Soluble Cytochrome $b_{-}c_l$ Complex and the Reconstitution of Succinate-Cytochrome $c$ Reductase*

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SUMMARY

The cytochrome $b_{-}c_l$ complex has been "solubilized" and purified to a stage containing 0.5 nmoles of cytochrome $b$ per mg of protein. Seven bands are resolved on a polyacrylamide gel electrophoretic column in sodium dodecyl sulfate-P-mercaptoethanol medium. Five of these seven bands have been identified as cytochromes $b$ and $c_l$ and a non-heme iron protein. The remaining two bands might be associated with these components or, less likely, might be impurities but do not belong to the so-called structural proteins. The cytochrome $b_{-}c_l$ complex is enzymatically active and can reconstitute with soluble succinate dehydrogenase to form an integral entity of antimycin A-sensitive succinate-cytochrome $c$ reductase. The reconstituted reductase shows the same structural and functional characteristics as the intact reductase. The total number of the p-hydroxymercuribenzoate (p-MB) titratable groups in the cytochrome $b_{-}c_l$ complex has been found to be $11 \pm 1$ moles per mole of cytochrome $b$. The p-MB-reacted complex is inactive in reconstitution but shows the same catalytic activity in the oxidation of reduced ubiquinone by cytochrome $c$.

One approach in pursuing mitochondrial electron transport involves the sequential cleavage of the respiratory chain and subsequent reconstitution (1). From such tactics, the path of electrons from succinate to cytochrome $c$ has been elucidated relatively well at the succinate dehydrogenase end (cf. for example, Ref. 1). Reconstitutively active succinate dehydrogenase contains two moieties, an iron-flavoprotein and an iron protein (see Refs. 1 and 2 and references cited therein) which may be differentiated, among other means, kinetically (3) and by redoximetric titration (2). Although, as expected, trichloroacetate or guanidine hydrochloride does not cleanly cleave these two moieties even under denaturing conditions (4), a sharp separation can be obtained by polyacrylamide gel electrophoresis in a medium containing sodium dodecyl sulfate and $\beta$-mercaptoethanol (for example, Refs. 5 and 6).²

* This work was supported by grants from the United States Public Health Service.


2 Unpublished observations from this laboratory.

On the other hand, the respiratory segment containing cytochromes $b$ and $c_l$ is rather poorly defined. Reconstitution of succinate cytochrome $c$ reductase was accomplished several years ago (7, 8) using soluble succinate dehydrogenase and a cytochrome $b_{-}c_l$ particle. The latter preparation is very insoluble in aqueous media even in the presence of surface-active agents. This characteristic has hampered an understanding of the structure and function of the components in the cytochromes $b$ and $c_l$ region. Although a preparation known as Complex III is available (9), its usefulness is somehow limited, not only because of its gross contaminations of other components and low yield but also because its unexpected and very undesirable inability to reconstitute with soluble succinate dehydrogenase.

We have been continuing our effort in the reconstitution approach of bioenergetics using well defined systems. Recently we have been able to obtain a preparation of the cytochrome $b_{-}c_l$ complex, which is "soluble" enough to give optical clarity suitable for circular dichroism experiments.³ The preparation is highly active enzymatically when tested by reconstitution with soluble succinate dehydrogenase. Of special interest as reported in this paper, the sulphydryl groups of the complex are not essential for its catalytic activity in the oxidation of ubiquinone nor for binding with the dehydrogenase. However, these sulphydryl groups are indispensable for reconstitution.

EXPERIMENTAL PROCEDURES

Materials—Cytochrome $c$ (type III), bovine serum albumin, sodium dodecyl sulfate, and sodium $\pmb{p}$-hydroxymercuribenzoate were procured from Sigma. ⁴ Enzyme grade sodium cholate and ammonium sulfate were from Mann. All other chemicals in the highest available purity were obtained commercially. The heart muscle preparation was prepared from bovine heart, according

³ Complex III was structurally defined (9) as the segment of the respiratory chain containing cytochromes $c_l$ and $b$ and other essential factors (but free of flavoproteins) for the transfer of electrons from reduced coenzyme Q to cytochrome $c$. It is prepared from mitochondria through a stage of DPNH-cytochrome $c$ reductase. However, the Complex III thus prepared always shows gross contaminations of other respiratory components, especially succinate-cytochrome $c$ reductase and succinate dehydrogenase. Moreover, the yield based on mitochondria is very low.

⁴ For convenience, we call the p-MB titratable (under the conditions tested) groups as the sulphydryl groups. We do not have evidence to exclude the so-called labile sulfide. The latter, indeed, may react readily with p-MB. Labile sulfide in succinate dehydrogenase or succinate-cytochrome $c$ reductase cannot be removed by aeration at pH 5 as can some other iron-sulfide proteins.
to an adaptation (10, 11) used in this laboratory of the original method of Keilin and Hartree (12). Soluble succinate dehydrogenase was prepared as before (13, 11) and stored at -150°C. Complex III was prepared by the original method (15) or subsequent modifications (16-18). Coenzyme Q derivatives were kindly supplied by Dr. Karl Folkert of the University of Texas, Austin.

Methods—Concentrations of cytochromes c, c1, and b were determined spectrophotometrically. The millimolar extinction coefficients used were 18.5 for A100-succinate cytochrome c 17.5 for A105-succinate cytochrome c and 19.5 for the difference spectra was used (20). Total iron content was determined by alkaline pyridine hemochromogen methods (24, 25). Total iron content was determined by atomic absorption spectrophotometry. Non-heme iron was released from the reductase of the b-cl complex by incubating the samples in 1 M HCl at room temperature for 30 min with vigorous shaking and was then determined colorimetrically (21, 22).

Flavin was determined by a fluorescent method reported previously (23). The acid-acetone-extractable hem and the acid-acetone-nonextractable hem, i.e. covalently bound, were determined by the alkaline pyridine method (24, 25). Protein was estimated by the biuret method in the presence of hydrogen peroxide (26) using crystalline bovine serum albumin as a standard. The concentration of bovine serum albumin was determined spectrophotometrically with an extinction coefficient of A1% = 6.6 (27). Sulphydryl groups of the cytochrome b-cl complex in the presence and absence of sodium dodecyl sulfate were determined by the method of Boyer (28). All spectrophotometric measurements were conducted with a Cary spectrophotometer, model 14 or 16, at approximately 22°C.

Enzymic activities of succinate-cytochrome c reductase and succinate dehydrogenase were assayed according to the previous method (7) in a 1 ml reaction mixture without addition of Triton X-100 or lipid. Analytical sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out according to Osborn and Weber (29) except that one-third of the ammonium persulfate prescribed was used.

Improved Method for Preparation of Succinate-Cytochrome c Reductase—All operations were conducted at 0-4°C unless otherwise specified. The principal steps involved in the isolation of a highly purified succinate-cytochrome c reductase were similar to but not identical with that previously reported (7). The concentration of ammonium sulfate was increased to a protein concentration of 20 mg per ml with 0.1 M sodium borate-phosphate buffer, pH 7.8 (11). To 1.5 liters of the heart muscle preparation 78.8 ml of 1 M succinate for 30 min. The suspension was again adjusted to pH 10.5 and the precipitate was collected by centrifugation immediately. The precipitate was washed once with 50 mM phosphate buffer, pH 7.4, and finally suspended in 0.9% KCl to a final protein concentration of approximately 10 mg per ml.

The suspension, 10% potassium deoxycholate (pH 9.0) was added up to a concentration of 0.3 mg per mg of protein. The insoluble material, if any, was removed by centrifugation. The supernatant fraction was brought to 50% ammonium sulfate saturation by adding a neutralized saturated ammonium sulfate. The precipitate formed was dissolved in 25 mM phosphate buffer, pH 7.4, containing 0.25 M sucrose and the insoluble material, if any, was removed by centrifugation. The clear bright red solution containing cytochromes b and c1 was designated as the soluble cytochrome b-cl complex.

The yield was found to be approximately 80% based on the cytochrome b content of the succinate-cytochrome c reductase used. The preparation can be stored at -20°C for more than 2 weeks without losing reconstitutive activity if most of the residual detergents have been removed from the preparation. Removal was usually accomplished by dialyzing the sample against 20 mM phosphate buffer containing 0.25 M sucrose overnight with three changes of the solvent.

RESULTS

Succinate-Cytochrome c Reductase—The specific activity of succinate-cytochrome c reductase was found to be between 1.2 and 3.3 umoles of succinate oxidized per min per mg of protein depending upon the lipid content of the preparation. The low activity preparations could be “activated” by addition of phospholipids and ubiquinone (cf. Ref. 30) up to a maximal activity approaching 3.3. But the high activity preparation was not further stimulated by phospholipids; and the activity of 3.3 seemed to be the limit. The reductase preparation contained cytochromes b and c1, acid-nonextractable flavin, and non-heme iron in addition to a small amount of ubiquinone and about 20% lipid, as shown in Table I. The reductase contained no acid-nonextractable flavin and the ratio of cytochromes b to c1 was practically the same as that obtained from our original method (7). However, both the specific activity and the acid-nonextractable flavin content were more than double those of the preparation previously reported in 1964, although the turnover number based on the flavin remained the same for both preparations.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Intact</th>
<th>Reconstituted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid-nonextractable flavin, nmole/mg</td>
<td>0.9</td>
<td>1.1</td>
</tr>
<tr>
<td>Non-heme iron, nanomol/mg</td>
<td>12 14.0</td>
<td></td>
</tr>
<tr>
<td>Cytochrome b, nmole/mg</td>
<td>4.0</td>
<td>5.8</td>
</tr>
<tr>
<td>Cytochrome c1, nmole/mg</td>
<td>2.3</td>
<td>3.4</td>
</tr>
<tr>
<td>Ratio of flavin to non-heme iron</td>
<td>1:13</td>
<td>1:13</td>
</tr>
<tr>
<td>Lipids, weight per cent.</td>
<td>30</td>
<td>Not determined</td>
</tr>
</tbody>
</table>

The present study was supported by the National Heart Institute (Grant 7911) and by the National Science Foundation (GP-2322).
Table II

Composition of soluble cytochrome b-c1 complex

<table>
<thead>
<tr>
<th>Components</th>
<th>Composition (nmols/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome b</td>
<td>6.5</td>
</tr>
<tr>
<td>Cytochrome c1</td>
<td>4.1</td>
</tr>
<tr>
<td>Non-heme iron</td>
<td>6.0</td>
</tr>
<tr>
<td>Flavin</td>
<td>0.0</td>
</tr>
<tr>
<td>Ubiquinone</td>
<td>1.7</td>
</tr>
<tr>
<td>Lipids</td>
<td>17% (weight)</td>
</tr>
</tbody>
</table>

Fig. 1. Absorption spectra of the cytochrome b-c1 complex. The sample contained 0.54 mg of protein per ml of 50 mM phosphate buffer, pH 7.4; optical path, 1 cm; temperature, approximately 25°. The longer curve represents the oxidized form and the shorter curve represents the dithionite-reduced form.

Composition of Cytochrome b-c1 Complex—The cytochrome b-c1 complex previously reported was very insoluble and thus it was called the cytochrome b-c1 particle (7, 8). It was not suitable for a number of experiments, not even for the measurement of electron paramagnetic resonance spectra in the frozen condition. We have now succeeded in obtaining a soluble preparation of the cytochrome b-c1 complex possessing a high specific activity. This method of preparation is reproducible and is based actually on the principle of sequential cleavage of the respiratory chain (7) by alkaline solubilization of succinate dehydrogenase under anaerobic conditions (13).

The composition of the cytochrome b-c1 complex thus obtained is shown in Table II. It contained no flavin and the ratio of non-heme iron to cytochrome b is about unity. With respect to cytochrome c1, the purity of the complex was approximately double that of the reductase. It is difficult to visualize the structure of the complex with a ratio of b to c1 of 1.6. Possibly the extinction coefficients used for the calculation were not precise enough for accurate estimation of these cytochromes under these conditions. Whether the actual ratio should be 1:1, or 2:1, or 3:2 remains to be rigorously ascertained.

Spectral Properties of Soluble Cytochrome b-c1 Complex—Since the cytochrome b-c1 complex, as prepared, was in soluble form, the absorption spectra of both the oxidized and the reduced forms could be obtained easily. As shown in Fig. 1, the oxidized form of the cytochrome b-c1 complex exhibited maxima at 278 (with a distinct shoulder at 290), 355, and 415 nm, and a very broad band between 565 and 530 nm. The ratio of the Soret to ultraviolet bands was found to be 0.8, indicating high purity of the preparation. Upon reduction by dithionite, the Soret absorption shifted to 429 nm with a shoulder at 418 nm; apparently the 429-nm absorption peak belongs to cytochrome b (31) and the 418-nm peak belongs to cytochrome c1 (19). In the reduced form, more distinguishable absorption maxima were observed in the visible region, discernible at 562, 552, 531, and 592 nm. The maxima of 562 and 531 nm belonged to cytochrome b and 552 and 522 nm belonged to cytochrome c1. Carbon monoxide had no effect on the spectral properties of either the reduced or the oxidized form of the complex.

Catalytic Activity of Cytochrome b-c1 Complex—The b-c1 complex catalyzed the oxidation of reduced ubiquinone by cytochrome c. The specific activity was found to be 14 nmols of CoQH2 oxidized (2 electron eq) per min per mg of protein at room temperature in a system containing 50 mM phosphate buffer (pH 7.4), 1.25 mM EDTA, 25 mM sodium azide, 5 mg of bovine serum albumin, 50 μg of CoQH2, and 0.5 mM cytochrome c. Under the same conditions, Complex 111 showed a similar or lower specific activity.

Reconstitutive Activity of Cytochrome b-c1 Complex—The cytochrome b-c1 complex showed neither succinate-cytochrome c reductase nor succinate dehydrogenase activities with cytochrome c and phenazine methosulfate as electron acceptors, respectively. Addition of succinate dehydrogenase to the cytochrome b-c1 complex restored the succinate-cytochrome c reductase activity in the presence of phospholipid and ubiquinone. Such kind of reconstitution was, in general, done as described previously (7, 8) or as in the legend to Fig. 2, and the reconstituted activity thus obtained was completely sensitive to antimycin A.

The catalytic activity of the reconstituted system was found to be dependent on both the concentrations of the cytochrome b-c1 complex and succinate dehydrogenase (cf. Fig. 2). When the dehydrogenase was titrated with the cytochrome b-c1 complex, the reconstitutive activity rapidly increased as the cytochrome b-c1 complex was added. Similar results were obtained when the activity of the system was determined in the presence of a constant concentration of the cytochrome b-c1 complex and increasing amounts of the dehydrogenase.

The turnover number, based on cytochrome b, was similar to that of the intact succinate-cytochrome c reductase. However, the turnover number based on the flavin was approximately only half that of the intact system. The disparity between these turnover numbers of the reconstituted and intact reductase led us eventually to conduct the following experiment. When the reconstituted succinate-cytochrome c reductase was diluted and centrifuged at 200,000 × g for 1 hour more than 85% of the succinate-cytochrome c reductase activity was recovered in the pellet. The recovery of the flavoprotein was only 50%. This result indicated that the denatured or slightly modified flavoprotein was unable to bind the cytochrome b-c1 complex and thus remained in the supernatant. The turnover numbers, based on either cytochrome b or flavin, were then virtually the same as those of the intact system. The chemical composition of the reconstituted reductase was comparable to the intact system as shown in Table I. It may be noted from the table that the reconstituted preparation had a slightly higher content of cytochrome b, cytochrome c1, flavin, and non-heme iron but the stoichiometric ratio of these components remained virtually

* The abbreviations used are: CoQ, coenzyme Q; p-MB, p-hydroxymethylbenzoate.
Fig. 2. Reconstitution of succinate-cytochrome c reductase. 
A, reconstitutive activity as a function of the cytochrome b-c₁ complex. The systems contained 1.74 mg of soluble succinate dehydrogenase (SDH) and indicated amounts of the cytochrome b-c₁ complex in final volumes of 1 ml of 50 mM phosphate buffer, pH 7.4, 0.1 mg of asolectin, and 20 μg of CoQ₄. CoQ₄ was added to the asolectin micelle solution prior to admixing with the complex. The reconstituted system was assayed directly without incubation. B, reconstitutive activity as a function of soluble succinate dehydrogenase. The system contained 1.47 mg of the cytochrome b-c₁ complex and indicated amounts of soluble succinate dehydrogenase. Other conditions and operations were the same as those of A.

constant. The increase in purity might result simply from the removal of the protein impurity during the preparation. As a matter of fact, a protein with a molecular weight of 80,000 which was often seen in the intact reductase was not detected in the reconstituted preparation.

It must be mentioned that the reconstitution reported here differs dramatically from the work of Tzagoloff et al. (32). These investigators have used respiratory fragments labeled as Complexes I, II, III, and IV for their reconstitution. They have found the composition of the reconstituted particles to vary according to the proportions of the Complexes used in the initial mixture. Their reconstituted particles are obtained as precipitates by dilution of such mixtures.

Non-identity of Cytochrome b-c₁ Complex with Complex III—Complex III possesses catalytic activity for the oxidation of reduced ubiquinone by cytochrome c (15-18). It was interesting, therefore, to ascertain whether Complex III could replace our cytochrome b-c₁ complex in the reconstitution of succinatecytochrome c reductase. We found that the Complex III prepared by either the original method (15), or modifications (16-18), did not reconstitute nor react with the soluble succinate dehydrogenase.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis of Cytochrome b-c₁ Complex and Succinate-Cytochrome c Reductase—As indicated in Fig. 3, the cytochrome b-c₁ complex shows seven protein bands on the analytical sodium dodecyl sulfate gel electrophoretic column after staining with Coomassie blue. The molecular weights of these bands were found to be, in thousands, approximately 53, 50, 37, 30, 28, 17, and 15.¹ These values were obtained from a "calibrated plot" of molecular weights versus electrophoretic mobilities of the proteins of known molecular weights; the slope of our calibrated plot was the same as that reported by Weber and Osborn (29). Nonetheless, it may be emphasized that the so-called molecular weights described in this paper are more significant for comparative purpose than the true values; for example, those obtained from the classical hydrodynamics. The proteins used were cytochrome c (12 × 10³ daltons), trypsin (23 × 10³ daltons), lactate dehydrogenase (36 × 10³ daltons), ovalbumin (43 × 10³ daltons), and bovine serum albumin (68 × 10³ daltons). We found that lipoproteins behaved somewhat differently from simple proteins in electrophoresis on polyacrylamide gel in a medium containing sodium dodecyl sulfate and β-mercaptoethanol.

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The time course of p-MB reaction with the cytochrome b-cl complex. A sample of the cytochrome b-cl complex was diluted to a protein concentration of 1 mg per ml (cytochrome b, 6.5 nmoles per mg) in 0.1 M phosphate buffer, pH 7.4, containing 0.5% sodium cholate. Two ml of the diluted b-cl complex, 30 µl of 5.8 mM p-MB solution were added. The absorption increase at 250 nm was followed in a Gilford spectrophotometer. Two blanks, p-MB, and the cytochrome b-cl complex were used. A millimolar extinction coefficient of 7.6 was used to calculate the amount of titrated sulfhydryl groups. The point marked 1 at zero time was the reaction between p-MB and the cytochrome b-cl complex which had been treated with 1% of sodium dodecyl sulfate.

Effect of p-MB on Reconstitution of Succinate-Cytochrome c Reductase—We are especially interested in examining the correlation between the p-MB titratable groups and the reconstitutive activity. The reconstitution experiments were consequently conducted in systems containing soluble succinate dehydrogenase and a limiting quantity of the cytochrome b-cl complex which had been reacted with increasing amounts of p-MB. The results are summarized in Fig. 5. As shown in the figure, the decrease of the reconstitutive activity was directly proportional to the extent of p-MB immediately titratable groups of the cytochrome b-cl complex. When the complex with all the p-MB immediately titratable groups (or 34 nmoles per mg of protein) reacted, no reconstituted succinate-cytochrome c reductase activity was observed.

When the p-MB reacted, cytochrome b-cl complex was treated with cysteine (approximately 2 mM followed by dialysis to remove the cysteine), the resultant complex showed more than 80% of its reconstitutive activity. This experiment substantiated the essentiality of sulfhydryl groups or p-MB immediately titratable groups in the reconstitution of succinate-cytochrome c reductase.

Effect of p-MB on Catalytic Activity of Complex and Binding with Soluble Succinate Dehydrogenase—The cytochrome b-cl complex catalyzed antimycin A-sensitive oxidation of reduced ubiquinone by cytochrome c. This activity was not impaired by p-MB treatment of the complex.

The binding of soluble succinate dehydrogenase to the cytochrome b-cl complex was also not affected by the p-MB treatment. Table III shows that the amount of succinate dehydrogenase incorporated into the p-MB-treated b-cl complex was the same as that incorporated into the untreated preparation.

**DISCUSSION**

It is remarkable to note that the gel electrophoretic pattern of the cytochrome b-cl complex is so simple and only seven bands...
were observed. As described earlier in the paper, only two of these seven bands are somewhat uncertain—$53 \times 10^3$ and $28 \times 10^3$. The details of the analysis and the assignment of these bands will be reported elsewhere. Here it suffices to emphasize that the cytochrome $b_{-}c_{1}$ complex preparation has been purified to a stage that has no gross contamination of alien proteins essential to electron transport. However, more important is the fact that this preparation is highly active in reconstitution with soluble succinate dehydrogenase to form antimycin A-sensitive succinate-cytochrome $c$ reductase. In other words, the transfer of electrons from the substrate (succinate) to cytochrome $c$ requires only these known components plus the $53 \times 10^3$ and $28 \times 10^3$ peptides. The latter two may be subunits associated with known components or, less likely, impurities. But they cannot belong to the so-called structural proteins (34, 35) associated with known components or, less likely, impurities. But they cannot belong to the so-called structural proteins (34, 35) because the structural proteins or proteins of similar nature as they cannot belong to the so-called structural proteins (34, 35) associated with known components or, less likely, impurities. But they cannot belong to the so-called structural proteins (34, 35).

The cytochrome $b_{-}c_{1}$ complex possesses a large number of p-MB titratable groups and for convenience we call them sulfurhydrl sulfhydryl groups. About half of them are believed to extend toward the outside or are located on the surface of the complex and the other half are buried judging from their distinctively biphasic reactivity toward p-MB. Reaction of the immediately titratable sulfurhydrl groups resulted in the loss of reconstitutive activity indicating that such sulfurhydrl groups are essential in some way. Perhaps they are involved in the transfer of electrons between succinate dehydrogenase and the cytochrome $b_{-}c_{1}$ complex, because the p-MB-treated $b_{-}c_{1}$ complex showed the same catalytic activity for the oxidation of reduced ubiquinone by cytochrome $c$ as the unreacted preparation. It is interesting that Complex III has protein components and a catalytic function similar to the $b_{-}c_{1}$ complex, and yet is reconstitutively inactive. Perhaps this could be due to the lack or modification of some sulfurhydrl groups required to channel the electron from succinate dehydrogenase to the $b_{-}c_{1}$ moiety.

Technically, it is very difficult to examine the p-MB reactive behavior of the intact or reconstituted succinate-cytochrome $c$ reductase in order to pull out the p-MB sites on the cytochrome $b_{-}c_{1}$ complex, because succinate dehydrogenase itself reacts with p-MB or related compounds (40–42). However, judging from the lack of action by p-MB on reduced ubiquinone oxidation, it may be tentatively concluded that the p-MB sites are on a component at the substrate side of cytochrome $b$. It is interesting to note that the p-MB reaction does not affect the binding of cytochrome $b_{-}c_{1}$ complex to soluble succinate dehydrogenase. Yet only the native succinate dehydrogenase (not even mildly denatured enzyme) can physically bind the cytochrome $b_{-}c_{1}$ complex.

REFERENCES


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