Novikov, D., FitzPatrick, D. R., Vihinen, M., Valle, D., and Hiltunen, J. K. (1998) J. Biol. Chem. 273, 349–355

15. Kiema, T. R., Engel, C. K., Schmitz, W., Filppula, S. A., Wierenga, R. K., and Hiltunen, J. K. (1999) Biochemistry 38, 2991–2999

16. Palosaari, P. M., and Hiltunen, J. K. (1991) J. Biol. Chem. 266, 2507–2527

17. Yang, S. Y., Cuebas, D., and Schulz, H. (1986) J. Biol. Chem. 261, 15390–15395

18. Miles, E. W., Rhee, S., and Davies, D. R. (1999) J. Biol. Chem. 274, 12193–12196

19. Raushel, F. M., Thoden, J. B., and Holden, H. M. (2003) Acc. Chem. Res. 36, 539–548

20. Holden, H. M., Thoden, J. B., and Raushel, F. M. (1998) Curr. Opin. Struct. Biol. 8, 679–685

21. Hyde, C. C., Ahmed, S. A., Padlan, E. A., Miles, E. W., and Davies, D. R. (1988) J. Biol. Chem. 263, 17857–17871

22. Manjasetty, B. A., Powlowski, J., and Vrielink, A. (2003) Proc. Natl. Acad. Sci. U.S.A. 100, 6992–6997

23. Ecock, A. H., Potter, M. J., Matthews, D. A., Knighton, D. R., and McCammon, J. A. (1996) J. Mol. Biol. 262, 370–374

24. Attrey, C. E., Johnson, E. F., Williamson, J., Chang, S. Y., Liang, P. H., and Anderson, K. S. (2003) J. Biol. Chem. 278, 28901–28911

25. Ishikawa, M., Tsuchiya, D., Oyama, T., Tsunaka, Y., and Morikawa, K. (2004) EMBO J. 23, 2745–2754

26. Hamed, R. B., Batchelar, E. T., Clifton, I. J., and Schofield, C. J. (2008) Cell. Mol. Life Sci. 65, 2507–2527

27. Barycki, J. J., O’Brien, L. K., Strauss, A. W., and Banaszak, L. J. (2000) J. Biol. Chem. 275, 27186–27196

28. Barycki, J. J., O’Brien, L. K., Birkoft, J. J., Strauss, A. W., and Banaszak, L. J. (1999) Protein Sci. 8, 2010–2018

29. Taskinen, J. P., Kiema, T. R., Koivuranta, K. T., Wierenga, R. K., and Hiltunen, J. K. (2002) Acta Crystallogr. D Biol. Crystallogr. 58, 690–693

30. Leslie, A. G. (1992) Joint CCP4 + ESF-EAMCB Newsletter on Protein Crystallography, No. 26

31. Evans, P. (2006) Acta Crystallogr. D Biol. Crystallogr. 62, 72–82

32. Collaborative Computational Project, No. 4 (1994) Acta Crystallogr. D Biol. Crystallogr. 50, 760–763

33. McCoy, A. J., Grosse-Kunstleve, R. W., Storoni, L. C., and Read, R. J. (2005) Acta Crystallogr. D Biol. Crystallogr. 61, 458–464

34. Emsley, P., and Cowtan, K. (2004) Acta Crystallogr. D Biol. Crystallogr. 60, 2126–2132

35. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) Acta Crystallogr. D Biol. Crystallogr. 53, 240–255

36. Davis, I. W., Leaver-Fay, A., Chen, V. B., Block, J. N., Kapral, G. J., Wang, X., Murray, L. W., Arendall, W. B., 3rd, Snoeyink, J., Richardson, J. S., and Richardson, D. C. (2007) Nucleic Acids Res. 35, W375–W83

37. Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., and Higgins, D. G. (2007) Bioinformatics 23, 2947–2948

38. Partanen, S. T., Novikov, D. K., Popov, A. N., Mursula, A. M., Hiltunen, J. K., and Wierenga, R. K. (2004) J. Mol. Biol. 342, 1197–1208

39. Deleted in proof

40. Engel, C. K., Kiema, T. R., Hiltunen, J. K., and Wierenga, R. K. (1998) J. Mol. Biol. 275, 847–859

41. Mursula, A. M., Hiltunen, J. K., and Wierenga, R. K. (2004) FEBS Lett. 557, 81–87

42. Modis, Y., Filppula, S. A., Novikov, D. K., Norledge, B., Hiltunen, J. K., and Wierenga, R. K. (1998) Structure 6, 957–970

43. Krissinel, E., and Henrick, K. (2004) Acta Crystallogr. D Biol. Crystallogr. 60, 2262–2268

44. Tschiaya, D., Shimizu, N., Ishikawa, M., Suzuki, Y., and Morikawa, K. (2006) Structure 14, 237–246

45. Engel, C. K., Mathieu, M., Zeeden, J. P., Hiltunen, J. K., and Wierenga, R. K. (1996) EMBO J. 15, 5135–5145

46. Gurvitz, A., Mursula, A. M., Firzinger, A., Hamilton, B., Kilpeläinen, S. H., Hartig, A., Ruis, H., Hiltunen, J. K., and Rottensteiner, H. (1998) J. Biol. Chem. 273, 31366–31374

47. Hofstein, H. A., Feng, Y., Anderson, V. E., and Tonge, P. J. (1999) Biochemistry 38, 9508–9516

48. Bahnson, B. J., Anderson, V. E., and Petsko, G. A. (2002) Biochemistry 41, 2621–2629