25-Hydroxylation of C_{27}-Steroids and Vitamin D_{3} by a Constitutive Cytochrome P-450 from Rat Liver Microsomes*

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A constitutive cytochrome P-450 catalyzing 25-hydroxylation of C_{27}-steroids and vitamin D_{3} was purified from rat liver microsomes. The enzyme fraction contained 16 nmol of cytochrome P-450/mg of protein and showed only one protein band with a minimum molecular weight of 51,000 upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

The purified cytochrome P-450 catalyzed 25-hydroxylation of 5\beta-cholostane-3\alpha,7\alpha,12\alpha-triol, and 1\alpha-hydroxyvitamin D_{3} up to 50 times more efficiently, and 25-hydroxylation of vitamin D_{3} about 150 times more efficiently than the microsomes. The catalytic 25-hydroxylase activity towards vitamin D_{2} and was inactive in cholesterol 7\alpha-hydroxylation as well as in 12\alpha- and 26-hydroxylations of C_{27}-steroids. It catalyzed hydroxylations of testosterone and demethylation of ethylmorphine at the same rates as, or lower rates than, microsomes.

The 25-hydroxylation of 5\beta-cholostane-3\alpha,7\alpha,12\alpha-triol and vitamin D_{3} with the purified cytochrome P-450 was not stimulated by addition of phospholipid or cytochrome b_{5} to the reconstituted system. Emulgen inhibited 25-hydroxylase activity towards both substrates.

The possibility that 25-hydroxylation of C_{27}-steroids and vitamin D_{3} is catalyzed by the same species of cytochrome P-450 is discussed.

During the last five years, several groups have reported on the purification and characterization of a number of cytochrome P-450 species from rabbit and rat liver microsomes (1-10). Most efforts have been concentrated on the purification of the major species which are induced by various xenobiotics. There are only a few reports concerning the purification of constitutive species of cytochrome P-450. The catalytic properties of the isolated cytochrome P-450 fractions have been studied with exogenous substrates and with steroid hormones, such as testosterone, androstenedione, and progesterone (6-9). Hydroxylations of these substrates in the liver generally lead to products of minor physiological importance.

Cholesterol and other C_{27}-steroids involved in the biosynthesis of bile acids as well as vitamin D_{3} are important physiological substrates for the microsomal cytochrome P-450 system in the liver (11-15). In a previous report, we showed that a cytochrome P-450 fraction prepared from liver microsomes of untreated rats catalyzed efficient 25-hydroxylation of 5\beta-cholostane-3\alpha,7\alpha,12\alpha-triol as well as of vitamin D_{3} (16). The fraction was not pure and showed at least two protein bands upon gel electrophoresis. Thus, it was not possible to define the species of cytochrome P-450 involved in 25-hydroxylation of the two substrates. The present communication reports the isolation from this fraction of a constitutive, apparently homogeneous cytochrome P-450 active in 25-hydroxylation of C_{27}-steroids and vitamin D_{3}.

**EXPERIMENTAL PROCEDURES**

**Materials**—5\beta-[\text{3-}^{14}C]-Cholesterol-3\alpha,7\alpha,12\alpha-triol, 5\beta-[\text{3-}^{14}C]\text{-cholestane-3,7,10,12a-triol, and 25,26,-}{\text{2H}}\text{Hodrocortisone vitamin D}_{3} were synthesized as described previously (17, 18). [\text{4,4,4-}^{2}H_{3}\text{-cholesterol and [4,4,4-}^{2}H_{3}\text{-testoster}}

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and purified by preparative high performance liquid chromatography. Testosterone, and ethylmorphine were performed as described previously (29).

In the incubations with la-hydroxyvitamin D3, [26-'H3]cholestane-3a,7a-diol and decyl sulfate with Pharmacia gradient slab gels, PAA 4/30, as described by Yasukochi and Masters (22) and described by Omura and Sato (25), Spatz and Strittmatter (26) and Lowry et al. (27).

Optical spectra were measured as described by Haugen and Coon (1) using a Shimadzu multipurpose recording spectrophotometer.

Gel electrophoresis was performed in the presence of sodium dodecyl sulfate with Pharmacia gradient slab gels, PAA 4/30, as described previously (12). 5β-Cholesterol-3a,7a-diol, 5β-cholesterol-3a,7a,12a-triol, vitamin D3, 1α-hydroxyvitamin D3, and vitamin D2, 125 nmol in 10–25 μl of acetone, were incubated for 10 min with 0.1 nmol of cytochrome P-450, 2.0 units of NADPH-cytochrome P-450 reductase, and 1–2 amol of NADPH in a total volume of 1 ml of 150 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA. The column was washed with the equilibrating buffer until no absorption at 276 nm (due to Emulgen) was detected. The resulting cytochrome P-450 was diazylated against 50 mM phosphate buffer, pH 7.25, containing 20% glycerol and 0.1 mM EDTA.

A major phenobarbital-inducible form of cytochrome P-450 (fraction C) was isolated from phenobarbital-treated rats as described by West et al. (21).

NADPH-cytochrome P-450 reductase was prepared from phenobarbital-treated rats as described by Yasukochi and Masters (22) and had a specific activity of 50 units/mg of protein.

Cytochrome b5, which was obtained in the octylamine-Sepharose chromatography step (23), was further purified to electrophoretical homogeneity as described by Strittmatter et al. (24).

The purified cytochrome P-450, cytochrome b5, and protein were determined as described by Omura and Sato (25), Spatz and Strittmatter (26) and Lowry et al. (27).

RESULTS

A summary of the purification of cytochrome P-450 from liver microsomes of untreated rats is shown in Table I. Chromatography of solubilized microsomal cytochrome P-450 on octylamine-Sepharose, hydroxylapatite, and DEAE-Sepharose resulted in the isolation of cytochrome P-450 fraction A of similar purity as described previously (16). Upon gel electrophoresis, this fraction showed one major protein band and one minor band of lower molecular weight. The eluted fractions from the DEAE-Sepharose column which were enriched with respect to the major band were pooled and applied to octyl-Sepharose chromatography. This chromatographic step removed the contaminating protein of lower molecular weight. The specific content of cytochrome P-450 increased from 0.8 nmol/mg of protein in the microsomes to 15.6 nmol/mg of protein in the purified cytochrome. The purified cytochrome P-450 represented 2% of the total microsomal cytochrome P-450.

Fig. 1 shows the results of gel electrophoresis experiments with the purified cytochrome P-450. The purified cytochrome P-450 was then eluted with 500 mM phosphate buffer in the buffer. The resulting cytochrome P-450 was isolated from phenobarbital-treated rats as described previously (28).

TABLE I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Cytochrome P-450 (specific content, nmol/mg protein)</th>
<th>Cytochrome P-450 (yield, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomes</td>
<td>3200</td>
<td>0.8</td>
<td>100</td>
</tr>
<tr>
<td>Octylamine-Sepharose</td>
<td>208</td>
<td>3.8</td>
<td>31</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>32</td>
<td>6.7</td>
<td>8.4</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td>10.5</td>
<td>11.0</td>
<td>4.5</td>
</tr>
<tr>
<td>Octyl-Sepharose</td>
<td>3.4</td>
<td>15.6</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Fig. 1. Polyacrylamide gel electrophoresis of purified cytochrome P-450 from liver microsomes. Electrophoresis was performed on Pharmacia gradient slab gels. The protein samples were pretreated with sodium dodecyl sulfate and mercaptoethanol at 100 °C for 2 min and submitted to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Migration was from top to bottom. A, purified cytochrome P-450 from untreated rats, 5 μg (1); and 1 μg (2); mixture of phosphorylase b (M, = 94,000), albumin (M, = 67,000), ovalbumin (M, = 43,000), carbonic anhydrase (M, = 30,000), trypsin inhibitor (M, = 20,100), and α-lactalbumin (M, = 14,400); 3 μg of each (3). B, phenobarbital-induced cytochrome P-450 from rat liver, 2 μg (1); mixture of cytochrome P-450 from untreated rats, 2 μg (3). C, purified cytochrome P-450 from untreated rats, 1 μg (1); phenobarbital-induced cytochrome P-450 from rat liver, 1 μg (2).
showed only one protein band with an apparent M, = 51,000. The electrophoretic mobility differed from that of the phenobarbital-induced cytochrome P-450 from rat liver (M, = 50,000) as well as from that of cytochrome P-450m (M, = 48,000) and P-450b (M, = 53,000) from rabbit liver microsomes.

Fig. 2 shows the spectral properties of the purified cytochrome P-450. The fraction showed absorbance maxima in the oxidized state at 418, 540, and 570 nm, and in the reduced state at 416 and 550 nm. The absorbance maximum for the reduced carbon monoxide complex was at 452 nm.

The catalytic activities of the original microsomes and of the purified cytochrome P-450 from untreated rats are summarized in Table II. The hydroxylations studied were the 7α-hydroxylation of cholesterol, the 12α-hydroxylation of 5β-cholestan-3α,7α-diol, the 25-hydroxylation of 5β-cholestan-3α,7α-diol, 5β-cholestan-3α,7α,12α-triol, vitamin D₃, 1α-hydroxyvitamin D₃, and vitamin D₂, the 26-hydroxylation of vitamin D₃, and the 1α-hydroxylation of vitamin D₂. The purified cytochrome P-450 was inactive in 7α-hydroxylation, 12α-hydroxylation, and 25-hydroxylation of vitamin D₁. With the methods used, no 25-hydroxylation of vitamin D₁ could be detected. The purified cytochrome P-450 was inactive in 7α-hydroxylation, 12α-hydroxylation, and 25-hydroxylation of the C₂₇-steroids and showed very low capacity to catalyze 6β- and 7α-hydroxylations of testosterone. The cytochrome P-450 catalyzed efficient 25-hydroxylation of 5β-cholestan-3α,7α-diol, 5β-cholestan-3α,7α,12α-triol, vitamin D₂, and 1α-hydroxyvitamin D₂. The 25-hydroxylation of vitamin D₁ was not detectable. It also catalyzed 28- and 16α-hydroxylations of testosterone as well as demethylation of ethylmorphine. The rate of 25-hydroxylation of the C₂₇-steroids and of 1α-hydroxyvitamin D₁ was 20 to 50 times higher, and the rate of 25-hydroxylation of vitamin D₂ was about 150 times higher, with the purified cytochrome P-450 than with the original microsomes. On the other hand, the testosterone hydroxylase activities and the ethylmorphine demethylase activity were lower than, or the same as, with the microsomes. The rate of 25-hydroxylation was two to three times higher with 5β-cholestan-3α,7α,12α-triol as substrate than with 1α-hydroxyvitamin D₃ and five to

![Absolute spectra of purified cytochrome P-450 from liver microsomes of untreated rats.](http://www.jbc.org/)

**Fig. 2.** Absolute spectra of purified cytochrome P-450 from liver microsomes of untreated rats. The concentration of the cytochrome was 1 nmol of cytochrome P-450/mL of 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol. ---, oxidized; ----, reduced; -----, reduced CO complex.
10 times higher than with 5β-cholestan-3α,7α-diol and vitamin D$_3$ as substrates.

The reconstitution conditions for the 25-hydroxylation of 5β-cholestan-3α,7α,12α-triol and of vitamin D$_3$ were analyzed in a series of experiments and the results are shown in Table III. As expected, the 25-hydroxylase activity towards both substrates showed an absolute requirement for the cytochrome P-450, the NADPH-cytochrome P-450 reductase as well as for NADPH. However, addition of phospholipid was not required for 25-hydroxylation of the two substrates. Rather, it inhibited 25-hydroxylation of the C$_{27}$-steroid by 15 to 20%. Addition to the incubations of Emulgen 913 in a concentration of 0.06% (w/v) inhibited the 25-hydroxylase activity towards both substrates by more than 95%. Similarly, sodium deoxylcholate, 60 μM, inhibited 25-hydroxylation of both substrates by about 30%. The inclusion of an equimolar amount of cytochrome b$_5$ in the reconstituted system had no stimulatory effect on the 25-hydroxylation of any of the substrates.

**DISCUSSION**

The 25-hydroxylation reactions carried out by the present constitutive cytochrome P-450 are involved in pathways leading to physiologically active products. Thus, the formation of active forms of vitamin D$_3$ requires a 25-hydroxylation as the initial step in the liver (30). It has also been established that 25-hydroxylated C$_{27}$-steroids can be converted into bile acids in vivo (31). The microsomal 25-hydroxylation appears less important than the mitochondrial 26-hydroxylation under normal conditions. However, the reaction may function as an alternative pathway for bile acid synthesis under certain pathological conditions (11, 32, 33).

Recent studies with purified cytochrome P-450 fractions from rabbit and rat liver microsomes have provided evidence for the participation of multiple species of cytochrome P-450 in hydroxylations of C$_{27}$-steroids (34-36). Work with highly purified cytochrome P-450$_{LM}$, fractions from rabbit liver microsomes has indicated that cholesterol 7α-hydroxylation as well as 12α-hydroxylation of 5β-cholestan-3α,7α,12α-triol are catalyzed by specific species of cytochrome P-450 (34, 36). However, it has previously not been possible to obtain a separation of the 12α-hydroxylase activity from the 25-hydroxylase activity. The present results, showing that a cytochrome P-450 catalyzing exclusively 25-hydroxylation of C$_{27}$-steroids can be obtained, provide strong evidence for the concept that 12α-hydroxylation and 25-hydroxylation of 5β-cholestan-3α,7α,12α-triol are catalyzed by separate species of cytochrome P-450. It should be mentioned that cholesterol 7α-hydroxylation, 12α-hydroxylation, and 26-hydroxylation were catalyzed by other cytochrome P-450 fractions obtained in the purification procedure.

Hiwatashi and Ichikawa (15) have reported that a cytochrome P-450 fraction isolated from bovine liver microsomes catalyzed 25-hydroxylation of vitamin D$_3$ as efficiently as vitamin D$_3$ and 1α-hydroxylamine D$_3$. In contrast, the rat liver microsomes as well as the purified cytochrome P-450 used in the present study did not show detectable 25-hydroxylase activity towards vitamin D$_3$. It should be pointed out that the catalytic activity towards vitamin D$_3$ and 1α-hydroxylamine D$_3$ was much higher with the present cytochrome P-450 than with that reported by Hiwatashi and Ichikawa.

In consonance with the results of previous studies, the 25-hydroxylase activity towards vitamin D$_3$ was low in whole microsomes (16, 37). However, upon solubilization and purification of microsomal cytochrome P-450, the catalytic activity increased drastically. On the basis of yield and catalytic activity of the cytochrome P-450, it could be calculated that the purified enzyme represented about four times as much vitamin D$_3$ 25-hydroxylase activity than was present in microsomes. Thus, it seems that inhibitory factors of the vitamin D$_3$ 25-hydroxylase activity have been removed during the purification procedure.

It is generally considered that all reconstituted systems show a significant dependency of lipid or detergent for catalytic activity, provided that the level of detergent is sufficiently low in the enzyme preparations (38). However, the present cytochrome P-450 did not require phospholipid or detergent to catalyze 25-hydroxylation of 5β-cholestan-3α,7α,12α-triol or vitamin D$_3$. In fact, Emulgen almost fully inhibited 25-hydroxylase activity. It should be pointed out that the preparations of cytochrome P-450 used were extensively treated on hydroxylapatite columns to remove Emulgen. No definite explanation for this lack of lipid dependency can be offered at present. It is possible that these lipophilic substrates may behave in the reconstituted system in a manner similar to lipid and thus replace the lipid (38).

Cytochrome b$_5$ has been reported to stimulate certain hydroxylations catalyzed by purified cytochrome P-450 (39, 40). Noshiro et al. (41) have reported that antibodies to protease-solubilized cytochrome b$_5$ inhibit 25-hydroxylation of 5β-cholestan-3α,7α,12α-triol catalyzed by crude rat liver microsomes. However, cytochrome b$_5$ was found to have no stimulatory effect on 25-hydroxylation of 5β-cholestan-3α,7α,12α-triol or vitamin D$_3$ catalyzed by the purified cytochrome P-450 used in the present investigation. It might be mentioned that in a separate experiment the same preparation of cytochrome b$_5$ was found to stimulate 16α-hydroxylation of testosterone by a phenobarbital-induced cytochrome P-450 from rat liver microsomes three times.

The C$_{27}$-steroid and vitamin D$_3$ 25-hydroxylase activities copurified and were enriched in the apparently homogeneous cytochrome P-450. Further, it is obvious that the properties of the cytochrome P-450 with respect to 25-hydroxylation of C$_{27}$-steroids and vitamin D$_3$ were very similar. Thus, the present results indicate that the same species of cytochrome P-450 catalyzes 25-hydroxylation of C$_{27}$-steroids and vitamin D$_3$. It should be mentioned that recent work with electrophoretically homogeneous cytochrome P-450$_{LM}$, fractions from rabbit liver microsomes has resulted in the separation of two subfractions with different substrate specificity towards C$_{27}$-steroids (34). Therefore, it can not be excluded that the present, apparently homogeneous, cytochrome P-450 fraction might contain two closely related isozymes with different substrate specificity.

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**REFERENCES**


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