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RESOLUTION AND RECONSTITUTION OF SUCCINATE-CYTOCHROME *c* REDUCTASE

PREPARATIONS AND PROPERTIES OF HIGH PURITY SUCCINATE DEHYDROGENASE AND UBIQUINOL—CYTOCHROME *c* REDUCTASE

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Summary

An improved method was developed to sequentially fractionate succinate-cytochrome *c* reductase into three reconstitutive active enzyme systems with good yield: pure succinate dehydrogenase, ubiquinone-binding protein fraction and a highly purified ubiquinol-cytochrome *c* reductase (cytochrome b-c_1 III complex).

An extensively dialyzed succinate-cytochrome *c* reductase was first separated into a succinate dehydrogenase fraction and the cytochrome b-c_1 complex by alkali treatment. The resulting succinate dehydrogenase fraction was further purified to homogeneity by the treatment of butanol, calcium phosphate gel adsorption and ammonium sulfate fractionation under anaerobic condition in the presence of succinate and dithiothreitol. The cytochrome b-c_1 complex was separated into cytochrome b-c_1 III complex and ubiquinone-binding protein fractions by careful ammonium acetate fractionation in the presence of deoxycholate.

The purified succinate dehydrogenase contained only two polypeptides with molecular weights of 70 000 and 27 000 as revealed by the sodium dodecyl sulfate polyacrylamide gel electrophoretic pattern. The enzyme has the reconstitutive activity and a low K_m ferricyanide reductase activity of 85 μmol succinate oxidized per min per mg protein at 38°C.

Chemical composition analysis of cytochrome b-c_1 III complex showed that the preparation was completely free of contamination of succinate dehydro-

Abbreviations: Q, ubiquinone; SDS, sodium dodecyl sulfate.

genase and ubiquinone-binding protein and was 30% more pure than the available preparation.

When these three components were mixed in a proper ratio, a thenoyltrifluoroacetone- and antimycin A-sensitive succinate-cytochrome *c* reductase was reconstituted.

Introduction

Succinate-cytochrome *c* reductase [1], an enzyme complex of the mitochondrial respiratory chain, catalyzes the thenoyltrifluoroacetone- and antimycin A-sensitive electron transfer from succinate to cytochrome *c*. The complex is composed of two enzyme systems: succinate-ubiquinone reductase (Complex II) [2] and ubiquinol-cytochrome *c* reductase (Complex III) [3]. Although reconstitution of a functional succinate-cytochrome *c* reductase from these two complexes, which are prepared from independent procedures, is a well know fact, resolution of isolated succinate-cytochrome *c* reductase into Complex II and Complex III has not yet been shown. However, the reductase is easily split into succinate dehydrogenase [4] and the soluble cytochrome *b*-c_1 complex by treatment with alkali at approximately pH 10 [5]. The resulting cytochrome *b*-c_1 complex differs from the well known Complex III [3], in that the former can reconstitute with soluble succinate dehydrogenase to form succinate-cytochrome *c* reductase and the latter is inactive in this reconstitution. Aside from this difference in function, the differences in the chemical compositions are rather small, and many investigators use the terms the *b*-c_1 complex and Complex III interchangeably. Our efforts to resolve the difference between Complex III and our soluble *b*-c_1 complex led to the recent isolation of the ubiquinone-binding protein [6] from the *b*-c_1 complex [5] and in the reconstitution of succinate-ubiquinone reductase from ubiquinone-binding protein [7] and soluble succinate dehydrogenase. These results have provided a clear-cut difference between the two complexes. Complex III does not contain ubiquinone-binding protein and therefore does not reconstitute with isolated soluble succinate dehydrogenase.

For years, soluble succinate dehydrogenase has been routinely prepared from heart muscle preparation by the 'butanol' method [4] in many laboratories. The inferior purity of the product has been outweighed by the high yield and operational simplicity of the method. The methods developed to isolate succinate dehydrogenase from Complex II [2] by Davis and Hatefi [8] and lately by Ackrell et al. [9] yielded succinate dehydrogenase with high purity. These procedures are satisfactory for those interested only in succinate dehydrogenase.

Recently, we have developed a simple procedure to resolve succinate-cytochrome *c* reductase into succinate dehydrogenase, ubiquinone-binding protein and ubiquinol-cytochrome *c* reductase quantitatively. With these components, we have successfully reconstituted succinate-cytochrome *c* reductase. In this report we wish to present the results of the resolution and reconstitution of succinate-cytochrome *c* reductase, and the detailed isolation procedure of pure succinate dehydrogenase and a highly purified ubiquinol-cytochrome *c* reductase (*b*-c_1 III complex). Part of these results have been reported [10].

Materials and Methods

Keilin Hartree muscle preparation [11], succinate-cytochrome *c* reductase [5], and reconstitutively active, soluble succinate dehydrogenase [4] were prepared and assayed according to the reported methods. Succinate-cytochrome *c* reductase as prepared was dialyzed against 50 mM phosphate buffer, pH 7.4, containing 0.25 M sucrose overnight with two changes of buffer and frozen at −70°C until used. Isolation of ubiquinone-binding protein and reconstitution of succinate-ubiquinone reductase were carried out according to Yu et al. [7]. Essential components, such as flavin [12], non-heme iron [13], cytochrome *b* [14], cytochrome c_1 [15], phospholipids [16] and ubiquinone [17] were determined according to the method described previously. Protein concentration was determined by biuret method in the presence [18] or absence [19] of H_2O_2 depending on the heme concentration of the preparations.

Ubiquinol-cytochrome *c* reductase was assayed spectrophotometrically by reduction of cytochrome *c* using the same assay mixture as used for succinate-cytochrome *c* reductase [5] except that succinate was replaced by 5 μl of Q_2H_2. The Q_2H_2 (15 mM) was made in 95% ethanol, 1 mM HCl. Succinate-low K_m ferricyanide reductase of succinate dehydrogenase was assayed in a mixture containing 40 mM succinate, 300 μM ferricyanide and 50 mM phosphate, pH 7.8. All the enzymatic activity assays and spectrophotometric measurements were done at room temperature unless otherwise specified. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was carried out according to the procedure described [20].

Cytochrome *c*, type III, sodium cholate and deoxycholate were products of Sigma. Other chemicals were purchased commercially at highest available purity.

Results

Separation of succinate-cytochrome c reductase into the cytochrome b-c_1 complex and succinate dehydrogenase

Separation of the *b*-c_1 complex and succinate dehydrogenase from succinate-cytochrome *c* reductase was achieved essentially according to the method described [5] except that a slightly lower pH and a more strictly anaerobic condition were used in order to get a better recovery of succinate dehydrogenase. Succinate-cytochrome *c* reductase, 10 mg per ml, was centrifuged at 170 000 × *g* for 45 min and the collected precipitate was resuspended in 50 mM phosphate-borate buffer, pH 7.8, to a protein concentration of 15 mg/ml and incubated with 20 mM succinate in an argon atomosphere in the presence of 1 mM dithiothreitol for 30 min before the pH of the solution was brought to 10.0 by anaerobic addition of N NaOH. The mixture was further incubated at 0°C for 20 min before being centrifuged at 170 000 × *g* for 45 min. Succinate dehydrogenase was collected from the supernatant and the *b*-c_1 complex was recovered from the precipitate. The supernatant solution was immediately adjusted to pH 9.0 by anaerobic addition of 2 N acetic acid and succinate dehydrogenase was further purified to homogeneous state (see next

section). The collected precipitate containing the cytochrome b-c_1 complex was resuspended and homogenized in 50 mM phosphate buffer, pH 7.4, and then brought to pH 10.3 once more by the addition of N NaOH. After incubation at 0°C for 20 min, the mixture was centrifuged at 170 000 × g for 40 min. The b-c_1 complex was collected in the precipitate, and the supernatant solution, containing a small amount of succinate dehydrogenase, was discarded. The precipitate was suspended in 50 mM Tris-HCl buffer, pH 8.0, containing 0.67 M sucrose and kept at −70°C for the isolation of ubiquinone-binding protein and b-c_1 III complex (ubiquinol-cytochrome c reductase).

Purification of succinate dehydrogenase

The purification of succinate dehydrogenase was essentially according to the method reported by King [4] with a modification introduced to preserve enzymatic activity and to ensure purity. All the steps involved in the isolation of succinate dehydrogenase were carried out anaerobically. The supernatant solution (pH 9.0) obtained in the previous step was used as starting material for the isolation of pure succinate dehydrogenase. The calcium phosphate gel precipitate was washed with an argon saturated solution containing 10 mM succinate and 1 mM dithiothreitol before the succinate dehydrogenase was eluted by 0.1 M phosphate buffer. The purification date of succinate dehydrogenase is given in Table I. About 50% of the succinate dehydrogenase present in succinate-cytochrome c reductase was recovered in purified form. The enzymatic properties and chemical composition of the isolated succinate dehydrogenase are given in Table II. Data on the preparations of Davis and Hatefi [8] and that of Ackrell et al. [9] are included for comparison. It should be mentioned that the activity given for the present method is an average value rather than the highest one obtained. In our laboratory, succinate dehydrogenase prepared from Complex II according to Ackrell et al. [9] showed a similar activity as that of succinate dehydrogenase prepared by the present method.

Perhaps it is worthwhile here to clarify that succinate dehydrogenase prepared according to the method reported by King [4] and its modified version, is often mistakenly referred as 'butanol solubilized or extracted' succinate dehydrogenase. In fact the butanol is used to denature the other solubilized

TABLE I

PURIFICATION OF SUCCINATE DEHYDROGENASE FROM SUCCINATE-CYTOCHROME c REDUCTASE

Treatment	Total protein (mg)	Specific activity *	Yield (%)
Succinate-cytochrome c reductase	1000	12	100
Supernatant solution	200	51	85
'Butanol extract'	110	70	65
Calcium sulfate gel eluate	74	84	52
Ammonium sulfate precipitate	70	85	50

* μmol succinate oxidized per min per mg protein at 38°C.

TABLE II

CHEMICAL COMPOSITION AND ENZYMATIC ACTIVITIES OF SUCCINATE DEHYDROGENASE PREPARATIONS

Succ. PMS, succinate phenazine methosulfate.

Preparations	Chemical composition (nmol/mg protein)			Enzyme activity (μmol succinate oxidized/min per mg protein at 38°C)		
	Flavin	Non-heme iron	Acid-labile sulfide	Succ. PMS (V)	Low K_m ferri-cyanide	Reconstitution activity
Present procedure	9.9	78	78	85	85	85 *
Davis and Hatefi [8]	10.3	70—80	70—80	100—110	—	10
Ackrell et al. [9]	9.7	77	77	120	120	—

* Refers to the reconstituted activity of succinate-ubiquinone reductase after reconstitution with excess ubiquinone-binding protein. The calculation of specific activity is based only on the protein concentration of succinate dehydrogenase. Similar results were obtained when soluble cytochrome b-c_1 complex [5] was used and the activity of succinate-cytochrome c reductase was measured.

proteins which are subsequently removed in the precipitate upon centrifugation. Not butanol, but alkali extracts the succinate dehydrogenase.

Separation of ubiquinone-binding protein and b-c_1 III complex (ubiquinol-cytochrome c reductase) from the b-c_1 complex

The frozen b-c_1 complex was thawed and protein concentration adjusted to 20 mg/ml with 50 mM Tris-HCl buffer, pH 8.0, containing 0.67 M sucrose. The suspension was solubilized with potassium deoxycholate at a concentration of 0.5 mg per mg of protein, and fractionated with ammonium acetate solution, which was prepared according to Hatefi and Rieske [21] by dissolving 454 g of solid ammonium acetate in 613 ml of H_2O. Ammonium acetate solution was added to the deoxycholate-solubilized b-c_1 complex, 11 ml of 50% saturated ammonium acetate solution per 100 ml solution, to give 5% ammonium acetate saturation. The mixture was stirred at 0°C for 20 min and then centrifuged at 50 000 × g for 30 min. The supernatant was collected and brought to 10% ammonium acetate saturation (12 ml of ammonium acetate solution to 100 ml of solution). The pellet, which contained ubiquinone-binding protein was collected by centrifuging at 50 000 × g for 30 min and dissolved in 50 mM Tris-HCl buffer, pH 8.0, containing 0.67 M sucrose. Two main contaminants were present in the crude ubiquinone-binding protein preparation: a cytochrome b with molecular weight of 17 000 [22], and a protein with molecular weight of 30 000. The crude ubiquinone-binding protein preparation can be frozen at −70°C for several weeks without significant loss of activity. The crude ubiquinone-binding protein thus obtained can be further purified by a rather complicated procedure, which will be published elsewhere [33].

The supernatant solution was collected and brought to 18.5% ammonium acetate saturation (20 ml of 50% saturated ammonium acetate solution to 100 ml solution). After stirring for 20 min, the solution was centrifuged at 170 000 × g for 30 min. The pellet which contained purified ubiquinol-cytochrome c reductase (b-c_1 III complex), was dissolved in 50 mM Tris-HCl,

pH 8.0, containing 0.67 M sucrose, diluted to a protein concentration of approximately 20 mg per ml, and frozen at −70°C. The ubiquinol-cytochrome *c* reductase thus obtained can be further purified by ammonium sulfate fractionation in the presence of 2 M urea and 0.5% sodium cholate at a protein concentration about 10 mg per ml. The highly purified ubiquinol-cytochrome *c* reductase was recovered in the precipitate formed between 55% and 70% ammonium sulfate saturation. The summary of the resolution data is given in Table III. About 50% of succinate dehydrogenase, 85% of ubiquinone-binding protein and 60% of ubiquinol-cytochrome *c* reductase activities present in succinate-cytochrome *c* reductase were recovered in the corresponding fractions. No cross-contamination among the three fractions, i.e., succinate dehydrogenase, ubiquinone-binding protein and b-c_1 III complex was observed based on their enzymatic activities. From the result of the SDS-polyacrylamide gel electrophoresis, b-c_1 III complex was also found to be free from contamination by succinate dehydrogenase protein.

Characterization of succinate dehydrogenase

As can be seen from Table II, the purity and reconstitutive activity of succinate dehydrogenase prepared by the present procedure are comparable to those of the succinate dehydrogenase preparation reported by Ackrell et al. [9] from Complex II. Their preparation was reported to be virtually homogeneous with full reconstitutive activity. The succinate dehydrogenase we obtained has an identical SDS-polyacrylamide gel electrophoretic pattern to that of the preparation obtained by Davis and Hatefi [8] which showed only two protein bands with molecular weights of 70 000 and 27 000.

It has been shown recently that active succinate dehydrogenase possesses both high and low K_m ferricyanide reductase activities and the latter has been

TABLE III

SUMMARY OF THE RESOLUTION OF SUCCINATE-CYTOCHROME *c* REDUCTASE

Treatments	Protein (mg)	Cyt. *b* (%)	Enzyme activity, 23°C			
			Ubiquinone-binding protein* Succinate → Q		Cyt. b-c_1 III complex Q_2H_2 → cyt. *c*	
			Spec. act. **	%	Spec. act. ***	%
Succinate-cytochrome *c* reductase	1000	100	4.0	100	46.8	100
Deoxycholate-solubilized cytochrome b-c_1 complex	800	95	5.0	99	60.0	99
Ammonium acetate fractionation (% saturation)						
0—5	160	7.6	2.4	9.6	—	—
5—10	149	11.4	23.0	85.7	—	—
10—18.5	305	73.0	—	—	86.0	56

* Assayed after reconstituted with soluble succinate dehydrogenase.

** The specific activity was expressed by μmol succinate oxidized per min per mg.

*** The specific activity was expressed by μmol Q_2H_2 oxidized per min per mg.

shown to have direct correlation with the reconstitutive activity [23]. The K_m for low and high ferricyanide reductase activities of succinate dehydrogenase prepared by the present method was found to be 60 μM and 3.2 mM, respectively. The former value was obtained through the stop-flow determination of the enzymatic activity. The low K_m ferricyanide reductase activity is very sensitive to ferricyanide concentration [23] and is difficult to measure at various substrate concentrations in the conventional spectrophotometer.

Characterization of ubiquinone-binding protein

The fraction obtained between 5% and 10% ammonium acetate saturation possessed practically no enzymatic activity. However, when the fraction was mixed with soluble succinate dehydrogenase, a succinate-ubiquinone reductase was formed. The SDS-polyacrylamide gel electrophoresis showed that the enzyme was in a partially purified form (see Fig. 1), with three major polypeptides of molecular weights 30 000, 17 000 and 15 000. The further purification of this protein has shown that only one of the polypeptides, molecular weight 15 000, is responsible for ubiquinone-binding protein activity (see Fig. 1) [6] although a smaller molecular weight cytochrome *b* (17 000), has been co-purified until the last step by a recently developed method [33]. A significant amount (5 nmol/mg) of none-heme iron was detected in the crude ubiquinone-binding protein preparation, but was eliminated upon further purification with no effect on the enzymatic activity. This indicates that part of the non-heme iron present in the original soluble *b*-c_1 complex plays no functional role in this segment of electron transfer.

Purified ubiquinone-binding protein contains 20 nmol Q and 0.6 μmol phospholipids per mg protein. A trace amount of cytochrome *b* was also detected. The isolated protein is soluble in aqueous solution, in a highly aggregated form. It became partially deaggregated upon reconstitution with succinate dehydrogenase in the absence of added detergent. In the presence of detergent the reconstituted complex dissociates further to a monomeric form, with a molecular weight of about 120 000.

Characterization of b-c_1 III complex (ubiquinol-cytochrome c reductase)

Table IV compares the chemical compositions of *b*-c_1 III complex, Complex III and soluble cytochrome *b*-c_1 complex. The cytochrome *b*-c_1 III complex has a significantly higher concentration of the essential components, with the exception of non-heme iron content, than the other active preparations of mammalian cytochrome *b*-c_1 complex. The significance of this observation is currently under investigation in light of the recent success in isolation of an active non-heme iron sulfur protein from succinate-cytochrome *c* reductase by Trumpower and Edwards [29]. The relatively low content of none-heme iron in *b*-c_1 III complex does not seem to affect the specific activity of this preparation, which is 86 μmol of Q_2H_2 oxidized per min per mg at 23°C. Polypeptide composition as revealed by SDS-polyacrylamide gel electrophoresis, is similar to that of soluble cytochrome *b*-c_1 complex, with the exception of a significant decrease in the protein with molecular weight 15 000. Fig. 1 shows the protein tracing of succinate-cytochrome *c* reductase, *b*-c_1 III complex, ubiquinone-binding protein and purified succinate dehydrogenase after SDS-

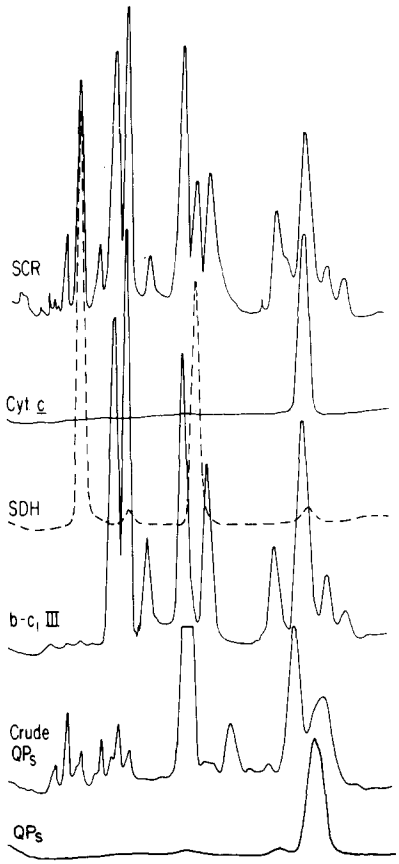

Fig. 1. SDS-polyacrylamide gel electrophoretic patterns of succinate dehydrogenase (SDH), cytochrome *b*-c_1 III complex (*b*-c_1) III, ubiquinone-binding protein (QPs), succinate-cytochrome *c* reductase (SRC) and cytochrome *c*. The isolated enzymes were dialyzed against phosphate buffer, pH 7.0, overnight to remove the detergent used in the preparations. The dialyzed samples were then dissociated with 1% SDS and 1% β-mercaptoethanol at protein concentration of 2 mg/ml at 45°C for 1 h. The protein quantities used in electrophoresis were 20 μg for succinate-cytochrome *c* reductase and cytochrome *b*-c_1 III complex, 15 μg for crude ubiquinone-binding protein, 5 μg for purified ubiquinone-binding protein, 10 μg for purified succinate dehydrogenase, 2 μg for cytochrome *c*. The tracking dye was located at the end of the tracing at right. The absorption tracings were monitored at 600 nm.

polyacrylamide gel electrophoresis. Horse cytochrome *c* was included as the mobility reference.

Fig. 2 shows the absorption spectra of cytochrome *b*-c_1 III complex. The solid line represents the oxidized form which showed a Soret peak at 416 nm and a rather undefined α absorption at 530 nm. Upon reduction by dithionite as shown by the dotted line, α and Soret absorptions for cytochrome *b* were at 562 nm and 428 nm and those for cytochrome c_1 were 552 nm and 418 nm.

Reconstitution of succinate-cytochrome c reductase from the isolated succinate dehydrogenase, ubiquinone-binding protein and cytochrome b-c_1 III complex

The reconstitution of succinate ubiquinone reductase from soluble succinate

TABLE IV

CHEMICAL COMPOSITION OF CYTOCHROME b-c_1 III COMPLEX, COMPLEX III AND SOLUBLE CYTOCHROME b-c_1 COMPLEX PREPARATIONS *

Components	Concentration (nmol/mg protein)		
	Cytochrome b-c_1 complex	Complex III [24]	Soluble cytochrome b-c_1 complex [28]
Cytochrome b	10 —10.5	6.8 ± 0.4	6.5
Cytochrome c_1	5.7— 6.0	3.4 ± 0.2	4.1
Non-heme iron	6.6— 7.2	6.2 ± 0.5	6.0
Flavin	0	0.15—0.5	0
Ubiquinone	2—3	1—4	3.7
Phospholipids	250	257	220

* There are several other cytochrome b-c_1 complex preparations from various sourced such as yeast [25], neurospora [26] and beef [27] with different degrees of purity and activity.

dehydrogenase and ubiquinone-binding protein has been briefly described previously [7]. The maximum activity was achieved by mixing both enzymes in an equimolar protein ratio. The reconstituted succinate-ubiquinone reductase is sensitive to thenoyltrifluoroacetone. When the reconstituted succinate-ubiquinone reductase was mixed with cytochrome b-c_1 III complex or the three resolved components, i.e., succinate dehydrogenase, ubiquinone-binding protein and cytochrome b-c_1 III complex were mixed, an antimycin A-sensitive succinate-cytochrome c reductase activity was obtained immediately. The

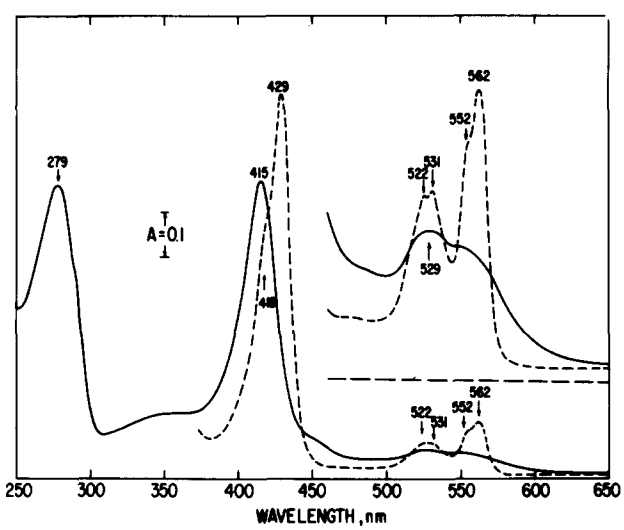

Fig. 2. Absorption spectra of cytochrome b-c_1 III complex. Purified cytochrome b-c_1 III complex was diluted to 1.7 mg/ml in 50 mM phosphate buffer, pH 7.4, containing 0.5% sodium cholate. Spectra were measured on Cary 14 spectrophotometer at room temperature. A 0.2-cm light path curvette was used. The solid line (———) indicates oxidized form and the broken line (- - - - - -) represents the reduced form. The reduction was effected by $Na_2S_2O_4$. The insert spectra were obtained with the same sample when 1.0-cm light path cuvettes were used.

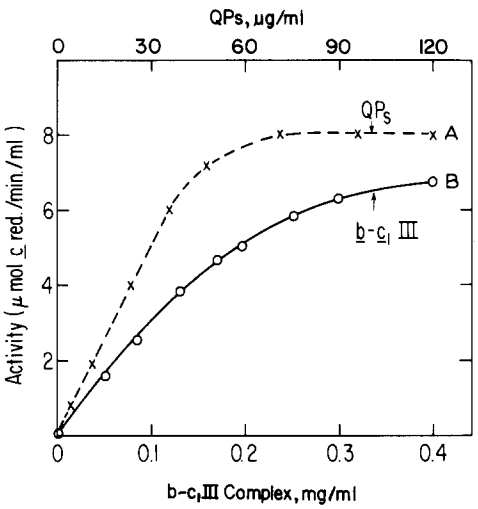

Fig. 3. Reconstitution of succinate-cytochrome *c* reductase from cytochrome *b*-c_1 III complex, succinate dehydrogenase, and ubiquinone-binding protein (QPs). (A). Purified cytochrome *b*-c_1 III complex, 0.39 mg was mixed with varying amounts of purified ubiquinone-binding protein (X- - - - -X) and then diluted to 0.45 ml with 50 mM phosphate, pH 7.4, before addition of 0.05 ml of soluble succinate dehydrogenase (7.7 mg/ml). Aliquots of the mixture were then used for assay at room temperature. (B). Same as (A) except a constant amount of purified ubiquinone-binding protein, 40 µg in one ml of reconstituted system, and varying amounts of cytochrome *b*-c_1 III complex (o———o) were used in the reconstitution.

dotted curve of Fig. 3 shows the reconstitution of succinate-cytochrome *c* reductase from a given amount of succinate dehydrogenase and cytochrome *b*-c_1 III complex and varying amounts of purified ubiquinone-binding protein, and the solid curve shows the reconstitution carried out with varying amounts of cytochrome *b*-c_1 III complex and constant succinate dehydrogenase and ubiquinone-binding protein. The reconstituted enzyme has the same kinetic parameters as those of intact succinate-cytochrome *c* reductase.

Discussion

The separation procedure described in this paper clearly demonstrates that succinate-cytochrome *c* reductase can be sequentially fractionated into three reconstitutively active enzymes without cross-contamination; soluble succinate dehydrogenase, ubiquinone-binding protein and cytochrome *b*-c_1 III complex. Although isolation procedures for those enzymes are described in the literature, this separation scheme offers several advantages for the study of electron transfer reactions in the succinate to cytochrome *c* region. First, it prevents waste of materials, since using succinate-cytochrome *c* reductase as a starting material simultaneously gives three components with good yield. Second, since these three enzyme preparations are free of cross contamination, it provides a great advantage in study of the interaction and reconstitution. The reconstitutive results from these components completely rule out the possibility that the reconstituted enzymatic activity is due to the activation of residual enzyme present in the preparations. Third, the cytochrome *b*-c_1 III complex thus

obtained is suitable for the study of the ubiquinone-binding protein existing in the cytochrome b-c_1 region. In addition, it serves as a good starting material for the isolation of cytochromes b and c_1.

Of the four electron transfer complexes introduced by Green and co-workers [30], Complex II [2] is the most poorly defined structurally. The cytochrome b present in Complex II is reducible by dithionite, but not by succinate. Its status in Complex II is not clear. Whether it represents a new species of cytochrome b [31], different from the cytochrome b in the cytochrome b-c_1 complex, or a denatured form of cytochrome b, is an unsettled question. The cytochrome c_1 present in Complex II has been more definitely shown to be a denatured form as it is not reduced by ascorbate. The presence of denatured cytochromes in Complex II is probably due to the organic solvent treatment during isolation. Successful reconstitution of succinate-ubiquinone reductase from succinate dehydrogenase and ubiquinone-binding protein has ruled out the functional participation of cytochromes in Complex II.

The cytochrome b-c_1 III complex preparation described in this paper is considered to have higher purity than other available preparations of ubiquinol-cytochrome c reductase from the same source, as it is free from contamination with succinate dehydrogenase and ubiquinone-binding protein. In addition to cytochromes b and c_1, non-heme iron sulfur protein and phospholipids, this complex contains ubiquinone. Whether or not this ubiquinone is bound to a specific protein subunit is an important question to be solved. Our recent observations [32] that when cytochrome b-c_1 III complex is reduced by succinate in the presence of catalytic amounts of succinate-ubiquinone reductase, an ubisemiquinone free radical is formed [32] and that the appearance of this radical is concurrent with the reduction of cytochrome b [32], indicates strongly that ubiquinone is bound to a specific protein, which we have tentatively labelled, ubiquinone-binding protein [32]. Final proof of the existence of this ubiquinone-binding protein will, of course, await successful isolation.

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