Activities of Recombinant Human Cytochrome P450c27 (CYP27) Which Produce Intermediates of Alternative Bile Acid Biosynthetic Pathways*

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The primary physiological significance of cytochrome P450c27 (CYP27) has been associated with its role in the degradation of the side chain of C27 steroids in the hepatic bile acid biosynthesis pathway, which begins with 7α-hydroxylation of cholesterol in liver. However, recognition that in humans P450c27 is a widely or ubiquitously expressed mitochondrial P450, and that there are alternative pathways of bile acid synthesis which begin with 27-hydroxylation of cholesterol catalyzed by P450c27, suggests the need to reevaluate the role of this enzyme and its catalytic properties. 27-Hydroxycholesterol was thought to be the only product formed upon reaction of P450c27 with cholesterol. However, the present study demonstrates that recombinant human P450c27 is also able to further oxidize 27-hydroxycholesterol giving first an aldehyde and then 3β-hydroxy-5-cholestenoic acid. Kinetic data indicate that in a reconstituted system, after 27-hydroxycholesterol is formed from cholesterol, it is released from the P450 and then competes with cholesterol for reentry the enzyme active site for further oxidation. Under saturating substrate concentrations, the efficiencies of oxidation of 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid to the acid by human P450c27 are greater than the efficiency of hydroxylation of cholesterol to 27-hydroxycholesterol indicating that the first hydroxylation step in the overall conversion of cholesterol into 3β-hydroxy-5-cholestenoic acid is rate-limiting. Interestingly, 3β-hydroxy-5-cholestenoic acid was found to be further metabolized by the recombinant human P450c27, giving two monohydroxylated products with the hydroxyl group introduced at different positions on the steroid nucleus.

The major pathway for the metabolism and excretion of cholesterol in mammals is the formation of the bile acids in liver. Cholesterol can be degraded to bile acids in mammalian liver via two different routes, one starting with 7α-hydroxylation, catalyzed by the microsomal cytochrome P450 cholesterol 7α-hydroxylase (P450c7), and the other one initiated by 27-

hydroxylation, catalyzed by the mitochondrial cytochrome P450c27 (1). The route that begins with 7α-hydroxylation (the classical pathway) is well described and is believed to be quantitatively most important, whereas the sequence of reactions leading to bile acids after 27-hydroxylation of cholesterol (alternative pathways) are only now being investigated (2–8).

Discovery that cultured human macrophages can efficiently convert cholesterol into 27-hydroxycholesterol and its oxidation product 3β-hydroxy-5-cholestenoic acid and secrete both products into the medium (9) initiated a series of experiments showing that there is a continuous flux of these 27-oxigenated products from peripheral tissues to the liver where they are rapidly converted into bile acids (10). The net uptake by the human liver of circulating 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid was measured and found to correspond to ~4% of the total bile acid formation, assuming quantitative conversion into bile acids (10). Investigation of the quantitative importance of this pathway in extrahepatic tissues in relation to high density lipoproteins (HDL)-mediated reverse cholesterol transport, the most important mechanism for cholesterol removal from extrahepatic cells, also indicates that the 27-hydroxylation pathway is an alternative and/or a complement to HDL-mediated reverse cholesterol transport (11, 12). Finding that P450c27 mRNA and enzyme activity are present in most if not all tissues (13–17) indicates that this 27-hydroxylation pathway is not limited to macrophages and probably represents a general mechanism for removal of intracellular cholesterol.

Although P450c27 appears to be the only enzyme responsible for the conversion of cholesterol into 27-hydroxycholesterol (18–21), it has not been proven that P450c27 can efficiently oxidize 27-hydroxycholesterol into 3β-hydroxy-5-cholestenoic acid. One hypothesis is that this reaction may be catalyzed by alcohol and aldehyde dehydrogenases (22). Moreover, even though comparable amounts of 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid are formed upon incubation of human macrophages with cholesterol, purified rabbit liver P450c27 has been found only to produce 27-hydroxycholesterol from cholesterol with no detectable 3β-hydroxy-5-cholestenoic acid (23). Recently, we overexpressed human P450c27 in Escherichia coli yielding the highly purified enzyme and in initial experiments we also did not observe the formation of 3β-hydroxy-5-cholestenoic acid (24). Herein, we describe the conditions under which 3β-hydroxy-5-cholestenoic acid is formed from cholesterol in a reconstituted system demonstrating for cytochrome P450 catalyzing the multiple oxidation reactions at the C-27 atom of steroids, the product of the CYP27 gene; P450sc, cholesterol side-chain cleavage cytochrome P450, the product of the CYP11A gene (55); HPLC, high pressure liquid chromatography; GC-MS, gas chromatography-mass spectrophotometry; HDL, high density lipoprotein; Adx, adrenodoxin; Adr, adrenodoxin reductase.

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‡ The abbreviations used are: P450c7, cytochrome P450 catalyzing 7α-hydroxylation of cholesterol, the product of the CYP7 gene; P450c27, cytochrome P450 catalyzing the multiple oxidation reactions at the C-27 atom of steroids, the product of the CYP27 gene; P450sc, cholesterol side-chain cleavage cytochrome P450, the product of the CYP11A gene (55); HPLC, high pressure liquid chromatography; GC-MS, gas chromatography-mass spectrophotometry; HDL, high density lipoprotein; Adx, adrenodoxin; Adr, adrenodoxin reductase.
the first time that P450c27 is able to catalyze multiple oxidation reactions at the C-27 atom of cholesterol. We also show that under identical in vitro conditions 27-hydroxycholesterol and 3β-hydroxy-5-cholesten-7-one are more efficiently converted into 3β-hydroxy-5-cholesten-7-one by P450c27 than is cholesterol into 27-hydroxycholesterol. Because 27-hydroxycholesterol has a number of potnet biological activities that relate to regulation of cholesterol synthesis (25, 26), cytotoxicity to different cell types (27–30), and platelet aggregation (31), discovery of factors influencing the level of 27-hydroxycholesterol is important. Because a single enzyme catalyzes both the formation and the degradation of 27-hydroxycholesterol, studies identifying factors influencing the rates of both the oxidation of cholesterol into 27-hydroxycholesterol, and the conversion of 27-hydroxycholesterol into 3β-hydroxy-5-cholesten-7-one by P450c27, provide insight as to how the level of these two oxygenated cholesterol metabolites and the ratio between them may differ within extrahepatic tissues. These studies establish the initial sequence of reactions in alternative bile acid biosynthetic pathways and expand our knowledge of the diversity of the substrates utilized by this enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials—Reagents for bacterial growth were from Difco.** [4,6-14C]cholesterol was from DuPont; tritium-labeled and unlabeled 27-hydroxycholesterol were synthesized as described previously (32). 3β-Hydroxy-5-cholesten-7-one was synthesized via standard Wittig Horner reaction of 3β,5-prenyltetralin to trimethylsilyloxycholesterol-24-al (33). Tritium-labeled 3β-hydroxy-5-cholesten-7-one was obtained by oxidizing the unlabeled free acid with commercial 3β-hydroxycholesterol dehydrogenase (obtained from Boehringer Mannheim) under the conditions previously described (34) to yield the 3-oxo-4-unaturated analogue of cholesterol. This compound was methylated and converted into the corresponding aldehyde and hydroxyester (34). The crude aldehyde was immediately reduced with NaBH₄ (obtained from Amersham International, UK) in isopropanol. The resultant methyl ester of 3β-hydroxy-5-cholesten-7-one was hydrolyzed by alkaline saponification and extracted with ether from acidified water phase. The ether extract was washed with water until neutral, and the ether was evaporated. The material was pure as judged by radio thin layer chromatography with the following solvent systems: chloroform/methanol (w/v) 2:1 (v/v) as a moving phase, and the identity was confirmed by combined gas chromatography-mass spectrometry (GC-MS) of the trimethylsilyl ether-methyl ester. The specific radioactivity was 5 × 10⁶ cpm/μg, and it was diluted with unlabeled 3β-hydroxy-5-cholesten-7-one to a specific radioactivity of 600,000 cpm/μg prior to incubation. Cholesterol-27-al was prepared from unlabeled and labeled 3β-hydroxy-5-cholesten-7-one by methylation and acetylation followed by reduction with disubutyltin hydride (35). The aldehyde was purified by preparative thin-layer chromatography, using toluene/ethyl acetate 3:7 (v/v) as a moving phase. The identity of the compound was confirmed by GC-MS of the trimethylsilyl ether-methyl ester derivative. There was a continuous degradation of the aldehyde into the corresponding acid and alcohol due to non-enzymatic dismutation. As a consequence, only newly synthesized and newly purified material could be used in the experiments, and in general the purified aldehyde used contained a minimum of 4% of the corresponding acid and 4% of the corresponding alcohol.

Recombinant human P450c27, recombinant bovine adrenodoxin (Adx), and recombinant adrenodoxin reductase (Adr) were expressed and purified as described previously (24). P450c27 with a heme content of 17.6 nmol/mg protein showing a single band upon silver-stained SDS-polyacrylamide gel electrophoresis was used in this study. Prior to enzymatic assays P450c27 was dialyzed overnight against 100 volumes of 40 mm phosphate buffer (pH 7.4) containing 20% glycerol to remove 1 M NaCl and 0.5% sodium cholate present in the elution buffer used during the last purification step, and concentrated to 70 μg/mL using ultrafiltration membranes (Amicon). Spectral purity indexes of recombinant Adx (A₂₈₀/₂₈₀) and recombinant Adr (A₅₅₀/₅₅₀) were 0.95 and 7.5, respectively. Adx and Adr are considered to be pure with the spectral purity indexes beginning from 0.86 for Adx and 7.5 for Adr (36).

**Enzyme Assays—** P450c27 activities using either cholesterol, 27-hydroxycholesterol or 3β-hydroxy-5-cholesten-7-one as a substrate were assayed in 40 mm phosphate buffer (pH 7.4), containing 0.1% Tween 20 (24). When 3β-hydroxycholesterol was used as a substrate, the buffer did not contain detergent. Reaction mixtures (1 mL) contained variable amounts of P450c27, Adx, Adr, and substrate. Enzymatic assays were initiated by addition of NADPH (final 1 mM), carried out at 37°C for different times, and terminated by adding 2 mL of CH₃CO₂H. Steroids, after extraction with CH₂Cl₂, were evaporated, dissolved in methanol and analyzed by HPLC. The conditions used for separation of cholesterol from 27-hydroxycholesterol and 3β-hydroxy-5-cholesten-7-one were the same as described previously (37). The retention times for 3β-hydroxy-5-cholesten-7-one, 27-hydroxycholesterol, and cholesterol were 9.25, 10.95, and 18.6 min, respectively. Under these conditions, however, 27-hydroxycholesterol and 3β-hydroxy-5-cholesten-7-one were not completely separated, and the two products appeared as one homogenous peak. To separate 27-hydroxycholesterol and 3β-hydroxy-5-cholesten-7-one, another HPLC system was developed. Steroids were separated using a YMC-PACK-ODS-A (4.6 × 250 mm) (YMC Co., Ltd.) column and isocratic elution with methanol/water/acetic acid 85:15:0.01 (v/v/v). The flow rate was 1 mL/min. The retention times for 3β-hydroxy-5-cholesten-7-one, 27-hydroxycholesterol, and 3β-hydroxy-5-cholesten-7-one were 40, 46, and 51 min, respectively. The products obtained after the incubation of P450c27 with different substrates were identified using GC-MS.

**RESULTS**

Previous studies of enzymatic activities showed no formation of 3β-hydroxy-5-cholesten-7-one by incubation of purified P450c27 with cholesterol (23, 24). In these experiments, relatively low (0.1–0.2 nmol) amounts of P450c27 were used in reconstituted systems. Results using cultured human macrophages (9) and human aortic endothelial cells (17) demonstrating conversion of cholesterol into 3β-hydroxy-5-cholesten-7-one led us to further investigate metabolism of cholesterol by P450c27 using greater (up to 3 nmol) amounts of enzyme. These experiments were carried out in the presence of 0.1% Tween 20 because addition of non-ionic detergents substantially increases solubility of cholesterol in aqueous solutions (38, 39). To be able to compare catalytic properties of P450c27 toward different substrates under similar conditions, 0.1% Tween 20 was also included in the reaction mixture when 27-hydroxycholesterol and 3β-hydroxy-5-cholesten-7-one were used as substrates.

**Product Formation upon Incubation of Cholesterol with P450c27—** As judged by HPLC separation (Fig. 1), two major products are formed from cholesterol when 1.5 μM P450c27 is used in a reconstituted system in the presence of 0.1% Tween 20. No products were formed when either Adx or NADPH were omitted (data not shown). GC-MS analysis performed after preliminary HPLC separation showed that the first peak is 3β-hydroxy-5-cholesten-7-one, the second peak besides 27-hydroxycholesterol may contain small amounts of 3β-hydroxy-5-cholesten-7-one, and the third peak is cholesterol. Finding that second peak is not homogeneous was surprising because 3β-hydroxy-5-cholesten-7-one was not detected when lower concentrations of P450c27 (0.04 μM or 0.2 μM) were used (Fig. 1A). To check whether 3β-hydroxy-5-cholesten-7-one is really not separated from 27-hydroxycholesterol under the chromatographic conditions used and to develop an HPLC system allowing separation of all three reaction products, 3β-hydroxy-5-cholesten-7-one was synthesized (see “Experimental Procedures”). Analysis of the chromatographic behavior of chemically synthesized 27-hydroxycholesterol and 3β-hydroxy-5-cholesten-7-one revealed that these two products are indeed not separated from each other under the conditions described in Fig. 1. The new chromatographic system (see “Experimental Procedures”) separates 3β-hydroxy-5-cholesten-7-one, 27-hydroxycholesterol, and 3β-hydroxy-5-cholesten-7-one (Fig. 2). The retention times of the chemically synthesized 3β-hydroxy-5-cholesten-7-one was virtually identical to that of one of the biologically generated products. However, the amounts of this product after preliminary HPLC separation were too small to allow clear identification by GC-MS. The final proof that 3β-hydroxy-5-cholesten-7-one is indeed the intermediate in the conversion of cholesterol into 3β-hydroxy-
Novel Activities of P450c27

5-hydroxy-5-cholestenoic acid catalyzed by P450c27 was obtained from the incubations with 27-hydroxycholesterol described below. Because the new chromatographic system does not allow quantitation of unmetabolized cholesterol, the patterns of product formation (Fig. 1B) were generated by first determining the total conversion of cholesterol into 3β-hydroxy-5-cholestenal and (27-hydroxycholesterol + 3β-hydroxy-5-cholestenal) using the initial chromatographic system, and then the ratio between the three products was determined using the second chromatographic system.

Kinetic analysis of the reaction using 1.5 μM P450c27 and 50 μM cholesterol reveals that there is a lag phase in the formation of 3β-hydroxy-5-cholestenoic acid (Fig. 1B), indicating that a certain amount of 27-hydroxycholesterol should be accumulated before it can be further metabolized. The presence of the lag phase and the fact that the ratio between 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid is not constant but changes during time course of the reaction (Fig. 1B) indicates that after 27-hydroxycholesterol is formed from cholesterol, it is released from the P450c27, and then competes with cholesterol to reenter the enzyme active site for further oxidation. The more 27-hydroxycholesterol formed, the more efficiently it competes with cholesterol, changing a ratio between 27-hydroxycholesterol and 3β-hydroxy-5-cholestenolic acid from 10.96 after 10 min of reaction to 1.51 after 40 min under the conditions used in Fig. 1B. If 27-hydroxycholesterol were not released from the P450c27 active site, the formation of 3β-hydroxy-5-cholestenolic acid would parallel the formation of 27-hydroxycholesterol.

**Product Formation upon Incubation of 27-Hydroxycholesterol with P450c27**—In the reconstituted system containing 1.5 μM P450c27 and 50 μM 27-hydroxycholesterol as initial substrate, the major product formed was 3β-hydroxy-5-cholestenolic acid (Fig. 2). The amount of 3β-hydroxy-5-cholestenolic acid was increased with reaction time (Fig. 2, inset), no 3β-hydroxy-5-cholestenolic acid was detected when NADPH was omitted from the reaction mixture, and formation of 3β-hydroxy-5-cholestenolic acid was substantially reduced in the presence of ketoconazole, which is an inhibitor of many P450s.

Identification of the aldehyde as an intermediate establishes the expected reaction sequence in the overall conversion of cholesterol into 3β-hydroxy-5-cholestenolic acid by P450c27.

**Product Formation upon Incubation of 3β-Hydroxy-5-cholestenol with P450c27**—Due to the instability, only small amount of 3β-hydroxy-5-cholestenolic acid was synthesized. This amount, however, was enough to show that synthetic 3β-hydroxy-5-cholestenolic acid can be efficiently converted by P450c27 into 3β-hydroxy-5-hydroxycholestenol and to compare under identical conditions the rates of conversion of 3β-hydroxy-5-hydroxycholestenol into 3β-hydroxy-5-hydroxycholesterol and cholesterol into 27-hydroxycholesterol (Table I). Because 3β-hydroxy-5-hydroxycholestenol can dismutate nonenzymatically, the formation of 3β-hydroxy-5-cholestenolic acid was also determined in the absence of NADPH. As is seen from Table I, the capacity of P450c27 to convert 3β-hydroxy-5-hy-
droxycholestenal into 3β-hydroxy-5-cholestenal is at least 5 times higher than that to convert cholesterol into 27-hydroxycholesterol. The fact that 79% of 3β-hydroxy-5-hydroxycholestenal was converted into 3β-hydroxy-5-cholestenoic acid within 10 min of reaction also indicates that experimental conditions using this substrate were not optimal for kinetic analysis and the concentration of the substrate could be rate-limiting. The rate of conversion of 3β-hydroxy-5-hydroxycholestenal into 3β-hydroxy-5-cholestenoic acid may be even higher than in Table I if optimal conditions are used.

Product Formation upon Incubation of 3β-Hydroxy-5-cholestenal and cholesterol catalyzed by P450c27—

As is seen from Fig. 2, there is a minor polar product eluting from the column earlier than 3β-hydroxy-5-cholestenal which is formed upon incubation of P450c27 with 27-hydroxycholesterol. To check the origin of this product, 3β-hydroxy-5-cholestenoic acid was used as substrate at very low concentrations to maximize conversion. Fig. 4 shows that 3β-hydroxy-5-cholestenoic acid can be metabolized by P450c27. The formation of the product is time-dependent and requires the complete reconstituted system consisting of P450c27, Adx, Adr, and NADPH (data not shown). Isolation of the polar product fraction by HPLC and analysis of the trimethylsilylated methyl ester showed that it consists of one major and one minor monohydroxylated metabolite (Fig. 5, A and B). The mass spectrum of both products was similar to the mass spectrum of the corresponding derivative of 3β-hydroxy-5-cholestenoic acid with prominent peaks at m/z 79, m/z 129 (3β-hydroxy-5-unsaturated steroid), and m/z 213. The ions at

![Mass spectrum of trimethylsilyl (TMS) ether of the synthetic 3β-hydroxy-5-cholestenal (A) and one of the metabolites eluting at 51 min (Fig. 2) upon incubation of 27-hydroxycholesterol with P450c27 (B).](image)

![HPLC separation of products formed after 30 min reaction of 1.5 nmol of human recombinant P450c27 with 2.3 nmol of 3β-hydroxy-5-cholestenoic acid (27-COOH). The reaction conditions and HPLC separation are as described in Fig. 1.](image)

**Table I**

Comparison of the rates of metabolism of 3β-hydroxy-5-cholestenal and cholesterol catalyzed by P450c27

| Reaction catalyzed | Conversion into product<sup>a</sup> (%) |
|-------------------|----------------------------------------|
|                   | 10 min | 20 min |
| 3β-Hydroxy-5-cholestenal → 3β-hydroxy-5-cholestenoic acid | Full system | 73 | 78 |
|                   | NADPH | 4 | 11 |
| Cholesterol → 27-hydroxycholesterol | Full system | 13 | 28 |
|                   | NADPH | ND | ND |

<sup>a</sup> The reactions with both substrates were carried out simultaneously and under identical conditions using 12.5 μM substrate and 1.5 μM P450c27.

<sup>b</sup> ND, not detectable.
m/z 255 (nucleus – trimethylsilyl ether), m/z 345 (M – side chain), m/z 373 (M – 129), m/z 412 (M – 90), and m/z 502 (M) in the mass spectrum of cholestenoic acid derivative were shifted in the two products by two mass units to m/z 253, m/z 343, m/z 371, m/z 410, and m/z 500 as shown in Fig. 5. This is consistent with loss of one trimethylsilyl ether group from the steroid nucleus in both products. Although it was not possible to define the specific location of the hydroxyl group introduced in the steroid nucleus by P450c27 in each product from the fragmentation pattern, it is possible to exclude certain positions due to the absence of characteristic peaks obtained with standards. P450c27 has been reported to catalyze hydroxylations in positions 24, 25, and 1 (40, 41). All these three positions are, however, excluded in the metabolites in Fig. 5. Because mass spectra of cholestenoic acid derivatives are not identical to that of 3β,7α-dihydroxy-5-cholestenoic acid or 3β,7β-dihydroxy-5-cholestenoic acid, 7α- and 7β-positions can also be excluded as possible sites of hydroxylation.

Kinetic Analysis of Cholesterol and 27-Hydroxycholesterol Metabolism Catalyzed by P450c27—Because accurate determination of $K_m$ and $V_{max}$ requires linear rates of product formation with respect to enzyme concentrations, we determined the effect of varying P450c27 concentrations on product formation when cholesterol and 27-hydroxycholesterol were used as substrates. Fig. 6 shows that the rates of formation of 27-hydroxycholesterol from cholesterol and 3β-hydroxy-5-cholestenoic acid from 27-hydroxycholesterol are linear within P450c27 concentration ranges of 0.3–0.75 $\mu$M and 0.3–1.5 $\mu$M, respectively. As is also seen from Fig. 6, when the concentration of the substrate is 50 $\mu$M, there is a direct correlation between the amount of the 3β-hydroxy-5-cholestenoic acid formed either from cholesterol or 27-hydroxycholesterol and the concentration of P450c27. The fact that, upon incubation of cholesterol with P450c27, there is a threshold concentration of the enzyme below which no 3β-hydroxy-5-cholestenoic acid is detected explains why this metabolite was not identified in the previous studies in which lower concentrations of both P450c27 and cholesterol were used.

We tried to determine $K_m$ and $V_{max}$ for both cholesterol and 27-hydroxycholesterol. In kinetic experiments, the maximal concentration of steroid used was 30 $\mu$M with no turbidity being observed after addition of the steroid to the reaction mixture. In the case of cholesterol, the reaction conditions (amount of P450c27 and reaction time) have been optimized for only one product, 27-hydroxycholesterol, with no formation of 3β-hydroxy-5-cholestenoic acid. The conversion of either cholesterol or 27-hydroxycholesterol into product did not exceed 8%. The relationship between the initial velocity ($v_i$) of the P450c27-catalyzed reaction and the concentration of the substrate (S) (Fig. 7) shows that the reactions are first-order with respect to substrate concentration indicating that the range of the substrate concentrations used is much lower than $K_m$. Because determination of $K_m$ requires use of a range of substrate concentrations between 0.33 and 2.0 $K_m$ (42), it becomes rather
problematic to accurately determine $K_m$ for either cholesterol or 27-hydroxycholesterol because of their limited solubilities in aqueous solutions even in the presence of detergent. From double-reciprocal plots (data not shown), we could only make a rough estimate of $K_m$ of 100 μM or higher for both cholesterol and 27-hydroxycholesterol. However, in the case when $[S] < K_m$, it is possible to determine the enzyme catalytic efficiency ($k_{cat}/K_m$) using Equation 1 (43)

$$v_o = \frac{k_{cat}/K_m}{[E]}[S]$$ (Eq. 1)

$k_{cat}$ is a catalytic constant of the enzyme, and $[E]$ is the concentration of the enzyme. Because in our experiments $[E]$ is constant and known, this equation can be rearranged into that shown in Equation 2.

$$v_o/[E] = \frac{k_{cat}/K_m}{[S]}$$ (Eq. 2)

($k_{cat}/K_m$) are determined by plotting $v_o/[E]$ as the ordinate and $[S]$ as the abscissa (Fig. 7). The fact that ($k_{cat}/K_m$) for cholesterol is approximately 3 times lower than that for 27-hydroxycholesterol (Table II) indicates that in the overall conversion of cholesterol into 3β-hydroxy-5-cholestenic acid from 27-hydroxycholesterol is approximately 3 times lower than that for 27-hydroxycholesterol (Table II).

P450c27 has a wide substrate specificity (44). The best endogenous substrate for P450c27 is 5β-cholestone-3α,7α,12α-triol, which is formed during the hepatic bile acid synthesis pathway initiated by 7α-hydroxylation of cholesterol. Using 5β-cholestone-3α,7α,12α-triol, rabbit and human enzymes are able to hydroxylate the terminal methyl group three times to give at first 5β-cholestone-3α,7α,12α,27-tetrol, the intermediate aldehyde, and subsequently 3α,7α,12α-trihydroxy-5β-cholestoneic acid (35, 45). Because $k_{cat}$ is also known as the turnover number (43), the value ($k_{cat}/K_m$) for these substrates can be calculated from previous experiments where these kinetic parameters were determined. Comparison of the values of ($k_{cat}/K_m$) for different substrates of P450c27 as well as for cholesterol of another cytochrome P450 (P450scc, catalyzing the conversion of cholesterol to pregnenolone in mitochondria of steroidogenic tissues) revealed that enzyme’s catalytic efficiency ($k_{cat}/K_m$) appeared to be the highest for 5β-cholestone-3α,7α,12α-triol followed by 5β-cholestone-3α,7α,12α,27-tetrol, 27-hydroxycholesterol, and cholesterol (Table II). Comparison of the ($k_{cat}/K_m$) values between the two mitochondrial P450s that utilize cholesterol as a substrate (P450c27 and P450scc) (Table II) indicates that at subsaturating substrate concentrations cholesterol is a much better substrate for P450scc than for P450c27.

**DISCUSSION**

The present study clearly and conclusively demonstrates for the first time that recombinant human P450c27 is able to hydroxylate the terminal methyl group of cholesterol three times to give at first 27-hydroxycholesterol, then 3β-hydroxy-5-cholestenic, and finally 3β-hydroxy-5-cholestenic acid with the first hydroxylation step being rate-limiting. In addition to the finding that at low, subsaturating, concentrations 27-hydroxycholesterol is an even better substrate for P450c27 than cholesterol, we demonstrate that after P450c27 finishes complete oxidation of the C-27 atom of cholesterol to form 3β-hydroxy-5-cholestenic acid, it can further introduce a hydroxyl group at different positions of the steroid nucleus but not at the steroid side chain. Such activity toward steroid substrates has not been reported previously, and indicates that regioselectivity of hydroxylations catalyzed by P450c27 is determined by the state of the oxidation of the C-27 atom of the side chain, and if C-27 is completely oxidized, P450c27 will introduce hydroxyl groups in the steroid nucleus. Because the intracellular concentration of 3β-hydroxy-5-cholestenic acid is very low (12) and because this acid is easily transported out of the cells, it is not possible to ascribe a physiological importance to these novel hydroxylations.

Finding that $K_m$ for both cholesterol and 27-hydroxycholesterol are high and that the concentration of both substrates used in previous reconstitution experiments was much lower than $K_m$, provides an explanation for the inability to detect 3β-hydroxy-5-cholestenic acid in previous studies. When [S] << $K_m$, very little enzyme-substrate complex [ES] is formed, and consequently very little product is formed within the time of the assay. In the present study, using higher amounts of P450c27, we increased the concentration of [ES], thus increasing the formation of the intermediate product, 27-hydroxycholesterol. This allowed demonstration that P450c27 can efficiently convert cholesterol into 3β-hydroxy-5-cholestenic acid. Because 3β-hydroxy-5-cholestenic acid is found in human serum at concentrations 6.72 ± 2.79 μg/100 ml (3), this reaction is of physiological significance.

Our data also indicate that in the reconstituted system after

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**Table II**

| Reaction catalyzed | $k_{cat}/K_m$ | Source |
|--------------------|---------------|--------|
| P450c27 | | |
| Cholesterol → 27-hydroxycholesterol | 0.005 ± 0.001 | Present work |
| 27-Hydroxycholesterol → 3β-hydroxy-5-cholestenic acid | 0.014; 0.018 | Present work |
| 5β-Cholesterol-3α,7α,12α-triol → 5β-cholostane-3α,7α,12α,27-tetrol | 2.94 | (23) |
| 5β-Cholostane-3α,7α,12α,27-tetrol → 3α,7α,12α-trihydroxy-5β-cholestenic acid | 0.81 | (23) |
| P450scc | | |
| Cholesterol → pregnenolone | 0.53 | (56) |
| 27-Hydroxycholesterol → pregnenolone | 0.028 | (56) |

* Results are means of triplicate experiments.
* Results of duplicate experiments.
27-hydroxycholesterol is formed from cholesterol, it is released from P450c27 and competes with cholesterol to reenter the enzyme active site for further oxidation. We speculate that a similar sequence of events occurs in vivo. Indeed, if 27-hydroxycholesterol formed from cholesterol in vivo, does not leave P450c27 active site and is directly oxidized to 3β-hydroxy-5-cholestanolic acid, no 27-hydroxycholesterol would be found in the serum. The concentration of 27-hydroxycholesterol in human serum ranges from 12.5 to 29.4 μg/100 ml (46). P450c27 is the only enzyme in humans responsible for the conversion of cholesterol to 27-hydroxycholesterol (18–21). In patients with cerebrotendinous xanthomatosis, a hereditary sterol storage disorder disease caused by the mutations in CYP27, serum levels of 27-hydroxycholesterol as well as 3β-hydroxy-5-cholestanolic acid are significantly reduced if detected at all (21).

The metabolic fate of 27-hydroxycholesterol and 3β-hydroxy-5-cholestanolic acid formed in the alternative bile acid pathway in nonhepatic tissues is under study. Obviously, it is not possible to identify all the factors which determine what fraction of 27-hydroxycholesterol formed in the inner mitochondrial membrane is further metabolized into 3β-hydroxy-5-cholestanolic acid from reconstitution experiments. Present data do, however, indicate that the concentration of cholesterol in the inner mitochondrial membrane and the level of expression of P450c27 will establish the product profile (the ratio between 27-hydroxycholesterol and 3β-hydroxy-5-cholestanolic acid) in a given tissue. Experiments with different types of cultured cells (human lung alveolar macrophages, human monocyte-derived macrophages, human umbilical endothelial cells, bovine aortal endothelial cells, and human fibroblasts) (12) as well as rat liver mitochondria (47) indicate that cholesterol content in the inner mitochondrial membrane in these types of cells is low, subsaturating for P450c27, and availability of cholesterol to the enzyme limits synthesis of 27-hydroxycholesterol. Under these circumstances, as it follows from our studies using reconstituted system, the total production of 27-hydroxycholesterol and 3β-hydroxy-5-cholestanolic acid and the ratio between them in a given tissue will be determined by the level of expression of P450c27. Indeed, macrophage preparations (human lung alveolar macrophages and human monocyte-derived macrophages) have considerably higher capacity to secrete 27-hydroxycholesterol as well as 3β-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid formed in the alternative bile acid biosynthetic pathways (4, 51–54). The present study establishes the enzymatic basis for production of the initial intermediates in alternative bile acid biosynthetic pathways (27-hydroxycholesterol and 3β-hydroxy-5-cholestanolic acid) and how the ratio of production of the intermediates may vary between different extrahepatic cells.

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