Peroxisomal multifunctional enzyme type 2 from the fruitfly: dehydrogenase and hydratase act as separate entities, as revealed by structure and kinetics

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All of the peroxisomal β-oxidation pathways characterized thus far house at least one MFE (multifunctional enzyme) catalysing two out of four reactions of the spiral. MFE type 2 proteins from various species display great variation in domain composition and predicted substrate preference. The gene CG3415 encodes for Drosophila melanogaster MFE-2 (DmMFE-2), complements the Saccharomyces cerevisiae MFE-2 deletion strain, and the recombinant protein displays both MFE-2 enzymatic activities in vitro. The resolved crystal structure is the first one for a full-length MFE-2 revealing the assembly of domains, and the data can also be transferred to structure–function studies for other MFE-2 proteins. The structure explains the necessity of dimerization. The lack of substrate channelling is proposed based on both the structural features, as well as by the fact that hydration and dehydrogenation activities of MFE-2, if produced as separate enzymes, are equally efficient in catalysis as the full-length MFE-2.

Key words: crystal structure, fatty acid metabolism, hydroxyster-oid (17-β) dehydrogenase 4 homologue, multifunctional enzyme type 2 (MFE-2), peroxisome, substrate channelling.

INTRODUCTION

Acyl-CoA esters are oxidized in the β-oxidation spiral that is the major pathway for the breakdown of fatty acids and is found, depending on the species, in peroxosomes and mitochondria. The mitochondrial system seems to be a property of mammals only, whereas peroxisomal β-oxidation is found in all eukaryotes studied thus far [1].

The second and third reactions in the peroxisomal β-oxidation cycle (Figure 1) are performed by an MFE (multifunctional enzyme) that consists of one single polypeptide chain harbouring both dehydrogenase and hydratase activities. In the mammalian peroxisomes, there are two structurally non-related MFEs involved in fatty acid breakdown, with different stereospecificities towards the 3-hydroxy intermediate. The subject of the present study, MFE-2 (MFE type 2), is active towards the R-isomers of the substrate intermediates [2,3]. Both peroxisomal MFE-1 and the membrane-associated mitochondrial trifunctional enzyme complex house (3S)-hydroxy-specific dehydrogenase [4,5]. MFE-2 has been identified from various species, first in fungi [2] and later on in mammals [3,6–9]. Curiously, MFE-2 deficiency forms the larger subgroup among peroxisomal disorders in humans [10]. The protein has variable domain organizations depending on the source, as summarized in Figure 2.

The dehydrogenase domain of MFE-2 belongs to the SDR (short-chain dehydrogenase/reductase) family [11]. The structure from the rat domain [12] includes the ligand NAD+ and, in addition to the typical ‘Rossman fold’ nucleotide-binding core, it also contains an extended C-terminal subdomain. This 60-residue part is unique: other proteins in the SDR family do not have it. In the dehydrogenase homodimer the C-terminal subdomain from one monomer folds on top of the substrate-binding tunnel of the other monomer, and vice versa [12]. In this way the monomers contribute to the active-site architectures of each other in a co-operative manner, and monomer–monomer interactions are strengthened. The dehydrogenases in yeast MFE-2 form a structural dimer within the polypeptide (Figure 2), with the two domains preferring different chain-length fatty acyl-CoA substrates [13,14].

The hydratase domain structures of MFE-2 (which is R-specific) from humans and Candida tropicalis [15,16] are structurally different from the S-specific hydratases (hydratase 1, crotonase) from mitochondria [17] and peroxisomal MFE-1 [18]. Hydratase 2 from MFE-2 folds into a hot-dog fold with a long α-helix wrapped inside a four-stranded β-sheet. The bacterial R-hydratase [19], however, has common structural features with the MFE-2 hydratase. Comparison of the two has revealed the structural basis for the adaptation of the eukaryotic enzyme to accept long-chain substrates: the absence of the α-helix from one of the hot-dog fold subdomains creates space for bulky substrates [15].

Mammalian species and species such as Danio rerio MFE-2s have a compact 13 kDa domain at their C-terminus [20], which is structurally homologous with SCP-2 (sterol carrier protein 2) (Figure 2). It is a non-specific lipid-binding protein displaying broad ligand-binding properties. Drosophila, however, is one of the species lacking this domain from its MFE-2 protein (Figure 2).

Despite the structurally well-characterized domains of MFE-2, it is not known how the full-length protein is assembled and how the enzymatically active domains possibly interact in catalysis. In the case of multifunctional proteins possessing more than one enzymatic activity responsible for consecutive steps in a pathway, substrate channelling is always worth considering. There are well-characterized substrate channelling enzymes [21]; however, an emerging picture of substrate channelling is that there may be real tunnels for small molecules inside proteins, but for bulkier...
substrates, such as fatty acyl-CoAs, the molecular mechanisms are not as obvious, as exemplified by studies on the mitochondrial trifunctional enzyme [22] and peroxisomal MFE-1 [18].

In the present study we characterize the DmMFE-2 (Drosophila melanogaster MFE-2) as an enzyme both in vitro and in vivo. We show that DmMFE-2 complements a defect of peroxisomal MFE-2 in yeast. We also present the first crystal structure of a full-length MFE-2 protein revealing the assembly of domains and dimerization. Both the structure and the kinetic properties do not support a substrate-channelling mechanism in DmMFE-2.

EXPERIMENTAL

Construction of the bacterial overexpression plasmids

The D. melanogaster cDNA clone (GH14720) encoding a 598 amino acid polypeptide that possesses a stretch of amino acid sequence similar to SDR and maoC-like dehydratase enzymes (FlyBase accession code CG3415) was purchased from the Drosophila Genomics Resource Center (Bloomington, IN, U.S.A.). In the present study, the 64.1 kDa expression product is referred to as the peroxisomal multifunctional enzyme type 2 (DmMFE-2). The 1797 bp DNA fragment was amplified using the primers 5′-tttttaaatATGTCCTCATCGGATGG-3′ and 3′-tttttaacagCACGTTG-5′ with the restriction enzyme recognition sites underlined and the template-specific sequence in uppercase. The fragment was cloned into modified pET23b(+) expression vector [23] resulting in plasmid pET23b::DmMFE-2. The same plasmid-construct strategy was used when preparing the expression plasmids of the separate dehydrogenase (DmDH) and hydratase 2 (DmH2) domains of the DmMFE-2, resulting in plasmids pPAL7::DmDH and pET15b::DmH2 covering amino acids 1–309 and 310–598 respectively.

Overexpression and purification

The plasmid pET23b::DmMFE-2 was transformed into Escherichia coli BL21(DE3) pLysS. A pre-culture for overexpression was cultured overnight at 37 °C in Luria–Bertani broth supplemented with 50 μg/ml carbenisillin and 34 μg/ml chloramphenicol. The pre-culture (20 ml) was transferred to 1.0 litre of M9ZB medium and cultivated at 37 °C under aerobic conditions. The production of recombinant DmMFE-2 was induced by the addition of IPTG (isopropyl β-D-thiogalactopyranoside) to 0.4 mM at D_{600} of 0.8, and the cells were harvested by centrifugation (4000 g for 25 min at 4 °C) after 4 h of induction. The cells were washed with PBS, and 5 g (wet weight) was suspended in 20 ml of Ni-NTA (Ni2+–nitrilotriacetate) lysis buffer [50 mM Tris/HCl (pH 8.0), 300 mM NaCl and 1.0 M urea], frozen using liquid nitrogen and stored at −70 °C until use.

The frozen cell suspension was thawed out at 30 °C and the cell lysis was completed by the addition of 500 μg/ml lysozyme, 50 μg/ml DNase I and 50 μg/ml RNase A. After 30 min of incubation at 30 °C, the lysate was cleared by centrifugation (30000 g for 45 min at 4 °C). The soluble fraction was applied at 1 ml/min to a 2.5 ml Ni-NTA affinity column (Qiagen) equilibrated in lysis buffer prior to use. After flushing with Ni-NTA wash buffer [50 mM Tris/HCl (pH 8.0), 500 mM NaCl and 20 mM imidazole], the target protein was released from the column using an elution buffer [50 mM Tris/HCl (pH 8.0),

Figure 2 Comparison of peroxisomal MFE-2s from various species

The Figure demonstrates the versatility of the domain organization between MFE-2s from the selected species. Black corresponds to the 3R-hydroxyacyl-CoA dehydrogenase domain, vertical black stripes corresponds to the 2E-enoyl-CoA hydratase 2 domain, and the SCP-2L domain is white. Human (H. sapiens), rat (R. norvegicus) and zebrafish (D. rerio) MFE-2 share the same domain composition and organization. DmMFE-2 (D. melanogaster) lacks the SCP-2L domain. Nematodes (Caenorhabditis elegans) and yeasts (C. tropicalis) have yet different domain compositions. In C. elegans the enzymes of MFE-2 have been separated from each other such that hydratase 2 is a separate protein, whereas dehydrogenase and SCP-2L domains form a united polypeptide. The yeast MFE-2 is also missing the SCP-2L domain and has duplicated the dehydrogenase domain which has evolved altered substrate specificities in the two domains [13]. The N-terminal dehydrogenase domain displays the highest activity toward medium- and long-chain (3R)-hydroxy-CoAs, and the middle dehydrogenase domain shows the preference toward the short chain (C_{3}) substrates [13].
500 mM NaCl and 500 mM imidazole. A 14 ml Red Sepharose CL-6B (GE Healthcare) dye-affinity column was equilibrated in 50 mM potassium phosphate buffer (pH 7.0). The protein sample was diluted with 10 vol. with distilled water and was applied to the column at 0.5 ml/min. The bound protein was eluted with a linear gradient of KCl (0–2.0 M in 140 ml). Fractions containing DmMFE-2 were pooled, stabilized with 10% (v/v) glycerol and concentrated. The sample was then applied to a Superdex 200 10/300 GL size-exclusion column (GE Healthcare) equilibrated in 50 mM sodium phosphate (pH 7.5), 200 mM NaCl, 1 mM Na2EDTA, 5% glycerol and 1 mM sodium azide. Major peaks were fractionated, and DmMFE-2-containing fractions were pooled and finally concentrated to 2.9 mg/ml.

The separate domains were expressed using E. coli BL21(DE3) and EnBase Flo medium (BioSilta). The high-yield recombinant protein expression was carried out in a volume of 500 ml according to the instructions provided with the kit. The recombinant protein was induced for 24 h at 30°C by adding 0.4 mM or 1 mM IPTG to the culture medium. In a separate study, the use of EnBase Flo significantly improved the expression of the full-length enzyme [24].

The purification of the 2E-enoyl-CoA hydratase 2 from DmMFE-2 followed the protocol for purifying the full-length DmMFE-2, except that the step with Red Sepharose CL-6B was omitted. The final yield of recombinant DmH2 from a single 500 ml expression was 113 mg of purified protein. The recombinant (3R)-hydroxyacyl-CoA dehydrogenase of DmMFE-2 was initially purified using a 5 ml Profinity eXact affinity column (Bio-Rad Laboratories). After the expression, the cells were suspended in Profinity eXact lysis buffer [0.1 M sodium phosphate (pH 7.2) and 0.3 M sodium acetate] and treated as described in the purification of the full-length DmMFE-2 to obtain the cleared lysate for the first affinity-purification step.

After binding the dehydrogenase in the Profinity eXact column, the weakly bound proteins were flushed out using a wash buffer [0.1 M sodium phosphate (pH 7.2) and 0.15 M sodium acetate]. The elution of the tag-free recombinant protein was triggered using elution buffer [0.1 M sodium phosphate (pH 7.2) and 0.2 M NaCl]. After the elution, the purification of the DmDH proceeded as described for DmH2. In total, 5 mg of purified protein was obtained from the 500 ml expression.

**SLS (static light scattering) experiments**

A Superdex 200 HR 10/300 GL (GE Healthcare) size-exclusion column connected to an ÄKTA purifier (GE Healthcare) and a multi-angle light-scattering device (miniDAWN TREOS; Wyatt Technology), was used for analysing four different samples of DmMFE-2 using the SLS technique. The column was equilibrated in 50 mM sodium phosphate (pH 7.5), 200 mM NaCl, 1 mM Na2EDTA, 5% glycerol and 1 mM sodium azide prior to use. Sample concentrations were 7.4 mg/ml DmMFE-2, 5.7 mg/ml DmDH, 6.1 mg/ml DmH2, and 5.7 mg/ml DmDH+DmH2 (in a stoichiometric ratio). The samples were prepared in the same phosphate buffer in which the column was equilibrated. A 50 μl aliquot of protein was used for one injection, and during the separation the system flow was 250 μl/min at 17°C. The results were analysed using Astra V software (Wyatt Technology).

**In vivo studies, enzyme kinetics and substrate synthesis**

The DNA fragment encoding DmMFE-2 was cloned into the pYE352::CTAI Saccharomycys cerevisiae expression vector [25], replacing the CTAI-gene encoding the yeast peroxisomal catalase and resulting in pYE352::DmMFE-2. The plasmid was transformed [26] into S. cerevisiae deletion strain BY4741 Δfox2 (YKR009c, Euroscarf, accession code Y0508) for the complementation studies. In addition, the Δfox2 deletion strain was also transformed with pYE352::ScMFE-2 [13].

For the yeast complementation study, the BY4741 Δfox2+pYE352::DmMFE-2 dilution series (10⁻¹–10⁻⁵) was pipetted on thin YPD-plates supplemented with oleic acid [0.67% yeast nitrogen base without amino acids, 0.1% yeast extract, 0.5% potassium dihydrogen phosphate (pH 6.0), 0.5% Tween 80, 0.14% oleic acid, 0.2% dextrose, 50 μg/ml ampicillin and 2.0% agar]. BY4741 Δfox2 and BY4741 Δfox2+pYE352::CTAI served as the negative controls and BY4741 wt (wt is wild-type) and BY4741 Δfox2+pYE352::ScMFE-2 as positive controls on the examined plates. The cells for the dilution series were obtained from fresh saturated liquid cultures. The cells were washed with sterile water prior to use. The D₆₀₀ of the cultures was evened out to 1.0 for preparing the dilution series in sterile water. The plates were incubated first at 30°C for 1 week to force the yeast to utilize the oleic acid as the sole carbon source for growth, and an additional week at 4°C for optimizing the visualization of the clearing zones obtained around the yeast growth.

The enzyme activity assays were carried out according to Hiltunen et al. [27] using (2E)-butenoyl-CoA, (2E)-hexenoyl-CoA and (2E)-decenoyl-CoA as substrates. Substrates were synthesized as described by Qin et al. [13]. All of the measurements were carried out as a double determination using 0.5 ml quartz cuvettes at 22°C, and Kₘ, Vₘₐₓ and kₐₜ values were calculated with GraFit 5 (Erithacus Software).

**Crystallization**

The method described by Jancarik and Kim [28] was used for the initial crystallization screening. Sitting and hanging-drop vapour-diffusion methods were used at 21°C and set up using a Tecan Freedom Evo liquid-handling robot operated via the Gemini software. The crystallization drops contained equal volumes of protein solution and mother liquor (1 μl). A tetragonal shaped crystal grown in 100 mM Tris/HCl (pH 8.0), 1.0 M NaCl, 20% (v/v) PEG [poly(ethylene glycol)] 5000 MME and 5 mM NaD+ was the most suitable for X-ray analysis. The crystal was approximately 0.23 mm × 0.21 mm × 0.05 mm in dimensions. The crystal was transferred into a cryo-protecting mother liquor containing 20% (v/v) glycerol in addition to the original mother liquor, incubated for 1 min and flash-frozen at 100 K using liquid nitrogen.

**Data collection and structure determination**

A dataset (90° of diffraction data in 180 frames) was collected at 100 K using synchrotron radiation [wavelength 0.9310 Å (1 Å = 0.1 nm)] at the ID29 beamline [ESRF (European Synchrotron Radiation Facility), Grenoble]. The images were processed using XDS [29] with the high-resolution limit set to 2.15 Å (1 Å = 0.1 nm). The data fitted best to the space group P4₃2₁2. The structure was solved by molecular replacement using Phaser [30]. Molecule A of the (3R)-hydroxyacyl-CoA dehydrogenase fragment of rat peroxisomal MFE-2 (PDB code 1GZ6) and molecule A of the (2E)-enoyl-CoA hydratase 2 domain of C. tropicalis MFE-2 complexed with (3R)-hydroxydecanoyl-CoA (PDB code 1PN4) were used as models in the rotation and translation search. The first search cycle was performed using the hydratase 2 molecule and the dehydrogenase molecule was used in the second cycle. After solving the structure, the amino acid sequences of the homology models were modified by...
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understanding of MFE-2 proteins from a domain level to domain assembly level. According to the calculated Matthew’s coefficient (2.25), the asymmetric unit is occupied by a single DmMFE-2 monomer. The monomer consists of one N-terminal (3R)-hydroxyacyl-CoA dehydrogenase domain, a C-terminal (2E)-enoyl-CoA hydratase 2 domain and a short loop which connects the two catalytic domains. The monomer is approximately L-shaped and it is hourglass-shaped in the side view. The constituent domain structures bear high similarity to the previously published MFE-2 single-domain structures from rat and yeast.

The monomer of DmMFE-2 reveals the contacts between the two enzymatic domains within the polypeptide. As shown in Figure 4, three salt bridges (Lys281 to Asp116, and Lys256 and Lys305 to Glu115) are found between the DmDH and the DmH2 domains, and one between the connecting loop and the DmH2 (Asp312 to Lys412). These contacts reside at the narrowest section of the molecule. From the residues mentioned, only Lys305 is conserved among the MFE-2 family.

It is known, however, that MFE-2 is active as a dimer, and many features in the new structure also support this. The DmMFE-2 homodimer is formed at the two-fold symmetry axis in such a way that the two polypeptides align each other crosswise, creating a complex interaction network between the two dehydrogenase and hydratase monomers, as shown previously [12,15]. Dimerization could also be understood such that both identical catalytic domains are forming their own dimers which are connected via short polypeptide linkers in the centre of the molecule. Sequence alignment using ClustalX [39] revealed that the linker region in the DmMFE-2 appeared to be shorter than in human MFE-2. In DmMFE-2 the unfolded linker region consisted of six residues (determined using ClustalX and PROCHECK [39,40]), but in human MFE-2 there are additional 22 amino acids forming a gap between the dehydrogenase dimer and the hydratase dimer that, in practice, are absent in the DmMFE-2 sequence (Supplementary Figure S1). The overall shape of the DmMFE-2 dimer is rather rectangular when viewing the molecule from the front (Figures 5A and 5B). When viewing the molecule from the side, the monomer in front covers the other monomer, and the silhouette resembles that of the monomer (Figure 5C). Surprisingly, the centre of the molecule is not filled, and there exists a clear physical hole (Figure 5B). The active sites of one polypeptide are facing to the same sides (Figure 5D).

The two DmMFE-2 monomers in the dimer are kept together by several hydrogen bonds, as well as hydrophobic and aromatic interactions, as shown previously [12,15,16]. The structure is further stabilized by salt bridges between the two polypeptides. In the dehydrogenase dimer, the interactions are mainly of a hydrophobic nature between the long helixes α5 and α5’ in addition to hydrogen bonds. However, a few salt bridges are formed that will give the dehydrogenase dimer additional stability. The first bonds are formed between Glu115 and Arg261, and between Glu215 and Arg245. Glu241 and Arg244 also form bonds, as well as Glu241 and Arg244. In the hydratase domain, a small hydrophobic patch is formed by residues Ile326, Leu330, Leu400 and Leu405. This patch defines the bottom area of a local groove which is filled with several water molecules. This patch is also responsible for the hydrophobic interaction between α10 and α13’, and α20 and α13. Two stabilizing salt bridges are formed between Arg492 and Glu497, and between Arg492 and Glu497. Monomers are possibly further cross-linked via Ser135 and Asp486, and between Ser135 and Asp486, since the side chains are only 2.6 Å apart.
Table 2 | Data collection, refinement statistics and quality of the structure

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| Value in parentheses refer to the highest-resolution shell. |
| Value in parentheses refer to the percentage of reflections taken randomly for the test calculations. |
| †The non-hydrogen atoms used in the refinement. |
| ‡The non-hydrogen atoms used in the refinement. |

When examining the central opening which is formed between the dehydrogenase and hydratase subunits more carefully, one further observation is worth mentioning. The opening is bordered with lysine and histidine residues such that they create a positively charged patch in the middle of the dimer. The residues involved are His256, Lys285, Lys287, Lys305 and Lys372 from both of the polypeptides. The locations of positively charged residues in the DmMFE-2 dimer are shown in Figure 5(D).

(3R)-Hydroxacyl-CoA dehydrogenase domain of DmMFE-2

The (3R)-hydroxacyl-CoA dehydrogenase domain of DmMFE-2 consists of amino acids 1–307 such that residues 1–244 display a classical Rossmann fold with an α/β doubly wound structure, and residues 245–307 constitute the extension specific to MFE-2 proteins (Figure 5A). Five β-sheets (β1–β5) align in a row in the core of the catalytic domain, and α-helices (two or three depending on the side) border the β-sheets in the middle of the core. Presumably, the missing loop regions and the partially unfolded part of the N-terminus both contribute to the binding of the cofactor NAD⁺. The phosphate-binding consensus sequence GXXGXG is itself ordered in the structure, but the non-conserved loop (between β2 and α2) also participating in the NAD⁺ binding is completely absent. In addition, the polypeptide after helix α2 does not form a clear secondary structure as it does in the previously published MFE-2 dehydrogenase domain structures (PDB codes 1GZ6 and 2ET6). The NAD⁺-binding region is open in the structure, which would permit cofactor binding. However, owing to molecular packing within the crystal lattice, the binding of NAD⁺ probably becomes inhibited by a loop between sheets β12 and β13 from a symmetry-related hydratase molecule. Gln474 especially is only 3.62 Å apart from the location of the superimposed NAD⁺ molecule of the rat dehydrogenase. The crystal packing may also partially explain the weaker electron density in the N-terminal part of the structure.

The overall structural topology of the DmMFE-2 dehydrogenase is very similar to the previously published rat dehydrogenase domain (PDB code 1GZ6) [12]. The C-terminal extension of the rat dehydrogenase domain also exists well-ordered in the DmMFE-2. The extension is not common in the SDR superfamily [11], but the feature is shared between the different MFE-2 homologues. According to the sequence alignments, the human and rat homologues share the highest similarity in the C-terminal region of the domain. When examining the rat C-terminal extension, the β-sheets from two monomers clearly intersect at Trp246. The corresponding region in the present structure shows an intersection between Met332 and Met335 (Supplementary Figure S1). Amino acids Ser334, Tyr335 and Lys337 form the widely conserved SDR family catalytic triad in DmMFE-2 (Supplementary Figure S1). This triad, needed in the hydride transfer reaction, is well-ordered in the present structure.

The dehydrogenase dimer of DmMFE-2 superimposed with the rat MFE-2 dehydrogenase is shown in Figure 6(A).

(2E)-enoyl-CoA hydratase 2 domain of DmMFE-2

The (2E)-enoyl-CoA hydratase 2 domain is built from amino acids 314–598 in DmMFE-2. The DmH2 subunit consists of seven α-helices (α10–α16) and ten β-sheets (β8–β17) that are well-defined (Figure 6B). The subunit can further be divided into two separate subdomains that are connected via a long loop region between sheets β12 and β13 (Figure 5A). A typical hot-dog fold [38] is formed in the cores of the subdomains: in the N-terminal subdomain helix α12 is covered by five β-sheets (β8–β12) and the C-terminal hot-dog fold is formed by α-helix 16 and β-sheets β13–β17.

The DmMFE-2 hydratase domain possesses an interesting structural difference in comparison with the known yeast and human hydratases, and the Aeromonas caviae R-hydratase [19]. We have shown previously that the ability of the eukaryotic hydratase 2 to accept long-chain substrates is due to extra space in the core of the domain created by the lack of the central α12-helix. This helix is present in the bacterial counterpart and it fills the whole interior space of the β-sheet structure, thus rendering the protein active for short-chain substrates only. In Drosophila hydratase the α12-helix is of intermediate length (Figure 7B). In this respect, the present structure resembles more the bacterial protein, but on the other hand retains the ability to utilize a broader spectrum of substrates as in eukaryotic MFE-2s. In fact, when the liganded yeast structure (PDB code 1PN4) is superimposed on top of the DmH2, it can immediately be seen that the fatty acid tail is in close physical contact with the helix α12. Despite this, the DmMFE-2 prefers the C₁₀ substrate over shorter substrates (see above). The structural comparison of known R-hydratase structures is presented in Figure 7.
The assembly of a full-length MFE-2 has been unknown. The DmMFE-2 dimer is dominated by dehydrogenase and hydratase subdimers that are connected via a short loop (residues 308–313, Supplementary Figure S1). The loop, which is short enough to limit large domain movements, is involved in domain–domain contacts within each MFE-2 monomer. The monomers in turn contribute to the rigid assembly with the extensive dimerization contacts between monomers. The centre of the dimeric molecule contains a hole. Both dehydrogenase and hydratase catalytic sites within one polypeptide are accessible on the same side: the CoA-binding region of the dehydrogenases is facing the centre of the dimer, whereas the hydratase active site is pointing out from the centre.

Owing to the structural similarity of the enzymatic domains of all known MFE-2 proteins, and due to the extensive dimerization present in DmMFE-2, it is very likely that assembly of the enzymatic domains in mammalian and yeast counterparts will be similar, despite variation in the connecting linkers and the fact that DmMFE-2 lacks the SCP-2L domain.

The hydratase subunit of DmMFE-2 shares only a 17% sequence identity with the A. caviae R-specific hydratase and 38% with the yeast C. tropicalis hydratase 2 from MFE-2. It is staggering to discover that all of these proteins have such a similar structure, even though the sequence similarity shows such variation. The most visible difference between the three structures is the hot-dog fold α-helix (in the Drosophila hydratase labelled as α12) that varies in length. The helix is virtually absent in both the liganded and unliganded forms of the C. tropicalis hydratase 2 domain [15]. In the bacterial hydratase homodimer the α-helix extends up to the upper part of the β-sheet bundle at the N-terminal end of a monomer. The α-helix in the DmH2 domain is not as long as in the A. caviae R-hydratase monomer, and it also slightly leans away from the active site. It is also clear from the kinetic data (Table 1) that DmMFE-2 uses the C18 substrate efficiently.

The structure of the A. caviae R-hydratase suggests the existence of two active sites, and activity against short-chain enoyl-CoAs that are up to six carbon atoms in length. The substrate-binding pocket in C. tropicalis hydratase is much more capacious owing to replacement of the long hot-dog α-helix (corresponding to α12 in DmMFE-2) with a highly mobile polypeptide and a short α-helix. This has resulted in the broader substrate utilization range in the yeast counterpart. Similarly to the yeast, in the present structure there exists only one active site in a hydratase 2 monomer. The size of the substrate-binding pocket is restricted by the hot-dog helix α12. When superimposing the hydratase domain with the liganded yeast hydratase domain, the end of the fatty acid tail of the substrate is in close contact with the hot-dog α-helix. This could indicate narrower substrate specificity for the Drosophila hydratase domain, unless the hot-dog α-helix is exceptionally flexible.

In the catalytic reactions by MFE-2, the (2E)-enoyl-CoA substrate is first hydrated by the hydratase 2 domain and the (3R)-hydroxy intermediate then serves as the substrate for the dehydrogenase domain for the subsequent oxidation. However, the assembly does not indicate whether the substrate leaving from a hydratase is further catalysed in the dehydrogenase of the same MFE-2 polypeptide or whether it proceeds to the dehydrogenase of the other polypeptide. The latter possibility, together with the subdimer assembly mode, well explains the necessity of dimerization for activity found for MFE-2 proteins.

Enzyme kinetic data do not support efficiency-enhancing substrate channelling between the enzymatic domains. The structure, however, has some features that can be discussed relative to a channelling mechanism being present. The surface of the central opening of the dimer has a number of positively charged residues that could bind and route the phosphate-containing substrates. However, the hole appears too narrow (approximately 9 Å) to let a bulky (and possibly non-extended [15]) fatty acyl-CoA substrate pass through. In addition, there is also a track of positively charged residues leading from the hydratase active site towards the dimer centre. One pathway towards the centre opening could be the residues which lie at the hydratase dimer interface. A small opening from the hydratase substrate-binding pocket exists in the subunit that has access to the positively charged residues in the dimer interface. However, an alternate pathway should be considered since the CoA molecule is too monolithic to transfer through the opening. The amount of charged residues on the surface of the hydratase 2 domain on the
way from the hydratase active site to the dehydrogenase active site via the outer edge of the hydratase appear to form just a narrow path. It is tempting to see these residues providing access between the active sites and thus contributing to the enzymatic efficiency. We attempted to test this by mutating two to five of these residues to glutamate residues (T. J. K. Haataja, J. K. Hiltunen and T. Glumoff, unpublished work). Dihydrofolate reductase–thymidylate synthase [41] is proposed to contain such an ‘electrostatic highway’, but it consists of a larger, generally negative surface instead of a few residues.

**Concluding remarks**

*DmMFE-2* is a peroxisomal protein active in the β-oxidation of fatty acyl-CoAs. *DmMFE-2* domains show amino acid sequence, as well as fold similarities to those of other MFE-2s. *DmMFE-2* complements functionally the *S. cerevisiae* peroxisomal MFE-2 *in vivo*, and the recombinant domains display the predicted catalytic activities *in vitro*. The assembly of domains renders *DmMFE-2* a ‘double-dimeric’ protein, where dimerization can equally well be seen as the dimer of polypeptides or the dimer of like enzymes coming from different polypeptides. There is a physical hole in the middle of the protein, but it is not large enough to allow fatty acyl-CoA molecules from using it as a channel between active sites. Positive surface-charge distribution is also not extensive enough to convincingly support the hypothesis of an electrostatic substrate channelling mechanism. In contrast, strong evidence against the presence of substrate channelling in *DmMFE-2* comes from enzyme kinetic data. The turnover number and the
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Figure 6 Comparison of the dehydrogenase and hydratase domains with known structures of separate MFE-2 domains

(A) Stereoview of the Drosophila dehydrogenase monomer of MFE-2 superimposed with the rat dehydrogenase monomer of MFE-2 (PDB code 1GZ6) which is in complex with NAD$^+$. The Drosophila dehydrogenase monomer is coloured in marine blue and the rat dehydrogenase monomer is in silver. Black arrows indicate locations of the flexible loops, which are absent in the DmMFE-2 structure. The N- and C-termini have also been indicated in the Figure. (B) Stereoview of the Drosophila 2E-enoyl-CoA hydratase 2 dimer of MFE-2 superimposed with the homologous hydratase 2-dimer from the yeast C. tropicalis (PDB code 1PN4) and hydratase 2 from human MFE-2 (PDB code 1S9C). The substrate-binding site in each hydratase monomer is evident since the C. tropicalis protein is a complex with the reaction product (3R)-hydroxydecanoyl-CoA. The Drosophila hydratase is coloured in marine blue, and human and yeast counterparts are coloured in pale cyan and in silver respectively.

Figure 7 Structural comparison of known R-hydratase structures with the hydratase domain of DmMFE-2

(A) The homodimeric R-hydratase from A. caviae (PDB code 1IQ6) consists of two 14 kDa subunits. (B) DmH2 domain of DmMFE-2. The black arrow indicates the front loop. Helix $\alpha12$ is also labelled. The monomeric domain has a molecular mass of 30.8 kDa. (C) The yeast C. tropicalis hydratase 2 domain of MFE-2 (PDB code 1PN4) in complex with (3R)-hydroxydecanoyl-CoA. The hydratase monomer has a molecular mass of 31.5 kDa. Although the interior of the bacterial hydratase is completely filled, there are different amounts of free space left for differently sized substrates in the insect and yeast hydratases. The eukaryotic hydratases (B and C) have only one catalytic site, whereas the bacterial hydratase possesses two catalytic sites.

catalytic efficiency for separate hydratase and dehydrogenase enzymes are of the same order of magnitude as for the native full-length protein. Thus the question remains as to why the two enzymatic activities reside together in a multifunctional protein. Possibly the answer is simply the need for stability and dimerization for efficient catalysis. Should, however, a substrate-channelling phenomenon be present, it could serve the purpose of achieving something other than efficiency, such as the protection of an intermediate.

AUTHOR CONTRIBUTION
Tatu Haataja contributed to the experimental design, carried out the experiments and analysed the results, and drafted and wrote the paper. Kristian Koski contributed to
the experimental design and analysis of the results, supervised computational methods and contributed to drafting the paper. Kalervo Hiltunen contributed to the experimental design, analysis of the results and writing of the paper. Tuomo Glumoff contributed to the experimental design, analysis of the results, drafting and writing of the paper, and supervised the project.

ACKNOWLEDGEMENTS

We thank Päivi Pirilä, Tuula Kurvinen and Marja Lajunen for synthesizing the substrates. Biochemistry student Henri Uurpilainen is acknowledged for preparing the plasmid constructs for the separate dehydrogenase and hydratase enzymes of DmMFE-2 during a laboratory rotation study. The ESFR (European Synchrotron Radiation Facility) in Grenoble is acknowledged for beamtime on the beamline ID29.

FUNDING

This work was supported by the Academy of Finland [grant number 24300182 (to T.G.)] and the Sigrid Jusélius Foundation [grant number 24000336 (to K.H.).]

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SUPPLEMENTARY ONLINE DATA

Peroxisomal multifunctional enzyme type 2 from the fruitfly: dehydrogenase and hydratase act as separate entities, as revealed by structure and kinetics

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Figure S1 Amino acid sequence alignment of MFE-2s from various species

DmMFE-2 has been aligned using ClustalX [1] together with human (HsMFE-2), rat (RnMFE-2), zebrafish (DrMFE-2) and yeast C. tropicalis (3R)-hydroxyacyl-CoA dehydrogenase domain B and (2E)-enoyl-CoA hydratase 2 domain fragment (CtDhB+H2). The N-terminal (3R)-hydroxyacyl-CoA dehydrogenase domain A of C. tropicalis MFE-2 has been omitted from the comparison for clarity.

The UniProtKB identifiers of the selected sequences are Q9VXJ0, P51659, P97852, Q8AYH1 and P22414 respectively. The secondary structure elements of a monomer have been highlighted above the sequences with bars and arrows. The rectangular box indicates the linker region between the catalytic domains. The black vertical arrows indicate the location of residues that are essential for enzyme catalysis [2,3]. Complete identity or similarity of residues between the sequences has been indicated with black shading, whereas decreasing greyness is indicating regions with four or three matching residues.

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Atomic co-ordinates and structure factors for the crystal structure of DmMFE-2 have been deposited in the PDB under code 3OML.
Figure S2  Investigation of heterogenic protein complex formation in vitro using the SLS technique

The formation of a protein complex between the dimers of catalytic domains of DmMFE-2 was studied using a multi-angle light scattering device (miniDAWN TROS; Wyatt Technology). The curves in the chromatogram each represent a single injection. Red indicates the full-length DmMFE-2, blue indicates the 3R-hydroxyacyl-CoA dehydrogenase domain of DmMFE-2, green indicates the 2E-enoyl-CoA hydratase 2 domain of DmMFE-2, and black indicates both of the catalytic domains as separate polypeptides in the same injection. The data demonstrate that the DmMFE-2 dehydrogenase and hydratase domains do not form a complex in vitro when expressed as separate polypeptides. According to the numeric data provided by the experiment, in the conditions used, the DmMFE-2 dimer has a molecular mass of 122 kDa, the dehydrogenase dimer has a molecular mass of 62 kDa, the hydratase 2 dimer has a molecular mass of 69 kDa and the mixture of the two dimers has a molecular mass of 65 kDa. With the exception of the mixed sample, this is in agreement with the theoretical values when taking into account the monodispersity of each of the samples.

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Received 8 October 2010/10 February 2011; accepted 14 February 2011 Published as BJ Immediate Publication 14 February 2011, doi:10.1042/BJ20101661