Isolation and Reconstitution of the Iron-Sulfur Protein in Ubiquinol-Cytochrome c Oxidoreductase Complex

PHOSPHOLIPIDS ARE ESSENTIAL FOR THE INTEGRATION OF THE IRON-SULFUR PROTEIN IN THE COMPLEX*

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An iron-sulfur protein has been purified from beef heart ubiquinol-cytochrome c oxidoreductase (Complex III) of the mitochondrial respiratory chain by phenyl-Sepharose column chromatography and Sephacryl S-200 gel chromatography. Depletion of most of the endogenous phospholipids in the complex was a prerequisite to the dissociation of the protein from the complex in the former chromatography. The iron-sulfur protein was nearly homogeneous as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and contained 76 ng atoms of nonheme iron and 66 nmol of acid-labile sulfide/mg of protein. When this preparation was incubated with an iron-sulfur protein-depleted complex in the presence of soybean phospholipids, the enzymic activity was restored up to 90% of that of the parent Complex III, whereas the recovery of the activity was marginal in the absence of the phospholipids. Thus it is clear that the iron-sulfur protein is integrated into the complex with the aid of phospholipids.

Ubiquinol-ferricytochrome c oxidoreductase (Complex III), which mediates electron transport from NADH-ubiquinone oxidoreductase and succinate-ubiquinone oxidoreductase to cytochrome c oxide of the mitochondrial respiratory chain, contains cytochrome b, cytochrome c1, and an iron-sulfur protein as the main redox-active components (1). The iron-sulfur protein was first isolated in a succinylated form from Complex III and shown to possess a 2Fe-2S cluster by Rieske et al. (2). Trumpower et al. (3, 4) purified the iron-sulfur protein from succinate-cytochrome c reductase complex and demonstrated that ubiquinol-cytochrome c reductase activity is restored by reconstituting the protein into the reductase complex which had been depleted of it. Their purification method consisted of many steps, and guanidine was used to isolate the iron-sulfur protein from the reductase complex. The reconstituted activity was not high compared to the activity of the parent reductase complex, probably due to the denaturing effect of guanidine. Recently, Engel et al. (5) have reported a relatively simple method for purification of the protein from Complex III. Their method of dissociation of the complex was similar, in use of a chaotropic agent, to that of Trumpower and Edwards (3) in that urea was used in place of guanidine.

In the course of our study on the interaction between Complex III and phospholipids, we have recently used phenyl-Sepharose column chromatography to deplete the complex of phospholipids under mild conditions (6) and observed that when the complex from which most of the phospholipids have been removed is further subjected to affinity chromatography on cytochrome c-Sepharose, the complex is deprived of the iron-sulfur protein (7). This finding means that the removal of phospholipids facilitates the release of the iron-sulfur protein from Complex III.

In the present study, we have developed a novel method for purifying the iron-sulfur protein from Complex III by the hydrophobic interaction chromatography without using any chaotropic agent. Since an iron-sulfur protein-depleted complex with a limited amount of phospholipids has also been obtained by the same chromatography, it has become possible to test the effect of phospholipids on the reconstitution. In this paper, evidence is presented to show that phospholipids are essential for the incorporation of the iron-sulfur protein into the depleted complex and for the recovery of ubiquinol-cytochrome c reductase activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Complex III was prepared from beef heart mitochondria by the method of Rieske et al. (8), suspended at approximately 50 mg of protein/ml in 25 mM Tris-Cl, pH 8.0, containing 20% glycerol, and stored at −80°C until use. This preparation contained 3.7 nmol of cytochrome c1/mg of protein and 15.3% (w/w) phospholipids, and the enzymic activity was 194 µmol of cytochrome c reduced/min/mg of protein at ambient temperature (−22°C) when ubiquinol-2 was used as a substrate. Deoxycholate, cholate, antimycin A, cytochrome c (Type III), and a soybean phospholipid mixture (containing 22% L-α-phosphatidylcholine) were obtained from Sigma. The deoxycholate and cholate were recrystallized from 50% hot ethanol. The soybean phospholipid mixture was sonicated at a concentration of 5 mg/ml in 25 mM potassium phosphate, pH 7.5, containing 25 µM EDTA beforehand. Phenyl-Sepharose CL-4B, Sephacryl S-200 superfine, and Sephacryl S-300 superfine were the products of Pharmacia Fine Chemicals, Uppsala. Ultralifier UP-20 for ultracentrifugation was obtained from Toyo Kagaku Sangyo Co., Ltd., Osaka, and Tween 20 from Wako Pure Chemical Industries, Ltd., Osaka. Ubiquinone-2 was supplied from Fuso Co., Ltd., Tokyo, and ubiquinol-2 was prepared by the method of Rieske (9). Other chemicals used were of reagent grade.

**Analytical Methods**—The quantities of cytochromes b and c1 were estimated from the difference absorption spectrum of the dithionite-reduced minus ascorbate-reduced form and that of the ascorbate-reduced minus ferricyanide-oxidized form, respectively. Absorbance measurements were performed using a Shimadzu UV-VIS recording spectrophotometer, model UV-250. The millimolar extinction coefficients used were 28.5 for A553-540 for cytochrome b (10) and 17.5 for A450-460 for cytochrome c1 (11). Nonheme iron and total iron were

*This investigation was supported in part by Grants-in-Aid for Special Project Research (311909) from the Ministry of Education, Science and Culture, Japan. 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.
determined by the method of Brumby and Massey (12), and acid-
labile sulfide by the method of Rabizowit (13). Protein was assayed by
the Lowry method modified by Hartree (14) using bovine serum
albumin as a standard. The total phospholipid content was calculated
from the amount of total phosphorus determined by the method of
Chen et al. (15), assuming the average phospholipid molecular weight as
720 (16). SDS-polyacrylamide gel electrophoresis was performed
on disc gels as described by Merle and Kadenbach (17) or on slab
gels according to the method of Kadenbach et al. (18). Samples for
electrophoresis were treated in 10 mM Tris-phosphate buffer, pH 8.3,
containing 4% SDS, 8 M urea, 40 mM dithiothreitol, and 10% glycerol
at 37 °C for 1 h. After electrophoresis, the gels were stained with
Coomassie Brilliant Blue.

Separation of Complex III into the Iron-Sulfur Protein and the
Iron-Sulfur Protein-depleted Complex—All the following procedures
were carried out at 0-4 °C, unless otherwise stated. Complex III (113
mg of protein) was applied to a phenyl-Sepharose column (1.5 x 11
cm) equilibrated with 25 mM Tris-Cl, pH 8.0, containing 0.25%
deoxycholate, 0.2 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, and
20% glycerol. The complex bound to the column was washed with
700 ml of the same buffer to remove phospholipids, and then brown-
ish-pink-colored protein (iron-sulfur protein) was eluted with 25 mM
Tris-Cl, pH 8.0, containing 1% deoxycholate, 1 mM dithiothreitol, and
20% glycerol. Approximately 40% of the applied protein was eluted in
a peak at the exclusion volume of the column, most of the proteins bound
to the column were eluted as the iron-sulfur protein-depleted complex with
25 mM Tris-Cl, pH 7.5, containing 3% Tween 20, 1 mM dithiothreitol,
and 20% glycerol. The proteins still remaining on the column were
eluted at room temperature with 25 mM Tris-Cl, pH 7.5, containing
2% SDS. The iron-sulfur protein fraction was concentrated by ultra-
filtration using an ultrafilter UP-20 and chromatographed on a
Sephacryl S-200 superfine column (1.6 x 75 cm) equilibrated with 0.1
M Tris-Cl, pH 7.5, containing 0.5% cholate, 0.1 M NaCl, 1 mM
dithiothreitol, and 20% glycerol. Approximately 40% of the applied
protein was eluted in a peak at the exclusion volume of the column,
and the rest of the protein was eluted subsequently in a broad peak.
Although both of the peaks contained the iron-sulfur protein, the frac-
tions at the exclusion volume which were rich in this protein were
combined and concentrated by ultrafiltration and used for analyses
and reconstitution experiments. The final preparation was stable for
at least 1 month at -30 °C. A portion of the iron-sulfur protein-
depleted complex was stored after mixing with a soybean phospholipid
mixture (1.7 mg of lipids/mg of protein) at -30 °C until use, because
the reconstitutive ability of the depleted complex was better retained
when the phospholipid mixture was present during the storage.

Reconstitution of the Iron-Sulfur Protein with the Iron-Sulfur
Protein-depleted Complex—A standard reconstitution experiment
was performed as follows. The purified iron-sulfur protein (20 μg)
was mixed at 0 °C with the depleted complex (76 μg) and a soybean phospholipid mixture (275 μg), and the volume of the mixture was
brought to 150 μl with 25 mM potassium phosphate buffer, pH 7.5,
containing 25 mM EDTA, and then the resulting mixture was incuba-
ted at 30 °C for 30 min.

Assay of Ubiquinol-Cytochrome c Reductase Activity—This activity
was assayed at ambient temperature (-22 °C) in 1 ml of reaction
medium containing 25 mM potassium phosphate, pH 7.5, 25 μM
EDTA, 25 μg of a soybean phospholipid mixture, 0.02% Tween 20,1
and 30 μM cytochrome c. Before starting the enzymic reaction, ubi-
quinal-2 was added to a concentration of 63 μM from a concentrated
ethanolic solution, and nonenzymic reduction of cytochrome c was
measured at 550 nm for about 30 s with a Gilford spectrophotometer,
model 250. Then the reaction was started by adding the enzyme, and the absorption of cytochrome c at 550 nm was recorded. The activity
of the enzyme was estimated from the rate of an initial linear portion
of the trace using the millimolar extinction coefficient of 18.5 at 550
nm for the reduced minus oxidized form of cytochrome c (19).

RESULTS

Separation of Complex III into the Iron-Sulfur Protein and
the Iron-Sulfur Protein-depleted Complex—Complex III is bound to a phenyl-Sepharose column equilibrated with buffer

1 The abbreviation used is: SDS, sodium dodecyl sulfate.
2 The addition of 0.02% Tween 20 in the assay medium increased
the enzyme activity by approximately 1.5-fold and decreased the
nonsense reduction of cytochrome c by ubiquinol-2.

containing 0.25% deoxycholate and 0.2 M NaCl, and most of
the phospholipids in the complex are washed out by passing
the same buffer through the column as reported previously
(7). In this study we examined the conditions for eluting the
iron-sulfur protein and the iron-sulfur protein-depleted com-
plex separately and found that buffer containing 1% deoxy-
cholate and no salt can elute brownish-pink-colored protein
(Fig. 1A). SDS-polyacrylamide gel electrophoresis indicated
that this protein preparation was mostly the iron-sulfur protein
(Mi, 27,000) contaminated with minor amounts of core
proteins (designated by Silman et al. (20), M, 49,000 and
47,000) (Fig. 1B). When buffer containing 3% Tween 20 was
subsequently passed through the column, the majority of the
proteins bound to the column were eluted (Fig. 1A). This
preparation contained only a trace of the iron-sulfur protein,
but the other polypeptides were present in the same com-
position as those of the original Complex III, as demonstrated
by SDS-gel electrophoresis (Fig. 2, A and D).

To examine the effect of depletion of phospholipids on the
dissociation of the iron-sulfur protein, chromatography on
phenyl-Sepharose was carried out in the same manner as in
the above experiment except that the column was washed to
a limited extent prior to elution of proteins. The results
indicated that only a small amount of the iron-sulfur protein
could be eluted by the buffer containing 1% deoxycholate and
no salt (Fig. 1B) and that the proteins eluted with the buffer
containing 3% Tween 20 contained a substantial amount of

FIG. 1. Phenyl-Sepharose column chromatography of Com-
plex III. A, Complex III bound to a phenyl-Sepharose column was
washed with 700 ml of buffer containing 0.25% deoxycholate and 0.2
M NaCl to deplete phospholipids. Then, the iron-sulfur protein was
eluted with buffer containing 1% deoxycholate (DCA) and no salt,
and the iron-sulfur protein-depleted complex was subsequently eluted
with buffer containing 3% Tween 20. The proteins still remaining on
the column were eluted with buffer containing 2% SDS. Approxi-
ately 5 ml of fractions were collected at a flow rate of 30 ml/h. B,
Complex III is chromatographed by the same procedure as in exper-
inent A, except for the washing step in which only 30 ml of the buffer
were passed through the column.
The Iron-Sulfur Protein of Complex III

A, original Complex III (9.0 μg); B, the iron-sulfur protein fraction eluted from a phenyl-Sepharose column (fractions 143–145 in Fig. 1A) (1.5 μg); C, the iron-sulfur protein purified by Sephacryl S-200 column chromatography (9.1 μg); and E, the complex eluted from a phenyl-Sepharose column without the intensive washing to deplete phospholipids (fractions 19–22 in Fig. 1B) (9.1 μg).

the iron-sulfur protein in addition to the rest of the polypeptides of Complex III (Fig. 2E). It is clear from these results that a critical condition for the dissociation of the iron-sulfur protein from Complex III is the extensive depletion of phospholipids.

When the iron-sulfur protein preparation obtained in the former chromatography (Fig. 1A) was subjected to gel chromatography on Sephacryl S-200, the iron-sulfur protein was eluted at the exclusion volume of the column, indicating that it exists in an aggregated state. The preparation thus obtained was nearly homogeneous as judged by SDS-gel electrophoresis (Fig. 2C).

Properties of the Purified Iron-Sulfur Protein and the Depleted Complex—Table I gives the purification profile of the iron-sulfur protein. The protein was purified 9.2-fold from Complex III with a yield of 16%. It contains 76.2 ng atoms of nonheme iron and 66.3 nmol of acid-labile sulfide/mg of protein. The molecular weight calculated from these values is in the range of 26,000–30,000 and is consistent with the value of 27,000 estimated from SDS-gel electrophoresis (Fig. 2C).

Reconstitution of the Isolated Iron-Sulfur Protein into the Depleted Complex—Since the iron-sulfur protein-depleted complex almost totally loses the enzymic activity to transport electrons from ubiquinol to cytochrome c (Table II), the reconstitution of the iron-sulfur protein into the depleted complex can be examined by monitoring recovery of the activity. When a mixture of the purified iron-sulfur protein, the depleted complex, and the soybean phospholipids was incubated at 30 °C as specified under “Experimental Procedures,” the activity increased with incubation time and reached the maximum in 30 min. The reconstituted activity increased gradually with increasing the concentration of the phospholipids, and the maximal activity was attained at more than 2 mg of the phospholipids/mg of protein of the depleted complex. The activity then increased further upon addition of FMN but the activity thus obtained was in the range of Control 100%.

![Fig. 3. Spectra of the purified iron-sulfur protein. Spectra were taken on a solution of the purified iron-sulfur protein (0.62 mg of protein/ml) in 0.1 M Tris-Cl, pH 7.5, containing 0.5% cholate, 0.1 M NaCl, 1 mM dithiothreitol, and 20% glycerol. Since the isolated iron-sulfur protein was in the reduced form, the spectrum of the oxidized form was obtained by addition of a minimum amount of ferricyanide. ---, the oxidized form; -- - - - , the reduced form. The inset shows the difference spectrum of the oxidized minus reduced form of the protein.](image-url)

showed that it contains 7.3 nmol of cytochrome b and 3.9 nmol of cytochrome c1/mg of protein and 0.6% (w/w) phospholipids and that the only phospholipid remaining in this complex is cardiolipin (1 mol of cardiolipin/mol of cytochrome c1) as reported previously (7).

### Table I

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein</th>
<th>Nonheme iron</th>
<th>Acid-labile sulfide</th>
<th>Purification (%)</th>
<th>Recovery (%)</th>
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<tr>
<td>Complex III</td>
<td>112.8</td>
<td>7.4</td>
<td>7.2</td>
<td>1</td>
<td>100</td>
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<tr>
<td>Phenyl-Sepharose column</td>
<td>4.5</td>
<td>62.0</td>
<td>51.3</td>
<td>7.1</td>
<td>28</td>
</tr>
<tr>
<td>Sephacryl S-200 superfine column</td>
<td>1.9</td>
<td>76.2</td>
<td>66.3</td>
<td>9.2</td>
<td>16</td>
</tr>
</tbody>
</table>

* Degree of purification and recovery are calculated from the specific content of acid-labile sulfide.

* This value is calculated on the basis of total iron content, because the nonheme iron could not be measured by contamination of EDTA in this preparation.
TABLE II

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Enzymic activity (μmol cytochrome c reduced/min/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent Complex III</td>
<td>52.4</td>
</tr>
<tr>
<td>Depleted complex</td>
<td>6.6</td>
</tr>
<tr>
<td>Depleted complex + iron-sulfur protein</td>
<td>46.8</td>
</tr>
<tr>
<td>+ phospholipids</td>
<td>0.8</td>
</tr>
<tr>
<td>Depleted complex + iron-sulfur protein + phospholipids</td>
<td>6.6</td>
</tr>
<tr>
<td>+ antimycin A</td>
<td>2.6</td>
</tr>
</tbody>
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The iron-sulfur protein-depleted complex (0.51 mg/ml) was incubated with either a soybean phospholipid mixture (1.83 mg/ml) or the purified iron-sulfur protein (0.13 mg/ml), or both, at 30 °C for 30 min. When the effect of antimycin A was tested, it was added to approximately 0.2 μmol/mg of protein of the depleted complex after the incubation.

The purified iron-sulfur protein-depleted complex and a soybean phospholipid mixture were mixed with varying amounts (0-40 pg) of the purified iron-sulfur protein at 0 °C. The volume of the mixture was brought to 150 μl with 25 μM phosphate buffer, pH 7.5, containing 25 μM EDTA and then incubated at 30 °C for 30 min. The amount of the iron-sulfur protein added is expressed in milligrams/mg of protein of the depleted complex. Enzymic activity is expressed per mg of protein of the depleted complex used in the assay.

Fig. 4 shows a typical result of the experiment in which recovery of the enzymic activity was examined with varying amounts of the iron-sulfur protein. In a reconstitution mixture containing phospholipids, the activity with no additional iron-sulfur protein gave approximately 10% of the activity of the parent Complex III. The activity increased linearly by increasing the amount of the iron-sulfur protein up to 0.23 mg/mg of protein of the depleted complex and leveled off at this point. This means that approximately 2 mol of the iron-sulfur protein should be added per mol of the depleted complex to reconstitute the maximal activity. It may be that about half of the purified iron-sulfur protein is reconstitutively active.

To confirm the reconstitution on the molecular basis, poly-peptide composition of the reconstituted complex was tested by SDS-polyacrylamide gel electrophoresis after separating the unincorporated iron-sulfur protein by gel filtration. A mixture of the iron-sulfur protein, the depleted complex, and the phospholipids was incubated at 30 °C for 30 min and was chromatographed at 4 °C on a Sephacryl S-300 column. As a control, the complete mixture minus the depleted complex was carried through the same procedure. In the latter case (Fig. 5A) a peak of the iron-sulfur protein appeared at fraction 27, and only a trace of the protein was observed after fraction 31. On the other hand, in the case of the complete mixture (Fig. 5B), the complex that apparently contained the iron-sulfur protein-depleted complex with various amounts of the iron-sulfur protein. Seventy-six micrograms of protein of the iron-sulfur protein-depleted complex and 275 μg of the phospholipid mixture were mixed with varying amounts (0-40 μg) of the purified iron-sulfur protein at 0 °C. The volume of the mixture was brought to 150 μl with 25 μM phosphate buffer, pH 7.5, containing 25 μM EDTA and then incubated at 30 °C for 30 min. The amount of the iron-sulfur protein added is expressed in milligrams/mg of protein of the depleted complex. Enzymic activity is expressed per mg of protein of the depleted complex used in the assay.

complex. The maximum activity (183 μmol of cytochrome c reduced/min/mg of protein of the depleted complex or 46.8 μmol/min/mmol of cytochrome c) amounted up to 90% of the activity of the parent Complex III. The reconstituted activity was sensitive to antimycin A (Table II) as was the case with the parent complex. On the other hand, when the complete reconstitution mixture minus the iron-sulfur protein was incubated under the same conditions as above, the depleted complex showed approximately 10% of the activity of the parent complex (Table II). This activity may be due to the residual iron-sulfur protein in the depleted complex preparation (Fig. 2D). When the complete mixture minus the phospholipids was incubated, the restored activity was only 5% of that of the parent complex (Table II). These results indicate that the phospholipids are indispensable for reconstitution.

Fig. 5. SDS-polyacrylamide gel electrophoresis of the reconstituted complex eluted from a Sephacryl S-300 column. A mixture (1.58 ml) of the iron-sulfur protein (0.7 mg), the depleted complex (2.1 mg), and the phospholipids (4.1 mg) was incubated at 30 °C for 30 min and chromatographed on a Sephacryl S-300 column (1.5 × 117 cm) equilibrated with 0.1 M Tris-Cl, pH 7.5, containing 0.2% Tween 20, 0.1 M NaCl, 1 mM dithiothreitol, and 20% glycerol. Fractions (2.4 ml) were collected at a flow rate of 5 ml/h. Elution profile of the chromatography was monitored by the determination of proteins in each fraction. As a control, the mixture without the depleted complex was incubated and chromatographed in the same way. Proteins in 0.2 ml of each fraction were precipitated by adding 0.8 ml of cold acetone, collected by centrifugation at 10,000 × g for 5 min, and treated in the sample solvent for gel electrophoresis as described under "Experimental Procedures." A, the complete reconstitution mixture minus the depleted complex; B, the complete reconstitution mixture. The enlarged photograph of the gel pattern for fraction 33 in B is shown in C. The arrows indicate the position of the iron-sulfur protein.
sulfur protein (Fig. 5C) was eluted from the column after the unincorporated iron-sulfur protein.

**DISCUSSION**

In the present study, separation of Complex III into an iron-sulfur protein and a complex devoid of this protein was achieved by phenyl-Sepharose column chromatography (Fig. 1), and the iron-sulfur protein could be further purified to near homogeneity by gel chromatography (Fig. 2). The purified preparation contains 76 ng atoms of nonheme iron and 66 nmol of acid-labile sulfide/mg of protein (Table I) and shows typical oxidized and reduced spectra of iron-sulfur protein (Fig. 3). The protein is very stable when stored at -30 °C and is reconstitutively active, the maximal reconstituted activity being 90% of that of the parent complex (Table II and Fig. 4).

Trumpower et al. (3, 4) isolated a reconstitutively active preparation of the iron-sulfur protein from succinate-cytochrome c reductase complex. The reconstituted activity, however, was in a range of 20–40% of the activity of the parent complex. Since their isolation method included a procedure in which guanidine chloride was used to dissociate the iron-sulfur protein from the complex, the resulting iron-sulfur protein may have lost its catalytic activity and/or reconstitutive capability to some extent; in fact, it was reported to be very unstable, and the specific contents of nonheme iron and acid-labile sulfide (56 ng atoms and 36 nmol/mg of protein, respectively) were considerably lower than those in our preparation. Engel et al. (5) have recently reported the purification of a reconstitutively active iron-sulfur protein preparation from Complex III. It was obtained by dissociating Complex III with urea and described as unstable. Although their data indicated that the maximal reconstituted activity attained was comparable to the activity of the parent Complex III, their observation was made at a very low level of specific activity with ubiquinol-9 as a substrate (3 μmol of cytochrome c reduced/min/nmol of the complex at 25 °C), and at least 7 mol/mol of the depleted complex was required to obtain the maximal enzymic activity.

In the course of development of our purification method for the iron-sulfur protein, we noticed that extensive removal of phospholipids was necessary for successful separation of the protein from Complex III (Fig. 1) and reasoned that phospholipids played an important role in the retention of this protein in the complex. In connection with this view, it is noted that Complex III preparations obtained by other workers, which contained only a trace of phospholipids, were devoid of the iron-sulfur protein (22, 23).

In order to substantiate the above reasoning, it should be experimentally demonstrated that reconstitution of the iron-sulfur protein into the depleted complex requires phospholipids. The results of the reconstitution experiments showed that the enzymic activity was almost completely restored upon incubation of the protein with the depleted complex in the presence of soybean phospholipids, whereas the recovery of the activity was marginal in the absence of the phospholipids (Table II). Furthermore, SDS-polyacrylamide gel electrophoresis of the reconstituted complex after separation of the unincorporated iron-sulfur protein by gel chromatography showed that the iron-sulfur protein was present in the reconstituted complex (Fig. 5), indicating that the protein is actually incorporated into the depleted complex. Thus, these findings developed a concept that phospholipids not only cover the hydrophobic surface of Complex III but also play a role in integrating subunit(s) of the enzyme.

We previously reported that cardiolipin has a higher affinity for Complex III than other mitochondrial phospholipids (6), based on the observation that phospholipids other than cardiolipin were preferentially removed from Complex III. This led us to theorize that cardiolipin is important to retain the iron-sulfur protein in the complex. However, the results presented here indicated that cardiolipin was not necessarily required in reconstitution of the isolated iron-sulfur protein into the depleted complex, because the reconstitution was accomplished with soybean phospholipids alone. The role of cardiolipin in the complex remains to be clarified.

**REFERENCES**