Organization of the multifunctional enzyme type 1: interaction between N- and C-terminal domains is required for the hydratase-1/isomerase activity

Tiila-Riikka KHEMA*, Jukka P. TASKINEN*, Päivi L. PIRILÄ*, Kari T. KOIVURANTA*†, Rik K. WIERENGA* and J. Kalervo HILTUNEN*¹

*Biocenter Oulu and Department of Biochemistry, University of Oulu, P.O. Box 3000, FIN-90014, Finland, and †VTT Biotechnology, P.O. Box 1500, FIN-02044 VTT, Finland

Rat peroxisomal multifunctional enzyme type 1 (perMFE-1) is a monomeric protein of β -oxidation. We have defined five functional domains (A, B, C, D and E) in the perMFE-1 based on comparison of the amino acid sequence with homologous proteins from databases and structural data of the hydratase-1/isomerases (H1/I) and (3S)-hydroxyacyl-CoA dehydrogenases (HAD). Domain A (residues 1–190) comprises the H1/I fold and catalyses both 2-enoyl-CoA hydratase-1 and Δ^3 - Δ^2 -enoyl-CoA isomerase reactions. Domain B (residues 191–280) links domain A to the (3S)-dehydrogenase region, which includes both domain C (residues 281–474) and domain D (residues 480–583). Domains C and D carry features of the dinucleotide-binding and the dimerization domains of monofunctional HADs respectively. Domain E (residues 584–722) has sequence similarity to domain D of the perMFE-1, which suggests that it has

evolved via partial gene duplication. Experiments with engineered perMFE-1 variants demonstrate that the H1/I competence of domain A requires stabilizing interactions with domains D and E. The variant His-perMFE (residues 288–479) Δ , in which the domain C is deleted, is stable and has hydratase-1 activity. It is proposed that the extreme C-terminal domain E in perMFE-1 serves the following three functions: (i) participation in the folding of the N-terminus into a functionally competent H1/I fold, (ii) stabilization of the dehydrogenation domains by interaction with the domain D and (iii) the targeting of the perMFE-1 to peroxisomes via its C-terminal tripeptide.

Key words: β -oxidation, lipid metabolism, peroxisome, protein engineering, structure–function.

INTRODUCTION

Multifunctional enzyme type 1 (MFE-1) has been found in mammalian mitochondria (as a membrane-associated complex) [1], in mammalian [2] and plant peroxisomes [3], and also in bacteria [4]. MFE-1 catalyses the second (hydration) and third (dehydrogenation) reactions of the fatty acid β -oxidation cycle. The conversion of *trans*-2-enoyl-CoA esters to 3-oxo intermediates proceeds via (3S)-hydroxyacyl-CoA esters [2]. In addition to 2-enoyl-CoA hydratase-1 and (3S)-hydroxyacyl-CoA dehydrogenase (HAD) activities, MFE-1 can catalyse other reactions, depending on the source of the protein [1,3,5,6]. Mammalian peroxisomal MFE-1 (perMFE-1) also catalyses the Δ^3 - Δ^2 -isomerization of enoyl-CoA esters [7] and this activity is suggested to contribute to the metabolism of polyunsaturated fatty acids when they are oxidized via the dienoyl-CoA isomerase-dependent pathway [8]. The proposal that isomerase activity of MFE-1 can play a physiological role is further supported by the observation that heterologous expression of rat MFE-1 in *eci1* Δ *Saccharomyces cerevisiae* strain allows the strain to grow on unsaturated fatty acids [9].

The rat perMFE-1, which is one of the most abundant proteins in peroxisomes, is a monomeric protein of 722 amino acid residues. Multiple-sequence alignment with the monofunctional homologues of perMFE-1 suggests that it is a multidomain protein. The N-terminal part of perMFE-1 belongs to the low sequence-similarity hydratase-1 isomerase (H1/I) family [10]. This family includes mechanistically diverse enzymes [11], catalysing different reactions but utilizing the same stabilization

strategy of an oxyanion intermediate of a CoA *O*-ester [12]. The crystal structure has been solved for five members of this family [13–17]. Among them, 4-chlorobenzoyl-CoA dehalogenase [13] is a homotrimer, whereas others are hexamers assembled as dimers of trimers. The N-terminal core domain of each subunit consists of a H1/I fold and a long C-terminal helix. The H1/I fold, formed by four $\beta\beta\alpha$ -spirals and a $\beta\alpha\beta\alpha$ motif, binds the substrate and contains the active site. The long, ‘connecting’ terminal helix links the H1/I fold to the C-terminal domain called the trimerization domain, which is formed by three α -helices. The known structures of the H1/I family show that the subunits in the trimeric discs tightly interact with the neighbouring subunits, stabilizing and completing the active site of the H1/I domain.

The middle part of the perMFE-1 sequence (HAD part of MFE-1, mfeHAD) is related to the mitochondrial NAD⁺-dependent HADs [18], the sequence identity being 34 % with both pig and human-soluble mitochondrial HADs. Previously published structures of human and pig heart homodimeric HADs [19,20] show that they are composed of two identical subunits, each having two domains. The larger N-terminal domain contains the Rossmann fold [21] with the dinucleotide-binding $\beta\alpha\beta$ -unit [22], whereas the smaller, mostly α -helical, domain mediates subunit–subunit interactions. CoA *O*-ester substrates are bound in a cleft between the domains.

The HAD domains in bacteria and both perMFE-1 and mitochondrial MFE-1 in mammals are followed by an extension of approx. 140 amino acids in length (139 in rat perMFE-1). The function of these extensions remains unknown except for the three extreme C-terminal amino acid residues (Ser-Lys-Leu-OH)

Abbreviations used: H1/I, hydratase-1/isomerase; HAD, (3S)-hydroxyacyl-CoA dehydrogenase; MFE-1, multifunctional enzyme type 1; mfeHAD, HAD part of MFE-1; perMFE-1, mammalian peroxisomal MFE-1; PTS-1, peroxisomal targeting signal type 1.

¹ To whom correspondence should be addressed (e-mail Kalervo.Hiltunen@oulu.fi).

in the mammalian perMFE-1, which act as peroxisomal targeting signal type 1 (PTS-1) [23].

Our aim was to elucidate the structure–function relationship of multifunctional β -oxidation proteins, and we show that perMFE-1 can be divided into five separate domains or parts. Studies of the functional organization of perMFE-1 using deletion variants show that interaction between the N- and C-terminal regions is required to fold an enzymically active H1/I domain. The C-terminal domain E has intrinsic homology with the dimerization domain of the mfeHAD. The possible roles of the C-terminal domain of the perMFE-1 are discussed and a model of the structural organization of the perMFE-1 is proposed.

EXPERIMENTAL

Cloning of constructs

The oligonucleotides used in the present study are shown in Table 1. DNA was synthesized by PCR using 900 ng of rat perMFE-1 cDNA [24] as a template in 10 mM KCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 20 mM Tris/HCl (pH 8.8), 2 mM MgSO_4 , 0.1% Triton X-100, 0.1 mg/ml BSA buffer containing 1 μM of forward and reverse primers, 200 μM dNTP and 1.25–1.4 units of *Pfu* polymerase (Stratagene, La Jolla, CA, U.S.A.; Promega, Madison, WI, U.S.A.). The *pUC18::perMFE-1wt* was used as a template in all DNA synthesis and site-directed mutagenesis reactions unless otherwise stated. Deletion variants of the perMFE-1 [*perMFE*(281–467) Δ , *perMFE*(281–467) Δ (584–719) Δ SKL, *perMFE*(281–495) Δ , *perMFE*(288–472) Δ , *perMFE*(281–583) Δ and *perMFE*(191–479) Δ] were prepared with the overlap-extension PCR method as described in [25]. All the subcloning reactions were performed with the Sure-Clone ligation kit (Amersham Pharmacia Biotech). The deletion or mutagenesis sites were confirmed by sequencing with an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.). The restriction enzymes and the DNA-modifying enzymes were purchased from Amersham Pharmacia Biotech (*Sa*II, calf intestinal phosphate), Fermentas, Vilnius, Lithuania (*Mun*I, *Bam*HI, T4DNA ligase) and New England Biolabs, Beverly, MA, U.S.A. (*Nco*I).

pUC18::perMFE-1wt, *pAO815::perMFE-1wt* and *pET3a::perMFE-1wt*

The open reading frame of rat MFE-1 was cloned as three copies into the pAO815 yeast expression vector (*pAO815::perMFE-1wt*) via *Mun*I restriction sites, which were introduced by PCR using Rkokoap, Rkokokp as primers (*pUC18::perMFE-1wt*) and applying a Multi-copy *Pichia* expression kit (Invitrogen, Groningen, The Netherlands). For expression in *Escherichia coli*, the open reading frame was cloned into the pET3a expression vector (Novagen, Madison, WI, U.S.A.). The required *Nde*I restriction site on the 5'-end was introduced by using a Quick-Change site-directed mutagenesis kit (Stratagene) using MFEf and its reverse primer (*pET3a::perMFE-1wt*) and the required *Bam*HI site originated from the pUC18 vector, leaving the *Mun*I site intact for subsequent cloning. To clone the deletion variants into the pET3a vector, the *pET3a::perMFE-1wt* was digested using an intrinsic *Nco*I site at position 211 in the perMFE-1 cDNA on the 5'-side and the *Mun*I site on the 3'-side.

C-terminal truncations of the perMFE-1

Deletion of the nucleotides (1771–2187) coding for the residues Pro⁵⁸⁴-Leu⁷²² was achieved by site-directed mutagenesis using *pET3a::perMFE-1wt* as a template and Bcl140del and its reverse

primer as mutagenic primers, creating an additional stop codon after Lys⁵⁸³ [*pET3a::perMFE*(584–722) Δ]. The deletion of the nucleotides (1144–2187) coding for residues Asp³⁷⁵-Leu⁷²² was achieved by PCR using Rkokoap and Handle as 5'- and 3'-primer respectively. The PCR product was cloned into pUC18 [*pUC18::perMFE*(375–719) Δ SKL], and the insert was digested with *Nco*I and *Mun*I restriction enzymes and ligated to the similarly digested *pET3a::perMFE-1wt* vector [*pET3a::perMFE*(375–719) Δ SKL]. The deletion of the nucleotides (862–2173) coding for the residues Ser²⁸¹-Leu⁷²² was achieved by PCR using RevH1 and M13fwd as 5'- and 3'-primer respectively, whereas *pUC18::per*(281–467) Δ served as a template. The PCR product was digested with *Nco*I and *Mun*I restriction enzymes and ligated into the similarly digested *pET3a::perMFE-1wt* vector [*pET3a::perMFE*(281–722) Δ].

Generation of internally deleted variants of perMFE-1

The deletion of the nucleotides (862–1770) coding for the dehydrogenase domain (Ser²⁸¹-Lys⁵⁸³) of the perMFE-1 was achieved by overlap-extension PCR using Rkokoap, Rkokokp, DHGdel and its reverse primer as 5', 3' and deletion primers respectively. The final PCR product was cloned into pUC18 [*pUC18::perMFE*(281–583) Δ]. The insert was digested with *Nco*I and *Mun*I restriction enzymes and cloned into similarly digested *pET3a::perMFE-1wt* [*pET3a::perMFE*(281–583) Δ]. The deletion of the nucleotides (862–1506) coding for residues Ser²⁸¹-Leu⁴⁹⁵ was achieved by overlap-extension PCR using *pUC18::perMFE*(281–467) Δ as a template and Rkokokp, Rkokoap, NADB8H10 and its reverse primer as 5', 3' and deletion primers respectively. The resulting PCR product was cloned into pUC18 [*pUC18::perMFE*(281–495) Δ] and into pET3a, as described above [*pET3a::perMFE*(281–495) Δ]. A fragment coding for the dinucleotide-binding domain (Ser²⁸¹-Lys⁴⁶⁷) of perMFE-1 (nucleotide residues 862–1422) was deleted with overlap-extension PCR using Rkokoap, Rkokokp, NADdelfwd and its reverse primer as 5', 3' and deletion primers respectively. By using 140del as a 3'-primer, a C-terminal truncation was generated leading to the additional deletion of the residues Pro⁵⁸⁴-Leu⁷²². For cloning into pET3a, the inserts were digested out from *pUC18::perMFE*(281–467) Δ and *pUC18::perMFE*(281–467) Δ (584–719) Δ SKL vectors with *Nco*I and *Mun*I enzymes. The inserts were then ligated to the similarly digested *pET3a::perMFE-1wt* construct, resulting in *pET3a::perMFE*(281–467) Δ and *pET3a::perMFE*(281–467) Δ (584–719) Δ SKL constructs. The deletion of the nucleotides (883–1437) coding for the amino acid residues Trp²⁸⁸-Val⁴⁷², leaving out seven extra amino acids to the N-terminal side of the deletion and removing five residues from the C-terminal side, was achieved by overlap-extension PCR using Rkokoap, M13fwd, NAD-linkerfwd and its reverse primer as 5', 3' and deletion primers respectively. The *Nco*I- and *Mun*I-digested PCR product was directly cloned into the *pET3a::perMFE-1wt* expression vector as described above [*pET3a::perMFE*(288–472) Δ]. The deletion of the nucleotide residues 883–1458 corresponding to the amino acid residues Trp²⁸⁸-Val⁴⁷⁹ was achieved by overlap-extension PCR using Rkokoap, M13fwd and NADB8 as 5', 3' and deletion primers respectively. The PCR product was cloned as described above, resulting in a *pET3a::perMFE*(288–479) Δ construct. For cloning into the pET15b vector, the insert was digested with *Nde*I and *Bam*HI restriction enzymes and ligated to the similarly digested pET15b expression vector [*pET15b::perMFE*(288–479) Δ]. The deletion of the nucleotide residues (592–1458) coding for the amino acid residues Pro¹⁹¹-Val⁴⁷⁹ was achieved by overlap-extension PCR using Rkokoap, M13fwd, linkerNADB8 and its

Table 1 Oligonucleotides used in generating the variants of the rat perMFE-1

Restriction sites are underlined, start and stop codons are in boldface.

Primer name	Sequence in 5' → 3' orientation	Application
Rkokoap	CACACAATTGA AATG GCTGAGTATCTGAG	5'-Primer with <i>MunI</i> site and start codon
Rkokokp	GCACTCAATTG TCAC AGTTTGCTGCCGT	3'-Primer with stop codon and <i>MunI</i> site
MFEf*	CCACACAATCAT ATG GCTGAG	5'-Primer with <i>NdeI</i> site and start codon
Handle	TCAATTGTCAC AGCTTGGACACAGTTGAAAGTTCC	3'-Primer stop codon after Val ³⁴⁷ with <i>MunI</i> site
140del	TCAATTGTCAC AGCTTGGACTTGTCTACTG	3'-Primer with stop codon after Lys ⁵⁸³
Bcl140del*	GTATGACAAG TGATC AGGTCGCATC	3'-Primer creating stop codon after Lys ⁵⁸³ with <i>BclI</i> site
RevH1	CCAAGACGCTCCTGACAA TTGTCAC CACTTATTTGC	3'-Primer with stop codon after Trp ²⁸⁰ with <i>MunI</i> site
NADdel4wd*	GTCTGCAAATAAGTGGATTGGAGTAGTGGTTGGC	Deletion of amino acids 281–467
DHdel4fwd*	GTCTGCAAATAAGTGGCACTGGGTGCGATCC	Deletion of amino acids 281–583
NADB8H10*	GTCTGCAAATAAGTGGGAGGAAGGTAGCAAGCC	Deletion of amino acids 281–495
NADlinkerfwd*	CAACTCCCTCAGGAGCGTCTGGCAACTGCTATGGATTG	Deletion of amino acids 288–472
NADB8*	CAACTCCCTCAGGAGCGTCTGGGAATCGGATGTTGGC	Deletion of amino acids 288–479
LinkerNADB8*	GAAGATTATAGATAAAGGGAATCGGATGTTGGC	Deletion of amino acids 191–479
M13fwd	GTAAACGACGGCCAGT	3'-Flanking primer

* The reverse primer has a complementary sequence.

reverse primer as 5', 3' and deletion primers respectively. The PCR product was directly cloned to the *pET3a::perMFE-1wt* expression vector as described above [*pET3a::perMFE(584–722)Δ*].

Expression of the perMFE-1wt in *Pichia pastoris* and purification

The *pAO815::perMFE-1wt* linearized with *SaI* restriction enzyme was transformed to the *P. pastoris* SMD1168 strain with electroporation according to the manufacturer's instructions (Invitrogen). The screening of the transformants and the expression of the target gene were performed by using *HIS4* transformants according to the manufacturer's instructions (Invitrogen). Tricine (25 mM; pH 7.6), 1 mM EDTA, 0.5 mM benzamidine (BA), 0.1 mM PMSF and 0.02 % Na₂S₂O₃ were added to the cells (2.5 g wet wt.) in a final volume of 30 ml. After addition of (NH₄)₂SO₄ to a final concentration of 0.75 M, the cells were disrupted with a French press (Spectronic, Rochester, NY, U.S.A.). The cell lysate was centrifuged at 12000 *g* for 10 min at 4 °C and the resulting supernatant was recentrifuged at 100000 *g* for 1 h at 5 °C. The supernatant was filtered through glass wool to remove lipids and applied on to a butyl-Sepharose Fast-Flow column (2.5 cm × 10 cm; Amersham Pharmacia Biotech) equilibrated with 25 mM Tricine (pH 7.6), 1 mM EDTA and 0.75 M (NH₄)₂SO₄. The bound proteins were eluted in 25 mM Tricine (pH 7.6) and 1 mM EDTA with a linear gradient of 0.75–0 M (NH₄)₂SO₄, with concomitant increase in ethylene glycol from 0 to 30 % in 75 ml. Fractions containing the hydratase-1 activity were pooled, concentrated to 15 ml on a Macrosep 10 K ultrafiltration membrane (Pall Filtron Corporation, Northborough, MA, U.S.A.) and dialysed against 1.5 litres of 50 mM potassium phosphate buffer (pH 6.8) and 1 mM EDTA. The sample was applied on to a 6 ml Resource S column (Amersham Pharmacia Biotech) equilibrated with the dialysis buffer. The protein was eluted with a linear gradient of KCl from 0 to 0.1 M in 60 ml. The activity containing fractions were pooled, concentrated with a Biomax-10K centrifugal filter (Millipore Corporation, Bedford, MA, U.S.A.) and applied on to a Superdex-200 HR 10/30 size-exclusion column (Amersham Pharmacia Biotech) equilibrated with 25 mM Tricine (pH 8.0), 1 mM EDTA, 0.2 M NaCl, 0.02 % Na₂S₂O₃ and 15 % glycerol. The perMFE-1wt-containing fractions were pooled and concentrated on centrifugal filters.

Expression of perMFE-1 variants in *E. coli* and purification of His-perMFE(288–479)Δ

The *E. coli* pET expression constructs were transformed to the BL21pLysS cells and the expression was performed according to the manufacturer's instructions (Novagen). The expressions were conducted for 3 h at 30 °C. The cells were harvested, washed with PBS buffer and stored at –70 °C until use. The cell pellets were suspended in cold lysis buffer [25 mM Hepes (pH 8.0), 100 mM NaCl, 1 mM EDTA, 15 % glycerol, 0.05 % Triton X-100, 100 µg/ml lysozyme supplemented with Complete Protein Inhibitor Cocktail Tablets (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions], incubated at 30 °C for 10 min and broken by sonication. The insoluble material was removed by centrifugation at 20000 *g* for 30 min and the supernatants were assayed for 2-enoyl-CoA hydratase-1 activity. For the purification of His-perMFE(288–479)Δ, a culture of 1 litre was used, yielding 7.4 g of cells. The cells were resuspended in 60 ml of 5 mM imidazole, 0.5 M NaCl, 20 mM Tris/HCl (pH 7.9), 15 % glycerol containing 0.02 mg/ml DNase I, 0.002 mg/ml RNase A, 0.1 mg/ml lysozyme, 0.05 % Triton X-100 and 5 mM MgCl₂ and incubated at 37 °C for 15 min. The sample was centrifuged at 30000 *g* for 30 min at 4 °C, and the supernatant was applied on to a His-Bind Resin column (1 cm × 3.5 cm; Novagen) equilibrated according to the manufacturer's instructions. The bound proteins were eluted with a linear gradient of imidazole from 5 to 150 mM in 0.5 M NaCl, 20 mM Tris/HCl (pH 7.9) and 15 % glycerol. The fractions containing hydratase-1 activity were pooled, supplemented with (NH₄)₂SO₄ to a final concentration of 1.2 M and applied on to a 1 ml Resource isopropyl hydrophobic interaction column (Amersham Pharmacia Biotech) equilibrated with 25 mM Tricine (pH 8.0), 1 mM EDTA, 1.2 M (NH₄)₂SO₄ and 15 % glycerol. The bound proteins were eluted from the column by decreasing the (NH₄)₂SO₄ concentration from 1.2 to 0 M in a 10 ml linear gradient. Fractions containing the hydratase-1 activity were pooled and concentrated with an ultrafiltration device. The purification was completed by size-exclusion chromatography on a Superdex 75 HR 10/30 column (Amersham Pharmacia Biotech) equilibrated with 25 mM Tricine (pH 8.0), 1 mM EDTA, 200 mM NaCl, 15 % glycerol and 0.02 % Na₂S₂O₃. The fractions containing His-perMFE(288–479)Δp were pooled and concentrated as described above.

Figure 1 Division of the rat perMFE-1 based on multiple-sequence alignment with H/I proteins and HADs

(a) Sequence alignment of the N-terminal part of the rat perMFE-1 and the H/I protein family members with known structures (1DUB, 2-enoyl-CoA hydratase-1; 1DCI, Δ^3 - Δ^2 -dienoyl-CoA isomerase; 1NZY, 4-chlorobenzoyl-CoA dehalogenase; 1EF8, methylmalonyl-CoA decarboxylase; 1HNO, Δ^3 - Δ^2 -enoyl-CoA isomerase). The region showing sequence similarity (residues 1-190) to the H/I protein is indicated by a grey bar and denoted by the letter 'A'. The active-site residues of 1DUB, Glu¹⁴⁴ and Glu¹⁶⁴, are marked with black dots above the sequences. The next 89 amino acid residues (residues 191-280), indicated by a white bar and marked with the letter 'B', do not show sequence similarity to H/I proteins. (b) The alignment shows a stretch of amino acid residues of the rat perMFE-1 with sequence similarity to HADs. 3HAD and 3HDH represent human and pig mitochondrial soluble HADs respectively. The region aligned to the dinucleotide-binding domain (residues 281-476) is indicated by a grey bar and marked with the letter 'C'. The region showing sequence similarity to the dimerization domain of HAD is indicated by a white bar and marked with the letter 'D'. A stretch of three amino acids (Tyr-Gly-Phe) serves as a linker region between these two domains and is marked with the letter 'L'. (c) The C-terminal extension of the rat perMFE-1, indicated by a grey bar and marked with the letter 'E', can be aligned with the dimerization domain of HADs as well as with domain D. The vertical arrows correspond to the arrows in Figures 2(a) and 2(b).

Table 2 Putative domain architecture of the rat perMFE-1

Domain (size)	Amino acids	Description
A (190 residues)	1–190	H/I core domain plus connecting helix
B (90 residues)	191–280	Domain with unknown function, no homologous domain could be found
C (194 residues)	281–474	Shows homology to dinucleotide-binding domain of the short-chain acyl-CoA dehydrogenase
D (104 residues)	480–583	Shows homology to dimerization domain of the short-chain acyl-CoA dehydrogenase
E (139 residues)	584–722	Domain with unknown function, shows homology to domain D and carries PTS-1

Figure 2 Ribbon drawings showing one subunit of 2-enoyl-CoA hydratase-1 and HAD

(a) The H1/I fold is shown in dark grey, the connecting helix in black and the trimerization domain located at the C-terminus in light grey. (b) The dinucleotide-binding site is shown in dark grey, the connecting strand in black and the dimerization domain in light grey. In (a), the arrow indicates the position where sequence similarity to perMFE-1 stops; in (b), the open and closed arrows point to the deletion sites of the constructs perMFE(281–467) Δ and His-perMFE(288–479) Δ respectively (see legend to Figure 3). Ribbon diagrams were generated using the PyMOL program [DeLano, W. L. (2002) The PyMOL Molecular Graphics System, DeLano Scientific, San Carlos, CA, U.S.A., <http://www.pymol.org>]. The PDB codes are 1DUB and 3HAD respectively.

Enzyme assay

The 2-enoyl-CoA hydratase-1 activity was measured by following the rate of generation of (3*S*)-hydroxyacyl-CoA ester, which was oxidized in the presence of HAD and NAD⁺ as described previously [26], by using *trans*-2-decenoyl-CoA or *trans*-2-hexenoyl-CoA as substrate. To measure the combined activity of the perMFE-1 (combined action of 2-enoyl-CoA hydratase-1 and HAD), the externally added HAD was omitted. Δ^3 - Δ^2 -enoyl-CoA isomerase activity was measured as described previously [7],

except that the monitoring of NADH was replaced by monitoring the Mg²⁺–3-oxoacyl-CoA complex at 303 nm. The kinetic parameters of the 2-enoyl-CoA hydratase-1 and isomerase activities of perMFE-1wt and His-perMFE (288–479) Δ were determined with *trans*-2-decenoyl-CoA and *trans*-2-hexenoyl-CoA, where the substrate concentrations varied from 10 to 290 μ M and from 10 to 180 μ M respectively, whereas the kinetic parameters for the Δ^3 - Δ^2 -enoyl-CoA isomerase activity of perMFE-1wt and His-perMFE(288–479) Δ were determined using 10–250 μ M of *trans*-3-hexenoyl-CoA concentrations. The measurements were made in 50 mM Tris/HCl (pH 8.0), 50 mM KCl containing 1 mM NAD⁺, 1 mM sodium pyruvate, 12.5 mM MgCl₂ and, as auxiliary enzymes, 10 μ g HAD and 20 μ g L-lactate dehydrogenase at 22 °C. The data were analysed with GraFit software (Sigma-Aldrich).

CD spectroscopy

A JASCO J-715 spectropolarimeter (JASCO Corporation, Hachioji City, Tokyo, Japan) was used for CD spectroscopy measurements at 20 °C. The far-UV spectra of the proteins were measured from 195 to 260 nm in 25 mM Tricine (pH 8.0), 150 mM NaCl, 15% glycerol, with the following instrument settings: response, 1 s; sensitivity, 20 mdeg; speed, 50 nm/min; path length, 1 mm; and average of 16 scans. The melting curves were generated by monitoring the protein solution at 222 nm over a temperature gradient of 20–80 °C in a CD spectrometer with the following instrument settings: temperature increase, 50 °C/h; response, 16 s; sensitivity, 20 mdeg; and path length, 1 mm.

Other methods

Protein concentrations were determined by the Bradford method (Bradford reagent; Bio-Rad Laboratories). The concentrations of the purified proteins were estimated from the molar absorption coefficient at 280 nm [27,28]. Proteins were separated on SDS/PAGE (12% gel) and blotted on to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) [29]. For immunodetection with an ECL[®] kit (Amersham Pharmacia Biotech), antibody raised against rat perMFE-1 [7] was used as the primary antibody and anti-rabbit IgG conjugated with horseradish peroxidase was used as the secondary antibody (Bio-Rad Laboratories). The acyl-CoA esters were synthesized as described previously [26].

RESULTS AND DISCUSSION

H1/I proteins of known structure are either homotrimers or homo-hexamers in which the subunits are assembled into either trimeric discs [13] or doublets of trimers [14–17]. However, perMFE-1 is a monomeric enzyme and is thus unique in the H/I family. The present study examines the domain organization of perMFE-1 with a special emphasis on the domain organization and interactions required for hydratase-1 and isomerase activities.

Figure 3 Schematic representation of the wild-type and engineered variants of the rat perMFE-1

The number and a description of each construct are given on the left-hand side. The number in parentheses indicates the deleted amino acid residue. This scheme follows the domains defined in Figure 1. The numbering indicates the last residue of the domain or the last residue included in the construct. The italic numbers on the C-terminal flanking regions indicate the first amino acid residue included in the construct. The construct ending with domain E also contains the PTS-1 (Ser-Lys-Leu-OH). If the Ser-Lys-Leu is included in the C-terminus in other constructs, it is indicated by 'SKL'. The N-terminal extension of six histidine residues is indicated by the letter 'H'. The '+' or '-' signs on the right-hand side denote the observed hydratase-1 activity of the constructs.

Figure 1 shows the sequence alignment of perMFE-1 with the homologous proteins having known structures. Based on the domain organization of the H1/I and HADs as well as the sequence similarity, perMFE-1 has been divided into five parts: A, B, C, D and E (Table 2). In Figure 1(a), the N-terminal part of perMFE-1 is aligned with 2-enoyl-CoA hydratase [14], $\Delta^{3,5}$ - $\Delta^{2,4}$ -dienoyl-CoA isomerase [15], 4-chlorobenzoyl-CoA dehalogenase [13], methylmalonyl-CoA decarboxylase [16] and Δ^3 - Δ^2 -enoyl-CoA isomerase [17], and in Figure 1(b), the C-terminal half of perMFE-1 is aligned with human HAD [19] and pig HAD [20]. Domain A (residues 1–190) has sequence similarity with the H1/I fold and the connecting helix in other H1/I proteins. The alignment also shows that H1/I similarity stops after the connecting helix, and other members of the H1/I family have an additional 100 residues that fold into a trimerization domain. In perMFE-1, the corresponding position is occupied by a stretch of 89 amino acid residues (residues 191–280, domain B) separating domain A from the region similar to HADs (residues 281–583 of the perMFE-1; mfeHAD). Although the fold and the

function of the domain B of the perMFE-1 remain unknown, the secondary structure prediction of perMFE-1 (PHD secondary structure prediction [30,31]) suggests that, like the trimerization domain of the multimeric H1/I, domain B is mainly α -helical.

The mfeHAD comprises a sequence related to dinucleotide binding and dimerization domains of the HADs (Figure 2b), suggesting that the mfeHAD also folds into two domains, which are subsequently marked as C (residues 281–474) and D (residues 480–583) separated by the residues 474–479. Domain C includes a conserved Gly-X-Gly-X-X-Gly motif, known to be located in a turn between a β -strand and an α -helix of the dinucleotide-binding $\beta\alpha\beta$ architecture [22]. In the soluble dimeric HADs, the dimerization domains of the subunits create a contact site between the two monomers. As suggested by sequence identity (34%) and comparison of the predicted secondary structure elements with the known elements of the HADs, domain D can adopt a fold similar to the dimerization domain of the HAD. However, in monomeric perMFE-1, a stabilizing counterpart (the other subunit in HAD) is missing; consequently, this structural feature

Table 3 Specific hydratase-1 activities of rat perMFE-1 and the deletion variants in *E. coli*

The variants given in the Experimental section were expressed in a pET3a expression system. Hydratase-1 activity was measured from a crude cell extract with 60 μM *trans*-2-hexenoyl-CoA as a substrate.

perMFE-1 variant	Specific activity ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)*
pET3a	0.010 $\pm$ 0.003
pET3a::perMFE-1wt	0.332 $\pm$ 0.087
pET3a::perMFE(281–722) Δ	0.013 $\pm$ 0.004
pET3a::perMFE(584–722) Δ	0.016 $\pm$ 0.001
pET3a::perMFE(375–719) Δ SKL	0.011 $\pm$ 0.003
pET3a::perMFE(281–583) Δ	0.008 $\pm$ 0.001
pET3a::perMFE(281–467) Δ	0.177 $\pm$ 0.040
pET3a::perMFE(281–495) Δ	0.011 $\pm$ 0.001
pET3a::perMFE(281–467) Δ MFE(584–719) Δ SKL	0.008 $\pm$ 0.003
pET3a::perMFE(288–472) Δ	1.293 $\pm$ 0.415
pET3a::His-perMFE(288–479) Δ	1.309 $\pm$ 0.077
pET3a::perMFE(191–479) Δ	0.011 $\pm$ 0.005

* Means $\pm$ S.D.; $n = 3$.

Figure 4 Immunoblot analysis of the rat perMFE-1 and the deletion variants in *E. coli*

Each lane contained approx. 1.5 μg of protein. On lane N is the cell lysate from *E. coli* transformed with pET3a only. The predicted molecular masses of the expressed polypeptides are: lane 1, 78.7 kDa (perMFE-1wt); lane 2, 28.8 kDa [perMFE(281–722) Δ]; lane 3, 62.8 kDa [perMFE(584–722) Δ]; lane 4, 40.0 kDa [perMFE(375–719) Δ SKL]; lane 5, 46.1 kDa [perMFE(281–583) Δ]; lane 6, 58.8 kDa [perMFE(281–467) Δ]; lane 7, 55.8 kDa [perMFE(281–495) Δ]; lane 8, 43.0 kDa [perMFE(281–467) Δ MFE(584–719) Δ SKL]; lane 9, 58.9 kDa [perMFE(288–472) Δ]; lane 10, 60.4 kDa [His-perMFE(288–479) Δ]; lane 11, 47.5 kDa [perMFE(191–479) Δ]. The positions of molecular-mass standards (kDa) are indicated on the left: 31.0 kDa, carbonic anhydrase; 45.0 kDa, ovalbumin; 66.2 kDa, serum albumin; 97.4 kDa, phosphorylase b.

has to be supplemented by another region of the perMFE-1 polypeptide. The domain D is followed by an extension of residues 584–722 with a C-terminal Ser-Lys-Leu-OH triplet and a canonical PTS-1 [32]. When this extension, marked here as domain E, was searched against PDB, the dimerization domain of HADs appeared with 32.5% similarity. The subsequent sequence alignment of domain E with perMFE-1 (Figure 1c) also revealed 18% similarity to domain D (residues 479–526), which suggests that domain E could have evolved by gene duplication from domain D and, consequently, functions as a complementary part similar to the dimerization domain of HAD. Experimental evidence also suggests a tight interaction between domains D and E. For example, it has been shown that the perMFE-1 truncated

Figure 5 CD spectroscopy of the recombinant wild-type rat perMFE-1 and His-perMFE(288–479) Δ

(a) Far-UV spectra of the proteins. (b) Temperature titration curves followed at 222 nm. —, perMFE-1wt; ---, perMFE(288–479) Δ .

for the N-terminal 259 residues is a fully active dehydrogenase [33], whereas variants having a deletion of ten C-terminal residues or domain E are completely inactive.

In the H1/I family, the trimerization domain is an essential component of the active site in the trimeric disc. As this domain is absent from perMFE-1, the question arises how domain A is stabilized. To investigate this aspect further, we engineered modified variants of perMFE-1 and then tested for hydratase-1 activity, an indicator of functional competence. Initially, seven modified variants were designed by deleting domains, as tentatively defined by sequence alignments (Figure 1) and structural data (Figures 2a and 2b). The cDNAs encoding these variants (Figure 3, variants 2–6) were cloned into pET3a and the vectors were transformed into *E. coli* cells. When immunoblot analysis was performed using antibody raised against the rat perMFE-1 [7], protein bands of the predicted sizes of the deletion variants were visualized in the homogenates from cells expressing the constructs, but not in the homogenates from cells transformed with the pET3a vector alone (Figure 4). During initial screening, the bacterial cell lysate showing increased hydratase-1 activity compared with a negative control ($0.177 \pm 0.040 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ versus $0.010 \pm 0.003 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$; Table 3) was from cells expressing pET3a::perMFE(281–467) Δ , a

Figure 6 Schematic representation of the domain organization of perMFE-1

Both models agree with the experimental data. As explained in the text, we favour model (2) as it assigns functions to each domain.

variant in which domain C was deleted (Figure 3). Furthermore, perMFE(281–467) Δ had lost HAD activity. An additional deletion of the 28 amino acids at the N-terminus of domain D [perMFE(281–495) Δ] or a deletion of domain B [perMFE(191–479) Δ] led to a loss of hydratase-1 activity (Table 3). Similarly, truncation of the extreme C-terminal domain E [perMFE(584–722) Δ] resulted in an inactivation of the hydratase-1 activity.

To characterize further the properties of the perMFE(281–467) Δ variant, the soluble extract from *E. coli* cells transformed with *pET3a::perMFE(281–467) Δ* was applied on to a DEAE column at pH 8.8. The hydratase-1 activity was found in the flow-through. However, the activity was lost despite the addition of glycerol or salt, indicating instability of the expressed polypeptide, and this hampered further purification attempts. Therefore, additional modifications to perMFE(281–467) Δ were studied. Among the variants tested, the His-perMFE(288–479) Δ p (containing Ser²⁸¹-Thr-Pro-Ser-Gly-Ala-Ser²⁸⁸ at the N-terminal deletion site, but not Ile⁴⁶⁸-Gly-Val-Val-Ser-Gly-Asn-Cys-Tyr-Gly-Phe-Val⁴⁷⁹ at the C-terminal deletion site) was active and maintained the hydratase-1 activity with increased stability. To facilitate purification, a His tag with six histidine residues was added to the N-terminus; using metal-binding affinity, hydrophobic interaction and size-exclusion chromatography, His-perMFE(288–479) Δ was purified. SDS/PAGE resulted in a single protein band of 60 kDa, and size-exclusion chromatography indicated that His-perMFE(288–479) Δ is a monomeric protein. Expression of the wild-type perMFE-1 (perMFE-1wt) from pET3a in *E. coli* yielded an enzymically active protein, but the expression level was low. Subsequently, perMFE-1wt, over-expressed in yeast *P. pastoris* as described in the Experimental section, was purified and used for the characterization.

The kinetic characterization of the hydratase-1 activity of His-perMFE(288–479) Δ and perMFE-1wt showed that His-perMFE(288–479) Δ had higher k_{cat} values than perMFE-1wt with both the substrates *trans*-2-decenoyl-CoA and *trans*-2-hexenoyl-CoA, the values being $130.1 \pm 4.8 \text{ s}^{-1}$ versus $80.1 \pm 4.8 \text{ s}^{-1}$ and $209.0 \pm 2.5 \text{ s}^{-1}$ versus $102.1 \pm 4.1 \text{ s}^{-1}$ respectively. However, when isomerase activity was measured with *trans*-3-hexenoyl-CoA as a substrate, the k_{cat} values were lower for His-perMFE(288–479) Δ ($0.085 \pm 0.004 \text{ s}^{-1}$) than for perMFE-1wt ($0.41 \pm 0.03 \text{ s}^{-1}$). Interestingly, the K_m value for both substrates *trans*-3-hexenoyl-CoA and *trans*-2-hexenoyl-CoA was higher in His-perMFE(288–479) Δ

than in perMFE-1wt ($46.9 \pm 7.2 \mu\text{M}$ versus $23.6 \pm 6.4 \mu\text{M}$ and $38.7 \pm 1.4 \mu\text{M}$ versus $9.1 \pm 1.7 \mu\text{M}$ respectively), whereas with *trans*-2-decenoyl-CoA as substrate, no significant change was observed ($16.6 \pm 2.5 \mu\text{M}$ versus $21.3 \pm 5.2 \mu\text{M}$).

Because the deletion variant from perMFE-1 caused dissimilar changes in the kinetic parameters of hydratase-1 and isomerase activities, the results imply that upon deletion of domain C, the environment in the catalytic site changes, favouring the hydratase-1 activity over the Δ^3 - Δ^2 -isomerase activity. There are two conserved active-site residues assigned for the hydratase-1 activity in perMFE-1, namely Glu¹⁰³ and Glu¹²³ (corresponding to Glu¹⁴⁴ and Glu¹⁶⁴ in 1DUB). The postulated active-site residue for the isomerase activity is the Glu¹²³ in perMFE-1. In line with this proposal, site-directed mutagenesis experiments showed that the hydratase activities of the Glu¹⁰³-Ala and Glu¹²³-Ala variants are reduced by 3200 and 1900 times and their isomerase activities by 77 and 260 times respectively.

The secondary structure elements of perMFE-1wt and His-perMFE(288–479) Δ were analysed with CD spectroscopy in the far-UV region (195–250 nm) (Figure 5a). The spectrum of His-perMFE(288–479) Δ showed that it is a properly folded protein. The close similarity between the spectra of His-perMFE(288–479) Δ and perMFE-1wt indicates that the deletion of the dinucleotide-binding domain (domain C) did not cause a major change in the composition of secondary-structure elements [Jfit analysis: His-perMFE(288–479) Δ 58% α -helix and perMFE-1wt 69% versus calculated from the predicted domain structure and secondary structure elements (Figure 1) within domains 69 and 63% respectively]. The melting points, 53.3 and 44.3 °C, were derived from the second derivatives of the melting curves for perMFE-1wt and His-perMFE(288–479) Δ respectively (Figure 5b). The difference of 9.0 °C in the melting points indicates that the His-perMFE(288–479) Δ is not as stable as perMFE-1wt.

Our studies with perMFE-1 variants suggest two modes of assembly for the five domains (Figure 6). From the structural homology of domain A with enzymes of the H1/I family, it appears that the active site of domain A must be completed by interaction with another domain. This could be either domain B or the C-terminal domains D and/or E, or both. Sequence comparison suggests that domains D and E have a similar fold. Domain D is the dimerization domain of HAD, suggesting that domains D and E also interact in perMFE-1 and, therefore,

form a compact structure stabilizing the whole protein. Our studies indicate that the D/E domain is required for full hydratase activity as well as for dehydrogenase activity [33]. In model (1) (Figure 6), domain A tightly interacts with the D/E domain, whereas in model (2), domain A tightly interacts with the domain B. Both models agree with our results. In model (2), a function can be assigned to all the domains, including domain B. Model (2) also agrees with the notion that the D/E domain is important for both the H1/I fold of domain A and the catalytic activity of domain C. Further characterization of MFE-1, aimed at crystallizing the full-length and fragments of MFE-1, are in progress. The crystal structure of the C/D/E construct [33] will give further insight into the domain organization of MFE-1.

CONCLUSIONS

Domain-deletion experiments with the perMFE-1 indicate that the variant of domains A and B does not have H1/I activity. However, the variant (AB, DE) in which domain C has been deleted is fully active. Additional deletion of any of those domains results in hydratase-1 inactivation. The most stable deletion variant requires additional trimming at the boundary of the deleted domain C. This trimming includes the deletion of a short linker [His-perMFE(288–479) Δ] formed by residues 475–479 (corresponding to 202–206, which is the region between the two HAD domains). At least three separate functions can be assigned for domain E: (i) stabilization of the H1/I fold of the MFE-1 together with domain D, (ii) stabilization of the domain D of a perMFE-1 variant with HAD activity and (iii) carrying the PTS-1 required for transport to the peroxisomes.

We thank Marika Kamps and Ville Ratas for their technical assistance. The Academy of Finland and the Sigrid Jusélius Foundation are acknowledged for financial support.

REFERENCES

- Uchida, Y., Izai, K., Orii, T. and Hashimoto, T. (1992) Novel fatty acid β -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.
- Osumi, T. and Hashimoto, T. (1979) Peroxisomal β -oxidation system of rat liver. Copurification of enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase. *Biochem. Biophys. Res. Commun.* **89**, 580–584.
- Gühnemann-Schäfer, K. and Kindl, H. (1995) Fatty acid β -oxidation in glyoxysomes. Characterization of a new tetrafunctional protein (MFP III). *Biochim. Biophys. Acta* **1256**, 181–186.
- Binstock, J. F., Pramanik, A. and Schulz, H. (1977) Isolation of a multi-enzyme complex of fatty acid oxidation from *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **74**, 492–495.
- Pramanik, A., Pawar, S., Antonian, E. and Schulz, H. (1979) Five different enzymatic activities are associated with the multienzyme complex of fatty acid oxidation from *Escherichia coli*. *J. Bacteriol.* **137**, 469–473.
- Yang, S. Y., Li, J. M., He, X. Y., Cosloy, S. D. and Schulz, H. (1988) Evidence that the *fadB* gene of the *fadAB* operon of *Escherichia coli* encodes 3-hydroxyacyl-coenzyme A (CoA) epimerase, Δ^3 -cis- Δ^2 -trans-enoyl-CoA isomerase, and enoyl-CoA hydratase in addition to 3-hydroxyacyl-CoA dehydrogenase. *J. Bacteriol.* **170**, 2543–2548.
- Palosaari, P. M. and Hiltunen, J. K. (1990) Peroxisomal bifunctional protein from rat liver is a trifunctional enzyme possessing 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and Δ^3 , Δ^2 -enoyl-CoA isomerase activities. *J. Biol. Chem.* **265**, 2446–2449.
- Zhang, D., Yu, W., Geisbrecht, B. V., Gould, S. J., Sprecher, H. and Schulz, H. (2002) Functional characterization of Δ^3 , Δ^2 -enoyl-CoA isomerases from rat liver. *J. Biol. Chem.* **277**, 9127–9132.
- Gurvitz, A., Mursula, A. M., Firzinger, A., Hamilton, B., Kilpeläinen, S. H., Hartig, A., Ruis, H., Hiltunen, J. K. and Rottensteiner, H. (1998) Peroxisomal Δ^3 -cis- Δ^2 -trans-enoyl-CoA isomerase encoded by EC11 is required for growth of the yeast *Saccharomyces cerevisiae* on unsaturated fatty acids. *J. Biol. Chem.* **273**, 31366–31374.
- Müller-Newen, G., Janssen, U. and Stoffel, W. (1995) Enoyl-CoA hydratase and isomerase form a superfamily with a common active-site glutamate residue. *Eur. J. Biochem.* **228**, 68–73.
- Gerlt, J. A. and Babbitt, P. C. (1998) Mechanistically diverse enzyme superfamilies: the importance of chemistry in the evolution of catalysis. *Curr. Opin. Chem. Biol.* **2**, 607–612.
- Babbitt, P. C. and Gerlt, J. A. (1997) Understanding enzyme superfamilies. Chemistry as the fundamental determinant in the evolution of new catalytic activities. *J. Biol. Chem.* **272**, 30591–30594.
- Benning, M. M., Taylor, K. L., Liu, R. Q., Yang, G., Xiang, H., Wesenberg, G., Dunaway-Mariano, D. and Holden, H. M. (1996) Structure of 4-chlorobenzoyl coenzyme A dehalogenase determined to 1.8 Å resolution: an enzyme catalyst generated via adaptive mutation. *Biochemistry* **35**, 8103–8109.
- Engel, C. K., Mathieu, M., Zeelen, J. P., Hiltunen, J. K. and Wierenga, R. K. (1996) Crystal structure of enoyl-coenzyme A (CoA) hydratase at 2.5 Å resolution: a spiral fold defines the CoA-binding pocket. *EMBO J.* **15**, 5135–5145.
- Modis, Y., Filippula, S. A., Novikov, D. K., Norledge, B., Hiltunen, J. K. and Wierenga, R. K. (1998) The crystal structure of dienoyl-CoA isomerase at 1.5 Å resolution reveals the importance of aspartate and glutamate sidechains for catalysis. *Structure (London)* **6**, 957–970.
- Benning, M. M., Haller, T., Gerlt, J. A. and Holden, H. M. (2000) New reactions in the crotonase superfamily: structure of methylmalonyl CoA decarboxylase from *Escherichia coli*. *Biochemistry* **39**, 4630–4639.
- Mursula, A. M., van Aalten, D. M., Hiltunen, J. K. and Wierenga, R. K. (2001) The crystal structure of Δ^3 - Δ^2 -enoyl-CoA isomerase. *J. Mol. Biol.* **309**, 845–853.
- Noyes, B. E. and Bradshaw, R. A. (1973) L-3-hydroxyacyl coenzyme A dehydrogenase from pig heart muscle. I. Purification and properties. *J. Biol. Chem.* **248**, 3052–3059.
- Barycki, J. J., O'Brien, L. K., Bratt, J. M., Zhang, R., Sanishvili, R., Strauss, A. W. and Banaszak, L. J. (1999) Biochemical characterization and crystal structure determination of human heart short chain L-3-hydroxyacyl-CoA dehydrogenase provide insights into catalytic mechanism. *Biochemistry* **38**, 5786–5798.
- Barycki, J. J., O'Brien, L. K., Birktoft, J. J., Strauss, A. W. and Banaszak, L. J. (1999) Pig heart short chain L-3-hydroxyacyl-CoA dehydrogenase revisited: sequence analysis and crystal structure determination. *Protein Sci.* **8**, 2010–2018.
- Rossmann, M. G., Moras, D. and Olsen, K. W. (1974) Chemical and biological evolution of nucleotide-binding protein. *Nature (London)* **250**, 194–199.
- Wierenga, R. K., Terpstra, P. and Hol, W. G. (1986) Prediction of the occurrence of the ADP-binding $\beta\alpha\beta$ -fold in proteins, using an amino acid sequence fingerprint. *J. Mol. Biol.* **187**, 101–107.
- Gould, S. J., Keller, G. A., Hosken, N., Wilkinson, J. and Subramani, S. (1989) A conserved tripeptide sorts proteins to peroxisomes. *J. Cell Biol.* **108**, 1657–1664.
- 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 β -oxidation in yeast. *J. Biol. Chem.* **270**, 27453–27457.
- Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. and Pease, L. R. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**, 51–59.
- Kiema, T. R., Engel, C. K., Schmitz, W., Filppula, S. A., Wierenga, R. K. and Hiltunen, J. K. (1999) Mutagenic and enzymological studies of the hydratase and isomerase activities of 2-enoyl-CoA hydratase-1. *Biochemistry* **38**, 2991–2999.
- Pace, C. N., Vajdos, F., Fee, L., Grimsley, G. and Gray, T. (1995) How to measure and predict the molar absorption coefficient of a protein. *Protein Sci.* **4**, 2411–2423.
- Gill, S. C. and von Hippel, P. H. (1989) Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.* **182**, 319–326.
- Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685.
- Rost, B. and Sander, C. (1993) Prediction of protein secondary structure at better than 70% accuracy. *J. Mol. Biol.* **232**, 584–599.
- Rost, B. and Sander, C. (1994) Combining evolutionary information and neural networks to predict protein secondary structure. *Proteins* **19**, 55–72.
- Gould, S. J., Keller, G. A. and Subramani, S. (1988) Identification of peroxisomal targeting signals located at the carboxy terminus of four peroxisomal proteins. *J. Cell Biol.* **107**, 897–905.
- Taskinen, J. P., Kiema, T.-R., Koivuranta, K. T., Wierenga, R. K. and Hiltunen, J. K. (2002) Crystallization and characterization of the dehydrogenase domain from rat peroxisomal multifunctional enzyme type 1. *Acta Crystallogr. D Biol. Crystallogr.* **58**, 690–693.