Subcellular localization and physiological role of α-methylacyl-CoA racemase

Sacha Ferdinandusse,* Simone Denis,* Lodewijk IJlst,* Georges Dacremont,§ Hans R. Waterham,† and Ronald J. A. Wanders[1,*,†]

Departments of Clinical Chemistry* and Pediatrics,† Emma Children's Hospital, Academic Medical Center, University of Amsterdam, 1105 AZ Amsterdam, The Netherlands; and Department of Pediatrics,§ University of Ghent, 9000 Ghent, Belgium

Abstract **α-Methylacyl-CoA racemase plays an important role in the β-oxidation of branched-chain fatty acids and fatty acid derivatives because it catalyzes the conversion of several (2*R*)-methyl-branched-chain fatty acyl-CoAs to their (*S*)-stereoisomers. Only stereoisomers with the 2-methyl group in the (*S*)-configuration can be degraded via β-oxidation. Patients with a deficiency of α-methylacyl-CoA racemase accumulate in their plasma pristanic acid and the bile acid intermediates di- and trihydroxycholestanoic acid, which are all substrates of the peroxisomal β-oxidation system. Subcellular fractionation experiments, however, revealed that both in humans and rats α-methylacyl-CoA racemase is bimodally distributed to both the peroxisome and the mitochondrion. Our findings show that the peroxisomal and mitochondrial enzymes are produced from the same gene and that, as a consequence, the bimodal distribution pattern must be the result of differential targeting of the same gene product. In addition, we investigated the physiological role of the enzyme in the mitochondrion. Both in vitro studies with purified heterologously expressed protein and in vivo studies in fibroblasts of patients with an α-methylacyl-CoA racemase deficiency revealed that the mitochondrial enzyme plays a crucial role in the mitochondrial β-oxidation of the breakdown products of pristanic acid by converting (2*R*,6)-dimethylheptanoyl-CoA to its (*S*)-stereoisomer.**—Ferdinandusse, S., S. Denis, L. IJlst, G. Dacremont, H. R. Waterham, and R. J. A. Wanders. **Subcellular localization and physiological role of α-methylacyl-CoA racemase. *J. Lipid Res.* 2000.** 41: **1890–1896.**

Supplementary key words branched-chain fatty acid β-oxidation • stereospecificity • (2*R*,6)-dimethylheptanoyl-CoA

Peroxisomes in mammals harbor two distinct pathways for fatty acid β-oxidation. The first pathway catalyzes the β-oxidation of very long-chain fatty acids, such as C26:0, and the second pathway catalyzes the β-oxidation of branched-chain fatty acids and fatty acid derivatives, such as pristanic acid and the bile acid intermediates di- and trihydroxycholestanoic acid (DHCA and THCA, respectively). The central role of peroxisomes in the oxidation of branched-chain fatty acids and fatty acid derivatives is clearly demonstrated by studies in patients with Zellweger syndrome, who lack functional peroxisomes. Analysis of plasma from these patients reveals a series of abnormalities including the accumulation of DHCA, THCA, phytanic acid, and pristanic acid, which is derived from phytanic acid after one cycle of α-oxidation in the peroxisome (1). Previous studies have shown that the peroxisomal β-oxidation system is stereospecific (2–4), because the peroxisomal oxidases [branched-chain acyl-coenzyme A (CoA) oxidase in humans and trihydroxycholestanoyl-CoA (THC-CoA) oxidase and pristanoyl-CoA oxidase in rat] can handle only the (*S*)-stereoisomer of 2-methyl-branched acyl-CoAs (2, 3). Because both phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) and pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) naturally occur as a mixture of two different diastereomers [(2*S*,6*R*,10*R*) and (2*R*,6*R*,10*R*) in the case of pristanic acid] (5), the (2*R*)-pristanic acid first needs to be converted to its (*S*)-stereoisomer to become substrate for the peroxisomal β-oxidation (**Fig. 1**). This conversion is catalyzed by a racemase called α-methylacyl-CoA racemase, which catalyzes the interconversion of a large variety of (*R*)- and (*S*)-2-methyl-branched-chain fatty acyl-CoAs (6–9). The same racemase is also essential for the degradation of DHCA and THCA (7, 9), of which only the (25*R*)-stereoisomers are produced via (*R*)-specific mitochondrial 27-hydroxylation (10) (Fig. 1).

Studies on the subcellular localization of α-methylacyl-CoA racemase revealed that the enzyme activity is not only localized in peroxisomes but is also present in mitochondria, at least in humans (7, 8). In rat, however, the localization is controversial. Conzelmann and co-workers, who

Abbreviations: DHCA, dihydroxycholestanoic acid; EDTA, ethylenediaminetetraacetic acid; HPLC, high performance liquid chromatography; LCAD, long-chain acyl-CoA dehydrogenase; MBP, maltose-binding protein; MOPS, morpholinepropane sulfonic acid; THCA, trihydroxycholestanoic acid; THC-CoA, trihydroxycholestanoyl coenzyme A.

[1] To whom correspondence should be addressed.

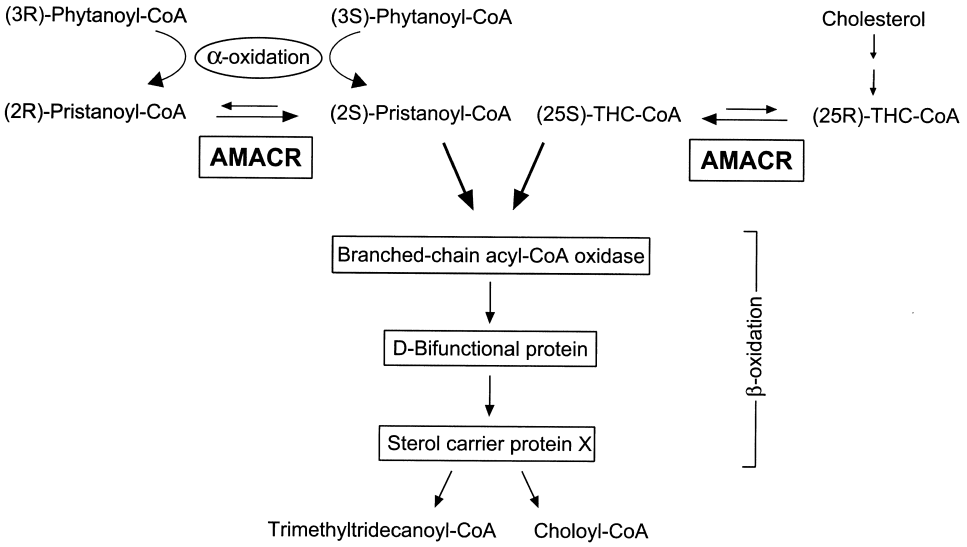

Fig. 1. Schematic representation of the steps involved in the oxidation of (3*R*)- and (3*S*)-phytanic acid as derived from dietary sources and (25*R*)-THCA produced from cholesterol in the liver. After the activation of (3*R*)- and (3*S*)-phytanic acid to their corresponding CoA esters, they both become substrates for the peroxisomal α-oxidation system, which produces (2*R*)- and (2*S*)-pristanoyl-CoA. Because branched-chain acyl-CoA oxidase, the first enzyme of the β-oxidation system, can handle only the (*S*)-stereoisomer, (2*R*)-pristanoyl-CoA needs to be converted by α-methylacyl-CoA racemase (AMACR) into its (2*S*)-stereoisomer. The bile acid intermediates DHCA and THCA are exclusively produced as (25*R*)-stereoisomers. To be β-oxidized, the CoA esters of the (25*R*)-stereoisomer also need to be converted by α-methylacyl-CoA racemase (AMACR) into their (25*S*)-stereoisomers.

purified the enzyme from rat liver, reported that it is exclusively localized in mitochondria (7), while Van Veldhoven and co-workers also detected racemase activity in rat liver peroxisomes although the distribution among the two organelles was quite different from that in human liver (8).

The peroxisomal localization of this enzyme is obvious in view of the importance of peroxisomes in the degradation of branched-chain fatty acids. It is less clear why mitochondria would need α-methylacyl-CoA racemase activity. It has been hypothesized, however, that this is necessary for the further oxidation of the breakdown products of pristanic acid (8, 11) because pristanic acid contains three chiral carbon atoms. The methyl groups at positions 6 and 10 of naturally occurring pristanic acid have the (*R*)-configuration. Therefore, (2*R*,6*R*,10)-trimethylundecanoyl-CoA, which is formed from pristanic acid after two β-oxidation cycles, requires racemization before it can be further degraded (see **Fig. 2**). After three cycles of β-oxidation (4*R*,8)-dimethylnonanoyl-CoA is exported from the peroxisome as a carnitine ester (12, 13) and subsequently further β-oxidized in the mitochondrion. As in peroxisomes, the dehydrogenating enzymes in the mitochondrion have been shown to be absolutely specific for the (2*S*)-isomer (4, 14). As a consequence, (2*R*,6)-dimethylheptanoyl-CoA, which is formed after four β-oxidation cycles (Fig. 2), first needs to be converted to its (*S*)-stereoisomer before further degradation is possible. It is unknown which racemase is responsible for this conversion, but α-methylacyl-CoA racemase is a good candidate.

In this article, we have studied the subcellular localization of α-methylacyl-CoA racemase in both humans and rat, and show that α-methylacyl-CoA racemase is the enzyme that is responsible for the racemase activity measured in both peroxisomes and mitochondria. Furthermore, we have studied the physiological role of α-methylacyl-CoA racemase in the mitochondrion and demonstrate that this enzyme is the main if not the only racemase that converts (2*R*,6)-dimethylheptanoyl-CoA into its (*S*)-stereoisomer.

MATERIALS AND METHODS

Subcellular fractionation of liver homogenates

Livers obtained from male Wistar rats that had been fed a standard laboratory diet supplemented with 1% (w/w) di-(2-ethylhexyl)-phthalate for 7 days, were homogenized in 250 mM sucrose, 5 mM morpholinepropane sulfonic acid (MOPS), and 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (final pH 7.4). A postnuclear supernatant was produced by centrifugation of the homogenate at 600 *g* for 10 min at 4°C and subjected to differential centrifugation as described previously (15). The light mitochondrial fraction, enriched in peroxisomes and lysosomes, was subfractionated by equilibrium density gradient centrifugation in a linear Nycodenz gradient as described (16). Pieces of human liver were obtained from patients undergoing liver resection. The tissue was homogenized in 250 mM sucrose, 2 mM MOPS, and 0.5 mM ethylenediaminetetraacetic acid (EDTA) (final pH 7.4), and subcellular fractionation was carried out as described for rat liver. Catalase (17) and glutamate dehydrogenase (18) were used as marker enzymes for peroxisomes and mitochondria, respectively.

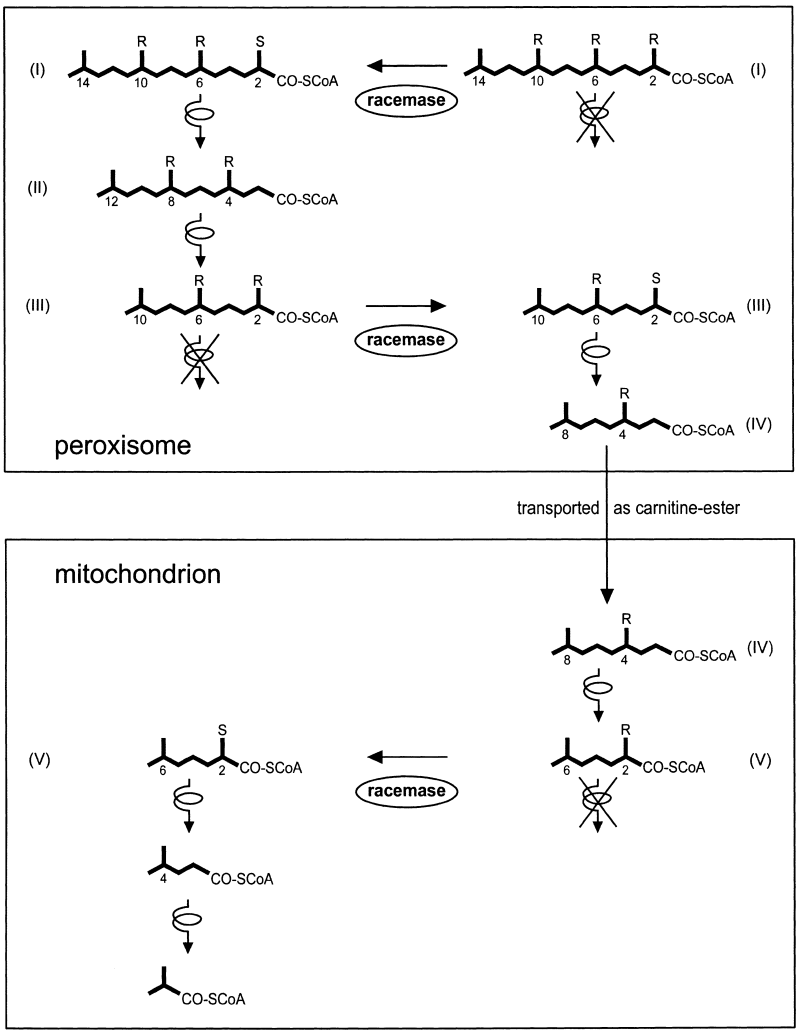

Fig. 2. Schematic representation of the pristanic acid β-oxidation and the involvement of racemase activity in mitochondria and peroxisomes. (2*R*,6*R*,10*R*,14)-pristanoyl-CoA (**I**), which is the configuration of half of the naturally occurring pristanoyl-CoA, needs to be converted to its (*S*)-stereoisomer before it can enter the β-oxidation spiral because the peroxisomal oxidases, the first enzymes of the β-oxidation system, can handle only (*S*)-stereoisomers. The resulting product, (4*R*,8*R*,12)-trimethyltridecanoyl-CoA (**II**), can be β-oxidized without any problem, but the next intermediate in the breakdown process of pristanic acid is again a 2-methyl-branched fatty acyl-CoA [(2*R*,6*R*,10)-trimethylundecanoyl-CoA (**III**)] with the (*R*)-configuration and requires therefore a racemase to convert it to its (*S*)-stereoisomer. After another cycle of β-oxidation (4*R*,8)-dimethylnonanoyl-CoA (**IV**) is transported from the peroxisome to the mitochondrion as carnitine ester for further oxidation. One cycle of mitochondrial β-oxidation results in the production of (2*R*,6)-dimethylheptanoyl-CoA (**V**) and a racemase is needed to form the (*S*)-stereoisomer, which can be β-oxidized to completion.

Expression of α-methylacyl-CoA racemase in *Escherichia coli*

The cDNA encoding human α-methylacyl-CoA racemase (GenBank accession number AF158378) was expressed as a fusion protein with maltose-binding protein (MBP) as described previously (9). The fusion protein was purified from *Escherichia coli* lysate by one-step affinity chromatography according to the manufacturer protocol (New England BioLabs, Beverly, MA).

Synthesis of the substrates for the enzyme assays

The CoA thioesters of THCA (19) and 2,6-dimethylheptanoic acid (20) were chemically synthesized by the method described by Rasmussen, Börchers, and Knudsen (21). The two stereoisomers of THCA were purified by high performance liquid chromatography (HPLC) as described previously (9).

Patient cell lines

The cell lines used in this study were from two patients with a defined deficiency of α-methylacyl-CoA racemase caused by mutations in the encoding gene. Racemase activity in fibroblasts of these patients as measured with THC-CoA as substrate was completely deficient (9).

Enzyme assays

α-Methylacyl-CoA racemase activity in the subcellular fractions obtained by differential centrifugation of rat and human liver homogenates was measured with (25*R*)-THC-CoA as substrate. The production of (25*S*)-THC-CoA was monitored by HPLC essentially as described previously (9) with one minor modification: 100 mM Bis-Tris-Propane (pH 7.5) was used as buffer in the incubation. Racemase activity measurements of the purified human α-methylacyl-CoA racemase-MBP fusion protein with (25*S*)-THC-CoA as substrate were performed as described (9).

Racemase activity of the purified human α-methylacyl-CoA racemase-MBP fusion protein was also determined with 2,6-dimethylheptanoyl-CoA as substrate. Because the two stereoisomers of 2,6-dimethylheptanoyl-CoA could not be separated by our HPLC method, we developed a coupled assay with purified long-chain acyl-CoA dehydrogenase (LCAD) to measure the activity. Purified LCAD (2.6 μU, determined with C8-CoA as substrate) was incubated with a racemic mixture of 2,6-dimethylheptanoyl-CoA in the absence or the presence of 3 μg of purified α-methylacyl-CoA racemase-MBP fusion protein. The incubation mixture consisted of 100 mM sodium phosphate-0.1 mM EDTA (pH 7.2), 0.4 mM hexafluorophosphate, 20 μM FAD, and 50 μM 2,6-dimethylheptanoyl-CoA. Reactions were allowed to proceed for 15, 30, or 60 min at 37°C and terminated by the addition of 0.18 M HCl. Production of 2,6-dimethylheptenoyl-CoA was followed by HPLC. This was done with a reversed-phase C_{18} column (Alltima 250 × 4.6 mm; Alltech, Deerfield, IL) and optimal resolution was achieved by elution with a linear gradient of methanol in 50 mM potassium phosphate buffer (pH 5.3).

The coupled assay was also used to determine racemase activity for 2,6-dimethylheptanoyl-CoA in fibroblast homogenates. In-

stead of a racemic mixture, however, 50 μM purified (2*R*,6)-dimethylheptanoyl-CoA was used as substrate in the assay. The protein concentration was 0.5 mg/ml and the reactions were allowed to proceed for 30 min at 37°C.

Purification of LCAD

Purified LCAD was a generous gift from T. Hashimoto (Shinshu University School of Medicine, Matsumoto, Japan). Purification was performed as described (22).

Preparation of antibodies

α-Methylacyl-CoA racemase-MBP fusion protein expressed in *E. coli* was purified from the lysate, subjected to preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis, isolated from the gel, and used to raise antibodies. To this end, a female New Zealand White rabbit was injected subcutaneously with 250 μg of the antigen mixed with an equal volume of Freund's complete adjuvant. After 1 month, the immunization was continued by booster injections (each containing 250 μg of antigen in Freund's incomplete adjuvant) until a satisfactory antibody titer was obtained.

Immunoblot analysis

Thirty microliters of each fraction of the Nycodenz density gradients from rat or human liver was subjected to electrophoresis on a 10% (w/v) SDS-polyacrylamide gel essentially as described by Laemmli (23) and transferred to a nitrocellulose sheet. After blocking of nonspecific binding sites with Protifar (50 g/l; Nutricia, Zoetermeer, The Netherlands) in Tween 20 at 1 g/l in phosphate-buffered saline (Tween 20-PBS) for 1 h, the blot was incubated for 2 h with rabbit polyclonal antibodies raised against human α-methylacyl-CoA racemase diluted 1:5,000 in Protifar (10 g/l). Goat anti-rabbit IgG antibodies conjugated to alkaline phosphatase and CDP-star were used for detection according to the manufacturer instructions (Boehringer Mannheim Biochemicals, Indianapolis, IN).

RESULTS

Subcellular localization of α-methylacyl-CoA racemase in rat liver

Thus far, the subcellular localization of α-methylacyl-CoA racemase in rat has been controversial. To resolve this, we have performed both differential and density gradient centrifugation experiments. A peroxisome-enriched fraction was first prepared by differential centrifugation and subsequently further fractionated by Nycodenz equilibrium density gradient centrifugation. The distinct activity patterns for the marker enzymes catalase (peroxisomes) and glutamate dehydrogenase (mitochondria) demonstrate a good separation between the various subcellular organelles (**Fig. 3A**). When racemase activity was measured in the gradient fractions with (25*R*)-THC-CoA as substrate, most of the activity was associated with the mitochondrial fractions and some activity was found in the peroxisomal fractions (Fig. 3B).

To study the possibility that the racemase activities in peroxisomes and mitochondria are derived from the same enzyme, we used a specific antiserum raised against recombinant human α-methylacyl-CoA racemase expressed in and purified from *E. coli*. Immunoblotting experiments revealed that this antiserum cross-reacts with the rat liver enzyme and specifically recognizes a single protein species of ~44 kDa, which is in good agreement with the predicted molecular mass of rat α-methylacyl-CoA racemase (data not shown). Immunoblot analysis of the fractions from the density gradient revealed a similar distribution pattern for the 44-kDa protein as for the racemase activity (Fig. 3B–D), suggesting that α-methylacyl-CoA racemase could be responsible for both the mitochondrial and the peroxisomal racemase activity measured.

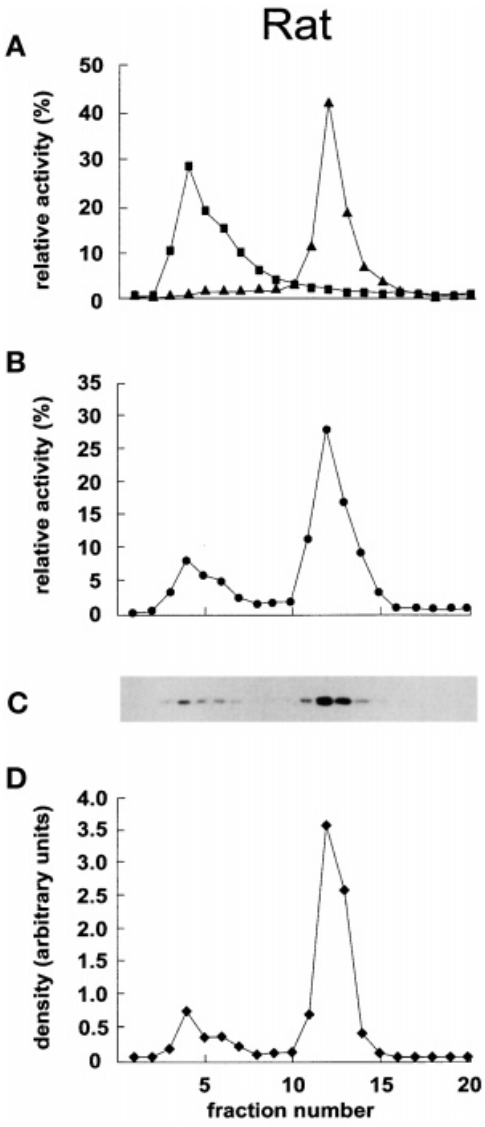

Fig. 3. Rat liver subcellular fractions were obtained by equilibrium density gradient centrifugation as described in Materials and Methods. Fractions were analyzed for the activity of the peroxisomal marker enzyme catalase (solid squares) and the mitochondrial marker enzyme glutamate dehydrogenase (solid triangles) (A), and α-methylacyl-CoA racemase (solid circles) measured with THC-CoA as substrate (B). Relative activities are expressed as a percentage of total gradient activity present in each fraction. (C) Immunoblot analysis with an antibody raised against human α-methylacyl-CoA racemase. (D) Densitometric analysis of the immunoblot (solid diamonds). The pattern of distribution of racemase activity and the mean density of the cross-reactive immunological material were similar.

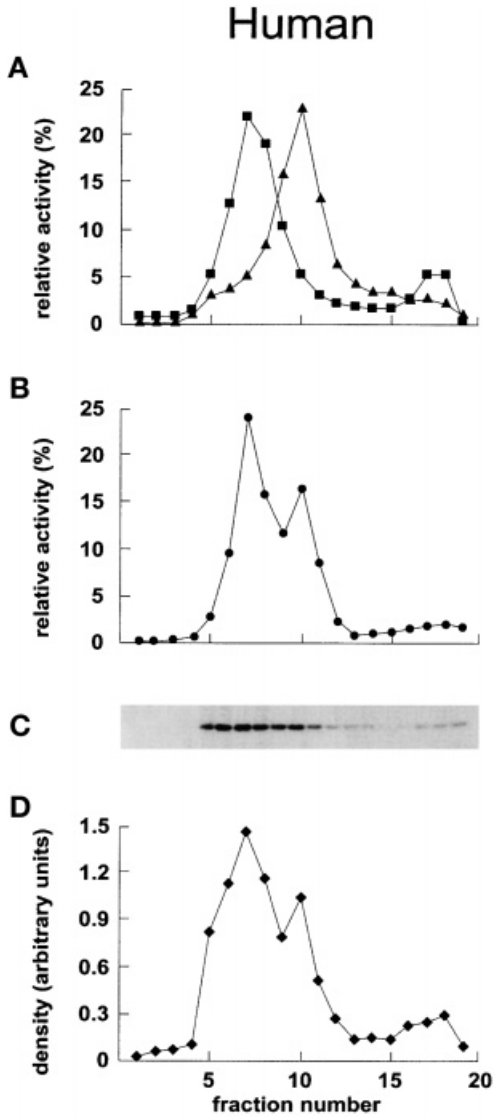

Fig. 4. Human liver subcellular fractions were obtained by equilibrium density gradient centrifugation as described in Materials and Methods. Fractions were analyzed for the activity of the peroxisomal marker enzyme catalase (solid squares) and the mitochondrial marker enzyme glutamate dydrogenase (solid triangles) (A), and α-methylacyl-CoA racemase (solid circles) measured with THC-CoA as substrate (B). Relative activities are expressed as a percentage of total gradient activity present in each fraction. (C) Immunoblot analysis with an antibody raised against human α-methylacyl-CoA racemase. (D) Densitometric analysis of the immunoblot (solid diamonds). The pattern of distribution of racemase activity and the mean density of the cross-reactive immunological material were similar.

Subcellular localization of α-methylacyl-CoA racemase in human liver

Measurement of racemase activity in the different fractions of the human gradient also revealed a bimodal activity profile as observed for rat liver. In contrast to the situation in rat, however, the activity associated with peroxisomes was higher than the mitochondrial activity (**Fig. 4B**). In addition, some racemase activity was measured in the upper part of the gradient. This activity is most likely due to α-methylacyl-CoA racemase released from peroxisomes broken during the homogenization process because these fractions also contain catalase, a peroxisomal matrix protein. The pattern of distribution obtained by immunoblot experiments using the antiserum against α-methylacyl-CoA racemase was similar to the distribution of the activity measured in the gradient fractions (Fig. 4B–D). Furthermore, the antiserum recognized only one protein species of ~44 kDa in the various fractions, which is the predicted molecular mass of human α-methylacyl-CoA racemase.

In vitro study of the mitochondrial function of α-methylacyl-CoA racemase

A possible physiological function of α-methylacyl-CoA racemase in the mitochondrion is that it is involved in the mitochondrial β-oxidation of the breakdown products of pristanic acid, notably 2,6-dimethylheptanoyl-CoA (Fig. 2). We first tested this hypothesis by measuring the activity of purified human α-methylacyl-CoA racemase expressed as a fusion protein with MBP in *E. coli*, using 2,6-dimethylheptanoyl-CoA as substrate. To this end, we developed a coupled assay making use of purified rat LCAD, which was previously shown to dehydrogenate 2,6-dimethylheptanoyl-CoA into its corresponding enoyl-CoA ester (20) and to be stereospecific for (*S*)-stereoisomers (4, 14). Indeed, on incubation of a chemically synthesized racemic mixture of 2,6-dimethylheptanoyl-CoA with LCAD, only half the mixture was converted to the enoyl-CoA ester (**Fig. 5**). When the mixture was incubated with LCAD in the presence of purified α-methylacyl-CoA racemase, however, an increasing amount of 2,6-dimethylheptenoyl-CoA was formed over time (Fig. 5). These results show that α-methylacyl-CoA racemase is able to convert (2*R*,6)-dimethylheptanoyl-CoA into its (*S*)-stereoisomer, which can then be desaturated by LCAD. The calculated activity of the purified α-methylacyl-CoA racemase-MBP fusion protein with 2,6-dimethylheptanoyl-CoA was comparable to the activity measured with THC-CoA as substrate (17.9 and 15.4 nmol/min/mg, respectively).

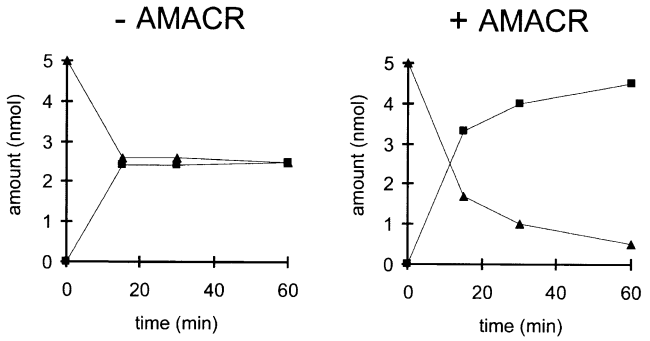

Fig. 5. 2,6-Dimethylheptanoyl-CoA was incubated with purified rat LCAD in the absence (−AMACR) or in the presence (+AMACR) of purified human α-methylacyl-CoA racemase-MBP fusion protein. The production of the enoyl-CoA esters, 2,6-dimethylheptenoyl-CoA (solid squares), and the consumption of the substrate, 2,6-dimethylheptanoyl-CoA (solid triangles), was monitored over time by HPLC analysis.

TABLE 1. Activity measurements of α-methylacyl-CoA racemase in homogenates of cultured skin fibroblasts using (25*S*)-THC-CoA and (2*R*,6)-dimethylheptanoyl-CoA as substrates

	(25*S*)-THC-CoA[a]	(2*R*,6)-Dimethylheptanoyl-CoA[a]
Controls	92 ± 30 (n = 11)	85 ± 18 (n = 5)
Patient 1[b]	ND	ND
Patient 2[b]	ND	ND

[a] Expressed in pmol/min/mg.
[b] Patients 1 and 2 correspond to patients 2 and 3 described previously (9). Results represent the mean ± SD, n represents the number of measurements. ND, Not detectable.

In vivo study of the mitochondrial function of α-methylacyl-CoA racemase

To examine the putative mitochondrial function of α-methylacyl-CoA racemase in vivo, we first measured the racemase activity using 2,6-dimethylheptanoyl-CoA as substrate in fibroblast lysates from control subjects and from patients with an established α-methylacyl-CoA racemase deficiency. Previously, we already showed that fibroblast lysates of these patients were no longer able to convert (25*R*)-THC-CoA into the (25*S*) form (9; **Table 1**). Using 2,6-dimethylheptanoyl-CoA as substrate, racemase activity was also fully deficient in fibroblasts of the patients, in contrast to control fibroblasts, in which we measured ample activity with this substrate (Table 1). This confirms that 2,6-dimethylheptanoyl-CoA is a physiological substrate of α-methylacyl-CoA racemase and that there is no other racemase involved in racemization of 2,6-dimethylheptanoyl-CoA.

Next, we measured racemase activity in the mitochondrial and peroxisomal peak fractions of the density gradients from rat and human liver using 2,6-dimethylheptanoyl-CoA as substrate to determine the subcellular localization of the activity. We found a similar bimodal distribution to both peroxisomes and mitochondria as observed with the substrate THC-CoA, suggesting that the same enzyme is involved in the racemization of the two substrates. This is supported by the similar ratios of racemase activities measured with THC-CoA and 2,6-dimethylheptanoyl-CoA in the different organelle fractions. In the rat gradient this ratio was 1.3 in the mitochondrial fraction and 1.0 in the peroxisomal fraction. In the human gradients the ratios were 2.0 and 2.2 in the mitochondrial and peroxisomal fractions, respectively.

DISCUSSION

In this study we investigated the localization of α-methylacyl-CoA racemase in subcellular fractions of human and rat liver obtained by equilibrium density gradient centrifugation. In both organisms we found a bimodal distribution pattern, in contrast to the results obtained by Conzelmann and co-workers, who reported that in rat α-methylacyl-CoA racemase is exclusively localized in the mitochondrion (7). There was, however, a considerable difference in distribution between humans and rats. In rat liver the enzyme activity was mainly associated with mitochondria, while in human liver the highest racemase activity was measured in the peroxisomal fractions. This species-dependent difference in distribution is remarkable and may be related to a different contribution of peroxisomes and mitochondria to branched-chain fatty acid oxidation in humans and rats. Indeed, according to Schmitz and Conzelmann (4) mitochondria contribute much more to whole cell branched-chain oxidation in the rat as compared with humans. In this respect it is also important to mention the data from Vanhove et al. (24), who studied the oxidation of 2-methylpalmitate in rat liver. According to these authors oxidation of this branched-chain fatty acid is shared between peroxisomes and mitochondria. Furthermore, they showed that the contribution of peroxisomes and mitochondria to whole cell 2-methylpalmitate oxidation is dependent on the nutritional status of the animal because clofibrate was found to induce mitochondrial much more than peroxisomal 2-methylpalmitate oxidation.

The similar distribution of racemase activity with only one immuno crossreactive 44-kDa racemase protein in density gradients of both rat and human liver suggested that both the mitochondrial and the peroxisomal racemase activities are produced by the same enzyme, namely α-methylacyl-CoA racemase. Unequivocal evidence that both the mitochondrial and peroxisomal enzyme activity is derived from a single gene was provided by our findings in fibroblasts from patients with an established α-methylacyl-CoA racemase deficiency caused by missense mutations in the encoding gene (9). In homogenates of these fibroblasts we found a complete absence of racemase activity both for THC-CoA and for 2,6-dimethylheptanoyl-CoA.

The finding that α-methylacyl-CoA racemase is encoded by one gene but localized in two different subcellular compartments implies differential targeting of the same gene product. This phenomenon is not unprecedented: for example, the cDNAs encoding mitochondrial and peroxisomal carnitine acetyltransferase originate from alternative splicing of one single gene (25). Another example is $\Delta^{3,5}\Delta^{2,4}$-dienoyl-CoA isomerase, first identified by Luo and co-workers (26), which has both a mitochondrial targeting signal at the amino terminus and a peroxisomal targeting signal at the carboxy terminus (27). The molecular basis of the differential targeting of α-methylacyl-CoA racemase, however, is still unknown. The human and rat enzyme both contain a carboxy-terminal peroxisomal targeting signal type 1 (−KASL in humans, −KANL in rats). Inspection of the amino terminus of both rat and human racemase does not reveal an obvious mitochondrial targeting signal. By using software predicting cleavage site motifs in mitochondrion-targeting peptides (28), however, a weak potential mitochondrial transit peptide was predicted at positions 1−34 of human α-methylacyl-CoA racemase.

Patients with a deficiency of α-methylacyl-CoA racemase accumulate pristanic acid and the bile acid intermediates DHCA and THCA in plasma (9). This clearly demonstrates that racemase activity is needed in the peroxisome for β-oxidation of pristanic acid, DHCA, and THCA. To elucidate the putative mitochondrial function of α-methylacyl-CoA racemase, we first studied in vitro whether the purified enzyme is able to convert (2*R*,6)-dimethylheptanoyl-CoA to its

(*S*)-stereoisomer, the only stereoisomer that can be β-oxidized in the mitochondrion (4, 14), and found that this was indeed the case. Subsequently, we studied whether this is the true physiological function of the enzyme. We found that fibroblasts from patients with an established α-methylacyl-CoA racemase deficiency were not able to convert (2*R*,6)-dimethylheptanoyl-CoA to its (*S*)-stereoisomer, which confirms that α-methylacyl-CoA racemase activity is essential at several steps in the degradation of pristanic acid to CO_2 and H_2O in the peroxisome as well as in the mitochondrion.

To obtain additional in vivo evidence of the role of mitochondrial racemase in the oxidation of 2,6-dimethylheptanoyl-CoA, we performed acylcarnitine analysis in α-methylacyl-CoA racemase-deficient patients. These studies did not reveal accumulation of 2,6-dimethylheptanoylcarnitine (data not shown). The reason for this is most probably that even though half the pristanic acid can enter the β-oxidation spiral in these patients, as it naturally occurs as a racemic mixture (see Fig. 2), it cannot proceed beyond 2,6,10-trimethylundecanoyl-CoA, of which all methyl groups have the (*R*)-configuration. For this compound to be further β-oxidized, the (2*R*)-methyl group needs to be converted to the (*S*)-configuration, which is most likely also catalyzed by α-methylacyl-CoA racemase (see Fig. 2). This is supported by the finding of 2,6,10-trimethylundecanoyl-carnitine in the plasma of one of the racemase-deficient patients.

We thank R. Ofman, F. M. Vaz, and C. J. Dekker for technical assistance. This work was supported by the Princess Beatrix Fund (The Hague, The Netherlands).

Manuscript received 3 March 2000 and in revised form 19 May 2000.

REFERENCES

1. Wanders, R. J. A., R. B. H. Schutgens, and P. G. Barth. 1995. Peroxisomal disorders: a review. *J. Neuropathol. Exp. Neurol.* **54:** 726–739.
2. Van Veldhoven, P. P., K. Croes, S. Asselberghs, P. Herdewijn, and G. P. Mannaerts. 1996. Peroxisomal β-oxidation of 2-methyl-branched acyl-CoA esters: stereospecific recognition of the 2*S*-methyl compounds by trihydroxycoprostanoyl-CoA oxidase and pristanoyl-CoA oxidase. *FEBS Lett.* **388:** 80–84.
3. Pedersen, J. I., T. Veggan, and I. Björkhem. 1996. Substrate stereospecificity in oxidation of (25*S*)-3α,7α,12α-trihydroxy-5β-cholestanoyl-CoA by peroxisomal trihydroxy-5β-cholestanoyl-CoA oxidase. *Biochem. Biophys. Res. Commun.* **224:** 37–42.
4. Schmitz, W., and E. Conzelmann. 1997. Stereochemistry of peroxisomal and mitochondrial β-oxidation of α-methylacyl-CoAs. *Eur. J. Biochem.* **244:** 434–440.
5. Ackman, R. G., and R. P. Hansen. 1967. The occurrence of diastereomers of phytanic and pristanic acids and their determination by gas-liquid chromatography. *Lipids.* **2:** 357–362.
6. Schmitz, W., R. Fingerhut, and E. Conzelmann. 1994. Purification and properties of an α-methylacyl-CoA racemase from rat liver. *Eur. J. Biochem.* **222:** 313–323.
7. Schmitz, W., C. Albers, R. Fingerhut, and E. Conzelmann. 1995. Purification and characterization of an α-methylacyl-CoA racemase from human liver. *Eur. J. Biochem.* **231:** 815–822.
8. Van Veldhoven, P. P., K. Croes, M. Casteels, and G. P. Mannaerts. 1997. 2-Methylacyl racemase: a coupled assay based on the use of pristanoyl-CoA oxidase/peroxidase and reinvestigation of its subcellular distribution in rat and human liver. *Biochim. Biophys. Acta.* **1347:** 62–68.
9. Ferdinandusse, S., S. Denis, P. T. Clayton, A. Graham, J. E. Rees, J. T. Allen, B. N. McLean, A. Y. Brown, P. Vreken, H. R. Waterham, and R. J. A. Wanders. 2000. Mutations in the gene encoding peroxisomal α-methylacyl-CoA racemase cause adult-onset sensory motor neuropathy. *Nature Genet.* **24:** 188–191.
10. Batta, A. K., G. Salen, S. Shefer, B. Dayal, and G. S. Tint. 1983. Configuration at C-25 in 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid isolated from human bile. *J. Lipid Res.* **24:** 94–96.
11. Wanders, R. J. A., P. Vreken, M. E. J. den Boer, F. A. Wijburg, A. H. van Gennip, and L. IJlst. 1999. Disorders of mitochondrial fatty acyl-CoA β-oxidation. *J. Inher. Metab. Dis.* **22:** 442–487.
12. Verhoeven, N. M., D. S. Roe, R. M. Kok, R. J. A. Wanders, C. Jakobs, and C. R. Roe. 1998. Phytanic acid and pristanic acid are oxidized by sequential peroxisomal and mitochondrial reactions in cultured fibroblasts. *J. Lipid Res.* **39:** 66–74.
13. Ferdinandusse, S., J. Mulders, L. IJlst, S. Denis, G. Dacremont, H. R. Waterham, and R. J. A. Wanders. 1999. Molecular cloning and expression of human carnitine octanoyltransferase: evidence for its role in the peroxisomal β-oxidation of branched-chain fatty acids. *Biochem. Biophys. Res. Commun.* **263:** 213–218.
14. Battaile, K. P., M. Mcburney, P. P. van Veldhoven, and J. Vockley. 1998. Human long chain, very long chain and medium chain acyl-CoA dehydrogenases are specific for the S-enantiomer of 2-methylpentadecanoyl-CoA. *Biochim. Biophys. Acta.* **1390:** 333–338.
15. Wanders, R. J. A., G. J. Romeyn, R. B. H. Schutgens, and J. M. Tager. 1989. L-Pipecolate oxidase: a distinct peroxisomal enzyme in man. *Biochem. Biophys. Res. Commun.* **164:** 550–555.
16. Wanders, R. J. A., C. W. T. van Roermund, D. S. M. Schor, H. J. ten Brink, and C. Jakobs. 1994. 2-Hydroxyphytanic acid oxidase activity in rat and human liver and its deficiency in the Zellweger syndrome. *Biochim. Biophys. Acta.* **1227:** 177–182.
17. Wanders, R. J. A., M. Kos, B. Roest, A. J. Meijer, G. Schrakamp, H. S. A. Heymans, W. H. Tegelaers, H. van den Bosch, R. B. H. Schutgens, and J. M. Tager. 1984. Activity of peroxisomal enzymes and intracellular distribution of catalase in Zellweger syndrome. *Biochem. Biophys. Res. Commun.* **123:** 1054–1061.
18. Wanders, R. J. A., C. W. T. van Roermund, C. T. de Vries, H. van den Bosch, G. Schrakamp, J. M. Tager, A. W. Schram, and R. B. H. Schutgens. 1986. Peroxisomal beta-oxidation of palmitoyl-CoA in human liver homogenates and its deficiency in the cerebrohepato-renal (Zellweger) syndrome. *Clin. Chim. Acta.* **159:** 1–10.
19. Van Eldere, J. R., G. G. Parmentier, H. J. Eyssen, R. J. A. Wanders, R. B. H. Schutgens, J. Vamecq, F. van Hoof, B. T. Poll-Thé, and J. M. Saudubray. 1987. Bile acids in peroxisomal disorders. *Eur. J. Clin. Invest.* **17:** 386–390.
20. Wanders, R. J. A., S. Denis, J. P. N. Ruiter, L. IJlst, and G. Dacremont. 1998. 2,6-Dimethylheptanoyl-CoA is a specific substrate for long-chain acyl-CoA dehydrogenase (LCAD): evidence for a major role of LCAD in branched-chain fatty acid oxidation. *Biochim. Biophys. Acta.* **1393:** 35–40.
21. Rasmussen, J. T., T. Börchers, and J. Knudsen. 1990. Comparison of the binding affinities of acyl-CoA-binding protein and fatty-acid-binding protein for long-chain acyl-CoA esters. *Biochem. J.* **265:** 849–855.
22. Furuta, S., S. Miyazawa, and T. Hashimoto. 1981. Purification and properties of rat liver acyl-CoA dehydrogenases and electron transfer flavoprotein. *J. Biochem.* **90:** 1739–1750.
23. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680–685.
24. Vanhove, G., P. P. van Veldhoven, F. Vanhoutte, G. Parmentier, H. J. Eyssen, and G. P. Mannaerts. 1991. Mitochondrial and peroxisomal β oxidation of the branched chain fatty acid 2-methylpalmitate in rat liver. *J. Biol. Chem.* **266:** 24670–24675.
25. Corti, O., S. DiDonato, and G. Finocchiaro. 1994. Divergent sequences in the 5′ region of cDNA suggest alternative splicing as a mechanism for the generation of carnitine acetyltransferases with different subcellular localizations. *Biochem. J.* **303:** 37–41.
26. Luo, M. J., T. E. Smeland, K. Shoukry, and H. Schulz. 1994. Delta3,5-delta2,4-dienoyl-CoA isomerase from rat liver mitochondria. Purification and characterization of a new enzyme involved in the beta-oxidation of unsaturated fatty acids. *J. Biol. Chem.* **269:** 2384–2388.
27. Filppula, S. A., A. I. Yagi, S. H. Kilpelainen, D. Novikov, D. R. Fitz-Patrick, M. Vihinen, D. Valle, and J. K. Hiltunen. 1998. Delta3,5-delta2,4-dienoyl-CoA isomerase from rat liver. Molecular characterization. *J. Biol. Chem.* **273:** 349–355.
28. Gavel, Y., and G. von Heijne. 1990. Cleavage-site motifs in mitochondrial targeting peptides. *Protein Eng.* **4:** 33–37.