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Site-directed mutagenesis to enable and improve crystallizability of *Candida tropicalis* (3*R*)-hydroxyacyl-CoA dehydrogenase

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Abstract

The N-terminal part of *Candida tropicalis* MFE-2 (MFE-2(h2Δ)) having two (3*R*)-hydroxyacyl-CoA dehydrogenases with different substrate specificities has been purified and crystallized as a recombinant protein. The expressed construct was modified so that a stabile, homogeneous protein could be obtained instead of an unstabile wild-type form with a large amount of cleavage products. Cubic crystals with unit cell parameters $a = 74.895$, $b = 78.340$, $c = 95.445$, and $\alpha = \beta = \gamma = 90°$ were obtained by using PEG 4000 as a precipitant. The crystals exhibit the space group $P2_12_12_1$ and contain one molecule, consisting of two different (3*R*)-hydroxyacyl-CoA dehydrogenases, in the asymmetric unit. The crystals diffract to a resolution of 2.2 Å at a conventional X-ray source.

Keywords: 17β-HSD; MFE-2; β-Oxidation; Peroxisome; SDR

Fatty acid degradation in living organisms occurs mainly via β-oxidation, which is a spiral pathway consisting of four enzymatic steps. Following oxidation of fatty acyl-CoA esters at the β-carbon, the spiral continues by addition of a hydroxy group to the β-carbon; this hydroxyl is further oxidized in the third step. The multifunctional enzymes (MFEs) catalyzing these steps contain the latter two activities, 2-enoyl-CoA hydratase (''hydratase'') and hydroxyacyl-CoA dehydrogenase (''dehydrogenase''). MFEs can also have additionally either Δ^3-Δ^2-enoyl-CoA isomerase or 3-ketoacyl-CoA thiolase activities, depending on species and cellular origin of the enzyme. Each cycle of the spiral shortens the CoA-activated fatty acid by two carbons, since the last step of β-oxidation involves a thiolytic cleavage at the β-carbon and re-entering of the remaining fatty acyl body into the cycle.

Two types of multifunctional enzymes exist in mammalian peroxisomes: MFE type 1 and 2. They are not sequence related. Based on kinetic in vitro properties of the enzyme, MFE-1 was originally thought to be responsible for the β-oxidation of straight chain fatty acids [1–3]. However, MFE-1 null mutant mice are both clinically and biochemically healthy, leaving the function of MFE-1 under in vivo conditions an enigma [4,5]. In contrast to MFE-1, a number of MFE-2 deficiencies have been identified in peroxisomal disease patients suffering from psychomotor dysfunction, hypotonia, and craniofacial dysmorphosis. These patients show accumulation of both very long chain and α-methyl branched chain fatty acids as well as C27 intermediates of bile acid synthesis, demonstrating a key role of MFE-2 in the degradation of these metabolites in mammalian peroxisomes [6–8]. A striking difference between MFE-1 and MFE-2 is that the two-enzyme step from *trans*-2-enoyl-CoA to 3-keto-metabolites uses opposite stereochemistry with respect to the (3*R*)-hydroxyacyl-CoA intermediate [9,10]. Because the peroxisomal

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MFE-2 is the only protein catalyzing the second and third reactions in yeast, the β-oxidation in yeast proceeds only via (3*R*)-hydroxyacyl-CoA intermediates. Kinetic differences among the MFE proteins are also seen as differences in their structure, and are not limited to those between MFE-1 and MFE-2; structural variation exists also between yeast and mammalian peroxisomal MFE-2, the former having two copies of the (3*R*)-hydroxyacyl-CoA dehydrogenase instead of one in the same polypeptide and lacking a sterol carrier protein type 2 like domain.

The two copies of (3*R*)-hydroxyacyl-CoA dehydrogenase from *Candida tropicalis* show different substrate specificities. The two dehydrogenases have 40% identity with each other at the amino acid sequence level. The first domain, domain A or dehydrogenase A, catalyzes the reaction for medium and long chain fatty acids whereas domain B, or dehydrogenase B, shows preference for short chain substrates, although it also accepts substrates of medium and long chain lengths. Both of these domains belong to the short chain dehydrogenase/reductase (SDR) family, with domain A being more similar in amino acid sequence to the mammalian enzymes [11].

Short chain dehydrogenases/reductases constitute a large family of NAD(P)(H) dependent enzymes. Presently, over 60 enzymes belonging to the SDR family have been characterized from human [12–14]. Although the sequence identity (10–30%) is low between enzymes, the conformational patterns of over 20 available three-dimensional structures are highly similar α/β folds [15–21]. Enzymes belonging to this family can be identified by a certain set of conserved residues, namely the N-terminal Gly-X-X-X-Gly-X-Gly motif, which forms part of the nucleotide cofactor binding region, and the Ser-Tyr-Lys triad in the active site. The catalytic Tyr is the most conserved residue within the whole family.

As part of our effort to understand how MFE-2s work, we have produced a recombinant, truncated variant of yeast MFE-2 (MFE-2(h2Δ)) containing only both (3*R*)-hydroxyacyl-CoA dehydrogenases, but lacking the 2-enoyl-CoA hydratase 2 domain. Via stabilizing modifications the protein could be purified to homogeneity and crystallized. Results are presented on preliminary crystallographic analysis as well as on protein engineering to arrive at a crystallizable species.

Materials and methods

Modifications of the construct. The cloning of yMFE-2(1–591) and yMFE-2(1–612) encoding amino acid residues 1–591 and 1–612 of *C. tropicalis* MFE-2 into pET3a has been described previously [11,22]. The 8 aa long hydrophilic tail at the C-terminus of the latter was removed to get yMFE-2(1–604) by introducing a stop codon by site-directed mutagenesis with the primers: 5′-G TCT GCC GTT GGT GGT GAT **TGA** GAT GAT GAT GAC GAA GAC G-3′ and 3′-C GTC TTC GTC ATC ATC ATC **TCA** ATC ACC ACC AAC GGC AGA C-5′. The stop codon is marked with bold letters. Site-directed mutagenesis was performed according to the instructions of QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The cleaved peptide was separated from the rest of the protein by reverse phase chromatography and the cleavage site of the protein was analyzed by N-terminal sequencing of the cleaved peptide. A C-terminal hexahistidine tag was introduced by site-directed mutagenesis. A cleavage site for Factor Xa was introduced in front of the eight histidine residues. Primers for the mutagenesis were 5′-GCC GTT GGT GGT GAT att gag ggt cgc cac cac cac cac cac cac cac **tga** GAT GAT GAT GAC G-3′ and 3′-C GTC ATC ATC ATC **tca** gtg gtg gtg gtg gtg gtg gtg gtg gcg acc ctc aat ATC ACC ACC AAC GGC-5′. The Factor Xa cleavage site is marked with underlined letters, stop codon with bold letters, and the lowercase letters indicate the mismatches to the (3*R*)-hydroxyacyl-CoA dehydrogenase gene.

Candida tropicalis (3*R*)-hydroxyacyl-CoA dehydrogenase tends to be cleaved at the substrate binding site of domain B in three positions: after amino acids Leu505, Ile507 or Phe508. To prevent the unwanted cleavage of the (3*R*)-hydroxyacyl-CoA dehydrogenase during purification, the cleavage site was mutated to resemble more the consensus between the site in human, rat, *Saccharomyces cerevisiae* (domain A), and *C. tropicalis* (domain A) sequences. $T_{506}IF_{508}$ to $S_{506}IM_{508}$-mutations were introduced to the cleavage site by site-directed mutagenesis to get the variant yMFE-2(1–604dm). Primers for the mutagenesis were 5′-CCA CAC GCT GAA ACT GCC ATG ACC TTG agc ATC atg AGA GAA CAA GAC AAG AAC TTG-3′ and 3′-CAA GTT CTT GTC TTG TTC TCT cat GAT gct CAA GGT CAT GGC AGT TTC AGC GTG TGG-5′, with the lowercase letters indicating the mismatches to the gene of (3*R*)-hydroxyacyl-CoA dehydrogenases.

Expression and purification. Expression of (3*R*)-hydroxyacyl-CoA dehydrogenase was performed by propagating BL21 Codon Plus (DE3)-RIL *Escherichia coli* cells (Stratagene, La Jolla, CA, USA) harboring pET3a::(3*R*)-hydroxyacyl-CoA dehydrogenase according to manufacturer's instructions. Induction was carried out for 3 h at 306 K in the presence of 0.4 mM isopropyl-1-thio-β-D-galactoside. Cells were collected by centrifugation at 4100*g* for 20 min, washed with 16 mM potassium phosphate, 120 mM NaCl, pH 7.4, and recentrifuged and the cell pellet was stored in −70 °C until used.

Bacterial cells from 0.5 L of cell culture (3.5 g) were suspended in 35 ml ice-cold lysis buffer (20 mM Hepes, pH 7.9, 500 mM NaCl, and 5% glycerol) and broken by sonication. The sonication was done in ~15 ml batches for 10 × 30 s, with 30 s resting intervals in plastic tubes cooled on ice using Soniprep 150 Ultrasonic Disintegrator (SANYO Gallenkamp PLC, Berkshire, UK) equipped with a probe 9.5 mm in diameter. The suspension was then centrifuged at 20,000*g*, for 45 min in 4 °C to remove cell debris. The supernatant was applied on a Ni–NTA column (0.75 cm × 1.3 cm, Novagen, Madison, WI, USA) equilibrated with the lysis buffer. The column was washed with the same buffer, and (3*R*)-hydroxyacyl-CoA dehydrogenase was eluted from the column by 80 ml linear gradient of 0–200 mM imidazole (pH 7.9). Fractions containing (3*R*)-hydroxyacyl-CoA dehydrogenase were pooled, diluted 2-fold with 20 mM Mes buffer (pH 6.0, 40 mM NaCl, 5% glycerol), and dialyzed against the same buffer overnight. After that the sample was applied to a 1 ml Resource S column (Amersham Biosciences, Freiburg, Germany) also equilibrated with the MES buffer. (3*R*)-hydroxyacyl-CoA dehydrogenase bound to the column was eluted by a 30 ml linear gradient of 0–150 mM NaCl. As the final step of purification the sample containing (3*R*)-hydroxyacyl-CoA dehydrogenase was applied to a Superdex 200 HR 10/30 column (Amersham Biosciences) in equilibrium of 30 mM Pipes buffer (pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1 mM NaN_3). The fractions containing pure (3*R*)-hydroxyacyl-CoA dehydrogenase were pooled, concentrated to 5 mg/ml, and stored at 283 K. Substrate synthesis and enzyme activity measurements were done as described earlier [11].

Crystallization and data collection. Crystallization was carried out by hanging drop vapor-diffusion technique at 293 K. Crystallization trials were made by using the sparse matrix approach [23] using Crystal Screen and Crystal Screen II kits (Hampton Research, Aliso Viejo, CA, USA). Each drop contained 2 μl protein solution (5 mg/ml) and 2 μl precipitant solution. Crystals were optimized by adjusting the pH. Cubic crystals with a hollow in them grew in 30% PEG 4000, 0.1 M sodium acetate, pH 4.2, 0.2 M ammonium acetate. X-ray diffraction data were collected from crystals flash-frozen in liquid nitrogen at 100 K using a rotating-anode generator and a MAR345 image-plate detector. Glycerol (11% (v/v)) added to mother liquor was found to be a suitable cryo-protectant. Intensity data were processed using XDS [24].

Results

The MFE-2 from *C. tropicalis* was truncated for C-terminal hydratase 2, and the remaining part, containing two (3*R*)-hydroxyacyl-CoA dehydrogenases, was cloned aiming to obtain a dehydrogenase with two modules having different substrate specificities. However, it turned out that the production of a stabile recombinant MFE-2(h2Δ) was not trivial due to occurring proteolysis and determination of boundaries for functional (3*R*)-hydroxyacyl-CoA dehydrogenases. The roadmap leading over these obstacles is summarized in Fig. 1.

Candida tropicalis (3*R*)-hydroxyacyl-CoA dehydrogenase domains A and B were first expressed as separate domains, but because they were not active (unpublished results), new constructs were planned by comparing the secondary structure prediction of *C. tropicalis* MFE-2 with the sequences of *C. tropicalis*, *S. cerevisiae*, and rat MFE-2 in the multiple amino acid sequence alignment (Fig. 2). (3*R*)-hydroxyacyl-CoA dehydrogenase from rat liver has been purified as two different N-terminal cleavage products of MFE-2. These two alternative cleavage sites in rat MFE-2 were after amino acids 312 and 316, as shown in Fig. 2 [2,25,26]. Using this piece of information, *C. tropicalis* yMFE-2(1–591) containing amino acids 1–591 was generated and purified [22]. Although fresh yMFE-2(1–591) was active, the preparation was inactivated within a few days. Thus, the construct should contain at least the last α-helix, but probably need not exceed amino acid 617, which represents the physiological cleavage site with respect to the rat enzyme (Fig. 2B). The variant yMFE-2(1–612) (67 kDa) was stabile, but possessed properties that adversely affected its crystallizability: the purified protein

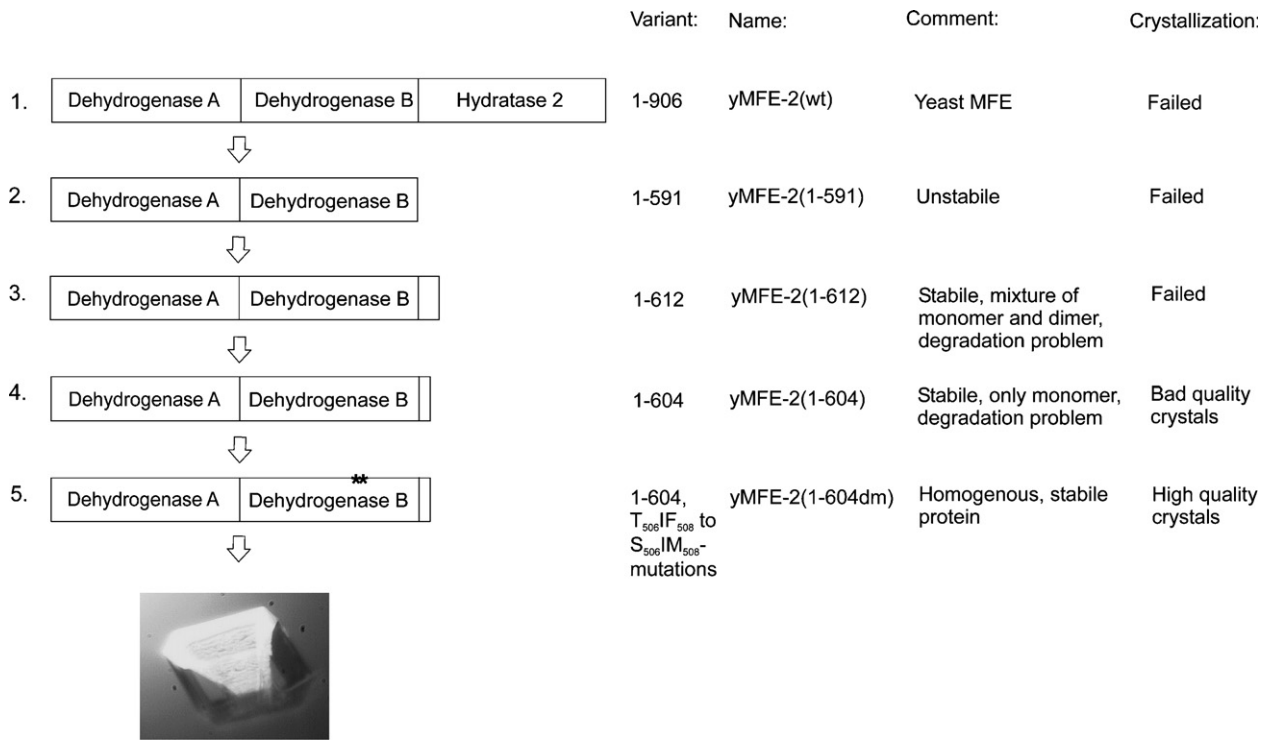

Fig. 1. Roadmap to generate crystals of MFE-2 dehydrogenases suitable for X-ray crystallography. Numbers indicate the amino acids included in the wild-type and different variants of *C. tropicalis* MFE-2. In addition to modifying the length of the construct, two point mutations were also needed to get high-quality crystals. The second construct, yMFE-2(1–591), yielded an active but unstabile protein. The problem was solved by extending the protein with 21 C-terminal amino acid residues. The new variant, yMFE-2(1–612), did, however, have other problems: it existed as a mixture of monomer and dimer, and additionally it was cleaved at the substrate binding area of domain B. Shortening the construct with eight hydrophilic amino acids helped to keep the protein monomeric. This construct, yMFE-2(1–604), yielded disordered crystals not suitable for data collection. Double mutation, yMFE-2(1–604dm), was introduced in the cleavage site, and this solved the degradation problem, and high-quality, cubic crystals shown in the figure were grown as described in the text.

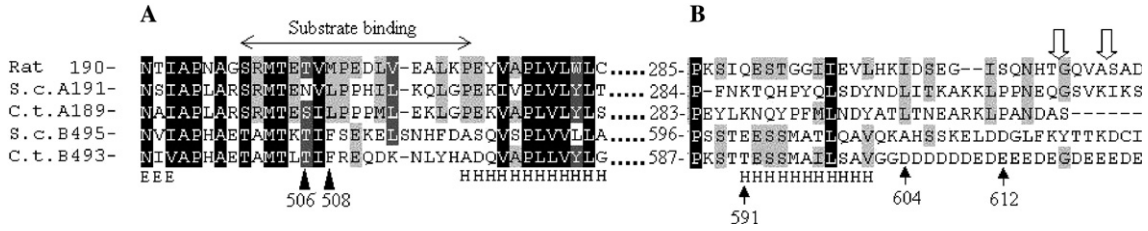

Fig. 2. Multiple sequence alignment of the (3*R*)-hydroxyacyl-CoA dehydrogenases from yeast and rat MFE-2s including the substrate binding area and the C-terminal part of dehydrogenases. Both domains A and B of *C. tropicalis* and *S. cerevisiae* (3*R*)-hydroxyacyl-CoA dehydrogenases were taken into the alignment as separate entities, although they occur as one polypeptide in nature. Secondary structure prediction is made by JPred server [31] for the domain B of *C. tropicalis* (3*R*)-hydroxyacyl-CoA dehydrogenase with E standing for β-strands and H for α-helices. (A) Substrate binding area is predicted by comparing these sequences with the substrate binding area of *E. coli* 7α-hydroxysteroid dehydrogenase [20], a member of the SDR family with the known 3D structure. *C. tropicalis* (3*R*)-hydroxyacyl-CoA dehydrogenase tends to be cleaved at the substrate binding site after amino acids Leu505, Ile507 or Phe508. To avoid the cleavage of this protein, Thr506 was mutated to Ser and Phe508 to Met (triangles). (B) Constructs of *C. tropicalis* (MFE-2(h2Δ)) were planned by comparing secondary structure prediction of *C. tropicalis* MFE-2 with the sequences of *C. tropicalis*, *S. cerevisiae*, and mammalian MFE-2s. N-terminal cleavage products of rat MFE-2 (open arrows) including amino acids 1–312 and 1–316 with (3*R*)-hydroxyacyl-CoA dehydrogenase activity have been purified from rat liver [2,25,26]. Last amino acids of different constructs (Fig. 1) of *C. tropicalis* MFE-2(h2Δ) are also marked (filled arrows). Two longer constructs yMFE-2(1–604) and yMFE-2(1–612) were stabile, but they tend to be cleaved at the substrate binding area. The only construct which could be purified to apparent homogeneity and yielded good-quality crystals included amino acids 1–604 and contained Thr(506)IlePhe(508) to Ser(506)IleMet(508) mutations (yMFE-2(1–604dm)).

preparation equilibrated as a mixture of monomers and dimers, and it was also prone to cleavage to a 55 kDa species. Removing eight residues from the C-terminus totally excluded the charged amino acid segment (Fig. 2B) and brought the C-terminus closer to the end of the last predicted α-helix. The resulting construct yMFE-2(1–604) was stabile, remained a monomer, justified by size exclusion chromatography, and could be crystallized. The crystals were, however, disordered and the space group could not be determined. The quality of the crystals was adversely affected by the persisting problem of degradation at the substrate binding area of the (3*R*)-hydroxyacyl-CoA dehydrogenase domain B (Fig. 2A). The cleavage site was not very specific, being alternatively after residues Leu505, Ile507 or Phe508. After such cleavage (3*R*)-hydroxyacyl-CoA dehydrogenase was not active with short chain substrates, because the protein was lacking the substrate-binding loop of domain B.

Because the protease inhibitors tested (0.5 mM benzamidine hydrochloride hydrate, 0.5 mM dithiothreitol, 5 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycolbis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, and 1 mM phenylmethylsulfonyl fluoride) did not prevent the degradation, mutations were planned to the cleavage site to prevent the cleavage of the protein. Mutations were designed by comparing the N-terminal parts of rat, *S. cerevisiae*, and *C. tropicalis* peroxisomal MFE-2 with each other (Fig. 2A). Thr506 was mutated to Ser according to the *C. tropicalis* domain A. Phe508 was mutated to Met, which makes this site more like those in rat and human MFE-2. These mutations ($T_{506}IF_{508}$ to $S_{506}IM_{508}$) solved the problem of degradation. If purification is carried out in 1 week and temperatures >24 °C are avoided during purification, it is possible to purify yeast MFE-2 double mutant yMFE-2(1–604dm) with only a very minor degree of degradation. The purification protocol yielded yMFE-2(1–604dm) to apparent homogeneity as justified by SDS–polyacrylamide gel analysis. About 5 mg pure protein was obtained per 0.5 L of bacterial culture using this method.

The crystals of the yMFE-2(1–604dm) grew in 1 month to a maximum size of 0.27 mm × 0.2 mm and diffracted to 2.2 Å resolution. The properties of these crystals are summarized in Table 1. Since a minor amount of cleavage was detected as a function of time, crystallizations had to be made immediately after the protein was purified. In order to measure the activity of the modified domain B, domain A was inactivated by G15S mutation at the NAD-binding site according to the procedure we used earlier [11]. Although the stabilizing mutations were done on the substrate binding

Table 1
Data collection statistics

Space group	$P2_12_12_1$
Unit cell parameters	
a (Å)	74.895
b (Å)	78.340
c (Å)	95.445
Oscillation range (°)	0.5
Matthews coefficient V_m (Å^3/Da)	2.1
Solvent content (%)	41
Resolution (Å)	20–2.22 (2.36–2.22)
R_{merge} (%)[a]	10.2 (29.7)
Completeness (%)	98.7 (96.4)
$I/\sigma I$	12.56 (4.74)
Unique reflections	27948
Redundancy	4.4
Mosaicity (°)	3.0

Values in parentheses are for the highest resolution shell.
[a] $R_{merge} = \sum_h \sum_i |I - I_i| / \sum_h \sum_i I_i$.

area of domain B, this domain had maintained part of its enzymatic activity. The activity of the modified domain B with a short chain substrate, (3*R*)-hydroxybutyryl-CoA, was 5.9 % compared to the wild type domain B (k_{cat} = 1.7 s^{-1} vs. 29 s^{-1}), whereas with a medium-chain substrate, (3*R*)-hydroxydecanoyl-CoA, it had remained at the same level (k_{cat} = 19.6 s^{-1} vs. 17 s^{-1}).

The native data set for crystal structure determination was collected by the rotation method with 0.5° rotations per frame at a wavelength of 1.54179 Å. Statistics are given in Table 1. The crystals belong to the orthorhombic space group $P2_12_12_1$ with unit cell dimensions a = 74.895 Å, b = 78.340 Å, c = 95.445 Å, and $\alpha = \beta = \gamma = 90°$. The assumption of one molecule per asymmetric unit was made based on Matthews coefficient value of 2.1 $Å^3$/Da. This indicates the solvent content to be 41%, which is typical for protein crystals [27].

Discussion

The strategies to overcome the steric problems of β-oxidation of a large variety of fatty acyl-CoA substrates with various modifications and chain lengths are intimately linked to the evolution of β-oxidation enzymes as paralogues and adaptive active site pockets. In this respect, the yeast peroxisomal MFE-2 is a unique protein, since it contains two (3*R*)-hydroxyacyl-CoA dehydrogenases (domains A and B) in the same polypeptide showing different substrate specificities instead of one dehydrogenase of a broader substrate specificity, like mammals (1). Both dehydrogenases belong to the short chain dehydrogenase/reductase family (SDR-family), dehydrogenase A being more similar to the dehydrogenase in the mammalian MFE-2, and dehydrogenase B bearing more resemblance to non-MFE-2 dehydrogenases. Consequently, domain A is active with medium and long chain (3*R*)-hydroxyacyl-CoA substrates, whereas domain B shows the highest activity with short-chain substrates [11].

Our long-term goal is to understand the structural basis for the observed substrate specificity difference of dehydrogenase domains A and B. In the beginning we noticed that the modified MFE-2, where the hydratase has been removed and where the dehydrogenases A and B were produced as one polypeptide, tends to be cleaved at the substrate binding area of domain B. Furthermore, the protein also occurred as a mixture of monomers and dimers. Careful comparison of yeast MFE-2 sequence to sequences of other MFE-2s led us to design and subsequently screen a construct that appeared only as a monomer, was not degraded, and was inclined to purification to apparent homogeneity. Due to homogeneity and stability of the protein, well-diffracting crystals could be obtained.

It should also be noted that domain B was on purpose not made identical to domain A at the cleavage site to preserve as much as possible of the determinants of substrate specificity. The fact that the modified domain B is still active towards a short-chain substrate, although with decreased catalytic efficiency, indicates that the specificity determinants still apply.

We have recently solved the structures of the dehydrogenase of rat MFE-2 [28] and hydratase 2 of *C. tropicalis* MFE-2 [29]. In these works, as well as in a previous study of the SCP-2-like domain of human MFE-2 [30], we followed the same approach, where the functional subunits of MFE under study were cleaved from the full-length enzyme and crystallized as separate proteins. As a result of the generation of stabile *C. tropicalis* (3*R*)-hydroxyacyl-CoA dehydrogenase, we are in the process of solving the structure of *C. tropicalis* (3*R*)-hydroxyacyl-CoA dehydrogenase using the rat dehydrogenase structure as a model. Parallel to structure determination, constructing several variations of the mutated substrate binding area of the domain B is in process in order to increase its activity for short-chain substrates towards the level of the native protein.

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