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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

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Abstract Trihydroxycoprostanoyl-CoA oxidase and pristanoyl-CoA oxidase, purified from rat liver, both catalyse the desaturation of 2-methyl-branched acyl-CoAs. Upon incubation with the pure isomers of 2-methylpentadecanoyl-CoA, both enzymes acted only on the *S*-isomer. The *R*-isomer inhibited trihydroxycoprostanoyl-CoA oxidase but did not affect pristanoyl-CoA oxidase. The activity of both enzymes was suppressed by 3-methylheptadecanoyl-CoA. Valproyl-CoA and 2-ethylhexanoyl-CoA, however, did not influence the oxidases. Although only one isomer of 25*R*,*S*-trihydroxycoprostanoyl-CoA was desaturated by trihydroxycoprostanoyl-CoA oxidase, isolated peroxisomes were able to act on both isomers, suggesting the presence of a racemase in these organelles. Given the opposite stereoselectivity of the 26-cholesterol hydroxylase and of the oxidase, the racemase is essential for bile acid formation.

Key words: Bile acid; Isoprenoid; Pristanic acid; Phytanic acid; Racemase; Zellweger syndrome

1. Introduction

In mammals, peroxisomes are responsible for the degradation of various lipophilic carboxylates such as very long chain fatty acids, dicarboxylic acids, arachidonic acid metabolites (prostaglandins, thromboxanes, and leukotrienes), isoprenoid-derived fatty acids such as pristanic acid (2,6,10,14-tetramethylpentadecanoic acid), the bile acid intermediates di- and trihydroxycoprostanic acids (3α,7α-dihydroxy- and 3α,7α,12α-trihydroxy-5β-26-cholestanoic acids) and some xenobiotics (see [1–3]). The CoA esters of trihydroxycoprostanic acid and pristanic acid, which both possess a 2-methyl branch, are desaturated in rat liver by trihydroxycoprostanoyl-CoA oxidase [4,5] and pristanoyl-CoA oxidase [5–7], respectively. Whereas trihydroxycoprostanoyl-CoA oxidase is a liver-specific enzyme [4], pristanoyl-CoA oxidase is also present in extra-hepatic tissues such as kidney and intestinal mucosa [6,8,9]. Purified trihydroxycoprostanoyl-CoA oxidase also has some activity towards pristanoyl-CoA and the synthetic 2-methylacyl-CoAs derived from hexanoic and hexadecanoic acids [5,10]. Pristanoyl-CoA oxidase does not recognize trihydroxycoprostanoyl-CoA but oxidizes, in addition to short and long chain 2-methylacyl-CoAs, straight chain acyl-CoAs and 4-methylacyl-CoAs [4,5,9].

In human liver peroxisomes, a single enzyme, called branched chain acyl-CoA oxidase, appears to act on both 2-methyl-branched compounds, pristanoyl-CoA and trihydroxycoprostanoyl-CoA [11]. In addition, the purified oxidase has also some activity towards straight chain acyl-CoAs [11].

Based on their meric composition, substrate spectrum, sensitivity to *N*-ethylmaleimide, and the observed cross-reactivity towards antisera, rat trihydroxycoprostanoyl-CoA oxidase and human branched chain acyl-CoA oxidase appear to be related enzymes [4,5,10,11]. In contrast to trihydroxycoprostanoyl-CoA oxidase, however, the branched chain acyl-CoA oxidase is expressed in all tissues examined thus far [11].

During the characterisation and purification of the above-described oxidases racemic 2-methylacyl-CoAs, pristanoyl-CoA and trihydroxycoprostanoyl-CoA were used [5–11]. Although the optically active methyl groups of phytol, the precursor of phytanic acid which in turn gives rise to pristanic acid, possess the *R*-configuration, both 3*R*- and 3*S*-forms of phytanic acid and 2*R*- and 2*S*-forms of pristanic acid occur naturally [12,13]. Both 25*S*- and 25*R*-isomers of di- and trihydroxycoprostanic acid are formed from cholesterol [14] and in vivo and in vitro studies showed that both isomers are equally well converted to the C24 bile acids in rat and man [15–18]. In peroxisomal disorders, both 25*R*- and 25*S*-C27 bile acid intermediates accumulate [19,20].

The presence of the isomers of these 2-methyl branched compounds is likely due to the action of a racemase specific for 2-methylacyl-CoAs, which was localized to the mitochondria in rat liver and to peroxisomes and mitochondria in human liver [21,22]. The enzyme accepts also the CoA esters of trihydroxycoprostanic acid and 2-arylpropionic acids [21,22]. Yet another racemase activity acting on trihydroxycoprostanoyl-CoA has been found in purified rat liver peroxisomes [23], a finding confirmed in this report. On the other hand, the 24-hydroxy-intermediate formed during the peroxisomal β-oxidation of di- and trihydroxycoprostanic acids, irrespectively of the configuration at C25, has been assigned the 24*R*,25*S* configuration [24–26]. This would implicate that the involved enzymes, either the oxidase and/or the enoyl-CoA hydratase, are stereospecific. If the oxidase acts only on the 25*S*-isomer of trihydroxycoprostanoyl-CoA, the racemase(s) might be essential enzymes in C27 bile acid (and pristanic acid) degradation since through the action of the mitochondrial 26-cholesterol hydroxylase only the 25*R*-isomer of 26-hydroxycholesterol (and its further breakdown products) is produced [27–30]. We, therefore, investigated the stereospecificity of purified trihydroxycoprostanoyl-CoA oxidase and pristanoyl-CoA oxidase using the CoA esters of the synthetic 2*R*- and 2*S*-methylpentadecanoic acids.

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2. Materials and methods

The synthesis of 2*S*- and 2*R*-methylpentadecanoic acid was based on the condensation of 1-bromododecane with *R*-3-bromo-2-methylpropan-1-ol and *S*-3-bromo-2-methylpropan-1-ol (both from Fluka), respectively, followed by oxidation of the formed alcohols, analogous to the synthesis of the isomers of 2-methyltetradecanoic acid [21]. Mass spectra of the alcohols and acids were in agreement with their proposed structure. Phytanic acid and 3-methylheptadecanoic acid were synthesized as described before [31]. A 4-methyl substituted fatty acid (4,8,12-trimethyltridecanoic acid) was synthesized from farnesol (Across Chimica) [9]. The above-described synthetic fatty acids and commercially obtained valproic acid and 2-ethylhexanoic acid (Aldrich) were converted to their CoA esters after activation to acylimidazoles [32]. 26-[^{14}C]Trihydroxycoprostanoyl-CoA was prepared as described before [33]. [2,4-^{3}H]Cholic acid was obtained from Dupont-New England Nuclear.

Trihydroxycoprostanoyl-CoA oxidase [10] and pristanoyl-CoA oxidase [8] were purified from rat liver as described before. Peroxisomes were isolated from liver of clofibrate-treated rats by subfractionation of a light mitochondrial fraction by means of Percoll and Nycodenz gradients [34]. Oxidase measurements were based on coupling of the substrate-dependent hydrogen peroxide production to the formation of dimeric homovanillic acid, a reaction catalyzed by peroxidase. Assay conditions were similar to those described before [8,10] and measurements were performed in either 125 or 250 µl final assay volumes.

In a radioactive oxidase assay, aliquots of purified trihydroxycoprostanoyl-CoA oxidase or purified peroxisomes (80 µl) were pipetted into glass tubes and mixed with 20 µl of 100 µM FAD-5 mM DTT. After a preincubation period of 5 min at 0°C, reactions were started by adding 0.4 ml of assay mixture containing 50 mM K-phosphate buffer pH 8.3, 125 µM 26-[^{14}C]trihydroxycoprostanoyl-CoA, 0.3% (w/v) defatted albumin, 5 mM ATP and 5 mM $MgCl_2$. After 1 h incubation at 37°C, 10 µl of 2 mM [^{3}H]cholate (3 µCi/µmol) was added, followed by 40 µl of 2 N KOH and the tubes were placed at 50°C for 40 min. After acidification by the addition of 30 µl of 5 N HCl, the hydrolysed bile acid intermediates were extracted twice in 1 ml of ethyl acetate. The combined organic phases were dried, dissolved in 150 µl of methanol/water/30 mM trifluoroacetate pH 2.9 (40:36:24, v/v) and 50 µl was injected on a Novapak C18 column (4 µm; 60 Å; 150×3.9 mm; Waters) equilibrated in methanol/30 mM trifluoroacetate pH 2.9 (76:24, v/v) [35]. The bile acid intermediates were eluted isocratically at 0.8 ml/min and monitored for radioactivity on line (Radiomatic A500 detector, Canberra Packard) simultaneously in two channels. One channel measured both ^{3}H and ^{14}C signals (energy detection limits 0–156 keV), the other measuring only ^{14}C signals (20–156 keV). The flow rate of the scintillation fluid (Ultima-Flo M, Canberra Packard) was 3 ml/min. Radiodetector update time was 6 s and signals were analysed by using the Flo-one\Data computer program (Canberra Packard).

3. Results and discussion

As shown in Fig. 1, purified trihydroxycoprostanoyl-CoA oxidase was active only on 2*S*-methylpentadecanoyl-CoA. In agreement with earlier observations, the activity of the oxidase was dependent on the presence of albumin. In the presence of 0.12% albumin, half-maximal activity was observed at 40 µM substrate. Irrespective of the amounts of albumin added, no peroxide formation could be measured upon addition of the *R*-form of the substrate. Therefore, it was possible to investigate the influence of the *R*-stereoisomer on the desaturation of the *S*-form. The *R*-isomer was weakly inhibitory (Fig. 2). The CoA ester of 3-methylheptadecanoic acid, which was not a substrate, was a more potent inhibitor, especially in the absence of albumin (Fig. 2). Also, racemic 2-ethylhexanoyl-CoA and valproyl-CoA (2-propylpentanoyl-CoA) were not oxidized and they did not influence the oxidation of 2*S*-methylpentadecanoyl-CoA. Depending on the assay conditions, straight chain acyl-CoAs (hexanoyl- and palmitoyl-CoA)

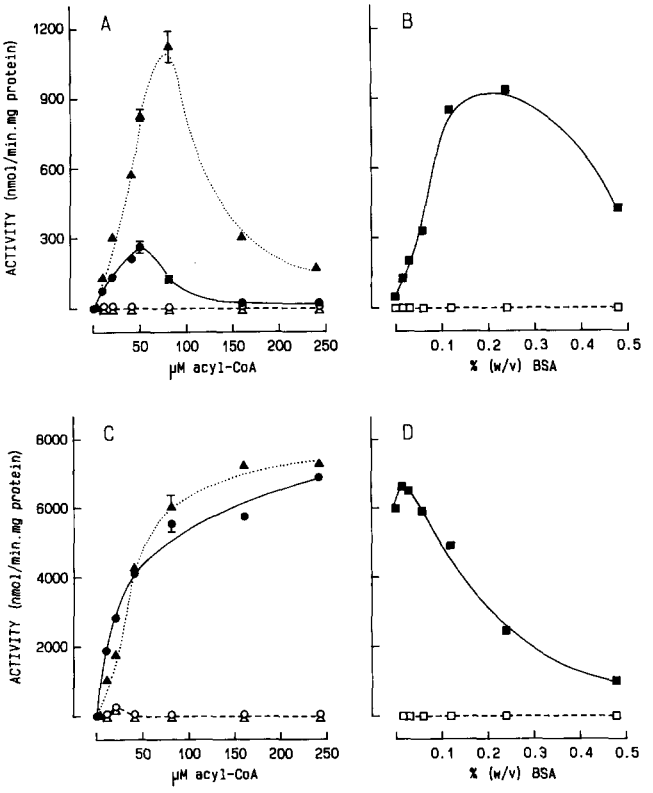

Fig. 1. Stereospecific desaturation of 2-methyl-branched acyl-CoAs by trihydroxycoprostanoyl-CoA oxidase and pristanoyl-CoA oxidase. Purified rat liver trihydroxycoprostanoyl-CoA oxidase (A,B) and pristanoyl-CoA oxidase (C,D) were incubated with increasing amounts of 2*S*-methylpentadecanoyl-CoA (closed symbols) or 2*R*-methylpentadecanoyl-CoA (open symbols; broken lines) in the absence (circles; solid lines; A,C) or presence of albumin (triangles; dashed lines; 0.12% (w/v) in A; 0.06% (w/v) in C). In B and D, the acyl-CoA concentration was fixed at 100 µM while varying amounts of albumin, final assay concentration indicated, were added (squares). Values, for assay conditions resulting in optimum activity, represent mean ± S.D. of three or more separate determinations; other values are from one experiment.

and 4-methyl-branched acyl-CoA (4,8,12-trimethyltridecanoyl-CoA) either stimulated (at low concentrations) or suppressed (at high concentrations) the oxidation of the 2*S*-compound (data not shown). The stimulatory effect appeared to be caused by the desaturation of these acyl-CoAs by trihydroxycoprostanoyl-CoA oxidase, a finding which is in disagreement with earlier observations [4,5]. With hexanoyl-CoA as substrate Michaelis-Menten kinetics were observed. The K_m and V_{max} values (in the presence of 0.12% (w/v) albumin) were 40 µM and 60 nmol/min per mg protein. With palmitoyl-CoA and 4,8,12-trimethyltridecanoyl-CoA complex kinetics were seen, the velocities being dramatically influenced by the albumin and substrate concentrations (data not shown). Maximal activities were observed between 50 and 125 µM palmitoyl-CoA and 4,8,12-trimethyltridecanoyl-CoA when the molar substrate/albumin ratio (v) approached 3.0 (130–160 and 340–400 nmol/min per mg protein, respectively). Although it is impossible to prove the total absence of traces of contaminating oxidases able to act on these acyl-CoAs (e.g. palmitoyl-CoA oxidase and (dimeric) pristanoyl-CoA oxidase) in our purified enzyme preparation, the following lines of evidence indicate that 4-methyl-branched and straight chain

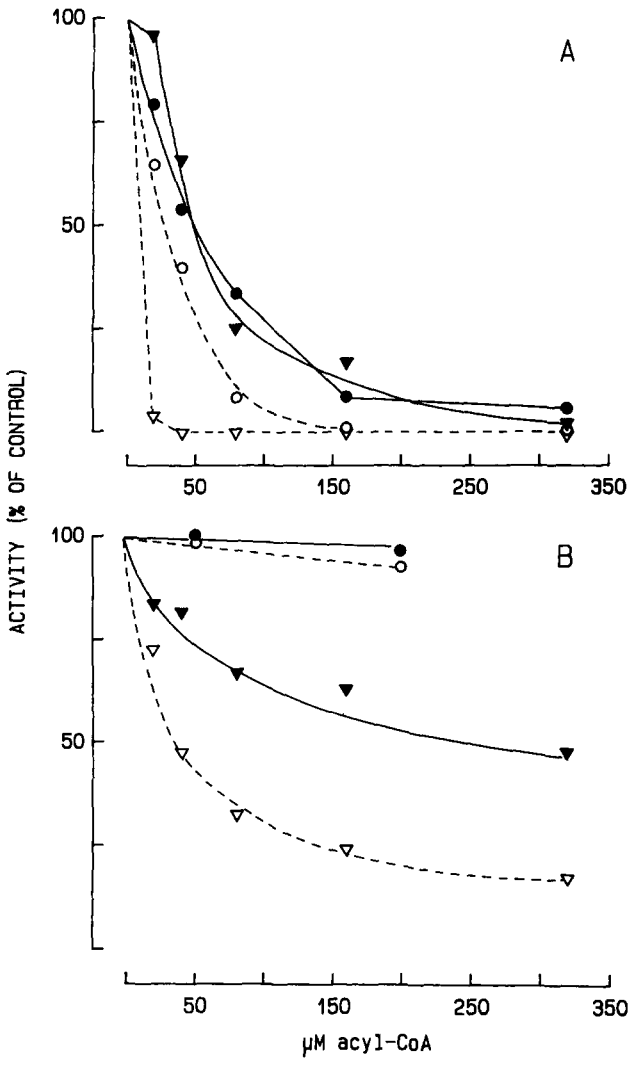

Fig. 2. Inhibition of trihydroxycoprostanoyl-CoA oxidase and pristanoyl-CoA oxidase by 2*R*-methyl- and 3*R*,*S*-methyl-branched acyl-CoAs. Activities of purified rat liver trihydroxycoprostanoyl-CoA oxidase (A) and pristanoyl-CoA oxidase (B) were measured with 80 μM 2*S*-methylpentadecanoyl-CoA as substrate in the absence (open symbols) and presence of albumin (closed symbols; 0.12% (w/v) for A; 0.06% (w/v) for B) and assay mixtures were fortified with increasing amounts of 2*R*-methylpentadecanoyl-CoA (circles) or 3-methylheptadecanoyl-CoA (triangles). Control values (expressed in nmol/min per mg protein and determined in duplicate in the absence of inhibitor) were 1226 (presence of albumin) and 131 (absence of albumin) for trihydroxycoprostanoyl-CoA oxidase, the corresponding values being 6021 and 5550 for pristanoyl-CoA oxidase. Data from one representative experiment are shown but similar effects were observed under slightly different assay conditions.

acyl-CoA oxidase activities are intrinsic to the trihydroxycoprostanoyl-CoA oxidase: (1) the activity towards these acyl-CoAs was blocked by *N*-ethylmaleimide while the alkylating agent does not affect palmitoyl-CoA oxidase and pristanoyl-CoA oxidase [4]; (2) the activity towards straight long chain acyl-CoA and 4-methylacyl-CoA was albumin dependent, like that observed before with trihydroxycoprostanoyl-CoA and 2-methylacyl-CoAs as substrate [5]. The (inducible) palmitoyl-CoA oxidase is maximally active at much lower albumin concentrations ($v \approx 10$) [5] while pristanoyl-CoA oxidase is hardly affected by the presence of albumin [5].

Like trihydroxycoprostanoyl-CoA oxidase, purified pristanoyl-CoA oxidase acted solely on 2*S*-methylpentadecanoyl-CoA, but the activity was only weakly influenced by albumin (Fig. 1). In the absence of albumin, a K_m of 40 μM was determined. The CoA esters of 2*R*-methylpentadecanoic acid, trihydroxycoprostanic acid, valproic acid, 2-ethylhexanoic acid, and 3-methylheptadecanoic acid were not oxidized.

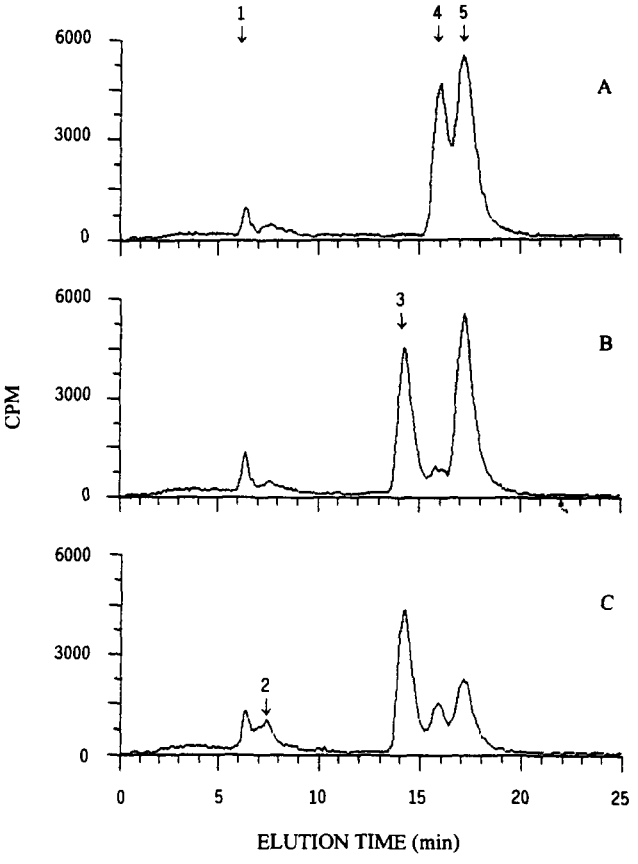

Fig. 3. Desaturation of 26-[^{14}C]trihydroxycoprostanoyl-CoA by purified trihydroxycoprostanoyl-CoA oxidase and purified peroxisomes. Purified trihydroxycoprostanoyl-CoA oxidase (18 μg of protein) (B) or purified peroxisomes (540 μg of protein) (C) were incubated with 100 μM of 26-[^{14}C]trihydroxycoprostanoyl-CoA as described in Section 2. In A, no oxidase or peroxisomes were present during the incubation. After addition of tritiated cholic acid as internal standard, the reaction products were hydrolysed and analysed by reversed-phase HPLC. The panels show only the radioactivity measured in the first channel (both ^{3}H and ^{14}C signals). The numbered arrows indicate the elution of the following compounds (elution time between parentheses): 1, cholic acid (6.40 min); 2, varanic acid (7.46 min); 3, (24*E*)-trihydroxycoprostenic acid (14.23 min); 4, 25*S*-trihydroxycoprostanic acid (16.05 min); 5, 25*R*-trihydroxycoprostanic acid (17.25 min). Based on the stereoselectivity observed with 2-methylpentadecanoyl-CoA, the earlier eluting peak of racemic trihydroxycoprostanic acid is assigned the 25*S*-configuration. According to Farrants et al. [35], the more polar isomer would correspond to the 25*R*-form, whereas Une et al. [47] claimed that the 25*R*-form (as the phenacyl derivative) eluted later than the 25*S*-isomer from a reversed-phase column (TSK gel LS410, 5 μm; 300×4 mm). In our hands, using a Novapak C18 or a Econosphere C18 (5 μm; 150×4.6 mm; Alltech), no separation of the phenacyl derivatives was achieved. Therefore, the ethyl acetate extracts were analysed by TLC on silica G plates. Autoradiography of the plates, developed in chloroform/acetone/methanol (70:50:10, v/v) [48], revealed that the faster migrating trihydroxycoprostanic acid isomer, assigned the *S*-configuration by Batta et al. [48], disappeared after incubation with the oxidase (data not shown).

Except for 3-methylheptadecanoyl-CoA, which was weakly inhibitory, the other CoA esters did not interfere with the desaturation of the 2*S*-methyl compound (Fig. 2).

Our findings imply that during the desaturation of 2-methyl-branched fatty acids by peroxisomes the 2*R*-H group is removed. Since it has been shown that the pro-*R* hydrogens at C2 and C3 are *anti*-eliminated during the oxidation of straight chain acyl-CoAs by palmitoyl-CoA oxidase [36], as well as by the mitochondrial acyl-CoA dehydrogenases (see [37]), it becomes apparent that the basic mechanism underlying these reactions ((pro)*R*-proton abstraction at C2 by an acidic amino acid and a hydride transfer from the pro*R*-position at C3 to the flavin cofactor) must be similar in all these enzymes. In the dehydrogenases essential glutamate residues have been linked to the proton abstraction [37–40]. Comparison of the amino acid sequence of acyl-CoA oxidases, presently known (14 in toto), reveals a stretch of similar amino acids (corresponding to amino acids 389–427 of rat palmitoyl-CoA oxidase [41]), containing a fully conserved glutamate residue (amino acid 421 of palmitoyl-CoA oxidase) and resembling a consensus sequence found in the C-terminal part of most mitochondrial dehydrogenases (Van Veldhoven and Mannaerts, unpublished data). In contrast to the peroxisomal oxidases, the mitochondrial acyl-CoA dehydrogenases acting on short 2-methyl-branched substrates also oxidize the 2*R*-isomers [42]. In this case, however, dehydrogenation occurs on the methyl branch, resulting in the formation of 2-alkyl-acrylyl-CoA. Hence, the reaction occurred with the same stereospecificity but the catalytic site of the dehydrogenase appears less restrictive with regard to the length of the branch on C2 and even allows the entry of a 2-propyl branch (valproyl-CoA) [43,44]. Although the presence of a valproyl-CoA oxidase in rat liver has been reported [45], the oxidases used in this study do not act on valproyl-CoA or 2-ethylhexanoyl-CoA. Moreover, with the substrate used in this study, no activity towards 2*R*-isomers was found, indicating that only a methyl branch has access to the catalytic site of the oxidases. However, it has been shown that both di- or trihydroxycoprostanic acid isomers are converted to chenodeoxy- or cholic acid in man and rat at comparable rates and that their CoA esters are degraded at similar rates in rat or human liver homogenates or isolated rat liver peroxisomes [15–18]. When racemic trihydroxycoprostanoyl-CoA was incubated with excess purified trihydroxycoprostanoyl-CoA oxidase, only one isomer, presumably the 25*S*-isomer, was desaturated (Fig. 3). However, when the racemic substrate was given to purified peroxisomes, both isomers were converted (Fig. 3). Therefore, one must conclude that a racemase (at least acting on bile acyl-CoAs) is present in the purified peroxisomal fractions used in these studies, which is in agreement with recent data reported by Ikegawa et al. [23]. Whether the racemase is truely peroxisomal, or associated with contaminating organelles (endoplasmic reticulum vesicles, mitochondria) remains to be investigated. According to Conzelmann and co-workers [21,22], a racemase acting on 2-methylacyl-CoAs, but also active towards the bile acid intermediates, is confined to the mitochondria in rat liver but present in mitochondria and peroxisomes in human liver. Regardless of the localisation and nature of the racemase(s), our data indicate that this activity is essential in order to convert the 25*R*-forms of the bile acid intermediates, the formation of which is controlled by the 26-hydroxylation of cholesterol [27–29], to the 25*S*-forms before they can be degraded by the peroxisomal β-oxidation system. Most likely, the same is true for the racemisation of pristanic acid.

Due to the stereoselectivity of peroxisomal β-oxidation, a deficiency of racemase activity will likely result in elevated levels of bile acid intermediates (and pristanic acid) but normal levels of very long chain fatty acids, a picture that would mimic a branched chain acyl-CoA oxidase deficiency. Finally, our data also point to an inhibitory effect of 2*R*-methylacyl-CoA on trihydroxycoprostanoyl-CoA oxidase and of racemic 3-methyl-branched acyl-CoA on pristanoyl-CoA oxidase and trihydroxycoprostanoyl-CoA oxidase. Elevated phytanic acid levels, as seen in Refsum patients and reported in some other patients with a peroxisomal disorder [46], may therefore interfere with the degradation of bile acid intermediates (via inhibition of the branched chain acyl-CoA oxidase by increased phytanoyl-CoA levels).

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