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PARTIAL PURIFICATION AND CHARACTERIZATION OF STEROID 12α-MONOOXYGENASE

Kuniko Murakami and Kyuichiro Okuda

Department of Biochemistry, Hiroshima University
School of Dentistry, Hiroshima 734, Japan

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SUMMARY

Steroid 12α-monooxygenase was partially purified from rabbit liver microsomes. The enzyme was solubilized with cholate, fractionated with polyethylene glycol and chromatographed on a Sepharose 4B column with cholate as ligand. The enzyme activity was eluted from the column with 300 mM phosphate buffer containing 0.4% cholate. The enzyme activity was reconstituted from the partially purified cytochrome P-450, highly purified NADPH-cytochrome P-450 reductase, dilauroylglyceryl-3-phosphorylcholine and NADPH. It was shown that the cytochrome P-450 of the enzyme was highly deteriorative in low buffer concentration, which could be prevented by keeping the cytochrome at higher buffer concentrations.

Steroid 12α-monooxygenase is an important microsomal enzyme which regulates the ratio of cholic acid and chenodeoxycholic acid in the bile. The enzyme was solubilized from rat liver microsomes and the activity was reconstituted from the fraction containing cytochrome P-450 and that containing NADPH-cytochrome P-450 reductase by Bernhardsson et al. (1). However, the reconstituted enzyme was not significantly inhibited by CO, and therefore a possibility that an enzyme other than cytochrome P-450 which had been contained in the cytochrome P-450 fraction was responsible for the reaction could not be ruled out. This ambiguity about the nature of the enzyme was not dissolved by further purification of the enzyme by Hansson and Wickvall (2). They purified cytochrome P-450 from liver microsomes of both rat and rabbit to an electrophoretically homogenous state, and reconstituted the enzyme activity from the purified cytochrome P-450 and NADPH-cytochrome P-450 reductase which had been also highly purified from the same source according to the method described by Yasukochi and Masters (3). However, the reconstituted

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enzyme did not show any elevated turnover number compared to that in microsomal preparations.

It was recently found in this laboratory that the enzyme is highly deteriorative and is significantly inactivated during the process of enzyme purification, and this finding prompted us to seek a method to stabilize the enzyme. In this paper we describe a method of partial purification of the enzyme which is carried out with avoiding enzyme deterioration.

MATERIALS AND METHODS

Cholic acid, NADPH and cytochrome c were obtained from Sigma Chem. Co., and Sepharose 4B from Pharmacia Co. Polyethylene glycol was purchased from Baker Chem. Co., and 3-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl from Peptide Institute (Osaka, Japan). BrCN and ethylenediamine were from commercial sources. 7α-[4-^{14}C]Hydroxy-4-cholesten-3-one was synthesized according to the method described by Shimasue in this laboratory (4). 7α,12α-Dihydroxy-4-cholesten-3-one was synthesized according to the method described by Berseus et al. (5). Cholate-Sepharose 4B was prepared according to the method described elsewhere (6).

Methods of Preparations

Cytochrome P-450 was prepared as follows: Male albino rabbits weighing about 2 to 2.5 kg were allowed to drink 0.1% solution of sodium phenobarbital for 6 days and then fasted for 20 h. Liver microsomes were prepared and extracted with 0.1 M sodium pyrophosphate containing 1.0 mM EDTA according to the method described by Hoeven and Coon (7). The pyrophosphate treated microsomes were solubilized with sodium cholate, and fractionated with polyethylene glycol. The fractions precipitated by 8-10, 10-12, and 12-15% were collected and dialyzed against 100 mM phosphate buffer, pH 7.0 containing 1 mM EDTA and 20% glycerol. Either one of these dialyzed fraction was applied to a cholate-Sepharose 4B column previously equilibrated with 0.1 M potassium phosphate buffer, pH 7.0, containing 1 mM EDTA and 20% glycerol The protein was then eluted with the same buffer, and this was followed with the equilibration buffer containing 0.2% cholate. The enzyme was eluted with the 0.3 M phosphate buffer containing 1 mM EDTA, 20% glycerol and 0.4% cholate. The fractions were collected until no more protein was eluted.

NADPH-cytochrome P-450 reductase from phenobarbital treated rabbit was Prepared by an affinity chromatographic method which was essentially the same as described by Yasukochi and Masters (3). The solubilization procedure and the initial chromatographic step were carried out according to the modified method described by Vermillion and Coon (8). The enzyme thus prepared had a specific activity of 31.9 U/mg of protein when assayed according to the method described by Yasukochi and Masters.

Analytical Procedures

The concentration of cytochrome P-450 was determined from the CO difference spectrum of dithionite-reduced sample, using an extinction coefficient of 91 $mM^{-1}cm^{-1}$ between 450 and 490 nm (9).

The steroid 12α-monooxygenase was assayed as follows: A typical incubation mixture contained 50 nmol of 7α-[4-^{14}C]hydroxy-4-cholesten-3-one, 0.5-1.5 nmol of cytochrome P-450, 3.0 unit of NADPH-cytochrome P-450 reductase, 30

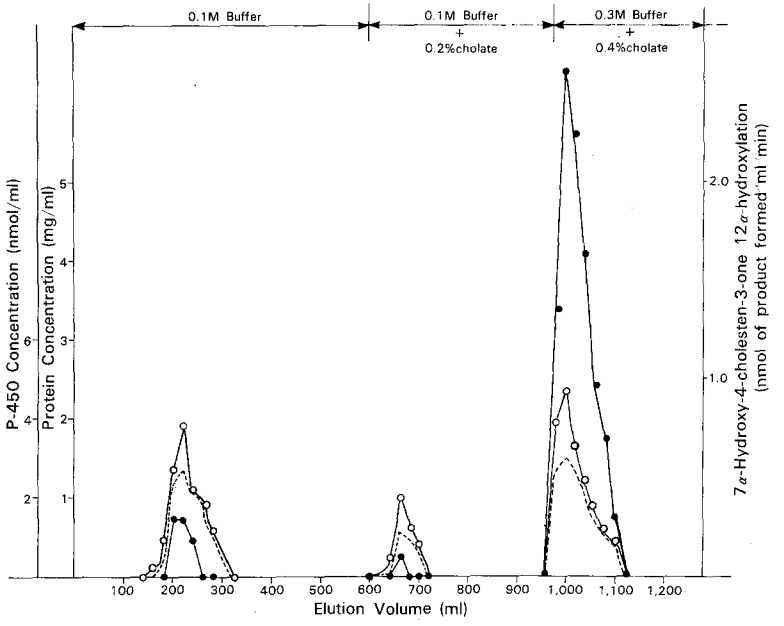

Fig. 1. Affinity chromatogram of rabbit liver microsomal cytochrome P-450 precipitated by 10 to 12% polyethylene glycol. Assays were performed as described in the text.
Column: cholate-Sepharose 4B (2.2 x 27 cm)
·····: protein concentration, o——o : cytochrome P-450 concentration,
●——● : 7α-hydroxy-4-cholesten-3-one 12α-monooxygenase activity

μg of dilauroylglyceryl-3-phosphorylcholine, 500 nmol of NADPH and 150 μmol of potassium phosphate buffer, pH 7.0, in a total volume of 1.5 ml. Incubations were conducted at 30° for 30 min and were terminated by adding 0.2 ml of dilute HCl and the mixture was extracted with 5 ml of ethyl acetate. The extract was washed twice with water and the solvent was evaporated to concentrate the solution. The residue was subjected to thin layer chromatography with solvent system: benzene:ethyl acetate, 3:7 (v/v) on a high performance thin layer plate (Merck Co., West Germany). The distribution of radioactivity was measured by a thin layer chromatogram scanner (Aloka Co., Japan) and the extent of conversion was calculated by estimating the ratio of the product area to the whole.

RESULTS

Purification of Cytochrome P-450 of Steroid 12α-Monooxygenase: The steroid 12α-monooxygenase in the solubilized extract of rabbit liver microsomes was coprecipitated with cytochrome P-450 by 8 to 15% polyethylene glycol (Table I), and it was eluted from the affinity column in the fraction eluted with 0.3 M buffer containing 0.4% cholic acid together with cytochrome P-450(s) (Fig. 1). Table II summarizes the extent of purification and the yield of the enzyme pre-

Table I

Steroid 12α-Monooxygenase Activities in the Fractions Precipitated by Varying Concentrations of Polyethylene Glycol

Fraction	Protein	Cytochrome P-450	Specific content[a]	7α-Hydroxy-4-cholesten-3-one 12α-monooxygenase			Yield
				Total activity[b]	Specific activity[c]	Turnover number	
	(mg)	(nmol)					
Microsomes	5400	10039	1.86	1104	0.20	0.11	100
Concentration of polyethylene glycol							
0 - 6	392	115	0.29	5	0.01	0.04	0.5
6 - 8	170	164	0.96	16	0.10	0.10	1.4
8 - 10	900	2834	3.15	340	0.38	0.12	31
10 - 12	505	1597	3.16	383	0.76	0.24	35
12 - 15	743	1987	2.64	258	0.34	0.13	23

[a] nmol of cytochrome P-450/mg of protein; [b] nmol of product formed/min; [c] nmol of product formed/min/mg of protein; [d] nmol of product formed/min/nmol of cytochrome P-450

Table II

Purification of Cytochrome P-450 of Steroid 12α-Monooxygenase

Fraction	Cytochrome P-450		Total protein	Specific content[a]	7α-Hydroxy-4-cholesten-3-one 12α-monooxygenase		
	Amount	Yield			Total activity[b]	Specific activity[c]	Turnover number[d]
	(nmol)	(%)	(mg)				
Microsomes	10039	100	5400	1.86	1104	0.20	0.11
Polyethylene glycol 10-12%	1597	15.9	505	3.16	383	0.76	0.24
Cholate-Sepharose 4B (non-bound fraction)	457	4.5	180	2.54	55	0.30	0.12
Cholate Sepharose 4B (bound fraction)	631	6.3	156	4.04	360	2.30	0.57

[a] nmol of cytochrome P-450/mg of protein; [b] nmol of product formed/min; [c] nmol of product formed/min/mg of protein; [d] nmol of product formed/nmol of cytochrome P-450/min

parations. The specific content of the final preparation was about 4 nmol/mg of protein, corresponding to 2.2 fold purification from microsomes and the specific activity of the preparation was about 2.3 nmol of product/min/mg of protein, which corresponded to about 11.5 fold purification on the basis of specific activity. An important trick for the purification of the enzyme was to prevent the enzyme from keeping in lower buffer concentration. Thus in the present experiment we have tried to keep the enzyme solution in at least more than 300 mM of buffer concentration. When lower ionic strength was necessary as in the case of affinity chromatography, the enzyme solution was diluted to 100 mM buffer concentration immediately before chromatography, but the eluate was brought back to 300 mM of buffer concentration as soon as possible after chromatography, otherwise the enzyme activity was rapidly deteriorated as described in the following section. Another important point in the purification of the enzyme was concerning the use of non-ionic detergent. Since non-ionic detergent was inhibitory to the enzyme activity it was necessary to remove it in the final preparation, but this procedure might in turn be an another cause of enzyme deterioration. It was therefore desirable to minimize the chances to use non-ionic detergent in the process of purification.

Stability of Enzyme: To study the stability of the cytochrome P-450 of the enzyme, aliquots of 6 to 12% polyethylene glycol fraction of cytochrome P-450s of rabbit liver microsomes were stored at 4° under varying concentrations of phosphate buffer. As shown in Fig. 2 the enzyme activity was rapidly reduced in the fraction containing 50 mM buffer, while the amount of cytochrome P-450s, measured by CO difference spectrum of the reduced form, was not reduced significantly. Contrarily, the enzyme activity in the fraction containing 300 mM buffer was not lost significantly for at least 4 weeks.

Reconstitution of Steroid 12α-Monooxygenase: As shown in Table III the enzyme activity was reconstituted from the partially purified cytochrome P-450 by the addition of the highly purified NADPH cytochrome P-450 re-

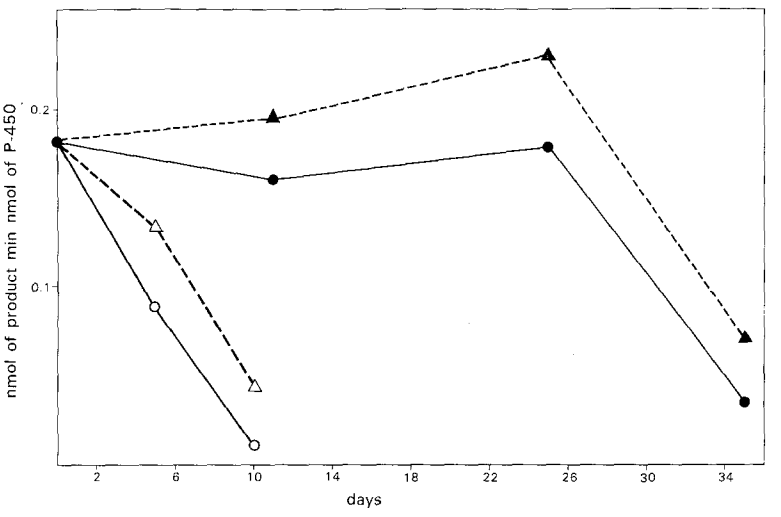

Fig. 2. Stability of the cytochrome of 7α-hydroxy-4-cholesten-3-one 12α-monooxygenase under varying buffer concentrations. A fraction precipitated by 6 to 12% polyethylene glycol was stored in the cold under varying buffer concentrations and the enzyme activity was checked after varying periods.
o—o : 50 mM phosphate buffer containing 20% glycerol
Δ···Δ : 50 mM phosphate buffer containing 20% glycerol and 0.5% cholate
●—● : 0.3 M phosphate buffer containing 20% glycerol
▲···▲ : 0.3 M phosphate buffer containing 20% glycerol and 0.5% cholate

ductase, dilauroylglyceryl-3-phosphorylcholine and NADPH. The omission of either cytochrome P-450 or NADPH-cytochrome P-450 reductase resulted in complete loss of activity, whereas the omission of phospholipid caused 23% loss of activity.

Table III

Reconstitution of 7α-Hydroxy-4-cholesten-3-one 12α-Monooxygenase

System	nmol of product formed/nmol of cytochrome P-450/min
Complete[a]	0.86
Minus cytochrome P-450	0
Minus reductase	0
Minus phospholipid	0.66
Minus NADPH	0

[a] Experimental conditions are described in the text

DISCUSSION

The steroid 12α-monooxygenase was precipitated together with cytochrome P-450(s) which were precipitated by 8 to 15% polyethylene glycol and it was eluted from the affinity column with 0.3 M phosphate buffer containing 0.4% cholate together with cytochrome P-450(s). Furthermore, Table II clearly shows that the specific activity of the steroid 12α-monooxygenase was increased at each step of purification of cytochrome P-450 from microsomes, and the final preparation showed about 5 fold increase of turnover number from that in microsomes. These results seem to suggest that cytochrome P-450(s) are involved in the reaction despite its insensibility to CO. Further purification of the enzyme was, however, hampered by its instability in lower buffer concentration. Attempts to purify the enzyme by ion exchange or hydroxyl apatite column chromatographies were difficult because the enzyme kept in higher buffer concentrations could not be adsorbed by those columns.

Although Hansson and Wickvall (2) have shown that cytochrome P-450 LM_4 of rabbit liver microsomes is the cytochrome involved in the steroid 12α-hydroxylation, our results are not compatible with theirs because the steroid 12α-monooxygenase was not induced by methylcholanthrene (data not shown), furthermore cytochrome P-450 LM_4 purified according to the method described by Coon et al. (10) did not show the 12α-monooxygenase activity when reconstituted as described in the experimental section.[1]

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[1] Unpublished data obtained by K. Okuda and M.J. Coon when the former was staying at Department of Biological Chemistry, the University of Michigan Medical School, Ann Arbor, Michigan, as a visiting Professor in 1979.

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