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Cytochrome *P*450 CYP27-catalyzed Oxidation of C_{27}-steroid into C_{27}-acid

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Rabbit liver cytochrome *P*450 CYP27 has been previously shown to catalyze the complete conversion of 5β-cholestane-3α,7α,12α-triol into 3α,7α,12α-trihydroxy-5β-cholestanoic acid. This study compares some properties of the reactions involved, the 27-hydroxylation of 5β-cholestane-3α,7α,12α-triol and the further oxidation of 5β-cholestane-3α,7α,12α,27-tetrol. The K_m was the same for the two substrates, whereas the V_{max} was three times higher for 27-hydroxylation than for the oxidation of 5β-cholestane-3α,7α,12α,27-tetrol. Ketoconazole inhibited both reactions, whereas disulfiram did not. Carbon monoxide inhibited the 27-hydroxylation of 5β-cholestane-3α,7α,12α-triol but not the further oxidation of 5β-cholestane-3α,7α,12α,27-tetrol. There was no difference in sensitivity to varying oxygen concentrations between the two reactions. The present study shows that CYP27 also converts, although less efficiently, 5β-cholestane-3α,7α-diol into 3α,7α-dihydroxy-5β-cholestanoic acid and cholesterol into 3β-hydroxy-5-cholestenoic acid. The rate of oxidation of cholesterol into C_{27}-acid was very low—less than 1% of that with the other C_{27}-steroids.

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INTRODUCTION

The first step in the degradation of the cholesterol side chain in bile acid biosynthesis is the 27-hydroxylation of C_{27}-steroids [1]. The reaction is catalyzed by cytochrome *P*450 CYP27 (sterol 27-hydroxylase), a mitochondrial cytochrome *P*450 [2–4]. CYP27 has also been shown to catalyze the complete conversion of 5β-cholestane-3α,7α,12α-triol into 3α,7α,12α-trihydroxy-5β-cholestanoic acid [4–6]. In a recent study, the formation and isolation of the C_{27}-aldehyde intermediate was reported [7]. It was also shown, with the use of $^{18}O_2$, that the three oxidation steps from 5β-cholestane-3α,7α,12α-triol into 3α,7α,12α-trihydroxy-5β-cholestanoic acid are monooxygenation reactions. In the present work, apparently homogeneous CYP27 was used to compare some properties of the 27-hydroxylation of 5β-cholestane-3α,7α,12α-triol with those of the further oxidation of the tetrol into 3α,7α,12α-trihydroxy-5β-cholestanoic acid. So far, studies on the involvement of CYP27 in the formation of C_{27}-acid have only concerned 3α,7α,12α-trihydroxy-5β-cholestanoic acid. Considering the various pathways described for the formation of chenodeoxycholic acid in mammals and man [1], it should be of interest to study the oxidation also of other C_{27}-steroids into C_{27}-acids. In the present communication, the ability of CYP27 to oxidize cholesterol and 5β-cholestene-3α,7α-diol is reported.

EXPERIMENTAL

Materials

Ketoconazole was a generous gift from Janssen Pharmaceutical N.V. (Beerse, Belgium). Disulfiram was obtained from Sigma. Octylamine-Sepharose 4B was prepared as described previously [2]. Hydroxylapatite (Bio-Gel) was from Bio-Rad. Bio-Gel was mixed with an equal amount of Whatman CF-1 cellulose powder before chromatography. The remaining chemicals were reagent grade. Unlabeled 5-cholestene-3β,27-diol was prepared from kryptogenin as previously described [8] and 3β-hydroxy-5-cholestenoic acid was a generous gift from Professor Jan Sjövall, Stockholm.

Labeled compounds

[4-^{14}C]Cholesterol (55 Ci/mmol) was from Amersham International (Amersham, Bucks, U.K.). 5β-[7β-^{3}H]Cholestane-3α,7α-diol (200 Ci/mol) and 5β-[7β-^{3}H]cholestane-3α,7α,12α-triol (200 Ci/mol) were synthesized as described previously [9]. 5β-[7β-^{3}H]-

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Cholestane-3α,7α,27-tetrol (200 Ci/mol) and 5β-[7β-^{3}H]cholestane-3α,7α,12α,27-tetrol (200 Ci/mol) were prepared biosynthetically as described [7].

Enzyme purification

CYP27 from rabbit liver mitochondria and adrenodoxin and adrenodoxin reductase from bovine adrenal mitochondria were prepared as described previously [10]. Protein concentration was determined according to Lowry *et al.* [11] and cytochrome *P*450 content according to Omura and Sato [12]. Adrenodoxin and adrenodoxin reductase concentrations were determined as described by Huang and Kimura [13] and Chu and Kimura [14], respectively.

Incubation procedures and analyses of incubation mixtures

Incubations with rabbit liver CYP27 were carried out for 10–60 min at 37 C. 5β-Cholestane-3α,7α,27-triol and 5β-cholestane-3α,7α,12α,27-tetrol (5–12.5 nmol in 10 μl acetone) were incubated with 0.1 nmol of CYP27, 2 nmol of adrenodoxin, 0.2 nmol of adrenodoxin reductase and 1 μmol of NADPH in a total volume of 1 ml of 50 mM Tris–acetate buffer, pH 6.0. Incubations were terminated by the addition of 5 ml of ethanol and extracted with diethyl ether acidified with 6 M HCl. The combined extracts were washed with water until neutral. The organic phase was evaporated and the residue was subjected to thin-layer chromatography. The developing solvent was toluene–isopropyl alcohol–acetic acid (75:25:2.5). The chromatoplates were analyzed by radioactivity scanning [15]. 5-Cholestene-3β,27-diol was incubated and incubations were terminated as described above. To the terminated incubations, 0.3 μg of 4-androstene-3,17-dione were added as an internal recovery standard, the mixture was extracted with acidified ethyl ether and the organic phase was dried under nitrogen. The samples were dissolved in 25 μl of acetone and 875 μl of 100 mM phosphate buffer, pH 7.4, containing 0.1 mM EDTA. A second reaction was then started with addition of 0.4 units of cholesterol oxidase (Sigma Chemicals Co.) dissolved in 0.1 ml of the incubation buffer. After 20 min the reaction was terminated with 5 ml of trichloroethane–methanol (2:1) and the organic phase was dried under nitrogen and dissolved in 0.1 ml of mobile phase, hexane–isopropanol (96:4). The samples were then subjected to straight-phase HPLC on a 150 × 3 mm silica column (LiChrosorb Si 60, 5 μm) at a flow rate of 0.7 ml/min. Steroids with a 3-oxo-Δ^4-structure were monitored at 240 nm. The retention time was 6.2 min for 27-hydroxy-4-cholesten-3-one, 7.8 min for 3-oxo-4-cholestenoic acid and 14.0 min for 4-androstene-3,17-dione. Incubations with 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol were performed in the same way as described above except that the Tris–acetate buffer had pH 7.4 and the developing solvent for extracts of incubations was ethyl acetate–trimethylpentane–acetic acid (50:50:15, v/v/v). Incubations with cholesterol were performed and analyzed as described [2].

Incubations with CO and with varying O_2 concentrations in N_2

The Tris–acetate buffer used was saturated with nitrogen. The incubation mixture was prepared without NADPH, sealed by a rubber membrane and put under a stream of the pertinent gas for at least 10 min. The incubation was then started by adding NADPH through the rubber membrane. The gas was on during the entire incubation. After 10–60 min the incubation was stopped by the addition of 5 ml of ethanol followed by extraction with diethyl ether as described above.

RESULTS AND DISCUSSION

Some properties of reactions involved in the CYP27-catalyzed oxidation of 5β-cholestane-3α,7α,12α-triol into 3α,7α,12α-trihydroxy-5β-cholestanoic acid

The properties of CYP27 in the 27-hydroxylation of 5β-cholestane-3α,7α,12α-triol and the further oxidation of 5β-cholestane-3α,7α,12α,27-tetrol were compared. The results obtained show similarities but also differences between the two reaction steps.

Kinetic constants and pH optimum

K_m and V_{max} were determined by double reciprocal plot of the kinetic data (Fig. 1). These values show that the rate of product formation differed substantially between the two reactions. The rate of hydroxylation of 5β-cholestane-3α,7α,12α-triol was three times

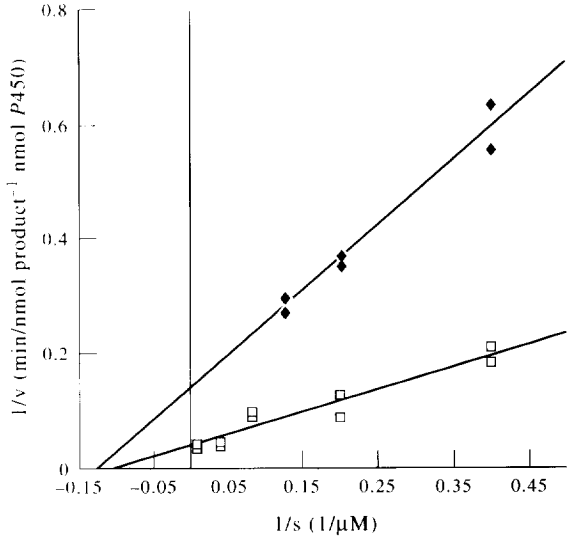

Fig. 1. CYP27 substrate kinetics for 5β-cholestane-3α,7α,12α-triol (□) and 5β-cholestane-3α,7α,12α,27-tetrol (◆). Enzyme activities were evaluated with concentrations of the two substrates of 2.5–25 and 2.5–7.5 μM, respectively. The lines are from the theoretical fit, whereas the data points are experimental. Details of the incubation and analysis procedures are given in the Experimental section.

higher than the rate of oxidation of 5β-cholestane-3α,7α,12α,27-tetrol (25 nmol min^{-1} nmol $P450^{-1}$ compared to 7.3 nmol min^{-1} nmol $P450^{-1}$). The affinity of sterol 27-hydroxylase for 5β-cholestane-3α,7α,12α-triol was on the other hand essentially the same as for 5β-cholestane-3α,7α,12α,27-tetrol (8.5 and 9.7 μM, respectively). The pH optima for the formation of 3α,7α,12α-trihydroxy-5β-cholestanoic acid from 5β-cholestane-3α,7α,12α,27-tetrol and for the hydroxylation of 5β-cholestane-3α,7α,12α-triol into 5β-cholestane-3α,7α,12α,27-tetrol were 6.0 and 7.4, respectively.

Effect of carbon monoxide (CO) on CYP27

The sensitivity to CO-inhibition of the monooxygenation reactions in the conversion of 5β-cholestane-3α,7α,12α-triol into 5β-cholestane-3α,7α,12α,27-tetrol and the further oxidation of the tetrol into 3α,7α,12α-trihydroxy-5β-cholestanoic acid was also compared. In general, CO is an inhibitor of cytochrome *P*450 reactions. The results in Table 1 show that in a $CO–O_2$-atmosphere (98:2) the conversion of 5β-cholestane-3α,7α,12α-triol into 5β-cholestane-3α,7α,12α,27-tetrol was inhibited by about 75%, but the further conversion of 5β-cholestane-3α,7α,12α,27-tetrol into 3α,7α,12α-trihydroxy-5β-cholestanoic acid was unaffected. The finding that the oxidation of 5β-cholestane-3α,7α,12α,27-tetrol was insensitive to CO is surprising. However, cytochrome *P*450 reactions may not be uniformly susceptible to such inhibition. Several previous reports have shown that carbon monoxide has no effect upon the rate of aromatization of androstenedione. Thompson and Siiteri [16] speculated that this lack of inhibition could be due to a limited rate of entry of the first electron into the oxidized cytochrome *P*450–substrate complex resulting in a lowered steady state concentration of the reduced cytochrome *P*450-substrate complex. Since CO acts upon the reduced complex, the sensitivity of the reaction to CO would be expected to be low. Zachariah and Juchau [17] proposed another explanation. They suggested that androstenedione is capable of preventing the complexing of CO with NADPH-reduced cytochrome *P*450. Furthermore, Estabrook *et al.* [18] demonstrated that under conditions in which low concentrations of NADPH were present in reaction mixtures, cytochrome *P*450-dependent oxidations were not inhibited by CO. Whether any of these possibilities applies to the lack of CO-inhibition of the oxidation of 5β-cholestane-3α,7α,12α,27-tetrol remains to be determined.

Another possible explanation for the difference in inhibition could be different sensitivity to the low oxygen tension used (2% O_2). In order to investigate this, the enzyme activities were determined in presence of different concentrations of O_2 in N_2. There was no significant difference in O_2-sensitivity between the two reactions (Fig. 2).

Table 1. Effect of carbon monoxide on the two CYP27-catalyzed reactions in the conversion of 5β-cholestane-3α,7α,12α-triol into 3α,7α,12α-trihydroxy-5β-cholestanoic acid

Assay conditions	Activity
5β-Cholestane-3α,7α,12α-triol + adrenodoxin + adrenodoxin release + NADPH	
+ N_2/O_2 (98:2); control	100%
+ CO/O_2 (98:2)	24% ± 13.5
5β-Cholestane-3α,7α,12α,27-tetrol + adrenodoxin + adrenodoxin reductase + NADPH	
+ N_2/O_2 (98:2); control	100%
+ CO/O_2 (98:2)	117% ± 9.0

Details of the incubation and analysis procedures are given in the Experimental section. The activity is expressed as % of that in control experiments (= 100%). The results from 6 experiments are given as the means ± SD.

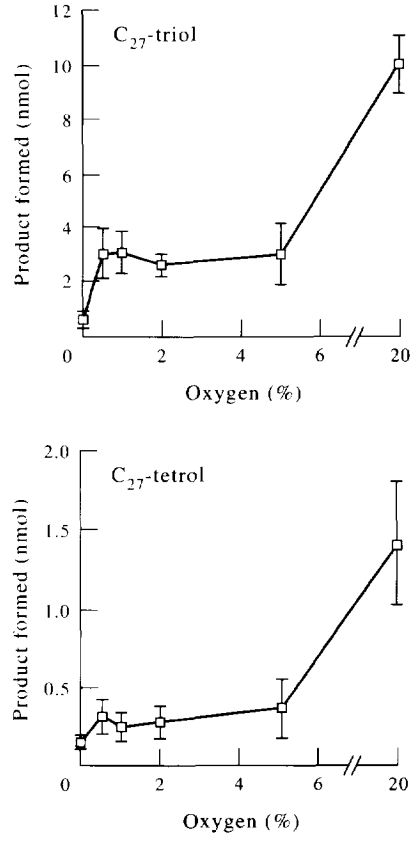

Fig. 2. Product formation upon incubation of 5β-cholestane-3α,7α,12α-triol and 5β-cholestane-3α,7α,12α,27-tetrol in an atmosphere of oxygen/nitrogen of various concentrations. The Tris–acetate buffer used was saturated with nitrogen. The incubation mixture was prepared without NADPH, sealed by a rubber membrane and put under a stream of the pertinent gas for at least 10 min. The incubation was then started by adding NADPH through the rubber membrane. The gas was on during the entire incubation. After 10–60 min the incubation was stopped by the addition of 5 ml of ethanol followed by extraction with diethyl ether. Details of the incubation and analysis procedures are given in the Experimental section.

Effect of ketoconazole and disulfiram

Ketoconazole is an inhibitor of cytochrome *P*450 reactions. It inhibits the demethylation of lanosterol by lanosterol 14α-demethylase, a cytochrome *P*450 that catalyzes a sequence similar to that of the CYP27 [19, 20]. Ketoconazole, in concentrations up to 50 μM inhibited the 27-hydroxylation of 5β-cholestane-3α,7α,12α-triol by 60%, and further oxidation by 80% (Fig. 3).

Disulfiram is an inhibitor of the aldehyde dehydrogenase in cytosol catalyzing oxidation of C_{27}-aldehyde into C_{27}-acid. Disulfiram in concentrations of up to 50 μM did not affect the activity of 27-hydroxylase-catalyzed reaction steps (Fig. 3). The results with ketoconazole and disulfiram support the previous observations of involvement of cytochrome *P*450 in the complete reaction sequence.

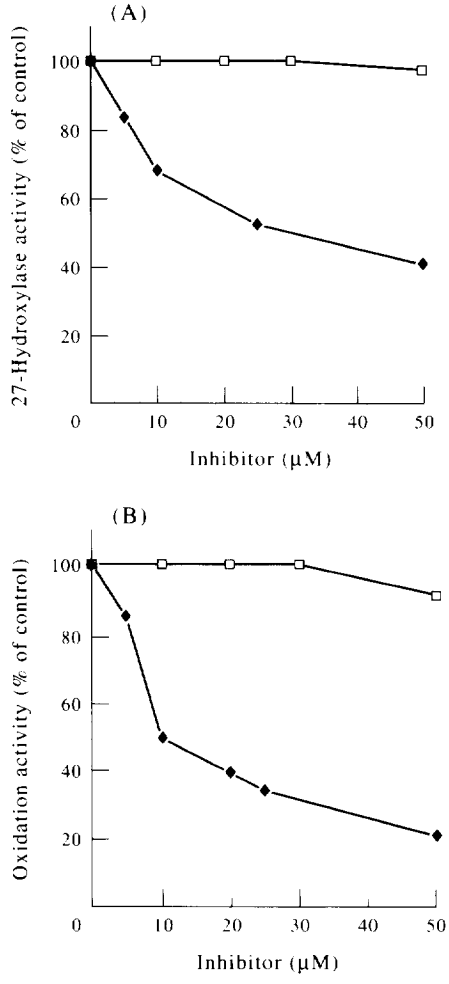

Fig. 3. Effect of ketoconazole (◆) and disulfiram (□) on the 27-hydroxylation of 5β-cholestane-3α,7α,12α-triol (A) and the further oxidation into 3α,7α,12α-trihydroxy-5β-cholestanoic acid (B). Varying amounts of ketoconazole dissolved in 25 μl of dimethyl sulfoxide or disulfiram dissolved in 25 μl of acetone were added to incubations with 5β-cholestane-3α,7α,12α-triol or 5β-cholestane-3α,7α,12α,27-tetrol (5–12.5 nmol in 20 μl of acetone), 0.2 nmol of CYP27, 2 nmol of adrenodoxin, 0.2 nmol of adrenodoxin reductase and 1 μmol of NADPH in a total volume of 1 ml of 50 mM Tris–acetate buffer. The pH of the buffer and incubation time were 7.4 and 40 min, respectively, for incubations with 5β-cholestane-3α,7α,12α-triol and 6.0 and 60 min, respectively, for 5β-cholestane-3α,7α,12α,27-tetrol. Control incubations without inhibitor contained 25 μl dimethyl sulfoxide or acetone, respectively. Details of incubation and analysis procedures are given in the Experimental section.

Table 2. Conversion of various C_{27}-steroids into 27-hydroxy-steroids and 27-carboxysteroids by CYP27

Reactions measured	Activity (pmol min^{-1} nmol cytochrome *P*450^{-1})
Cholesterol	
27-Hydroxylation	60 ± 15
Oxidation to acid	N.D.
5β-cholestane-3α,7α-diol	
27-Hydroxylation	750 ± 77
Oxidation to acid	131 ± 34
5β-cholestane-3α,7α,12α-triol	
27-Hydroxylation	1088 ± 205
Oxidation to acid	1512 ± 260

Incubations were performed as described in the Experimental section except that the substrate concentration was 15 μM and incubation time was 40 min. The results from four experiments are given as the means ± SD. N.D., not detectable.

Oxidation of various C_{27}-steroids into C_{27}-acids by CYP27

Several 27-hydroxylated sterols have been described as intermediates in chenodeoxycholic acid biosynthesis in mammals and man [1]. It has also been suggested that conversion of cholesterol into 3β-hydroxy-5-cholestenoic acid represents a general defense mechanism for macrophages and possibly other peripheral cells exposed to cholesterol [21]. Tables 2 and 3 compare the ability of CYP27 to convert some C_{27}-steroids in bile acid biosynthesis into their corresponding 27-hydroxy- and 27-carboxysteroids. Incubation with 5β-cholestane-3α,7α,12α-triol resulted in the formation of 3α,7α,12α-trihydroxy-5β-cholestanoic acid as the major product (Table 2). 5β-Cholestane-3α,7α-diol was also converted, although less efficiently, into both

Table 3. Oxidation of various 27-hydroxysteroids into corresponding 27-carboxysteroids by CYP27

Reactions measured	Activity (pmol min^{-1} nmol cytochrome *P*450^{-1})
5-cholestene-3β,27-diol oxidation to acid	2.5 ± 1.1
5β-cholestane-3α,7α,27-triol oxidation to acid	275 ± 80
5β-cholestane-3α,7α,12α,27-tetrol oxidation to acid	495 ± 66

Incubations were performed as described in the Experimental section except that the substrate concentration was 5 μM and the incubation time 60 min.

The results from four (5β-cholestane-3α,7α,27-triol and 5β-cholestane-3α,7α,12α,27-tetrol) and seven (5-cholestene-3β,7α-tiol) experiments are given as the means ± SD.

27-hydroxy- and 27-carboxysteroids. 5β-Cholestane-3α,7α,27-triol was the major product. Cholesterol incubated under the same conditions resulted in the formation of 5-cholestene-3β,27-diol at a low rate but no detectable 3β-hydroxy-5-cholestenoic acid (Table 2). Incubations with the 27-hydroxysteroids as substrates confirmed that 5β-cholestane-3α,7α,12α,27-tetrol was more efficiently oxidized into the C_{27}-acid than 5β-cholestane-3α,7α,27-triol. The rate of oxidation of 5-cholestene-3β,27-diol into 3β-hydroxy-5-cholestenoic acid was 100–200 times lower than the oxidation of the other two substrates (Table 3). Considering the very low efficiency of CYP27 to oxidize 5-cholestene-3β,27-diol into C_{27}-acid, other enzyme systems might also be involved in the formation of 3β-hydroxy-5-cholestenoic acid.

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