Side chain hydroxylation of C_{27}-steroids and vitamin D_3 by a cytochrome P-450 enzyme system isolated from human liver mitochondria

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Abstract The present study was undertaken to obtain information on the involvement of cytochrome P-450 in the 26-hydroxylation of bile acid intermediates and in the 25-hydroxylation of vitamin D_3 in human liver mitochondria. Cytochrome P-450 was solubilized from human liver mitochondria and purified two times to a specific content of 0.125 nmol per mg protein. Furthermore, a ferredoxin was isolated from the mitochondria and partly purified. This iron-sulfur protein had properties similar to bovine adrenal ferredoxin. A mitochondrial NADPH-ferredoxin reductase was also isolated and purified to homogeneity. This enzyme was a flavoprotein with properties very similar to the bovine adrenal NADPH-ferredoxin reductase. The cytochrome P-450 preparation catalyzed 26-hydroxylation of C_{27}-steroids and 25-hydroxylation of vitamin D_3 when reconstructed with NADPH, the ferredoxin and the ferredoxin reductase. With different substrates the following turnover numbers (nmol product × nmol P-450^{-1} × min^{-1}) were found: cholesterol, 8; 5-cholestene-3β,7α-diol, 10; 7α-hydroxy-4-cholesten-3-one, 23; 7α,12α-dihydroxy-4-cholesten-3-one, 27; 5β-cholestane-3α,7α-diol, 28; 5β-cholestane-3α,7α,12α-triol, 41; and vitamin D_3, 0.16. The hydroxylation reactions were inhibited by CO and metyrapone. The human liver mitochondrial ferredoxin and ferredoxin reductase could be replaced by adrenal ferredoxin and adrenal ferredoxin reductase without reduction of activity, but they could not be replaced by microsomal NADPH-cytochrome P-450 reductase. It is concluded that human liver mitochondria contain cytochrome P-450 involved in the oxidation of the side chain of C_{27}-steroids and vitamin D_3.—**Oftebro, H., K. Saarem, I. Björkhem, and J. I. Pedersen.** Side chain hydroxylation of C_{27}-steroids and vitamin D_3 by a cytochrome P-450 enzyme system isolated from human liver mitochondria. *J. Lipid Res.* 1981. **22:** 1254–1264.

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The mitochondrial fraction of both rat (1–3) and human (4) liver catalyzes 26-hydroxylation of cholesterol and a number of C_{27}-steroids that are intermediates in the biosynthesis of bile acids. Rat liver mitochondria also have the ability to convert vitamin D_3 into 25-hydroxyvitamin D_3 (5, 6). In the rat liver, solubilization and reconstitution experiments have demonstrated that both the C_{27}-steroid 26-hydroxylase (7–11) and the vitamin D_3 25-hydroxylase (12, 13) are cytochromes P-450.

A distinctive feature of liver mitochondrial cytochrome P-450 (as opposed to the microsomal cytochrome P-450) is the specific requirement of a ferredoxin (an iron-sulfur protein) and NADPH-ferredoxin reductase (a flavoprotein) for catalytic activity (7–9, 13, 14).

26-Hydroxylation is considered to be the first step in the normal oxidative degradation of the cholesterol side chain in the formation of bile acids (15, 16). In the human liver this enzymic activity appears to be located exclusively in the mitochondria (4). Recently we reported that mitochondria isolated from the liver of a patient with the rare lipid storage disease, cerebrotendinous xanthomatosis, had no ability to catalyze 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol (17) and other C_{27}-steroids (18). Since the formation

Abbreviations: HPLC, high pressure liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry; DPPD, N,N′-diphenyl-p-phenylene diamine; DTT, dithiothreitol; EGTA, ethylene glycol bis (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid; Hepes, 4-(2-hydroxyethel)-1-piperazineethanesulfonic acid; Mops, 4-morpholino propane sulfonic acid. For a discussion of the nomenclature used in this paper, see ref. 17.

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of both cholic acid and chenodeoxycholic acid is considerably reduced in this disease (19, 20), we concluded that the mitochondrial 26-hydroxylase must be involved in the main pathway in bile acid biosynthesis in man. The nature of the 26-hydroxylase in human liver mitochondria has not yet been established. In the present work we describe the isolation and partial purification of cytochrome P-450, a ferredoxin, and a ferredoxin reductase from human liver mitochondria. We demonstrate that when supplemented with NADPH these enzymes are reconstitutively active in the 26-hydroxylation of several bile acid intermediates and in the 25-hydroxylation of vitamin D_3.

METHODS

Chemicals

ATP, malic acid, glucose-6-phosphate, NADP, and 4-morpholino propane sulfonic acid (Mops) were purchased from Sigma Chemical Co., St. Louis, MO. Glucose-6-phosphate dehydrogenase was from Boehringer & Soehne, Mannheim, West Germany. N,N′-diphenyl-p-phenylene diamine (DPPD) was from Eastman Kodak Co., Rochester, NY, and metyrapone was from Ciba-Geigy AG, Basel, Switzerland. Other chemicals and solvents were standard commercial high purity materials.

Unlabeled steroids

Vitamin D_3 (cholecalciferol) and cholesterol were obtained from Sigma Chemical Co. and were purified by HPLC prior to use. 25-Hydroxyvitamin D_3 was from Philips-Duphar B.V., Veenendaal, Holland. 5-Cholestene-3β,7α-diol, 5β-cholestane-3α,7α-diol, 7α-hydroxy-4-cholesten-3-one, 7α,12α-dihydroxy-4-cholesten-3-one, and 5β-cholestane-3α,7α,12α-triol were synthesized as described previously (21–25).

Deuterium-labeled steroid

25-Hydroxy[26 ^{2}H]vitamin D_3 was synthesized (26) and purified on HPLC before use (13).

Tritium-labeled steroids

[7β-^{3}H]5-Cholestene-3β,7α-diol (6 Ci/mol), [7β-^{3}H]-5β-cholestane-3α,7α-diol (7 Ci/mol), and [7β-^{3}H]5β-cholestane-3α,7α,12α-triol (7 Ci/mol) were synthesized as described previously (1). All these steroids were converted biosynthetically into the corresponding 26-hydroxylated compounds as described previously (1). [7β-^{3}H]7α,12α-Dihydroxy-4-cholesten-3-one (7 Ci/mol) was prepared from [7β-^{3}H]5β-cholestane-3α,7α,12α-triol, using the method of synthesis described in ref. 23. [6β-^{3}H]7α-Hydroxy-4-cholesten-3-one (7 Ci/mol) was prepared as described previously (27) and was biosynthetically converted into the corresponding 26-hydroxylated compound as described in ref. 27. [7β-^{3}H]5β-Cholestane-3α,7α,12α,-25-tetrol was obtained biosynthetically from [7β-^{3}H]-5β-cholestane-3α,7α,12α-triol (28). [1,2(n)-^{3}H]Cholesterol (43 Ci/mol) was obtained from the Radiochemical Centre, Amersham, England, and was purified by HPLC (11) prior to use.

Preparation of subcellular fractions

Human liver material was made available from two renal donors, 30 and 32 year-old men, dead according to officially adopted criteria. In both cases the liver was removed immediately after transfer of the kidneys, cut into small pieces, and put on ice-cold 0.25 M sucrose, 15 mM Hepes, 1 mM EGTA buffer, pH 7.4.

Homogenization was performed in two steps, using a Waring blendor and then a Potter Elvehjem homogenizer with three strokes of the pestle. Mitochondria and microsomes were prepared without delay as previously described (14, 17). The mitochondrial preparations used for incubations were washed twice, and then resuspended in a small volume of 0.25 M sucrose, 25 mM Mops buffer, pH 7.4. The mitochondrial pellets used for enzyme preparation were stored in liquid N_2. After thawing, the pellets were washed two additional times before being further processed.

The protein concentrations of the subcellular fractions were determined by the method of Lowry et al. (29) using bovine serum albumin as a standard.

Solubilization of mitochondrial cytochrome P-450

The mitochondrial pellets were suspended in three volumes (w/v) of distilled water, and after 20 min on ice they were centrifuged at 25,500 g (at R_{max}) for 20 min. The pellets were suspended in 0.1 M potassium–phosphate buffer, pH 7.4, 25% glycerol, 1 mM DTT to a protein concentration of 10 mg per ml. A 10% cholate solution was added to a final concentration of 0.5 mg per mg protein. After gentle shaking on ice for 20 min, the suspension was centrifuged at 100,000 g for 90 min. The supernatant was fractionated with polyethyleneglycol (PEG 6,000) and centrifuged at 27,000 g for 30 min. The fraction that precipitated between 5 and 14% saturation was collected by centrifugation at 27,000 g (at R_{max}) for 30 min and suspended in a small volume of 0.1 M potassium–phosphate buffer, pH 7.4, 25% glycerol, 1 mM

DTT. The preparation was stored in liquid N_2 until used.

The amount of cytochrome P-450 was determined from the carbon monoxide reduced minus reduced difference spectrum using an extinction coefficient of 91 $mM^{-1} \times cm^{-1}$ (30). The amount of cytochrome P-450 in the crude mitochondrial suspension was determined as described (31) using an extinction coefficient of 104 $mM^{-1} \times cm^{-1}$ (32).

For comparison, cytochrome P-450 was solubilized from human liver microsomes by exactly the same procedure as described above. The specific content of cytochrome P-450 in this preparation was 1.83 nmol per mg protein.

Protein in the soluble preparations was determined by a modification of the method of Lowry et al. (29) after precipitation with trichloroacetic acid (33).

Preparation of reductase components

Human liver mitochondrial ferredoxin and NADPH-ferredoxin reductase were prepared essentially as described for bovine liver ferredoxin (7) and rat liver NADPH-ferredoxin reductase (14).

Bovine adrenal ferredoxin (adrenodoxin) and NADPH-ferredoxin reductase were prepared as described (14, 34). The adrenal ferredoxin exhibited a ratio of A_{414}/A_{280} of 0.71 and the adrenal ferredoxin reductase, a ratio A_{450}/A_{270} of 0.128. Both proteins were homogenous on SDS polyacrylamide gel electrophoresis.

Liver microsomal NADPH-cytochrome P-450 reductase was purified from phenobarbital-treated rats by combining the initial purification steps of Strobel and Dignam (35) with a final purification on 2′5′ ADP-Sepharose (36). The preparation was essentially pure on SDS gel electrophoresis; only a very faint band was detected in front of the main band. The specific NADPH-cytochrome *c* reductase activity was 27 μmol of cytochrome *c* reduced $\times$ $mg^{-1} \times min^{-1}$ measured at 30°C in 0.3 M potassium phosphate buffer, pH 7.7 (35).

An extinction coefficient of 11.3 $mM^{-1} \times cm^{-1}$ was used for the determination of the molar concentration of flavoprotein based on absorbance at 450 nm (37).

The molecular weights of human liver mitochondrial ferredoxin and NADPH-ferredoxin reductase were estimated by SDS gel electrophoresis using 15% polyacrylamide gels in a discontinuous system described in detail by LeStourgeon and Beyer (38) and by HPLC (M-45-Solvent delivery system, Waters Associates, Inc. Maple Street, Milford, MA) using the protein analysis column I-125 (Waters Associates) and 0.15 M KCl, 10 mM Tris, pH 7.4, as eluting buffer (0.5 ml per min). The absorbance at 280 nm in the eluate was monitored by a Dual Path Monitor UV-2 detector from Pharmacia (Pharmacia Fine Chemicals AB, Uppsala, Sweden). The retention times of the ferredoxin and the NADPH-ferredoxin reductase were compared to those of the corresponding proteins from bovine adrenals.

The optical spectra of the preparations were recorded at room temperature on a Cary 219 spectrophotometer (Varian Instrument Div., Palo Alto, CA).

The electron spin resonance (ESR) spectrum of the human liver ferredoxin preparation was recorded as described (31).

Incubation, extraction, and chromatographic procedures

All incubations with mitochondria were performed immediately after the subcellular fractionation processes were completed. The incubations contained the following in 1.0 ml 0.25 M sucrose, 27 mM Mops, pH 7.4: 2.7 μmol ATP, 3 μmol potassium malate, 0.7 μmol NADP, 0.6 μmol glucose-6-phosphate, 0.5 unit glucose-6-phosphate-dehydrogenase, 10 μmol $MgCl_2$, and 0.5 mg mitochondrial protein.

The incubations with the soluble enzymes contained the following in 1.5 ml 25 mM Mops, pH 7.6: 0.5 μmol NADP, 1 μmol glucose-6-phosphate, 1.0 unit glucose-6-phosphate-dehydrogenase, 7.5 μmol $MgCl_2$, 4.3 nmol bovine adrenal ferredoxin, 30 pmol bovine adrenal ferredoxin reductase, and 7–10 pmol cytochrome P-450 from human liver mitochondria. When vitamin D_3 was the substrate, each incubation mixture contained 25 pmol cytochrome P-450 and 2 μg DPPD. Alterations are given in the tables.

The reactions were started by the addition of substrate (100 μg vitamin D_3 or 10 μg of a C_{27}-steroid) in 6–18 μl of ethanol. With vitamin D_3 or cholesterol as substrate the reaction was allowed to proceed at 37°C under oxygen for 60 min, and was terminated with 10 ml of methanol–chloroform 2:1. To the incubations that contained vitamin D_3 was then added either 50 nmol [^{2}H]25-hydroxyvitamin D_3 as internal standard, or about 10,000 cpm [^{3}H]25-hydroxyvitamin D_3 to correct for recovery during extraction and chromatographic procedures. With the other steroids the incubations were allowed to proceed at 37°C under oxygen for 20 min, terminated with 100 μl of 1 N HCl, followed by 5 ml of ethyl acetate. HCl was omitted, however, when 5-cholestene-3β,7α-diol was the substrate, to avoid degradation of the product.

The incubations were extracted with chloroform–methanol (7) or ethyl acetate (2) as described. The

chloroform or the ethyl acetate was evaporated under N_2 and the residue was redissolved in 50 μl (vitamin D_3) or 150 μl (C_{27}-steroids) of methanol.

Aliquots of the C_{27}-steroid samples were injected into a Spectra Physics HPLC instrument fitted with a Rheodyne injector and a Zorbax ODS column (4.6 × 250 mm). The eluting solvent was 100% methanol when cholesterol was the substrate, 7.5% water in methanol when 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol were the substrates, 10% water in methanol when 5-cholestene-3β,7α-diol and 7α-hydroxy-4-cholesten-3-one were the substrates, and 13% water in methanol when 7α,12α-dihydroxy-4-cholesten-3-one was the substrate. The flow rate was 1 ml per min and 1-ml fractions were collected. Counting solution was added (Insta-Gel II, Packard Instrument Co., Inc., Downers Grove, IL) and the samples were counted in a Packard Tri-Carb liquid scintillation spectrometer at 60% efficiency. The rate of product formation was calculated from the percentage distribution of the radioactivity.

The extracts of the incubations with vitamin D_3 were separated on two consecutive HPLC systems, Zorbax ODS column (4.6 × 250 mm) using 7.5% water in methanol as eluting solvent (1 ml/min) and Zorbax silica column (2.1 × 250 mm) using 2.5% isopropanol in hexane as eluting solvent (1 ml/min). (See also ref. 13). 25-Hydroxyvitamin D_3 was quantitated by comparing the height of the absorbance peak at 254 nm to a standard curve obtained with various amounts of authentic 25-hydroxyvitamin D_3, and correcting for the recovery of the added radioactive 25-hydroxyvitamin D_3. When compared to the previously published mass fragmentographic assay (5, 13), good agreement was found between the two methods.

Identification of products

The criteria used for product identification were described in detail previously (17, 39). The products were separated on HPLC, converted to their trimethylsilyl ethers (40), and analyzed by a method based on gas–liquid chromatography–mass spectrometry, using a 1.5% SE column at 280°C and the LKB 9000 instrument (LKB Instrument Inc., Stockholm, Sweden), equipped with a multiple ion detector. A product was regarded as identical with a certain authentic standard compound when the product and the standard co-chromatographed on HPLC and GLC and contained one or several characteristic ions in common in their mass spectra.

For the identification of the following products the multiple ion detector was focused at certain characteristic ions: 5-cholestene-3β, 26-diol, m/e 546, 531, 456, and 441; 5-cholestene-3β,7α,26-triol, m/e 544 and 529; 7α,26-dihydroxy-4-cholesten-3-one, m/e 380; 5β-cholestane-3α,7α,26-triol, m/e 456 and 441; 5β-cholestane-3α,7α,12α,26-tetrol, m/e 544 and 529. The 25-hydroxylated products were identified by focusing the multiple ion detector at m/e 131, a characteristic ion of all steroids with a trimethylsilyl oxo function at C-25 (41, 42) in addition to the characteristic ions of the corresponding 26-hydroxylated compounds.

7α,12α,26-Trihydroxy-4-cholesten-3-one was identified after biological conversion into 5β-cholestane-3α,7α,12α,26-tetrol by incubation with rat liver cytosol (100,000 *g* supernatant) and NADPH (1.5 μg of steroid was incubated in 3.0 ml of Tris buffer, pH 7.4, containing 10 mg of cytosol protein and 3 μmol NADPH) for 30 min at 37°C as described (43). After extraction with chloroform–methanol, the extract was converted into the trimethylsilyl ether and analyzed by GLC–MS. The multiple ion detector was focused at m/e 544 and 529. The transformation of 7α,12α,26-trihydroxy-4-cholesten-3-one into 5β-cholestane-3α,-7α,12α,26-tetrol was calculated to be more than 50% by this method.

25-Hydroxyvitamin D_3 was also purified and identified by a combination of HPLC (see Methods) and GLC–MS. This method is described in detail previously (12, 13). The trimethylsilyl/t-butyl-dimethylsilyl derivative was analyzed by GLC–MS using an LKB 2091 instrument equipped with a multiple ion detector and focused at the ion m/e 586.

RESULTS

Isolation of hydroxylase enzyme components

The cytochrome P-450 solubilized from human liver mitochondria had a specific content of 0.125 nmol/mg protein (**Table 1**). Compared to the content determined in isolated mitochondria, this corresponds to a twofold purification and a yield of 22%.

The reduced carbon monoxide-difference spectrum of the preparation is shown in **Fig. 1.** The absorbance peak is at 450 nm, and the small shoulder at 420 nm indicates only trace amounts of cytochrome P-420. No hemoglobin was detected in the preparation.

A ferredoxin was isolated and purified 860-fold from an extract of sonicated human liver mitochondria. The specific adrenodoxin reductase-dependent NADPH-cytochrome-*c* reductase activity of

TABLE 1. Solubilization of cytochrome P-450 from human liver mitochondria

	Total Protein	Specific Content of Cyt. P-450	Total Amount of Cyt. P-450	Purification	Yield
	mg	*nmol/mg protein*	*nmol*		%
Mitochondrial fraction	258	0.056	14.4	1	100
Soluble preparation after PEG fractionation	26	0.125	3.2	2.2	22

The solubilization and fractionation procedures are given in Methods.

the preparation was 15.5 μmol × min^{-1} × mg protein^{-1} (**Table 2**). After reduction by NADPH and adrenal ferredoxin reductase, the ESR spectrum of the preparation was typical of a ferredoxin-type iron-sulfur protein (44), with g values at 1.94 and 2.02. The optical spectrum was characteristic for a ferredoxin with peaks at 416 and 455 nm (not shown) (44). The preparation was not pure in that three main bands and several faint bands were observed on SDS-polyacrylamide gel electrophoresis. One main band revealed the same migration distance as purified bovine adrenal ferredoxin. On the I-125 column (HPLC), only one main peak was seen. This peak had the same retention time as adrenal ferredoxin (M_r = 12.500 (44)).

An NADPH-ferredoxin reductase was purified about 4,000-fold after solubilization from human liver mitochondria (**Table 3**). The specific adrenodoxin-dependent NADPH-cytochrome *c* reductase activity of the purified enzyme was about 14 μmol × min^{-1} × mg protein^{-1}. The optical spectrum was characteristic for a flavoprotein (**Fig. 2**) with peaks at 271 and 450 nm and shoulders at 420 and 470 nm. The spectrum was very similar to those reported for NADPH-ferredoxin reductases from rat liver mitochondria (14) and bovine adrenal mitochondria (45–47). The ratio of the absorbance at 450 nm to that at 271 nm was 0.135, which indicates a high degree of purity of the preparation. The purified protein revealed only one band on SDS-polyacrylamide gel electrophoresis with the same migration distance as bovine adrenal ferredoxin reductase (not shown) (M_r 49.500–54.000 (45–47)).

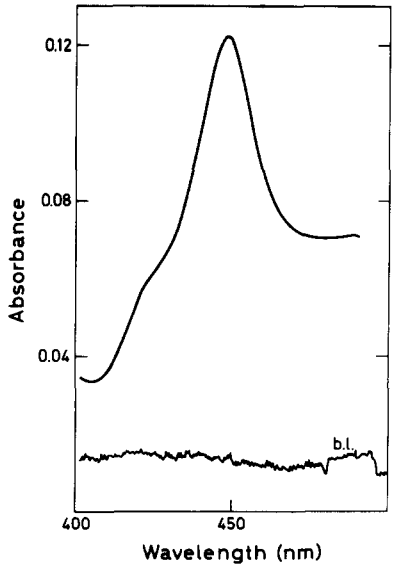

Fig. 1. Difference spectrum of the carbon monoxide complex of reduced cytochrome P-450 solubilized from human liver mitochondria. Protein (4.6 mg/ml) in 0.1 M potassium phosphate buffer, pH 7.4, 25% glycerol, and 1 mM dithiothreitol was equally divided into a sample and a reference cuvette. The baseline (b.l.) was recorded after addition of 0.6 mM dithionite to both cuvettes. After bubbling CO through the sample for 60 sec, the difference spectrum was recorded.

Reconstitution of steroid hydroxylase activity

In the presence of an NADPH-generating system, the isolated enzyme components were able to catalyze the conversion of 5β-cholestane-3α,7α,12α-triol and vitamin D_3 into more polar products (**Table 4**). With 5β-cholestane-3α,7α,12α-triol as a substrate, the product co-chromatographed on HPLC with authentic 5β-cholestane-3α,7α,12α,26-tetrol. The identity was verified by GLC-MS (see Methods). The amount of 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid formed was negligible (less than 0.5% of the polar products). With vitamin D_3 as a substrate, the product co-chromatographed with authentic 25-hydroxyvitamin D_3 in two consecutive HPLC systems (see Methods). The identity was verified by GLC–MS as described above (see Methods).

The rates of hydroxylation of 5β-cholestane-3α,7α,-12α-triol and of vitamin D_3 were found to be linear with time up to 60 min and to the amount of cytochrome P-450 up to 20 and 50 pmol, respectively.

The ferredoxin and the flavoprotein from human liver mitochondria could be replaced by the corresponding proteins isolated and purified from bovine adrenal mitochondria without reduction in hydroxylation rates. Due to small amounts of enzymes available, we have, in the following experiments, used the ferredoxin and the flavoprotein isolated from bovine

TABLE 2. Purification of ferredoxin from human liver mitochondria

Purification Step	Total Protein	Total Activity[a]	Specific Activity	Purification	Yield
	mg	*units*	*units/mg protein*		%
Mitochondrial fraction	65,790				
Supernatant of sonicated mitochondrial fraction	39,830	716,963	18	1	100
First and second DEAE-cellulose column	230	501,870	2,195	122	70
Sephadex G-100	13	122,579	9,429	524	17
Third DEAE-cellulose	6.6	102,147[b]	15,477	860	14

[a] NADPH-cytochrome *c* reductase activity in the presence of saturating amount of adrenal NADPH-ferredoxin reductase measured at room temperature (7). One unit is defined as the amount of enzyme catalyzing the reduction of 1 nmol cytochrome *c* per min.

[b] Total activity at this purification step corresponded to 53.6 nmol when titrated with adrenodoxin as a standard (7).

The purification procedure is described in Methods.

adrenals to reconstitute the hydroxylation activities of the cytochrome P-450 isolated from human liver mitochondria.

Substrate specificity of the soluble human liver mitochondrial cytochrome P-450

A number of C_{27}-steroids, intermediates in bile acid formation, and vitamin D_3 were found to act as substrates for the soluble cytochrome P-450. 5β-Cholestane-3α,7α,12α-triol, 5β-cholestane-3α,7α-diol, 7α-hydroxy-4-cholesten-3-one, and 7α,12α-dihydroxy-4-cholesten-3-one were all transformed into more polar products at high rates, while 5-cholestene-3β,7α-diol and cholesterol were converted more slowly (**Table 5**). The main product of each of these C_{27}-steroids was identified as the corresponding 26-hydroxylated derivative. Vitamin D_3 was converted into 25-hydroxyvitamin D_3 at a relatively high rate (see ref. 13). With all C_{27}-steroids, but not with vitamin D_3, the specific activity of the reconstituted enzyme system was considerably higher than that of isolated mitochondria (Table 5).

Inhibition of the hydroxylation activities by CO and metyrapone

Both the 26-hydroxylation of 5β-cholestane-3α,7α,-12α-triol and the 25-hydroxylation of vitamin D_3 were strongly inhibited when the incubations were performed in an atmosphere containing 40% carbon monoxide (**Table 6**). Metyrapone (1.5 μM) in the incubation mixtures inhibited the 25-hydroxylation of vitamin D_3 by 50%, whereas the 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol was reduced only 10% (Table 6).

Evidence for a mitochondrial origin of the reconstructed hydroxylation activities

As previously discussed (13), it is virtually impossible to prepare mitochondria free from microsomal contamination. It was therefore considered

TABLE 3. Purification of NADPH-ferredoxin reductase from human liver mitochondria

Purification Step	Total Protein	Total Activity[a]	Specific Activity	Purification	Yield
	mg	*units*	*units/mg protein*		%
Mitochondrial fraction	17,600				
Mitochondrial extract after DEAE-treatment	6,150	22,500	3.6	1	100
Ammonium sulfate fractionation	1,024	14,880	15	4	66
Sephadex G-100	260	11,087	43	12	49
Adrenodoxin-Sepharose 4B-affinity column (peak fraction)	0.23[b]	3,281	14,265	3,898	17

[a] NADPH-cytochrome *c* reductase activity in the presence of saturating amount of adrenal ferredoxin measured at room temperature (14). One unit is defined as the amount of enzyme catalyzing the reduction of 1 nmol cytochrome *c* per min.

[b] Total amount of flavoprotein 4.2 nmol.

The purification procedure is described in Methods.

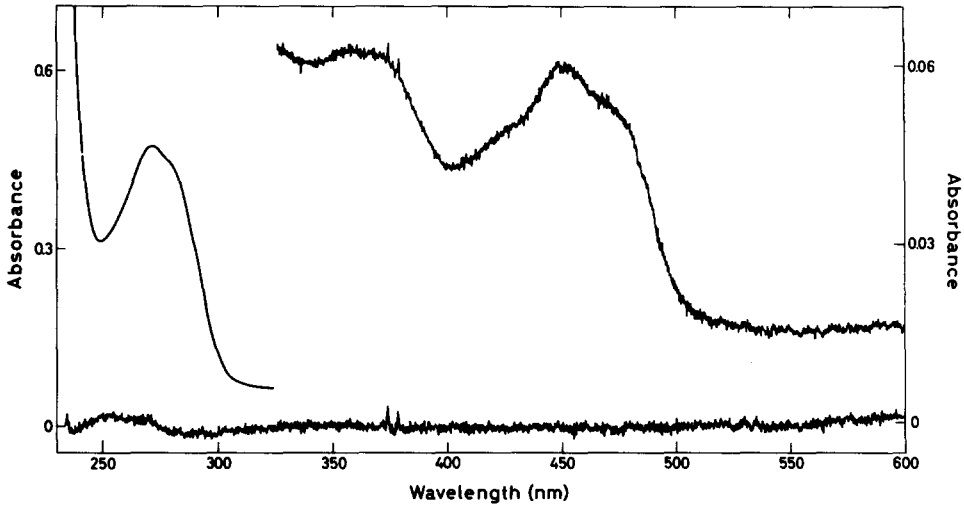

Fig. 2. Absorption spectrum of oxidized NADPH-ferredoxin reductase purified from human liver mitochondria. The visible part of the spectrum is expanded ten times compared to the ultraviolet region.

important to exclude the possibility that the enzymic activities catalyzed by the soluble mitochondrial cytochrome P-450 preparation were due to contaminating microsomal cytochrome P-450.

The results presented in **Table 7** demonstrate that the cytochrome P-450 preparation is almost completely dependent upon both the mitochondrial ferredoxin and the ferredoxin reductase for catalytic conversion of 5β-cholestane-3α,7α,12α-triol into 5β-cholestane-3α,7α,12α,26-tetrol and vitamin D_3 into 25-hydroxyvitamin D_3. Replacement of the ferredoxin and the NADPH-ferredoxin reductase with NADPH-cytochrome P-450 reductase isolated from rat liver microsomes reduced the conversion of 5β-cholestane-3α,7α,12α-triol and vitamin D_3 to the detection limit (i.e., <0.5% and <2% of the conversion observed with the intact mitochondrial reconstituted system for 5β-cholestane-3α,7α,12α-triol and vitamin D_3, respectively). Furthermore, in this case the product of the incubation with 5β-cholestane-3α,7α,12α-triol was identified as 5β-cholestane-3α,7α,12α,25-tetrol. This compound was also identified as the main product when cytochrome P-450 isolated from human liver microsomes was incubated with NADPH-cytochrome P-450 reductase from rat liver microsomes (expt. 6). The small formation of 5β-cholestane-3α,7α,12α,26-tetrol by the microsomal cytochrome P-450 in combination with the mitochondrial reductase components (expt. 7) can be explained by a small amount of contaminating mitochondrial cytochrome P-450. The vitamin D_3 25-hydroxylase activity of the microsomal cytochrome P-450 preparation in the presence

TABLE 4. Conversion of vitamin D_3 to 25-hydroxyvitamin D_3 and of 5β-cholestane-3α,7α,12α-triol to 5β-cholestane-3α,7α,12α,26-tetrol by soluble enzyme components from human liver mitochondria

Additions to the Incubation Mixture	Vitamin D_3 as Substrate, Experiment No.				5β-Cholestane-3α,7α,12α-triol as Substrate, Experiment No.				
	1	2	3	4	1	2	3	4	5
Human liver mitochondrial cytochrome P-450 (pmol)	25	25	25	25	7	7	7	7	7
Human liver mitochondrial ferredoxin (nmol)			1.3	1.3			0.07	0.7	2.7
Human liver mitochondrial NADPH-ferredoxin reductase (pmol)		30		30		30	30	30	30
Product formation (nmol × nmol cyt. P-450^{-1} × min^{-1})	0	0.002	0.003	0.093	0	0	12	25	43

The incubation conditions were as described in Methods with alterations as given in the table. The results represent two sets of single experiments.

TABLE 5. Rates of hydroxylation of different substrates by mitochondria and by solubilized mitochondrial cytochrome P-450 from human liver

Substrate	Mitochondria		Solubilized Mitochondrial Cytochrome P-450	
	nmol × mg protein^{-1} × hr^{-1}	nmol × nmol cyt. P-450^{-1} × min^{-1}	nmol × mg protein^{-1} × hr^{-1}	nmol × nmol cyt. P-450^{-1} × min^{-1}
5β-Cholestane-3α,7α,12α-triol	18.2	5.4	306.9	40.9
5β-Cholestane-3α,7α-diol	13.0	3.9	211.6	28.2
7α-Hydroxy-4-cholesten-3-one	15.8	4.7	174.6	23.3
7α,12α-Dihydroxy-4-cholesten-3-one	19.5	5.8	204.3	27.2
5-Cholestene-3β,7α-diol	4.7	1.4	72.4	9.7
Cholesterol	5.2	1.6	60.8	8.1
Vitamin D_3	0.41	0.13	1.17	0.16

The incubations were performed as described in Methods. The results represent the mean of two sets of experiments.

of either the microsomal or the mitochondrial reductase components (expts. 10 and 11) was only about 0.1% of that of the reconstructed mitochondrial enzyme system (expt. 8). This excludes the possibility that the vitamin D_3 25-hydroxylase activity of the mitochondrial cytochrome P-450 preparation is due to a microsomal contamination.

DISCUSSION

Previous studies have revealed that the mitochondrial fraction of human liver catalyzes 26-hydroxylation of a number of C_{27}-steroids that are intermediates in bile acid formation (4). Also, rat liver mitochondria contain a 5β-cholestane-3α,7α,12α-triol 26-hydroxylase (1, 2), and reconstitution experiments (7–11) as well as spectrophotometric studies (48) have established that this enzyme is a cytochrome P-450 that requires ferredoxin (7, 49) and NADPH-ferredoxin reductase (14, 49) for activity. The cytochrome P-450 preparation from rat liver mitochondria also catalyzed the conversion of vitamin D_3 into 25-hydroxyvitamin D_3 (12, 13).

In the present study we have demonstrated that human liver mitochondria contain cytochrome P-450 that is catalytically active both in the 26-hydroxylation of several C_{27}-steroids and in the 25-hydroxylation of vitamin D_3. The cytochrome P-450 nature of the enzyme(s) was evident from the inhibitory action of carbon monoxide and metyrapone. The isolated cytochrome P-450 is clearly distinct from the microsomal cytochrome P-450 because of the specific requirement of a ferredoxin and an NADPH-ferredoxin reductase of mitochondrial origin for catalytic activity. Because of the small amounts of partly purified liver ferredoxin available, purified bovine adrenal ferredoxin (adrenodoxin) was used in most of the reconstitution experiments reported herein (Tables 5–7). It cannot be excluded that ferredoxin and NADPH-ferredoxin reductase of liver mitochondria might have properties somewhat different from the corresponding proteins of the adrenal cortex. When the results of Tables 4 and 5 are compared, it is seen that partly purified liver ferredoxin and adrenodoxin supported 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol to the same extent when added in saturating amounts. (The amount of ferredoxin was measured as stated in the footnotes to Table 2.) We therefore consider it unlikely that substitution of adrenodoxin for liver mitochondrial ferredoxin would influence the main conclusions of our results very much.

The enzyme system that we have described in this work is thus in all respects similar to the adrenal mitochondrial cytochrome P-450, active in the cholesterol side-chain cleavage and steroid 11β-hydroxylation (50). In fact, no 5β-cholestane-3α,7α,12α-triol 26-hydroxylase activity was observed when the mitochondrial reductase components were replaced by the

TABLE 6. Effect of carbon monoxide and metyrapone on the formation of 5β-cholestane-3α,7α,12α,26-tetrol and 25-hydroxyvitamin D_3 by human liver mitochondrial cytochrome P-450

	5β-Cholestane-3α,7α,12α-triol as Substrate	Vitamin D_3 as Substrate
	nmol product × nmol cyt. P-450^{-1} × min^{-1} (%)	
100% O_2	53.8 (100)	0.12 (100)
40% CO, 55% N_2, 5% O_2	15.9 (30)	0.04 (33)
5% O_2, 95% N_2	48.2 (90)	0.14 (117)
Metyrapone (1.5 μM)	48.6 (90)	0.06 (50)

Standard incubation conditions were used (see Methods) with gas phases as given in the table. The results represent one typical out of four sets of experiments.

TABLE 7. Enzyme specificity of reconstituted 5β-cholestane-3α,7α,12α-triol 26-hydroxylase and vitamin D_3 25-hydroxylase

Components in the Incubation Medium	Experiment No.										
	1	2	3	4	5	6	7	8	9	10	11
Human liver mitochondrial cytochrome P-450 (pmol)	10	10	10	10				25	25		
Human liver microsomal cytochrome P-450 (pmol)					190	190	190			1900	1900
Bovine adrenal ferredoxin (nmol)		4.2	4.2				4.2	4.2			28
Bovine adrenal ferredoxin reductase (pmol)	32		32				32	32			240
Rat liver microsomal cytochrome P-450 reductase (pmol)				72		360			180	940	
Phosphatidylcholine (μg)				20	20	20	20		20	20	
Vitamin D_3 (μg)								100	100	100	100
5β-Cholestane-3α,7α,12α-triol (μg)	10	10	10	10	10	10	10				
Product formation (nmol × nmol cyt. P-450^{-1} × min^{-1})	0	0	48	<0.2	0.05[a]	0.10[a]	0.10[a]	0.16	<0.01	$2.2 \cdot 10^{-4}$	$2.0 \cdot 10^{-4}$

[a] Percentage distribution of products between 5β-cholestane-3α,7α,12α,25-tetrol and 5β-cholestane-3α,7α,12α,26-tetrol: expt. 5, 95% and 5%; expt. 6, 100% and 0%; expt. 7, 15–20% and 80–85%.

The incubation conditions were as described in Methods with alterations as given in the table. The results represent one typical out of three sets of experiments.

microsomal NADPH-cytochrome P-450 reductase. The small hydroxylation activity detected in this case was shown to be due to the formation of 5β-cholestane-3α,7α,12α,25-tetrol. This could be explained by a small contamination of microsomal cytochrome P-450, since this tetrol was the predominant product when 5β-cholestane-3α,7α,12α-triol was incubated with soluble microsomal cytochrome P-450 in the presence of NADPH and microsomal NADPH-cytochrome P-450 reductase. This is in accordance with previous findings that the mitochondrial fraction of human liver hydroxylates 5β-cholestane-3α,7α,12α-triol in the 26-position while the microsomal fraction predominately hydroxylates this triol in the 25-position (4).

The bile acid intermediates, 5β-cholestane-3α,7α-diol, 7α-hydroxy-4-cholesten-3-one, 5β-cholestane-3α,7α,12α-triol, and 7α,12α-dihydroxy-4-cholesten-3-one, were all hydroxylated very efficiently by the reconstituted mitochondrial cytochrome P-450 enzyme system, whereas cholesterol and 5-cholestene-3β,7α-diol were 26-hydroxylated at lower rates. For all of these substrates, the turnover numbers were considerably higher than those previously observed with cytochrome P-450 isolated from rat liver mitochondria (11). This may reflect a greater importance of the mitochondrial 26-hydroxylase in human liver than in rat liver where 26-hydroxylase activity has been detected also in the endoplasmic reticulum (1). The important role of the mitochondrial 26-hydroxylase in the formation of bile acids was clearly demonstrated in our studies on a patient with the lipid storage disease cerebrotendinous xanthomatosis. The mitochondrial fraction isolated from the liver of this patient had no ability to hydroxylate either 5β-cholestane-3α,7α,12α,triol (17) or several precursors of chenodeoxycholic acid (18). Furthermore, very high levels of 5β-cholestane-3α,7α,12α-triol, 7α,12α-dihydroxy-4-cholesten-3-one, and other bile acid intermediates were detected in subcellar fractions of the liver (17, 39). We concluded that the basic metabolic defect in this disease is a lack of the mitochondrial C_{27}-steroid 26-hydroxylase and that this can explain the reduced formation of cholic acid and the almost complete absence of chenodeoxycholic acid from the bile of these patients (20).

All the C_{27}-steroids that were tested as substrates were 26-hydroxylated at much higher rates by the reconstituted soluble enzyme components than by the parent mitochondrial fraction. Transport of the substrates through the mitochondrial membranes to the active site of the cytochrome P-450 enzyme may thus be a rate limiting step in the C_{27}-steroid side chain oxidation. Similar differences in rates of 26-hydroxylation were also observed with soluble cytochrome P-450 and mitochondria isolated from rat liver (11). As in a previous study with rat liver (13), no significant difference in the rate of 25-hydroxylation of vitamin D_3 by the soluble enzymes and the mitochondrial fraction was observed.

It should be noted that vitamin D_3 was very efficiently converted into 25-hydroxyvitamin D_3 by the reconstituted mitochondrial enzyme system. The rates

observed are higher than any previously reported vitamin D_3 25-hydroxylase activity. The rate of 25-hydroxylation of vitamin D_3 by the mitochondrial fraction was about five times higher than that obtained with the microsomal fraction (0.41 versus 0.08 nmol × mg protein^{-1} × h^{-1}). Since we have not determined the K_m values for the substrate, we cannot from these rates evaluate the relative physiological importance of the mitochondrial and the microsomal vitamin D_3 25-hydroxylase in human liver. In rat liver, an apparent K_m for vitamin D_3 of 6×10^{-8} M has been determined for the crude microsomal enzyme (51) and 10^{-5} M for the crude and the reconstituted mitochondrial enzyme (5, 13). It is reasonable to assume, however, that, at least at high substrate concentration, the mitochondrial vitamin D_3 25-hydroxylase may be of importance in the physiological conversion of vitamin D_3 in man.

It is now well established that the microsomal fraction of both rabbit and rat liver contains several species of cytochrome P-450 with affinity for different substrates (52). Human liver microsomes probably also contain several species of cytochrome P-450 (53, 54). Recently we have published some evidence that the 26-hydroxylation of bile acid intermediates and the 25-hydroxylation of vitamin D_3 in rat liver are catalyzed by two different species of cytochrome P-450 (13). The liver mitochondria of the patient with cerebrotendinous xanthomatosis catalyzed 25-hydroxylation of vitamin D_3, but had no ability to carry out 26-hydroxylation of bile acid intermediates (17). This suggests that the 26-hydroxylation of C_{27}-steroids and the 25-hydroxylation of vitamin D_3 in human liver mitochondria are carried out by different enzymes. The different degrees of inhibition by metyrapone of the 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol (10%) and the 25-hydroxylation of vitamin D_3 (50%) observed in the present study are also in agreement with this contention.

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