

Substrate specificities of peroxisomal members of short-chain alcohol dehydrogenase superfamily: expression and characterization of dehydrogenase part of *Candida tropicalis* multifunctional enzyme

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Abstract In addition to several other enzymes, the short-chain alcohol dehydrogenase superfamily includes a group of peroxisomal multifunctional enzymes involved in fatty acid and cholesterol side-chain β -oxidation. Mammalian peroxisomal multifunctional enzyme type 2 (perMFE-2) is a 2-enoyl-CoA hydratase-2/(R)-3-hydroxyacyl-CoA dehydrogenase. As has been shown previously, perMFE-2 hydrates (24E)-3 α ,7 α ,12 α -trihydroxy-5 β -cholest-24-enoyl-CoA to (24R,25R)-3 α ,7 α ,12 α ,24 ξ -tetrahydroxy-5 β -cholestanoyl-CoA, which has been characterized as a physiological intermediate in cholic acid synthesis. Out of four possible stereoisomers of 3 α ,7 α ,12 α ,24 ξ -tetrahydroxy-5 β -cholestanoyl-CoA, the mammalian perMFE-2 dehydrogenates only the (24R,25R)-isomer. The yeast peroxisomal multifunctional enzyme (MFE) was first described as 2-enoyl-CoA hydratase-2/(R)-3-hydroxyacyl-CoA dehydrogenase. To investigate the stereospecificity of yeast peroxisomal MFE, the two dehydrogenase domains of *C. tropicalis* MFE were expressed in *E. coli* as a 65 kDa recombinant protein. This protein catalyzes the dehydrogenation of straight-chain (R)-3-hydroxyacyl-CoAs, but it is devoid of (S)-3-hydroxyacyl-CoA dehydrogenase and 2-enoyl-CoA hydratase activities. The protein dehydrogenates (24R,25R)- and (24R,25S)-isomers of 3 α ,7 α ,12 α ,24 ξ -tetrahydroxy-5 β -cholestanoyl-CoA. Interestingly, the protein also shows 17 β -estradiol dehydrogenase activity. ■ As a monofunctional (R)-specific 3-hydroxyacyl-CoA dehydrogenase is currently unavailable, this recombinant enzyme can be used to study the stereochemistry of bile acid synthesis.—Qin, Y.-M., M. H. Poutanen, and D. K. Novikov. **Substrate specificities of peroxisomal members of short-chain alcohol dehydrogenase superfamily: expression and characterization of dehydrogenase part of *Candida tropicalis* multifunctional enzyme.** *J. Lipid Res.* 2000. 41: 93–98.

Supplementary key words short-chain alcohol dehydrogenase superfamily • peroxisomes • fatty acids • bile acids

The short-chain alcohol dehydrogenase (SCAD) superfamily is a phylogenetically related group of enzymes that act on substrates as diverse as steroids, fatty acids, bile acids, aro-

matic hydrocarbons, sugars, antibiotics, and compounds involved in nitrogen metabolism (1). Multiple sequence alignment of proteins of the SCAD superfamily has revealed 372 sequences in the ProDom database (ProDom 36, domain 16) (2). The family contains a subfamily of closely related proteins (Fig. 1) involved in peroxisomal β -oxidation of fatty acids in *Saccharomyces cerevisiae* and *Candida tropicalis* (3, 4), as well as in mammals (5–9). The mammalian sequence was recently found to be an integral part of the peroxisomal multifunctional (hydratase-dehydrogenase) enzyme type 2 (perMFE-2) (6, 8). The N-terminal part of perMFE-2 displays dehydrogenase activity toward (R)-3-hydroxyacyl-CoAs and the (24R,25R)-stereoisomer of 3 α ,7 α ,12 α ,24 ξ -tetrahydroxy-5 β -cholestanoyl-CoA (24-OH-THCA-CoA), which is an intermediate in cholic acid synthesis (6, 7).

Both the peroxisomal and mitochondrial β -oxidation systems catalyze the same set of reactions of the β -oxidation cycle (10). Shortening of fatty acids in each turn of the cycle occurs via four consecutive reactions: 1) the acyl-CoAs are desaturated to 2-*trans*-enoyl-CoAs; 2) a hydration reaction converts the enoyl-CoAs to 3-hydroxyacyl-CoAs; 3) an NAD⁺-linked dehydrogenation forms 3-ketoacyl-CoAs from the 3-hydroxyacyl-CoAs; and 4) a thiolitic cleavage yields acetyl-CoA and an acyl-CoA shortened by two carbon atoms that can re-enter the β -oxidation cycle (10).

Although peroxisomal and mitochondrial β -oxidation pathways are similar, the particular enzymes in each system are different. Furthermore, there is a difference in the stereo-

Abbreviations: SCAD, short-chain alcohol dehydrogenase; yeast MFE, yeast multifunctional 2-enoyl-CoA hydratase-2/(R)-3-hydroxyacyl-CoA dehydrogenase; perMFE-1, peroxisomal multifunctional Δ^3,Δ^2 -enoyl-CoA isomerase/2-enoyl-CoA hydratase-1/(S)-3-hydroxyacyl-CoA dehydrogenase; perMFE-2, peroxisomal multifunctional 2-enoyl-CoA hydratase-2/(R)-3-hydroxyacyl-CoA dehydrogenase; 24-OH-THCA, 3 α ,7 α ,12 α ,24 ξ -tetrahydroxy-5 β -cholestanic acid; HAD, 3-hydroxyacyl-CoA dehydrogenase.

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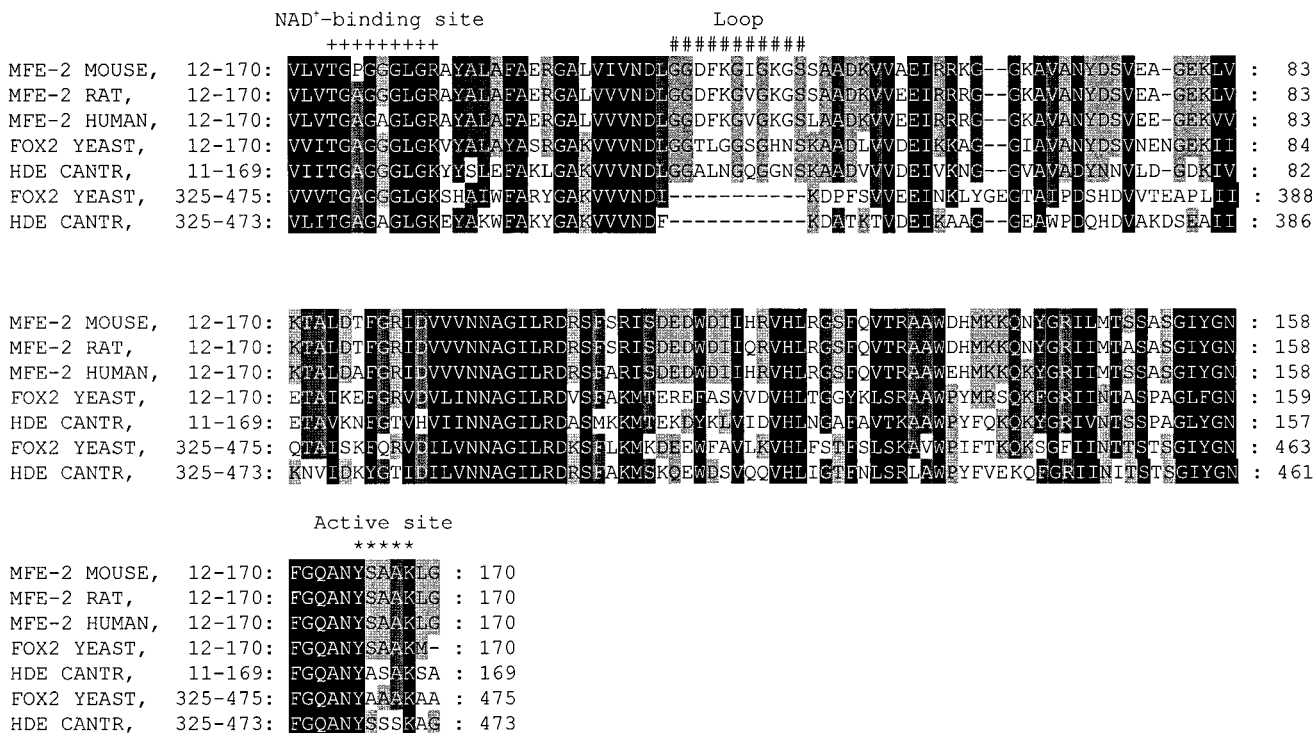


Fig. 1. Homology of dehydrogenase domains of mammalian peroxisomal MFE-2 and yeast (*S. cerevisiae* and *C. tropicalis*) MFE. The alignment reveals 372 members of short chain alcohol dehydrogenase super family (<http://www.toulouse.inra.fr/prodom>) (2). Only peroxisomal members of the family are shown on the figure. These members possess an GG×××G×G××S loop region missing from the non-peroxisomal SCAD proteins. The NAD⁺-binding and active sites are highly conserved throughout the SCAD family.

chemistry of the two mammalian β -oxidation systems. It has been shown that mitochondrial 2-enoyl-CoA hydratase-1 converts 2-enoyl-CoA esters to (S)-3-hydroxyacyl-CoA esters, whereas the peroxisomal β -oxidation system contains two multifunctional enzymes, perMFE-1 and perMFE-2, which display different stereochemistry (6–9). Peroxisomal MFE-1 displays 2-enoyl-CoA hydratase-1 and (S)-3-hydroxyacyl-CoA dehydrogenase activities (6–9, 11) and peroxisomal MFE-2 displays 2-enoyl-CoA hydratase-2 and (R)-3-hydroxyacyl-CoA dehydrogenase activities (6–9).

Substrates of mammalian perMFE-2 are CoA derivatives of bile acid intermediates, straight- and branched-chain fatty acids (5–9). Surprisingly, as has been shown by Adamski et al. (12), the dehydrogenase domain of perMFE-2 additionally displays 17 β -steroid dehydrogenase activity.

In lower eukaryotes such as yeast and fungi, peroxisomes appear to be the only subcellular organelles involved in β -oxidation (13, 14). The (R)-3-hydroxyacyl-CoA dehydrogenase has been described as an integral part of the peroxisomal multifunctional enzyme (MFE) in *C. tropicalis* and *S. cerevisiae* (3, 4). Furthermore, amino acid sequence comparison of the duplicated dehydrogenase domain of yeast MFEs reveals high homology to mammalian perMFE-2 (Fig. 1). In the present study we expressed and purified the recombinant dehydrogenase region of *C. tropicalis* MFE. The results demonstrate that the expressed 65 kDa protein shows substrate specificity similar to perMFE-2, suggesting a common ancestor for the yeast MFE and mammalian perMFE-2.

In comparison with mitochondrial (S)-3-hydroxyacyl-CoA dehydrogenase, there is no known monofunctional (R)-3-hydroxyacyl-CoA dehydrogenase available. Thus, the expressed monofunctional (R)-3-hydroxyacyl-CoA dehydrogenase can be useful for studies on stereochemical pathways of fatty and bile acid metabolism.

EXPERIMENTAL PROCEDURES

Materials

Sure-Clone ligation kit, restriction enzymes, T7 sequencing kit, coenzyme A, DNase, and RNase were obtained from Pharmacia Biotech (Uppsala, Sweden). Lactate dehydrogenase (from rabbit muscle) (S)-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) (from pig heart), NAD⁺, (R)- β -hydroxybutyric acid, acetoacetyl-CoA were obtained from Sigma Chemicals (St. Louis, MO). The plasmid pMK 22/HDE50 encoding the *Candida tropicalis* MFE was a gift from Dr. R. A. Rachubinski. Pure isomers of 3 α ,7 α ,12 α ,24 ξ -tetrahydroxy-5 β -cholestanoyl-CoA were synthesized and purified as previously described (5, 6). 3-Hydroxyacyl-CoAs and 2-*trans*-enoyl-CoAs were prepared by the mixed anhydride method (15). Estradiol and estrone were obtained from Steraloids and [³H]estrone and [³H]estradiol from DuPont (NEN, Boston, MA).

Expression of (R)-3-hydroxyacyl-CoA dehydrogenase (HAD)

The DNA encoding amino acid residues 1–591 of *Candida tropicalis* peroxisomal MFE was obtained by PCR from the plas-

mid pMK22/HDE50 containing the full-length *Candida tropicalis* MFE gene. Primers used were the following: 5'-primer F1, 5'-cactcatATGCTCTCCAGTTGATTTTAAA-3' and 3'-primer R1, 5'-cactctggatccttaGGTGGTGTAGATTTTGATTTTC-3', which include restriction sites for NdeI and BamHI (underlined). Upper case letters indicate identity with the *Candida tropicalis* MFE gene. The resulting 1773 bp PCR fragment was subcloned into pUC18 using the SureClone ligation kit. The 1773 bp-fragment was then digested out of pUC18 with NdeI and BamHI and subsequently cloned into the pET-3a expression vector (Novagen, Milwaukee, WI) yielding the plasmid pET-HAD. The insert was verified using the sequencing by dideoxynucleotide chain termination method and the pET-HAD plasmid was subsequently transformed into *E. coli* BL21 (DE3) plysS cells.

Enzyme assays

The (S)- and (R)-3-hydroxyacyl-CoA dehydrogenase activities were measured spectrophotometrically by following the formation of the magnesium complex of 3-ketodecanoyl-CoA at 303 nm using an absorption coefficient of $13,500 \text{ M}^{-1} \times \text{cm}^{-1}$ (4). The racemic mixture of 3-hydroxyacyl-CoA was first incubated with (S)-3-hydroxyacyl-CoA dehydrogenase as an auxiliary enzyme to remove the (S)-isomer. The assay mixture consisted of 50 μmol Tris-HCl, pH 9.0, 50 μg of defatted BSA, 50 μmol KCl, 25 μmol MgCl_2 , 1 μmol pyruvate, 1 μmol NAD^+ , 10 μg lactate dehydrogenase, 10 μg (S)-3-hydroxyacyl-CoA dehydrogenase and (R,S)-3-hydroxyacyl-CoA (5–200 μM) in a total volume of 1 ml. The reaction was allowed to proceed until the (S)-isomer was completely oxidized. Subsequently, a sample containing (R)-3-hydroxyacyl-CoA dehydrogenase was added to convert the remaining (R)-isomer.

Fluorimetric monitoring of NAD^+ reduction was used for the measurement of the 3-hydroxyacyl-CoA dehydrogenase activity with pure stereoisomers of $3\alpha,7\alpha,12\alpha,24\xi$ -tetrahydroxy-5 β -cholestanoyl-CoA (5). Reactions were started by adding 0.1 ml of enzyme solution, appropriately diluted in 0.125 M Tris-HCl, pH 8.0, to the incubation mixture in a microfluorescence cell resulting in a total volume of 0.5 ml and a final concentration of 25 mM Tris-HCl, pH 8.0, 1 mM NAD^+ , 60 mM hydrazine, pH 8.0, 50 mM KCl, 0.01% (w/v) Triton X-100, 0.05% (w/v) defatted BSA, and 25 μM substrate (5). Fluorescence readings (excitation 340 nm, slit 2 nm; emission 460 nm, slit 8 nm) were standardized by means of 0.5 and 1 μM NADH solutions. Appropriate blanks, measured in the absence of enzyme, were always performed.

Acetoacetyl-CoA reductase (reverse 3-hydroxyacyl-CoA dehydrogenase reaction) activity was measured by monitoring the acetoacetyl-CoA-dependent oxidation of NADH at 340 nm, as described by Furuta et al. (16).

Activity measurements of 17 β -hydroxysteroid dehydrogenase were performed in vitro as previously described (17). The final substrate concentration was 40 nM to 400 μM .

Hydratase activity was measured as the hydration of 2-*trans*-enoyl-CoAs of straight-chain fatty acids as described previously (6). The reaction mixture consisted of 0.3 M Tris-HCl, pH 7.5, 0.05% (w/v) defatted BSA and 50 μM 2-*trans*-decanoyl-CoA or 2-*trans*-hexenoyl-CoA in a final volume of 250 μl . The reaction was started by adding the enzyme and the absorbance of the double bond was monitored at 263 nm ($\epsilon = 7600 \text{ M}^{-1} \times \text{cm}^{-1}$).

Purification of recombinant monofunctional (R)-3-hydroxyacyl-CoA dehydrogenase

M9ZB medium supplemented with ampicillin (50 $\mu\text{g}/\text{ml}$) and chloroamphenicol (30 $\mu\text{g}/\text{ml}$) was used for expression experiments. A 100-ml portion of an overnight culture of host cell containing the pET-HAD was used to inoculate 4 l of culture. The cells were grown at 37°C under aerobic conditions until an OD_{600}

of 0.6 was reached. The culture was supplemented with additional ampicillin (50 $\mu\text{g}/\text{ml}$), and expression of HAD was induced by addition of 0.1 M IPTG to a final concentration of 0.4 mM. After 3 h of additional incubation at 33°C, the cells were harvested by centrifugation (10 min, 5,000 g, 4°C), washed with PBS and stored at -20°C .

The bacterial cell pellet (20 g wet weight) was re-suspended in 100 ml of 10 mM potassium phosphate, 1 mM DTT, 1 mM benzamidine, 0.1 mM PMSF, 10% glycerol, pH 7.2. After the cell wall had been digested with lysozyme (100 $\mu\text{g}/\text{ml}$) for 20 min at 37°C, the viscosity of the cell lysate was reduced with DNase (25 $\mu\text{g}/\text{ml}$) and RNase (25 $\mu\text{g}/\text{ml}$) in the presence of 10 mM MgCl_2 . Cell debris was sedimented by centrifugation at 16,000 g for 30 min at 4°C. Proteins precipitated from the supernatant with 35–60% of ammonium sulfate were dissolved in 50 ml of 10 mM potassium phosphate, 0.8 M ammonium sulfate, 0.5 mM EDTA, 0.5 mM EGTA, 0.5 mM DTT, and 20% glycerol, pH 7.2, and applied to butyl-Sepharose 4 Fast Flow column (Pharmacia) equilibrated with 10 mM potassium phosphate, 1.2 M ammonium sulfate, 0.5 mM EDTA, 0.5 mM EGTA, 0.5 mM DTT, 20% glycerol, pH 7.2 (buffer A). Bound proteins were eluted in a linear gradient of 0–20% ethylene glycol (v/v) in 200 ml of buffer A. Peak activity fractions were pooled, dialyzed against 10 mM potassium phosphate, 0.1 M KCl, 15% glycerol, pH 7.2 (buffer B) and applied to a hydroxylapatite column (1.6 \times 7.0 cm) equilibrated with the same buffer. The bound proteins were eluted with a linear gradient of 300 mM potassium phosphate, 15% glycerol, pH 7.2, buffer in a total volume of 400 ml. The fractions containing HAD (150–240 ml of potassium phosphate) were pooled and concentrated against polyethylene glycol 20000. The sample from the hydroxylapatite column was applied to a Superdex 200 (HR 10/30) gel filtration column (Pharmacia) equilibrated with 200 mM potassium phosphate, 1 mM EDTA, 1 mM EGTA, 15% glycerol, pH 7.2, and eluted at 0.5 ml/min. Peak activity fractions were pooled and stored at -20°C .

RESULTS

Expression of recombinant (R)-3-hydroxyacyl-CoA dehydrogenase of *Candida tropicalis* MFE

The expression of recombinant protein was induced by 0.4 mM IPTG. The soluble protein fraction of the bacterial lysate, containing the major portion of (R)-3-hydroxyacyl-CoA dehydrogenase activity, was recovered by centrifugation and used as starting material for enzyme purification.

Ammonium sulfate precipitation followed by three chromatographic steps on hydrophobic interaction, hydroxylapatite, and gel filtration columns resulted in an overall yield of about 32% (Table 1). In the enzyme assay, the racemic mixture of 3-hydroxyacyl-CoA was first incubated with commercial (S)-3-hydroxyacyl-CoA dehydrogenase in order to remove the (S)-isomer. Subsequently, a sample containing (R)-3-hydroxyacyl-CoA dehydrogenase activity was added to convert the remaining (R)-isomer to 3-ketodecanoyl-CoA. The formation of the Mg^{2+} complex of 3-ketodecanoyl-CoA was monitored at 303 nm.

The molecular mass of the recombinant (R)-3-hydroxyacyl-CoA dehydrogenase was estimated to be 65 kDa by SDS-PAGE (Fig. 2A), which agrees with the molecular mass deduced from the amino acid sequence (64.35 kDa). Immunoblot analysis confirmed the crossreactivity of the recombinant protein with antibodies raised against MFE

TABLE 1. Purification of recombinant 65 kDa (R)-3-hydroxyacyl-CoA dehydrogenase

Purification Step	Total Protein	Specific Activity	Total Activity	Yield
	mg	$\mu\text{mol}/\text{min}$ per mg	$\mu\text{mol}/\text{min}$	
Supernatant	639	0.36	230	100
(NH ₄) ₂ SO ₄ fractionation	385	0.57	219	95
Butyl-Sepharose	143	0.78	112	47
Hydroxylapatite	16	5.50	88	38
Superdex 200 (HR10/30)	2.8	26.8	75	33

The cDNA fragment of *Candida tropicalis* MFE encoding the dehydrogenase portion was expressed in *E. coli* cells. The purification was started from 20 g (wet weight) of bacterial cells and performed as described in Experimental Procedures.

of *Candida tropicalis* (Fig. 2B). The protein does not include the hydratase domain of yeast MFE and, as expected, 2-enoyl-CoA hydratase activity was not detectable (data not shown).

Table 2 summarizes the kinetic constants of the 65 kDa HAD with (R)-3-hydroxyacyl-CoA substrates. The K_m value for (R)-3-hydroxydecanoyl-CoA (C₁₀) was 8-times lower than that measured with (R)-3-hydroxybutyryl-CoA (C₄). The K_{cat} ratio with C₁₀/C₄ substrates was 4.8 and the specificity constant (K_{cat}/K_m) ratio was 40.

Another member of the SCAD superfamily, mammalian peroxisomal multifunctional enzyme 2 (perMFE-2), shows high homology (Fig. 1) with both yeast proteins in the cofactor binding and catalytic regions (6, 8, 12). The N-terminal part of perMFE-2 displays (R)-3-hydroxyacyl-CoA dehydrogenase activity with straight- and branched 3-hydroxyacyl-CoAs, cholic acid intermediate 24-OH-THCA-CoA and 17 β -hydroxysteroids (6–9, 12). In addition to specificity towards the 24R-hydroxy group of 24-OH-THCA-CoA, which corresponds to the 3R-hydroxyl of acyl-CoA, strict specificity of perMFE-2 towards 25R-methyl group has been observed (6, 7). Out of four possible stereoisomers of 24-OH-THCA-CoA perMFE-2 dehydrogenates only the 24R,25R-isomer (6, 7). **Table 3** shows that the 65 kDa recombinant HAD dehydrogenates 24R,25R- and 24R,25S-isomers of 24-OH-THCA-CoA, indicating that there is not a strict stereospecificity towards the 25-methyl group, although activity with the 24R,25R-

TABLE 2. Kinetic constants of recombinant 65 kDa (R)-3-hydroxyacyl-CoA dehydrogenase

Substrate	K_m (μM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (M ⁻¹ s ⁻¹)
(R)-3-hydroxybutyryl-CoA	43	4.4	1.0×10^5
(R)-3-hydroxyhexanoyl-CoA	41	32.9	8.0×10^5
(R)-3-hydroxydecanoyl-CoA	5.3	21.1	4.0×10^6
Estradiol	370	6.2×10^{-3}	16.8

The (R)-3-hydroxyacyl-CoA dehydrogenase and 17 β -estradiol dehydrogenase activities were measured as described in Experimental Procedures. Kinetic parameters of the enzyme were measured with concentrations of (R,S)-3-hydroxyacyl-CoAs from 5 to 200 μM and from 40 nm to 400 μM of 17 β -estradiol, and calculated with the GraFit program.

isomer was 6.5 times higher than that found with 24R,25S-isomer.

Similarly to the perMFE-2, the yeast recombinant HAD also shows 17 β -estradiol dehydrogenase activity (Table 2). Kinetic parameters revealed that the 17 β -hydroxy group of estradiol is not a preferable target of the enzyme. As estradiol is not involved in any biochemical pathway in the yeast, we consider the 17 β -estradiol dehydrogenase activity as non-specific activity. However, the detected 17 β -HSD activity will be useful in structure–functional studies of the SCAD family.

DISCUSSION

Amino acid sequence comparison of the SCAD superfamily has identified a group of peroxisomal proteins with high homology to each other (Fig. 1). This group consists of enzymes involved in fatty acid and cholesterol side-chain β -oxidation. Another common feature of these enzymes, which could provide insight into the substrate-binding site, is the utilization of CoA derivatives as substrates.

The yeast *Candida tropicalis* provides a unique example in the evolution of the SCAD superfamily. The evolutionary process has produced a gene duplication resulting in a chimeric protein containing a tandem (R)-3-hydroxyacyl-CoA dehydrogenase repeat highly homologous to the SCAD family members. The resulting 906-residue protein includes a 2-enoyl-CoA hydratase domain and the two (R)-3-hydroxyacyl-CoA dehydrogenase repeats (3, 18). Both repeats appear to contain all of the domains required for

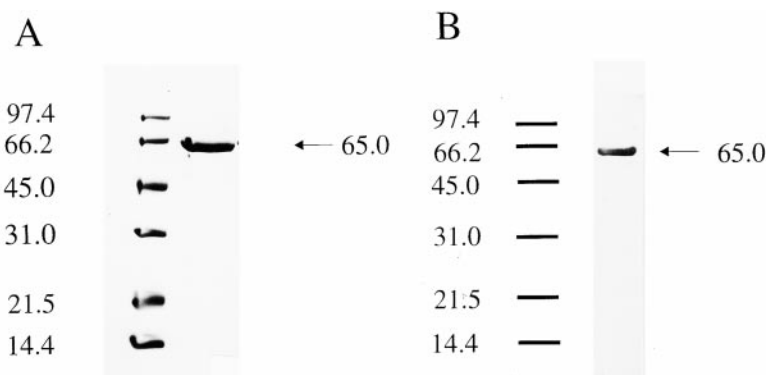


Fig. 2. Purification of recombinant 65 kDa (R)-3-hydroxyacyl-CoA dehydrogenase. Recombinant 65 kDa dehydrogenase was purified from *E. coli* cell extract. (A) Purified dehydrogenase (5 μg) was analyzed by SDS/PAGE. (B) Polyclonal antibodies raised against *C. tropicalis* MFE was used as the primary antibody, and affinity-purified goat anti-(rabbit IgG) conjugated with horseradish peroxidase as the second antibody. The recognized epitopes were revealed with 4-chloro-1-naphthol.

TABLE 3. Stereospecificity of recombinant 65 kDa (R)-3-hydroxyacyl-CoA dehydrogenase with cholic acid synthesis intermediate 3 α ,7 α ,12 α ,24 ξ -tetrahydroxy-5 β -cholestanoyl-CoA

Isomer	Activity
	<i>nmol/min per mg of protein</i>
24R, 25R	326
24R, 25S	50
24S, 25S	n.d.
24S, 25R	n.d.

The dehydrogenase activity was measured fluorimetrically with 25 μ M of pure diastereomers of 3 α ,7 α ,12 α ,24 ξ -tetrahydroxy-5 β -cholestanoyl-CoA as described in Experimental Procedures. 24R,25R-isomers is a physiological intermediate in cholic acid synthesis. The activities below the detection limits of the assay are given as n.d. (not detectable).

dehydrogenase activity (Fig. 1). The second dehydrogenase repeat is more closely related to the other SCAD enzymes than is the first, suggesting that the first peptide is the progenitor. On the other hand, the first repeat is more homologous to the mammalian perMFE-2 and the first repeat of FOX2 (Fig. 1). These domains contain an 11 amino acid loop region (GG \times \times G \times G \times S) located 19 amino acids downstream from the NAD⁺ binding site (Fig. 1). Non-peroxisomal members of the SCAD family do not possess this additional loop region. The function of the loop is still unknown. Possibly, the loop regulates substrate specificity towards the 25-methyl group of 24-OH-THCA-CoA. However, further site-directed mutagenesis studies should be carried out to clarify this proposal.

In order to characterize the activity of each HAD repeat, we mutated tyrosine residues in the active sites (19) of the first repeat (Y163A; A-mutant), second repeat (Y469A; B-mutant) or both of the repeats (Y163A, Y469A; AB-mutant). Mutants A and B had lower activity than the wild-type 65 kDa recombinant HAD (data not shown). In addition, the AB-mutant was inactive proving that both of the dehydrogenase repeats are functional (data not shown). All mutants displayed low stability during purification, and hence, could not be used in stereospecificity studies.

The 17 β -steroid dehydrogenase activity of the 65 kDa recombinant HAD (Table 2) is an interesting example of substrate overlapping. Based on previous data (3, 4) yeast MFE dehydrogenates CoA derivatives of fatty acids only. However, our results show that CoA derivatives of bile acid intermediates also fit the active site and can be used as substrates (Table 3). It is obvious that only a part of the substrate molecule adjacent to CoA is recognized by active site.

According to the sequence homology, substrate specificity and subcellular localization, yeast MFE and mammalian perMFE-2 have a common ancestor. In yeast, MFE is the only enzyme displaying 2-enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities, the second and third steps of fatty acid β -oxidation. The mammalian counterpart of yeast MFE plays a crucial role in bile acid synthesis as it catalyzes cholesterol side-chain oxidation (5–7, 9). The physiological role of 17 β -steroid dehydrogenase activity of perMFE-2, proposed by Adamski et al.

(12), is still questionable. Recently van Grunsven et al. (20) described a patient with a defect in the 3-hydroxyacyl-CoA dehydrogenase component of perMFE-2. The mutation (G16S) impaired the NAD⁺-binding site. As a result bile acid intermediates and very-long-chain fatty acids were highly elevated in plasma. Among the accumulated bile acid intermediates, 24-OH-THCA was the most abundant, supporting the involvement of perMFE-2 in bile acid synthesis.

A monofunctional enzyme catalyzing (S)-3-hydroxyacyl-CoA dehydrogenase from porcine heart is commercially available. In contrast, no monofunctional (R)-3-hydroxyacyl-CoA dehydrogenases have been characterized until now. In this study a monofunctional (R)-specific dehydrogenase was obtained by expression and purification of a truncated *C. tropicalis* peroxisomal MFE. This recombinant protein will be a useful tool to study the stereospecificity of cholesterol side-chain oxidation in bile acid synthesis. ■

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