Metabolism of 32-Oxo-24,25-dihydrolanosterols by Partially Purified Cytochrome P-450$_{14DM}$ from Rat Liver Microsomes

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Metabolism of 32-oxo-24,25-dihydrolanosterols (3β-hydroxylanost-8-en-32-al (4, Δ^8-CHO) and 3β-hydroxylanost-7-en-32-al (5, Δ^7-CHO)) was studied in a reconstituted system consisting of rat liver partially purified cytochrome P-450, which catalyzes lanosterol 14-demethylation (P-450$_{14DM}$), and reduced nicotineamide adenine dinucleotide phosphate (NADPH)–cytochrome P-450 reductase. The reconstituted system converted Δ^8-CHO (4) to 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol (2, 8,14-Diene), which corresponds to the 14-deformylated product. Δ^7-CHO (5), the isomer of Δ^8-CHO (4), was not converted to the corresponding 14-deformylated product. The apparent K_m value of cytochrome P-450$_{14DM}$ for Δ^8-CHO (4) was about 1/20 of that for 24,25-dihydrolanosterol (1, DHL). The metabolism of Δ^8-CHO (4) was inhibited by 7-oxo-24,25-dihydrolanosterol (6, 7-oxo-DHL), which is a potent inhibitor of cholesterol biosynthesis from lanosterol or DHL (1). However, the metabolism of Δ^8-CHO (4) was less inhibited by 7-oxo-DHL (6) than that of DHL (1).

Keywords lanosterol 14-demethylation; lanosterol 14-deformylation; P-450$_{14DM}$; 32-oxo-24,25-dihydrolanosterol; 7-oxo-24,25-dihydrolanosterol

The initial step of sterol biosynthesis from lanosterol (lanosta-8,24-dien-3β-ol) in yeast and mammals is oxidative removal of the 14-methyl group (C-32) of lanosterol catalyzed by a cytochrome P-450-containing enzyme system. The 14-methyl group is removed probably as formic acid *via* 32-oxygenated lanosterol. The product, 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol,[1] is further converted to cholesterol *via* several steps (Fig. 1). 24,25-Dihydrolanosterol (**1**, DHL) is also demethylated like lanosterol and converted to 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol (**2**, 8,14-Diene). 3β,32-Dihydroxylanost-8-ene (**3**, Δ^8-CH_2OH) and 3β-hydroxylanost-8-en-32-al (**4**, Δ^8-CHO) have been assumed to be intermediates of the 14α-demethylation sequence since both sterols[2-4] are converted to cholesterol by cell-free systems. Further, both sterols have been identified as metabolites in large-scale incubation mixtures of cell-free systems in the presence of 0.5 mM NaCN.[5]

We have reported[6] that the substrate specificity of partially purified cytochrome P-450$_{14DM}$ is high for the double bond position on the steroid nucleus. That is, the reconstituted system containing the P-450$_{14DM}$ converted Δ^8-CH_2OH (**3**) to 8,14-Diene (**2**), but the isomers of Δ^8-CH_2OH (**3**), Δ^6- and Δ^7-32-hydroxylated compounds, were not metabolized to the corresponding dehydroxymethylated products. In this report, we clarified the substrate specificity of cytochrome P-450$_{14DM}$ for double bonds positioned on the steroid nucleus with a 32-oxo group. In this experiment, Δ^8-CHO (**4**) and Δ^7-CHO (**5**) were used as test compounds (Fig. 2).

Experimental Procedures

Materials Δ^8-CHO (**4**)[7] and Δ^7-CHO (**5**)[7] were synthesized as described previously. 8,14-Diene (**2**) was synthesized by the method of Paik *et al.*[8] DHL (**1**) was prepared by hydrogenation of a commercial mixture of lanosterol and 24,25-dihydrolanosterol. 7-Oxo-DHL (**6**) was prepared by the method of Pinky *et al.*[9] Dilauroyl phosphatidylcholine (DLPC) was obtained from Sigma Chemical Co. Other chemicals and biochemicals were of the highest quality available commercially.

Preparation of Partially Purified P-450$_{14DM}$ This procedure followed the method of Iwasaki *et al.*[10] Microsomes were prepared from livers of male Wistar rats (220—230 g). Microsomes (specific content of cytochrome P-450, 0.76 nmol/mg protein) were solubilized with sodium cholate, and the solubilized supernatant fraction was chromatographed on an aminooctyl Sepharose column. The P-450 fraction eluted with 10 mM potassium phosphate buffer (KPB) (pH 7.4) containing 0.2% Emulgen 913 was subjected to DE-52 column chromatography at room temperature with a linear gradient of NaCl (0—180 mM), and the P-450 fraction eluted first was further subjected to hydroxyapatite column chromatography. The P-450 fraction eluted with 200 mM KPB was collected and the Emulgen 913 was removed by stirring with Biobeads. The remaining Emulgen 913 amounted to *ca.* 0.01%, as determined from its absorbance at 276 nm. The specific content of the cytochrome P-450 was 4.12 nmol/mg protein. The partially purified cytochrome P-450$_{14DM}$ was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-

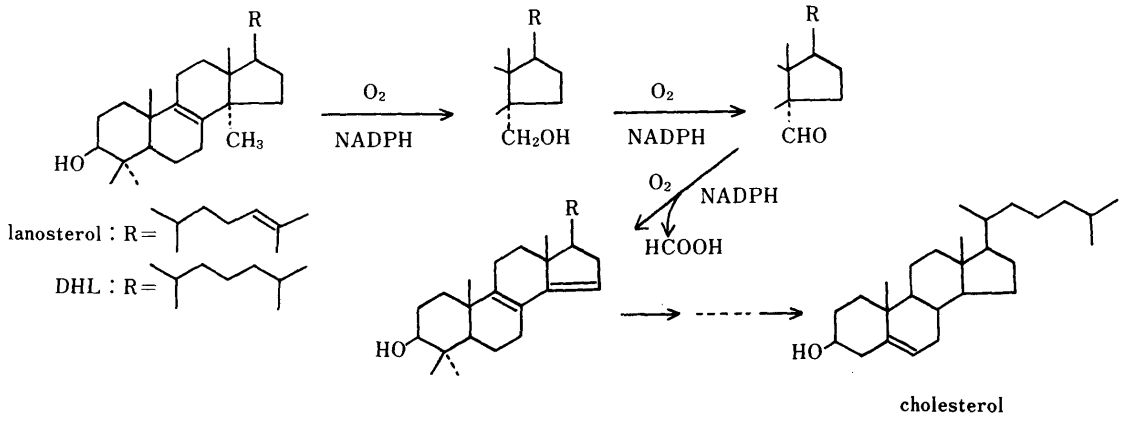

Fig. 1. Hypothetical Pathway for 14α-Demethylation of Lanosterol or DHL to Cholesterol

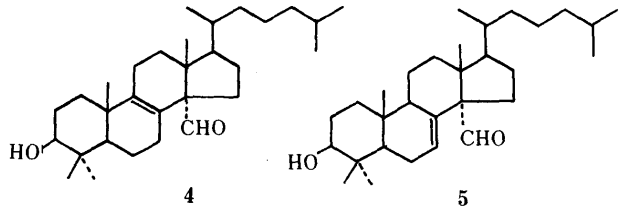

Fig. 2. The Structures of Δ^8-CHO (**4**) and Δ^7-CHO (**5**)

PAGE).[11] The major band of P-450 was found to have an apparent molecular weight of 51000[10] and was accompanied with small amounts of some other bands.

Preparation of Reduced Nicotineamide Adenine Dinucleotide Phosphate (NADPH)–Cytochrome P-450 Reductase This enzyme was prepared to a specific activity of 10 μmol of cytochrome c reduced/min/mg protein from rat liver microsomes according to the method of Yasukochi and Masters[12] and was observed as a single band on SDS-PAGE.[11]

Assay for 14-Demethylation and 14-Deformylation by the Reconstituted System The substrate was dispersed with DLPC as described by Aoyama *et al.*[1] Then, 30 μl of cytochrome P-450 (0.18 nmol) in 0.1 M KPB (pH 7.4) containing 20% glycerol, 5 μl of NADPH–cytochrome P-450 reductase (1 unit) in 10 mM KPB (pH 7.7), and 5 μl of substrate solution dispersed with DLPC were mixed and sonicated, and 0.36 ml of 0.1 M KPB (pH 7.4) consisting of glucose 6-phosphate (final 40 mM), $MgCl_2$ (final 0.4 mM), and glucose 6-phosphate dehydrogenase (0.2 unit) was added. The reaction was started by adding 0.1 ml of NADPH solution (final, 2 mM), and the mixture was incubated at 37 °C under air.

Analytical Methods The reaction was stopped by adding 2 ml of 20% (w/v) KOH and 1.5 ml of methanol, and the reaction mixture was saponified at 80 °C for 1 h. Sterols were extracted with CH_2Cl_2, the organic layer was dried over sodium sulfate, and the solvent was evaporated. In the case of Δ^8-CHO (**4**) and Δ^7-CHO (**5**), a half of the extract of the incubation mixture was subjected to treatment with an excess of $NaBH_4$ (6 mg) overnight at room temperature. The reaction mixture was poured into water, extracted with CH_2Cl_2, and processed as described above. A portion of the product was trimethylsilylated with bis(trimethylsilyl(TMS))-acetamide. The trimethylsilylated sterols were analyzed with a JEOL gas chromatograph–mass spectrometer equipped with a 10 m × 0.2 mm SP-2250 fused silica capillary column (Supelco Inc.) with helium as the carrier gas. Samples were injected at an initial column temperature of 50 °C. After 4 min, the temperature was raised to 255 °C at a rate of 32 °C/min. The injector, separator, and inlet temperatures were 270, 255, and 260 °C, respectively. The lowest limit of detection by mass chromatography was *ca.* 5 ng. The extracted sterols from the reaction mixture were analyzed by high-performance liquid chromatography (HPLC), which was performed on a μBondapak C_{18} reverse-phase column (3.9 mm × 30 cm), using a Waters pump (model 510) and a Waters detector (model 480 spectrometer, set at 214 or 248 nm). Acetonitrile–methanol–water (45 : 45 : 10, v/v/v) was employed as the eluent (flow rate, 1.0 ml/min). For calculation of the activity (nmol of product formed/min), ergosterol (5 μg) was added as an internal standard before extraction of the incubation mixture and analyzed by HPLC (248 nm). The activity was calculated from the areas of the peaks of ergosterol and the metabolite. Protein was determined by the method of Lowry *et al.*[13] using bovine serum albumin as a standard.

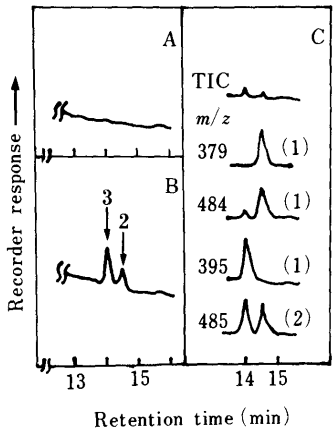

Fig. 3. Gas Chromatographic Detection of the Metabolite of Δ^8-CHO (**4**)

Panel A: incubation was carried out without substrate, as described for panel B. Panel B: Δ^8-CHO (**4**) (23 nmol) dispersed with DLPC (50 μg) was incubated with the reconstituted system as described under Experimental Procedures. Panel C: mass chromatogram of the product in panel B. The column effluent was monitored by mass chromatography at *m/z* 379, 484, 395, and 485 as well as by measuring the total ion current. Values in parentheses show relative magnifications.

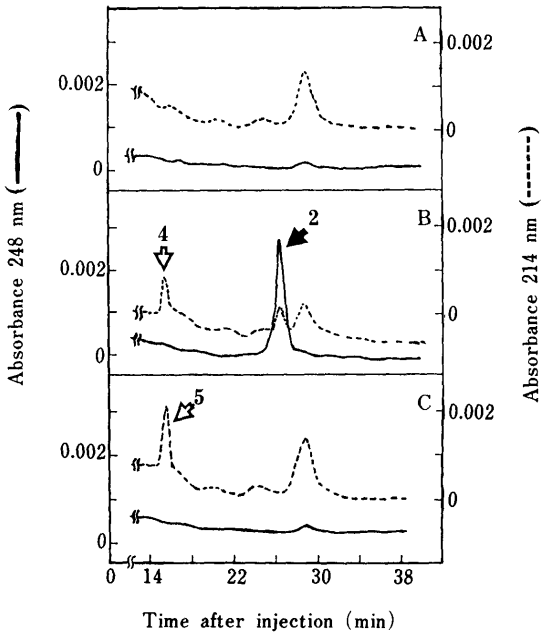

Fig. 4. Reverse-Phase HPLC Chromatograms with Detection at 214 and 248 nm of the Metabolite from Δ^8-CHO (**4**) and Δ^7-CHO (**5**) Formed by the Reconstituted System

Panel A: the reaction extract without substrate. Panel B: sterols in the reaction extract of Δ^8-CHO (**4**). Panel C: sterols in the reaction extract of Δ^7-CHO (**5**).

Results and Discussion

Metabolism of 32-Oxo-24,25-dihydrolanosterols We have previously shown[6] that the reconstituted system consisting of partially purified cytochrome $P\text{-}450_{14DM}$ and NADPH–cytochrome P-450 reductase catalyzes the 14-demethylation of DHL (**1**). The metabolite was identified as 8,14-Diene (**2**) by comparison with an authentic sample, and this compound was the sole metabolite formed from the substrate by the reconstituted system.

In this experiment, the metabolism of Δ^8-CHO (**4**) and Δ^7-CHO (**5**) was studied. Because Δ^8-CHO (**4**) and Δ^7-CHO (**5**) were decomposed on gas chromatography (GC), a half of the extract of the incubation mixture was treated with $NaBH_4$, giving Δ^8-CH_2OH (**3**) from Δ^8-CHO (**4**). Figure 3B shows a gas chromatogram of trimethylsililated derivatives of Δ^8-CH_2OH (**3**) and the metabolite, 8,14-Diene (**2**), after treatment with $NaBH_4$. It is clear from the chromatograms that Δ^8-CHO (**4**) was converted to 8,14-Diene (**2**), which showed the same gas-chromatographic behavior as that of the metabolite from DHL (**1**), and its mass spectrum coincided with that of an authentic sample. The ions at *m/z* 484 and 379 can be identified as the molecular ion (M^+) and the $M^+ - CH_3 - TMSOH$ of the 14-deformylated product, 8,14-Diene (**2**). The ions at *m/z* 485 and 395 can be identified as $M^+ - CH_2OTMS$ and $M^+ - CH_2OTMS - TMSOH$ of Δ^8-CH_2OH (**3**). However, **5** was not convert-

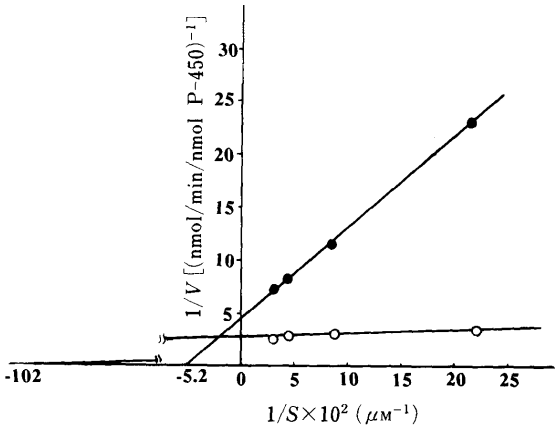

Fig. 5. Double-Reciprocal Plots of Metabolism of DHL (**1**) and Δ^8-CHO (**4**) by the Reconstituted System

Metabolism of DHL (**1**) and Δ^8-CHO (**4**) was assayed as described in the legend to Fig. 3 except that the concentrations of the substrates were varied as indicated and the extract was not treated with $NaBH_4$. (●), DHL; (○), 3β-hydroxylanost-8-en-32-al.

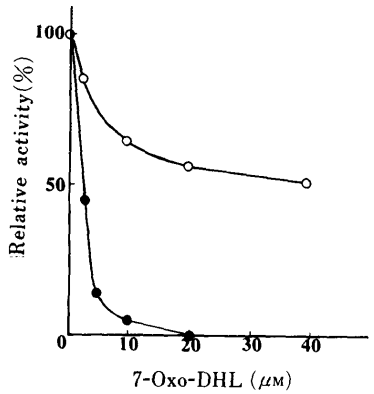

Fig. 6. Effect of 7-Oxo-DHL (**6**) on the Metabolism of DHL (**1**) and Δ^8-CHO (**4**) by the Reconstituted System

Metabolism of DHL (**1**) and Δ^8-CHO (**4**) was assayed as described in the legend to Fig. 5 in the presence of the indicated concentrations of 7-oxo-DHL (**6**). 7-Oxo-DHL was added to the reaction mixture as a DLPC emulsion (5 μl). A corresponding volume of the solvent was added in the control experiment. (●), DHL; (○), 3β-hydroxylanost-8-en-32-al.

ed to the corresponding 14-deformylated product and no other metabolite was detected on GC (data not shown). The double bond at a definite position thus appears to make a large contribution to the removal of the 14-formyl group of 32-oxo-24,25-dihydrolanosterols. However, Fried *et al.*[14] have reported that Δ^7-CHO (**5**) was converted into cholesterol by rat liver whole homogenate. Further, the metabolite generated by the reconstituted system can be detected by HPLC analysis (Fig. 4). Figures 4B and 4C show the sterols in the reaction extracts of Δ^8-CHO (**4**) and Δ^7-CHO (**5**), respectively. Figure 4A shows the chromatogram of the reaction extract without substrate. Detection at 248 nm presents unambiguous evidence for the presence of an 8,14- or 7,14-diene system. It can be concluded that the reconstituted system catalyzes the removal of the 14-formyl group of Δ^8-CHO (**4**) as well as the 14-methyl group of DHL (**1**), and both of these sterols are converted to the same metabolite, 8,14-Diene (**2**). These results are in accord with those on the metabolism of 32-hydroxylated compounds with a double bond at various positions.[6] Since 14α-demethylation is the essential step in cholesterol biosynthesis, the participation of another P-450$_s$ could be neglected, although the partially purified cytochrome P-450$_{14DM}$ used may be contaminated with small amounts of other P-450$_s$.

As shown in Fig. 5, the apparent K_m value of the reconstituted system for Δ^8-CHO (**4**) was determined as 0.98 μM while that for DHL (**1**) was 19.2 μM. Further, V_{max} values for Δ^8-CHO (**4**) and DHL (**1**) determined from Fig. 5 were 0.36 and 0.22 nmol/min/nmol cytochrome P-450, respectively. These results indicated that cytochrome P-450$_{14DM}$ showed higher affinity for Δ^8-CHO (**4**) than for DHL (**1**).

Recently, Trzaskos *et al.*[15] reported that 14α-demethylation intermediates are endogenously generated modulators of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity. Further, regulation of HMG-CoA reductase by inhibitors of lanosterol 14α-demethylation has been demonstrated in cultured cell system of various origins.[16-18] Further, Saucier *et al.*[19] reported that the addition to the culture medium of mevalonate to repress the reductase activity by 90% resulted in the accumulation of 32-oxygenated lanosterol.

Our present results indicated that cytochrome P-450$_{14DM}$ has high substrate specificity for the double bond position on the steroid nucleus, as demonstrated[6] in the metabolism of 32-hydroxylated 24,25-dihydrolanosterol. Also, Δ^8-CHO (**4**) was suggested to be the intermediate of lanosterol demethylation. Aoyama *et al.*[1] demonstrated that cytochrome P-450$_{14DM}$ from yeast catalyzes all the process of lanosterol demethylation, consisting of three monooxygenations. The present results with mammalian cytochrome P-450$_{14DM}$ suggest that the processes of demethylation are the same as in the case of *Saccharomyces cerevisiae* cytochrome P-450$_{14DM}$.

Effect of 7-Oxo-DHL (6) on the Metabolism of DHL (1) and Δ^8-CHO (4) Aoyama *et al.* have reported[20] recently that 7-oxo-DHL (**6**) has two characteristics as an inhibitor of lanosterol 14-demethylase from yeast; it is a typical competitive inhibitor and it acts as an inhibitor of electron transfer to the oxyferro intermediate. In this experiment, it was demonstrated that 7-oxo-DHL (**6**) inhibited the removal of the 14-formyl group from Δ^8-CHO (**4**) by the reconstituted system, as shown in Fig. 6. 7-Oxo-DHL (**6**) also inhibited the removal of the 14-methyl group from DHL (**1**), but its inhibitory effect on the metabolism of Δ^8-CHO (**4**) was weaker than that in the case of DHL (**1**). These results reflect a higher affinity of Δ^8-CHO (**4**) than DHL (**1**) for cytochrome P-450$_{14DM}$, and further suggest that Δ^8-CHO (**4**) is held at the active site cavity of the cytochrome and less effectively released, as in the case of the metabolism of Δ^8-CH_2OH (**3**).

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