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NADPH–Cytochrome P-450 Reductase from Rat Liver: Purification by Affinity Chromatography and Characterization†

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ABSTRACT: (NADPH)-cytochrome P-450 reductase was purified to apparent homogeneity by a procedure utilizing nicotinamide adenine dinucleotide phosphate (NADP)-Sepharose affinity column chromatography. The purified flavoprotein has a molecular weight of 79 700 and catalyzes cytochrome P-450 dependent drug metabolism, as well as reduction of exogenous electron acceptors. Aerobic titration of cytochrome P-450 reductase with NADPH indicates that an air-stable reduced form of the enzyme is generated by the addition of 0.5 mol of NADPH per mole of flavin, as judged by spectral characteristics. Further addition of NADPH causes no other changes in the absorbance spectrum. A K_m value for NADPH of 5 μM was observed when either cytochrome P-450 or cytochrome *c* was employed as electron acceptor. A K_m value of 8 ± 2 μM was determined for cytochrome *c* and a K_m value of 0.09 ± 0.01 μM was estimated for cytochrome P-450.

The mixed-function oxidase system of liver endoplasmic reticulum, which catalyzes the hydroxylation of a variety of drugs, polycyclic hydrocarbons, alkanes, fatty acids, steroids, and other xenobiotics, is the subject of wide interest and has been reviewed by Brodie et al. (1958), Conney (1967), and Gillette et al. (1972). Omura et al. (1965) demonstrated the role of cytochrome P-450, the carbon monoxide binding pigment of hepatic microsomes (Omura and Sato, 1962), in hydroxylation of various substrates. Lu and Coon (1968) reconstituted the mixed function oxidase system from its resolved components (cytochrome P-450, cytochrome P-450 reductase and a heat-stable lipid factor) using laurate as a substrate, and later Lu et al. (1970) showed a number of other compounds, including drugs, alkanes, and fatty acids, were also hydroxylated by the reconstituted system. Subsequent to the reconstitution of activity from components, efforts were made to purify and characterize the individual components of the mixed-function oxidase system. The lipid component was identified as phosphatidylcholine (Strobel et al., 1970), and cytochrome P-450 has been purified to homogeneity (van der Hoeven et al., 1974; Ryan et al., 1975a). Characterization of the oxidation-reduction properties (Ballou et al., 1974; Guengerich et al., 1975) and the existence of multiple forms of cytochrome P-450 (Ryan et al., 1975b; Haugen et al., 1975) have also been reported. Early efforts to solubilize the reductase with proteolytic (Phillips and Langdon, 1962; Pederson et al., 1973) or lipolytic (Williams and Kamin, 1962) enzymes yielded a flavoprotein capable of reducing electron acceptors such as cytochrome *c* but unable to support cytochrome P-450 dependent substrate hydroxylation (Masters et al., 1975). This flavoprotein, NADPH–cytochrome *c* reductase (EC 1.6.2.4.), has been extensively studied and characterized (Masters et al., 1965a,b; Baggot and Langdon, 1970; Iyanagi et al., 1974; Masters et al., 1975).

Partially purified detergent-solubilized preparations of cytochrome P-450 reductase from rabbit and rat liver have been reported by van der Hoeven and Coon (1974), Satake et al. (1972), and Vermilion and Coon (1974). Recently, our laboratory reported the purification of cytochrome P-450 reductase to apparent homogeneity by solubilization of rat liver microsomes with the detergent Renex 690 and column chro-

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matography on DEAE[1]-Sephadex A-25, agarose 0.5m, and QAE-Sephadex A-25 (Dignam and Strobel, 1975). This detergent-solubilized preparation supports cytochrome P-450 dependent drug metabolism and also catalyzes the reduction of various artificial electron acceptors (e.g., cytochrome *c*). This preparation was shown to contain both FMN and FAD in agreement with the work of Iyanagi and Mason (1973) who showed that protease-solubilized cytochrome *c* reductase contained FMN and FAD in equimolar quantities.

This paper reports the purification by NADP-affinity chromatography of cytochrome P-450 reductase, which supports cytochrome P-450 drug metabolism, and describes some of the kinetic and catalytic characteristics of the purified enzyme. Yasukochi and Masters (1976) have independently reported on the purification of this enzyme using 2′,5′-ADP-Sepharose.

Experimental Procedure

Materials. Renex 690 (poly(oxyethylene(10)) nonylphenol ether) was purchased from ICI. Horse heart cytochrome *c*, FMN, FAD, hydrocortisone-21-sodium succinate, NADP, and NADPH were obtained from Sigma. Dichlorophenolindophenol and dithiothreitol were obtained from Calbiochem, potassium ferricyanide from J. T. Baker, and dilauroylphosphatidylcholine was purchased from Serdary Research Laboratories. Protamine sulfate was obtained from Nutritional Biochemical Corp. Benzphetamine hydrochloride was the gift of Dr. J. W. Hinman of the Upjohn Company. Sodium phenobarbital was obtained from Merck. DEAE-Sephadex and Sepharose 4B were purchased from Pharmacia. All other chemicals were reagent grade or better.

Analytical Methods. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Samples were prepared for protein determination by precipitation with 6% trichloroacetic acid, followed by an acetone wash of the precipitates. Polyacrylamide gel electrophoresis was performed under nondenaturing conditions at 25 °C in the presence of 0.1% Renex 690 according to the method of Davis (1964) and under denaturing conditions in the presence of sodium dodecyl sulfate and dithiothreitol according to the procedures of Weber and Osborn (1969) or Laemmli (1970). Protein bands were detected by staining with Coomassie Blue. Spectra were recorded on an Aminco Chance DW-2 scanning spectrophotometer. Kinetic determinations were made on a GILFORD 24]]-S recording spectrophotometer equipped with a multiple sample changer.

Enzyme Assays. Benzphetamine hydroxylation was determined at 30 °C by measuring the benzphetamine-dependent oxidation of NADPH spectrophotometrically at 340 nm, as described by Lu et al. (1969), with reductase as the limiting component. The concentration of NADPH stock solutions was determined from the absorptivity at 340 nm using an extinction coefficient of 6.2 cm^{-1} mM^{-1}. Cytochrome P-450 concentration was calculated from the $\Delta A_{450-490}$ in a reduced carbon monoxide difference spectrum using an extinction coefficient of 91 cm^{-1} mM^{-1} (Omura and Sato, 1964). NADPH-cytochrome *c* reductase activity was assayed by a modification of the method of Phillips and Langdon (1962) at 30 °C in 1.0-mL reaction mixtures containing 0.3 M potassium phosphate buffer, pH 7.7, by reduction of cytochrome *c* using an extinction coefficient of 21 cm^{-1} mM^{-1} at 550 nm (Williams and Kamin, 1962) and is reported as micromoles of cytochrome *c* reduced per minute per milligrams of protein. In similar reaction mixtures, the reduction of dichlorophenolindophenol was measured spectrophotometrically at 30 °C at 600 nm using an extinction coefficient of 21 cm^{-1} mM^{-1} (Steyn-Parve and Beinert, 1958). Ferricyanide reduction was determined spectrophotometrically at 30 °C in reaction mixtures similar to those used for cytochrome *c* reduction according to the method of Schellenberg and Hellerman (1958) using an extinction coefficient at 420 nm of 1.02 cm^{-1} mM^{-1}. The concentration of stock solutions of ferricyanide was determined spectrophotometrically at 420 nm. When highly purified preparations of cytochrome P-450 reductase were used in these assays, 200 μg/mL bovine serum albumin was added to protect the dilute reductase.

Preparation of Affinity Resin. The affinity column used in our procedure has an NADP ligand covalently attached to Sepharose 4B through an adipic acid dihydrazide spacer arm. Sepharose 4B was activated by cyanogen bromide according to the method of Cuatrecasas et al. (1968), and Cuatrecasas (1970). Addition of NADP ligand and adipic acid dihydrazide to Sepharose was accomplished with some modification of the procedure of Lamed et al. (1973). Two hundred milliliters of packed cyanogen bromide activated Sepharose 4B was added to 200 mL of saturated adipic acid dihydrazide (18 g) in 0.1 N $NaHCO_3$ (pH 9.5) at 4 °C. The mixture was stirred overnight in the cold and washed the next day with approximately 2 L of 2.0 M NaCl. The side arm resin was stored at 4 °C until used. NADP was prepared for attachment to the side-arm resin by oxidation for 3 h at 5 °C in a 20-mL reaction volume containing 0.1 M potassium phosphate buffer (pH 7.0), 0.01 M NADP, and 0.04 M sodium periodate. Five milliliters of glycerol was then added and the mixture was stirred overnight in the cold. Twenty milliliters of the oxidized nucleotide and 40 mL of packed adipic acid dihydrazide-Sepharose were combined with 25 mL of 0.4 M sodium acetate buffer (pH 5.0) in a final volume of 100 mL. The suspension was stirred in the cold for 4 h and then washed with 2 L of 2.0 M sodium chloride and 500 mL of deionized water. A typical preparation of the affinity resin contains about 4 μmol of NADP bound per mL of Sepharose-adipic acid dihydrazide (Lamed et al., 1973).

The structure of the affinity resin is shown in Figure 1. Evidence supporting this proposal as the best representation of the affinity resin is presented in the infrared, mass spectral, and NMR studies of Hansske et al. (1974) on the structure of the products of the reaction between periodate-oxidized AMP and carboxylic acid dihydrazides. Parikh et al. (1974) have also studied the structure of the linkage of side-arm dihydrazides to cyanogen bromide activated Sepharose.

Preparation of Microsomes. Male, Sprague-Dawley, rats, 75-100-g body weight (obtained from Flow Laboratories, Rockville, Md.), were injected with phenobarbital (75 mg/kg of body weight) and hydrocortisone (50 mg/kg of body weight) every 8 h for 2 days before sacrifice to induce cytochrome P-450 and cytochrome P-450 reductase. Livers were homogenized in a Waring Blendor in 1.14% KCl and 10 mM EDTA, to which phenylmethanesulfonyl fluoride was added to a final concentration of 0.25 mM immediately prior to cell disruption. The microsomal subfraction, prepared by centrifugation at 100 000*g*, was washed once in 10 mM EDTA and 1.14% KCl and stored in 0.25 M sucrose at −70 °C.

Preparation of Cytochrome P-450. Cytochrome P-450 used

[1] Abbreviations used are: DEAE, diethylaminoethyl; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced NADP; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; DTT, dithiothreitol; DOC, deoxycholate; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance.

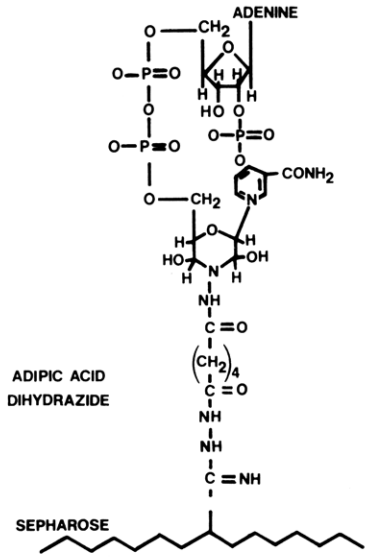

FIGURE 1: Proposed structure for NADP-Sepharose 4B affinity resin.

TABLE I: Purification of Cytochrome P-450 Reductase.

Step	Vol (mL)	Total Protein (mg)	Total Act.	Sp Act.[a]	Recovery (%)
(1) Solubilized Microsomes	700	5670	2667	0.47	100
(2) DEAE-Sephadex A-25	520	208	2650	12.73	99
(3) NADP-Sepharose	55	24.5	1700	69.4	64

[a] Micromoles of cytochrome *c* reduced per minute per milligram of protein at 30 °C in 0.3 M potassium phosphate buffer, pH 7.7.

in these experiments was prepared from rat liver microsomes using the procedure of Levin et al. (1974) through the DEAE-cellulose chromatography step (step IV). The specific content of these preparations was 8 nmol/mg of protein.

Purification of Cytochrome P-450 Reductase. Cytochrome P-450 reductase was solubilized from microsomes and chromatographed on DEAE-Sephadex A-25 by modification of methods previously developed in this laboratory (Dignam and Strobel, 1975). All procedures were carried out at 4 °C. Five to six grams of microsomal protein was suspended in 0.1 M Tris-HCl buffer (pH 7.7), containing 30% glycerol (v/v), 1.0 mM EDTA, and 0.1 mM dithiothreitol. The final volume of the suspension was 600 mL, and the protein concentration was approximately 10 mg/mL. A 10% (v/v) stock solution of Renex 690 was added slowly with stirring at 4 °C to a final concentration of 1.4% (v/v). To the clear solubilized microsomal suspension (700 mL), 12 mL of 1.5% (v/v) solution of protamine sulfate was added slowly with stirring. The turbid suspension was centrifuged at 100 000*g* for 1 h and the pellet was discarded. The supernatant solution was loaded at 150 mL/h onto a DEAE-Sephadex A-25 column (5 × 55 cm) previously washed with equilibration buffer (0.1 M Tris-HCl (pH 7.7), 1.0 mM EDTA, 0.1 mM dithiothreitol, 20% glycerol, and 0.15% (v/v) Renex 690). The loaded column was washed with 800 mL of equilibration buffer; the cytochrome P-450 reductase activity was eluted at 200 mL/h with a linear 3 L 0–0.4 M KCl gradient in equilibration buffer. The fractions containing reductase activity were identified by cytochrome

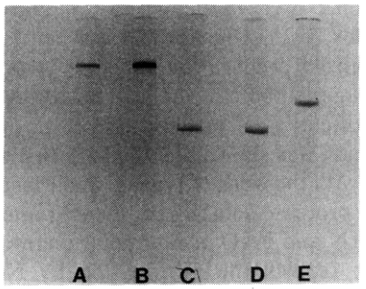

FIGURE 2: Polyacrylamide gel electrophoresis of cytochrome P-450 reductase. Samples were migrated from top to bottom. (A and B) Native gels run under nondenaturing conditions: (A) 7 μg; (B) 30 μg; (C and D) gels run under denaturing conditions in the presence of sodium dodecyl sulfate according to the procedure of Laemmli (1970), (C) 5 μg; (D) 15 μg; (E) gel run under denaturing conditions in the presence of sodium dodecyl sulfate according to the procedure of Weber and Osborn (1969); 10 μg.

c reduction, pooled, and loaded at 100 mL/h onto an NADP-Sepharose affinity column (2 × 6 cm) previously washed with equilibration buffer. About 20 to 25% of the activity did not bind to the affinity column. The loaded column was washed with 200 mL of 0.05 M Tris-HCl buffer (pH 7.7) containing 20% glycerol, 1.0 mM EDTA, 0.1 mM dithiothreitol, and 0.1% (w/v) deoxycholate. The activity was eluted with 0.5 mM NADP in 0.05 M Tris-HCl buffer (pH 7.7) containing 20% glycerol, 1.0 mM EDTA, 0.1 mM dithiothreitol, and 0.1% deoxycholate. The reductase was either frozen immediately or concentrated by ultrafiltration and frozen at −70 °C; $NADP^+$ was removed from the enzyme before use by dialysis or by chromatography on Sephadex G-25.

Results

Preparation and Purity of Cytochrome P-450 Reductase. Table I summarizes the purification of NADPH-cytochrome P-450 reductase by chromatography on an NADP-adipic acid dihydrazide-Sepharose 4B column. Solubilization of microsomes with Renex 690 and treatment with protamine sulfate caused no loss in activity, as judged by cytochrome *c* reduction (Dignam and Strobel, 1975). Chromatography of the solubilized protamine sulfate treated suspension on DEAE-Sephadex A-25 removes cytochromes b_5, P-450, and P-420 from the cytochrome P-450 reductase with little or no loss of the reductase activity. At this stage in the procedure, the cytochrome P-450 reductase preparation contains heme contaminants which can be observed spectrally but are not attributable to cytochrome b_5, P-450, or P-420. Chromatography of the pooled fractions from the DEAE-Sephadex step on the affinity resin removes these heme contaminants and gives a five- to sixfold purification of the reductase. Affinity chromatography in this procedure replaces both the agarose and QAE-Sephadex chromatography steps in our former procedure (Dignam and Strobel, 1975). The recovery from microsomes of enzyme prepared by this method varies from 30 to 65%. This yield is heavily dependent on the success of the DEAE-Sephadex column step. The specific activity of cytochrome P-450 reductase ranges from 62 to 71 $\mu mol\ min^{-1}\ mg^{-1}$ of reductase, as judged by reduction of cytochrome *c*.

Polyacrylamide disc gel electrophoresis of cytochrome P-450 reductase under nondenaturing and denaturing conditions is shown in Figure 2. Under nondenaturing conditions (gels A and B), the enzyme shows a single band. In experiments not shown, parallel gels stained for protein or activity show a single

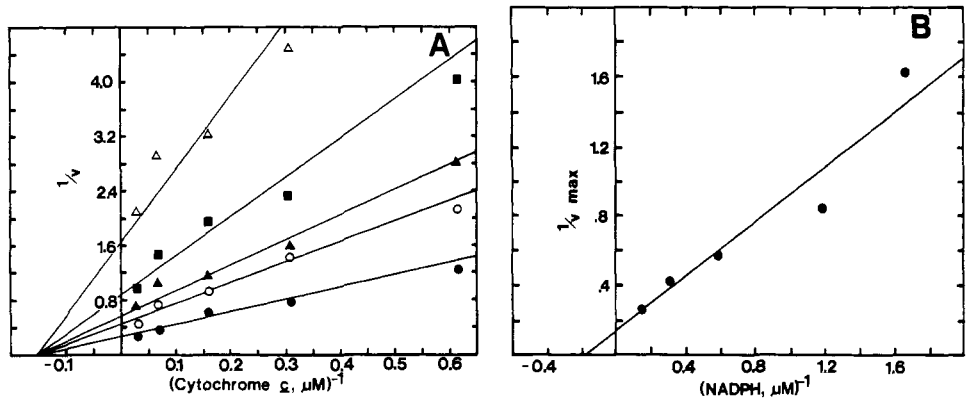

FIGURE 3: Double-reciprocal plots of initial velocity for the reduction of cytochrome *c*. Reactions were carried out in a 1.0-mL final volume containing 300 mM potassium phosphate (pH 7.7), varying amounts of cytochrome *c*, and 0.125 μmol of purified cytochrome P-450 reductase; velocity is expressed as μmol/min × 10^{-3}. Panel A: (●) 6.37 μM NADPH; (○) 3.19 μM NADPH; (▲) 1.71 μM NADPH, (■) 0.839 μM NADPH, (△) 0.613 μM NADPH. Panel B: secondary double-reciprocal plot of $1/v$ intercept from A vs. concentration of NADPH.

band with identical relative mobility, indicating that reductase activity and enzyme protein are coincident.

When gel electrophoresis is performed under denaturing conditions by the procedure of Weber and Osborn (1969) or by that of Laemmli (1970) in the presence of sodium dodecyl sulfate, cytochrome P-450 reductase migrates as a single band. Gels C and D show two concentrations of reductase in sodium dodecyl sulfate after electrophoresis by the procedure of Laemmli (1970), and gel E shows a single concentration of reductase after electrophoresis by the procedure of Weber and Osborn (1969). The reductase, prepared by affinity chromatography, appears homogeneous by the electrophoretic criteria cited.

Molecular Weight. Cytochrome P-450 reductase and six proteins of known molecular weight (β-galactosidase, phosphorylase a, bovine serum albumin, catalase, ovalbumin, and superoxide dismutase) were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate according to the procedure of Weber and Osborn (1969). Cytochrome P-450 reductase migrated with an R_f between that of phosphorylase a and bovine serum albumin and has an apparent molecular weight of 79 500. This approach to molecular-weight estimation was determined in other work to be valid for cytochrome P-450 reductase by the linearity of a Ferguson plot (Ferguson, 1964) of the reductase at various gel concentrations and the identity of the relative free mobility of the reductase with that of the marker proteins (Knapp et al., 1976). The minimum subunit molecular weight based on flavin content, determined by the procedure of Bessey et al. (1949), and on protein concentration determined according to Lowry et al. (1951), is 74 100. A minimum molecular weight of about 76 500, detemined in sedimentation equilibrium studies of the reductase in 6 M guanidine hydrochloride, is in agreement with these other estimations.

Activity of Cytochrome P-450 Reductase. Cytochrome P-450 reductase, prepared by affinity column chromatography, supported cytochrome P-450 dependent drug metabolism, as judged by benzphetamine hydroxylation in a reconstituted system containing the purified reductase, cytochrome P-450, phosphatidylcholine, and NADPH. The specific activity of the cytochrome P-450 reductase in the reconstituted system, measured spectrophotometrically by NADPH oxidation, was approximately 2.5 μmol of NADPH oxidized min^{-1} mg^{-1} of reductase. The rate is dependent on the presence of added lipid and cytochrome P-450. The cytochrome P-450 reductase preparation also supported the reduction of several artificial electron acceptors. Cytochrome *c*, dichlorophenolindophenol, and potassium ferricyanide are readily reduced by the reductase with K_m concentrations of 10, 10, and 11 μM, respectively. Thus, cytochrome P-450 reductase can catalyze cytochrome P-450 dependent drug hydroxylation, as well as the reduction of artificial electron acceptors (e.g., cytochrome *c*).

The effects of several possible inhibitors or cofactors on cytochrome P-450 reductase were also examined. A typical preparation of the reductase contained equal amounts of FMN and FAD (27.5 nmol of total flavin/mg of protein). Preincubation of the reductase with 10 μM FAD, FMN, or both flavins for 15 min at 0 °C did not affect the rate of cytochrome *c* reduction. Several possible inhibitors, SKF-525A (2-diethylaminoethyl 2,2-diphenylvalerate hydrochloride), sodium benzoate, and 8-hydroxyquinoline, had no effect on the rate of cytochrome *c* reduction catalyzed by cytochrome P-450 reductase.

Kinetic Properties of Cytochrome P-450 Reductase. Since the cytochrome P-450 reductase preparation will interact with both cytochrome P-450 as well as exogenous acceptors such as cytochrome *c*, an analysis of the kinetic properties of the reductase with the native acceptor, cytochrome P-450, and the exogenous acceptor, cytochrome *c*, was conducted. A kinetic analysis for the interaction of the cytochrome P-450 reductase with NADPH and cytochrome *c* is shown in Figure 3. When the concentration of cytochrome *c* was varied at fixed concentrations of NADPH, double-reciprocal plots of the rate of cytochrome *c* reduction vs. the concentration of cytochrome *c* at each concentration of NADPH (Figure 3A) extrapolated to a common point of intersection. These results are consistent with a sequential reaction mechanism for interaction of NADPH and cytochrome *c* with cytochrome P-450 reductase (Cleland, 1970). The apparent K_m value for cytochrome *c*, as determined from the point of intersection in Figure 3A, is 6.7 μM and is in good agreement with the K_m value for cytochrome *c* (10 μM) determined at saturating NADPH concentration. Kinetic analysis by other laboratories of the reaction between NADPH, cytochrome *c*, and cytochrome *c* reductase led to conflicting proposals for the reaction mechanism. Phillips and Langdon (1962) proposed a ternary complex of electron donor, electron acceptor, and enzyme formed by the random addition of substrates under equilibrium conditions. Masters et al. (1965a,b) observed parallel lines in double-reciprocal plots of NADPH concentration vs. activity at various concentrations of cytochrome *c* or dichlorophenolindophenol. They interpreted these data to be consistent with a mechanism in which one

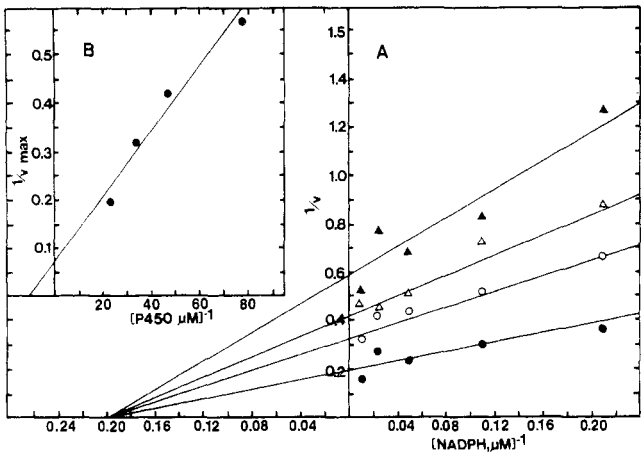

FIGURE 4: (A) Double-reciprocal plots for benzphetamine hydroxylation measured by NADPH oxidation. Velocity is expressed as nanomoles per minute. Each assay contained 100 μmol of potassium buffer (pH 7.7), 30 μg of dilauroylphosphatidylcholine, 15 μg of cytochrome P-450 reductase, and 1.0 μmol of benzphetamine in a final volume of 1.0 mL. Nanomoles of cytochrome P-450 per reaction mixture: (●) 0.0428 nmol; (○) 0.030 nmol; (△) 0.214 nmol; (▲) 0.0128 nmol. (B) Secondary double-reciprocal plot of the $1/v_{max}$ from A vs. the respective cytochrome P-450 concentrations.

substrate reacts with enzyme to form product and modified enzyme followed by reaction of the modified enzyme with the second substrate. Baggott and Landon (1970) replotted these data by the method of Woolf (1932) and observed no dependence of K_{NADPH} on cytochrome *c* concentration. The data presented here, obtained with detergent-solubilized reductase, exhibit intersecting lines in double-reciprocal plots, in conflict with the data of Masters et al. (1965a,b), which were obtained with a lipase-solubilized preparation. Yasukochi and Masters (1976) demonstrated that assay of the reductase under suboptimal conditions (i.e., low ionic strength) results in lower rates of reduction. Use of higher ionic strength (0.3 M phosphate vs. 0.05 M phosphate) in our standard assay mixtures might account for the disparity between our observations and those of Masters et al. (1965a,b) by making differences in the slopes of lines in double-reciprocal plots more apparent. A secondary double-reciprocal plot of the $1/v$ intercept of each line from Figure 3A vs. the fixed concentration of NADPH for each line is shown in Figure 3B. The points lie along a straight line and show that the apparent K_m for NADPH in this reaction is 5.0 μM.

The interaction of cytochrome P-450 reductase with its native acceptor, cytochrome P-450, and NADPH was determined in reconstituted benzphetamine hydroxylation reaction mixtures containing cytochrome P-450, dilauroylphosphatidylcholine, and cytochrome P-450 reductase. Benzphetamine hydroxylation was estimated spectrophotometrically as the rate of oxidation of NADPH or by formaldehyde formation (Cochin and Axelrod, 1959). As shown in Figure 4A, double-reciprocal plots of the effect of varying the concentration of NADPH at fixed concentrations of cytochrome P-450 on the rate of benzphetamine-dependent NADPH oxidation extrapolate to a common intersection. The apparent K_m value for NADPH is 5 μM. A secondary double-reciprocal plot of the $1/v$ intercepts vs. the concentrations of cytochrome P-450 used in Figure 4A is shown in Figure 4B. The apparent K_m value for cytochrome P-450 in this reaction is 0.1 M. An apparent K_m value for cytochrome P-450 was also determined directly at saturating concentrations of NADPH by measuring the amount of formaldehyde formation at various concentrations of cytochrome P-450 in a reconstituted system. The apparent K_m value for cytochrome P-450 determined in this fashion was 0.08 μM. This value is in good agreement with the apparent K_m of cytochrome P-450 determined from the data presented in Figure 4.

Aerobic Titration of Cytochrome P-450 Reductase with NADPH. The kinetic data presented in Figures 3 and 4 indicate that under our assay conditions the K_m value for NADPH is 5 μM, whether cytochrome P-450 or cytochrome *c* is used as the electron acceptor, and raise the question of the mechanism of reduction of the enzyme by NADPH. This question is of particular interest in view of the recent report that cytochrome P-450, the physiological electron acceptor for cytochrome P-450 reductase, requires 2 reducing equivalents for complete reduction (Ballou et al., 1974). The aerobic reduction by NADPH of cytochrome *c* reductase, the protease- or lipase-solubilized microsomal flavoprotein, has been studied extensively by Masters et al. (1965a,b), Iyanagi and Mason (1973), Iyanagi et al. (1974), and Masters et al. (1975). On the basis of spectroscopic back-titration of the air-stable partially reduced form with ferricyanide and EPR studies, Iyanagi and Mason (1973) and Iyanagi et al. (1974) concluded that the air-stable form contained 1 reducing equivalent per two flavins more than the fully oxidized form. However, Masters et al. (1975) maintain that generation of the air-stable reduced form requires 1 reducing equivalent per flavin. Although these two groups prepared cytochrome *c* reductase (inactive in cytochrome P-450-dependent reactions) by different procedures, the manner of solubilization in both procedures involved the use of proteolytic enzymes and the final product had a molecular weight of about 68 000. Nonetheless, results of the two groups are clearly different. Since our preparation of cytochrome P-450 reductase is active in the reduction of cytochrome *c* and cytochrome P-450, we have examined the ability of small increments of NADPH to reduce cytochrome P-450 reductase in the presence of air and the absence of any other electron acceptor. The flavin concentration of the cytochrome P-450 reductase preparations used in these experiments was determined spectrophotometrically using $\epsilon_{454} = 10.7\ mM^{-1}\ cm^{-1}$ in agreement with Iyanagi and Mason (1973). These preparations contained 1 mol each of FMN and FAD per mole of enzyme, as determined by the procedure of Bessey et al. (1949) in agreement with the data of Iyanagi and Mason (1973). As shown in Figure 5, each sequential addition of NADPH below a ratio of 0.5 mol of NADPH/mol of flavin causes a reduction in the absorbance of cytochrome P-450 reductase at 454 nm and a concomitant increase in absorbance at 585 nm. As seen in the insert in Figure 5, the equivalence point, as indicated by lack of any additional change in absorbance at either 454 or 585 nm when excess NADPH is added, occurs at a ratio of approximately 1 reducing equivalent/flavin. In data not shown, back-titration of the air-stable reduced form of cytochrome P-450 reductase was accomplished by the addition of approximately 1 mol of ferricyanide per mol of flavin. The requirement of 1 mol of ferricyanide per mol of flavin was the same whether the air-stable reduced form of the enzyme was generated by titrating the oxidized enzyme with discrete increments of NADPH or by adding excess NADPH and allowing the enzyme to oxidize to the air-stable state. This back-titration of the air-stable reduced form is consistent with the result of 1 reducing equivalent/flavin obtained by forward titration with NADPH.

Anaerobic Titration of Cytochrome P-450 Reductase. Aerobic addition of NADPH to cytochrome P-450 reductase beyond the equivalence point of 1 reducing equivalent per mol of flavin causes no further change in the absorbance values at

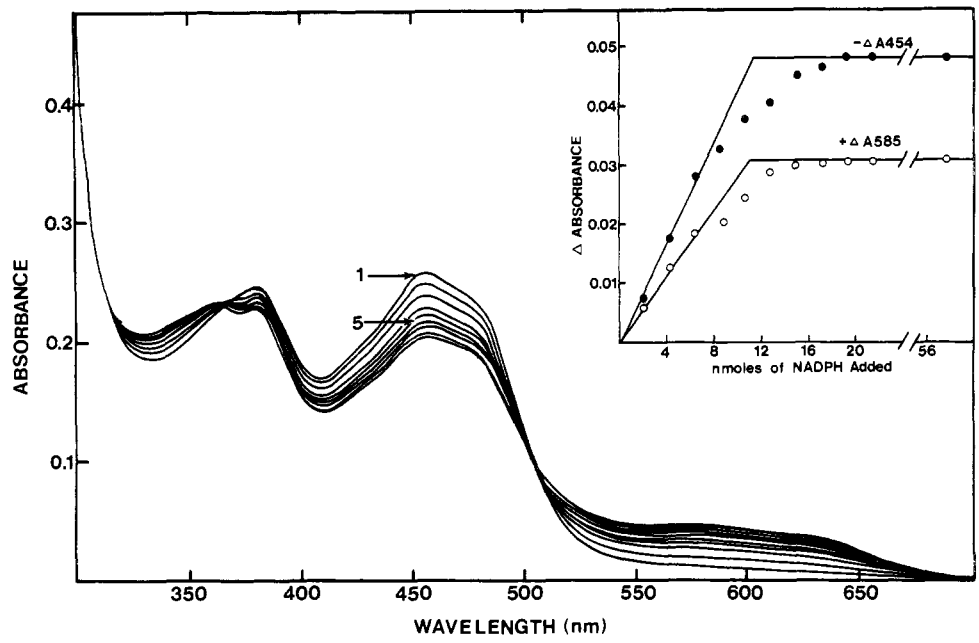

FIGURE 5: Aerobic titration of cytochrome P-450 reductase with NADPH. One milliliter of cytochrome P-450 reductase (23.9 μM in flavin) in 0.05 M Tris-HCl (pH 7.7), 20% glycerol, 1 mM EDTA, 0.1 mM DTT, and 0.1% deoxycholate was titrated by the addition of 3-μL aliquots of NADPH (0.718 mM). When no further spectral changes were observed after the addition of NADPH, excess NADPH was added and time was allowed for equilibration. Inset: the change in absorbance at 454 and 585 nm plotted against nmoles of NADPH added.

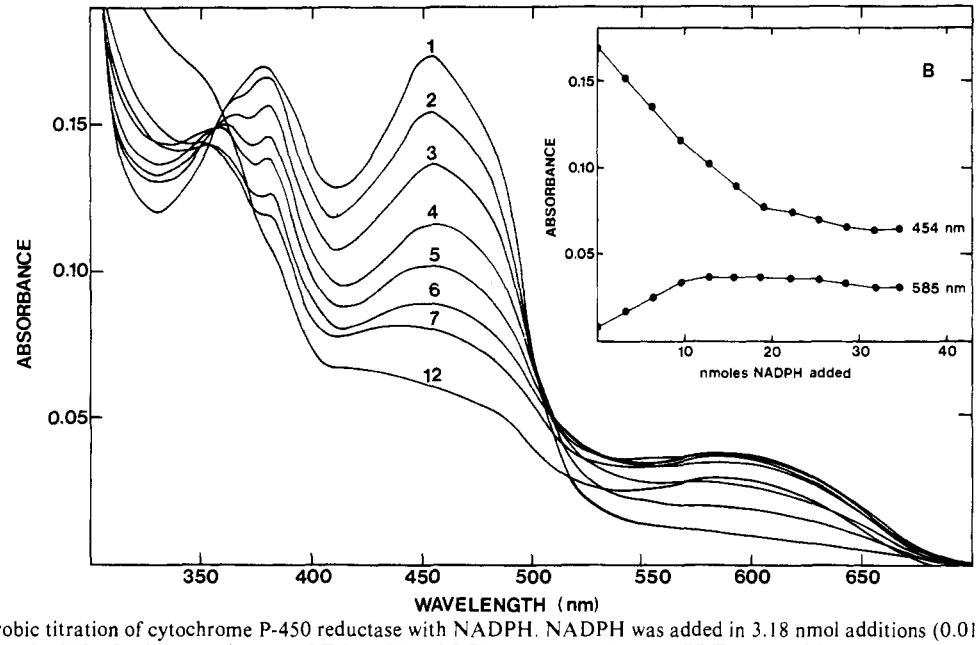

FIGURE 6: Anaerobic titration of cytochrome P-450 reductase with NADPH. NADPH was added in 3.18 nmol additions (0.0132 mL per addition) to an initial volume of 1.815 mL of enzyme in 0.05 M Tris-HCl (pH 7.7), 20% glycerol, 1 mM EDTA, 0.1 mM DTT, and 0.1% (w/v) DOC. Enzyme concentration was 14.33 μM (28.66 nmol of flavin). Final volume was 2.016 mL. Curves shown are not corrected for dilution. Inset: plot of absorbance at 454 and 585 nm against nanomoles of NADPH added.

454 or 585 nm, which indicates that further reduction of the flavoprotein does not occur under these conditions (Figure 5). Further titration of cytochrome P-450 reductase can only be achieved under anaerobic conditions. The effect of successive additions of NADPH to cytochrome P-450 reductase under anaerobic conditions is shown in Figure 6. Under anaerobic conditions, continued addition of NADPH beyond the level of 1 reducing equivalent per mol of flavin continues to affect the absorbance at both 454 and 585 nm, in contrast to the titration under aerobic conditions. Full reduction of the enzyme by NADPH under these conditions does not occur as indicated by the residual absorbance at 454 and 585 nm. NADPH is present in excess, as indicated by the rise in absorbance at 340 nm following addition 12. Iyanagi et al. (1974) have observed such an effect in their anaerobic titrations of reductase with NADPH and concluded that the oxidation-reduction potential of the lowest flavin couple is very near that of the NADPH/$NADP^+$ couple. Our data are consistent with this conclusion of Iyanagi et al. (1974).

As shown in the insert in Figure 6, no further change in the absorbance at 454 or 585 nm occurs after the addition of about 30 nmol of NADPH to the enzyme (28.66 nmol of flavin), or

in other words, at a ratio of approximately 2 reducing equivalents per mole of flavin. This observation is consistent with the anaerobic dithionite titration study of Iyanagi et al. (1974) and with the anaerobic NADPH titration study of Masters et al. (1975).

Discussion

Purification of membrane-bound enzymes has posed a major obstacle to definition at the molecular level of the functon and interaction of membrane proteins. The purification procedure for cytochrome P-450 reductase described in this paper can be used to provide highly purified enzyme in good yield. The affinity-column step in our procedure provides an important degree of specificity in purification of cytochrome P-450 reductase. This present procedure has a higher total recovery of enzyme at the end of the purification and requires only half the time to prepare the enzyme (2 days) than our previous procedure, which also yields apparently homogeneous enzyme (Dignam and Strobel, 1975). The availability of purified cytochrome P-450 reductase will facilitate precise characterization of the mechanism of electron transfer between the reductase and its native acceptor cytochrome P-450, as well as other acceptors present in the membrane environment.

The cytochrome P-450 reductase prepared by NADP-affinity chromatography has a molecular weight of about 79 500, whereas various preparations of cytochrome *c* reductase, which use proteolytic enzymes to solubilize the enzyme, have a molecular weight of about 68 000 (Iyanagi and Mason, 1973; Masters et al., 1975). This molecular weight difference is correlated with the inability of protease-solubilized enzyme to interact with cytochrome P-450. Thus, cytochrome *c* reductase cannot be analyzed for its activity in cytochrome P-450 dependent reactions. Aerobic titration of cytochrome P-450 reductase with NADPH, which shows the requirement of about 0.5 mol of NADPH for generation of the air-stable partially reduced form of the enzyme, is consistent with the cogent arguments of Masters et al. (1975) for generation of the air-stable reduced form of cytochrome *c* reductase and do not support the conclusions of Iyanagi and Mason (1973) and Iyanagi et al. (1974). On the other hand, anaerobic reduction of cytochrome P-450 reductase requires 4 reducing equivalents per mole of enzyme (approximately 1 mol of NADPH/mol of flavin) in agreement with the conclusions of Masters et al. (1975), Iyanagi and Mason (1973), and Iyanagi et al. (1974) for the requirement of 1 mol of NADPH/mol of flavin for reduction of protease-solubilized cytochrome *c* reductase. The similarity in NADPH titration of cytochrome P-450 reductase and cytochrome *c* reductase suggests that protease solubilization may not alter the NADPH binding site or flavin reduction mechanism, although this has not been directly demonstrated.

Determination of the K_m value for cytochrome *c* was conducted by direct spectrophotometric measurement of the rate of reduction of cytochrome *c*. The K_m value for cytochrome P-450 was determined in two assay procedures but both were indirect. Overall substrate-dependent hydroxylation was measured, rather than reduction of cytochrome P-450, which would be analogous to the direct determination for cytochrome *c* reduction. Thus, the kinetic constants determined represent the best approximation possible under the conditions, but must be regarded with some caution until they are confirmed by direct determination. Such direct determination is possible by measuring reduction of the cytochrome P-450–carbon monoxide complex by stopped-flow spectrometry (Coon et al., 1971).

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Mandelate Racemase from *Pseudomonas putida.* Absence of Detectable Intermolecular Proton Transfer Accompanying Racemization†

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ABSTRACT: An equimolar mixture of DL-[α-^{2}H]- and DL-[α-^{13}C]mandelate, when incubated with mandelate racemase (EC 5.1.2.2), shows conversion of singly labeled mandelate to unlabeled mandelate, due to solvent exchange of the α proton, while the level of doubly labeled mandelate remains at a constant low level. Similarly, an equimolar mixture of unlabeled and DL-[α-^{2}H,α-^{13}C]mandelate, when incubated with the enzyme, shows conversion of doubly labeled mandelate to singly labeled mandelate, due to solvent exchange, while the level of unlabeled mandelate remains constant at 50%. Incubation of an equimolar mixture of DL-[α-^{3}H]mandelate and DL-*p*-chloromandelate, both with similar properties as substrates for mandelate racemase, showed solvent exchange of the α-^{3}H of mandelate, but no ^{3}H appeared in the *p*-chloromandelate. These results indicate that mandelate racemase does not catalyze an intermolecular proton transfer to achieve racemization. These data are necessary, but not sufficient, results to indicate that mandelate racemase operates via a one-acceptor mechanism, in which the proton abstracted from one stereochemical face of a substrate molecule is returned to the opposite face of the same carbon of the substrate molecule.

Mandelate racemase (EC 5.1.2.2), an inducible enzyme isolated from *Pseudomonas putida* A.3.12 (ATCC 12633) grown at the expense of DL-mandelic acid or DL-*p*-hydroxymandelic acid (Gunsalus et al., 1953), has been shown to catalyze the racemization of mandelic acid without requiring an organic coenzyme (Hegeman et al., 1970). A divalent metal ion is required for activity, of which Mg^{2+} or Mn^{2+} (Fee et al., 1974) work best. Magnetic resonance studies (Maggio et al., 1975) have indicated that the metal ion assists in polarizing the carbon–hydrogen bond in order to promote abstraction of the α hydrogen as a proton. The enzyme has been shown to catalyze solvent exchange of the α proton concurrent with racemization and at a rate approximately 20% of the rate of racemization (Kenyon and Hegeman, 1970).

A primary deuterium isotope effect of approximately 5 has been observed (Kenyon and Hegeman, 1970) when the α proton is replaced with deuterium. Study of the reaction kinetics of a series of para-substituted mandelic acids as substrates for the enzyme has yielded results which suggest, when

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