Quantitative Resolution of Succinate-Cytochrome c Reductase into Succinate-Ubiquinone and Ubiquinol-Cytochrome c Reductases*

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L. Yü and C. A. Yü
From the Department of Chemistry, State University of New York at Albany, Albany, New York 12222

A purified, active succinate-ubiquinone reductase was prepared from succinate-cytochrome c reductase without damage to ubiquinol-cytochrome c reductase by 1.1% Triton X-100 solubilization at pH 8.0, and calcium phosphate column chromatography in 50 mM Tris-succinate buffer, pH 8.0, containing 30 mM potassium phosphate. Succinate-ubiquinone reductase thus obtained contains ubiquinone and catalyzes thenoyltrifluoroacetone-sensitive oxidation of succinate by 2,6-dichlorophenolindophenol in the absence of exogenous mediator. Addition of ubiquinone enhanced the activity about 50%. Analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that the enzyme contains four polypeptides. The high molecular weight polypeptide contaminants usually observed in the Complex II preparation obtained by the reported method were absent. The active succinate-ubiquinone reductase can reconstitute with the cytochrome b-III complex, or Complex III to form succinate-cytochrome c reductase in the absence of exogenous ubiquinone or with the resolved ubiquinol-cytochrome c reductase in the presence of ubiquinone and phospholipids. Under the proper conditions, all the original succinate-cytochrome c reductase was obtained, indicating that the resolution caused no damage to the protein, despite the removal of phospholipids and ubiquinone from the ubiquinol-cytochrome c reductase region.

Study of the electron transfer mechanism of the mitochondrial respiratory chain has been greatly simplified by the development of methods for preparation of individual electron transfer complexes such as NADH-ubiquinone (1), succinate-ubiquinone (3), ubiquinol-cytochrome c (3, 4) reductases, and cytochrome c oxidase (5). Among the methods reported for isolation of these complexes, the method for preparation of succinate-Q reductase (Complex II) has been the least satisfactory. The yield has usually been low and purification was complicated by the use of organic solvent fractionation. Therefore, an improved method for isolation of Complex II is needed in order to facilitate study of the electron transfer mechanism between succinate and ubiquinone.

Since succinate-cytochrome c reductase (6) is much easier to prepare than is Complex II and is composed of succinate-ubiquinone reductase and ubiquinol-cytochrome c reductase, one would expect that a resolution of succinate-cytochrome c reductase should yield two individual reductases. However, this expectation had never materialized. The recent successful fractionation of succinate-cytochrome c reductase (6) into succinate dehydrogenase, ubiquinone-binding protein and ubiquinol-cytochrome c reductase, and the reconstitution of the first two enzyme fractions to form an active succinate-ubiquinone reductase strengthened our confidence that succinate-cytochrome c reductase could be fractionated into succinate-ubiquinone and ubiquinol-cytochrome c reductases, and prompted us to undertake this investigation.

Following a careful examination of the conditions (7, 8) used for the reconstitution of succinate-ubiquinone reductase from soluble succinate dehydrogenase and purified QPs, and the relative stability of the reconstituted enzyme complex in various environments, we have developed a simple method to resolve succinate-cytochrome c reductase into succinate-ubiquinone reductase and ubiquinol-cytochrome c reductase quantitatively by calcium phosphate column chromatography in the presence of succinate and Triton X-100. Both resolved reductases were in active form, except that ubiquinone and phospholipids were partially depleted from the latter. In this paper we wish to report the method for preparation and some properties of succinate-ubiquinone reductase. No special attention will be given to the resolved ubiquinol-cytochrome c reductase as its properties are similar to those of phospholipid- and ubiquinone-depleted ubiquinol-cytochrome c reductase preparation (9).

MATERIALS AND METHODS

The submitochondrial particles from beef heart were prepared essentially according to the reported method (10) except that no sucrose was used during homogenation of ground trimmed heart meat and the submitochondrial particles were collected from the supernatant solution of homogenate by acid (pH 5.8) precipitation and centrifugation at 4,000 rpm in a Beckman J-6 centrifuge, rotor 4.2 for 30 min. The collected pellets were washed once with 10 mM Na/K-phosphate buffer, pH 7.4, suspended in 10 mM phosphate/borate buffer, pH 7.8, containing 0.25 M sucrose and stored at -20 °C until use.

The preparation of succinate-cytochrome c reductase from frozen submitochondrial particles was essentially as reported (6), with modifications. Frozen submitochondrial particles were thawed and washed with 0.1 mM borate/phosphate buffer, pH 7.8, and resuspended in the same buffer to a protein concentration of 20 mg/ml (or to cytochrome b content of 11 μM) and solubilized with Na cholate (4 μl of 20% Na cholate/100 ml of submitochondrial particles suspension). To this suspension, solid ammonium sulfate was added to 35% saturation (19.65 gm/100 ml), and stirred for 60 min before being centrifuged at 12,000 rpm for 90 min in a Beckman J-22 centrifuge, rotor JA-14. The supernatant thus collected was brought to 50% ammonium sulfate saturation (8.9 gm/100 ml) and centrifuged at 14,000 rpm in the same centrifuge for 20 min. The pellet thus collected was dissolved

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† Present address, Department of Biochemistry, Oklahoma State University, Stillwater, OK 74078.

1 The abbreviations used are: QPs, ubiquinone-binding protein in succinate-ubiquinone reductase; DCIP, 2,6-dichlorophenolindophenol; R, red relative electrophoretic mobility based on cytochrome c in the Swank and Munkres gel system.
in 50 mM Na/K-phosphate buffer, pH 7.4, containing 0.5% sodium cholate and 1 mM EDTA, to a cytochrome b content of approximately 37 μM and stored at 5 °C overnight. The solution was then centrifuged at 40,000 rpm for 30 min in a Beckman centrifuge L-50B, rotor 50-2-Ti. The dark red supernatant was collected and dialyzed against volumes of Ti. The dark red supernatant was collected and dialyzed against 37°C at centrifuge, model content of mM Tris-C1 buffer, pH cytochrome c reductase was obtained in the pellet following centrifugation of the dialyzed solution at 20,000 rpm for 1 h in the Beckman centrifuge, model J2-21, JA-20 rotor. The pellet was suspended in 50 mM Tris-Cl buffer, pH 7.8, containing 0.67 mM succrose. The succinate-cytochrome c reductase at this stage had a specific cytochrome b content of 2-2.5 nmol/mg of protein. Further purification of succinate-cytochrome c reductase was achieved by ammonium acetate fractionation in the presence of deoxycholate. The crude succinate-cytochrome c reductase was diluted in 50 mM Tris-Cl buffer, pH 7.8, containing 0.67 mM succrose to a protein concentration of approximately 20 mg/ml (estimated by 75% absorption at 278 nm in 1% sodium dodecyl sulfate), and the protein was solubilized with deoxycholate (0.5 mg/mg of protein). The solubilized reduced was incubated at 0°C for 20 min before fractionation with ammonium acetate. A 50% saturated solution of ammonium acetate was used (11). Fractions between 0-6, 8-13, 15-16.5% of ammonium acetate saturation were collected by centrifuging at 20,000 rpm for 30 min in a Beckman centrifuge, model J2-21, JA-20 rotor, and discarded. The purified succinate-cytochrome c reductase, which precipitated between 16.5 and 33.5% ammonium acetate saturation, was centrifuged at 35,000 rpm for 30 min in a Beckman centrifuge, L-50B, 50.2-Ti rotor, dissolved in 50 mM phosphate buffer, pH 7.4, containing 0.25 mM succinate, and dialyzed against the same buffer, overnight, before being stored at -70°C.

The enzymatic activity assays for succinate-ubiquinone reductase (6), succinate-cytochrome c reductase (5), and ubiquinol-cytochrome c reductase (6), and determination of protein concentration (6, 13), cytochrome c (13), cytochrome b (14), ubiquinone (15), phospholipids (16), non-heme iron (17), and flavin (18) content were carried out by the reported methods. Analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to both Weber and Osborn (19) and Swank and Munks (20) methods. Cytochrome c was used as an electrophoretic mobility reference.

The calcium phosphate was prepared according to Jenner (21), and usually aged at 4°C for at least 2 weeks before using. The calcium phosphate was mixed with an equal amount of cellulose powder (w/w) to ensure an adequate flow rate. The column was packed and equilibrated with 2 bed volumes of buffer at room temperature then placed in the cold room (4°C), and further equilibrated with one bed volume of buffer. The equilibrating buffer used was 50 mM Trissuccinate, pH 8.0, containing 30 mM potassium phosphate and 0.5% Triton X-100.

Horse cytochrome c, type III, potassium deoxycholate, and sodium cholate were purchased from Sigma; ammonium sulfate from Mann; and other chemicals were obtained commercially at the highest purity available.

The ubiquinone derivative, 6-(10-bromodecyl)ubiquinone (ubiqui-
one,(CH₂)₂Br), used in the enzymatic activity assay of succinate-ubiquinone reductase was synthesized in our laboratory (22).

RESULTS AND DISCUSSION

Properties of Succinate-Cytochrome c Reductase—The separation of succinate-cytochrome c reductase is based on the positive and negative absorption properties of succinate-ubiquinone reductase and ubiquinol-cytochrome c reductase on the calcium phosphate column under given conditions. Therefore, most of the impurities present in the succinate-cytochrome c reductase which are not absorbed on the calcium phosphate column will appear in the effluent with succinate-ubiquinone reductase. A high-purity succinate-cytochrome c reductase is necessary in order to obtain a pure preparation of succinate-ubiquinone reductase. The succinate-cytochrome c reductase used in this report, prepared by the described method, has a purity of cytochrome b of 4 to 4.5, and cytochrome c of 2.5 to 2.7 nmol/mg of protein; and an antimycin A-sensitive specific activity of 8 μmol of cytochrome c reduced/mg of protein/min at 23°C. The succinate-cytochrome c reductase is free from contamination by cytochrome c oxidase or NADH dehydrogenase.

Although the purity of succinate-cytochrome c reductase obtained by the present method is about the same as that of the original method (23), which starts from Keilin-Hartree heart muscle preparation, the activity is consistently higher than that of the original method. Since a second cycle of ammonium sulfate fractionation in the presence of Na cholate, which is known to cause removal of phospholipids (24) was involved in the original method, considerable care and rapidity were needed in order to get a highly active enzyme preparation. The insertion of an additional dialysis step in the modified method is obviously time consuming but is required for the following step in which deoxycholate and Tris-Cl buffer were used. Replacement of ammonium sulfate fractionation with ammonium acetate fractionation prevented the loss of phospholipids during fractionation and thus ensured a more active and more highly purified preparation.

The use of submicrosomal particles prepared by the acid precipitation method in the isolation of succinate-cytochrome c reductase of heart muscle preparation has some advantages over the well known Keilin-Hartree preparation: simplicity, and the ready availability of large scale apparatus. The described submicrosomal preparation can also be made from frozen heart muscle. The preparation can be stored at -20°C for months without loss of activity.

Separation of Succinate-Cytochrome c Reductase into Succinate-Ubiquinone Reductase and Ubiquinol-Cytochrome c Reductase—Five ml of frozen succinate-cytochrome c reductase (14 mg/ml) was thawed and mixed with 0.3 ml of 1 mM Tris-succinate, pH 8.0, and 0.4 ml of 15% Triton X-100 solution. The mixture was then incubated at 0°C for 1 h and applied to a calcium phosphate column (2.2 x 5 cm) equilibrated with 50 mM Tris-succinate, pH 8.0, containing 30 mM phosphate and 0.5% Triton X-100. The flow rate of the column was adjusted to about 0.3 ml/min. The sample was followed by 10 ml of equilibrating buffer, and the column was washed with 30 ml of 50 mM Tris-succinate buffer, pH 8.0, containing 30 mM KPO₄, 10% glycerol, and 0.2% Emasol-1130 (washing buffer). All the succinate-ubiquinone reductase activity was collected in the effluent (13 ml) and the ubiquinol-cytochrome c reductase was absorbed on the column. Succinate-ubiquinone reductase thus obtained was diluted with an equal volume of water, adjusted to pH 6.0 with 0.5 M acetic acid, and applied to a second calcium phosphate column (1.6 x 5 cm), which was equilibrated with 50 mM Tris-succinate buffer, pH 6.0. The succinate-ubiquinone reductase was absorbed on the column. The Triton X-100 present in the enzyme was washed off with 30 ml 50 mM Tris-succinate buffer, pH 6.0, containing 10% glycerol and 0.2% Emasol-1130. After washing, succinate-ubiquinone reductase was eluted with 0.2 M potassium phosphate, pH 7.4, in the same equilibrating buffer. In this way, a Triton-free, ubiquinone-containing succinate-ubiquinone reductase was obtained.

The ubiquinol-cytochrome c reductase which was absorbed on the first calcium phosphate column was eluted with 0.2 M KPO₄ in 50 mM Tris-succinate buffer, pH 8.0, containing 10% glycerol and 0.2% Emasol-1130. The ubiquinol-cytochrome c reductase thus obtained contains very little Triton X-100. If a Triton-free ubiquinol-cytochrome c reductase is desired, the amount of washing buffer used is increased to 45 ml before eluting the enzyme. If replacement of Triton X-100 with Emasol-1130 or Tween 80 is not desired, the washing step can be omitted and the ubiquinol-cytochrome c reductase can be eluted right after removal of succinate-ubiquinone reductase. The presence or absence of glycerol in the equilibrating buffer used to wash the column following the appli-
culation of sample made little difference in the recovery of succinate-ubiquinone reductase. The extensive washing, however, caused a more complete removal of ubiquinone and phospholipids from the absorbed ubiquinol-cytochrome c reductase, and thus destabilized the enzyme. The presence of glycerol in the washing buffer protected the ubiquinol-cytochrome c reductase from denaturation (9).

Enzymatic activity determination showed that all the activity of succinate-ubiquinone reductase and part of the ubiquinol-cytochrome c reductase was recovered in the corresponding fractions. The full ubiquinol-cytochrome c reductase activity, however, was restored after addition of exogenous ubiquinone and phospholipids, indicating that the preparation was partially delipidated. Table I summarizes the purification data of succinate-ubiquinone and ubiquinol-cytochrome c reductases. Upon mixing these two fractions, in proportion to their resolved volumes, an antimycin A-sensitive succinate-cytochrome c reductase activity was reconstituted from the resulting succinate-ubiquinone reductase and ubiquinol-cytochrome c reductase was less than the original succinate-cytochrome c reductase activity, in the absence of exogenous ubiquinone. In the presence of exogenous ubiquinone and phospholipids, however, the activity was the same as the original, indicating that under the described conditions no essential components of succinate-cytochrome c reductase have been irreversibly denatured, although ubiquinone and phospholipids are partially dissociated from ubiquinol-cytochrome c reductase.

Besides Triton X-100, other non-ionic detergents, such as Emasol-1130, were also found to be effective in resolving succinate-cytochrome c reductase into succinate-ubiquinone and ubiquinol-cytochrome c reductases. Table II compares the effectiveness of commonly used detergents in the resolution of succinate-cytochrome c reductase.

The use of the hydroxyapatite column and Triton X-100 in preparation of inactive (22) and active (26) ubiquinol-cytochrome c reductase from beef heart or succinate-ubiquinone reductase (27, 28) from microbial systems has been reported, but the simultaneous isolation of both succinate-ubiquinone reductase and ubiquinol-cytochrome c reductase has not been mentioned before. The calcium phosphate column has been used to prepare cytochrome c, QPs, and cytochrome b in our laboratory but under conditions which caused cytochrome b to separate from cytochrome c and appear with QPs in the effluent (8).

The success of the described method for separation of succinate-cytochrome c reductase into succinate-ubiquinone reductase and ubiquinol-cytochrome c reductase relies on the introduction of succinate, which apparently keeps succinate dehydrogenase, and perhaps ubiquinone, in the reduced form. This prevents the separation of succinate dehydrogenase from QPs and stabilizes the succinate-ubiquinone reductase during the calcium phosphate column chromatography.

Properties of Succinate-ubiquinone Reductase—Table III compares the chemical compositions of succinate-ubiquinone obtained by the present method and those prepared by the reported method (Complex II) (2). Succinate-ubiquinone reductase obtained by the present method contains 5.8 nmol of FAD, 2 nmol of ubiquinone, and 48 nmol of non-heme iron/mg of protein. These values are very close to those of the Complex II preparation.

The presence of ubiquinone in the reductase is not due to copurification or contamination. It is bound to protein and resists removal by the wash with 0.2% Emasol-1130 in 50 mM Tris-succinate buffer, pH 6.0. Since at least part of the ubiquinone and Triton X-100 were bound to succinate-ubiquinone reductase in the first calcium phosphate column effluent, the separation of Triton X-100 from protein without simultaneous removal of ubiquinone during the second calcium phosphate column chromatography indicates that ubiquinone is not only bound to protein but also shows that its binding to protein is stronger than that of Triton X-100. Since a 50% stimulation in activity was observed upon addition of exogenous ubiquinone in the assay mixture, the purified succinate-ubiquinone reductase is clearly somewhat ubiquinone-deficient.

The enzymatic activity tracings in the presence and absence of exogenous ubiquinone are given in Fig. 1. The reduction of DCIP by succinate catalyzed by succinate-ubiquinone reductase in the absence of exogenous ubiquinone is very DCIP concentration-dependent. The activity decreased rapidly as the substrate became partially reduced. In the presence of exogenous ubiquinone, no such DCIP concentration-dependent phenomenon was observed. These results suggest that DCIP accepts electrons more readily from free ubiquinone, which is available when exogenous ubiquinone is added in the assay mixture, than from succinate-ubiquinone reductase bound ubiquinone or even directly from the enzyme. In the absence of exogenous ubiquinone, succinate-ubiquinone reductase activity is also very slow.

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### Table III

**Comparison of chemical composition of succinate-ubiquinone reductase preparations from beef heart**

<table>
<thead>
<tr>
<th>Components</th>
<th>Succinate-Q reductase</th>
<th>Present method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ziegler &amp; Doeg (1962)</td>
<td>Present method</td>
</tr>
<tr>
<td>Flavin</td>
<td>4.8-5.0</td>
<td>5.8</td>
</tr>
<tr>
<td>Non-heme iron</td>
<td>36-38</td>
<td>48</td>
</tr>
<tr>
<td>Cytochrome $b$</td>
<td>4.5-4.8</td>
<td>1.2</td>
</tr>
<tr>
<td>Ubiquinone</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>0.2 mg/mg</td>
<td>0.38 mg/mg</td>
</tr>
</tbody>
</table>

**Fig. 1.** Kinetic assay of succinate-ubiquinone reductase and its inhibition by thenoyltrifluoroacetone (TTF-A). The activity was traced in the presence (---) and in the absence (- - -) of exogenous ubiquinone. The concentrations of DCIP, ubiquinone, and TTF-A used in the assay mixture were 50 $\mu$M, 38 $\mu$M, and 1 $\mu$M, respectively. 0.8 $\mu$g of purified succinate-Q reductase (SQ-R) was used.

Succinate can reduce DCIP only when the dye is in high concentration and in highly oxidized form.

Spectral properties of succinate-ubiquinone reductase are given in Fig. 2. The Soret absorption maximum of the oxidized form was at 412 nm, and shifted to 422 nm when the preparation was reduced by sodium dithionite. The absorption maximum of the reduced enzyme was found at 560 nm. In contrast to cytochrome $b_{560}$ of Complex II prepared according to Hatefi and Galante (29), the cytochrome $b$ of this preparation is not succinate reducible, even at high concentrations of succinate. The dithionite reduced enzyme was not sensitive to treatment with carbon monoxide. This differs from the cytochrome $b$ present in QPs, which shows a reduced cytochrome $b$ spectrum completely sensitive to carbon monoxide.

As indicated in Fig. 2, the spectrum of the reduced form of succinate-ubiquinone reductase shows a small shoulder at 552 nm, indicating that a trace amount of cytochrome $c_1$, which was not ascorbate reducible was present in the preparation. The amount of cytochrome $c_1$ was estimated to be about 0.1 nmol/mg of protein.

Since cytochrome $b$ in succinate-ubiquinone and ubiquinol-cytochrome $c$ reductases are not reactive with carbon monoxide, nor are the cytochromes $b$ in the starting material, succinate-cytochrome $c$ reductase, a quantitative recovery of cytochromes $b$ in succinate-ubiquinone reductase and ubiquinol-cytochrome $c$ reductase is expected, and indeed that was the case. The amount of cytochrome $b$ recovered in the succinate-ubiquinone reductase fraction, however, was very small, amounting to less than 10% of that present in succinate-cytochrome $c$ reductase. The majority of cytochrome $b$ (> 90%) and cytochrome $c_1$ was retained in ubiquinol-cytochrome $c$ reductase. As expected, all the flavin was recovered in succinate-ubiquinone reductase preparation. This highly uneven distribution of flavin and cytochrome $b$ between succinate-ubiquinone and ubiquinol-cytochrome $c$ reductases suggests that a catalytic role for cytochrome $b$ in the former is unlikely.

The quantitative recovery of cytochromes $b$ and $c_1$ from the resolved succinate-ubiquinone reductase and ubiquinol-cytochrome $c$ reductase indicates that no change in the molar extinction coefficients of the cytochromes has occurred during the separation of succinate-ubiquinone reductase and ubiquinol-cytochrome $c$ reductase. This result contrasts with the earlier observation that splitting of cytochrome $b$ from cytochrome $c_1$ is concomitant with a reduction of molar extinction coefficient (30), thus invalidating the unique stoichiometry between FAD and cytochrome $b$ observed in Complex II (29). The higher concentration of cytochrome $b$ in Complex II resulted partly from use of a lower molar extinction coefficient (29) in computations of cytochrome $b$ content.

The subunit structure of succinate-ubiquinone reductase was similar to that of the well known Complex II (31) except that the two major contaminants in Complex II, often referred to as core proteins of Complex III, are not present in the succinate-ubiquinone reductase prepared by the present method. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis pattern of succinate-ubiquinone reductase is given in Fig. 3. As can be seen in Fig. 3, in the Swank-Munkres gel electrophoresis pattern of succinate-ubiquinone reductase is given in Fig. 3. As can be seen in Fig. 3, in the Swank-Munkres gel...
Succinate Ubiquinone Reductase

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis pattern of isolated succinate-ubiquinone reductase. The scan was taken at 550 nm on a Cary spectrophotometer, model 219. The relative mobility is referenced to that of cytochrome c. A, the Weber and Osborn system; B, the Swank and Munkres system. 100 μg of protein was used in each electrophoretic column.

system (Fig. 3B), the two low molecular weight bands of succinate-ubiquinone reductase in the Weber and Osborn system (Fig. 3A) are further separated into three protein bands. The correlation between these three bands and the two bands of the Weber-Osborn system is not yet established. However, the third band (R = 1.11) in the Swank-Munkres gel system has been identified to be the ubiquinone-binding protein (8, 32).

Properties of Ubiquinol-Cytochrome c Reductase—As indicated in Table I, the purity of ubiquinol-cytochrome c reductase obtained by this method is lower than that obtained from the soluble cytochrome b-c₃ complex by ammonium acetate fractionation in the presence of deoxycholate (6). The activity, without addition of ubiquinone and phospholipids, is also lower than that of the preparation obtained by the reported method. Addition of ubiquinone and phospholipids restored the activity to original level, indicating that the preparation is ubiquinone and phospholipid-depleted. When the washing step before the elution of ubiquinol-cytochrome c reductase was deleted, the loss of phospholipids was much less and more activity was retained, although the activity was still much lower than that of the lipid-sufficient preparation (6).

Since the separation of succinate-ubiquinone reductase from ubiquinol-cytochrome c reductase under the described conditions caused the removal of phospholipids and ubiquinone from the latter, the presence of glycerol during the washing and eluting of ubiquinol-cytochrome c reductase is necessary in order to prevent irreversible denaturation of ubiquinol-cytochrome c reductase (9).

Although the purity of this preparation is inferior to the reported ubiquinol-cytochrome c reductase (the cytochrome b-c₃ complex) (6), its delipidated nature provides a good opportunity for the study of protein-lipid interaction. It is believed that this method can be extended to preparation of a lipid-depleted cytochrome b-c₃ complex if the cytochrome b-c₃ complex were to be subjected to the same treatment. Perhaps the combination of Triton X-100 and calcium phosphate column at a selected pH can be used for the removal of phospholipids from other lipoprotein complexes.

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