PREPARATION OF HOMOGENEOUS NADPH-CYTOCHROME P-450 REDUCTASE FROM RAT LIVER*

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SUMMARY: NADPH-cytochrome P-450 reductase with capacity to support cytochrome P-450-dependent drug metabolism and to reduce artificial electron acceptors has been purified to apparent homogeneity by solubilization with Renex 690 and chromatography on DEAE-Sephadex, Agarose and QAE-Sephadex. The purified protein migrates as a single band on native and SDS-polyacrylamide gel electrophoresis, exhibits a minimum molecular weight of 80,000 daltons and contains 1 molecule each of FAD and FMN per 80,000 molecular weight. The specific activity for cytochrome c as electron acceptor is 48.8 μmoles per min and for substrate hydroxylation of benzphetamine measured as NADPH oxidation in the presence of cytochrome P-450 and phosphatidylcholine is 2.5 μmoles per min.

The hydroxylation of many compounds in the mammalian liver including drugs, alkanes, fatty acids, steroids and polycyclic hydrocarbons is known to be catalyzed by a microsomal multienzymic system recently shown to consist of a flavoprotein, NADPH-cytochrome P-450 reductase, a cytochrome P-450, and a heat-stable lipid phosphatidylcholine (1-6). The latter, phosphatidylcholine, was shown to be required for the anaerobic reduction of cytochrome P-450 by pyridine nucleotide in the presence of the reductase (7). Two cytochromes of the P-450 type have been purified from liver microsomes. A cytochrome P-450 of apparent homogeneity was isolated from phenobarbital-induced rabbits by van der Hoeven, Haugen and Coon (8) and a highly purified cytochrome P-448, active on polycyclic hydrocarbons such as benzpyrene, by Lu and co-workers (9). Early efforts to prepare a solubilized form of the reductase using proteolytic or lipolytic agents (10, 11) yielded a protein capable of reducing a number of electron acceptors such as cytochrome c but unable to support cytochrome P-450-dependent substrate hydroxylation (2). Whereas, more recent

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procedures using a variety of ionic or nonionic detergents have yielded reductases of varying capacity in support of hydroxylation and of the reduction of artificial acceptors (1, 2, 6, 9, 12-14). This communication describes the purification to apparent homogeneity and characterization of NADPH-cytochrome P-450 reductase.

Materials and Methods: DEAE-Sephadex A-25 and QAE-Sephadex A-25 were obtained from Pharmacia and hydroxylapatite (Bio-Gel HTP) and agarose (Bio-Gel A-0.5m) were purchased from Bio-Rad Laboratories. Renex 690 (polyoxyethylene-alkylaryl ether), was obtained from Emulsion Engineering, Inc. Horse heart cytochrome c, NADPH, hydrocortisone-21-sodium succinate, were purchased from the Sigma Chemical Company and sodium deoxycholate from Schwarz-Mann. Dichlorophenolindophenol was obtained as a sodium salt from Calbiochem, ethylmorphine and phenobarbital from Merck, and ephedrine and cyclohexane from Aldrich Chemical Company. Benzphetamine was the gift of Dr. J. W. Hinman of the Upjohn Company and dilauroyl phosphatidylcholine was a gift from Dr. T. van der Hoeven and Dr. M. J. Coon. Cytochrome P-450 was prepared by the method of Lu, et al. (5). FAD and FMN content were determined by the method of Bessey et al. (15). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out using the method of Weber and Osborn (16). Cytochrome c reduction was measured at 550 nm using an extinction coefficient of 21,000 M^{-1} cm^{-1} (10); ferricyanide reduction at 420 nm, using an extinction coefficient of 1020 M^{-1} cm^{-1} (17); dichlorophenolindophenol reduction at 600 nm, using an extinction coefficient of 21,000 $M^{-1}cm^{-1}$ (18). Microsomes were prepared from the livers of rats treated for each of three days with a single injection of phenobarbital (75 mg per kg body weight) and for two days immediately prior to sacrifice with an injection of phenobarbital (75 mg/kg) and hydrocortisone (50 mg/kg) every eight hours. The microsomal pellet was resuspended in 0.25M sucrose and stored at -20°C.

Preparation of Reductase: The purification procedure for the reductase is summarized in Table I. All steps were carried out at 4°C. One gram of

TABLE I

Purification of NADPH-cytochrome P-450 reductase

Preparation	Protein (mg)	Total Activity	Specific Activity	Yield
Microsomes	1088	100	0.092	100
Solubilized and treated with protamine sulfate	1000	100	0.1	100
DEAE-Sephadex A-25 column eluate; hydroxylapatite column eluate	16.8	76.1	4.55	76
Agarose A-0.5m	3.82	59.5	15.5	60
QAE-Sephadex A-25 column eluate; hydroxylapatite column eluate	0.533	26	48.8	26

Total activity is measured as described in Table II and is reported as μmoles of cytochrome c reduced per minute; specific activity is reported as μmoles of cytochrome c reduced per minute per mg reductase protein.

microsomes was suspended and stirred for 10 min in 100 ml final volume of 0.1 M Tris-chloride buffer pH 7.7, containing 30% glycerol, 0.1 mM dithiothreitol and 1.0 mM EDTA. The solution was made 1% by volume in Renex 690 and stirred for 20 min. Three ml of 1.5% protamine sulfate were added dropwise and after stirring again for 20 minutes the solution was centrifuged at 100,000 xg for 60 minutes. The supernatant fraction was loaded onto a 450 ml bed volume DEAE-Sephadex A-25 column previously equilibrated with 0.1 M Tris buffer pH 7.7 and 0.12% (v/v) Renex 690. The equilibration buffer and all buffers used throughout the remainder of this procedure also contained 20% glycerol, 0.1 mM dithiothreitol and 1.0 mM EDTA. The column was washed with 400 ml of equilibration buffer. The column flow-through and wash contained almost all of the initial content of cytochrome P-450, cytochrome P-420, cytochrome b_5 and NADH-cytochrome b_5 reductase and negligible amounts of NADPH-

TABLE II

Electron acceptor reduction and cytochrome P-450-dependent substrate hydroxylation

The reaction mixtures for acceptor reduction (Exp. 1) contained, in a final volume of 1.0 ml, 300 µmoles potassium phosphate buffer pH 7.7, 0.1 µmoles NADPH, reductase (0.2 µg) and the acceptor: cytochrome c, dichlorophenolindophenol, or ferricyanide. The rates of reaction were measured at 30°C. Substrate hydroxylation (Exp. 2) was measured spectrophotometrically at 30° by NADPH oxidation in 1.0 ml reaction mixtures containing 100 µmoles of potassium phosphate buffer pH 7.7, 0.1 µmoles NADPH, cytochrome P-450 (0.75 nmoles, 0.26 mg), 30 µg phosphatidylcholine, reductase (0.10 unit, 0.002 mg) and substrate.

Expt. No.	Acceptor or Substrate	Final Concentration M	Activity µmoles/min/mg reductase
1	Cytochrome c	4.0×10^{-5}	48.8
	Dichlorophenolindophenol	1.0×10^{-4}	35.3
	Ferricyanide	1.0×10^{-3}	47.3
2	Benzphetamine	1.0×10^{-3}	2.5
	Ephedrine	1.0×10^{-3}	1.4
	Ethylmorphine	1.0×10^{-2}	0.86
	Cyclohexane	1.0×10^{-3}	1.09

cytochrome c reductase. The NADPH-cytochrome P-450 reductase activity was eluted from the column in a sharp peak at 0.1 M KCl when a linear 0 to 0.4 M KCl gradient in 0.1 M Tris buffer pH 7.7 and 0.12% Renex 690 was applied to the column. The peak reductase fractions were pooled and concentrated by loading onto a 20 ml hydroxylapatite column washed with 0.1 M Tris buffer containing 0.12% Renex 690 and eluting with 0.3 M potassium phosphate buffer pH 7.7 and 0.12% Renex 690. The reductase containing eluate was loaded onto a 600 ml Agarose A-0.5m column which was equilibrated and eluted with 0.1 M Tris buffer pH 7.7 containing 0.12% Renex 690 and 0.15 M KCl. The peak reductase fractions which eluted at the void volume were diluted with three volumes of 0.1 M Tris buffer pH 7.7 containing 0.12% Renex 690 and loaded onto a 200 ml QAE-Sephadex A-25 column equilibrated with the dilution buffer. The reductase was eluted with a 0 to 0.3 M KCl gradient in the same buffer. The peak fractions were pooled and concentrated with a 5 ml hydroxylapatite column prepared as previously described. The loaded column was washed with

100 ml of 0.1 M Tris buffer pH 7.7 containing 0.1% deoxycholate. The reductase was eluted with 0.3 M potassium phosphate buffer pH 7.7 containing 0.1% deoxycholate. The purified reductase is stable in deoxycholate containing buffer at -70°C for at least several months.

Properties of Reductase: Solubilization with Renex and treatment with protamine sulfate under the conditions described does not cause any significant loss in the total amount of reductase present initially as shown in Table I. Although the protamine sulfate cut does not produce a significant improvement in specific activity it enables elution of the reductase from the succeeding DEAE-Sephadex column as a sharp peak. The total yield for the purification procedure ranges from 15 to 33% of the initial amount. As shown in Table II this purified reductase retained its ability to transfer electrons to the artificial electron acceptors dichlorophenolindophenol, ferricyanide and cytochrome c as well as cytochrome P-450 as determined by its capacity to support the hydroxylation of a variety of substrates in a reconstituted system containing phosphatidylcholine and a partially purified cytochrome P-450 fraction which had a specific content of 2.9 nmoles/mg and contained no cytochrome c reductase activity.

The purified reductase showed a single protein band when submitted to sodium dodecyl sulfate disc gel electrophoresis. By including standards of known molecular weight (β-galactosidase, bovine serum albumin, ovalbumin and cytochrome c) with the reductase in SDS gel experiments not shown the minimum molecular weight of the reductase was estimated as 80,000 daltons. Furthermore, the purified reductase exhibited a single protein band on native polyacrylamide gels (19) containing 0.05% Renex 690 stained with Coomassie blue which was coincident with diaphorase activity (20).

The spectrum of the oxidized reductase, shown in Fig. 2, is that of a typical flavoprotein with absorbance maxima at 383 and 456 nm and a shoulder at 480 nm. The absence of any shoulder at 418 nm is consistent with our findings of no cytochrome P-450, cytochrome P-420 or cytochrome b_5 in the purified

Fig. 1. SDS disc gel electrophoresis of purified NADPH-cytochrome P-450 reductase. The protein load on this 7.0% acrylamide gel was 30 μg and migration was from top to bottom. The dye front is marked with a wire insert in the gel. The R_f for reductase protein under these conditions is 0.26. Densitometric traces indicate that all the protein in the gel is present in this band.

reductase. Fluorimetric analysis of the flavin content revealed the presence of 0.98 nmoles of FAD and 0.94 nmoles of FMN per 80,000 ng of protein. The molecular weight and flavin content determinations presented in this paper are in good agreement with determinations by others using less purified preparations of the reductase (12, 13, 21).

Our detergent-solubilized preparation of NADPH-cytochrome P-450 reductase, adjudged homogeneous by native and SDS-gel electrophoresis (Fig. 1),

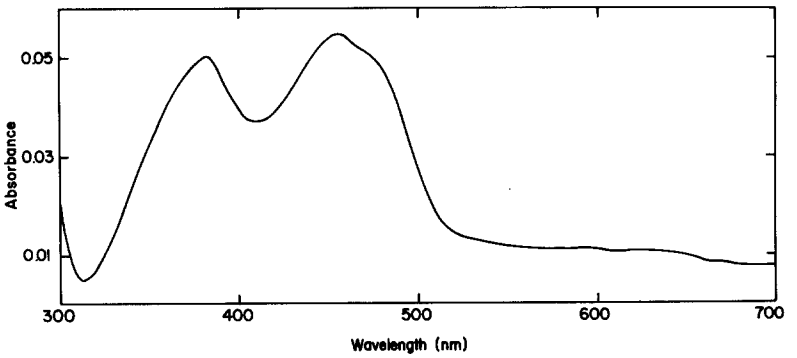

Fig. 2. Spectrum of purified NADPH-cytochrome P-450 reductase. The sample for the oxidized spectrum contained 0.205 mg of protein.

spectral characteristics, and flavin content, retains the ability to support cytochrome P-450-dependent substrate hydroxylation in contrast to protease-(11, 22) or lipase-(10,23) solubilized preparations. Our preparation also retains the ability to reduce artificial electron acceptors. The difference in the abilities of the detergent-and protease-or lipase-solubilized reductases may be correlated in some fashion with loss of a portion of the reductase molecule as suggested by the minimal molecular weight differences: 80,000 daltons reported in this paper and 79,000 daltons reported by Vermillion and Coon (13) for detergent solubilized preparations, 69,000 daltons for the lipase-solubilized preparation (10) and 71,000 daltons for a protease-solubilized preparation (22).

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