STEREOSPECIFIC FORMATION OF (24*R*,25*R*)-3 α ,7 α ,12 α ,24-TETRAHYDROXY-5 β -CHOLESTAN-26-OIC ACID CATALYZED WITH A PEROXISOMAL BIFUNCTIONAL D-3-HYDROXYACYL-CoA DEHYDRATASE/D-3-HYDROXYACYL-CoA DEHYDROGENASE

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The absolute configuration of 3 α ,7 α ,12 α ,24-tetrahydroxy-5 β -cholestan-26-oic acid CoA ester (V-CoA) produced by the incubation of (24*E*)-3 α ,7 α ,12 α -trihydroxy-5 β -cholest-24-en-26-oic acid CoA ester (24*E*-THC-CoA) with D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase (D-bifunctional protein) was investigated. When 24*E*-THC-CoA was incubated with D-bifunctional protein the formation of only one isomer (24*R*,25*R*-isomer) of four possible stereoisomers of V-CoA was observed, which suggested the cis-addition of water to a side chain double bond of 24*E*-THC-CoA. The dehydration reaction of V-CoA catalyzed by D-bifunctional protein was also observed when (24*R*,25*R*)-V-CoA was used as a substrate. The other three isomers (24*R*,25*S*-, 24*S*,25*R*- and 24*S*,25*S*-isomers) were not dehydrated with D-bifunctional protein. These results showed that D-bifunctional protein catalyzes stereospecifically the hydration and dehydration step in bile acid biosynthesis.

KEY WORDS bile acid biosynthesis; varanic acid; stereochemistry; D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase; HPLC

It is known that 3 α ,7 α ,12 α ,24-tetrahydroxy-5 β -cholestan-26-oic acid (VA) is one of the intermediates in the side chain degradation reaction of 3 α ,7 α ,12 α -trihydroxy-5 β -cholestan-26-oic acid (THCA).¹⁻³ VA is thought to be produced from (24*E*)-3 α ,7 α ,12 α -trihydroxy-5 β -cholest-24-en-26-oic acid (24*E*-THCA) by catalytic hydration of a bifunctional protein, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase.⁴ VA has two chiral centers at the C-24 and C-25 positions, and therefore four stereoisomers could be considered as possible intermediates in bile acid biosynthesis. There have been reports on the stereochemistry of VA produced in rat liver homogenate,^{5,6} and the reported results may indicate the presence of multiple enzymes responsible for the formation of VA. Recent studies have shown that there are two types of bifunctional enzyme in rat liver peroxisomes, bifunctional enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase (L-bifunctional protein)⁴ and D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase (D-bifunctional protein).⁷⁻¹⁰ The L-bifunctional protein was recently demonstrated to catalyze the hydration reaction of (24*E*)-THC-CoA, and gave (24*S*,25*S*)-V-CoA as the sole product.¹¹ However, the dehydrogenase component of the enzyme was inactive for (24*S*,25*S*)-V-CoA. Our recent study indicated that human D-bifunctional protein shows hydratase/dehydrogenase activities toward 24*E*-THC-CoA.¹² We also reported the absolute configurations of the intermediate VA as (24*R*,25*R*) and (24*S*,25*S*) from the results of incubation with THCA and 24*E*-THCA in both 800 x G supernatant and the light mitochondrial fraction of rat liver homogenate.¹³ It is of interest

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to elucidate the stereochemistry of VA produced by the catalytic hydration with D-bifunctional protein and to investigate the role and function of D-bifunctional protein in bile acid biosynthesis.

MATERIALS AND METHODS

The CoA esters of stereoisomeric VA (24*R*,25*R*-, 24*R*,25*S*-, 24*S*,25*R*- and 24*S*,25*S*-isomers) and 24*E*-THC-CoA were synthesized according to the reported methods.^{14,15} D-bifunctional protein and L-bifunctional protein were obtained by the reported procedure from rat liver homogenates.^{4,10} The enzymatic reactions were performed using 2.5 µg of D-bifunctional protein or 25 µg of L-bifunctional protein in the presence of a substrate (24*E*-THC-CoA and four isomers of V-CoA: 160 µM) in 0.1 M-Tris-HCl buffer (pH 8.0, 250 µl) at 30°C for 10 min. After the addition of an internal standard (homocholic acid CoA ester), acidification (pH 3), and centrifugal ultrafiltration, the filtrate was then analyzed by HPLC (flow rate: 1 ml/min., column: Mightysil 18, 4.6 x 250 mm, Kanto Co., detection at 254 nm) using solvent A: 30 mM acetate buffer (AcOH/AcONa, pH 5) - MeOH - CH₃CN [40:40:20] and B: 30 mM acetate buffer (AcOH/AcONa, pH 5) - CH₃CN [50/50] by linear gradient (from A/B =90/10 to A/B = 5/95 for 40 min.). The details of separation of the stereoisomeric V-CoA and related compounds by the above method will be reported separately.

RESULTS AND DISCUSSION

Figure 1a shows an HPLC chromatogram of the standard samples, in which each isomeric V-CoA was separated and their elution order was completely identical to the reported results.¹¹ Figures 1b and 1c show HPLC chromatograms obtained from the incubation mixture of (24*E*)-THC-CoA with D-bifunctional protein and L-bifunctional protein, respectively. D-bifunctional protein catalyzed the hydration of (24*E*)-THC-CoA to give only (24*R*,25*R*)-V-CoA as the sole product (Fig. 1b), while (24*S*,25*S*)-V-CoA was identified as a major component along with a small amount of (24*R*,25*R*)-isomer when L-bifunctional protein was used (Fig. 1c). The (24*R*,25*R*)-V-CoA found as a minor component in the case of incubation

Fig. 1 HPLC Chromatograms of CoA Esters

(a) Standard samples, (b) (24*E*)-THC-CoA with D-bifunctional protein, (c) (24*E*)-THC-CoA with L-bifunctional protein and (d) (24*R*,25*R*)-V-CoA with D-bifunctional protein. 1: (24*S*,25*R*)-V-CoA, 2: (24*R*,25*S*)-V-CoA, 3: (24*S*,25*S*)-V-CoA, 4: (24*R*,25*R*)-V-CoA, 5: Internal standard, 6: (24*E*)-THC-CoA.

with L-bifunctional protein was assumed to be due to the trace contamination of D-bifunctional protein or isomerization by the isomerase activity of L-bifunctional protein.¹⁶⁾ The activity for the formation of V-CoA with D-bifunctional protein (*ca.* 250 nmol/mg protein/min) was significantly higher than that of L-bifunctional protein (*ca.* 13 nmol/mg protein/min). These results indicated that L- and D-bifunctional enzymes catalyzed the addition of water from the opposite side on the plane of the side chain double bond.

The dehydratase activity of D-bifunctional protein was also investigated using isomeric V-CoA as a substrate. Only (24*R*,25*R*)-V-CoA was effectively converted into (24*E*)-THC-CoA (over 70 %) as shown in Fig. 1d. D-bifunctional protein did not catalyze the dehydration of the other three isomers (24*R*,25*S*-, 24*S*,25*R*-, and 24*S*,25*S*-isomers). This indicates that the dehydratase activity of D-bifunctional protein is highly stereospecific. The above results also suggest that hydration and dehydration proceed through the same reaction intermediate.

L-Bifunctional protein has been shown not to be sufficient for the formation of 24-oxo-intermediate in bile acid biosynthesis.¹¹⁾ However, our recent data showed that the hydratase/dehydrogenase activities of human D-bifunctional protein were over 1000 times higher than those of L-bifunctional protein.¹²⁾ This suggests that D-bifunctional protein may play a significant role in the side chain degradation of bile acid biosynthesis. Further experiments are underway to elucidate the specificity of the dehydrogenase activity of D-bifunctional protein.

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