Adrenocortical Cytochrome P-450 Side Chain Cleavage

PREPARATION OF MEMBRANE-BOUND SIDE CHAIN CLEAVAGE SYSTEM FROM PURIFIED COMPONENTS*

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Soluble cytochrome P-450 from bovine adrenocortical mitochondria, capable of side chain cleavage, can be incorporated into membranes prepared by dispersion of phospholipids in aqueous buffer in which cholate is added to the membrane suspension. In addition, the complete protein side chain cleavage system (i.e. including the ancillary proteins adrenodoxin and adrenodoxin reductase and the substrate cholesterol) can be incorporated into such membranes so that on addition of TPNH, pregnenolone is formed. These components remain in the membrane through gel filtration (which removes almost all the cholate) and sedimentation through sucrose density gradients which separate vesicles without protein and soluble enzyme from the membrane-bound P-450. P-450 remains associated with the membrane during and following lysis of vesicles. The vesicles which do not leak [14C]glucose were seen on electron microscopy to show a mean diameter of 350 to 450 Å. A number of phospholipids are capable of accommodating P-450 in this manner: mitochondrial lipid extracts, synthetic dipalmitoyl phosphatidylcholine, synthetic dipalmitoyl phosphatidylethanolamine, and egg lecithin, separately or in various combinations. Cholesterol is not necessary for incorporation of the side chain cleavage system, protein, and phospholipid.

* This investigation was supported by Grant CA14985-05 (to P.F.H.) from the National Cancer Institute and by Grant AM15621 from the National Institutes of Health, both from the Department of Health, Education, and Welfare. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
cases TPNH (10 mol) was added; (e) in other cases, sodium [\(^{13}C\)]
cholate (100,000 cpm) was added; (d) for some experiments, [\(^{3}H\)]
phosphatidylcholine was added to facilitate detection of fractions from column chromatography containing phospholipids; (c) vesicles
were sometimes prepared with P-450 but without the two ancillary
proteins; (b) in some cases, sonication of phospholipids for 30 min (5-
min treatments with 1-min intervals between treatments) at setting
6 on a sonicator (Heat Systems-Ultrasonics, Inc., model WIS5F) was
performed to disperse phospholipids instead of vortex mixing; (g)
some preparations were made on a larger scale; and (h) other phos-
pholipid mixtures were used.

When [\(^{13}C\)]Cholate was used, the vesicles were purified on Sephadex
G-25 and fractions were examined for [\(^{13}C\)]cholate. The fractions containing
vesicles were pooled and analyzed for [\(^{13}C\)], protein, and phospholipid,
and an aliquot (1.0 ml) was dialyzed against distilled water with five
changes of water (see above). After dialysis for a total of 1 h, the sample
was analyzed for [\(^{13}C\)], protein, and phospholipid.

The elution profile was virtually identical when the vesicles were
prepared from synthetic phospholipids (not shown). When material from the void volume of this column was
applied to Sepharose 4-B, the membrane-bound P-450 was again excluded and no free or soluble P-450 was observed (Fig. 2); this figure shows the elution volume of soluble P-450 (1), well separated from the void volume. When a nonvesicular preparation consisting of a mixture of P-450 and phospholipids (see “Experimental Procedures”) was examined successively by means of the same two columns, chromatography on
Sepharose 4B shows that much of the P-450 behaves as free
enzyme and some “smearing” of protein and phospholipid is seen (Fig. 2). It appears that some of the P-450 may be associated with the phospholipid while much of it remains
free or soluble.

**Centrifugation on Sucrose Density Gradients**

The membrane-bound P-450 (after removal of cholate on Sephadex G-50) was sedimented (150,000 \(\times\) g for 7 h) on linear
sucrose density gradients 0 to 20% (Fig. 3A). Enzyme activity
and phospholipid are to be found near the bottom of the
centrifuge tube while phospholipid without enzyme appears

**RESULTS**

**Column Chromatography of Membrane-bound Cytochrome P-450**

Fig. 1 shows that membrane-bound P-450 is excluded from
Sephadex G-50 in the void volume of the column while
[\(^{3}H\)]cholate is almost completely removed from the enzyme.

**Analytical Procedures**—Pregnenolone (product of side chain cleavage)
was determined by radioimmunoassay (13) as previously
applied to the present system (14). Unless otherwise stated, to determine
side chain cleavage activity, the following additions were made:
cholesterol 50 mol in \(N\),\(N\),dimethylformamide (5 \(\mu\)l); adrenodoxin,
adrenodoxin reductase, and P-450 (10 to 50 \(\mu\)g of protein). The reaction
was started by addition of TPNH (10 \(\mu\)mol/flask) and
stopped by boiling for 3 min. Incubation was routinely performed for
20 min; under the conditions used, the rate of the reaction is linear for
at least 60 min and is proportional to the concentration of P-450.
When vesicles included cholesterol, adrenodoxin reductase, and
adrenodoxin, these substances were not added to the incubation mixture
which contained only buffer, vesicles, and TPNH.

**Preparation of Mitochondrial Phospholipids**—Fresh adrenocor-
tical mitochondria prepared as described previously (1) were taken to
pH 11.0 by means of ammonium hydroxide and phospholipids were
extracted and washed twice by the Folch procedure (12). The extract
was taken to dryness and kept at -18°C under nitrogen.

**Electron Microscopy**—Electron microscopy was performed as de-
scribed elsewhere (18) using a JEM1. 100C microscope operated at 80
kV.

**Chemicals**—Details of the sources and characteristics of the chem-
icals used have been published (3, 5, 10) except for those that follow.
\(\delta\)-(\(^{5}\)\(^{13}C\))Glucose (3.0 mCi/mmol; batch 70) and \(\delta\)-(\(^{2,4}\)\(^{3}H\))cholic acid
(5 Ci/mmol; batch 146) were purchased from New England Nuclear.
Phospholipids were obtained from Applied Science.

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Membrane-bound Adrenal P-450 Side Chain Cleavage

FIG. 2. A, a sample of membrane-bound (mitochondrial phospholipid) side chain cleavage system (1.0 ml; 0.092 mg of protein) after elution from Sephadex G-50 was applied to a column of Sepharose 4B (0.9 × 50 cm). The column was washed with Tris/NaCl buffer (see “Experimental Procedures”) and fractions (0.9 ml) were examined for side chain cleavage activity and phospholipid content. B, a nonvesicular preparation was used for comparison with A (see “Experimental Procedures”). This preparation was made from the same phospholipid and protein mixture in the same volume of buffer as that used in A. ↓ indicates elution volume of soluble P-450.

FIG. 3. Sucrose density gradients with membrane-bound side chain cleavage system. The membrane-bound enzyme system was prepared from synthetic phospholipids (see “Experimental Procedures”) and after removal of cholate by chromatography on Sephadex G-50, a sample (0.2 ml; 460 μg of protein) was layered onto a sucrose density gradient (5.0 ml; 5 to 20%) and centrifuged at 150,000 × g for 7 h. Fractions (0.3 ml) were collected and analyzed for phospholipid, side chain cleavage, and specific gravity. A, vesicles with side chain cleavage system; B, vesicles without protein; and C, nonvesicular control (see “Experimental Procedures”). □ — □, side chain cleavage; △—△, phospholipid; ○—○, specific gravity.

FIG. 4. Chromatography of membrane-bound side chain cleavage system prepared from synthetic phospholipids on Sephadex G-50 (1.3 × 10.0 cm). The experiment was performed exactly as described under Fig. 1 except that [14C]glucose (105 cpm) was added to the 3.0 ml of buffer in which the vesicles were prepared; the same concentration of [14C]glucose was present in the mixture of proteins added to the phospholipid (see “Experimental Procedures”). Fractions (1.6 ml) were collected as before and examined for protein, enzyme activity, and 14C.

Properties of Membrane-bound Side Chain Cleavage System

Studies with [14C]Glucose—Vesicles containing P-450 or the side chain cleavage system were prepared in 3.0 ml of buffer containing 100,000 cpm of [14C]glucose and then applied to a column of Sephadex G-25. Fig. 4 shows a preparation using the complete side chain cleavage system with synthetic phospholipid. Free [14C]glucose is clearly separated from the membrane-bound enzyme, which contains some [14C]glucose. The peak of radioactivity associated with the vesicles shows no tailing so that the [14C]glucose is trapped within lipid vesicles; the absence of tailing indicates that vesicles are not leaky (20). Moreover, one preparation of P-450 in synthetic phospholipid with [14C]glucose showed a lipid/protein weight ratio of 0.51 and contained 4,800 cpm of 14C. One milliliter of this preparation was dialyzed against distilled water (1 liter with four changes). Following this treatment, the lipid/protein ratio was found to be 0.53 and no significant 14C was detected in the vesicles (measured as protein when P-450 alone was present) was 74 to 81% in eight determinations.

Since the peak of the phospholipid vesicles in Fig. 3B corresponding to a specific gravity of 1.027 (Fig. 3B) and when phospholipid alone is treated in the same way, the peak at 1.027 is seen (Fig. 3B). A nonvesicular preparation (also after Sephadex G-50) shows enzyme activity at the bottom of the tube where soluble P-450 sediments under these conditions (see arrow in Fig. 3A) and phospholipid is found at the top of the gradient (Fig. 3C). In addition, the nonvesicular preparation shows some “smearing” through the gradient (Fig. 3C) resembling that seen with similar preparations on Sepharose 4B (Fig. 2B).

The phospholipid of vesicles in Fig. 3B corresponding to a specific gravity of 1.027 was seen in fractions of the same density in other studies after 12 and 20 h of centrifugation, we conclude that the vesicles are banding at equilibrium. In eight determinations, the density of vesicles without protein was 1.027 ± 0.003 (range). By contrast, the membrane-bound enzyme moves further into the gradient with time and has been observed to penetrate 40% sucrose (density, 1.17). The maximal weight ratio of phospholipid to protein in membranes made from synthetic phospholipids and containing only P-450 was 0.6 ± 0.2 (mean and range for nine determinations). It has not so far been possible to determine amounts of each of the three proteins (P-450 and ancillary proteins) in side chain cleavage vesicles. Recovery of P-450 in the vesicles (measured as protein when P-450 alone was present) was 74 to 81% in eight determinations.

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(<100 cpm). In four separate preparations, the ratio did not change by more than 15% after lysis in distilled water. Since this treatment converted some of the P-450 to P-420, it was not possible to use CO spectra to measure P-450. However, the constancy of the lipid/protein ratio indicates that, when the vesicles were opened by lysis, the P-450 remained with the phospholipid and, unlike the [14C]glucose, was not merely trapped in the buffer within the vesicle.

**Enzyme Activity**—The enzymatic activity of membrane-bound P-450 is shown in Table I. The Vₘₐₓ for the soluble P-450 used here was found to be 2.9 nmol of product/min/mg of protein; this value is slightly higher than those observed with earlier preparations of the enzyme (3). Vₘₐₓ was decreased (2.2 nmol/min/mg of protein) by adding synthetic phospholipid vesicles, i.e. addition of the vesicles containing only phospholipid to the incubation mixture immediately before incubation (data not shown). In these studies, enzymatic activity is expressed per mg of protein rather than per nmol of P-450 for three reasons: (i) relatively large scale preparations are required to permit measurement of P-450 by CO-difference spectroscopy (this has been reserved for spectral studies; see below); (ii) it is difficult to concentrate vesicles; and (iii) some P-420 is present in the membrane-bound P-450 which limits the accuracy with which this substance can be determined by CO difference.

It will be seen (Table I) that the complete system is enzymatically active when TPNH is provided whether the phospholipid is synthetic or extracted from mitochondria; addition of adrenodoxin and adrenodoxin reductase does not increase side chain cleavage. There is, however, one major difference between vesicles prepared from the two different phospholipid mixtures, namely the system constructed from synthetic phospholipids requires, as expected, exogenous cholesterol as substrate. Exogenous cholesterol causes some inhibition of side chain cleavage when added to vesicles prepared from mitochondrial phospholipid (Table I). Again, when P-450 alone is inserted into vesicles of synthetic phospholipid, adrenodoxin, adrenodoxin reductase, cholesterol and TPNH must all be provided (Table I). The same is true (except for cholesterol) when mitochondrial phospholipid is used (data not shown); Folch extraction of mitochondria removes protein but not cholesterol (as expected) from the phospholipid. The values in Table I should be regarded as apparent Vₘₐₓ for reasons to be discussed. Fig. 5 shows a Lineweaver-Burk plot for synthetic phospholipid vesicles containing the complete side chain cleavage system. In this study (Fig. 5), Vₘₐₓ was 28.1 nmol of product/min/mg of protein.

Soluble P-450 at a concentration of 100 µg of protein/ml has a half-life of approximately 2 weeks at 0°C. No loss in activity was observed in the membrane-bound enzyme during 6 weeks under these conditions.

**Effect of Antibody**—Fig. 6 shows that anti-P-450 antiserum

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**Table I**

**Enzyme activity of membrane-bound cytochrome P-450**

Three preparations of membrane-bound P-450 were incubated under the conditions shown. The three preparations were P-450 with ancillary proteins in vesicles made from mitochondrial phospholipid, the same proteins in vesicles from synthetic phospholipids, and P-450 without ancillary proteins. The vesicles were purified by chromatography on Sephadex G-50 before use in this experiment (see "Experimental Procedures"). The vesicle preparation was made from synthetic phospholipid and was purified on Sephadex G-50 before use in this experiment (see "Experimental Procedures"). The reaction was started by addition of TPNH (10 µmol/flask). Following incubation, production of pregnenolone was measured by radioimmunoassay.

<table>
<thead>
<tr>
<th>Vesicles</th>
<th>Protein</th>
<th>Phospholipid</th>
<th>Additions</th>
<th>Pregnenolone (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Side chain cleavage system</td>
<td>None</td>
<td>None</td>
<td>Cholesterol</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>TPNH</td>
<td>Adrenodoxin and adrenodoxin reductase</td>
<td>21.2 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>Side chain cleavage system</td>
<td>PE and PC</td>
<td>PE and PC</td>
<td>Cholesterol</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Synthetic</td>
<td>TPNH</td>
<td>Adrenodoxin and adrenodoxin reductase</td>
<td>20.9 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>P-450</td>
<td>PE and PC</td>
<td>Complete system</td>
<td>Cholesterol + TPNH</td>
<td>24.2 ± 0.2</td>
</tr>
</tbody>
</table>

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**Fig. 5.** Kinetic study of membrane-bound side chain cleavage system. The vesicle preparation was made from synthetic phospholipid and was purified on Sephadex G-50 before use in this experiment (see "Experimental Procedures"). Each flask contained 20 µg of side chain cleavage protein and the additions described under "Experimental Procedures." The reaction was started by addition of TPNH (10 µmol/flask). Following incubation, production of pregnenolone was measured by radioimmunoassay.

**Fig. 6.** The effect of antibody on side chain cleavage activity of soluble P-450 and membrane-bound side chain cleavage system. Antiserum was mixed with the P-450 (soluble or membrane-bound 10 µg of side chain cleavage protein flask) for 3 h at 4°C; TPNH (20 nmol) was then added to the vesicle preparation, while adrenodoxin (0.2 nmol), adrenodoxin reductase (10.0 dichlorophenolindophenol units) (21), and TPNH (200 nmol) were added to soluble P-450 (10 µmol of protein/flask). The mixtures were incubated for 20 min. Following incubation, pregnenolone was measured by radioimmunoassay. The two different scales on the ordinate should be noticed.
inhibits enzyme activity of the membrane-bound side chain cleavage system. This antiserum was used to demonstrate the immunochemical homogeneity of soluble P-450 in a previous study. It will be seen that the antibody inhibits enzyme activity of both soluble and enzyme-bound P-450 over the same concentration range of antibody. Antibodies to P-450 11β-hydroxylase (22) were without effect on membrane-bound P-450 side chain cleavage.

Spectral Properties—Fig. 7 compares the absolute spectra of soluble and membrane-bound P-450 in the Soret region. It will be seen that in the oxidized form, the spin state of the P-450 has been altered from predominantly high spin in the soluble enzyme to predominantly low spin when the soluble enzyme becomes membrane-bound. The reduced CO spectra show the usual peak at 447 nm (3) in both forms (data not shown). The ratio \(A_{447}:A_{422}\) is 2.4 for the soluble enzyme and 2.3 for the membrane-bound form. A small amount of P-420 is seen in the membrane-bound form, but the soluble enzyme is free of P-420. No obvious difference was seen in the rate of reduction of the soluble and membrane-bound forms of the enzyme by dithionite. The membrane-bound P-450 could also be reduced by TPNH, adrenodoxin, and adrenodoxin reductase. All of the heme in both forms of the enzyme (measured as pyridine hemochromogen) was accounted for by CO-difference spectroscopy within the limits of experimental error using an extinction coefficient of 91,000 m\(^{-1}\) cm\(^{-1}\).

Electron Microscopy

The phospholipid vesicles were seen as spherical structures of 350 to 405 Å in diameter (Fig. 8). Some of the vesicles are approximately twice this size, suggesting fusion of two vesicles. Larger clumps result from more extensive fusion which occurs on standing. This example was examined 4 h after overnight dialysis. The appearance of the membrane is compatible with a bilayer organization (23).

DISCUSSION

The present experiments show that the highly purified soluble cytochrome P-450 from bovine adrenocortical mitochondria which catalyzes the conversion of cholesterol to pregnenolone can be attached to a phospholipid membrane in such a manner that a number of the properties of the enzyme are altered. The apparent \(V_{\text{max}}\) of the membrane-bound enzyme is an order of magnitude higher than that of the soluble enzyme and enzyme activity is more stable at 4°C when the P-450 is associated with a membrane. The term apparent \(V_{\text{max}}\) serves to emphasize the complexity of the side chain cleavage reaction, the insolubility of the substrate, and the additional step of association between cholesterol and the membrane. In spite of these complications, it can be seen that the enzyme is considerably more active in the membrane than in soluble form (Fig. 5). These findings are observed with membranes reconstituted from a crude extract of mitochondrial lipids (including cholesterol) and with membranes composed of synthetic phospholipids. The use of pure phospholipid provides an approach to studies concerning the structural requirements of these compounds for reconstituting catalytically active membrane-bound P-450 (with and without ancillary proteins) and of the role of cholesterol both as substrate for the enzyme and in modifying the properties of the membrane-bound side chain cleavage system.

Since the heme group of hemeproteins provides a sensitive index of the state of the protein in a given environment, this component of P-450 would be expected to reflect changes resulting from interaction with lipid. The soluble P-450 used in these studies show a mixed spin state (predominantly high) in aqueous media which changes to a predominantly low spin form in the membrane (Fig. 7). The membrane environment clearly influences the structure of the protein and this influence is communicated to the heme groups.

Although the details of the association between P-450 and the membrane are not known in molecular terms, the P-450 is not merely trapped within the interior of the vesicles since it remains with the membrane after lysis, in contrast to [\(^{14}C\)]glucose. Again, the P-450 is associated with the phospholipid during exclusion chromatography in two systems and during centrifugation through sucrose density gradients. These two procedures separate free cytochrome P-450 from that associated with the membranes while the density gradients also separate free vesicles from those containing P-450 (Fig. 3). Moreover, when P-450 is added to a phospholipid suspension without cholate, the protein and the lipid remain...
largely unassociated during chromatography and sedimentation (Figs. 2 and 3). In addition, the changes referred to above in the properties of the P-450 when it is associated with vesicles suggest that the immediate environment of the enzyme is considerably altered by association with the membrane so that it is reasonable to conclude that the P-450 molecule is at least partly inserted into the membrane structure.

The membrane-bound enzyme can interact with at least four substances introduced to the vesicles from without: cholesterol, adrenodoxin, dithionite, and anti-P-450. Presumably, that part of the P-450 which interacts with adrenodoxin is sufficiently exposed to be accessible to that protein without the aid of agents which would enable adrenodoxin to enter the membrane. As a result of this, membrane-bound P-450 can interact with the various factors necessary to permit catalytic activity (side chain cleavage) to occur when these factors are added to the water phase in which the vesicles are suspended (Table I). It is also apparent that membrane-bound P-450 presents to the external aqueous phase that part of the molecule which interacts with anti-P-450. Similarly, dithionite reduction of the membrane-bound enzyme occurs at about the same rate as that seen with soluble P-450; this rate is too rapid to be accounted for by diffusion of dithionite into the membrane. Finally, cholesterol can reach the enzyme whether it is incorporated into the membrane or added to the aqueous phase.

Electron microscopy reveals vesicles of mean diameter, approximately 350 to 450 Å. The vesicles appear relatively uniform in size and without added protein show a mean density of 1.027 ± 0.003 (range). Addition of P-450 to the membrane increases the density of the vesicles and enables them to be readily separated from free vesicles and from soluble P-450. The vesicles appear to be well sealed since [14C]glucose is associated with the vesicles and shows a symmetrical distribution without tailing which is a classical criterion for the absence of leaky vesicles in such preparations (19).

It is interesting to observe incorporation of adrenodoxin and adrenodoxin reductase into vesicles made either from pure phospholipids or mitochondrial lipid. A preliminary report has shown that hepatic microsomal P-460 and P-450 reductase can be incorporated into vesicles of phosphatidylcholine, although in this case, the enzyme must be kept in detergent when released from the original membrane (18). The nature of the association between the membrane and the two ancillary proteins is not known at present, but this association is maintained during sedimentation through sucrose gradients, and the high Vmax suggests that the side chain cleavage system may be organized in the vesicles in a manner which approaches that seen in mitochondria in vivo. The maximal lipid/protein weight ratio of 0.5 indicates that as many as 40 phospholipid molecules may be present for each protein subunit (53,000 daltons). This value is in the range observed with other enzymes inserted into membranes (19, 24).

The vesicular preparation described here is subject to many modifications, including variations in lipid mixtures in the ratio of lipid to protein and in the conditions used for the formation of the vesicles. Vesicles can be prepared by sonication following a well documented method (23); the properties of the enzyme associated with these vesicles are indistinguishable from those prepared by Vortex mixing. Our best preparations contain small amounts of P-420 revealed in absolute spectra of CO-reduced P-450, although P-420 is not detected in the less sensitive CO-difference spectra (data not shown). The changes in the properties of P-450 in the vesicles indicate the importance of a lipid environment for the function of the enzyme under conditions more closely resembling the situation in vivo and serve as a caution in interpreting data obtained with the soluble enzyme. Finally, advanced techniques of electron microscopy, such as freeze-etching together with antibodies to the three purified side chain cleavage proteins, can be used to explore the distribution of these proteins in the membrane.

Acknowledgment—We are grateful to Dr. Larry Vtckery for valuable discussions during the preparation of this manuscript.

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