HIGHLY PURIFIED DETERGENT-SOLUBILIZED NADPH-CYTOCHROME P-450 REDUCTASE FROM PHENOBARBITAL-INDUCED RAT LIVER MICROSOMES*

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Received August 19,1974

Summary: NADPH-cytochrome P-450 reductase was highly purified from liver microsomes of phenobarbital-induced rats by column chromatography on DEAE-cellulose, DEAE-Sephadex A-50, and hydroxylapatite in the presence of deoxycholate or Renex 690, a nonionic detergent. The purified enzyme gave a single major band with a molecular weight of 79,000 daltons on SDS-polyacrylamide gel electrophoresis. FMN and FAD were present in about equal amounts. The most active reductase preparation catalyzed the reduction of 40.9 μmoles of cytochrome c per min per mg of protein and, as an indirect measure of cytochrome P-450 reduction, the oxidation of 2.0 μmoles of NADPH per min per mg of protein in a reconstituted hydroxylation system containing benzphetamine as the substrate.

The liver microsomal enzyme system which catalyzes the hydroxylation of fatty acids, steroids, and a variety of drugs and other foreign compounds was solubilized and resolved in this laboratory into three components: cytochrome P-450, NADPH-cytochrome P-450 reductase, and phosphatidylcholine (1,2). The cytochrome P-450 of phenobarbital-induced rabbit liver microsomes has recently been purified to apparent homogeneity (3,4). The detergent-solubilized reductase has been partially purified from liver microsomes of several species (3,5-10), and the reductase from rat liver microsomes has been shown to couple effectively with cytochrome P-450 from a variety of sources (cf. 3). Whereas the detergent-solubilized reductase retains the ability to function in a reconstituted hydroxylation system (5), it cannot be replaced for this purpose by steapsin-solubilized NADPH-cytochrome c reductase (11). The present communication describes the purification of rat liver microsomal NADPH-cytochrome P-450 reductase in the presence of detergents to a nearly homogeneous state and the catalytic activity and other properties of such a preparation.

Materials and Methods: Cytochrome P-450 from rabbit liver microsomes was purified to a concentration of 11.1 nmoles per mg of protein as described elsewhere (3). Calcium phosphate gel was prepared according to

* This research was supported by Grant AM-10339 from the United States Public Health Service and Grant GB-30419X from the National Science Foundation.
† Predoctoral Trainee, United States Public Health Service, Grant GM-00187.

Swingle and Tiselius (12), and hydroxylapatite (Bio-Gel HTP) was obtained from Bio-Rad Laboratories and DEAE-cellulose (medium mesh; 0.85 meq per g) from Sigma. d-Benzphetamine was kindly furnished by Dr. J. W. Hinman of the Upjohn Co. and Renex 690, a nonionic detergent, by ICI America, Inc. FMN and FAD were determined according to the method of Faeder and Siegel (13) and by a modification of the method of King et al. (14). NADPH-cytochrome c reductase activity was measured at 550 nm as reported by Masters et al. (15), except that 0.3 M phosphate buffer was used and the temperature was 30°; the specific activity is expressed as μmoles of cytochrome c reduced per min per mg of protein. NADPH-cytochrome P-450 reductase activity was estimated from the rate of NADPH disappearance at 340 nm at 30° in a reconstituted enzyme system containing benzphetamine, purified cytochrome P-450, and phosphatidylcholine, with the reductase as the rate-limiting component. The original assay conditions (16) were modified (3) to give optimal hydroxylation rates. Protein concentrations were determined according to Lowry et al. (17).

Table I

Purification of liver microsomal NADPH-cytochrome P-450 reductase

Preparation	Protein (mg)	Activity		Ratio of activities	Yield %
		Cyt. c reduction	Cyt. P-450 reduction		
Microsomes	6,600	0.30 (0.27-0.31)			100
DEAE-cellulose column eluate (0.05% deoxycholate); calcium phosphate eluate	330	1.88 (1.49-2.08)	0.11	17.1	31
DEAE-cellulose column eluate (0.4% Renex); calcium phosphate eluate	25	15.1 (15.1-18.9)	0.89	17.0	19
DEAE-Sephadex A-50 column eluate (0.1% Renex); calcium phosphate eluate	11	21.5 (20.0-26.2)	1.22	17.6	12
Hydroxylapatite column eluate (0.1% Renex); calcium phosphate eluate	5	33.2 (30.4-40.9)	2.06	16.1	8

The activity of the enzyme, expressed as μmoles per min per mg of protein in reducing cytochrome P-450 (determined by NADPH oxidation) and cytochrome c was determined as described in the text. The values in parentheses indicate the range of activities in a series of such purifications. The yields were calculated from cytochrome c reduction.

Purification of Reductase: The procedure developed for the purification of the reductase from liver microsomes is given in Table I. Male Sprague-Dawley rats were given phenobarbital and hydrocortisone by intraperitoneal injection (18), and the liver microsomal fraction was prepared and stored at -20° at a protein concentration of 25 to 30 mg per ml. The microsomes were solubilized as described previously (1) except that the suspension was sonicated (19) before the addition of deoxycholate and centrifugation was omitted. The procedures were carried out at 4°, and all buffer solutions were at pH 7.7 and contained 0.1 mM EDTA and 10% glycerol unless otherwise indicated.

The microsomal extract was diluted with deionized water to a protein concentration of 3.3 mg per ml, and 300 ml were applied to a DEAE-cellulose column (3.7 x 35 cm) previously equilibrated with 0.1 M Tris-chloride buffer containing 0.1 mM dithiothreitol, 0.05% deoxycholate, and no glycerol. The column was washed with 1 liter of a similar buffer solution containing 0.2 M KCl, and the reductase was then eluted with 1 liter of a similar buffer solution containing 0.35 M KCl and 2.5% glycerol. Calcium phosphate gel (1.35 g, dry weight, in 45 ml) was added to the eluate, and the mixture was stirred for 10 min and centrifuged for 10 min. The reductase was eluted from the pellet by stirring for 20 min with 30 ml of 0.3 M potassium phosphate buffer, and the solution was dialyzed against 20 volumes of 0.05 M Tris buffer. Since attempts to scale up this column step resulted in poor recovery, the preparations obtained were stored at -20° for up to one month and then pooled before the next step.

The enzyme solution was diluted with 0.05 M Tris buffer to a protein concentration of 1.1 mg per ml, and Renex 690 was added to a final concentration of 0.4% ($^{w}/_{v}$). The mixture was applied to a DEAE-cellulose column (2.2 x 16 cm) previously equilibrated with the same buffer-Renex solution. The column was washed with 250 ml of a similar buffer mixture containing 0.1 M KCl, and the protein was then eluted with 400 ml of a similar solution in which the salt concentration was increased to 0.5 M in a linear gradient. The active fractions, which were eluted at about 0.2 M KCl, were pooled, calcium phosphate gel (1.0 mg per 20 ΔA_{550} per min) was added, and the mixture was stirred and centrifuged. The pellet was suspended in one-half the original volume (of DEAE-cellulose column eluate) of 0.01 M phosphate buffer, stirred 10 min, and centrifuged. The reductase was then eluted by stirring the pellet for 20 min with about 25 ml of 0.1M phosphate buffer. The elution was repeated if necessary with a smaller volume of buffer.

The enzyme solution was diluted with 0.05 M Tris buffer to a protein

concentration of about 0.25 mg per ml, and Renex and KCl were added to final concentrations of 0.1% and 0.15 M, respectively. The mixture was applied to a DEAE-Sephadex A-50 column (2.2 x 10 cm) previously equilibrated with 0.05 M Tris buffer containing 0.1% Renex and 0.15 M KCl. The column was washed with 200 ml of a similar buffer-Renex solution containing 0.2 M KCl, and the reductase was eluted with a similar solution containing 0.3 M KCl. The enzyme preparation was concentrated and freed of most of the detergent by treatment with calcium phosphate gel as described in the previous step, except that 1 mg of gel was added per 50 ΔA_{550} per min.

The preparation was dialyzed for 3 hours against 20 volumes of 0.01 M phosphate buffer, pH 7.4, the protein concentration was adjusted to about 0.25 mg per ml, and Renex was added to a final concentration of 0.1%. The solution was applied to a column of hydroxylapatite (1.5 x 6.0 cm) previously equilibrated with 0.01 M phosphate buffer, pH 7.4, containing 0.1% Renex. The column was washed with 100 ml of the same buffer mixture, and the reductase was eluted with 200 ml of a similar buffer mixture in which the phosphate concentration was increased to 0.2 M in a linear gradient. The yellow fractions eluted at about 0.05 M phosphate were pooled and dialyzed for 3 hours against 20 volumes of 0.01 M phosphate buffer, pH 7.4, containing no detergent. Calcium phosphate gel (1 mg per 500 ΔA_{550} per min) was added, the mixture was stirred and centrifuged, and the light brown pellet was discarded. This procedure was repeated. The enzyme solution was then dialyzed for 3 hours against 0.02 M Tris buffer, and the protein was concentrated and freed of excess detergent by treatment with calcium phosphate gel as after DEAE-Sephadex A-50 column chromatography except that before elution the pellet was washed with 0.02 M Tris buffer. The resulting yellow reductase preparation contained 1 mg of protein per ml and was stable for several months in the frozen state.

Properties of Purified Reductase: The purified, detergent-solubilized reductase retains activity toward cytochrome P-450, as shown by the rate of substrate hydroxylation in a reconstituted enzyme system containing purified, detergent-solubilized cytochrome P-450, phosphatidycholine, and the reductase as the rate-limiting component. As shown in Table I, the ratio of activities toward cytochrome c and cytochrome P-450 does not vary significantly throughout the purification procedure. Our most purified preparation catalyzed, as an indirect measure of cytochrome P-450 reduction, the oxidation of 2.0 μmoles of NADPH per min per mg of protein in the reconstituted enzyme system. Other activities, expressed as μmoles of acceptor reduced per min per mg of protein, were as follows: cytochrome c, 40.9; ferricyanide,

Fig. 1. SDS-polyacrylamide gel electrophoresis of purified reductase (5 µg of protein; specific activity, 40.9). The enzyme was treated with SDS and mercaptoethanol and submitted to electrophoresis by a slight modification of the method of Laemmli (20) with a 7.5% separating gel. Migration was from top to bottom, and the position of the tracking dye was marked by a stainless steel wire. The gel was stained with Coomassie Blue R250.

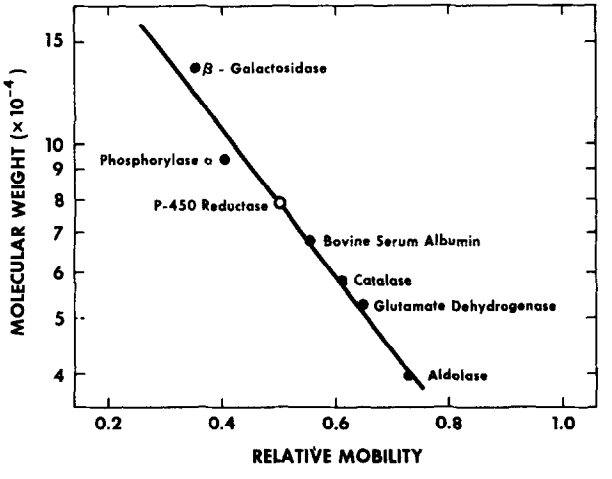

Fig. 2. Estimation of molecular weight of polypeptide chain of reductase by SDS-polyacrylamide gel electrophoresis in the presence of standard proteins. The procedure was that of Weber and Osborn (21) using a 5% gel.

42.9; and dichlorophenolindophenol, 30.7. No cytochrome P-450, cytochrome b_5, or NADH-cytochrome b_5 reductase (measured by its activity toward ferricyanide) could be detected in the purified enzyme.

As indicated in Fig. 1, SDS-polyacrylamide gel electrophoresis of the purified reductase revealed a single major band. In other experiments proteins of known molecular weight were included as standards, and the polypeptide chain of the reductase was estimated to have a molecular weight of about 79,000 daltons (Fig. 2). This value for the purified reductase is the same as found by Welton et al.(22) for NADPH-cytochrome c reductase obtained by immunoprecipitation of deoxycholate-solubilized rat liver microsomes.

FMN and FAD were determined fluorimetrically and found to be present in the amounts of 0.64 and 0.79 nmole, respectively, per 79,000 ng of protein in a purified reductase preparation having a specific activity of 33.4 toward cytochrome c. Since the preparation analyzed was about 75% pure, it appears that the enzyme may contain one molecule of each of these flavins per polypeptide chain. Both flavin nucleotides are also present in partially purified reductase preparations from rabbit liver microsomes, as shown by Iyanagi and Mason (7) and confirmed in this laboratory (3). The oxidized spectrum of the purified reductase from rat liver microsomes is given in Fig. 3. Absorption maxima occur at 456 and 385 nm, with a shoulder at about

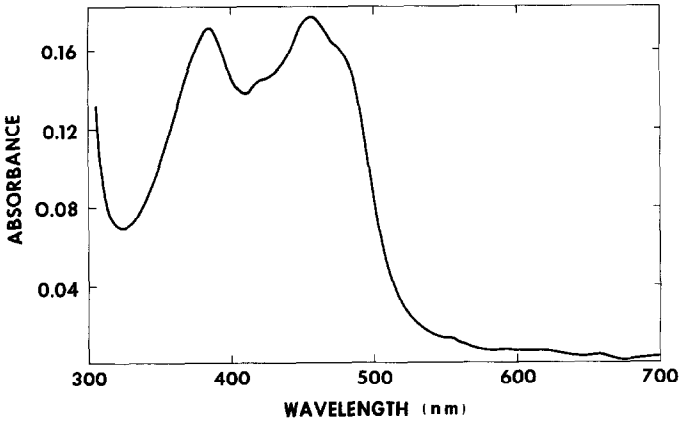

Fig. 3. Spectrum of reductase purified to a specific activity of 40.9. The enzyme was submitted to an additional column chromatographic step on DEAE-Sephadex A-50 in the presence of 0.1% Renex to remove most of the remaining heme. After application of the reductase the column was washed with 0.3 M potassium phosphate buffer, pH 7.7, containing 0.1% Renex, and the enzyme was then eluted and concentrated as already described in the purification procedure. This additional step did not alter the ratio of activities toward cytochrome c and cytochrome P-450.

479 nm. The small shoulder at about 418 nm is due to a trace of heme remaining in the preparation.

As reported elsewhere (23), NADPH-cytochrome c reductase preparations solubilized from liver microsomes with bromelain and purifed to homogeneity (24) or with steapsin and extensively purified (15) are inactive in the reconstituted hydroxylation system containing partially purified cytochrome P-450. Proteolytic solubilization apparently removes part of the polypeptide chain (6) or otherwise alters the ability of the reductase to couple with cytochrome P-450. Our studies show that upon detergent solubilization and extensive purification in the presence of detergents, the reductase retains its activity toward cytochrome P-450.

Acknowledgement: We are indebted to Joanne K. Heidema for skillful assistance in the purification of the reductase.

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