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Crystallization and characterization of the dehydrogenase domain from rat peroxisomal multifunctional enzyme type 1

Jukka P. Taskinen,[a] Tiila-Riikka Kiema,[a] Kari T. Koivuranta,[a,b] Rik K. Wierenga[a] and J. Kalervo Hiltunen[a]*

[a]Biocenter Oulu and Department of Biochemistry, University of Oulu, PO Box 3000, FIN-90014, Finland, and [b]VTT Biotechnology, PO Box 1500, FIN-02044 VTT, Finland

Correspondence e-mail: kalervo.hiltunen@oulu.fi

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Peroxisomal multifunctional enzyme type 1 from rat (perMFE-1) is a monomeric multidomain protein shown to have 2-enoyl-CoA hydratase/Δ^3-Δ^2-enoyl-CoA isomerase and (3*S*)-hydroxyacyl-CoA dehydrogenase domains followed by a C-terminal extension of 130 amino acids with unknown function apart from being a carrier of the peroxisomal targeting signal type 1. The truncated perMFE-1 without the N-terminal hydratase/isomerase domain (perMFE-1DH; residues 260–722) was overexpressed as an enzymatically active recombinant protein, purified and characterized. Using (3*S*)-hydroxydecanoyl-CoA as a substrate, the specific enzymatic activity of perMFE-1DH was determined to be 2.2 µmol min^{-1} mg^{-1}, comparable with that of perMFE-1 purified from rat liver (2.8 µmol min^{-1} mg^{-1}). The protein was crystallized in the apo form by the hanging-drop method and a complete data set to 2.45 Å resolution was collected using a rotating-anode X-ray source. The crystals have primitive tetragonal symmetry, with unit-cell parameters $a = b = 125.9$, $c = 60.2$ Å.

1. Introduction

All known fatty-acid β-oxidation pathways are found to have a multifunctional enzyme (MFE) consisting of multidomain polypeptides catalysing (i) the hydration of *trans*-2-enoyl-CoA to 3-hydroxyacyl-CoA and (ii) the NAD$^+$-dependent oxidation of the resultant 3-hydroxyacyl-CoA to 3-ketoacyl-CoA (Palosaari & Hiltunen, 1990; Reddy & Hashimoto, 2001). Depending on the species or the subcellular origin of the protein, the MFEs can also catalyse reactions such as Δ^3-Δ^2-enoyl-CoA isomerization and 3-hydroxyacyl-CoA epimerization and are sometimes associated with a subunit having 3-ketoacyl-CoA thiolase activity (Uchida *et al.*, 1992; Yang & Elzinga, 1993). The domains giving rise to enzyme activities in MFEs are often related in sequence to their monofunctional counterparts (Kamijo *et al.*, 1993; Qin *et al.*, 1997, 2000; Hiltunen & Qin, 2000).

The β-oxidation MFEs can be grouped into the non-homologous classes MFE type 1 and MFE type 2 (MFE-1 and MFE-2; Malila *et al.*, 1993; Jiang *et al.*, 1996). Members of the MFE-1 class convert *trans*-2-enoyl-CoA to 3-ketoacyl-CoA *via* (3*S*) intermediates, whereas members of the MFE-2 class utilize (3*R*) stereochemistry.

In mammals, MFE-1 has been found to be associated with the inner membrane of the mitochondria as well as with the peroxisomal matrix, whereas in plants and bacteria it is found in peroxisomes and cytosol, respectively. The mitochondrial MFE-1 is an $\alpha_4\beta_4$ hetero-octamer in which the α-subunits are MFE-1 monomers and the β-subunits are 3-ketoacyl-CoA thiolase monomers (Kamijo *et al.*, 1993). In peroxisomes the MFE-1 (perMFE-1) is a monomer, whereas its mitochondrial monofunctional homologues, *trans*-2-enoyl-CoA hydratase 1 (crotonase) and Δ^3-Δ^2-enoyl-CoA isomerase, form hexamers (Engel *et al.*, 1996) and (3*S*)-hydroxyacyl-CoA dehydrogenase occurs as a dimer (Furuta *et al.*, 1980; Barycki *et al.*, 1999). Rat perMFE-1 consists of 722 residues. Sequence similarity with the crotonase and (3*S*)-hydroxyacyl-CoA dehydrogenase families suggests that the hydratase activity is located at the N-terminus and the dehydrogenase activity is located in the middle of the polypeptide chain (Osumi & Hashimoto, 1979; Palosaari & Hiltunen, 1990). The perMFE-1 has a C-terminal extension of 130 amino acids that has a role in carrying a peroxisomal targeting signal type 1 (SKL), but is without other known functions.

So far, attempts to crystallize the perMFE-1 have failed and therefore trials for characterizing and crystallizing the domains separately have been initiated. The work described here shows that the dehydrogenase part together with the remaining C-terminal polypeptide from rat perMFE-1 can be produced in

bacteria as a catalytically active protein. Growth of well diffracting crystals of the protein is also reported.

2. Experimental methods

2.1. Overexpression and purification

The open reading frame of perMFE-1 was amplified using *Pfu* polymerase according to the manufacturer's instructions (Stratagen, La Jolla, CA, USA; Promega, Madison, WI, USA) using perMFE-1 cDNA (Filppula *et al.*, 1995) as a template and the primers 5′-CACACAATTGA**ATG**GCTGAGTATCTGAG (start codon in bold) and 5′-GCACTCAATTG**TCA**CAGTTTGCTGCCGT (stop codon in bold). The PCR product was subcloned into pUC18 vector applying a Sure-Clone ligation kit (Applied Biosystems, Foster city, CA), resulting in a pUC18::*MFE-1* vector which was subsequently used as a template for site-directed mutagenesis to introduce a 5′ *Nde*I restriction site (Quick-Change Site-directed Mutagenesis Kit, Stratagen) using the primer 5′-CCACACAATCAT**ATG**GCTGAG (*Nde*I restriction site underlined, start codon in bold) and a primer with a complementary sequence. The insert was digested out with *Nde*I and *Bam*HI and ligated to similarly digested pET3a (Novagen, Madison, WI, USA) resulting in the pET3a:per*MFE1*wt expression vector. An amino-terminally truncated MFE-1 (perMFE-1DH) was generated by introducing a new transcription start site embedded in the *Nde*I restriction site between nucleotides 798 and 799 applying the QuickChange mutagenesis kit using the primer 5′-GTTTATGTACCTCCGGCATA**TG**GCATCCGGGCAGGC (*Nde*I site underlined and the start codon in bold) and a primer with a complementary sequence, while pET3a::per*MFE-1*wt served as a template. The vector undergoing mutagenesis was digested with the *Nde*I restriction enzyme (New-England Biolabs, Beverly, MA) and religated, leading to deletion of the residues encoding amino-acid residues Met1–Arg259 of the perMFE-1. The resulting vector, pET3a::per*MFE-1DH*, coding for residues 260–722, was then transformed to *Escherichia coli* BL21(DE3)pLysS strain for overexpression.

A single colony was chosen and grown overnight in Luria–Bertani broth containing carbenicillin (50 µg ml^{-1}) and chloramphenicol (50 µg ml^{-1}). The culture was diluted 200-fold into 2 l of M9ZB medium (Studier *et al.*, 1990) and grown at 310 K until OD_{600} reached 0.8. Overexpression of rat perMFE-1 (3*S*)-hydroxyacyl-CoA dehydrogenase (perMFE-1DH) was initiated by adding isopropyl-β-D-thiogalactoside to 0.4 m*M* and was continued for 5 h at 303 K before harvesting the cells by centrifugation. Harvested cells were washed with PBS and stored at 203 K for later use.

The protein purification was started with thawed cells (approximately 5.0 g) that were suspended in cold lysis buffer (50 m*M* HEPES pH 7.6, 1 m*M* EDTA, 0.5 m*M* PMSF, 0.5 m*M* benzamidine–HCl, 0.5 m*M* DTT, 100 µg ml^{-1} lysozyme). The suspension was incubated for 15 min at 303 K and the cells were disrupted by sonication. The lysate was centrifuged (45 min, 35 000*g*, 277 K) to remove the debris and the soluble proteins were applied onto a DEAE-Sephacel column (2.5 × 20.0 cm) equilibrated with 50 m*M* HEPES pH 7.6, 1 m*M* EDTA (buffer *A*). The flowthrough containing perMFE-1DH was applied onto a 6 ml Resource S column (AP-Biotech) equilibrated with buffer *A*. The bound proteins were eluted with a linear NaCl gradient (0–1 *M*) over 150 ml. Fractions (1 ml) were collected from the eluate and measured for (3*S*)-hydroxyacyl-CoA dehydrogenase activity. The activity-containing fractions were pooled and subjected to dialysis against 50 m*M* HEPES pH 7.6, 50 m*M* NaCl, 1 m*M* EDTA (buffer *B*). The sample was loaded onto a POROS S column (PerSeptive Biosystems; bed volume 1.6 ml) equilibrated with buffer *B* and the bound proteins were eluted with a linear NaCl gradient (0–0.5 *M*). 1.0 ml fractions were collected to a total volume of 12 ml and assayed for enzyme activity. Fractions containing perMFE-1DH were pooled and concentrated using Vivapore 10/20 ml (Vivascience) concentrators and a 400 µl sample (1.5 mg protein) was loaded onto a SuperDex 75 HR (Amersham Pharmacia Biotech) column equilibrated with 50 m*M* tricine pH 7.6, 200 m*M* NaCl, 1.5 m*M* NaN_3. 300 µl fractions were collected from the eluate and analysed for dehydrogenase activity. SDS–PAGE analysis visualized a single band of 50.9 kDa indicating apparent homogeneity of the sample (Fig. 1). A total of 2.4 mg of pure protein was obtained from 2 l of cell culture.

The activity measurements for perMFE-1DH were performed at 295 K as described previously for wild-type MFE-1 by Palosaari & Hiltunen (1990). In brief, the assay mixture consisted of 50 m*M* KCl, 50 µg ml^{-1} bovine serum albumin and 1 m*M* NAD^+ in 50 m*M* Tris–HCl pH 9.0. To this assay mixture were added 30 ng ml^{-1} hydratase 1 and 60 µ*M* *trans*-2-decenoyl-CoA to

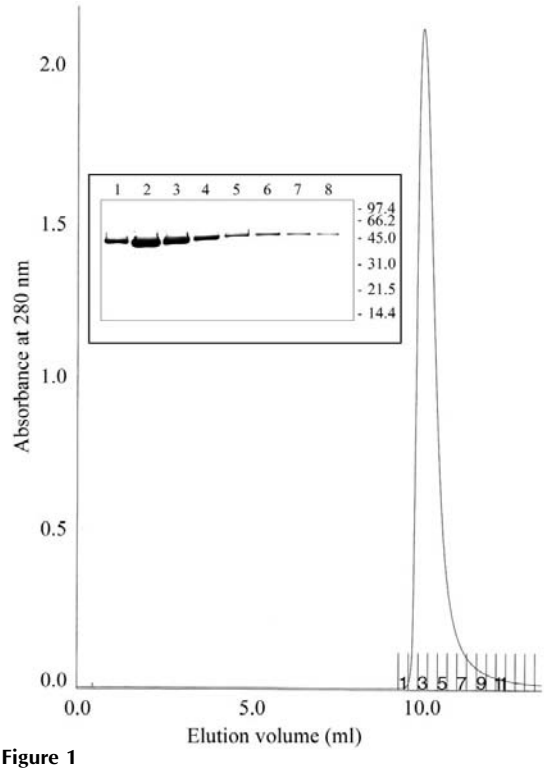

Figure 1
Size-exclusion chromatography and SDS–PAGE of rat perMFE-1DH. A sample containing 1.2 mg protein was applied onto a Superdex 75 HR column. The curve shows absorption at 280 nm in the eluate. The dehydrogenase activity profile co-eluted with the protein, peaking in fractions 1–8. The insert shows SDS–PAGE analysis of fractions 1–8. The molecular mass of perMFE-1DH is 50.9 kDa.

Table 1
Crystallization and preliminary X-ray analysis of the perMFE-1DH crystal.

Crystallizations were carried out at 293 K using the hanging-drop method.

Protein solution	25 m*M* tricine pH 7.6, 0.2 *M* NaCl, 1.5 m*M* NaN_3, 5.0 mg ml^{-1} perMFE-1DH
Precipitant solution	0.2 *M* sodium citrate pH 5.6, 20 m*M* ammonium acetate, 27%(*w*/*v*) PEG 4000
Crystal dimensions (mm)	0.35 × 0.2 × 0.2
Diffraction limit (Å)	2.45 Å (rotating anode)
Unit-cell parameters (Å)	$a = b = 125.9$, $c = 60.2$
Space group	Primitive tetragonal, $P4_12_12$ or $P4_32_12$
Molecules per asymmetric unit	1
Matthews coefficient (Å^3 Da^{-1})	2.3
Solvent content (%)	47

generate the substrate. After 3 min incubation, perMFE-1DH was added and the dehydrogenase activity was monitored as the change of the extinction at 340 nm.

2.2. Characterization and crystallization

Prior to crystallization experiments, the perMFE-1DH solution was concentrated to 5.0 mg ml^{-1} in 25 m*M* tricine buffer pH 7.6 (Table 1). The screening for crystallization conditions was initiated using Hampton Research Crystal Screen I (Jancarik & Kim, 1991) and the hanging-drop method at 295 K. In screening, 1 µl of protein solution was mixed with 1 µl of well solution and suspended over 500 µl of well solution. After 14 d, single crystals were observed from conditions containing 30% PEG 4000, 0.1 *M* sodium citrate, 0.2 *M* ammonium acetate pH 5.6. The crystal reached dimensions of 0.3 × 0.1 × 0.1 mm in 30 d. The conditions were optimized and crystals with a size of 0.35 × 0.2 × 0.2 mm could be grown in 7 d from conditions consisting of 27% PEG 4000, 0.2 *M* sodium citrate, 20 m*M* ammonium acetate pH 5.6 (Fig. 2, Table 1).

3. Results

The perMFE-1DH overproduced in *E. coli* gave soluble protein and was amenable to purification by ion-exchange, perfusion and size-exclusion chromatography. The protein eluted from the size-exclusion column as a monomer (Fig. 1) and was active towards (3*S*)-hydroxydecanoyl-CoA substrate. The specific dehydrogenase activity, catalysed by perMFE-1DH and using (3*S*)-hydroxydecenoyl-CoA as substrate, was 2.2 µmol min^{-1} mg^{-1}, comparable with that of the perMFE-1 purified from rat liver (2.8 µmol min^{-1} mg^{-1}; Palosaari & Hiltunen, 1990).

A single crystal grown from 27% PEG 4000, 0.2 *M* sodium citrate, 20 m*M* ammonium acetate pH 5.6 was cryoprotected by soaking in a solution containing 90%(*v*/*v*) well solution and 10%(*v*/*v*) glycerol for 1 min. The crystal was transferred to a nylon CryoLoop (Hampton Research) and flash-frozen in a 100 K nitrogen stream (Oxford Cryosystems Cryostream Cooler) prior to diffraction analysis using a Nonius FR591 rotating-anode source. A diffraction image of the crystal is shown in Fig. 3. The data collection was carried out to 2.45 Å resolution by exposing 0.5° oscillations per frame using a MAR345 image-plate detector (X-ray Research) and λ = 1.5418 Å. Indexing and scaling of the images, as carried out with the *HKL* package (Otwinowski & Minor, 1997), suggested the space group to be primitive tetragonal, with unit-cell parameters $a = b = 125.9$, $c = 60.2$ Å. The scaling of the data indicates 422 point-group symmetry. The systematic absences along *h*00 and 00*l* suggest space group $P4_12_12$ or $P4_32_12$. The statistics of the data collection are shown in Table 2.

4. Discussion

A catalytically active dehydrogenase fragment of the rat perMFE-1 has been characterized. When expressing either the dehydrogenase domain (amino acids 260–591) or the C-terminal domain (amino acids 592–722) separately in bacteria, the overproduced protein is localized into inclusion bodies and the dehydrogenase activity is lost. The dehydrogenase part folds into an active enzyme when expressed together with the C-terminal extension. Subsequently, several expression constructs containing the dehydrogenase domain and the C-terminal domain were tested. Ultimately, it was found that it was important to include all residues of the C-terminal domain, as a deletion of only ten amino acids at the C-terminus produces an inactive protein (unpublished results). The search for crystallization conditions was initiated using a protein containing a linker region and a hexahistidine tag at the N-terminus. As this resulted in only poorly diffracting (to 4.5 Å) crystals, the construct was later modified such that the N-terminus following the initiation methionine was made identical to the N-terminal sequence of a protein which is a biological degradation product and which has been characterized in purification experiments (Dieuaide-Noubhani *et al.*, 1996). The tag was removed and the transcription-initiation site was moved from nucleotide G797 to G779 to produce a longer translation product. This fragment (amino acids 260–722) is an active and stable monomeric protein suitable for crystallization. Crystals can be grown reproducibly in the pH range 5.5–7.0 using 27% PEG 4000 as a precipitant. Molecular-replacement calculations using *AMoRe* (Navaza, 1994) and *COMO* (Jogl *et al.*, 2001) have not been successful and therefore a search for suitable heavy-atom derivatives has been initiated.

Table 2
Data-collection statistics for perMFE-1DH crystals.

Values in parentheses refer to the highest resolution shell.

Temperature (K)	100
Wavelength (Å)	1.5418
Resolution range (Å)	25–2.45 (2.54–2.45)
Observed reflections	100253
Mosaicity (°)	0.30
Unique reflections	18346
Redundancy	5.5
Average $I/\sigma(I)$	9.5 (2.6)
Wilson *B* factor (Å^2)	37
Completeness (%)	99.6 (99.7)
$R_{\rm merge}$ (%)	11.6 (48.2)

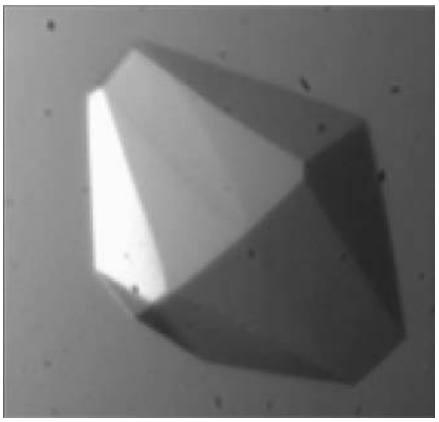

Figure 2
A tetragonal perMFE-1DH crystal grown with PEG 4000 as a precipitant at pH 5.6. The longest dimension of the crystal is 0.35 mm.

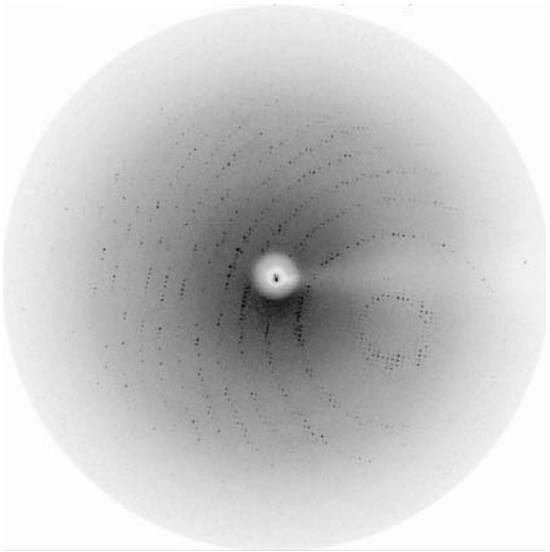

Figure 3
Diffraction image of the crystal recorded using a rotating Cu anode with a MAR345 image-plate detector. The edge of the picture corresponds to 2.4 Å resolution.

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