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CHARACTERIZATION OF THE LIVER MITOCHONDRIAL CYTOCHROME P-450 CATALYZING THE 26-HYDROXYLATION OF 5β-CHOLESTANE-3α,7α,12α-TRIOL

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The cytochrome P-450 catalyzing 26-hydroxylation of C_{27}-steroids (cytochrome P-450_{26}) was purified from rabbit liver mitochondria. The specific content of the cytochrome P-450 was 13.6 nmol per mg of protein and the 26-hydroxylase activity towards 5β-cholestane-3α,7α,12α-triol was 31,300 pmol/nmol of cytochrome P-450 x min^{-1}. The preparation also catalyzed 25-hydroxylation of vitamin D_3 at a rate of 350 pmol/nmol of cytochrome P-450 x min^{-1}. A monospecific monoclonal antibody raised against the 26-hydroxylating cytochrome P-450 was prepared. Experiments with the monoclonal antibody showed that cytochrome P-450_{26} is susceptible to proteolytic degradation during purification unless the protease inhibitor TPCK is included in the buffers. After coupling to Sepharose the antibody was able to bind to cytochrome P-450_{26} and to decrease the 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol. The 25-hydroxylation of vitamin D was not inhibited by the antibody. The results indicate that there are different species of cytochrome P-450 in rabbit liver mitochondria catalyzing 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol and 25-hydroxylation of vitamin D_3. The N-terminal amino acid sequence of the cytochrome P-450_{26} differed from those of hitherto isolated mammalian cytochromes P-450.

A 26-hydroxylation is the initial step in the degradation of the C_{27}-steroid side chain in bile acid biosynthesis (1). A 25-hydroxylation is the first step in the bioactivation of vitamin D_3 into its active, hormonal form (2). The reactions are catalyzed by liver mitochondrial cytochrome P-450 (3-8). In 1984, Wikvall reported the purification from rabbit liver mitochondria of an electrophoretically homogeneous cytochrome P-450 active in 26-hydroxylation of C_{27}-steroids (9). Recently, the catalytic properties of this cytochrome P-450 were described (10). The preparation catalyzed 26-hydroxylation of 5 -cholestane-3 ,7 ,12 -triol as well as 25-hydroxylation of vitamin D_3 (10). This finding raised the question whether the 26-hydroxylation of C_{27}-steroids and the 25-hydroxylation of vitamin D_3 are catalyzed by the same or

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different species of cytochrome P-450 in liver mitochondria. In the present communication the 26-hydroxylating mitochondrial cytochrome P-450 is further characterized by N-terminal amino acid sequence analysis and with a monoclonal antibody.

EXPERIMENTAL PROCEDURE

Enzyme purifications. Cytochrome P-450 catalyzing 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol (cytochrome P-450_{26}) was purified to electrophoretic homogeneity as described previously (9) with some modifications. Lubrol PX was used instead of Emulgen 913. After the octylamine-Sepharose and hydroxylapatite chromatography steps, the DEAE-chromatography was performed using high performance ion-exchange chromatography and an additional ion-exchange chromatography was performed as follows. The fractions containing the highest concentration of cytochrome P-450 were pooled and diluted four times with 20 mM Tris-acetate buffer, pH 7.4, containing 20 % (v/v) glycerol and 0.1 mM EDTA. Sodium cholate was added to a final concentration of 0.1 % (w/v). The sample was dialyzed overnight against 20 mM Tris-acetate buffer, pH 7.4, containing 20 % glycerol, 0.1 mM EDTA, 0.1 % sodium cholate and 0.05 % (w/v) Lubrol PX. The dialysate was then concentrated to a final volume of 10 ml. The sample was injected into a TSK DEAE-5PW column (21.5 x 150 mm) (Pharmacia, Sweden) equilibrated with the same buffer as used for dialysis. The column was eluted with linear gradients of sodium acetate (0-10 min, 0 M; 10-40 min, 0-0.15 M; 40-60 min, 0.15-0.3 M). The flow rate was 4 ml/min and the effluent was monitored at 405 and 280 nm. Chromatography was performed at room temperature using a Merck Hitachi 655 A-11 liquid chromatograph, Merck Hitachi L-5000 liquid chromatograph controller and a Dual Path Monitor UV-2 from Pharmacia. The chromatography resulted in four peaks of cytochrome P-450. The fractions with highest cytochrome P-450 content and with highest hydroxylase activities were obtained in peak 3. This peak was eluted at 0.12 M of sodium acetate in the gradient. The fractions in peak 3 were pooled and dialyzed overnight against 10 mM potassium phosphate buffer, pH 7.4, containing 20 % glycerol, 0.1 mM EDTA, 0.1 % sodium cholate and 0.05 % Lubrol PX. The dialysate was applied to a Mono S HR 10/10 column (Pharmacia, Sweden), equilibrated with the dialysis buffer. The column was eluted with linear gradients of sodium acetate (0-10 min, 0 M; 10-70 min, 0-0.15 M; 70-110 min, 0.15-0.3 M). The flow rate was 2 ml/min. The chromatography was performed using the same instruments as described above for the DEAE chromatography. The Mono S chromatography also resulted in four peaks of cytochrome P-450. The fractions with highest ratio of nmol of cytochrome P-450/mg of protein were pooled to give Pools S1, S2, S3, and S4. S1 was eluted with the equilibrating buffer, S2 at 0.08 M, S3 at 0.12 M, and S4 at 0.18 M of sodium acetate in the gradient. Pools S1, S2, S3, and S4 were dialyzed against 50 mM potassium phosphate buffer, pH 7.4, containing 20 % glycerol and 0.1 mM EDTA. The highest specific content of cytochrome P-450 and the highest hydroxylase activities were found in Pool S2. Pool S4 also showed 26-and 25-hydroxylase activities. In some preparations of liver mitochondria and partially purified cytochrome P-450 for immunoblotting experiments, 0.22 mM N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) was included in all buffers.
Production and purification of monoclonal antibodies. An 8-week old male mouse of the Balb/c strain was used. Cytochrome P-450_{26} was emulsified in an equal volume of Freund's complete adjuvant (Difco). Fifty μg of enzyme were injected subcutaneously and

intraperitoneally. Ten months later another fifty μg of the same antigen in 0.9 % sodium chloride were injected intraperitoneally. Four days later the mouse was killed and the spleen was used as source for antibody-producing cells. The fusion procedure and the etablishment of antibody-producing hybridomas were performed as described by Köhler and Milstein (11). Spleen cells were mixed 4:1 with sp2/0 (12) myeloma cells and fused using 50 % polyethylene glycol 1500 (13). The hybridoma cells were screened by ELISA after HAT-selection. Of 2,200 microwells resulting from a single fusion, 103 produced antibodies which recognized the cytochrome P-450. Five hybridomas of the 103 that produced antibodies were monospecific for the antigen. Cloning of positive cultures was carried out as described previously (14). Immunoglobulins were purified on a 1.5 x 6 cm Protein A-Sepharose (Pharmacia) column equilibrated with PBK (10 mM potassium phosphate buffer, pH 7.5, containing 150 mM KCl). The immunoglobulins were eluted in 0.1 M glycine buffer. The purified preparations were dialyzed against PBK. Subclass of the monoclonal antibody was determined by the Ouchterlony immunodiffusion technique.

Immunoadsorbent preparation, ELISA, SDS-polyacrylamide gel electrophoresis and immunoblotting were performed as described previously (14).

Incubation procedures. Incubations with cytochrome P-450 and antibody-coupled Sepharose were performed as follows. Cytochrome P-450 (0.05 nmol in 5β-cholestane-3α,7α,12α-triol incubations and 0.1 nmol in vitamin D_3 incubations) was incubated with the indicated amounts of Sepharose-bound Mab-S53 or Mab-2B4 in 0.4 ml 50 mM Tris--acetate buffer, pH 7.4, containing 20 % glycerol, 0.1 mM EDTA and 0.1 % 3- (3-cholamidopropyl)-dimethylammonio -1-propanesulfonate (CHAPS). After incubation for 1 h at room temperature on a rotating mixer, the mixture was centrifuged (4,000 x g, 10 min at + 4 °C) and the supernatant was used for assay of hydroxylase activity. The Sepharose pellet was washed twice with an appropriate amount of 50 mM Tris-acetate, pH 7.4. The cytochrome P-450-containing supernatant was assayed for hydroxylase activity as follows. 5β-Cholestane-3α,7α,12α-triol (62 nmol in 12 μl of acetone) was incubated for 3 min at 37 °C with 2 nmol of ferredoxin, 0.2 nmol of ferredoxin reductase and 1 μmol of NADPH in a total volume of 1 ml. Vitamin D_3 (312 nmol in 25 μl of acetone) was incubated for 15 min at 37 °C with 3 nmol of ferredoxin, 0.3 nmol of ferredoxin reductase and 5 μmol of NADPH in a total volume of 5 ml. Incubations were terminated and analyzed as described previously (10).

Analysis of N-terminal sequence. Sequence analysis was carried out in an Applied Biosystems gas-phase sequencer 790A and the phenylhydantoin derivates were identified by HPLC as described by Cederlund et al. (15).

Other analytical procedures. Assay of cytochrome P-450 and protein as well as preparation and assay of ferredoxin and ferredoxin reductase have been previously described (9).

RESULTS

Liver mitochondrial cytochrome P-450 catalyzing 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol was purified from untreated rabbits. The major amount of 26-hydroxylase activity was recovered in the second peak (S2) in the Mono S chromatography step (cf. Experimental Procedure). This preparation will be referred to as cytochrome $P\text{-}450_{26}$. The enzyme preparation had a specific content

of cytochrome P-450 of 13.6 nmol/mg of protein. It catalyzed 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol at a rate of 31,300 pmol/nmol of cytochrome P-450 x min^{-1} and 25-hydroxylation of vitamin D_3 at a rate of 350 pmol/nmol of cytochrome P-450 x min^{-1}. The cytochrome P-450 in Pool S4 was also active in the two hydroxylations but the rate of 26- and 25-hydroxylation was lower than that with cytochrome $P\text{-}450_{26}$. The preparation contained 9.2 nmol of cytochrome P-450/mg of protein. Fig 1A shows the results of SDS/polyacrylamide gel electrophoresis of cytochrome $P\text{-}450_{26}$ and Pool S4. The preparations each showed one protein band. The apparent M_r for cytochrome $P\text{-}450_{26}$ was about 52,000 whereas that of Pool S4 was about 51,000.

A monoclonal antibody, designated Mab-S53, directed against the cytochrome $P\text{-}450_{26}$, was prepared by immunization of a mouse with cytochrome $P\text{-}450_{26}$. Mab-S53 was found to belong to the IgG_1 subclass. To analyze the reactivity of this antibody with cytochrome $P\text{-}450_{26}$, the antibody was coupled covalently to Sepharose and incubated with cytochrome $P\text{-}450_{26}$. CHAPS (1 %) was included in all incubations with Mab-Sepharose to avoid unspecific binding. To avoid inhibition by CHAPS of 25-hydroxylase activity, the vitamin D_3 incubations were performed in 5 ml volumes. After

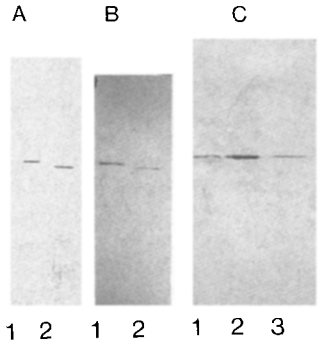

Fig. 1. A. SDS-polyacrylamide gel electrophoresis of purified mitochondrial cytochrome P-450 from rabbit liver (10). Migration was from top to bottom. Lane 1, cytochrome $P\text{-}450_{26}$, 0.7 μg; lane 2, Pool S4, 0.7 μg.

B and C. SDS-polyacrylamide gel electrophoresis and immunoblotting of cytochrome $P\text{-}450_{26}$, Pool S4, and hydroxylapatite eluate. Samples containing the indicated amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis, electrophoretic transfer and immunoblotting with Mab-S53 as described previously (14). Migration was from top to bottom. B. Lane 1, cytochrome $P\text{-}450_{26}$, 0.5 μg; lane 2, Pool S4, 0.5 μg. C. Lane 1, hydroxylapatite eluate, 25 μg; lane 2, cytochrome $P\text{-}450_{26}$, 0.5 μg; lane 3, hydroxylapatite eluate from a preparation with TPCK in all buffers, 25 μg.

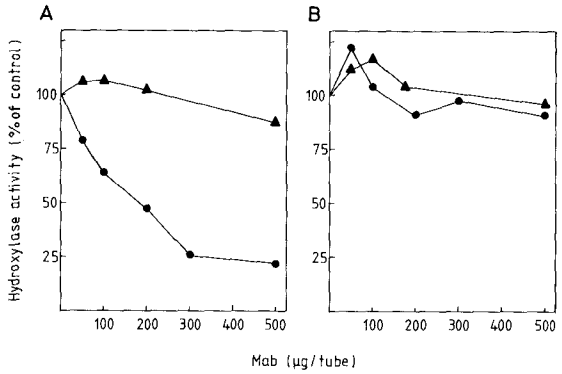

Fig. 2. Effects of Mab-S53 (anti-cytochrome P-450) on 26-hydroxylation of 5 -cholestane-3 ,7 ,12 -triol (A) and 25-hydroxylation of vitamin D_3 (B). Incubations were carried out as described in "Experimental Procedure" ▲, irrelevant IgG (Mab-2B4), ●, anti-cytochrome $P\text{-}450_{26}$ (Mab-S53). Results are given as % of control values for hydroxylase activities in incubations containing no antibody.

a 1 hour incubation, the antibody-Sepharose was removed by centrifugation, and the supernatant was assayed for hydroxylase activities towards 5β-cholestane-3α,7α,12α-triol and vitamin D_3. As a control, Sepharose was coupled to an irrelevant monoclonal antibody, Mab-2B4, directed against a rat liver microsomal protein of unknown function. Fig. 2 shows that increasing amounts of Mab-S53 but not Mab-2B4 were able to bind up to 80 % of the 26-hydroxylase activity. The monoclonal did not inhibit the 25-hydroxylation.

Fig. 1B shows the results of SDS/polyacrylamide gel electrophoresis and immunoblotting analyses of Mab-S53 and cytochrome $P\text{-}450_{26}$, Pool S4 and partially purified cytochrome P-450 (hydroxylapatite eluate) from rabbit liver mitochondria. The antibody recognized purified cytochrome $P\text{-}450_{26}$, lane 1, as well as the protein in Pool S4, lane 2. The antibody recognized proteins with the same apparent M_r as cytochrome $P\text{-}450_{26}$ and S4 in the hydroxylapatite eluate, lane 1 (Fig. 1 C). Since it was suspected that the protein in Pool S4 could be a proteolytically degraded product of cytochrome $P\text{-}450_{26}$, experiments were performed where the protease inhibitor TPCK was added in all buffers in the purification procedure. In hydroxylapatite eluate from such a preparation, the monoclonal antibody only recognized one protein corresponding to cytochrome $P\text{-}450_{26}$, lane 3 (Fig. 1 C)

The total amino acid composition of cytochrome P-450$_{26}$ was similar to that of other purified mammalian cytochromes P-450 (16). Partial N-terminal amino acid sequence analysis of purified cytochrome P-450$_{26}$ revealed the following sequence of the first 21 amino acids: Ala-Leu-Pro-Ala-Asp-Glu-Ala-Ala-Glu-Ala-Pro-Gly-Ala-Pro-Gly-Asp-Arg-Gly-Gly-Val.

DISCUSSION

In previous reports (9,10) the purification and catalytic properties of a mitochondrial cytochrome P-450 from rabbit liver catalyzing the 26-hydroxylation of C_{27}-steroids and the 25-hydroxylation of vitamin D_3 were presented. In the present work, a modified purification procedure was used, which resulted in an enzyme fraction with a higher content of cytochrome P-450, 13.6 nmol/mg of protein, and with the same catalytic properties. The amino acid composition was similar to that of other cytochromes P-450 (16). However, the N-terminal amino acid sequence of the cytochrome P-450$_{26}$ differed from those of hitherto isolated mammalian cytochromes P-450 (16).

There was also another electrophoretically homogeneous fraction of cytochrome P-450 active in 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol and 25-hydroxylation of vitamin D_3. This enzyme fraction had a lower molecular weight than the cytochrome P-450$_{26}$. The fact that the monoclonal antibody, Mab-S53, recognized both fractions of cytochrome P-450 indicates that the smaller protein could be a degradation product of the larger one. Therefore, another purification procedure was performed where the protease inhibitor TPCK was included in all buffers. The degradation product was absent in this experiment.

The results in the previous communication (10) indicated the possibility that the 26-hydroxylation of C_{27}-steroids and the 25-hydroxylation of vitamin D_3 were catalyzed by the same species of cytochrome P-450 in liver mitochondria. The enzyme fraction purified in this work was also active in both hydroxylations but there were some important differences. Firstly, the 25-hydroxylation was much more sensitive to detergents in the incubations than the 26-hydroxylation. Secondly, the monoclonal antibody, Mab-S53, bound about 80 % of the 26-hydroxylase activity but did not bind the 25-hydroxylase activity to any higher degree than the irrelevant antibody. These results indicate that different species of liver mitochondrial cytochrome P-450 are involved in

26-hydroxylation of C_{27}-steroids and 25-hydroxylation of vitamin D_3. This contention is supported by the work of Oftebro et al. (17) on the defect in cerebrotendinous xanthomatosis (CTX). Mitochondrial fractions of liver from CTX patients lacked 26-hydroxylase activity towards 5β-cholestane-3α,7α,12α-triol but had significant 25-hydroxylase activity towards vitamin D_3.

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