Role of Different Forms of Rabbit Liver Microsomal Cytochrome P-450 in Biosynthesis of Bile Acids*

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The hepatic microsomal cytochrome P-450 catalyzes the hydroxylation of both endogenous and exogenous substrates, such as fatty acids, steroids, drugs and carcinogens.[1] The biosynthesis of bile acids include a number of hydroxylations of the steroid nucleus and of the side chain catalyzed by the microsomal fraction of liver.[2] All these hydroxylations have been shown in systems containing purified cytochrome P-450 and NADPH-cytochrome P-450 reductase from rat and rabbit liver microsomes.[3–7] Several previous studies have shown that the hydroxylations differ from each other in several respects,[2–7] indicating the involvment of different species of cytochrome P-450.[6] Recently, different forms of cytochrome P-450 have been highly purified from rabbit liver microsomes.[1,8]

The present communication describes catalytic properties of different, highly purified forms of cytochrome P-450 from liver microsomes of phenobarbital-treated rabbits towards C_{27}-steroids.

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Experimental. Liver microsomes were prepared from phenobarbital-treated rabbits of the New Zealand strain. After treatment with pyrophosphate[1] the microsomes were solubilized and chromatographed on DEAE-cellulose as described by Yasukochi and Masters.[9] DEAE-cellulose chromatography resulted in subfractionation of cytochrome P-450. It was first eluted in a sharp peak, giving pool I and then in a broad flat peak, giving pool II. Each pool was applied to octylamine-Sepharose 4B columns and eluted as described by Guengerich.[8] The fractions from the octylamine-Sepharose columns were applied to hydroxyapatite columns and cytochrome P-450 was eluted stepwise with buffers of increasing phosphate concentration. Three different fractions of cytochrome P-450 in high yield were eluted. Two of these were apparently identical with cytochromes P-450 LM_2 and LM_4 described by Coon and associates.[1] They had similar apparent molecular weights, similar spectral properties, similar solubility properties upon storage in buffers with different ionic strength and similar catalytic activities. A third cytochrome P-450 fraction, cytochrome P-450 III, was also obtained.

Cytochromes P-450 LM_2 and LM_4 were also purified as described by Coon *et al.*[1] The purification involved solubilization of the microsomes, polyethylene glycol precipitation, DEAE-cellulose and hydroxyapatite chromatography. Cytochromes P-450 LM_2 and LM_4 showed similar apparent molecular weights and spectral properties as described previously.[1] The different fractions were treated with Amberlite XAD-2 and cytochromes P-450 LM_2 and III were dialyzed against 50 mM Tris acetate buffer, pH 7.4, containing 20 % glycerol and 0.1 mM

Table 1. Hydroxylase activities in preparation of liver microsomal cytochrome P-450 from phenobarbital-treated rabbits. The substrates were incubated under standard conditions at 37 °C for 20 min with 0.1–1 nmol of cytochrome P-450, 0.8–3.0 units of NADPH-cytochrome P-450 reductase, 50 μg of dilauroylglycero-3-phosphorylcholine, 50 μg of sodium deoxycholate and 2 μmol of NADPH in a total volume of 1–2 ml of 50 mM Tris-acetate buffer, pH 7.4. Incubations with ethylmorphine contained in addition 10 μmol $MgCl_2$ and were run for 10 min.

Fraction	Apparent molecular weight	Cytochrome P-450 (specific content) nmol/mg protein	Cytochrome P-450 (yield) %	5β-cholestane-3α,7α-diol 12α pmol/(nmol heme)min	5β-cholestane-3α,7α-diol 25 pmol/(nmol heme)min	5β-cholestane-3α,7α-diol 26 pmol/(nmol heme)min	5β-cholestane-3α,7α,12α-triol 25 pmol/(nmol heme)min	5β-cholestane-3α,7α,12α-triol 26 pmol/(nmol heme)min	Demethylation of ethylmorphine nmol/(nmol heme) min
Microsomes	–	2.9	100	37	22	<1	193	<1	10
Cytochrome P-450 LM_2	49 000	18.8	4.4	<1	<1	<1	<1	<1	15
Cytochrome P-450 LM_4	53 000	19.0	3.0	48	12	<1	33	<1	4
Cytochrome P-450 III	51 000	12.0	1.0	13	<1	<1	<1	<1	16

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EDTA. Cytochrome P-450 LM_4 was dialyzed against 150 mM phosphate buffer, pH 7.4, containing 20 % glycerol and 0.1 mM EDTA.

Results. Table 1 summarizes some physical properties of cytochrome P-450 LM_2, LM_4 and III. In the two different types of preparations of the cytochromes, the specific content of cytochrome P-450 LM_2 varied between 13 – 20 nmol of heme per mg of protein in five different preparations. The specific content of cytochrome P-450 LM_4 varied between 12 and 19 nmol mg^{-1} protein in four different preparations. Cytochrome P-450 III had a specific content of 12 nmol of heme per mg protein. All three cytochromes were apparently homogenous on sodium dodecyl sulfate – polyacrylamide gel electrophoresis and the apparent molecular weights of the different cytochromes were estimated to be 49 000, 53 000 and 51 000 for cytochrome P-450 LM_2, LM_4 and III, respectively. Cytochrome P-450 III might be identical with either cytochrome P-450 LM_{3a} or cytochrome P-450 LM_{3b} described by Coon and associates.[10,11] The specific content of this form of cytochrome P-450 was higher than that reported for cytochrome P-450 LM_{3b}.[11] Cytochrome P-450 III had spectral properties different from those of both cytochromes P-450 LM_2 and LM_4.

Table 1 also summarizes the catalytic properties towards different C_{27}-steroids and ethylmorphine of the original microsomal fraction and the purified cytochrome P-450 fractions combined with NADPH-cytochrome P-450 reductase, prepared as described by Yasukochi and Masters.[9] The microsomes were able to catalyze 12α- and 25-hydroxylation of 5β-cholestane-3α,7α-diol, 25-hydroxylation of 5β-cholestane-3α, 7α, 12α-triol and demethylation of ethylmorphine. Cholesterol 7α-hydroxylation and C_{27}-steroid 26-hydroxylation were very low. Cytochrome P-450 LM_2 showed no detectable catalytic activity towards the C_{27}-steroids tested but was able to catalyze the demethylation of ethylmorphine. This reaction was more efficient with cytochrome P-450 LM_2 than with microsomes. Cytochrome P-450 LM_4 catalyzed 12α- and 25-hydroxylation of 5β-cholestane-3α,7α-diol as efficiently as or more efficiently than the microsomes. The 25-hydroxylation of 5β-cholestane-3α, 7α, 12α-triol and demethylation of ethylmorphine were less efficient than with microsomes. Cytochrome P-450 III catalyzed 12α-hydroxylation but at a slower rate than microsomes. Demethylation of ethylmorphine with this fraction occurred at the same rate as with cytochrome P-450 LM_2. Cytochrome P-450 III was not able to catalyze 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol.

The results suggest the involvement of at least two different forms of cytochrome P-450 in the biosynthesis of bile acids. One of these forms, cytochrome P-450 LM_4, has been highly purified by two different methods and had a specific content of 19.0 nmol of heme per mg protein. The maximal content has been calculated to be around 20 nmol mg^{-1} of protein.

Several studies have shown that 12α- and 25-hydroxylations in contrast to many other hydroxylations are not stimulated by phenobarbital treatment of rabbits. The present results provide an explanation of this lack of stimulation. Cytochrome P-450 LM_2 occurs in very low concentrations in liver microsomes from untreated rabbits [10] but is the predominant form in microsomes from phenobarbital-treated rabbits. Cytochrome P-450 LM_4 is not affected by phenobarbital treatment. The finding that cytochrome P-450 LM_2 showed no significant catalytic activity towards the C_{27}-steroids and that cytochrome P-450 LM_4 was able to catalyze 12α- and 25-hydroxylations of the steroids can explain the lack of stimulation by phenobarbital treatment. The fact that cytochrome P-450 LM_4 in the presence of highly purified NADPH-cytochrome P-450 reductase catalyzed an efficient 12α-hydroxylation is the first conclusive evidence that this important hydroxylation in bile acid biosynthesis is dependent on cytochrome P-450.

1. Coon, M. J., van der Hoeven, T. A., Dahl, S. B. and Haugen D. A. *Methods Enzymol. C 52* (1978) 109.
2. Danielsson, H. and Sjövall, J. *Annu. Rev. Biochem. 44* (1975) 223.
3. Bernhardsson, C., Björkhem, I., Danielsson, H. and Wikvall, K. *Biochem. Biophys. Res. Commun. 54* (1973) 1030.
4. Björkhem, I., Danielsson, H. and Wikvall, K. *J. Biol. Chem. 249* (1974) 6439.
5. Björkhem, I., Danielsson, H. and Wikvall, K. *J. Biol. Chem. 251* (1976) 3495.
6. Cottman, J., Danielsson, H., Hansson, R. and Wikvall, K. In Paumgartner, G. and Stiehl, A., Eds., *Bile Acid Metabolism in Health and Disease*, MTP Press, Lancaster 1977, p. 1.
7. Hansson, R. and Wikvall, K. *Eur. J. Biochem. 93* (1979) 419.
8. Guengerich, F. P. *J. Biol. Chem. 252* (1977) 3970.
9. Yasukochi, Y. and Masters, B.S.S. *J. Biol. Chem. 251* (1976) 5337.
10. Haugen, D. A. and Coon, M. J. *J. Biol. Chem. 251* (1976) 7929.
11. Fasco, M. J., Vatsis, K. P., Kaminsky, L. S. and Coon, M. J. *J. Biol. Chem. 253* (1978) 7813.

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