

D. melanogaster full-length MFE-2 structure

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

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Abbreviations used: CoA, coenzyme A; DmMFE-2, *Drosophila melanogaster* multifunctional enzyme type 2; DH, 3R-hydroxyacyl-CoA dehydrogenase; H2, 2E-Enoyl-CoA hydratase 2; SCP-2L, sterol carrier protein-2-like; NAD⁺, nicotinamide adenine dinucleotide; IPTG, isopropyl β-D-1-thiogalactopyranoside; C4, 2E-butenoyl-CoA; C6, 2E-hexenoyl-CoA; C10, 2E-decenoyl-CoA.

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All the peroxisomal β -oxidation pathways characterized thus far house at least one multifunctional enzyme (MFE) catalyzing 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 *D. melanogaster* MFE-2 (*DmMFE-2*), complements the *S. 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.

Keywords: multifunctional enzyme type 2 (MFE-2), hydroxysteroid (17-beta) dehydrogenase 4 homolog, crystal structure, peroxisomes, fatty acid metabolism, substrate channelling

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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 peroxisomes and mitochondria. The mitochondrial system seems to be a property of mammals only, whereas peroxisomal β -oxidation is found in all eukaryotes studied this far [1].

The second and third reactions in the peroxisomal β -oxidation cycle (Figure 1) are performed by a multifunctional enzyme that consists of one single polypeptide chain harbouring both dehydrogenase and hydratase activities. In the mammalian peroxisomes, there are two structurally nonrelated multifunctional enzymes involved in the fatty acid breakdown with different stereo specificities towards the 3-hydroxy intermediate. The subject of the current study, multifunctional enzyme type 2 (MFE-2), is active towards the R-isomers of the substrate intermediates [2,3]. Both peroxisomal MFE type 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 short chain dehydrogenase/reductase (SDR) family [11]. The structure from the rat domain [12] includes the ligand NAD^+ , and in addition to the typical 'Rossmann 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 cooperative 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 human and *C. 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 4-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 such as *Danio rerio* MFE-2s have a compact 13 kDa domain in their C-terminus [20], which is structurally homologous to 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, a substrate channelling is always worth considering. There are well-characterized substrate channelling enzymes (review by Weeks et al.) [21]; however, an emerging picture of substrate channeling is that there may be real tunnels for small molecules inside proteins, but for bulkier substrates, like 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].

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Here we characterize the *Drosophila* MFE-2 (*DmMFE-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 channeling mechanism in *DmMFE-2*.

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Accepted Manuscript

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D. melanogaster full-length MFE-2 structure**EXPERIMENTAL****Construction of the bacterial over-expression plasmids**

The *D. melanogaster* cDNA clone (GH14720) encoding a 598 amino acid polypeptide that possesses a stretch of amino acid sequence similar to short chain dehydrogenase/reductase (SDR) and maoc-like dehydratase enzymes (accession code CG3415) was purchased from *Drosophila* Genomics Resource Center, Bloomington, IN, USA. The 64.1 kDa expression product is here referred to as the peroxisomal multifunctional enzyme type 2 (*DmMFE-2*). The 1797 bp DNA fragment was amplified using the primers 5' tttataaaacatATGTCCTCATCCGATGGAAAACCTCGTTACGAT 3' and 3' tttaaactcgagCAGCTTGGCCTGTGAGCTCTTCAGGTGCGACATA 5' with the restriction enzyme recognition sites underlined and the template specific sequence in capitals. The fragment was cloned into modified pET23b(+) expression vector [23] resulting in plasmid pET23b::*DmMFE-2*. The same plasmid construction 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.

Over-expression and purification

The plasmid pET23b::*DmMFE-2* was transformed into *E. coli* BL21(DE3) pLysS. A pre-culture for over-expression was cultured over night at 37 °C in Luria Bertani broth supplemented with 50 µg ml⁻¹ carbenicillin and 34 µg ml⁻¹ chloramphenicol. 20 ml of the pre-culture was transferred in 1.0 l of M9ZB medium and cultivated at 37 °C under aerobic conditions. The production of recombinant *DmMFE-2* was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 0.4 mM at OD₆₀₀ of 0.8 and the cells were harvested by centrifugation after 4 hours of induction. The cells were washed with PBS (137 mM NaCl, 10 mM Na₂HPO₄, 2.7 mM KCl, 1.8 mM KH₂PO₄ pH 7.4), 5 g (wet weight) suspended in 20 ml of Ni-NTA Lysis buffer (50 mM Tris pH 8.0, 300 mM NaCl, 1.0 M Urea) and frozen using liquid nitrogen and stored at -70 °C until used.

The frozen cell suspension was thawed out at 30 °C and the cell lysis was completed by the addition of 500 µg ml⁻¹ lysozyme, 50 µg ml⁻¹ Dnase I and 50 µg ml⁻¹ Rnase A. After 30 min of incubation at 30 °C, the lysate was cleared by centrifugation (30 000g, 45 min, 4 °C). The soluble fraction was applied at 1 ml min⁻¹ to a 2.5 ml Ni-NTA affinity column (Qiagen) equilibrated in the lysis buffer prior to use. After flushing with Ni-NTA wash buffer (50 mM Tris pH 8.0, 500 mM NaCl, 20 mM imidazol), the target protein was released from the column using an elution buffer (50 mM Tris pH 8.0, 500 mM NaCl, 500 mM imidazol). 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 by 10 volumes with distilled water and was applied to the column at 0.5 ml min⁻¹. 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 by 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, 1 mM NaN₃. Major peaks were fractionated, *DmMFE-2* containing fractions pooled and finally concentrated to 2.9 mg ml⁻¹.

The separate domains were expressed using *E. coli* BL21(DE3) and EnBase Flo medium (BioSilta, Oulu, Finland). The high-yield recombinant protein expression was carried out in 500 ml volume according to the instructions provided with the kit. The recombinant protein was induced for 24 hours at 30°C by adding of 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].

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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 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). After the expression, the cells were suspended in Profinity eXact lysis buffer (0.1 M sodium phosphate; pH 7.2, 0.3 M sodium acetate) and treated as 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, 0.15 M sodium acetate). The elution of the tag-free recombinant protein was triggered using the elution buffer (0.1 M sodium phosphate; pH 7.2, 0.2 M NaF). After the elution, the purification of the *Drosophila* dehydrogenase proceeded as in the case of the *DmH2*. 5 mg of purified protein was obtained from the 500 ml expression.

Static light scattering (SLS) experiments

Superdex 200 HR 10/300 GL (GE Healthcare) size exclusion column connected to ÄKTA purifier (GE Healthcare) and multi-angle light scattering device, miniDAWN TREOS (Wyatt Technology Corp.), was used for analysing four different samples of *DmMFE-2* by SLS-technique. The column was equilibrated in 50 mM sodium phosphate pH 7.5, 200 mM NaCl, 1 mM Na₂EDTA, 5 % glycerol, 1 mM NaN₃ prior to use. Sample concentrations were *DmMFE-2* 7.4 mg/ml, *DmDH* 5.7 mg/ml, *DmH2* 6.1 mg/ml and *DmDH* + *DmH2* (in stoichiometric ratio) 5.7 mg/ml. The samples were prepared in the same phosphate buffer in which the column was equilibrated. 50 µl 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 Corp.).

In vivo studies, enzyme kinetics and substrate synthesis

The DNA fragment encoding *DmMFE-2* was cloned into pYE352::*CTAI Saccharomyces 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 $\Delta fox2$ (YKR009c, Euroscarf, accession code Y0508) for the complementation studies. In addition, the $\Delta fox2$ deletion strain was also transformed with pYE352::*CTAI* [25] and pYE352::*ScMFE-2* [13].

For the yeast complementation study, the BY4741 $\Delta fox2$ + pYE352::*DmMFE-2* dilution series (10^{-1} – 10^{-4}) was pipetted on thin YPD-plates supplemented with oleic acid (0.67 % yeast nitrogen base w/o amino acids, 0.1 % yeast extract, 0.5 % potassium dihydrogen phosphate; pH 6.0, 0.5 % (v/v) Tween 80, 0.14 % oleic acid, 0.2 % dextrose, ampicillin 50 µg/ml and 2.0 % Agar). BY4741 $\Delta fox2$ and BY4741 $\Delta fox2$ + pYE352::*CTAI* served as the negative controls and BY4741 wt and BY4741 $\Delta fox2$ + 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 OD₆₀₀ 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 a 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 obtained clearing zones 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 the measurements were carried out as a double determination using

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0.5 ml quartz cuvettes at 22°C, and K_M , V_{max} and k_{cat} values were calculated with GraFit 5 (Erithacus Software).

Crystallization

The method 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 by 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 μ l). A tetragonal shaped crystal grown in 100 mM Tris pH 8.0, 1.0 M NaCl, 20 % (w/v) PEG 5000 MME, 5 mM NAD⁺ was the most suitable for X-ray analysis. The crystal was approximately 0.23 mm x 0.21 mm x 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 one minute 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) at the ID29 beamline, ESRF, Grenoble. The images were processed using XDS [29] with the high resolution limit set to 2.14 Å. The data fitted best to the space group $P4_32_12$. The structure was solved by molecular replacement using Phaser [30]. Molecule A of the 3R-hydroxyacyl-CoA dehydrogenase fragment of rat peroxisomal multifunctional enzyme type 2 (PDB ID 1GZ6) and molecule A of the 2E-enoyl-CoA hydratase 2 domain of *C. tropicalis* multifunctional enzyme type 2 complexed with 3R-hydroxydecanoyl-CoA (PDB ID 1PN4) were used as models in the rotation and translation search. The first search cycle was done 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 CHAINSAW [31] to match the *Dm*MFE-2 amino acid sequence. Iterative cycles were used for building the structure manually using coot [32]. PHENIX [33] and Refmac [34,35] was used for TLS refinement cycles. The final structure factors were validated using MolProbity [36].

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D. melanogaster full-length MFE-2 structure**RESULTS****Identification of *D. melanogaster* peroxisomal MFE-2**

The NCBI BLASTP was used to identify amino acid sequences from *D. melanogaster* in the RefSeq protein database that show high similarity to the human peroxisomal MFE-2 (NCBI protein accession code NP_000405). Among the 43 sequences, CG3415 clone, encoding a polypeptide with a predicted molecular mass of 64073 Daltons, possessed the highest score in the blast results with a 632 bits score and 0.0 e-value. The obtained amino residues showed similarity to the Rossmann fold that is common among the short chain alcohol dehydrogenase/reductase (SDR) family [11]. The C-terminus displayed a hydratase 2 motif [37] and similarities to amino acid sequences in the R-hydratase-like hot-dog fold [38]. Sequence alignments (Supplementary Figure S1) to the CG3415 clone with the separate domains of MFE-2 from human, rat (*Rattus norvegicus*) and yeast (*Candida tropicalis*) demonstrate high sequence identities between the various species at the amino acid level. The N-terminus of the CG3415 clone (residues 1-316) shares 55 % identity at the amino acid level with the 3R-hydroxyacyl-CoA dehydrogenase from the human and rat. The C-terminal part (residues 317-598) shares 50 % identity with the human enoyl-CoA hydratase 2 and 38 % identity with the yeast enoyl-CoA hydratase 2. Similar to the *C. tropicalis* MFE-2, the *Dm*MFE-2 also lacks the C-terminal SCP-2L domain. Another interesting feature is the linker between the dehydrogenase and hydratase domains, which is 22 amino acid residues shorter in the *Dm*MFE-2 than in human MFE-2 (Supplementary Figure S1).

Localization and enzymatic properties of *Dm*MFE-2

Kinetic properties of *Dm*MFE-2 were determined (Table 1). K_M and k_{cat} values show that *Dm*MFE-2 prefers the medium chain C10 substrate over the short chain C4 and C6 substrates. *Dm*MFE-2 is also active *in vivo* since it rescues the growth of yeast *S. cerevisiae* $\Delta fox2$ strain devoid of the endogenous peroxisomal MFE-2 on oleic acid as the carbon source (Figure 3A). Catalytic domains of MFE-2s are known to be active also if expressed and purified as stand-alone proteins. Our data from static light scattering (SLS) experiments indicate that the catalytic domains do not assembly into heteromeric complexes *in vitro* (Supplementary Figure S2). We wanted to find support for a possible substrate channelling phenomenon being present in *Dm*MFE-2 and therefore repeated the kinetic measurements by using the dehydrogenase and the hydratase 2 domains in 1:1 mixture instead of the full-length protein (Table 1 and Figure 3B and 3C). Most interestingly, it was found that the full-length *Dm*MFE-2 is not more efficient in catalysis compared to individual enzymes. Furthermore, this applies in a similar fashion to all the short and medium chain substrates accessible to us.

The structure determination of *Dm*MFE-2

Molecular replacement was done using the *C. tropicalis* 2E-enoil-CoA hydratase 2 domain of MFE-2 (1PN4) and the rat 3R-hydroxyacyl-CoA dehydrogenase domain of MFE-2 (1GZ6) as the starting models for the initial phase determination using a native dataset from *Dm*MFE-2 crystals. A successful molecular replacement solution was obtained using the space group $P4_32_12$ in the rotation and translation search. The asymmetric unit consists of one *Dm*MFE-2 monomer in a unit cell with dimensions of 114.48 x 114.48 x 89.11 Å with corresponding 90° angles. After solving the phases, the amino acid side chains were mutated to match the *Dm*MFE-2 sequence and the missing residues were built in the positive electron density. Clear electron density was discovered from the hydratase 2 domain as well as from the C-terminal part of the dehydrogenase domain. 80 % of the total sequence

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was comprised of the clear density that initially enabled the tracking of the correct position of the atoms in the main chain. However, the density was poor in some areas of the map that are known to contain flexible regions in the homologous structures. The missing polypeptide fragments include regions 1 – 6, 44 – 55, 88 – 98 and 200 – 204 in the dehydrogenase domain and 373 – 387, 451 – 457 and 593 – 598 in the hydratase domain. Despite the fact that the protein was crystallized in the presence of cofactor NAD⁺, no positive density was detected at the known cofactor binding region of the dehydrogenase domain. Structural refinement statistics are summarized in table 2.

The overall structure of *Dm*MFE-2 monomer and dimer

The current structure described here is the first crystal structure of a full-length MFE-2 protein thus extending the 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 *Dm*MFE-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 that connects the two catalytic domains. The monomer is roughly 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 the rat and yeast.

The monomer of *Dm*MFE-2 reveals the contacts between the two enzymatic domains within the polypeptide. As detailed in figure 4, three salt bridges (Lys285 to Asp316, and Lys256 and Lys305 to Glu315) are found between the dehydrogenase and the hydratase 2 domains, and one between the connecting loop and the hydratase 2 (Asp312 to Lys412). These contacts reside at the narrowest section of the molecule. From the mentioned residues, 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 *Dm*MFE-2 homodimer is formed at the 2-fold symmetry axis in a way that the two polypeptides align each other crosswise creating a complex interaction network between the two dehydrogenase and hydratase monomers as previously shown [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 fruit fly MFE-2 appears to be shorter than in human MFE-2. In *Dm*MFE-2 the unfolded linker region consists of 6 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 *Dm*MFE-2 sequence (Supplementary figure S1). The overall shape of the *Dm*MFE-2 dimer is rather rectangular when viewing the molecule from the front (Figure 5A and 5B). When viewing the molecule from the side, the monomer in front covers the other monomer and the silhouette resembles the silhouette 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 *Dm*MFE-2 monomers in the dimer are kept together by several hydrogen bonds as well as hydrophobic and aromatic interactions as shown before [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 helices $\alpha 5$ and $\alpha 5'$ 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 Glu215 and Arg261' and between Glu215' and Arg261. Glu241 and Arg254' are also forming bonds as well as Glu241' and Arg254. In the hydratase domain, a small hydrophobic patch is formed by residues Ile326, Leu330, Leu490 and Leu493. This patch defines the bottom area of a local groove that is filled with several water molecules. This patch is also responsible

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for the hydrophobic interaction between $\alpha 10$ to $\alpha 13'$ and $\alpha 10'$ to $\alpha 13$. Two stabilizing salt bridges are formed between Arg492 and Glu347' and between Arg492' and Glu347. Monomers are possibly further cross-linked via Ser335 and Asp486' and between Ser335' and Asp486 since the side chains are only 2.6 Å apart.

When examining the central opening that 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 *Dm*MFE-2 dimer are shown in figure 5D.

3R-hydroxyacyl-CoA dehydrogenase domain of *Dm*MFE-2

The 3R-hydroxyacyl-CoA dehydrogenase domain of *Dm*MFE-2 consists of amino acids 1-307 such that residues 1-244 display a classic 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 ($\beta 1 - \beta 5$) align in a row in the core of the catalytic domain and α -helices (2 or 3 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 GXXXGXG itself is ordered in the structure, but the non-conserved loop (between $\beta 2$ and $\alpha 2$) also participating in the NAD^+ binding is completely absent. In addition, the polypeptide after the helix $\alpha 2$ is not forming a clear secondary structure as it is in the previously published MFE-2 dehydrogenase domain structures (1GZ6, 2ET6). The NAD^+ binding region is open in the structure, which would permit the cofactor binding. However, due to molecular packing within the crystal lattice the binding of NAD^+ probably becomes inhibited by a loop between sheets $\beta 12$ and $\beta 13$ from a symmetry-related hydratase molecule. Especially Gln474 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 *Dm*MFE-2 dehydrogenase is very similar to the previously published rat dehydrogenase domain (1GZ6) [12]. The C-terminal extension of the rat dehydrogenase domain also exists well-ordered in the *Dm*MFE-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 current structure shows intersection between Met252 and Met252' (Supplementary figure S1). Amino acids Ser154, Tyr167 and Lys171 form the widely conserved SDR family catalytic triad in *Dm*MFE-2 (Supplementary figure S1). This triad, needed in the hydride transfer reaction, is well-ordered in the current structure. The dehydrogenase dimer of *Dm*MFE-2 superimposed with the rat MFE-2 dehydrogenase is presented in figure 6A.

2E-Enoyl-CoA hydratase 2 domain of *Dm*MFE-2

2E-Enoyl-CoA hydratase 2 domain is built from amino acids 314-598 in *Dm*MFE-2. The *Drosophila* hydratase 2 subunit consists of 7 α -helices ($\alpha 10$ - $\alpha 16$) and 10 β -sheets ($\beta 8$ - $\beta 17$) that are well-defined (Figure 6B). The subunit can further be divided in two separate subdomains that are connected via a long loop region between sheets $\beta 12$ and $\beta 13$ (Figure 5A). A typical hot-dog fold [38]

Abbreviations used: CoA, coenzyme A; *Dm*MFE-2, *Drosophila melanogaster* multifunctional enzyme type 2; DH, 3R-hydroxyacyl-CoA dehydrogenase; H2, 2E-Enoyl-CoA hydratase 2; SCP-2L, sterol carrier protein-2-like; NAD^+ , nicotinamide adenine dinucleotide; IPTG, isopropyl β -D-1-thiogalactopyranoside; C4, 2E-butenoyl-CoA; C6, 2E-hexenoyl-CoA; C10, 2E-decenoyl-CoA.

D. melanogaster full-length MFE-2 structure

is formed in the cores of the subdomains: in the N-terminal subdomain helix $\alpha 12$ is covered by five β -sheets ($\beta 8$ - $\beta 12$) and the C-terminal hot-dog fold is formed by α -helix 16 and β -sheets $\beta 13$ - $\beta 17$.

The *Dm*MFE-2 hydratase domain possesses an interesting structural difference in comparison to the known yeast and human hydratases and the *Aeromonas caviae* R-hydratase [19]. We have shown earlier that the ability of the eukaryotic hydratase 2's to accept long chain substrates is due to extra space in the core of the domain created by the lack of the central $\alpha 12$ -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 $\alpha 12$ -helix is of intermediate length (Figure 7B). In this respect the current 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 (1PN4) is superimposed on top of the fruit fly hydratase 2, it can immediately be seen that the fatty acid tail is in close physical contact to the helix $\alpha 12$. Despite this the *Dm*MFE-2 prefers the C10 substrate over shorter ones (see above). The structural comparison of known R-hydratase structures is presented in figure 7.

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DISCUSSION

The assembly of a full-length MFE-2 has been unknown. The *Dm*MFE-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 while the hydratase active site is pointing out from the centre.

Due to the structural similarity of the enzymatic domains of all known MFE-2 proteins, and due to the extensive dimerization present in *Dm*MFE-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 *Drosophila* MFE-2 lacks the SCP-2L domain.

The hydratase subunit of *Dm*MFE-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 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 fruit fly hydratase labelled as α 12) that is varying 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 in the N-terminal end of a monomer. The α -helix in the *D. melanogaster* hydratase 2 domain is not as long as in the *A. caviae* R-hydratase monomer and it is also slightly leaning away from the active site. It is also clear from the kinetic data in table 1 that *Dm*MFE-2 uses the C10 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 C6 in length. The substrate binding pocket in *C. tropicalis* hydratase is much more capacious due to replacement of the long hot-dog α -helix (corresponding α 12 in *Dm*MFE-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 current 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 α 12. 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 to the hot-dog α -helix. This could indicate narrower substrate specificity for the fruit fly 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 catalyzed 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 does 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 (ca. 9 Å) to let a bulky (and possibly non-extended [15]) fatty acyl-CoA

Abbreviations used: CoA, coenzyme A; *Dm*MFE-2, *Drosophila melanogaster* multifunctional enzyme type 2; DH, 3R-hydroxyacyl-CoA dehydrogenase; H2, 2E-Enoyl-CoA hydratase 2; SCP-2L, sterol carrier protein-2-like; NAD⁺, nicotinamide adenine dinucleotide; IPTG, isopropyl β -D-1-thiogalactopyranoside; C4, 2E-butenoyl-CoA; C6, 2E-hexenoyl-CoA; C10, 2E-decenoyl-CoA.

D. melanogaster full-length MFE-2 structure

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 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 glutamates, but the mutant proteins were insoluble. 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.

D. melanogaster full-length MFE-2 structure**CONCLUDING REMARKS**

The *D. melanogaster* MFE-2 is a peroxisomal protein active in the β -oxidation of fatty acyl-CoAs; (i) its domains show amino acid sequence as well as (ii) the fold similarities to those of other MFE-2s, (iii) *Dm*MFE-2 complements functionally the *S. cerevisiae* peroxisomal MFE-2 *in vivo*, and (iv) the recombinant domains display the predicted catalytic activities *in vitro*. The assembly of domains renders *Dm*MFE-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. Negative surface charge distribution is also not extensive enough to convincingly support the hypothesis of an electrostatic substrate channelling mechanism. On the contrary, forceful evidence against the presence of substrate channelling in *Dm*MFE-2 comes from enzyme kinetic data. The turnover number and the 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 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 else than efficiency, such as the protection of an intermediate.

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ACCESSION NUMBERS

Atomic coordinates and structure factors for the crystal structure of *Dm*MFE-2 have been deposited in the Protein Data Bank under the ID code 3OML.

ACKNOWLEDGEMENTS

We thank Päivi Pirilä, Tuula Kurvinen and Marja Lajunen for synthesizing the substrates. Biochemistry student Henri Urpilainen is acknowledged for preparing the plasmid constructs for the separate dehydrogenase and hydratase enzymes of *Dm*MFE-2 during a laboratory rotation study. The European Synchrotron Radiation Facility in Grenoble is acknowledged for beamtime on the beamline ID29. This research was carried out on grants from the Academy of Finland to T.G. and Sigrid Jusélius Foundation to K.H.

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D. melanogaster full-length MFE-2 structure

REFERENCES

- 1 Poirier, Y., Antonenkov, V. D., Glumoff, T. and Hiltunen, J. K. (2006) Peroxisomal beta-oxidation- a metabolic pathway with multiple functions. *Biochim. Biophys. Acta.* **1763**, 1413-1426
- 2 Hiltunen, J. K., Wenzel, B., Beyer, A., Erdmann, R., Fossa, A. and Kunau, W. H. (1992) Peroxisomal multifunctional beta-oxidation protein of *Saccharomyces cerevisiae*. Molecular analysis of the fox2 gene and gene product. *J. Biol. Chem.* **267**, 6646-6653
- 3 Adamski, J., Normand, T., Leenders, F., Monte, D., Begue, A., Stehelin, D., Jungblut, P. W. and de Launoit, Y. (1995) Molecular cloning of a novel widely expressed human 80 kDa 17 beta-hydroxysteroid dehydrogenase IV. *Biochem. J.* **311**, 437-443
- 4 Osumi, T. and Hashimoto, T. (1979) Peroxisomal beta oxidation system of rat liver. Copurification of enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase. *Biochem. Biophys. Res. Commun.* **89**, 580-584
- 5 Uchida, Y., Izai, K., Orii, T. and Hashimoto, T. (1992) Novel fatty acid beta-oxidation enzymes in rat liver mitochondria. II. Purification and properties of enoyl-coenzyme A (CoA) hydratase/3-hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase trifunctional protein. *J. Biol. Chem.* **267**, 1034-1041
- 6 Corton, J. C., Bocos, C., Moreno, E. S., Merritt, A., Marsman, D. S., Sausen, P. J., Cattley, R. C. and Gustafsson, J. A. (1996) Rat 17 beta-hydroxysteroid dehydrogenase type IV is a novel peroxisome proliferator-inducible gene. *Mol. Pharmacol.* **50**, 1157-1166
- 7 Qin, Y. M., Poutanen, M. H., Helander, H. M., Kvist, A. P., Siivari, K. M., Schmitz, W., Conzelmann, E., Hellman, U. and Hiltunen, J. K. (1997) Peroxisomal multifunctional enzyme of beta-oxidation metabolizing D-3-hydroxyacyl-CoA esters in rat liver: Molecular cloning, expression and characterization. *Biochem. J.* **321**, 21-28
- 8 Dieuaide-Noubhani, M., Novikov, D., Vandekerckhove, J., Veldhoven, P. P. and Mannaerts, G. P. (1997) Identification and characterization of the 2-enoyl-CoA hydratases involved in peroxisomal beta-oxidation in rat liver. *Biochem. J.* **321**, 253-259
- 9 Jiang, L. L., Kurosawa, T., Sato, M., Suzuki, Y. and Hashimoto, T. (1997) Physiological role of D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein. *J. Biochem.* **121**, 506-513
- 10 van Grunsven, E. G., van Berkel, E., Ijlst, L., Vreken, P., de Klerk, J. B., Adamski, J., Lemonde, H., Clayton, P. T., Cuebas, D. A. and Wanders, R. J. (1998) Peroxisomal D-hydroxyacyl-CoA dehydrogenase deficiency: Resolution of the enzyme defect and its molecular basis in bifunctional protein deficiency. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 2128-2133
- 11 Jörnvall, H., Persson, B., Krook, M., Atrian, S., Gonzalez-Duarte, R., Jeffery, J. and Ghosh, D. (1995) Short-chain dehydrogenases/reductases (SDR). *Biochemistry.* **34**, 6003-6013
- 12 Haapalainen, A. M., Koski, M. K., Qin, Y. M., Hiltunen, J. K. and Glumoff, T. (2003) Binary structure of the two-domain (3R)-hydroxyacyl-CoA dehydrogenase from rat peroxisomal multifunctional enzyme type 2 at 2.38 Å resolution. *Structure.* **11**, 87-97
- 13 Qin, Y. M., Marttila, M. S., Haapalainen, A. M., Siivari, K. M., Glumoff, T. and Hiltunen, J. K. (1999) Yeast peroxisomal multifunctional enzyme: (3R)-hydroxyacyl-CoA dehydrogenase domains A and B are required for optimal growth on oleic acid. *J. Biol. Chem.* **274**, 28619-28625
- 14 Ylianttila, M. S., Pursiainen, N. V., Haapalainen, A. M., Juffer, A. H., Poirier, Y., Hiltunen, J. K. and Glumoff, T. (2006) Crystal structure of yeast peroxisomal multifunctional enzyme: Structural basis for substrate specificity of (3R)-hydroxyacyl-CoA dehydrogenase units. *J. Mol. Biol.* **358**, 1286-1295

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D. melanogaster full-length MFE-2 structure

- 15 Koski, M. K., Haapalainen, A. M., Hiltunen, J. K. and Glumoff, T. (2004) A two-domain structure of one subunit explains unique features of eukaryotic hydratase 2. *J. Biol. Chem.* **279**, 24666-24672
- 16 Koski, M. K., Haapalainen, A. M., Hiltunen, J. K. and Glumoff, T. (2005) Crystal structure of 2-enoyl-CoA hydratase 2 from human peroxisomal multifunctional enzyme type 2. *J. Mol. Biol.* **345**, 1157-1169
- 17 Engel, C. K., Kiema, T. R., Hiltunen, J. K. and Wierenga, R. K. (1998) The crystal structure of enoyl-CoA hydratase complexed with octanoyl-CoA reveals the structural adaptations required for binding of a long chain fatty acid-CoA molecule. *J. Mol. Biol.* **275**, 847-859
- 18 Kasaragod, P., Venkatesan, R., Kiema, T. R., Hiltunen, J. K. and Wierenga, R. K. (2010) Crystal structure of liganded rat peroxisomal multifunctional enzyme type 1: A flexible molecule with two interconnected active sites. *J. Biol. Chem.* **285**, 24089-24098
- 19 Hisano, T., Tsuge, T., Fukui, T., Iwata, T., Miki, K. and Doi, Y. (2003) Crystal structure of the (R)-specific enoyl-CoA hydratase from *Aeromonas caviae* involved in polyhydroxyalkanoate biosynthesis. *J. Biol. Chem.* **278**, 617-624
- 20 Haapalainen, A. M., van Aalten, D. M., Meriläinen, G., Jalonen, J. E., Pirilä, P., Wierenga, R. K., Hiltunen, J. K. and Glumoff, T. (2001) Crystal structure of the liganded SCP-2-like domain of human peroxisomal multifunctional enzyme type 2 at 1.75 Å resolution. *J. Mol. Biol.* **313**, 1127-1138
- 21 Weeks, A., Lund, L. and Raushel, F. M. (2006) Tunneling of intermediates in enzyme-catalyzed reactions. *Curr. Opin. Chem. Biol.* **10**, 465-472
- 22 Ishikawa, M., Tsuchiya, D., Oyama, T., Tsunaka, Y. and Morikawa, K. (2004) Structural basis for channelling mechanism of a fatty acid beta-oxidation multienzyme complex. *EMBO J.* **23**, 2745-2754
- 23 Alanen, H. I., Williamson, R. A., Howard, M. J., Lappi, A. K., Jantti, H. P., Rautio, S. M., Kellokumpu, S. and Ruddock, L. W. (2003) Functional characterization of ERp18, a new endoplasmic reticulum-located thioredoxin superfamily member. *J. Biol. Chem.* **278**, 28912-28920
- 24 Krause, M., Ukkonen, K., Haataja, T., Ruottinen, M., Glumoff, T., Neubauer, A., Neubauer, P. and Vasala, A. (2010) A novel fed-batch based cultivation method provides high cell-density and improves yield of soluble recombinant proteins in shaken cultures. *Microb. Cell. Fact.* **9**, 11
- 25 Filppula, S. A., Sormunen, R. T., Hartig, A., Kunau, W. H. and Hiltunen, J. K. (1995) Changing stereochemistry for a metabolic pathway in vivo. Experiments with the peroxisomal beta-oxidation in yeast. *J. Biol. Chem.* **270**, 27453-27457
- 26 Chen, D. C., Yang, B. C. and Kuo, T. T. (1992) One-step transformation of yeast in stationary phase. *Curr. Genet.* **21**, 83-84
- 27 Hiltunen, J. K., Palosaari, P. M. and Kunau, W. H. (1989) Epimerization of 3-hydroxyacyl-CoA esters in rat liver. Involvement of two 2-enoyl-CoA hydratases. *J. Biol. Chem.* **264**, 13536-13540
- 28 Jancarik, J. and Kim, S.-H. (1991) Sparse matrix sampling: A screening method for crystallization of proteins. *J. App. Cryst.* **24**, 409-411
- 29 Kabsch, W. (2010) Xds. *Acta Crystallogr. D Biol. Crystallogr.* **66**, 125-132
- 30 McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. and Read, R. J. (2007) Phaser crystallographic software. *J. Appl. Crystallogr.* **40**, 658-674
- 31 Stein, N. (2008) CHAINSAW: A program for mutating pdb files used as templates in molecular replacement. *Journal of Applied Crystallography.* **41**, 641-643
- 32 Emsley, P., Lohkamp, B., Scott, W. G. and Cowtan, K. (2010) Features and development of coot. *Acta Crystallogr. D Biol. Crystallogr.* **66**, 486-501

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D. melanogaster full-length MFE-2 structure

- 33 Adams, P. D., Afonine, P. V., Bunkoczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L. W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., Richardson, J. S., Terwilliger, T. C. and Zwart, P. H. (2010) PHENIX: A comprehensive python-based system for macromolecular structure solution. *Acta Crystallogr. D Biol. Crystallogr.* **66**, 213-221
- 34 Murshudov, G. N., Vagin, A. A. and Dodson, E. J. (1997) Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr. D Biol. Crystallogr.* **53**, 240-255
- 35 Winn, M. D., Isupov, M. N. and Murshudov, G. N. (2001) Use of TLS parameters to model anisotropic displacements in macromolecular refinement. *Acta Crystallogr. D Biol. Crystallogr.* **57**, 122-133
- 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) MolProbity: All-atom contacts and structure validation for proteins and nucleic acids. *Nucleic Acids Res.* **35**, W375-83
- 37 Qin, Y. M., Haapalainen, A. M., Kilpeläinen, S. H., Marttila, M. S., Koski, M. K., Glumoff, T., Novikov, D. K. and Hiltunen, J. K. (2000) Human peroxisomal multifunctional enzyme type 2. Site-directed mutagenesis studies show the importance of two protic residues for 2-enoyl-CoA hydratase 2 activity. *J. Biol. Chem.* **275**, 4965-4972
- 38 Leesong, M., Henderson, B. S., Gillig, J. R., Schwab, J. M. and Smith, J. L. (1996) Structure of a dehydratase-isomerase from the bacterial pathway for biosynthesis of unsaturated fatty acids: Two catalytic activities in one active site. *Structure.* **4**, 253-264
- 39 Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. (1997) The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25**, 4876-4882
- 40 Laskowski, R. A., MacArthur, M. W., Moss, D. S. and Thornton, J. M. (1993) PROCHECK: A program to check the stereochemical quality of protein structures. *J. App. Cryst.* **26**, 283-291
- 41 Knighton, D. R., Kan, C. C., Howland, E., Janson, C. A., Hostomska, Z., Welsh, K. M. and Matthews, D. A. (1994) Structure of and kinetic channelling in bifunctional dihydrofolate reductase-thymidylate synthase. *Nat. Struct. Biol.* **1**, 186-194

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D. melanogaster full-length MFE-2 structure**Table 1** Kinetic parameters for the full-length *Dm*MFE-2 protein in comparison with the values obtained using the dehydrogenase (DH) and the hydratase 2 (H2) domains of *Dm*MFE-2 as separate monofunctional enzymes in a 1:1 ratio

C4, C6 and C10 stand for 2E-butenoyl-CoA, 2E-hexenoyl-CoA and 2E-decenoyl-CoA, respectively.

	Substrate		
	C4	C6	C10
<u>Full-length <i>Dm</i>MFE-2</u>			
V_{\max} ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	1.07 ± 0.3	15.0 ± 1.5	31.4 ± 0.5
K_M (μM)	85.3 ± 38.5	66.7 ± 11.8	1.14 ± 0.06
k_{cat} (s^{-1})	0.38	210	1100
k_{cat}/K_M	4.46×10^{-3}	3.2	970
<u>DH + H2</u>			
V_{\max} ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	0.36 ± 0.03	9.6 ± 2.6	56.0 ± 2.1
K_M (μM)	83.5 ± 15.1	70.7 ± 33.0	1.20 ± 0.15
k_{cat} (s^{-1})	7.5	200	1170
k_{cat}/K_M	9.0×10^{-2}	2.8	980

Abbreviations used: CoA, coenzyme A; *Dm*MFE-2, *Drosophila melanogaster* multifunctional enzyme type 2; DH, 3R-hydroxyacyl-CoA dehydrogenase; H2, 2E-Enoyl-CoA hydratase 2; SCP-2L, sterol carrier protein-2-like; NAD⁺, nicotinamide adenine dinucleotide; IPTG, isopropyl β -D-1-thiogalactopyranoside; C4, 2E-butenoyl-CoA; C6, 2E-hexenoyl-CoA; C10, 2E-decenoyl-CoA.

D. melanogaster full-length MFE-2 structure**Table 2** Data collection, refinement statistics and quality of the structure

^aValues in parentheses refer to the highest resolution shell. ^bValue in parentheses refer to the percentage of reflections taken randomly for the test calculations. ^cThe non-hydrogen atoms used in the refinement. ^dFull B-factors after applying TLS refinement in Refmac are given in accordance with the procedure required by the RCSB Protein Data Bank (PDB).

Beamline	ID29, ESRF, Grenoble, France
Data set	<i>Dm</i> MFE-2
<i>Data collection statistics</i>	
Wavelength (Å)	0.9310
Temperature (K)	100
Rotation range (°)	90
Space group	<i>P</i> 4 ₃ 2 ₁ 2
<i>a</i> (Å)	114.48
<i>b</i> (Å)	114.48
<i>c</i> (Å)	89.11
Resolution range (Å) ^a	28.5 - 2.15 (2.21 - 2.15)
Crystal mosaicity (°)	0.61
Hkl (observed/unique)	230188/32494
Redundancy	7.1
Completeness (%) ^a	99.4 (96.7)
Overall I/σI ^a	22.9 (3.0)
R _{merge} ^a	4.7 (63.1)
<i>Refinement statistics</i>	
R _{free} ^b	28.5 (5%)
R _{work}	23.4
Number of residues ^c	532
Number of atoms ^c	4173
Protein ^c	4035
Water ^c	138
<i>Geometry statistics</i>	
Root mean square deviations	
Lengths (Å)/angles (°)	0.011/1.20
Average B-factors ^d	
All atoms (Å ²)	65.8
Main chain atoms (Å ²)	68.8
Side chain atoms (Å ²)	62.8
Water molecules (Å ²)	52.9
Ramachandran plot	
Favored region (%)	97.3
Allowed regions (%)	2.7
Disallowed regions (%)	0.0

Abbreviations used: CoA, coenzyme A; *Dm*MFE-2, *Drosophila melanogaster* multifunctional enzyme type 2; DH, 3R-hydroxyacyl-CoA dehydrogenase; H2, 2E-Enoyl-CoA hydratase 2; SCP-2L, sterol carrier protein-2-like; NAD⁺, nicotinamide adenine dinucleotide; IPTG, isopropyl β-D-1-thiogalactopyranoside; C4, 2E-butenoyl-CoA; C6, 2E-hexenoyl-CoA; C10, 2E-decenoyl-CoA.

D. melanogaster full-length MFE-2 structure

FIGURE LEGENDS

Figure 1 Schematic view of β -oxidation cycle of an acyl-CoA molecule in the peroxisomes

The first step is FAD-dependent dehydrogenation that results to a carbon double bond at the Δ^2 -position. The formed 2E-enoyl-CoA intermediate becomes hydrated in the second step which is followed by nicotinamide adenine dinucleotide (NAD⁺)-dependent dehydrogenation that converts the intermediate to 3-ketoacyl-CoA. In the final step, the original carbon chain becomes shortened by two carbon atoms and acetyl-CoA is formed. The shortened substrate enters another degradative cycle. MFE-2 is responsible for the second and third steps of the cycle.

Figure 2 Comparison of peroxisomal multifunctional enzyme type 2s from various species

The figure demonstrates the versatility of the domain organization between MFE-2s from the selected species. Black colour corresponds to the 3R-hydroxyacyl-CoA dehydrogenase domain, vertical black stripes 2E-enoyl-CoA hydratase 2 domain and the sterol carrier protein 2-like domain (SCP-2L) is coloured in white. Human, rat and zebra fish MFE-2 share the same domain composition and organization. *Drosophila* MFE-2 is lacking the SCP-2L domain. Nematodes and yeasts 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, while 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 that has evolved altered substrate specificities in the two domains [13]. The N-terminal dehydrogenase domain displays the highest activity towards medium and long chain 3R-hydroxyacyl-CoAs and the middle dehydrogenase domain shows the preference towards the short chain (C4) substrates [13].

Figure 3 Activity of *Dm*MFE-2 *in vivo* and *in vitro*

(A) Rescue experiments with *Dm*MFE-2 in yeast mutant devoid of endogenous MFE-2. Yeast dilution series were prepared on YP-oleic acid plates (0.125 % oleic acid, 0.2 % D+ Glucose). 1. $\Delta fox2$ (halo-), 2. $\Delta fox2$ + pYE352::CTAI (halo-), 3. wt (halo+), 4. $\Delta fox2$ + pYE352::ScFOX2 (halo+), 5. $\Delta fox2$ + pYE352::DmMFE-2 (halo+). Samples 1. and 2. do not display development of halo due to the absence of a functional *fox2* gene. Samples 3. and 4. serve as positive controls in the experiment and demonstrate clearance of the oleic acid around the yeast cells. The *Dm*MFE-2 (Sample 5.) complements well the yeast null mutant strain. (B and C) Michaelis-Menten saturation curves and Lineweaver-Burk plots (inserts) of kinetic data for the full-length *Dm*MFE-2 (B) and for 1:1 mixture of the dehydrogenase domain and the hydratase 2 domain of *Dm*MFE-2 in stoichiometric ratio (C). 2E-decenoyl-CoA was used as the substrate, while the formation of the magnesium complex of 3-ketodecanoyl-CoA was monitored spectrophotometrically at 303 nm. GraFit 5 was used for fitting the data.

Abbreviations used: CoA, coenzyme A; *Dm*MFE-2, *Drosophila melanogaster* multifunctional enzyme type 2; DH, 3R-hydroxyacyl-CoA dehydrogenase; H2, 2E-Enoyl-CoA hydratase 2; SCP-2L, sterol carrier protein-2-like; NAD⁺, nicotinamide adenine dinucleotide; IPTG, isopropyl β -D-1-thiogalactopyranoside; C4, 2E-butenoyl-CoA; C6, 2E-hexenoyl-CoA; C10, 2E-decenoyl-CoA.

D. melanogaster full-length MFE-2 structure**Figure 4** The overall fold of *Dm*MFE-2 monomer offering a close up view into the dehydrogenase and hydratase 2 domain border

The C-terminal subdomain of the dehydrogenase (A; in the middle) and the hydratase 2 domain (A; on the bottom) share only a few contacts as indicated in (B) in stereo, and in the text.

Figure 5 Domain organization in the *Dm*MFE-2 dimer

(A) Standard view of the enzyme revealing the dehydrogenase and hydratase subdimers that are connected via short linkers (black arrows). The Rossmann fold region has been coloured in marine blue and C-terminal subdomain of the dehydrogenase subunit is in aquamarine. Lime green and dark gray colours represent the N- and C-terminal hot dog fold in the hydratase dimer. The central α -helix bundle in the hydratase dimer interface and the remaining loop regions are coloured in pale gray. (B) Surface presentation of the MFE-2 dimer in the same orientation as in (A). Gray and aquamarine are highlighting the crosswise dimerization of the two monomers. (C) Side view of the hourglass-shaped *Dm*MFE-2 dimer. The colouring corresponds to the colouring in (B) and the molecule has been turned 90° clockwise from the standard view of the protein. (D) Surface presentation of *Dm*MFE-2 dimer and the positively charged surface residues. The two polypeptides forming the dimer have been coloured in pale cyan and gray, and the dimeric dehydrogenase and hydratase 2 enzymes denoted by DH and H2, respectively. Of the positively charged residues (Lys, Arg and His), the conserved residues among MFE-2 family have been coloured in marine blue and the rest have been coloured in dark grey. The 3R-hydroxydecanoyl-CoA ligands have been positioned to the active sites by superimposing the liganded hydratase 2 domain structure of MFE-2 [13] and the proposed ligand-containing structure of dehydrogenase of yeast MFE-2 [14]. When viewing the molecule from the front a positively charged surface patch could be delineated leading from the hydratase active site (lower right corner, on the back of the enzyme) directly to the dehydrogenase CoA-binding site on the right side upwards from the central opening.

Figure 6 Comparison of the dehydrogenase and hydratase domains with known structures of separate MFE-2 domains

(A) Stereo view of the *Drosophila* dehydrogenase monomer of MFE-2 superimposed with rat dehydrogenase monomer of MFE-2 (1GZ6) that is in complex with NAD⁺. The *Drosophila* dehydrogenase monomer is coloured in marine blue and the rat dehydrogenase monomer in silver. Black arrows point out locations of the flexible loops, which are absent in the *Dm*MFE-2 structure. The N- and C-termini have been indicated in the figure as well. (B) Stereo view of the *Drosophila* 2E-enoyl-CoA hydratase 2 dimer of MFE-2 superimposed with the homologous hydratase 2-dimer from the yeast *C. tropicalis* (1PN4) and hydratase 2 from human MFE-2 (1S9C). 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, human and yeast counterparts are coloured in pale cyan and in silver, respectively.

Abbreviations used: CoA, coenzyme A; *Dm*MFE-2, *Drosophila melanogaster* multifunctional enzyme type 2; DH, 3R-hydroxyacyl-CoA dehydrogenase; H2, 2E-Enoyl-CoA hydratase 2; SCP-2L, sterol carrier protein-2-like; NAD⁺, nicotinamide adenine dinucleotide; IPTG, isopropyl β -D-1-thiogalactopyranoside; C4, 2E-butenoyl-CoA; C6, 2E-hexenoyl-CoA; C10, 2E-decenoyl-CoA.

D. melanogaster full-length MFE-2 structure**Figure 7 Structural comparison of known R-hydratase structures with the hydratase domain of *Dm*MFE-2**

(A) The homodimeric R-hydratase from *A. caviae* (1IQ6) consists of two 14 kDa subunits. (B) Hydratase 2 domain of *Dm*MFE-2. The black arrow points out the front loop. Helix $\alpha 12$ is also labelled. The monomeric domain has molecular mass of 30.8 kDa. (C) Yeast *C. tropicalis* hydratase 2 domain of MFE-2 (1PN4) in complex with 3R-hydroxydecanoyl-CoA. The hydratase monomer has a molecular mass of 31.5 kDa. While the interior of the bacterial hydratase is completely filled, there are different amount 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, while the bacterial hydratase possesses two catalytic sites.

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

The *Drosophila melanogaster* MFE-2 has been aligned using ClustalX [39] together with human (*Hs*MFE-2), rat (*Rn*MFE-2), zebra fish (*Dr*MFE-2) and yeast *C. tropicalis* 3R-hydroxyacyl-CoA dehydrogenase domain B and 2E-Enoyl-CoA hydratase 2 domain fragment (*Ct*DhB+H2). The N-terminal 3R-hydroxyacyl-CoA dehydrogenase domain A of *C. tropicalis* MFE-2 has been left out from the comparison for clarity. The UniProtKB identifiers of the selected sequences are Q9VXJ0, P51659, P97852, Q8AYH1 and P22414, respectively. The secondary structure elements have been highlighted above the sequences with bars and arrows. The rectangle box is pointing out the linker region between the catalytic domains. The black vertical arrows indicate the location of residues that are essential for the enzyme catalysis [12,15]. Complete identity or similarity of residues between the sequences has been indicated with black shade, while decreasing greyness is indicating regions with four or three matching residues.

Figure S2 Investigation of heterogenic protein complex formation *in vitro* using static light scattering (SLS) technique

The formation of protein complex between the dimers of catalytic domains of *Dm*MFE-2 was studied using multi-angle light scattering device, miniDAWN TREOS (Wyatt Technology Corp.). The curves in the chromatogram represent single injection each. Red colour is indicating the full-length *Dm*MFE-2, blue colour 3R-hydroxyacyl-CoA dehydrogenase domain of *Dm*MFE-2, green colour 2E-Enoyl-CoA hydratase 2 domain of *Dm*MFE-2 and black colour the both catalytic domains as separate polypeptides in the same injection. The data demonstrate that the *Dm*MFE-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 provided conditions the *Dm*MFE-2 dimer has a molecular mass of 122 kDa, the dehydrogenase dimer 62 kDa, the hydratase 2 dimer 69 kDa and the mixture of the two dimers 65 kDa. With the exception of the mixture sample, this is in agreement with the theoretical values when taking into account the monodispersity of each of the samples.

Abbreviations used: CoA, coenzyme A; *Dm*MFE-2, *Drosophila melanogaster* multifunctional enzyme type 2; DH, 3R-hydroxyacyl-CoA dehydrogenase; H2, 2E-Enoyl-CoA hydratase 2; SCP-2L, sterol carrier protein-2-like; NAD⁺, nicotinamide adenine dinucleotide; IPTG, isopropyl β -D-1-thiogalactopyranoside; C4, 2E-butenoyl-CoA; C6, 2E-hexenoyl-CoA; C10, 2E-decenoyl-CoA.

D. melanogaster full-length MFE-2 structure

ARTWORK

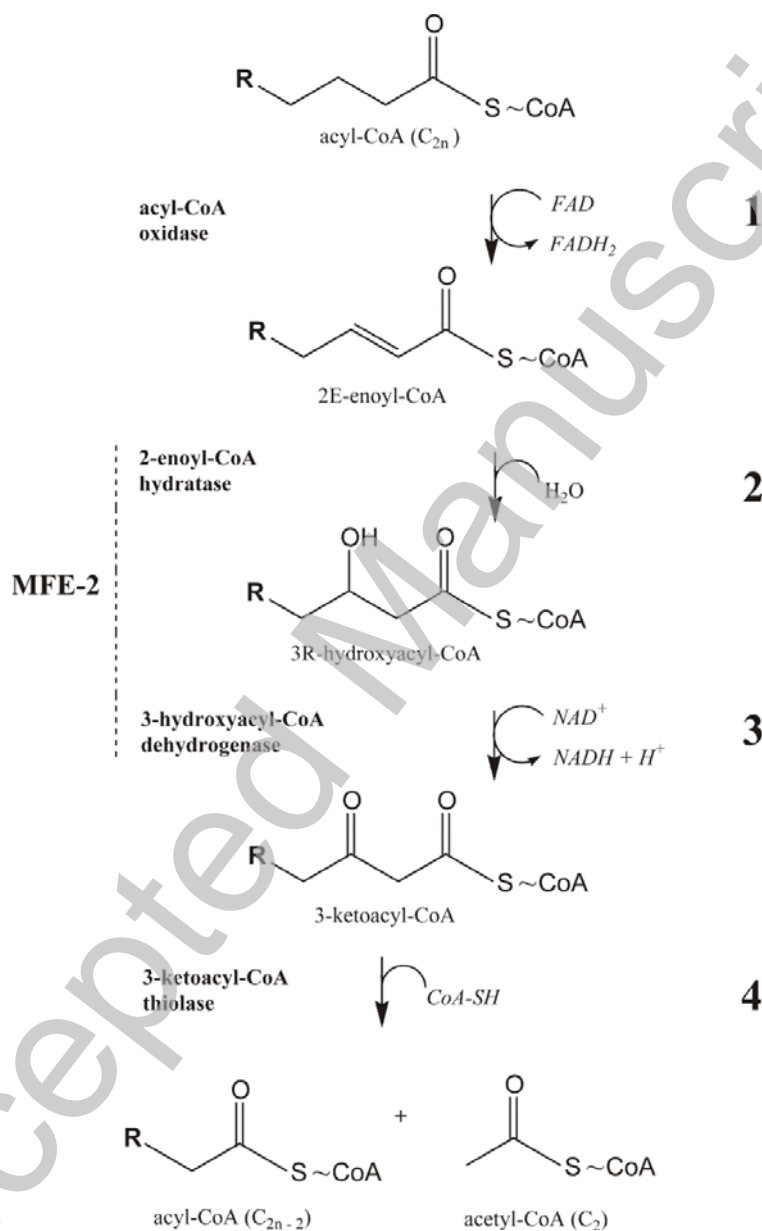


Figure 1

Abbreviations used: CoA, coenzyme A; DmMFE-2, *Drosophila melanogaster* multifunctional enzyme type 2; DH, 3R-hydroxyacyl-CoA dehydrogenase; H₂, 2E-Enoyl-CoA hydratase 2; SCP-2L, sterol carrier protein-2-like; NAD⁺, nicotinamide adenine dinucleotide; IPTG, isopropyl β-D-1-thiogalactopyranoside; C4, 2E-butenoyl-CoA; C6, 2E-hexenoyl-CoA; C10, 2E-decenoyl-CoA.

D. melanogaster full-length MFE-2 structure

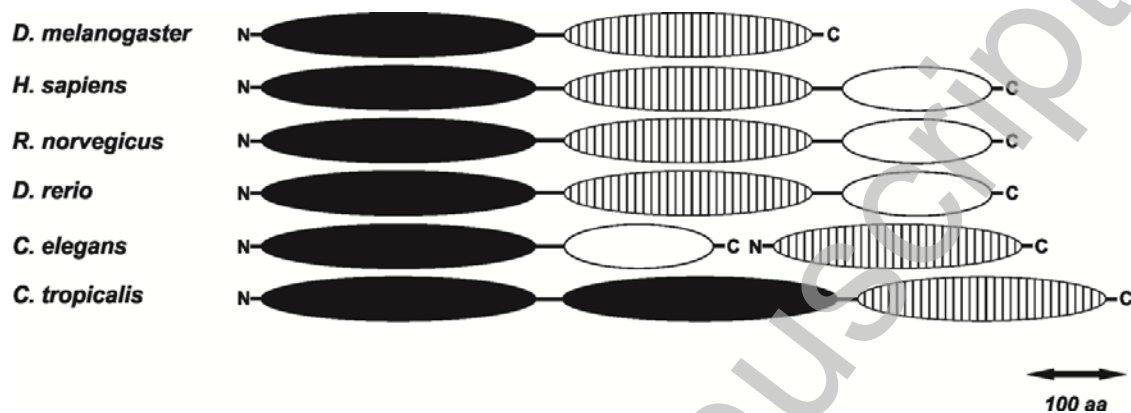


Figure 2

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Abbreviations used: CoA, coenzyme A; *DmMFE-2*, *Drosophila melanogaster* multifunctional enzyme type 2; DH, 3R-hydroxyacyl-CoA dehydrogenase; H2, 2E-Enoyl-CoA hydratase 2; SCP-2L, sterol carrier protein-2-like; NAD⁺, nicotinamide adenine dinucleotide; IPTG, isopropyl β-D-1-thiogalactopyranoside; C4, 2E-butenoyl-CoA; C6, 2E-hexenoyl-CoA; C10, 2E-decenoyl-CoA.

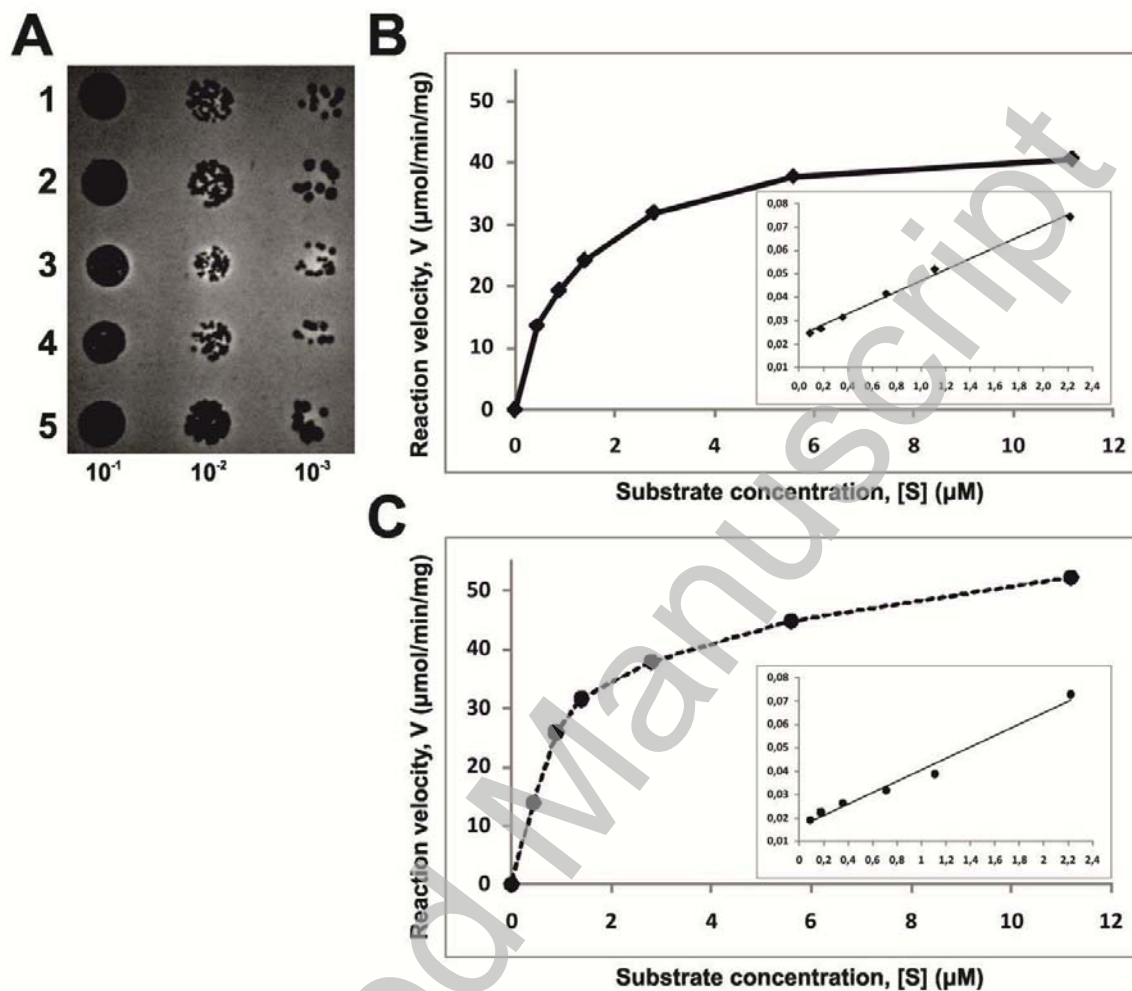
D. melanogaster full-length MFE-2 structure

Figure 3

Abbreviations used: CoA, coenzyme A; *DmMFE-2*, *Drosophila melanogaster* multifunctional enzyme type 2; DH, 3R-hydroxyacyl-CoA dehydrogenase; H2, 2E-Enoyl-CoA hydratase 2; SCP-2L, sterol carrier protein-2-like; NAD⁺, nicotinamide adenine dinucleotide; IPTG, isopropyl β -D-1-thiogalactopyranoside; C4, 2E-butenoyl-CoA; C6, 2E-hexenoyl-CoA; C10, 2E-decenoyl-CoA.

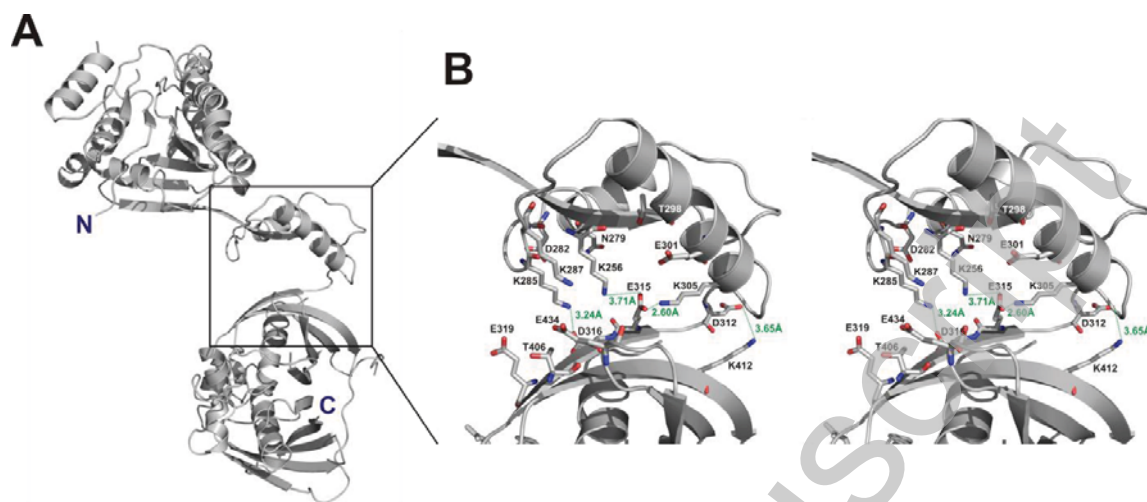
D. melanogaster full-length MFE-2 structure

Figure 4

Abbreviations used: CoA, coenzyme A; *DmMFE-2*, *Drosophila melanogaster* multifunctional enzyme type 2; DH, 3R-hydroxyacyl-CoA dehydrogenase; H2, 2E-Enoyl-CoA hydratase 2; SCP-2L, sterol carrier protein-2-like; NAD⁺, nicotinamide adenine dinucleotide; IPTG, isopropyl β-D-1-thiogalactopyranoside; C4, 2E-butenoyl-CoA; C6, 2E-hexenoyl-CoA; C10, 2E-decenoyl-CoA.

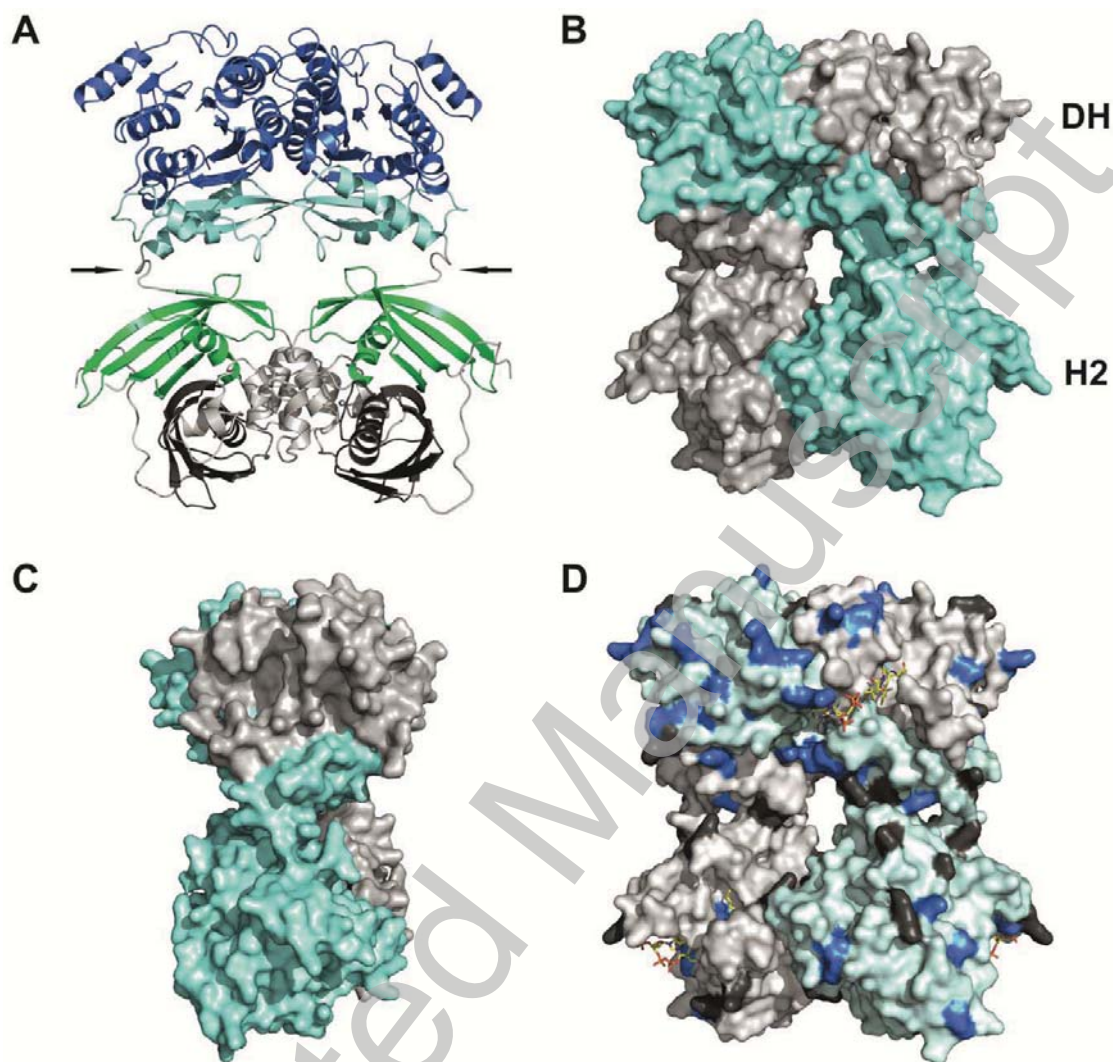
D. melanogaster full-length MFE-2 structure

Figure 5

Abbreviations used: CoA, coenzyme A; *DmMFE-2*, *Drosophila melanogaster* multifunctional enzyme type 2; DH, 3R-hydroxyacyl-CoA dehydrogenase; H2, 2E-Enoyl-CoA hydratase 2; SCP-2L, sterol carrier protein-2-like; NAD⁺, nicotinamide adenine dinucleotide; IPTG, isopropyl β-D-1-thiogalactopyranoside; C4, 2E-butenoyl-CoA; C6, 2E-hexenoyl-CoA; C10, 2E-decenoyl-CoA.

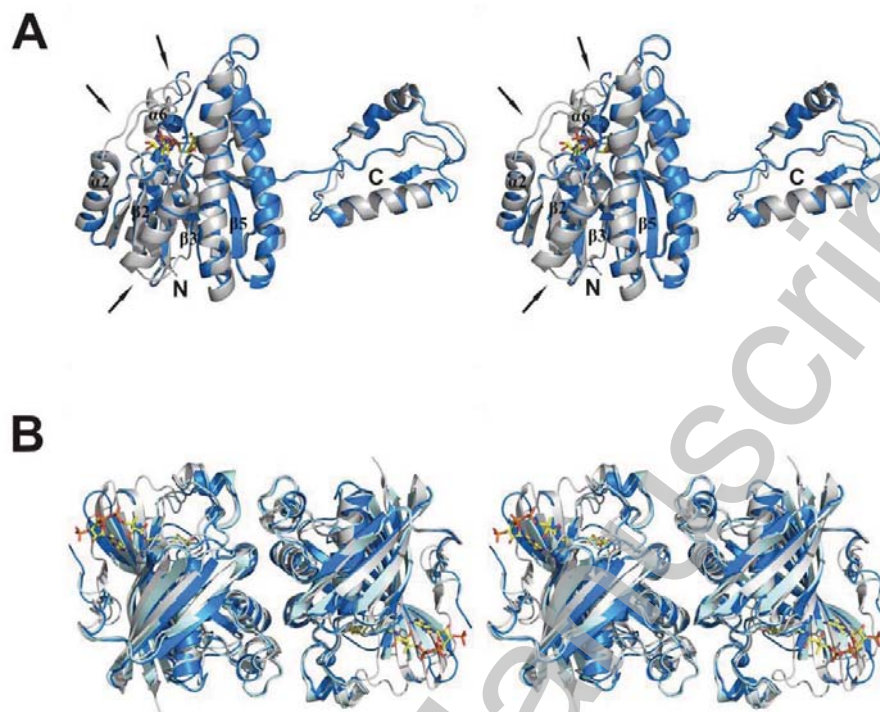
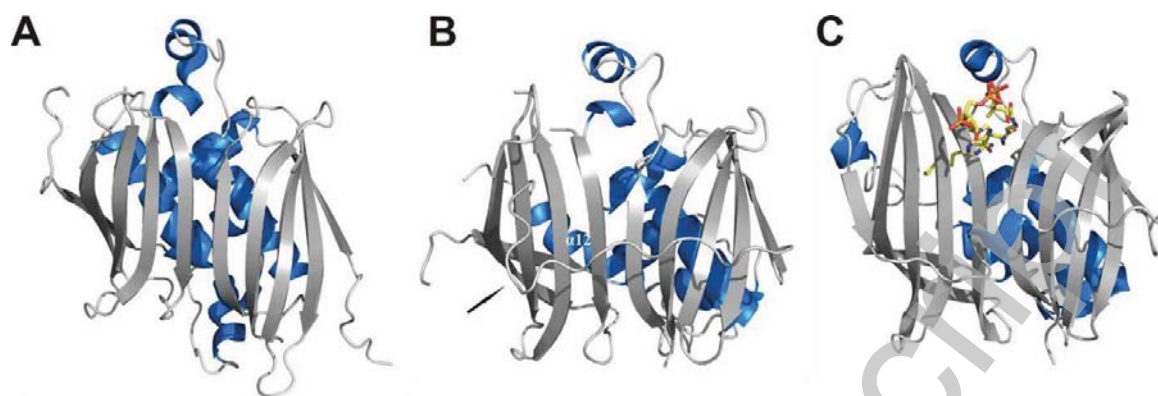
D. melanogaster full-length MFE-2 structure

Figure 6

Abbreviations used: CoA, coenzyme A; *DmMFE-2*, *Drosophila melanogaster* multifunctional enzyme type 2; DH, 3R-hydroxyacyl-CoA dehydrogenase; H2, 2E-Enoyl-CoA hydratase 2; SCP-2L, sterol carrier protein-2-like; NAD⁺, nicotinamide adenine dinucleotide; IPTG, isopropyl β -D-1-thiogalactopyranoside; C4, 2E-butenoyl-CoA; C6, 2E-hexenoyl-CoA; C10, 2E-decenoyl-CoA.

D. melanogaster full-length MFE-2 structure**Figure 7**

Abbreviations used: CoA, coenzyme A; *DmMFE-2*, *Drosophila melanogaster* multifunctional enzyme type 2; DH, 3R-hydroxyacyl-CoA dehydrogenase; H2, 2E-Enoyl-CoA hydratase 2; SCP-2L, sterol carrier protein-2-like; NAD⁺, nicotinamide adenine dinucleotide; IPTG, isopropyl β-D-1-thiogalactopyranoside; C4, 2E-butenoyl-CoA; C6, 2E-hexenoyl-CoA; C10, 2E-decenoyl-CoA.