Some Properties of a Detergent-solubilized
NADPH-Cytochrome c (Cytochrome P-450) Reductase Purified
by Biospecific Affinity Chromatography*

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NADPH-cytochrome c (cytochrome P-450) reductase (EC 1.6.2.4) has been purified to homogeneity, as
judged by sodium dodecyl sulfate disc gel electrophoresis, from detergent-solubilized rat and pig liver
microsomes using an affinity chromatography procedure. Treatment of microsomes with a polyethox-
ynylphenyl ether plus either cholate or deoxycholate and subsequent batch-wise DEAE-cellulose
chromatography followed by biospecific affinity chromatography on Sepharose 4B-bound N6-(6-aminohexyl)-adenosine 2',5'-bisphosphate (2',5'-ADP-Sepharose 4B) result in a > 30% yield of purified
reductase from microsomes. The enzyme contains 1 mol each of FAD and FMN and exhibits a molecular
weight of 78,000 g mol⁻¹ estimated by comparison with protein standards on sodium dodecyl sulfate
polyacrylamide gel electrophoresis. The turnover numbers calculated on the basis of flavin are 1360
min⁻¹ and 1490 min⁻¹ at 25° for the pig and rat liver enzyme, respectively.

Titration of these purified preparations aerobically with both NADPH and potassium ferricyanide
demonstrated unequivocally that the air-stable, reduced form of NADPH-cytochrome c (P-450)
reductase contains 2 electron equivalents, confirming recent results obtained by Masters et al. (Masters,
solubilized enzyme. In addition, these preparations are capable of reconstituting benzphetamine
N-demethylation activity in the presence of partially purified cytochrome P-450 and dilauroylphos-
phatidylcholine, as measured by formaldehyde formation from benzphetamine.

Numerous methods for the purification of hepatic micro-
somal constituents have been employed resulting in homogene-
ous preparations of NADPH-cytochrome c (P-450) reductase
(1, 2) and cytochrome P-450 (3-5). Various detergents have
been utilized, but the most successful combinations have
included the polyethoxyalkylaryl ethers, such as Emulgen 911
(3) or Renex 690 (1, 2, 4, 5). The use of such detergents has
produced preparations which are capable of reconstituting
microsomal hydroxylation reactions. A recent report by Golf et
al. (6) described the use of an affinity chromatography column
consisting of cytochrome c bound to Sepharose 4B, but the
final preparation was not homogeneous and the enzyme could
be eluted upon the addition of 1 M KCl to the equilibration
buffer. It has been, however, only with great difficulty that
purification to homogeneity has been achieved with any of
these preparations and the multiple steps required have
resulted in low percentage yields (1, 2).

The present paper will report purification procedures for rat
and pig liver NADPH-cytochrome c (P-450) reductase which,
in three and four chromatographic steps, respectively, from
colubilized microsomes produce spectrally pure enzyme prepa-
rations capable of reconstituting benzphetamine metabolism
with a partially purified preparation of cytochrome P-450 and
dilauroylphosphatidylcholine. The procedure involves the use
of Sepharose 4B-bound N6-(6-aminohexyl)-adenosine 2',5'-bis-
phosphate prepared by the method of Brodelius et al. (7).
Recent reports from this laboratory (8-10) confirmed the
earlier results obtained with proteolytically solubilized reduc-
tase demonstrating 2 electron equivalents in the air-stable,
half-reduced form of the reductase. The present results indi-
cate that the detergent-solubilized NADPH-cytochrome c
(P-450) reductase exhibits identical spectral properties with
the proteolytically solubilized flavoprotein. Aerobic titration
with NADPH to the air-stable, reduced form of the enzyme
and back titration with potassium ferricyanide to the fully
oxidized state reveals that it, too, contains 2 electron equiva-
lents, in agreement with previous results from this laboratory
(8-12) but in contrast to the reports of Iyanagi et al. (13-15).

EXPERIMENTAL PROCEDURE

Preparation of Pig Liver Microsomes—A pig liver homogenate was
prepared according to the procedure of Masters et al. (16) in a ratio of 1
g of liver/3 ml of 0.25 M sucrose, pH 7.4 (29% w/v). The supernatant
from centrifugation of this homogenate at 11,320 × g (Rmax) in the
Beckman J-21B was diluted 2.5-fold with cold distilled water
and mixed with a CaCl₂ solution yielding a final concentration of 8 mM.

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Welch Foundation.
Affinity-chromatographed NADPH-Cytochrome c (P-450) Reductase

according to the method of Cinti et al. (17). This mixture was centrifuged at 17,600 × g for 60 min. The microsomal pellet was washed by suspending in 0.1 M sodium pyrophosphate buffer, pH 7.4, containing 1 mm EDTA, and centrifuging in the Spinco L-2-65B at 105,000 × g for 60 min. The resulting supernatant fluid and the pellet were resuspended in 10 mm Tris-acetate buffer, pH 7.4, containing 20% glycerol and 0.1 mm EDTA, to a protein concentration of 25 mg ml⁻¹, and stored at -20°C. All centrifugations were performed at the g values determined at maximum loadings used.

Solubilization and Purification of Pig Liver NADPH-Cytochrome c (P-450) Reductase—The microsomal suspension, 500 ml (25 mg ml⁻¹), was rapidly mixed with 100 ml of 10% Renex 690, 100 ml of 5% sodium deoxycholate, and 300 ml of a 20% glycerol solution which contained 0.1 mM dithiothreitol. The mixture, which contained a final concentration of 1% Renex 690, 0.5% sodium deoxycholate, and 12.5 mg ml⁻¹ protein (a critical ratio of detergent to protein) was stirred for 30 min. The supernatant was centrifuged at 105,000 × g for 60 min. Fifty milliliters of the supernatant were applied directly to the 2',5'-ADP-Sepharose 4B as in Fig. 1 and the remainder was loaded onto a 1200-ml bed volume of DEAE-cellulose in a scinttered glass funnel previously equilibrated with 25 mm Tris buffer, pH 7.7, containing 0.6% Renex 690, 0.1% sodium deoxycholate, 0.05 mm EDTA, and 0.05 mm dithiothreitol. The DEAE-cellulose was washed with 4 liters of a similar buffer solution containing 0.1% EDTA, and the reductase was then eluted with a similar buffer solution containing 0.1% KCl. The eluate was diluted 2-fold with cold distilled water and calcium phosphate gel (100 ml of 80 mg equivalent of dry weight ml⁻¹) was added to it. The mixture was stirred for 10 min and centrifuged at 6370 × g for 10 min. The reductase was eluted from the pellet by stirring for 60 min with 500 ml of 0.3 mg potassium phosphate buffer, pH 7.7, containing 10% glycerol, 0.2% Renex 690, and 0.05 mm dithiothreitol. The elution from calcium phosphate gel was repeated with a small volume of the latter buffer. The resulting reductase fraction was concentrated in an Amicon concentrator and applied to two Ultrogel AcA 34 columns (5.0 × 100 cm) previously equilibrated with 50 mm Tris buffer, pH 7.7, containing 0.1% Renex 690, 0.2% sodium dodecyl sulfate, resulting in a single band upon electrophoresis. The reductase was further purified by sodium dodecyl-sulfate-polyacrylamide disc gel electrophoresis, and subsequently focused. For the titration experiment (Fig. 7), the final preparation was passed through a small column containing 3 mg of chelating and 16 mg of Celite to remove traces of 2'-AMP, a method which has been applied to the removal of NADPH⁺ from the reductase (8). Renex 690 was then removed by application of the reductase to a DEAE-cellulose column which had been equilibrated with 10 mm potassium phosphate buffer, pH 7.7, containing 20% glycerol. Fractions were monitored at 280 nm until the absorbance decreased to <0.03. The reductase was then eluted with 0.3 mM potassium phosphate, pH 7.7, containing 20% glycerol, and 0.1% deoxycholate. Finally, the reductase (2 to 5 ml) was dialyzed against 4 liters of 20 mM potassium phosphate buffer, pH 7.7, containing 20% glycerol, 0.1 mm EDTA, and 0.1% sodium deoxycholate.

Polyacrylamide Disc Gel Electrophoresis in Sodium Dodecyl Sulfate—Disc gels were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis, according to the method of Weber and Osborn (18), in order to determine purity of the reductase preparations. Samples of affinity-chromatographed reductase were applied to the gels and run for 4 h in potassium phosphate buffer, pH 7.2, containing 0.1% sodium dodecyl sulfate, resulting in a single band upon electrophoresis. Estimation of the molecular weight of NADPH-cytochrome c (P-450) reductase by sodium dodecyl-sulfate-polyacrylamide disc gel electrophoresis was performed by the procedure of Weber and Osborn (18) in the presence of the purified standard proteins: β-galactosidase, phosphorylase a, bovine serum albumin, catalase, and aldolase.

The Sepharose 4B-bound 2',5'-adenosyladenosine 2',5'-bisphosphate was kindly supplied by Dr. Robert Bywater of Pharmacia Fine Chemicals prior to its introduction on the commercial market. Dr. Klaus Mosbach, who published the original preparation procedure (7), was instrumental in developing the procedure for Pharmacia and in directing us to this source.

The partially purified cytochrome P-450 (specific content = 8.43 nmol mg⁻¹) and dilauroylphosphatidylcholine (20 mg ml⁻¹ in chloroform) were generously supplied by Dr. Anthony Y. H. Liu of Hoffman-LaRoche, Inc., Nutley, N. J. Renex 690 was obtained through the generosity of Mr. Henry Swab of ICI, United States, Inc., Wilmington, Del. NADPH+NADP⁺ and 2'-AMP were purchased from P.L. Biochemicals, Inc. FAD and FMN were purchased from Sigma Chemical Co. and purified by column chromatography as described previously (8). Cholic acid (A grade) and sodium deoxycholate (A grade) were purchased from Calbiochem. Ultrogel AcA 34 was obtained from LKB Instruments, Inc.

Determination of Protein Concentration—Protein concentration was determined by the method of Lowry et al. (19) as modified by the procedure of Dulley and Grieve (20) for solutions containing detergent.

Determination of NADPH-Cytochrome c Reductase Activity—All previous determinations of NADPH-cytochrome c reductase activity in these laboratories were performed in 0.05 M potassium phosphate buffer, pH 7.7, 0.1 mm EDTA at 25°C (16). For purposes of comparison, however, with the data of Vermilion and Coon (1) and Dignam and Stockel (2), it has been necessary in presenting these data to perform these assays also in 0.5 mM potassium phosphate, pH 7.7, 0.1 mm EDTA at 25°C. Under these conditions doubling of the activity obtained (see Table I). Therefore, it should be noted that the specific activity reported in Table II for liver microsomes from phenobarbital-pretreated rats is substantially higher (about twice) that obtained in most laboratories for NADPH-cytochrome c reductase activity.
This is demonstrated in the turnover numbers which are calculated on the highest specific activities obtainable, but have been deliberately
Pig liver reductase 17.8 1360 37.4 2856
Rat liver reductase 21.9 1365 43.8 2730
these turnover numbers with those obtained with proteolytically
the basis of flavin concentration in the preparations. A comparison of
of conditions.

TABLE I
Comparison of specific activities and turnover numbers of pig and rat liver microsomal NADPH-cytochrome c (P-450) reductase

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Specific activity ( \mu \text{mol} \text{ min}^{-1} \text{mg}^{-1} )</th>
<th>Turnover number ( \mu \text{mol} \text{ min}^{-1} \text{mg}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig liver reductase</td>
<td>17.8</td>
<td>1360</td>
</tr>
<tr>
<td>Rat liver reductase</td>
<td>21.9</td>
<td>1365</td>
</tr>
</tbody>
</table>

\( ^{a} \) NADPH-cytochrome c reductase activities were assayed as described under “Experimental Procedure” under the two separate sets of conditions.

\( ^{b} \) The enzyme preparations presented in this table do not represent the highest specific activities obtainable, but have been deliberately chosen to illustrate that the flavoprotein obtained by the affinity chromatography procedure outlined in this paper is spectrally pure. This is demonstrated in the turnover numbers which are calculated on the basis of flavin concentration in the preparations. A comparison of these turnover numbers with those obtained with proteolytically solubilized reductase in 0.05 \( M \) potassium phosphate buffer, pH 7.7, 0.1 \( M \) EDTA at 25\(^{\circ} \) (8, 11, 12) reveals that identical activities are obtained with the detergent-solubilized enzyme preparations.

TABLE II
Affinity Chromatography of Partially Purified Pig Liver Microsomal NADPH-Cytochrome c (P-450) Preparation—Fig. 2 illustrates the affinity chromatography of pig liver microsomal NADPH-cytochrome c (P-450) reductase which has been chromatographed batch-wise on DEAE-cellulose and subjected to gel filtration on Ultrogel AcA 34. It can be seen that approximately 3 times the amount of reductase has been bound to the same column of 2',5'-ADP-Sepharose 4B as was bound in the experiment of Fig. 1. The addition of 100 \( M \) potassium phosphate buffer, pH 7.7 (as in Fig. 1), was made as shown in Fig. 2, Arrow A. After re-equilibration (as in Fig. 1) with 10 \( M \) potassium phosphate, pH 7.7 (Fig. 2, Arrow B), a linear gradient from 0 to 5 \( M \) 2'-AMP was run in the same buffer as at B (Fig. 2, Arrow C).

The choice of 2'-AMP was made on the basis of the knowledge that 2'-AMP is a potent competitive inhibitor of NADPH-cytochrome c reductase activity (21) and that the group most crucial for the binding of NADPH to the reductase is most likely the 2'-phosphate group on the ribose moiety of the adenosine. Furthermore, it has been demonstrated (data not shown) that 5 \( M \) 5'-AMP is totally ineffective in eluting the reductase from the affinity column and that 5'-ADP is only partially effective at concentrations in excess of 2.5 \( M \). It was shown in Fig. 1 that NADH is also ineffective in eluting the reductase.

**RESULTS**

Pig liver microsomal NADPH-cytochrome c (P-450) reductase was purified on 2',5'-ADP-Sepharose 4B. The supernatant from a 100,000 \( \times g \) for 60 min centrifugation of microsomes solubilized by Renex 690 and sodium deoxycholate (“Experimental Procedure”) was applied to the Sepharose 4B column (1.0 \( \times \) 2.5 cm) pre-equilibrated with 10 \( M \) potassium phosphate buffer, pH 7.7, containing 20% glycerol, 0.1% Renex 690, 0.02 \( M \) EDTA, and 1.0 \( M \) dithiothreitol. In Fig. 1, it can be seen that only about 40% of the reductase is adsorbed onto the column, but the adsorbed enzyme could not be eluted with 100 \( M \) potassium phosphate, pH 7.7, containing 20% glycerol, 0.1% Renex, 0.2 \( M \) EDTA, and 1.0 \( M \) dithiothreitol (Fig. 1, Arrow A). After re-equilibration of the column with the original 10 \( M \) buffer system (Fig. 1, Arrow B), 1 \( M \) NADH was added to the buffer with no effect (Fig. 1, Arrow C). After re-equilibration (Fig. 1, Arrow D), 1 \( M \) NADPH was added (Fig. 1, Arrow E) resulting in elution of 70% of the bound reductase from the affinity column. The eluted enzyme represented a 100-fold purification from the microsome-bound state.

The amount of reductase which could be bound by the affinity column from solubilized pig liver microsomes was \( \frac{1}{3} \) to \( \frac{1}{2} \) as much as could be bound in a partially purified form, i.e., after two chromatography steps utilizing DEAE-cellulose and Ultrogel AcA 34, as will be illustrated in Fig. 9.

Affinity Chromatography of Partially Purified Pig Liver Microsomal NADPH-Cytochrome c (P-450) Preparation—Fig. 2 illustrates the affinity chromatography of pig liver microsomal NADPH-cytochrome c (P-450) reductase which has been chromatographed batch-wise on DEAE-cellulose and subjected to gel filtration on Ultrogel AcA 34. It can be seen that approximately 3 times the amount of reductase has been bound to the same column of 2',5'-ADP-Sepharose 4B as was bound in the experiment of Fig. 1. The addition of 100 \( M \) potassium phosphate buffer, pH 7.7 (as in Fig. 1), was made as shown in Fig. 2, Arrow A. After re-equilibration (as in Fig. 1) with 10 \( M \) potassium phosphate, pH 7.7 (Fig. 2, Arrow B), a linear gradient from 0 to 5 \( M \) 2'-AMP was run in the same buffer as at B (Fig. 2, Arrow C).

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**Determination of FAD and FMN Content of Purified Detergent-solubilized Pig Liver NADPH-Cytochrome c (P-450) Reductase**—The FAD and FMN content of detergent-solubilized pig liver microsomal NADPH-cytochrome c (P-450) reductase was determined by the method of Faeder and Siegel (22) as applied to the proteolytically solubilized, purified preparations of the flavoprotein from rat and pig liver (8, 10) and from pig kidney (23). The flavin content, determined on duplicate samples of pig liver reductase from which 80% of the flavin was released, was shown to be 1 mol each of FAD and FMN, in agreement with the results of Vermilion and Coon (1) and Dignam and Strobel (2).

**Affinity Chromatography of Partially Purified Rat Liver Microsomal NADPH-Cytochrome c (P-450) Reductase**—An aliquot containing 21.0 IU (25\(^{\circ} \)) of reductase, solubilized with 1.0% Renex 690 and 0.5% sodium cholate from rat liver microsomes and partially purified by gradient elution from DEAE-cellulose, was applied to the 2',5'-ADP-Sepharose 4B column (Fig. 3) pre-equilibrated with 10 \( M \) potassium phosphate buffer as described under “Experimental Procedure.”
raphy is biospecific and that the 2'-phosphate group of the ribose attached to the adenine ring is crucial for the binding of NADPH-Cytochrome c (P-450) reductase, but after re-equilibration with the original buffer, 12.5 ml of 0.7 mM potassium phosphate buffer, pH 7.7, did not elute the reductase. In another experiment, 5' AMP was applied to the column, and no reductase activity was eluted. In another experiment, not shown, 5' AMP eluted the reductase. In another experiment, 5' AMP was applied to the column, and no elution of the reductase was obtained. These data, with that obtained for the pig liver microsomal NADPH-cytochrome c reductase, support the conclusion that the chromatography is biospecific and that the 2'-phosphate group of the ribose attached to the adenine ring is crucial for the binding and subsequent elution from the 2',5'-ADP-Sepharose 4B column.

Typical Purification Scheme for Rat Liver Microsomal NADPH-Cytochrome c (P-450) Reductase—A typical purification scheme is shown in Table II for the rat liver microsomal NADPH-cytochrome c (P-450) reductase. The enzyme obtained after Step 4 has a slightly higher specific activity than either the preparation of Vermilion and Coon (1) or the one of Dignam and Strobel (2), measured under identical assay conditions, but still exhibits minor bands on sodium dodecyl sulfate polyacrylamide gel electrophoresis. The enzyme is homogeneous after gel filtration on LKB Ultrogel ACA 34 (Step 5), but there is little improvement in specific activity at this stage.

Disc Gel Electrophoresis of Affinity-chromatographed Reductase—Fig. 4 shows the typical disc gel electrophoresis patterns on sodium dodecyl sulfate gels of affinity-chromatographed rat liver NADPH cytochrome c (P-450) reductase after Step 4 (Fig. 4, tube 2) and Step 5 (Fig. 4, tubes 3 and 4) of the purification procedure of Table II. A comparison of the reductase preparations at these stages of purification shows that homogeneous reductase is obtained only after a final gel...
filtration step (Fig. 4, tubes 3 and 4). Tube 3 contained 30 μg and tube 4 contained 60 μg of purified reductase, and a control gel to which no protein was applied is shown in Fig. 4 (tube 1).

Estimation of Molecular Weight of NADPH-Cytochrome c (P-450) Reductase by Sodium Dodecyl Sulfate-Polyacrylamide Disc Gel Electrophoresis—In Fig. 5, an experiment to estimate the molecular weight of rat liver NADPH-cytochrome c (P-450) by sodium dodecyl sulfate-polyacrylamide-disc gel electrophoresis by the method of Weber and Osborn (18) is shown. The protein standards used were β-galactosidase (Mr = 130,000), phosphorylase a (Mr = 92,500), bovine serum albumin (Mr = 68,000), catalase (Mr = 57,500), and aldolase (Mr = 40,000). The molecular weight of the reductase was estimated to be 78,000 from the experiment of Fig. 5, a value within experimental error of that (79,000) obtained by Vermilion and Coon (1) and Dignam and Strobel (2). The proteolytically solubilized reductase exhibits an estimated molecular weight of 71,000 g mol⁻¹ on sodium dodecyl sulfate polyacrylamide disc gel electrophoresis (23).

Recombination of Affinity-chromatographed NADPH-Cytochrome c (P-450) Reductase with Cytochrome P-450 and Dilauroylphosphatidylcholine to Yield Benzphetamine N-Demethylation—Using a preparation of cytochrome P-450, generously supplied by Dr. Anthony Y. H. Lu of Hoffmann-LaRoche, Inc., the saturating amount of dilauroylphosphatidylcholine required for benzphetamine N-demethylation was determined at a level of cytochrome P-450 equal to 0.29 nmol (0.035 mg of protein) and of reductase activity equal to 0.286 μmol of reduced cytochrome c min⁻¹ (0.0052 mg of protein) (cytochrome P-450 to reductase ratio of 4.4:1; data not shown). In a subsequent experiment, shown in Table III, the effects of various omissions from the complete incubation system are shown. The omission of reductase, cytochrome P-450, or benzphetamine resulted in the zero time control activity, but omission of NADPH gave an even lower value. The absolute activity values obtained are strictly comparable to those obtained by Lu et al. (24) under similar conditions.

![Fig. 4. Electrophoretic homogeneity of purified rat liver NADPH-cytochrome c (P-450) reductase. The enzyme preparations were treated with sodium dodecyl sulfate and mercaptoethanol and submitted to polyacrylamide gel electrophoresis by the method of Weber and Osborn with a 10% separating gel. The direction of migration was top to bottom. The gels were stained with Coomassie brilliant blue in a mixture of 45% of methanol and 4.6 ml of glacial acetic acid, and destained in 7.5% acetic acid, 1. No protein applied; 2, eluate from 2',S'ADP-Sepharose 4B (about 50 μg); 3 and 4, eluate from Ultrogel AcA 34 (about 30 μg and 60 μg).](image-url)

![Fig. 5. Estimation of the molecular weight of rat liver NADPH-cytochrome c (P-450) reductase with sodium dodecyl sulfate polyacrylamide disc gel electrophoresis. Gels and samples were prepared as shown in Fig. 4. Migration of tracking dye was 10.7 cm. The marker proteins were β-galactosidase, phosphorylase a, bovine serum albumin, catalase, and aldolase. Electrophoresis was carried out on three gels containing all proteins together and on one gel containing phosphorylase a, NADPH-cytochrome c reductase, and bovine serum albumin, separately.](image-url)

**Table III**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Activity (nmol HCHO/10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Complete system</td>
<td>86</td>
</tr>
<tr>
<td>2. Minus reductase</td>
<td>20</td>
</tr>
<tr>
<td>3. Minus cytochrome P-450</td>
<td>20</td>
</tr>
<tr>
<td>4. Minus NADPH</td>
<td>12</td>
</tr>
<tr>
<td>5. Minus benzphetamine</td>
<td>24</td>
</tr>
<tr>
<td>6. Complete system at zero time</td>
<td>22</td>
</tr>
</tbody>
</table>
Aerobic Titration of Affinity-Chromatographed Pig Liver NADPH-Cytochrome c (P-450) Reductase

Affinity-chromatographed NADPH-cytochrome c reductase with K₃Fe(CN)₆. The reductase purified as described under "Experimental Procedure" (31.2 nmol of total flavin) in 0.05 mM potassium phosphate buffer, pH 7.7, containing 0.1 mM EDTA, 20% glycerol, and 0.1% sodium deoxycholate was titrated with 2.1 mM K₃Fe(CN)₆ in the above buffer at 25°C. To obtain the air-stable, half-reduced enzyme, 4 mol of NADPH/mol of flavin were added aerobically and the flavoprotein was allowed to reoxidize to the half-reduced state, which was confirmed by monitoring at 340 nm and 453 nm. Curve 1, half-reduced enzyme; curves 2 to 6 show equilibrium absorption spectra after the addition of 6.3, 12.6, 18.9, 25.2, and 34.7 nmol of K₃Fe(CN)₆, respectively. The total volume change was less than 5.5% and was not corrected for upon each addition. The inset shows the changes occurring at 453, 503, and 585 nm during the titration and the abscissa indicates total nanomoles of potassium ferricyanide, pH 7.7, and a turnover number of 1360 min⁻¹ at 25°C.

NADPH-Cytochrome c (P-450) Reductase Purified by Affinity Chromatography—In order to ascertain the electron transfer properties of the detergent-solubilized, affinity-chromatographed reductase, the experiment shown in Fig. 6 was performed. The flavoprotein was reduced with excess NADPH (4 mol of NADPH/mol of total flavin, i.e., 126 nmol of NADPH) aerobically and allowed to oxidize in air until there was no further change at 340 nm or 453 nm, indicating complete oxidation of the NADPH and no further oxidation of flavin, respectively. Potassium ferricyanide (2.1 mM) was added in aliquots resulting in the various equilibrium absorption spectra shown in Fig. 6. The changes in absorbance at 453, 503, and 585 nm are shown in the inset, and the abscissa indicates both total nanomoles of potassium ferricyanide added and the ratio of the oxidant to reducible flavin. The results show that 1 mol of potassium ferricyanide/mol of enzymically reducible flavin is required to reoxidize the flavoprotein to its fully oxidized state, indicating that 2 electrons remain in the stable, half-reduced form of the reductase (containing 1 mol each of FAD and FMN) in agreement with previous results of Masters et al. (8, 10) and Kamin (9). The previous titration results reported by Masters et al. (8, 10) and Kamin (9) were obtained with the proteolytically prepared reductase. The present data have been obtained with detergent-solubilized reductase, purified by affinity chromatography techniques to a specific activity with cytochrome c as electron acceptor of 30 μmol min⁻¹ mg⁻¹ as measured in 0.3 M potassium phosphate, pH 7.7, at 30°C, a specific activity comparable to that obtained by Vermilion and Coon (1) and

Aerobic Back Titration of Air-stable Half-reduced Pig Liver

Fig. 6. Aerobic titration of affinity-chromatographed pig liver detergent-solubilized NADPH-cytochrome c reductase with K₃Fe(CN)₆. The reductase purified as described under "Experimental Procedure" (31.2 nmol of total flavin) in 0.05 mM potassium phosphate buffer, pH 7.7, containing 0.1 mM EDTA, 20% glycerol, and 0.1% sodium deoxycholate was titrated with 2.1 mM K₃Fe(CN)₆ in the above buffer at 25°C. To obtain the air-stable, half-reduced enzyme, 4 mol of NADPH/mol of flavin were added aerobically and the flavoprotein was allowed to reoxidize to the half-reduced state, which was confirmed by monitoring at 340 nm and 453 nm. Curve 1, half-reduced enzyme; curves 2 to 6 show equilibrium absorption spectra after the addition of 6.3, 12.6, 18.9, 25.2, and 34.7 nmol of K₃Fe(CN)₆, respectively. The total volume change was less than 5.5% and was not corrected for upon each addition. The inset shows the changes occurring at 453, 503, and 585 nm during the titration and the abscissa indicates total nanomoles of potassium ferricyanide, pH 7.7, and a turnover number of 1360 min⁻¹ at 25°C.

Fig. 7. Aerobic titration of rat liver microsomal NADPH-cytochrome c (P-450) reductase with NADPH and K₃Fe(CN)₆. Absorbance changes at 453 (○), 500 (●), and 585 (□) nm are plotted against either NADPH (A) or K₃Fe(CN)₆ (B) added. Rat liver microsomal NADPH-cytochrome c (P-450) reductase was in 0.05 M potassium phosphate (pH 7.7) containing 0.1% sodium deoxycholate and 20% glycerol to a final concentration of 7.52 μM in a total volume of 3 ml and titrated with NADPH and K₃Fe(CN)₆ under aerobic conditions at 25°C. Experiments A and B used the same enzyme preparation but were carried out separately. The enzyme preparation was 85% reducible as determined by the addition of 50 μmol of NADPH/mol of flavin. Total volume change was less than 3.5% and was not corrected for upon addition. (Table II). The residual activity (about 20%) which is measured in the absence of reductase, or cytochrome P-450, or benzphetamine was also obtained in the absence of lipid and was not subtracted from the rate obtained with the complete incubation system.
Dignam and Strobel (2) for the purified rat liver reductase preparations.

Aerobic Titration of Rat Liver Microsomal NADPH-Cytochrome c (P-450) Reductase with NADPH and Potassium Ferricyanide—Fig. 7 illustrates a typical titration experiment in which oxidized reductase was titrated aerobically with aliquots of NADPH to the air-stable, reduced form (Fig. 7A), at which point the curve breaks and no further reduction of flavin is recorded at 453 nm. It is also seen that the isosbestic point at 585 nm for oxidized and half-reduced reductase is unaffected by the addition of NADPH. The longer wavelength absorption increases until maximal formation of the air-stable, half-reduced spectrum is obtained. When the amount of NADPH added equals 0.5 mol/mol of reducible flavin, the titration curves at both 453 and 585 nm are seen to break. In a separate experiment on the same enzyme preparation, excess NADPH (1.5 mol/mol of flavin) was added and the reductase was allowed to reoxidize to the air-stable, reduced form, at which time aliquots of K$_3$Fe(CN)$_6$ were added to reoxidize the enzyme to its fully oxidized state (Fig. 7B). Again, it can be seen that 1.0 mol of K$_3$Fe(CN)$_6$/mol of reducible flavin is required to reoxidize the flavoprotein from the air-stable reduced state to its fully oxidized state, confirming the results obtained in this paper (Fig. 6) on detergent-solubilized pig liver microsomal NADPH-cytochrome c reductase and earlier data on the proteolytically solubilized enzymes from pig and rat liver (8-10). These data unequivocally demonstrate that the spectral species which is obtained aerobically upon reduction with NADPH to an air-stable, partially reduced state and which results from reoxidation of the fully reduced enzyme with various electron acceptors (11, 12) does, indeed, contain 2 electron equivalents.

**DISCUSSION**

The foregoing paper has described purification procedures for pig and rat liver microsomal NADPH-cytochrome c (P-450) reductases utilizing biospecific affinity chromatography on Sepharose 4B-bound N°(6-aminohexyl)-adenosine 2',5'-bisphosphate (7). The procedures involve either prior batchwise chromatography on DEAE-cellulose plus gel filtration on Ultrogel AcA 34 for pig liver microsomal reductase or DEAE-cellulose column chromatography only for rat liver microsomal reductase. Both procedures produce spectrally pure NADPH-cytochrome c (P-450) reductase (such as that shown in Fig. 6) with a turnover number equal to that obtained with homogenous enzyme. With a subsequent chromatography step using gel filtration on Ultrogel AcA 34, it is possible to obtain homogenous flavoprotein as verified in Fig. 4 (tubes 3 and 4).

Electrophoresis on sodium dodecyl sulfate gels reveals minor contaminants with rat liver microsomal reductase purified by DEAE-cellulose column chromatography and subsequent affinity chromatography on 2',5'-ADP-Sepharose 4B as shown in Fig. 4 (tube 2), but final gel filtration on I.C.R. AcA 34 yields a preparation exhibiting a single band on disc gel electrophoresis (Fig. 4, tubes 3 and 4). The yields of reductase vary between 30 and 55%, depending on the purity of enzyme desired. It should be noted that the purification procedure through Step 4 in Table II is adequate for the production of spectrally pure reductase capable of reconstituting oxidative demethylation of drugs with partially purified cytochrome P-450 and dialuroylphosphatidylcholine (Table III). This preparation is also adequate for spectral titration studies, i.e., the turnover number, based on flavin, is equal to that obtained with any homogeneously purification. The molecular weight, estimated by the procedure of Weber and Osborn (18) on sodium dodecyl sulfate gel electrophoresis in the presence of standard proteins of known subunit molecular weight, was estimated to be 78,000 g mol$^{-1}$ (Fig. 5).

Aerobic titration of pig liver microsomal reductase from the air-stable, reduced form to the fully oxidized form with potassium ferricyanide resulted in a stoichiometry of 1 mol of ferricyanide/mol of reducible flavin (Fig. 6). In addition, aerobic titration of the fully oxidized rat liver reductase to the air-stable, reduced form with NADPH and back titration to the fully oxidized form with potassium ferricyanide confirm that 2 electron equivalents exist in this aerobically stable intermediate (Fig. 7). These results are in complete agreement with previous results of Masters et al. (8, 10) obtained with proteolytically solubilized reductase. It is essential to establish the mechanism of NADPH-cytochrome c (P-450) reductase purified from detergent-solubilized microsomes, since it is capable of recombining with preparations of cytochrome P-450 and dialuroylphosphatidylcholine to oxidatively demethylate drugs, a capacity which the proteolytically solubilized reductase did not possess. Indeed, it is cytochrome P-450 (not cytochrome c, potassium ferricyanide, or 2,6-dichlorophenolindophenol) which is the terminal acceptor of electrons donated through this flavoprotein (25).

The procedures outlined in this paper represent the first successful application of biospecific affinity chromatography to any microsomal electron transport component. The 2',5'-ADP-Sepharose-4B columns can be utilized repeatedly without loss of binding capacity. The affinity chromatography medium is stable in the presence of detergents, either nonionic or ionic. Since this improved, high yield procedure produces consistently pure enzyme preparations, we plan to utilize this technique for the production of the large quantities of flavoprotein required for reconstitution experiments involving cytochrome P-450, EPR titration studies, and spectrophotometric and fluorimetric studies on the reactivation of the enzyme with various flavins.

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