Plasma analysis of di- and trihydroxycholestanoic acid diastereoisomers in peroxisomal α-methylacyl-CoA racemase deficiency

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Abstract **We identified a new peroxisomal disorder caused by a deficiency of the enzyme α-methylacyl-coenzyme A (CoA) racemase. Patients with this disorder show elevated plasma levels of pristanic acid and the bile acid intermediates di- and trihydroxycholestanoic acid (DHCA and THCA), which are all substrates for the peroxisomal β-oxidation system. 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 (2*S*)-isomers. Only stereoisomers with the 2-methyl group in the (*S*)-configuration can be degraded via β-oxidation. In this study we used liquid chromatography/tandem mass spectrometry (LC-MS/MS) to analyze the bile acid intermediates that accumulate in plasma from patients with a deficiency of α-methylacyl-CoA racemase and, for comparison, in plasma from patients with Zellweger syndrome and patients with cholestatic liver disease. We found that racemase-deficient patients accumulate exclusively the (*R*)-isomer of free and taurine-conjugated DHCA and THCA, whereas in plasma of patients with Zellweger syndrome and patients with cholestatic liver disease both isomers were present. On the basis of these results we describe an easy and reliable method for the diagnosis of α-methylacyl-CoA racemase-deficient patients by plasma analysis. Our results also show that α-methylacyl-CoA racemase plays a unique role in bile acid formation.**—Ferdinandusse, S., H. Overmars, S. Denis, H. R. Waterham, R. J. A. Wanders, and P. Vreken. **Plasma analysis of di- and trihydroxycholestanoic acid diastereoisomers in peroxisomal α-methylacyl-CoA racemase deficiency.** ***J. Lipid Res.*** **2001.** 42: **137–141.**

Supplementary key words bile acid intermediates • peroxisomal β-oxidation • stereochemistry

Peroxisomes play an important role in the biosynthesis of bile acids from cholesterol because peroxisomal β-oxidation is responsible for chain shortening of the C_{27} bile acid intermediates di- and trihydroxycholestanoic acid [3α,7α-dihydroxy-5β-cholestan-26-oic acid (DHCA) and 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (THCA)], which results in formation of the primary bile acids chenodeoxycholic acid and cholic acid, respectively. The enzymes involved in this process not only handle DHCA and THCA as substrates but also other 2-methyl branched-chain fatty acids, such as pristanic acid (see **Fig. 1**). The first step of β-oxidation is catalyzed by branched-chain acyl-coenzyme A (CoA) oxidase (1, 2), which converts the 2-methyl branched-chain acyl-CoAs into their enoyl-CoA ester. These are subsequently hydrated into a hydroxyacyl-CoA and then dehydrogenated into a β-ketoacyl-CoA. Both these steps are catalyzed by D-bifunctional protein (3–6). Finally, sterol carrier protein X (SCPx) is responsible for the thiolytic cleavage of the β-ketoacyl-CoA esters of pristanic acid as well as DHCA and THCA (7–11).

It has been demonstrated that the peroxisomal β-oxidation system is stereospecific, because the first enzyme, branched-chain acyl-CoA oxidase, can handle only (2*S*)-isomers (12, 13). For this reason, a racemase called α-methylacyl-CoA racemase, identified by Conzelmann and coworkers (14, 15), is also involved in the β-oxidation of branched-chain fatty acids. This enzyme is able to convert (2*R*)-pristanoyl-CoA, (25*R*)-DHC-CoA, and (25*R*)-THC-CoA into their (*S*)-isomers (14, 15) (Fig. 1). This conversion is essential for degradation of these substrates, because naturally occurring pristanic acid is a mixture of two diastereomers, (2*R*,6*R*,10*R*) and (2*S*,6*R*,10*R*) (16), whereas in the case of DHCA and THCA only the (25*R*)-isomers are produced from cholesterol (17–20). As a consequence, patients who are unable to convert the (*R*)-isomer

Abbreviations: DHCA, 3α,7α-dihydroxy-5β-cholestan-26-oic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl; ESI, electrospray ionization; IS, internal standard; LC-MS/MS, liquid chromatography/tandem mass spectrometry; MRM, multiple reaction monitoring; SIR, single ion recording; THCA, 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid.

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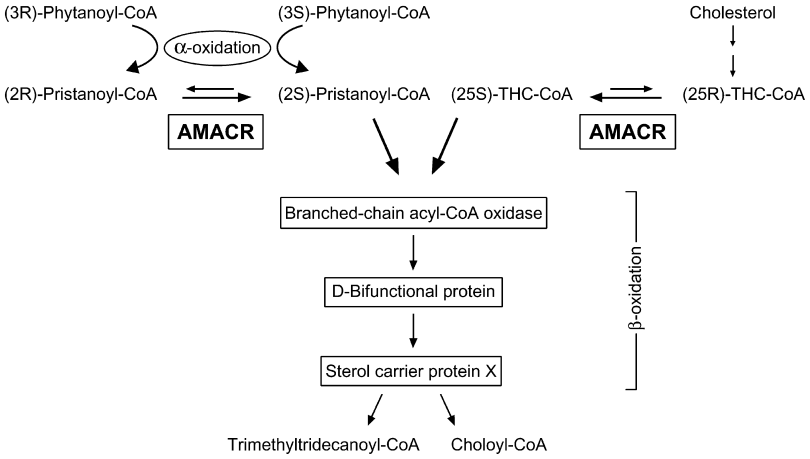

Fig. 1. Schematic representation of the steps involved in the oxidation of (3*R*)- and (3*S*)-phytanic acid as derived from dietary sources and of (25*R*)-THCA produced from cholesterol in the liver. After the activation of (3*R*)- and (3*S*)-phytanic acid to their corresponding coenzyme A (CoA) esters, 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 AMACR into their (25*S*)-stereoisomers.

of pristanoyl-CoA and the C_{27} bile acyl-CoAs to their respective (*S*)-isomers, which are the true substrates for the β-oxidation system, are predicted to accumulate these compounds in their plasma. We have identified three patients with a complete α-methylacyl-CoA racemase deficiency due to mutations in the encoding gene as shown by expression studies in *Escherichia coli* (21). The main clinical symptom in these patients was an adult-onset sensory motor neuropathy. As expected, plasma analysis in these patients revealed an accumulation of both pristanic acid and the bile acid intermediates DHCA and THCA.

In the present study we further analyzed the C_{27} bile acid intermediates accumulating in plasma from these patients and, for comparison, from patients with Zellweger syndrome and patients suffering from cholestatic liver disease, using liquid chromatography-tandem mass spectrometry (LC-MS/MS) to discriminate between the different diastereoisomers of DHCA and THCA. The results obtained indicate that α-methylacyl-CoA racemase is, indeed, indispensable for the oxidation of the bile acid intermediates and that there is no other racemase that takes over the role of the deficient enzyme. Furthermore, the plasma analysis we describe in this article provides an easy and reliable method by which to diagnose α-methylacyl-CoA racemase-deficient patients.

MATERIALS AND METHODS

Materials

The two diastereoisomers of THCA were obtained as described previously (21). Taurine was purchased from Serva (Heidelberg, Germany), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl (EDC) was from Sigma (St. Louis, MO), and [2,2,4,4-$^2H_4$]cholic acid was from J. H. Ritmeester BV (Utrecht, The Netherlands).

Methods

Patients. Plasma samples were obtained from three patients with a deficiency of α-methylacyl-CoA racemase, four patients with Zellweger syndrome, and five patients with cholestatic liver disease. The ages of the patients with cholestatic liver disease (three males and two females) and Zellweger syndrome (two males and two females) varied between 1 month and 3 years. The α-methylacyl-CoA racemase-deficient patients all had distinct mutations in the encoding gene, and racemase activity in fibroblasts of these patients as measured with THC-CoA as substrate was completely deficient (21). Patient 1, a boy, is now 7 years old, patient 2 is a 49-year-old man, and patient 3 is a 48-year-old woman. The patients with Zellweger syndrome all had the clinical and biochemical abnormalities described for Zellweger syndrome (22). Informed consent was obtained for all patients whose plasma was studied and the studies were approved by the Institutional Review Board of the Academic Medical Center, University of Amsterdam (Amsterdam, The Netherlands).

Derivatization of THCA with taurine. The two diastereoisomers of THCA were derivatized with taurine to allow determination of the stereospecificity of the different isomers of taurine-conjugated THCA in the plasma of patients. Derivatization of THCA was performed essentially as described by Zhang et al. (23). Briefly, 0.37 μmol of (25*R*)- or (25*S*)-THCA was dissolved in 0.2 ml of 0.1 M pyridine hydrochloride (pH 5.0). Fifty micromoles of EDC and 100 μmol of taurine were added and the mixture was left for 16 h at room temperature. It was then passed through an SPE-C_{18} column (1.5 × 0.8 cm) (J. T. Baker, Phillipsburg, NJ). After washing the column with water, taurine-conjugated THCA was eluted with methanol. The yield was approximately 70%.

Plasma sample preparation. Fifty microliters of the internal standards (IS) [2,2,4,4-$^2H_4$]cholic acid or [2,2,4,4-$^2H_4$]taurocholic acid was added to 50 μl of plasma. The mixture was deproteinized by addition of 500 μl of acetonitrile followed by subsequent centrifugation for 15 min at 20,000 g_{av} at 4°C. The supernatant was then evaporated under a stream of N_2 gas and the residue was redissolved in 100 μl of methanol–water 40:60 (v/v). Twenty-five microliters was injected into an LC-MS/MS system.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS). LC-MS/MS was carried out with a Hewlett-Packard (Palo Alto, CA) HP 1100 binary pump and a Micromass (Manchester, UK) Quattro II tandem mass spectrometer equipped with electrospray ionization (ESI). The LC separation was performed on an Alltima C_{18} reversed-phase (5 μm) column (250 × 2.1 mm) (Alltech, Deerfield, IL) and optimal resolution was achieved by elution with a linear gradient of methanol (70% → 100%) in 5 mM ammonium formate buffer (pH 5.0) at a flow rate of 0.3 ml/min. MS/MS parameters were as follows: negative ion mode, capillary voltage 3.1 kV, cone voltage 70 V, collision energy 60 eV, collision pressure 0.003 mBar. Argon was used as collision gas. Taurine conjugates were analyzed by multiple reaction monitoring, using

the following transitions (IS 518.3 → 79.8; tauro-DHCA 540.3 → 79.8; tauro-THCA 556.3 → 79.8); the free compounds were analyzed by single ion recording (IS 411.3; DHCA 433.3; THCA 449.3). The limit of detection of the bile acid intermediates was 0.05 μM.

RESULTS AND DISCUSSION

DHCA and THCA are obligatory intermediates in the major biosynthesis route of the primary bile acids chenodeoxycholic acid and cholic acid from cholesterol. They are produced from 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol, respectively. The mitochondrial 27-hydroxylase involved in this pathway has been shown to be stereospecific, which exclusively leads to the formation of the (25*R*)-isomer of DHCA and THCA (17–20). Activation of DHCA and THCA occurs at the membrane of the endoplasmic reticulum followed by transport of DHC-CoA and THC-CoA into the peroxisome via a mechanism yet unknown. In the peroxisome, (25*R*)-DHC-CoA and (25*R*)-THC-CoA are rapidly converted by α-methylacyl-CoA racemase (14, 15) into their (25*S*)-isomers, which can enter the β-oxidation spiral.

Three patients have been identified with a deficiency of α-methylacyl-CoA racemase due to mutations in the encoding gene. Plasma analysis revealed a marked increase in the levels of pristanic acid and of the C_{27} bile acid intermediates DHCA and THCA (21). These compounds, however, are known to accumulate in several other peroxisomal disorders, including isolated defects in the peroxisomal β-oxidation system and defects in peroxisomal biogenesis (22, 24–26). To examine the plasma C_{27} bile acids in closer detail, we developed a method to study the different diastereoisomers of DHCA and THCA in plasma from patients with Zellweger syndrome and patients with an isolated α-methylacyl-CoA racemase deficiency. In addition, we studied plasma from patients with cholestatic liver disease, who also accumulate bile acid intermediates in plasma but do not have a metabolic disorder affecting the oxidation of branched-chain fatty acids and fatty acid derivatives per se. The diastereoisomers of both free and taurine-conjugated C_{27} bile acids could be studied by our LC-MS/MS method. To determine the elution pattern of the diastereoisomers of taurine-conjugated THCA, (25*R*)- and (25*S*)-THCA were derivatized with taurine. Both free and taurine-conjugated (25*S*)-THCA eluted at a lower concentration of methanol than the (25*R*)-isomer (**Fig. 2**). Unfortunately, no standards were available for DHCA. Therefore, we can only speculate about the identification of the diastereoisomers of free and taurine-conjugated DHCA.

Examination of plasma from four different patients with Zellweger syndrome revealed the presence of two diastereoisomers of both free and taurine-conjugated DHCA and THCA (**Table 1** and Fig. 2). DHCA was mainly present as free acid, whereas in most patients more THCA was taurine conjugated than unconjugated. The predominant peak of both free and taurine-conjugated THCA corresponded to the (25*R*)-isomer. The mean values (±SD) for the (25*S*/25*R*)-isomer ratios in these four patients were 0.23 (±0.05) and 0.26 (±0.03) for free and taurine-conjugated THCA, respectively (Table 1). These results are in agreement with the (25*S*/25*R*)-THCA ratio found in urine from an infant with Zellweger syndrome by Une and coworkers (27). The presence of both isomers indicates that α-methylacyl-CoA racemase is enzymatically active in patients with Zellweger syndrome. A residual racemase activity of 10% for pristanoyl-CoA in fibroblasts from patients with Zellweger syndrome compared with control subjects has indeed been reported (15), and corresponds to the results we obtained with THC-CoA as substrate in fibroblasts from patients with Zellweger syndrome [control subjects, 97 ± 28 pmol/min/mg (n = 13); patients with Zellweger syndrome, 17 ± 5 pmol/min/mg (n = 3)]. For free and taurine-conjugated DHCA, respectively, the mean values (±SD) for the peak 1/peak 2 ratios in the four patients with Zellweger syndrome were 0.19 (±0.02) and 0.43 (±0.08) (Table 1).

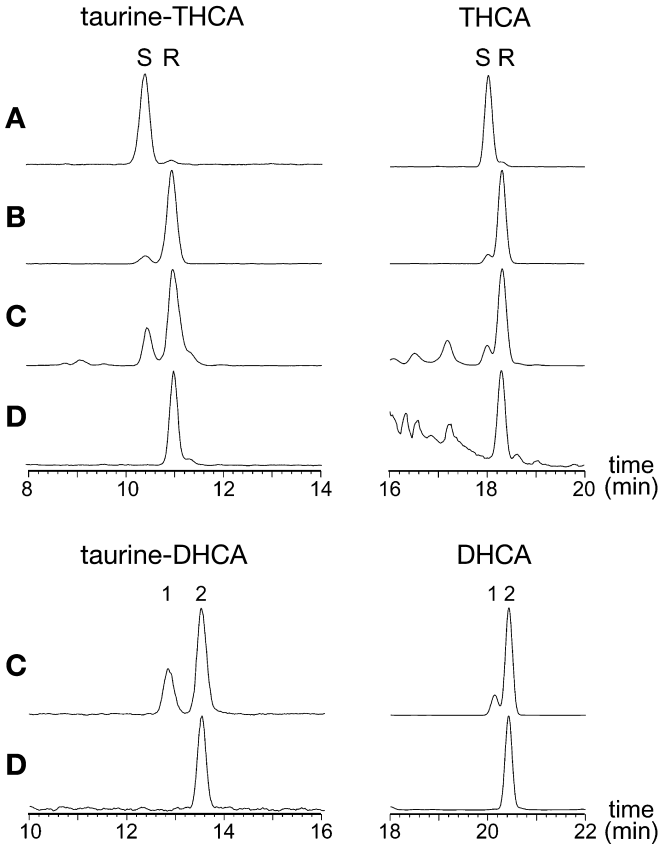

Fig. 2. Separation of the diastereoisomers of free and taurine-conjugated DHCA and THCA by LC-MS/MS. Analysis of the standards for (25*S*)- and (25*R*)-THCA in the free acid form and taurine-conjugated are shown (A and B, respectively). Plasma analysis of patients with Zellweger syndrome (C) revealed the presence of both diastereoisomers of free and taurine-conjugated THCA, whereas patients with a deficiency of α-methylacyl-CoA racemase (D) accumulate only the (25*R*)-isomer. No standards were available of the separate diastereoisomers of free and taurine-conjugated DHCA, but the exclusive accumulation of peak 2 for both compounds in racemase-deficient patients strongly suggests that peak 2 represents the (25*R*)-isomer.

TABLE 1. Analysis of the diastereoisomers of free and taurine-conjugated DHCA and THCA in plasma from five patients with cholestatic liver disease, four patients with Zellweger syndrome, and three patients with an α-methylacyl-CoA racemase deficiency

			α-Methylacyl-CoA Racemase Deficiency		
	Cholestatic	Zellweger	Patient 1	Patient 2	Patient 3
THCA free acid					
(*S*)-Isomer[a]	ND	1.9–13.6	ND	ND	ND
(*R*)-Isomer[a]	ND	9.7–75.5	2.2	2.2	0.1
(*S/R*)-Isomer[b]		0.23 ± 0.05	—	—	—
DHCA free acid					
Peak 1[a]	ND	4.4–14.3	ND	ND	ND
Peak 2[a]	ND	22.0–76.8	30.9	21.3	4.4
Peaks 1/2[b]		0.19 ± 0.02	—	—	—
THCA taurine conjugated					
(*S*)-Isomer[a]	0.06–0.43	3.8–10.5	ND	ND	ND
(*R*)-Isomer[a]	0.23–2.39	16.1–34.8	0.6	9.9	3.8
(*S/R*)-Isomer[b]	0.25 ± 0.08	0.26 ± 0.03	—	—	—
DHCA taurine-conjugated					
Peak 1[a]	ND	0.8–3.2	ND	ND	ND
Peak 2[a]	ND	1.7–7.0	ND	2.0	0.6
Peaks 1/2[b]		0.43 ± 0.08	—	—	—
Cholic acid[a,c]	14.1–74.8	0.3–13.0	0.1	0.2	0.2
Chenodeoxycholic acid[a,c]	32.2–100.9	4.3–37.0	0.2	0.7	0.5

ND, Not detectable. Cholestatic, cholestatic liver disease patients (n = 5); Zellweger, patients with Zellweger syndrome (n = 4); patient 1–3, α-methylacyl-CoA racemase-deficient patients.

[a] Range in micromoles per liter.

[b] Ratio mean ± SD.

[c] Sum of glycine- and taurine-conjugated species (normal range, 0.7–10 μM chenodeoxycholic acid; 0.1–4.7 μM cholic acid).

In the patients with cholestatic liver disease the mean value (±SD) for the (25*S*/25*R*)-isomer ratios for taurine-conjugated THCA was 0.25 (±0.08), which is similar to the ratio found in patients with Zellweger syndrome (0.26 ± 0.03, $P > 0.05$; *t*-test). These results confirm that plasma from Zellweger patients can be used as a control in this assay, even though the biogenesis of peroxisomes, where the racemase is localized, is disturbed in these patients. The amount of free THCA and free and taurine-conjugated DHCA in plasma of these patients was too low to draw any conclusions about the distribution of the different diastereoisomers.

Plasma analysis of C_{27} bile acid intermediates in the three patients with a defined α-methylacyl-CoA racemase deficiency revealed the exclusive accumulation of the (25*R*)-isomer of both free and taurine-conjugated THCA (Table 1 and Fig. 2). Only one diastereoisomer of DHCA was present in both free acid form and in taurine-conjugated form. This strongly suggests that, as for THCA, peak 2 of free and taurine-conjugated DHCA, which elutes at a higher methanol concentration than peak 1, represents the (25*R*)-isomer (Fig. 2). The concentrations of the normal C_{24} bile acids cholic acid and chenodeoxycholic acid were in the lower part of the normal range. These bile acids could be synthesized by the alternative 25-hydroxylation pathway (28), but the lack of 25-hydroxylated bile alcohols (data not shown) in plasma of racemase-deficient patients suggests that other pathways might be responsible for the residual C_{24} bile acid biosynthesis.

Routine plasma analysis of adult patients with sensory motor neuropathy usually does not include analysis of bile acids and branched-chain fatty acids. This, together with the fact that the clinical symptoms associated with α-methylacyl-CoA racemase deficiency are relatively mild, implies that thus far many patients with α-methylacyl-CoA racemase deficiency may have remained undiagnosed. The method described in this article provides a unique diagnostic tool for this disorder. Only a small amount of plasma is needed, the analysis takes little time, and the exclusive accumulation of the (25*R*)-isomer of free and taurine-conjugated DHCA and THCA indisputably reveals a deficiency of α-methylacyl-CoA racemase in the patient. Finally, our data indicate that α-methylacyl-CoA racemase plays an indispensable role in bile acid formation.

This work was supported by the Princess Beatrix Fund (The Hague, The Netherlands).

Manuscript received 10 July 2000 and in revised form 27 September 2000.

REFERENCES

1. Casteels, M., L. Schepers, P. P. van Veldhoven, H. J. Eyssen, and G. P. Mannaerts. 1990. Separate peroxisomal oxidases for fatty acyl-CoAs and trihydroxycoprostanoyl-CoA in human liver. *J. Lipid Res.* **31:** 1865–1872.
2. Vanhove, G. F., P. P. van Veldhoven, M. Fransen, S. Denis, H. J. Eyssen, R. J. A. Wanders, and G. P. Mannaerts. 1993. The CoA esters of 2-methyl-branched chain fatty acids and of the bile acid intermediates di- and trihydroxycoprostanic acids are oxidized by one single peroxisomal branched chain acyl-CoA oxidase in human liver and kidney. *J. Biol. Chem.* **268:** 10335–10344.
3. Dieuaide-Noubhani, M., D. Novikov, E. Baumgart, J. C. T. Vanhooren, M. Fransen, M. Goethals, J. Vandekerckhove, P. P. van Veldhoven, and G. P. Mannaerts. 1996. Further characterization of the peroxisomal 3-hydroxyacyl-CoA dehydrogenases from rat liver—relationship between the different dehydrogenases and evidence

that fatty acids and the C-27 bile acids di- and trihydroxycoprostanic acids are metabolized by separate multifunctional enzymes. *Eur. J. Biochem.* **240:** 660–666.
4. Jiang, L. L., A. Kobayaski, H. Matsuura, H. Fukushima, and T. Hashimoto. 1996. Purification and properties of human D-3-hydroxyacyl-CoA dehydratase: medium-chain enoyl-CoA hydratase is D-3-hydroxyacyl-CoA dehydratase. *J. Biochem.* **120:** 624–632.
5. Jiang, L. L., T. Kurosawa, M. Sato, Y. Suzuki, and T. Hashimoto. 1997. Physiological role of D-3-hydroxyacyl-CoA dehydratase D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein. *J. Biochem.* **121:** 506–513.
6. Qin, Y. M., M. H. Poutanen, H. M. Helander, A. P. Kvist, K. M. Siivari, W. Schmits, E. Conzelmann, U. Hellman, and J. K. Hiltunen. 1997. Peroxisomal multifunctional enzyme of beta-oxidation metabolizing D-3-hydroxyacyl-CoA esters in rat liver—molecular cloning, expression and characterization. *Biochem. J.* **321:** 21–28.
7. Seedorf, U., P. Brysch, T. Engel, K. Schrage, and G. Assmann. 1994. Sterol carrier protein X is peroxisomal 3-oxoacyl coenzyme A thiolase with intrinsic sterol carrier and lipid transfer activity. *J. Biol. Chem.* **269:** 21277–21283.
8. Antonenkov, V. D., P. P. van Veldhoven, E. Waelkens, and G. P. Mannaerts. 1997. Substrate specificities of 3-ketoacyl-CoA thiolase A and sterol carrier protein 2/3-oxoacyl-CoA thiolase purified from normal rat liver peroxisomes. *J. Biol. Chem.* **272:** 26023–26031.
9. Wanders, R. J. A., S. Denis, F. Wouters, K. W. A. Wirtz, and U. Seedorf. 1997. Sterol carrier protein X (SCPx) is a peroxisomal branched-chain β-ketothiolase specifically reacting with 3-oxopristanoyl-CoA: a new, unique role for SCPx in branched-chain fatty acid metabolism in peroxisomes. *Biochem. Biophys. Res. Commun.* **236:** 565–569.
10. Bun-Ya, M., M. Maebuchi, T. Kamiryo, T. Kurosawa, M. Sato, M. Tohma, L. L. Jiang, and T. Hashimoto. 1998. Thiolase involved in bile acid formation. *J. Biochem.* **123:** 347–352.
11. Wanders, R. J. A., S. Denis, E. van Berkel, F. Wouters, K. W. A. Wirtz, and U. Seedorf. 1998. Identification of the newly discovered 58 kDa peroxisomal thiolase SCPx as the main thiolase involved in both pristanic acid and trihydroxycholestanoic acid oxidation: implications for peroxisomal β-oxidation disorders. *J. Inher. Metab. Dis.* **21:** 302–305.
12. 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.
13. 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.
14. 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.
15. 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.
16. 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.
17. 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.
18. Gustafsson, J., and S. Sjostedt. 1978. On the stereospecificity of microsomal "26"-hydroxylation in bile acid biosynthesis. *J. Biol. Chem.* **253:** 199–201.
19. Shefer, S., F. W. Cheng, A. K. Batta, B. Dayal, G. S. Tint, and G. Salen. 1978. Biosynthesis of chenodeoxycholic acid in man: stereospecific side-chain hydroxylations of 5β-cholestane-3α,7α-diol. *J. Clin. Invest.* **62:** 539–545.
20. Shefer, S., F. W. Cheng, A. K. Batta, B. Dayal, G. S. Tint, G. Salen, and E. H. Mosbach. 1978. Stereospecific side chain hydroxylations in the biosynthesis of chenodeoxycholic acid. *J. Biol. Chem.* **253:** 6386–6392.
21. 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. *Nat. Genet.* **24:** 188–191.
22. Wanders, R. J. A., R. B. H. Schutgens, and P. G. Barth. 1995. Peroxisomal disorders: a review. *J. Neuropathol. Exp. Neurol.* **54:** 726–739.
23. Zhang, J., W. J. Griffiths, T. Bergman, and J. Sjövall. 1993. Derivatization of bile acids with taurine for analysis by fast atom bombardment mass spectrometry with collision-induced fragmentation. *J. Lipid Res.* **34:** 1895–1900.
24. Clayton, P. T., B. D. Lake, N. A. Hall, D. B. Shortland, R. A. Carruthers, and A. M. Lawson. 1987. Plasma bile acids in patients with peroxisomal dysfunction syndromes: analysis by capillary gas chromatography-mass spectrometry. *Eur. J. Pediatr.* **146:** 166–173.
25. Van Grunsven, E. G., E. van Berkel, L. IJlst, P. Vreken, J. B. C. de Klerk, J. Adamski, H. Lemonde, P. T. Clayton, D. A. Cuebas, and R. J. A. Wanders. 1998. Peroxisomal D-hydroxyacyl-CoA dehydrogenase deficiency: resolution of the enzyme defect and its molecular basis in bifunctional protein deficiency. *Proc. Natl. Acad. Sci. USA.* **95:** 2128–2133.
26. Van Grunsven, E. G., E. van Berkel, P. A. W. Mooijer, P. A. Watkins, H. W. Moser, Y. Suzuki, L. L. Jiang, T. Hashimoto, G. Hoefler, J. Adamski, and R. J. A. Wanders. 1999. Peroxisomal bifunctional protein deficiency revisited: resolution of its true enzymatic and molecular basis. *Am. J. Hum. Genet.* **64:** 99–107.
27. Une, M., Y. Tazawa, K. Tada, and T. Hoshita. 1987. Occurrence of both (25*R*)- and (25*S*)-3α,7α,12α-trihydroxy-5β-cholestanoic acids in urine from an infant with Zellweger's syndrome. *J. Biochem.* **102:** 1525–1530.
28. Duane, W. C., P. A. Pooler, and J. N. Hamilton. 1988. Bile acid synthesis in man. In vivo activity of the 25-hydroxylation pathway. *J. Clin. Invest.* **82:** 82–85.