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INTERACTION BETWEEN SUCCINATE DEHYDROGENASE AND UBIQUINONE-BINDING PROTEIN FROM SUCCINATE-UBIQUINONE REDUCTASE

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Summary

Purified ubiquinone-binding protein in succinate-ubiquinone reductase (QPs) reconstitutes with pure soluble succinate dehydrogenase to form succinate-ubiquinone oxidoreductase upon mixing of the two proteins in phosphate buffer at neutral pH. The maximal reconstitution was found with a weight ratio of succinate dehydrogenase to QPs of about 5, which is fairly close to the calculated value of 6.5, a value obtained by assuming one mole of QPs reacts with one mole of succinate dehydrogenase. Succinate-cytochrome *c* reductase was reconstituted when succinate dehydrogenase and QPs were added to Complex III or cytochrome b-c_1 III complex (a highly purified ubiquinol-cytochrome *c* reductase). The reconstituted enzyme possessed kinetic parameters which were identical to those of the native enzyme complex. Interaction between QPs and succinate dehydrogenase resulted in the disappearance of low K_m ferricyanide reductase activity from the latter. Unlike soluble succinate dehydrogenase, the reconstituted enzyme, as well as native succinate-cytochrome *c* reductase, reduced low concentration ferricyanide only in the presence of excess ubiquinone. The apparent K_m for ubiquinone was 6 μM for reduction of ferricyanide (300 μM) by succinate, which is similar to the K_m when ubiquinone was used as electron acceptor. When 2,6-dichlorophenolindophenol was used as electron acceptor for reconstitution of succinate-ubiquinone reductase very little or no exogeneous ubiquinone was needed to show the maximal activity

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Abbreviations: Q, ubiquinone; QPs, ubiquinone-binding protein in succinate-ubiquinone reductase; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; Taps, [tris(hydroxymethyl)methyl]aminopropanesulfonic acid; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

with QPs made by Method II, indicating that the bound ubiquinone in QPs is enough for enzymatic activity. In addition to restoring the succinate-ubiquinone reductase activity the interaction between QPs and succinate dehydrogenase not only stabilized succinate dehydrogenase but also partially deaggregated QPs. The reconstituted succinate-ubiquinone reductase had a minimal molecular weight of 120 000 when the reconstituted system was dispersed in 0.2% Triton X-100. The maximal reconstitution was observed at neutral pH in phosphate buffer, Tris-acetate or Tris-phosphate buffer. Tris-HCl buffer, however, produced a less efficient reconstitution. These results indicate that the interaction between QPs and succinate dehydrogenase may involve some cationic group which has a high affinity for Cl^-. Primary amino groups of QPs are not directly involved in the interaction as the reconstitution showed no significant difference when the amino groups of QPs were alkylated with fluorescamine. The Arrhenius plots of reconstituted succinate-ubiquinone reductase show that the enzyme catalyzes the reaction with an activation energy of 19.7 kcal/mol and 26.6 kcal/mol at temperatures above and below 26°C, respectively. These activation energies are similar to those obtained with native enzyme. The Arrhenius plots of the interaction between QPs and succinate dehydrogenase also have a break point at 26°C. The activation energy for this interaction was calculated to be 11.2 kcal/mol and 6.9 kcal/mol for the temperatures above and below the break-point. The significance of the difference in activation energies between the enzymatic reaction and the reconstitution reaction are further explored in the discussion.

Introduction

The isolation, chemical composition and properties of QPs from beef heart mitochondria have been reported [1]. We shall describe here the interaction between pure soluble succinate dehydrogenase and purified QPs. The reconstitution of succinate-ubiquinone reductase from soluble succinate dehydrogenase with the cytochrome b-c_1 complex [2], alkali treated heart muscle preparation [3], alkali treated Complex II [4] and other cytochrome b containing fractions [5—7] have been reported from several laboratories. Most of the reported reconstitution studies are based on partial restoration of enzymatic activity and little is known about the interaction at the molecular level. The key question, which is how the succinate-ubiquinone reductase activity is restored, remains unanswered. The interaction between QPs and succinate dehydrogenase can now be studied at the molecular level as both components are available in purified form. The unsettled question concerning the involvement of cytochrome b in this segment of electron transfer reaction is now resolved as purified QPs [1] contains little or no cytochrome b yet is active in converting pure succinate dehydrogenase into succinate-ubiquinone reductase. The reconstitution is not only evidenced by the restoration of succinate-ubiquinone reductase activity but also by the formation of a complex which is a distinct physical entity. The physical association of QPs and succinate dehydrogenase is confirmed by the complete loss of the low K_m ferricyanide reductase site of succinate dehydrogenase [8] which was exposed when succinate dehydrogenase was removed [9]

from succinate-cytochrome *c* reductase. Studies of enzyme kinetic parameters, inhibitor sensitivity and other properties of the reconstituted enzyme complex also confirm the true reconstitution of succinate-ubiquinone reductase.

Materials and Methods

Materials

Succinate-cytochrome *c* reductase and soluble cytochrome b-c_1 complex [2], Comple II [10], Complex III [11], cytochrome b-c_1 III complex [12], pure succinate dehydrogenase [12,13], purified QPs preparations [1] were prepared and assayed according to the reported methods. Q-2 was synthesized in this laboratory by the reported methods with the some modifications [1]. Triton X-100 was a product of Rohm and Haas, urea was obtained from Fisher, *p*-hydroxymercuribenzoate, fluorescamine and other buffers from Sigma.

Methods

Reconstitution between Qps and succinate dehydrogenase was carried out according to the method described previously [12]. Primary amine content in QPs and succinate dehydrogenase was determined by fluorescence measurement after the enzyme were treated with fluorescamine. The fluorescamine was made in acetone and the solvent was dehydrated by the treatment of anhydrous magnesium sulfate before use. Fluorescence measurement was carried out in a fluorescence spectrophotometer, model SF-1. The excitation wavelength was set at 390 nm and emission was at 475 nm [14]. The concentration of primary amine was estimated by assuming that all fluorescamine added reacted quantitatively with primary amino groups. The activation energy of the reconstitution reaction was determined from an Arrhenius plot. The time required to achieve a half maximal reconstitution at various temperatures was taken as $t_{1/2}$ for the formation of succinate-ubiquinone reductase from an excess succinate dehydrogenase and limited QPs. A plot of the reconstituted activity versus time after both components were mixed at a given temperature was used to obtain $t_{1/2}$. Assuming that under such conditions the formation of succinate-ubiquinone reductase follows pseudo first order kinetic then $t_{1/2}$ represents the rate constant for the formation of succinate-ubiquinone reductase.

Results

Reconstitution of succinate-ubiquinone oxidoreductase from purified QPs and pure succinate dehydrogenase

Curve A of Fig. 1 shows the reconstitution of succinate-ubiquinone reductase from a given amount of QPs and varying amounts of pure soluble succinate dehydrogenase. The maximal activity was obtained when the weight ratio of succinate dehydrogenase to QPs was 5. Theoretically, one would expect to obtain a ratio of 6.5 for succinate dehydrogenase and QPs if both proteins were in the pure form and no denaturation occurred during their isolation. However, as QPs and succinate dehydrogenase isolated are not absolutely native, a slight inactivation is inevitable. Therefore, the maximal reconstitution ratio of about 5 is reasonable. Furthermore, such a weight ratio indicates that the reconsti-

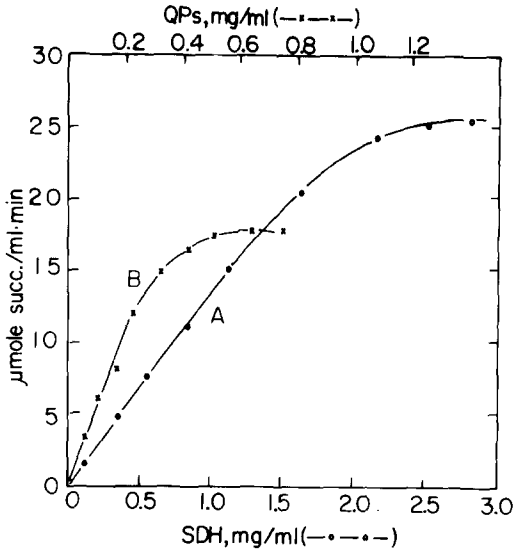


Fig. 1. Reconstitution of succinate-ubiquinone reductase. A. Reconstitution of succinate-ubiquinone reductase was made with a constant amount of QPs (prepared by Method II) and varying amounts of succinate dehydrogenase. QPs, 5 mg/ml, was diluted with 50 mM phosphate buffer, pH 7.4, to a protein concentration of 0.34 mg/ml. 0.2-ml aliquots of diluted QPs were then mixed with varying amounts (2 to 40 μl) of succinate dehydrogenase (20 mg/ml) and the reconstituted succinate-ubiquinone reductase was assayed after 10 min incubation at 0°C. B. Reconstitution of succinate-ubiquinone reductase was made with a constant amount of succinate dehydrogenase (SDH) (1.47 mg/ml) and varying amounts of QPs. 0.2 ml of the indicated concentration of QPs were mixed with 30 μl of succinate dehydrogenase (11.3 mg/ml) at 0°C. Other conditions were the same as in (A).

tuted succinate-ubiquinone reductase is composed of one molecule of succinate dehydrogenase and one molecule of QPs. Curve B of Fig. 1 shows the reconstitution of succinate-ubiquinone reductase at given amount of succinate dehydrogenase and varying amounts of QPs. The maximal reconstitution was again observed when the ratio between succinate dehydrogenase and QPs approached 5.

Reconstitution of succinate-cytochrome c reductase from succinate dehydrogenase, QPs and ubiquinol-cytochrome c reductase

It is a well known fact that the isolated Complex III can not reconstitute with succinate dehydrogenase to form succinate-cytochrome *c* reductase although the complex itself contains about 10 to 20% of succinate-cytochrome *c* reductase activity [15]. However, when QPs and succinate dehydrogenase were added to Complex III or cytochrome *b*-c_1 III complex, a succinate-cytochrome *c* reductase was reconstituted. Fig. 2 shows the reconstitution of succinate cytochrome *c* reductase from a given amount of cytochrome *b*-c_1 III complex and succinate dehydrogenase and varying amount of QPs. The maximal reconstitution was reached at a ratio of cytochrome *b*-c_1 III complex to QPs of about 6. If Complex III which is about 30% less pure than cytochrome *b*-c_1 III complex was used in the system, a ratio of about 7 for maximal activity was observed. This result clearly demonstrates that the inability of cytochrome *b*-c_1 III complex or Complex III to reconstitute with succinate dehydrogenase

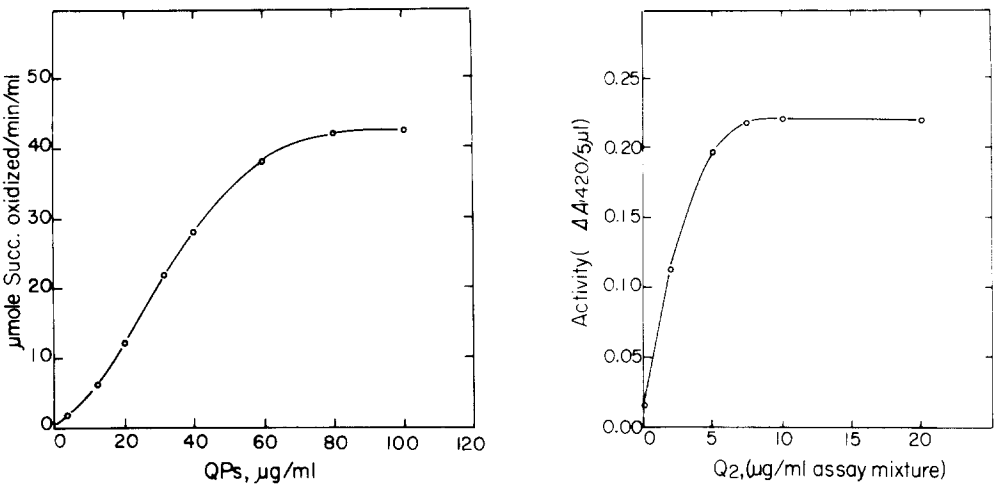


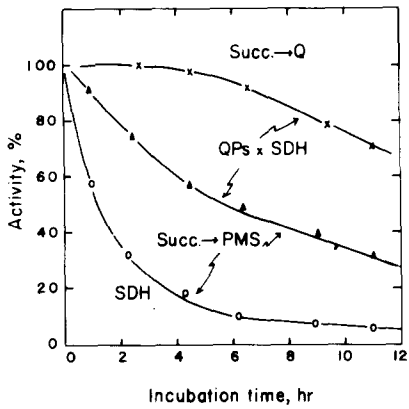
Fig. 2. Reconstitution of succinate-cytochrome *c* reductase from cytochrome *b*-c_1 III complex, succinate dehydrogenase and QPs. 0.2-ml aliquots of cytochrome *b*-c_1 III complex, 0.67 mg/ml in 50 mM phosphate buffer, pH 7.4, were mixed with 0.1 ml of various concentrations of QPs (prepared by Method II) and 30 μl of succinate dehydrogenase (8.5 mg/ml) were added. The reconstituted succinate-cytochrome *c* reductase was assayed after 30 min incubation at 0°C.

Fig. 3. Effect of Q-2 on the reduction of low concentration ferricyanide by succinate in the reconstituted succinate-ubiquinone reductase. Reconstitution of succinate-ubiquinone reductase was made with limited amounts of QPs (0.1 ml, 6.5 mg/ml) and excess succinate dehydrogenase (0.2 ml, 25 mg/ml). After mixing, the mixture was diluted with 0.9 ml of 50 mM phosphate buffer, pH 7.4, containing 0.1% deoxycholate. 5-μl aliquots were used for assay. The assay mixture (1 ml) contains indicated amounts of Q-2, 300 μM of ferricyanide, 50 mM phosphate buffer, pH 7.8, 1.5 mM KCN, 1 mg bovine serum albumin and 40 mM succinate.

to form succinate-cytochrome *c* reductase is due to a lack of QPs. In contrast to Complex III, soluble cytochrome *b*-c_1 complex [2] which contains QPs is fully active in reconstitution with succinate dehydrogenase. When QPs prepared by Method II was used for reconstitution with cytochrome *b*-c_1 III complex and soluble succinate dehydrogenase to form succinate-cytochrome *c* reductase, no external Q was needed to obtain maximal activity. However, when QPs prepared by Method I [1] was used in the reconstitution system, a 2- to 4-fold enhancement in activity was observed upon addition of external Q, indicating that the purified QPs is deficient in Q. The stimulation of activity by addition of Q in the reconstituted succinate-cytochrome *c* reductase was the same as that in the reconstituted succinate-ubiquinone reductase. Titration of Q in the reconstitution of succinate-cytochrome *c* reductase between QPs, soluble succinate dehydrogenase and cytochrome *b*-c_1 III complex showed that the maximal reconstitution was reached when 9 nmol of Q-2 were added to a system containing 18 nmol of QPs. This result indicates that purified QPs obtained by Method I [1] was more than 50% deficient in Q content as not all the protein in QPs is in an active form.

Proof of reconstitution

It is very important to prove that the system we are dealing with is a true reconstitution rather than an activation. Several approaches were used to



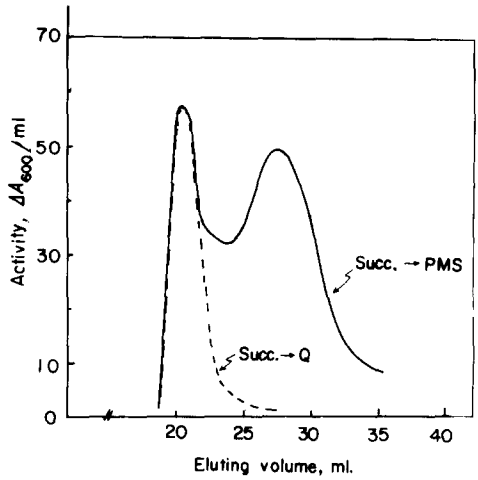


Fig. 4. Stabilizing effect of QPs on succinate dehydrogenase. One ml of diluted QPs, 50 μg per ml, in 50 mM phosphate buffer, pH 7.4, was mixed with 0.15 ml of succinate dehydrogenase (5 mg/ml) and incubated at 0°C. Succinate-ubiquinone (X———X) and succinate-phenazine methosulfate (PMS) (△———△) reductase activities were assayed at indicated times. An equal amount of free succinate dehydrogenase (SDH) in the absence of QPs (○———○) was also assayed under the same conditions.

Fig. 5. Eluting profile of the reconstituted succinate-ubiquinone reductase. A gel filtration column of Bio-Gel-A 0.5 m was used. QPs, 0.12 ml (6.5 mg/ml) was mixed with 0.3 ml of succinate dehydrogenase (20 mg/ml) and incubated at 0°C for 30 min before being applied to the column, (0.75 X 25 cm), which had been equilibrated with 0.1 M sodium/potassium phosphate buffer, pH 7.8, containing 6 mM succinate. The column was eluted with the same buffer at a flow rate of 0.1 ml/min. The broken line (- - - - - -) indicates the location of succinate-ubiquinone reductase activity and the solid line (———) represents the succinate-phenazine methosulfate (PMS) reductase activity.

demonstrate that the interaction between QPs and soluble succinate dehydrogenase is a true reconstitution of succinate-ubiquinone reductase.

1. Enzyme kinetic parameters. The K_m for succinate of the reconstituted succinate-ubiquinone reductase is 0.56 mM, identical to the K_m for succinate of intact succinate-cytochrome *c* reductase. To avoid complications, succinate-cytochrome *c* reductase used in K_m determination was treated with antimycin A to block electron transfer beyond succinate-ubiquinone reductase. In the phospholipid-depleted succinate-cytochrome *c* reductase, the K_m for succinate is 0.2 mM which is slightly lower than that of the intact system [16]. Since both the reconstituted enzyme and intact succinate-cytochrome *c* reductase have a similar apparent K_m for succinate, the succinate binding sites in the reconstituted and intact enzymes are probably similar if not identical.

2. Disappearance of low K_m ferricyanide reductase activity from soluble succinate dehydrogenase. The soluble, reconstitutively active succinate dehydrogenase is capable of reducing ferricyanide at both low and high concentrations [9]. Succinate dehydrogenase in succinate-cytochrome *c* reductase or in the submitochondrial particles, however, has only high K_m ferricyanide reductase activity. When the soluble, reconstitutively active succinate dehydrogenase was reconstituted with QPs, the low K_m ferricyanide reductase activity was abolished (see Fig. 1A of Ref. 8). When external Q was added to the reconstituted

system, the reduction of low concentration ferricyanide re-appeared and the reduction rate was proportionate to the amount of external Q added, as given in Fig. 3, indicating that an electron is transferred from succinate to ferricyanide through QPs and Q but not from succinate dehydrogenase directly. The apparent K_m for Q in such an electron transfer reaction was determined to be 6 μM. A Q-stimulated reduction of low concentration ferricyanide was observed in the intact succinate-cytochrome *c* reductase system. It should be mentioned here that the amount of Q required to monitor the electron transfer from succinate to low concentration ferricyanide in the reconstituted succinate-ubiquinone reductase is much more than that required to monitor electron transfer to 2,6-dichlorophenolindophenol through QPs.

3. Stabilization of succinate dehydrogenase by QPs. Soluble succinate dehydrogenase is unstable, especially in the absence of succinate and in the presence of oxygen. On the other hand, succinate dehydrogenase in succinate-cytochrome *c* reductase or in Complex II is very stable [11]. Addition of QPs to soluble succinate dehydrogenase led to the formation of a succinate-ubiquinone reductase complex which is stable under aerobic conditions for hours as shown in the upper curve of Fig. 4. Fig. 4 also compares the stability of free succinate dehydrogenase (lower curve) and succinate dehydrogenase in the presence of limiting amount of QPs (middle curve). When excess succinate dehydrogenase is added to QPs, only the succinate dehydrogenase which is bound to QPs is stabilized, and the unbound succinate dehydrogenase in the system undergoes the same denaturation pattern as free succinate dehydrogenase in the absence of QPs. This mixed denaturation behaviour is shown in the middle curve of Fig. 4. From comparison of the succinate-phenazine methosulfate reductase activity decay curves of a given amount of succinate dehydrogenase in the presence and absence of QPs, it is possible to estimate how much of the succinate dehydrogenase in the succinate dehydrogenase-QPs system is bound. It should be mentioned here that the loss of succinate-phenazine methosulfate reductase activity for free succinate dehydrogenase alone as shown in the lower curve of Fig. 4 is considerably faster than has been reported previously [17]. This is probably due to the greater dilutions and purer succinate dehydrogenase used in this study. In fact, a separate study indicates that the stability of the pure succinate dehydrogenase is protein concentration dependent (unpublished results).

4. Physical association between QPs and soluble succinate dehydrogenase. Pure succinate dehydrogenase has a molecular weight of 100 000 whereas purified QPs is in a highly aggregated form with a molecular weight of about one million. When excess succinate dehydrogenase and limiting QPs were mixed, succinate dehydrogenase was physically associated with QPs and unbound succinate dehydrogenase was separated from the reconstituted succinate-ubiquinone reductase when the mixture was passed through a gel filtration column of Bio-Gel-A 0.5 m (see Fig. 5). The reconstituted succinate-ubiquinone reductase appeared at void volume of the eluates, whereas unbound succinate dehydrogenase eluted out later. When the reconstituted system was prepared by mixing a stoichiometric amount of QPs and succinate dehydrogenase and was passed through a Bio-Gel-A 5 m column, the enzymatic activity was spread over a wide range of eluates. The reconstituted complex has a molecular weight ranging

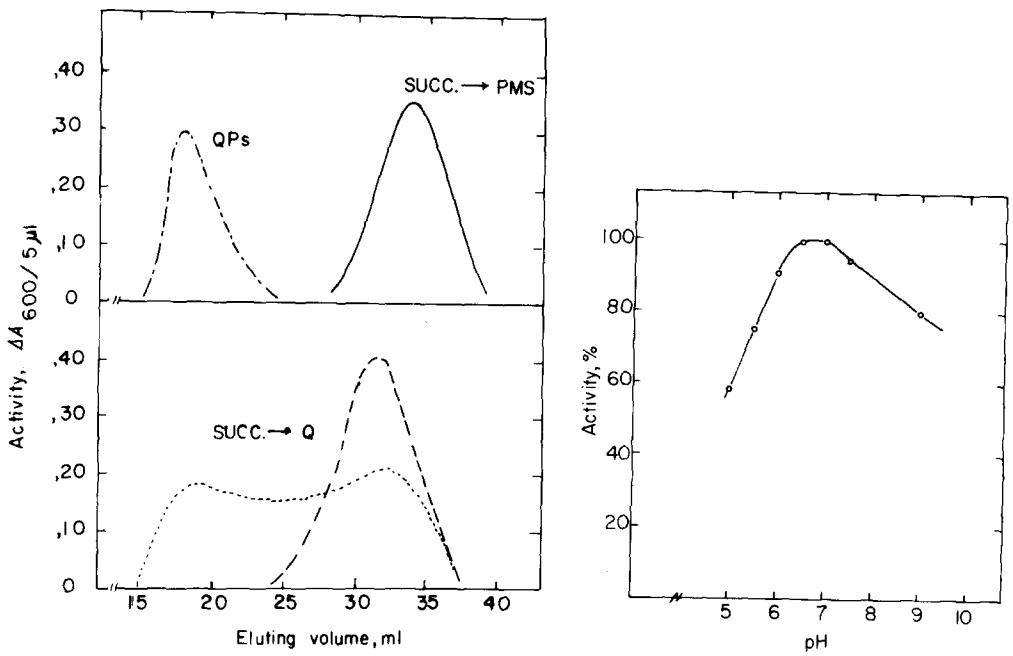


Fig. 6. Eluting profile of QPs, succinate dehydrogenase and reconstituted succinate-ubiquinone reductase from gel filtration column of Bio-Gel-A 5 m. QPs (· — · — ·), 2.5 mg/ml; SDH (———), 10 mg/ml; and reconstituted succinate-ubiquinone reductase in the absence of detergent (· · · · · ·). 0.3 ml each of the preparations were applied separately to Bio-Gel-A 5 m column (0.75 × 25 cm) which had been equilibrated with the buffer used in Fig. 5. The column was eluted with the same buffer at a flow rate of 0.1 ml per min. The broken line, (— — —), represents the reconstituted succinate-ubiquinone reductase made in 0.2% Triton X-100 and passed through the same column, which was equilibrated with 0.1 M phosphate buffer containing 6 mM succinate and 0.2% Triton X-100. The reconstituted succinate-ubiquinone reductase was prepared by mixing 0.1 ml of QPs (6.5 mg/ml) and 0.2 ml of succinate dehydrogenase (15 mg/ml).

Fig. 7. Effect of pH on the reconstitution of succinate-ubiquinone reductase. The reconstitution was made with 5 μl of QPs (6 mg/ml), 5 μl of soluble succinate dehydrogenase (30 mg/ml) and 0.5 ml of 0.1 M Tris-acetate buffer with indicated pH at 0°C. 5-μl aliquots of solution were withdrawn for assay without incubation. The assay mixture of succinate-ubiquinone reductase is in 0.1 M phosphate buffer, pH 7.0.

from 120 000 to 500 000. Fig. 6 shows the heterogeneity in molecular weight of the reconstituted succinate-ubiquinone reductase. In the absence of succinate dehydrogenase, QPs appeared immediately after the void volume of the column, as indicated in the dashed curve on the top of Fig. 6. The solid curve on the right indicates the eluting position of pure soluble succinate dehydrogenase in the same column in a separated run. When QPs was reconstituted with succinate dehydrogenase, the molecular weight of the reconstituted enzyme was significantly smaller than that of QPs itself. The dotted curve at the bottom of Fig. 6 represents the eluting profile of reconstituted succinate-ubiquinone reductase on Bio-Gel-A 5 m column. Some of the reconstituted enzyme showed a molecular weight slightly larger than that of free succinate dehydrogenase. When the reconstituted enzyme was passed through the same column in the presence of 0.2% Triton X-100, the major fraction of the reconstituted enzyme appeared at almost the same place as free succinate dehydrogenase,

indicating that a great degree of deaggregation had occurred. The molecular size of the deaggregated succinate-ubiquinone reductase is about 120 000 to 240 000. The eluting profile is shown by the broken curve at the bottom of Fig. 6. This indicates that succinate-ubiquinone reductase is formed by one mole of succinate dehydrogenase and one mole of QPs. When the deaggregated, reconstituted succinate-ubiquinone reductase was analyzed by SDS-polyacrylamide gel electrophoresis, three main protein bands with molecular weights of 70 000, 27 000 and 15 000 were observed and their blue color intensities were found to be directly proportional to their molecular weights.

Effects of pH on reconstitution

Fig. 7 shows the pH profile of the reconstitution. The maximal reconstitution was observed in the neutral pH range. The reconstitution efficiency decreased sharply when the pH of the solution was lower than 5.5. or higher than 9. The fact that reconstitution decreased more drastically on the acidic side than on the alkaline side of the profile can not be entirely attributed to the lower stability of free succinate dehydrogenase, QPs or the reconstituted succinate-ubiquinone reductase in the acidic medium, as succinate dehydrogenase was in excess in the reconstitution system, and very little time elapsed (less than 3 min) between the reconstitution and the assay. It has been shown that only active succinate dehydrogenase is capable of reacting with the soluble cytochrome b-c_1 complex [2], therefore it is not unreasonable to assume that only active succinate dehydrogenase will bind to QPs. Futhermore, brief exposure (2 min) of succinate dehydrogenase to pH 5.0 under the same conditions as those used in reconstitution, did not result in a decrease of low K_m ferricyanide reductase activity indicating the succinate dehydrogenase was not inactivated. No loss of reconstitutive activity of QPs was observed when it was incubated at pH 5.0 for a short period of time and then reconstituted with succinate dehydrogenase after being neutralized. Thus the possible explanation for the low reconstitution efficiency at low pH is the protonation of some ionized groups in either QPs or succinate dehydrogenase which would hinder the ionic interaction between the two proteins. More experimental support is needed for this explanation.

Effect of buffer systems on the reconstitution

Table I shows the effect of buffer systems on the reconstitution of succinate-ubiquinone reductase from QPs and soluble succinate dehydrogenase. Among the buffer systems tested phosphate buffer showed the best efficiency in reconstitution, and Tris-HCl and Taps were very poor buffer systems for the reconstitution. The result concurs with the observation that Tris-HCl was a better buffer system than phosphate for separation of QPs from cytochrome b-c_1 III complex during the ammonium acetate fractionation of soluble b-c_1 complex in the presence of deoxycholate. Another interesting point as shown in Table I, is that the efficiency of Tris buffers used in reconstitution decreased as the acidity of the anion of the Tris buffer increased, that is, Tris-acetate > Tris-phosphate > Tris-HCl. These results indicate that the reconstitution is more sensitive to the anion of the buffer than the cation. A possible explanation is that a cationic

TABLE I

EFFECT OF BUFFER SYSTEMS ON THE INTERACTION BETWEEN QPs AND SUCCINATE DEHYDROGENASE

Buffer systems *	Reconstituted activity (%)
Sodium/potassium phosphate buffer	100
Taps	44
Tes	82
Hepes	82
Tris-HCl	46
Tris-acetate	100
Tris-phosphate	82

* All the buffer systems used were 0.1 M at pH 7.4.

group on either QPs of succinate dehydrogenase, or both, is involved in the interaction between these two components, and the poor efficiency of Tris-HCl buffer could result from a strong binding between such a cationic group on the enzyme and the chloride ion of the buffer.

Effect of primary amino groups of QPs in the interaction between QPs and succinate dehydrogenase

As described in the previous section, a cationic group on the protein may be involved in the interaction between QPs and succinate dehydrogenase. Therefore, the primary amino group of lysine residues and the N-terminal amino group of the polypeptide automatically become candidates for these cationic groups. When QPs was treated with known amounts of fluorescamine before being reconstituted with succinate dehydrogenase, no decrease in the reconsti-

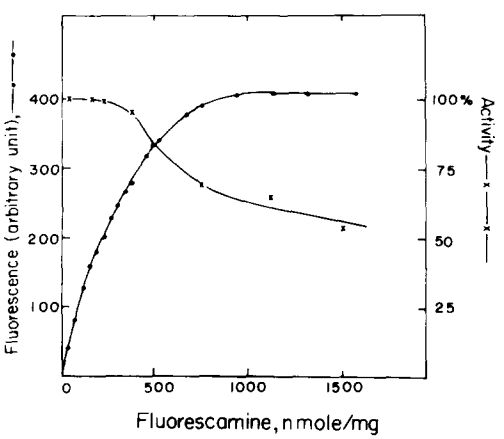


Fig. 8. Effect of primary amino groups of QPs on reconstitution. Purified QPs was dialyzed and diluted to a protein concentration of 0.23 mg/ml in 50 mM phosphate buffer, pH 7.4, containing 0.05% deoxycholate. Fluorescamine, 26 mM was made in anhydrous acetone. The indicated amount of fluorescamine was reacted with 3 ml of QPs solution at room temperature and the fluorescence of the mixture was measured immediately. The reconstitution of fluorescamine-treated samples was effected by mixing with excess succinate dehydrogenase. Under the conditions tested acetone itself showed no effect on the reconstitutive activity of QPs.

tutive activity of QPs was observed even when all of the available amino groups had been titrated (see Fig. 8). The slight decrease in the reconstitutive activity after excess fluorescamine was introduced can be explained as a secondary effect resulting from a conformation change of the protein. The study on modifying the primary amino groups of succinate dehydrogenase by fluorescamine has revealed that the inactivation of the low K_m ferricyanide reductase activity of succinate dehydrogenase is succinate dependent. Only when the enzyme was made in the presence of low concentrations of succinate (<0.2 mM succinate), a significant inactivation (>65%) was observed [18]. Under the usual experimental conditions, succinate dehydrogenase was prepared in a buffer system containing 20 mM succinate, therefore, no effect of fluorescamine on the interaction of QPs and succinate dehydrogenase can be observed.

Thermodynamic properties of succinate-ubiquinone reductase and the reconstituted succinate-ubiquinone reductase

Fig. 9 shows the Arrhenius plots of succinate-ubiquinone reductase activity of intact (broken line) succinate-ubiquinone reductase (succinate-cytochrome *c* reductase plus antimycin A) and of the reconstituted (solid line) succinate-ubiquinone reductase. Both native and reconstituted enzymes showed a greater activation energy at lower temperatures than at higher temperatures. A break point was found at approx. 26°C. This discontinuity in the plot might result from the association of the enzyme with phospholipids. Above 26°C, the succinate-ubiquinone reductase has an activation energy of 11.4 and 10.2 kcal/mol for intact and reconstituted enzymes, respectively. Below 26°C, the intact enzyme has an activation energy of 19.7 kcal/mol whereas the reconstituted enzyme has an activation energy of 26.7 kcal/mol. Since the phase transition temperatures of phospholipids in succinate-cytochrome *c* reductase and in QPs

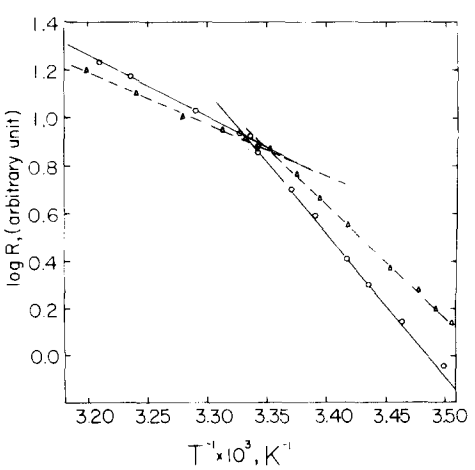


Fig. 9 Arrhenius plots of intact and reconstituted succinate-ubiquinone reductase. The assay mixture was preincubated at the indicated temperatures. The stock enzyme solutions of succinate-cytochrome *c* reductase plus antimycin A (△- - - - -△) and reconstituted succinate-ubiquinone reductase (○———○) were kept at 0°C during activity measurements. Since only 5 to 10 μl of enzyme solution was used, the temperature change caused by the addition of enzyme is negligible. *R* represents the reaction rates expressed by the changes of absorbance.

are not available, we are unable to correlate this biphasic Arrhenius plot with transition temperature at the present time. However, the similar break point of the Arrhenius plots of both intact and reconstituted systems and their identical activation energy at higher temperature indicates that their active sites are located in similar environments.

Activation energy of the interaction between QPs and succinate dehydrogenase

Although methods for precise measurement of the activation energy for the interaction between QPs and succinate dehydrogenase are not available, the activation energy can be estimated through indirect measurement of the formation of succinate-ubiquinone reductase at various temperatures, adapted from the method used by Nicholls et al. [20]. When limiting amounts of QPs were reacted with excess succinate dehydrogenase in a cuvette containing the assay mixture for succinate-ubiquinone reductase, the formation of succinate-ubiquinone reductase occurred proportionately to enzymatic activity, and was observed as time proceeded. From a plot of activity vs. time, we can obtain the time ($t_{1/2}$) required to reach half the maximum formation of succinate-ubiquinone reductase (activity) at a given temperature. From a plot of the log k_1 (which was calculated from $k_1 = 0.693/t_{1/2}$) against reciprocal temperature, one can calculate the apparent activation energy of the interaction. As can be seen in Fig. 10, the Arrhenius plot of the interaction between QPs and succinate dehydrogenase also shows a biphasic behaviour with a break point at 26°C. Above this temperature, the interaction between QPs and succinate dehydrogenase has an activation energy of 11.2 kcal/mol. At lower temperatures, an activation energy of 6.9 kcal/mol was calculated for this interaction. This is in con-

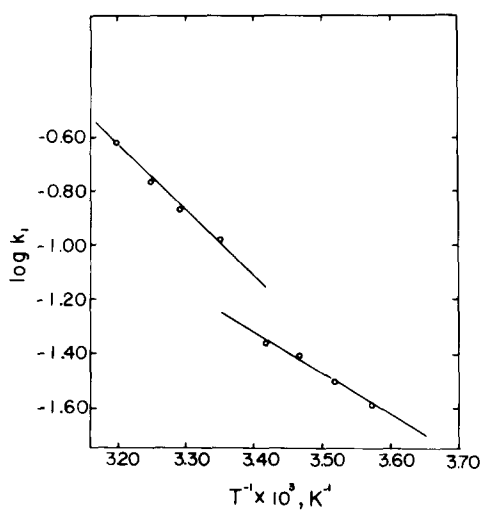


Fig. 10. Activation energy of the interaction between QPs and succinate dehydrogenase. The experimental conditions are the same as those in Fig. 9, except QPs and succinate dehydrogenase, both in small volumes, were added separately. QPs was first added to preincubated assay mixture at the indicated temperature and the reaction was then started with the addition of succinate dehydrogenase (in excess). The time ($t_{1/2}$) required to reach half of the maximal activity at a given temperature is used to calculate a rate constant using the equation: $k_1 = 0.69/t_{1/2}$.

trast to the activation energy observed for the enzymatic reaction itself. One possible explanation is that the binding between QPs and succinate dehydrogenase occurs in a more hydrophilic region, which may be on the outside of the QPs aggregate. With this orientation the binding between succinate dehydrogenase and QPs would be expected to be less affected by the physical state of the bound phospholipids or bound detergents. In fact, when the reaction temperature is lower than the transition temperature of bound phospholipids, QPs may be in a better orientation to bind succinate dehydrogenase, if the interaction, indeed, occurs in the hydrophilic region. It should be mentioned that the reported initial lag in the assay of succinate-dichlorophenolindophenol reductase activity of resolved succinate-cytochrome *c* reductase complex [19], is not observed in the reconstituted succinate-ubiquinone reductase or Complex II under our assay conditions. Therefore, the determination of the rate of interaction between QPs and succinate dehydrogenase by the above mentioned method should be valid.

Discussion

The successful reconstitution of succinate-ubiquinone reductase from pure soluble succinate dehydrogenase and purified QPs as described in this communication, clearly demonstrates that only three protein subunits are involved in succinate-ubiquinone reductase activity. Whether or not the other components found in the isolated Complex II [10,21] have an indirect effect on the stabilization of the enzyme complex remains to be seen. Although the reconstituted succinate-ubiquinone reductase possesses the same parameters as those of the intact enzyme system, the reconstituted activity is not as stable as the activity in Complex II or succinate-cytochrome *c* reductase. The reconstituted enzyme lost 20% of its activity after 10 h of incubation at 0°C, while under the same conditions, succinate-ubiquinone reductase activity in Complex II or succinate-cytochrome *c* reductase has almost no loss in activity. This result might indicate that the presence of other electron transferring components, even though these components are in the denatured state, might have some protective effect.

Since all three polypeptides of succinate-ubiquinone reductase are associated with functional prosthetic groups, they can not be considered so-called 'core' or 'structural' proteins [22]. Perhaps in the mitochondrial inner membrane, other electron transferring components such as cytochrome *b*, might have been used as a 'core protein' for the succinate-ubiquinone reductase complex. In fact it was suggested that cytochrome *b* protein has a structural role in succinate-ubiquinone reductase some years ago [5].

QPs as isolated exists in a highly aggregated form, becoming deaggregated when reacted with pure succinate dehydrogenase. The deaggregation could result from the introduction of a hydrophilic portion of SDH in the reconstituted complex, which would weaken the hydrophobic interaction between the aggregated molecules of the QPs complex that results from the solubilization from the membrane or other membrane protein complexes. In the presence of detergent the reconstituted succinate-ubiquinone reductase is indeed a separable entity. Whether or not QPs forms a complex with the next segment of elec-

tron transferring complex, i.e., ubiquinol-cytochrome *c* reductase remains to be elucidated through vigorous experimentation.

Reconstitution of succinate cytochrome *c* reductase from succinate dehydrogenase, QPs (prepared by Method II), and cytochrome b-c_1 III complex (or Complex III) requires no external Q, suggesting that both complexes are in close proximity, if not in a complex form. A 'Q pool' function of Q in NADH-cytochrome *c* reductase as suggested by Kröger and Klingenberg [23] and recently restated and modified by Heron et al. [24] probably is not present, at least, in the isolated succinate-cytochrome *c* reductase. It is likely that the interaction between QPs and succinate dehydrogenase in succinate-ubiquinone reductase involves both ionic and hydrophobic interactions. The described results indicated that a cationic group(s) in succinate dehydrogenase may play an important role. Such a group would be expected to bind a chloride ion much more strongly than a phosphate or acetate anion because less efficiency in the reconstitution was observed when the system was performed in Tris buffer containing chloride ions. One possible candidate could be the ϵ-amino group of lysine residues. Modification of primary amino groups of lysine residues of succinate dehydrogenase by fluorescamine [14] does appreciably affect its reconstitutive activity. The possibility of participation of the guanyl groups of arginine residues in the interaction remains to be verified experimentally. From the facts reported here and preciously [1] we can formulate a picture of succinate-ubiquinone reductase in the inner membrane of mitochondria. Succinate dehydrogenase and QPs form a three subunit complex through a protein-protein interaction, with the hydrophobic portion of QPs embedded in the matrix side of membrane. In that way succinate dehydrogenase itself has contact only with the polar group of phospholipid molecules and does not penetrate deeply into the hydrophobic region of the membrane. Such an arrangement would explain the observed facts about the solubility of succinate dehydrogenase. Succinate dehydrogenase is easily solubilized by slightly alkaline conditions [25] or by treatment with KCN [26], and the solubilized succinate dehydrogenase remains in monomer form and stays soluble in a neutral medium in the absence of detergent. The solubilization of succinate dehydrogenase by chaotropic reagents [13,27] may result from the disruption of hydrophobic interaction between succinate dehydrogenase and QPs. Although only the hydrophobic nature of the interaction of succinate dehydrogenase with the mitochondrial inner membrane or with other Complex II components has been stressed [13,28], it seems, at this moment, that both hydrophobic and ionic interactions are involved in the formation of succinate-ubiquinone reductase from succinate dehydrogenase and QPs. Further studies on the protein-protein interaction between succinate hydrogenase and QPs by chemical modification as well as by physical studies should be able to clarify which is the primary cause of the interaction, and are currently in progress in our laboratory.

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