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Substrate Stereospecificity in Oxidation of (25S)-3α,7α,12α-trihydroxy-5β-cholestanoyl-CoA by Peroxisomal Trihydroxy-5β-cholestanoyl-CoA Oxidase

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Partly purified 3α,7α,12α-trihydroxy-5β-cholestanoyl-CoA oxidase from rabbit liver peroxisomes was found to convert the 25S- but not the 25R diastereoisomer of 3α,7α,12α-trihydroxy-5β-cholestan-27-oyl-CoA into (24E)-3α,7α,12α-trihydroxy-5β-cholest-24-en-27-oic acid. In the presence of a peroxisomal THCA-CoA racemase, however, also the 25R isomer was oxidized. Since the mitochondrial steroid-27-hydroxylase, responsible for formation of THCA, is 25R specific a racemase seems to be obligatory for formation of cholic acid by the normal peroxisomal-dependent pathway.

3α,7α,12α-Trihydroxy-5β-cholestan-27-oic acid (THCA), an important intermediate in the biosynthesis of cholic acid, has an asymmetric carbon atom at position 25. Both the 25R and the 25S diastereoisomers of THCA have been isolated from biological sources (1). In human bile the natural isomer appears to be (25R)-THCA (2), while both (25R)- and (25S)-THCA were found to be excreted in a ratio of about 7 : 3 in urine from an infant with Zellweger syndrome (3). Patients with this disease are known to lack the peroxisomal THCA-CoA oxidase required for normal biosynthesis of bile acids (4).

Both isomers of THCA are converted into cholic acid in vivo and in vitro in man as well as in the rat (5-8). 5β-Cholestane-3α,7α,12α-triol 27- hydroxylase, the enzyme responsible for formation of THCA, has been found to be R specific (2,9). This raises the question whether the peroxisomal 3α,7α,12α-trihydroxy-5β-cholestanoyl-CoA oxidase (THCH-CoA oxidase) that catalyzes the first step in the oxidation of THCA is also R specific. A theoretical alternative could be conversion of (25R)-THCA into the 25S isomer by a recently demonstrated α-methylacyl-CoA racemase (10,11) prior to oxidation by a (25S)-THCA specific oxidase.

During our attempts to purify THCH-CoA oxidase from rabbit liver peroxisomes we noticed that at a certain step in the purification procedure one of the two isomers of THCA was more efficiently converted into (24E)-3α,7α,12α-trihydroxy-5β-cholest-24-en-27-oic acid (Δ24-THCA) than the other. We here report that THCA-CoA oxidase is specific for the 25S-diastereoisomer of THCA and that a THCA-CoA racemase is required in addition to the THCA-CoA oxidase for conversion of (25R)-THCA into Δ24-THCA.

MATERIALS AND METHODS

Chemicals. 3α,7α,12α-Trihydroxy-5β-[7^{3}H]cholestan-27-oic acid was prepared as described (12) using bile from Alligator mississipiensis as starting material. The natural form of THCA in bile of Alligator mississippiensis has been identified as the 25R-diastereoisomer (13) but the strong alkaline hydrolysis generally used during isolation causes isomerization at C25 and appearance of the 25S isomer (14). The isolated material was purified by HPLC on a 5 μm

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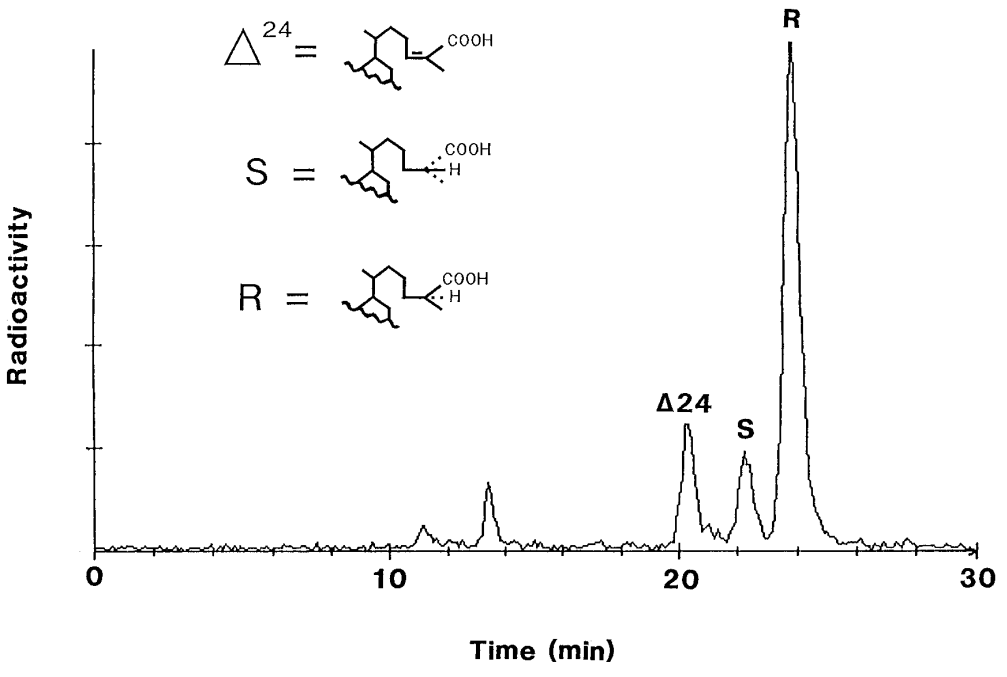

FIG. 1. HPLC radiochromatogram of an extract after incubation of tritium labeled (25R)+(25S)-THCA with peroxisomal THCA-CoA oxidase. The substrate was incubated with 4 μg of the partly purified oxidase preparation for 40 min as described in Methods. Extraction and chromatogaphy were as described in Methods. The product Δ24-THCA is marked by Δ24. Some polar material that eluted at 13 min was also seen in the blank incubation and probably does not represent any significant further conversion of Δ24-THCA. The structure of the side chain of the actual compounds is shown as inset.

C-18 Nucleosil column (0.5 $\times$ 25 cm) with 24% 30 mM trifluoroacetic acid (pH 2.9 with triethylamine) in methanol as eluting solvent. The 25R- and the 25S isomers of THCA were almost completely separated in this system (15). According to Une et al (16) (25S)-THCA is slightly more polar than the (25R) isomer and elutes in front of (25R)-THCA in a reversed phase HPLC system (3). (We have previously erroneously considered the R form to be the more polar (15)). The ratio between the 25R and the 25S isomers in the material primarily isolated was about 7 : 3. Both the combined fractions of (25R)+(25S)THCA and the purified (25R)-THCA were converted into their respective CoA esters using the same method as used for the synthesis of choloyl-CoA (17). (25R)+(25S)THCA-CoA consisted of 28.3% of the 25S- and 71.7% of the 25R isomer. (25R)-THCA-CoA was contaminated with less than 5% of the 25S isomer. Because of the small amounts of material available synthesis of sufficient amounts of (25S)-THCA-CoA was unsuccessful. Other chemicals were commercial high purity material.

Enzyme preparation. Liver peroxisomes were prepared from a male rabbit fasted for 24 h essentially as described for rat liver (18). The light mitochondrial fration was layered on a linear Nycodenz gradient ranging from 15% (w/v) in 0.25M sucrose, 1 mM EDTA and 1 mM Hepes at pH 7.4, to 45% in 1 mM EDTA, 1 mM Hepes at pH 7.4. The gradient tubes contained a prelayered 2 ml Maxidenz cushion and were centrifuged at 30.000 rpm for 30 min at 4°C in the Kontron TV 850 vertical rotor. Fractions of 1.7 ml were collected and assayed for catalase activity (18,19). The 5 fractions with the highest activity were combined, diluted with 0.25 M sucrose and centrifuged at 33.000 rpm (100.000g) for 45 min. The pellet (24 mg of protein) was resuspended in 10 ml of solubilization buffer consisting of 10mM sodium pyrophosphate, pH 9, 10% ethylene glycol, 0.1% Triton X-100, 10 μM FAD, 0.5 mM DTT. After stirring on ice for 1 h and centrifugation at 33.000 rpm for 45 min the supernatant was brought to pH 7.4 and applied to a hydroxylapatite column (vol. 5 ml, d. 1cm). The column was equilibrated in 10 mM phosphate buffer pH 7.4, 10 μM FAD, 0.5 mM DTT, 10% ethylene glycol, and eluted with the same buffer to remove unbound protein. The flow rate was 0.35 ml/min and 1.75 ml fractions were collected. Protein containing THCA-CoA oxidase (and devoid of palmitoyl-CoA oxidase activity) was eluted with 60 mM potassium phosphate buffer pH 7.4, 10 μM FAD, 0.5 mM DTT and 20% ethylene glycol. The active fractions (total vol. 8 ml) were concentrated to 1.2 ml in a dialysis bag covered with polyethylene glycol (mol.wt. 15-20.000, Sigma). Aliquots of the concentrated protein solution were subjected to gel filtration on a Superdex^{TM}200 HR 10/30 column connected to a Smart^{TM} chromatography system

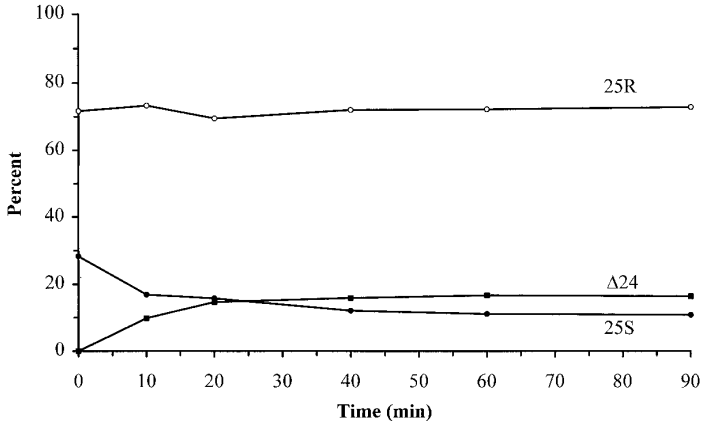

FIG. 2. Formation of 3α,7α,12α-trihydroxy-5β-cholest-24(E)-enoic acid (Δ24-THCA) from (25R)+(25S)-THCA-CoA by peroxisomal THCA-CoA oxidase. (25R)+(25S)-THCA-CoA was incubated with 4 μg of partly purified oxidase as described in Methods. The figures represent the percentage distribution of radioactivity between the product (Δ24) and the 2 isomers of THCA, (R) and (S). Product formation at 10 min corresponded to 2.5 nmol.

(Pharmacia Biotech, Uppsala, Sweden). Elution was performed with phosphate buffered saline containing 0.5 mM DTT and 20% ethylene glycol at a flow rate of 0.25 ml/min and fractions of 0.25 ml were collected. Maximum THCA-CoA oxidase activity was detected in the fraction that eluted between 13.00 and 13.25 ml which was immediately in front of the elution volume corresponding to albumin (13.5 ml), suggesting a molecular weight of the oxidase of about 70 kDa. Fractions between 12.75 and 13.50 ml were pooled and used as a source of the oxidase. No racemase activity was detected in this preparation. Maximum THCA-CoA racemase was found to be in a fraction between 14.75 and 15.00 ml corresponding to a molecular weight of about 40 kDa. This compares with the values given by Schmitz and coworkers of 45 kDa for a similar rat liver mitochondrial enzyme (10) and 47 kDa for a human liver peroxisomal enzyme (11) . Fractions between 14.5 and 15.5 ml were pooled. The racemase preparation was found to be contaminated with some THCA-CoA oxidase activity corresponding to about 22% of that in the oxidase preparation.

Enzyme assays and analytical procedures. The conversion of THCA into 3α,7α,12α-trihydroxy-5β-cholest-24(E)-enoic acid (Δ24-THCA) was measured at 30°C in a volume of 0.1 ml in an incubation medium that contained 0.1 M Tris-HCl, pH 8, 75 μM FAD, 1 mg/ml BSA, 0.01% Triton X-100, and 25 μM (25R)+(25S)-[^{3}H]THCA-CoA (60.000 c.p.m.). The incubations were stopped with 2.5 μl 6M KOH and hydrolyzed at 60°C for 30 min to remove bound CoA. After acidification and extraction with ethyl acetate, the samples were analyzed by reversed phase HPLC on a 5 μm YMC-Pack ODS-A column (250 $\times$ 4.6 mm I.D.) (YMC Co., Ltd. Kyoto, Japan). The eluting solvent was 20% 30 mM trifluoroacetic acid (pH 2.9 with triethylamine) in methanol. Radioactivity in the eluent was monitored by a Flo-One Beta Radio-Chromatography Detector (Radiomatic Instruments & chemical Co. Inc. Tampa, Florida, U.S.A.).

THCA-CoA racemase activity was assayed in the same incubation buffer by following the conversion of (25R)-THCA-CoA into (25S)-THCA after hydrolysis and extraction as above.

Protein was determined by the method of Bradford (21). Protein in the eluent from the gel filtration column was estimated based on the UV absorbance in the eluent compared to that of a fixed amount of serum albumin.

RESULTS

Incubation of (25R)+(25S)THCA-CoA with THCA-CoA oxidase resulted in the formation of a product previously identified as 3α,7α,12α-trihydroxy-5β-cholest-24(E)-enoic acid (Δ24-THCA) (22) (Fig. 1). No such product was seen in the absence of the oxidase preparation. From Fig. 1 it is seen that the HPLC system used separates completely the 25S- and the 25R isomers of THCA. With increasing time of incubation it was observed that the radioactive peak corresponding to (25S)-THCA decreased as the amount of product increased, while the amount of the 25R isomer remained constant (Fig.2). This indicates that the oxidase is stereo-specific for (25S)-THCA. In the presence of both THCA-CoA oxidase and THCA-CoA race-mase in the incubation mixture, both the 25S- and the 25R isomer decreased with increasing

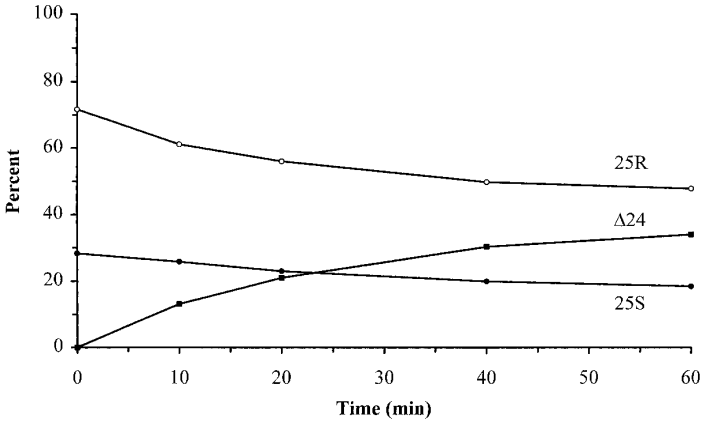

FIG. 3. Formation of 3α,7α,12α-trihydroxy-5β-cholest-24(E)-enoic acid (Δ24-THCA) from (25R)+(25S)-THCA-CoA by peroxisomal THCA-CoA oxidase in the presence of THCA-CoA racemase. The experiments were performed as in Fig. 3 but in the presence of 2.7 μg of the THCA-CoA racemase preparation.

product formation. Total product formation was also higher than in the absence of racemase (Fig.3). This indicates that the racemase catalyzes conversion of (25R)- into (25S)-THCA thus making more substrate available for the oxidase. The concentration of (25S)-THCA used in these experiments is certainly below saturation level since it has previously been shown that with isolated liver peroxisomes saturation is reached at about 10 μM when (25R)+(25S)-THCA is used as substrate (7). This may explain the increased rate of product formation in the presence of racemase. That the added racemase was indeed able to convert (25R)-THCA into (25S)-THCA was demonstrated by incubating (25R)-THCA-CoA with the racemase preparation. The 25R isomer was slowly converted into the 25S isomer and an equilibrium was reached at about 25% of (25S)-THCA and 75% (25R)-THCA (Fig. 4). In the presence of THCA-CoA oxidase the formed (25S)-THCA was further converted into Δ24-THCA (data not shown).

DISCUSSION

We have demonstrated here that partly purified liver peroxisomal THCA-CoA oxidase is stereospecific for (25S)-THCA and that a THCA-CoA racemase is required in addition for the oxidation of (25R)-THCA. Our results may explain the findings of Une et al. who demonstrated formation of (24R,25S)-3α,7α,12α,24-tetrahydroxy-5β-cholestan-27-oic acid when incubating either (25R)- or (25S)-THCA with a crude rat liver homogenate (23). More recently Ikegawa et al. demonstrated dehydrogenation of (25R)- and 25S-THCA into Δ24-THCA when incubated with rat liver light mitochondrial fraction and demonstrated elimination of a pro-R hydrogen at C-24 in both (25R)- and (25S)-THCA (24). If this is correct, there must be a syn-elimination of hydrogen from the 25R isomer and an anti-elimination from the latter unless there is an epimerization prior to oxidation. By analogy to previous findings that dehydrogenation of fatty acids by acyl-CoA oxidase is completely stereospecific and involves anti-elimination of a pro-R hydrogen at C-2 and C-3 (25) the authors discussed the possibility of the requirement of an THCA epimerase (or racemase). Such activity was in fact recently demonstrated in a rat liver peroxisomal fraction (26). Purification and characterization of an α-methylacyl-CoA racemase from rat liver mitochondria and from human liver peroxisomes active also with the two diastereoisomers of THCA have now been reported (10,11).

As discussed in detail elsewhere (27) 5β-cholestane-3α,7α,12α-triol 27-hydroxylase, the

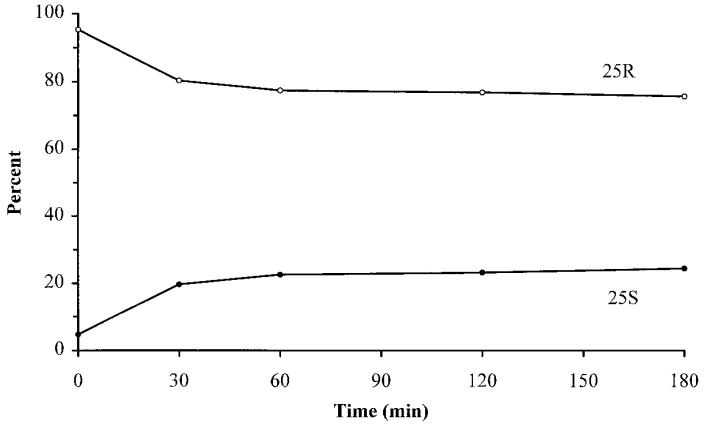

FIG. 4. Conversion of (25R)-THCA into (25S)-THCA by peroxisomal THCA-CoA racemase. Tritium labeled (25R)-THCA-CoA (16.000 cpm) was incubated with 2.7 μg of the racemase preparation for the indicated times as described in Methods. The figures represent the percentage distribution between the 2 isomers of THCA, (R) and (S).

enzyme involved in normal bile acid synthesis and responsible for formation of THCA ,appears to be R specific (2,9). A racemase is thus required for further conversion of (25R)-THCA into cholic acid. The presence of both (25R)- and (25S)-THCA in a ratio of 7 :3 in urine from an infant with Zellweger syndrome (3) may be explained if it is assumed that the racemase is at least partially active in this peroxisomal disorder and responsible for the equilibrium observed between the two isomers.

In conclusion, we have shown for the first time that THCA-CoA oxidase is specific for (25S)-THCA and that a (peroxisomal) THCA-CoA racemase seems to be obligatory for formation of cholic acid by the normal peroxisomal pathway.

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REFERENCES

1. Shah, P. P., Staple, E., and Rabinowitz, J. L. (1968) *Arch. Biochem. Biophys.* **123,** 427–428.
2. Batta, A. K., Salen, G., Shefer, S., Dayal, B., and Tint, G. S. (1983) *J. Lipid Res.* **24,** 94–96.
3. Une, M., Tazawa, Y., Tada, K., and Hoshita, T. (1987) *J. Biochem. (Tokyo)* **102,** 1525–1530.
4. Kase, B. F., Björkhem, I., Hågå, P., and Pedersen, J. I. (1984) *J. Clin. Invest.* **75,** 427–435.
5. Gustafsson, J. (1980) *Lipids*, **15,** 113–121.
6. Swell, L., Gustafsson, J., Danielsson, H., Schwartz, C. C., and Vlahcevic, Z. R. (1981) *J. Biol. Chem.* **256,** 912–916.
7. Kase, F., Björkhem, I., and Pedersen, J. I. (1983) *J. Lipid Res.* **24,** 1560–1567.
8. Kase, B. F., Pedersen, J. I., Strandvik, B., and Björkhem, I. (1986) *J. Clin. Invest.* **76,** 2393–2402.
9. Schefer, S., Cheng, F. W., Batta, A. K., Dayal, B., Tint, G. S., and Salen, G. (1978) *J. Clin. Invest.* **62,** 539–545.
10. Schmitz, W., Fingerhut, R., Conzelmann, E. (1994) *Eur. J. Biochem.* **222,** 313–223.
11. Schmitz, W., Albers, C., Fingerhud, R., Conzelmann, E. (1995) *Eur. J. Biochem.* **231,** 815–822.
12. Gustafsson, J. (1975) *J. Biol. Chem.* **250,** 3889–3893.
13. Batta, A. K., Salen, G., Cheng, F. W., Shefer, S. (1979) *J. Lipid Res.* **20,** 935–940.
14. Batta, A. K., Salen, G., Cheng, F. W., and Shefer, S. (1979) *J. Biol. Chem.* **254,** 11907–11909.
15. Prydz, K., Kase, B. F., and Pedersen, J. I. (1988) *J. Lipid Res.* **29,** 532–537.
16. Une, M., Nagai, F., and Hoshita, T. (1983) *J. Chromatogr.* **257,** 411–415.
17. Webster, L. T., Killenberg, P. G., (1970) *Methods Enzymol.* **77,** 430–436.

18. Prydz, K., Kase, B. F., Björkhem, I., Pedersen, J. I. (1986) *J. Lipid Res.* **27,** 622–628.
19. Baudhuin, P., Beaufay, H., Rahman-Li, H., Sellinger, O. Z., Wattiaux, R., Jacques, P., and deDuve, C. (1964) *Biochem. J.* **92,** 179–184.
20. Peters, T. J., Müller, M., and deDuve, C. (1972) *J. Exp. Med.* **136,** 1117–1139.
21. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
22. Östlund Farrants, A.-K., Björkhem, I., and Pedersen, J. I. (1989) *Biochim. Biophys. Acta.* **1002,** 198–202.
23. Une, M., Morigami, I., Kihira, K., and Hoshita, T. (1984) *J. Biochem. (Tokyo),* **96,** 1103–1107.
24. Ikegawa, S., Watanabe, H., Goto, T., Mano, N., Goto, J., and Nambara, T. (1995) *Biol. Pharm. Bull.* **18,** 1041–1044.
25. Kawaguchi, A., Tsubotani, S., Seyama, Y., Amakawa, T., Osumi, T., Hashimoto, T., Kikuchi, T., Ando, M., and Okuda, S. (1980) *J. Biochem. (Tokyo)* **88,** 1481–1488.
26. Ikegawa, S., Goto, T., Watanabe, H., and Goto, J. (1995) *Biol. Pharm. Bull.* **18,** 1027–1029.
27. Björkhem, I. (1985) *in* Sterol and Bile Acids (Danielsson, H., and Sjövall, J., Eds.), pp. 231–278, Elsevier Science Publishers B. V., Amsterdam.