Isolation and Properties of a Mitochondrial Protein That Converts Succinate Dehydrogenase into Succinate–Ubiquinone Oxidoreductase†

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ABSTRACT: A mitochondrial protein (QPs), which converts soluble succinate dehydrogenase into succinate–ubiquinone oxidoreductase, has been isolated from the soluble cytochrome b–c_1 complex by using two different methods. Method I involves treatment with Triton X-100 in the presence of 2 M urea, followed by calcium phosphate column chromatography and ammonium sulfate fractionation. This method gave a highly purified QPs which had relatively low specific activity and ubiquinone content. Method II involves ammonium acetate fractionation in the presence of deoxycholate, ammonium sulfate fractionation in the presence of urea, and differential centrifugation. This method yielded a QPs with high specific activity and higher recovery but with less purity. The major contaminant in QPs obtained by method II is denatured cytochrome b. The molecular (subunit) weight of QPs is 15000. QPs is stable at neutral pH and at low temperatures but denatures rapidly when the enzyme is incubated at room temperature or above. The isolated QPs is soluble in aqueous solution but is in a highly aggregated form. The molecular weight determined by Bio-Gel A-5m gel filtration is ~1 000 000. QPs obtained by method II contains about 25 nmol of ubiquinone/mg of protein whereas QPs obtained by method I contains less than 10 nmol of ubiquinone/mg of protein. The isolated QPs is very stable to detergent treatment, especially when detergent alone is used. No significant loss of activity was observed when QPs was incubated in 2% Tween-80, 1% Triton X-100, or 1% sodium dodecyl sulfate. In fact, the isolated QPs is more stable to sodium dodecyl sulfate treatment than QPs in the soluble b–c_1 complex or in succinate–cytochrome c reductase. This result may be due to the strong binding between QPs and the mild detergent used during the isolation. No loss of activity was observed when QPs or complex II was treated with purified phospholipase A_2.

Elucidation of the relationship between structure and function of a complicated membrane-bound enzyme complex requires full understanding of each participating component in the system. Careful analysis of the chemical composition of succinate–cytochrome c reductase (Takemori & King, 1964) reveals that the enzyme complex contains cytochromes b and c_1, non-heme iron sulfur proteins, flavoproteins, phospholipids (PL), and ubiquinone (Q).[1] Among these essential components, all the functional prosthetic groups except Q are found to be associated with a particular protein and have received great attention as holoprotein entities. Ubiquinone, on the other hand, until the recent identification of the succinate dehydrogenase succinate–ubiquinone reductase converting protein (QPs) (Yu et al., 1977a) and the observation of ubisemiquinone radical in the cytochrome b–c_1 region (Yu et al., 1978) has been believed to exist as a free mobile molecule in the mitochondrial inner membrane (Green, 1962; Kröger & Klingenberg, 1967).

The discovery of the QPs in succinate–Q reductase (Yu et al., 1977a) and the successful reconstitution of succinate–Q reductase from soluble succinate dehydrogenase (SDH) and QPs (Yu et al., 1977b) has modified the totally mobile status of ubiquinone (Green, 1962; Kröger & Klingenberg, 1967) to a partly protein-bound state during the electron transfer reactions. Since the amount of Q present in the mitochondrial inner membrane is much more than that of the other electron transfer components, it is obvious that a majority of Q exists in a mobile state. QPs is found in the soluble cytochrome b–c_1 complex (Yu et al., 1974), complex II (Ziegler & Doeg, 1962), and succinate–cytochrome c reductase (Takemore & King, 1964) but not in complex III (Hatefi et al., 1962) or the cytochrome b–c_1 III complex (Yu & Yu, 1980). Since preparations of complex III and the soluble b–c_1 complex have become available, investigators in the field have been puzzled concerning the real difference between them. In fact, many people have used both terminologies interchangeably despite our repeated stress on the differences in reconstitutive activity of the two preparations toward soluble SDH. The cytochrome b–c_1 complex can reconstitute with soluble SDH to form succinate–Q reductase or succinate–cytochrome c reductase while complex III cannot. Since the finding of QPs in the soluble b–c_1 complex it has become clear that the failure of complex III to react with SDH is due to a lack of QPs in the preparation. Upon addition of QPs and soluble SDH to complex III or cytochrome b–c_1 III complex, an antimycin A sensitive succinate–cytochrome c reductase activity was reconstituted.

In this communication, we report two isolation procedures for QPs and some properties of this protein.

Experimental Section

Materials. Succinate–cytochrome c reductase (Yu et al., 1974) and the soluble cytochrome b–c_1 complex (Yu et al., 1974) were prepared from beef heart muscle preparation (King, 1961) according to the methods reported from this

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[1] Abbreviations used: βME, β-mercaptoethanol; CTMAB, cetyltrimethylammonium bromide; DCIP, 2,6-dichlorophenolindophenol; DOC, deoxycholate; PHMB, *p*-(hydroxymercuri)benzoate; PUS, 50 mM sodium potassium phosphate buffer, pH 7.4, containing 2 M urea and 0.25 M sucrose; Q, ubiquinone; QPs, a mitochondrial protein which converts succinate dehydrogenase into succinate–Q reductase (QPs also refers to Q-binding protein in the authors' other publications); SDH, succinate dehydrogenase; $NaDodSO_4$, sodium dodecyl sulfate; TUS, 50 mM Tris-HCl buffer, pH 8.0, containing 2 M urea and 0.67 M sucrose; TUT, 50 mM Tris–acetate buffer, pH 7.8, containing 2 M urea and 1.5% Triton X-100.

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laboratory. Pure SDH (Yu & Yu, 1980), complex II (Ziegler & Doeg, 1962), complex III (Hatefi et al., 1962), and b–c_1–III (Yu & Yu, 1980) were prepared according to the reported methods. Q_2 was synthesized from Q_0 and geraniol essentially according to the method of Shunk et al. (1958) and Q_0 was synthesized from creosol by the method of Oberlin (1925) with some modifications. Triton X-100 was a product of Rohm and Haas; urea was obtained from Fisher; p-(hydroxymercuri)benzoate (PHMB), phospholipase A_2, and sodium cholate were from Sigma Chemical Co. Other chemicals were purchased commercially in the highest available purity.

Methods. Enzymatic activity of the QPs was assayed by succinate–Q oxidoreductase activity (Ziegler & Doeg, 1962) after reconstitution with excess soluble SDH. Experimentally, various amounts of QPs were diluted in 0.3 mL of 50 mM sodium potassium phosphate buffer, pH 7.4, before mixing with 20 μL of succinate dehydrogenase (10 mg/mL). Two to ten microliters of the mixture was used for assay after incubation for 10 min at 0 °C with 1 mL of an assay mixture containing 100 μmol of sodium potassium phosphate buffer, pH 7.0, 50 nmol of 2,6-dichlorophenolindophenol (DCIP), 20 μmol of succinate, 10 nmol of EDTA, 16 nmol of Q_2, and 0.1 mg Triton X-100.

For preparation of the calcium phosphate chromatographic column, a suspension of calcium phosphate was mixed and packed with an equal amount of cellulose powder in 50 mM Tris–acetate buffer, pH 7.8, containing 2 M urea and 1.5% Triton X-100. The use of cellulose powder ensures a good flow rate. The column was usually packed and equilibrated at room temperature with 2–3 volumes of the same buffer before it was placed in the cold room where the chromatography was done.

Analytical sodium dodecyl sulfate ($NaDodSO_4$) polyacrylamide gel electrophoresis was carried out according to the methods reported by Weber & Osborn (1969) and Swank & Munkres (1971). Protein content was estimated by biuret method (Gornall et al., 1949) using bovine albumin as the standard. Sulfhydryl groups were estimated according to Boyer (1964), and phospholipids were estimated by phosphorus content which was determined by the method of Ames & Dubin (1960). Individual phospholipids were detected essentially according to Rouser & Fleischer (1967) except the silica gel-H with SiO_2 as binder was used. The concentration of Q in QPs was estimated by the method of Redfearn (1967). The millimolar extinction coefficient of 12.25 was used for the difference in absorption of the oxidized and reduced forms of Q at 275 nm. Amino acid composition was determined according to the method reported by Moore & Stein (1963), and the analysis was performed on a Beckman 120 C amino acid analyzer.

Results

Purification of QPs. (*a*) *Method I.* Eight milliliters of the dialyzed cytochrome b–c_1 complex, 12 mg/mL, was precipitated with 2/3 volume of neutralized, saturated ammonium sulfate solution after the mixture was allowed to stand at 0 °C for 20 min. The precipitate was collected by centrifugation at 20000g for 20 min and dissolved in 50 mM Tris–acetate buffer, pH 7.8, containing 2 M urea and 1.5% Triton X-100 (TUT). The solution was kept at 0 °C for 20 min and frozen at −20 °C for at least 2 h. The sample was thawed and applied to a calcium phosphate column (2 × 10 cm), which was packed and equilibrated with TUT buffer. The column was eluted with the same buffer at a flow rate of 0.3 mL/min. The bright red effluent, which contains QPs and cytochrome b, was collected and subjected to ammonium sulfate fractionation. QPs was recovered in the floating precipitate formed between 25 and 35% ammonium sulfate saturation. QPs was then dissolved in 50 mM Tris–acetate buffer, pH 6.5, and dialyzed against the same buffer, overnight, with one change of buffer and stored in a deep freezer until use. Table I summarizes the purification data. About 15% of the QPs activity present in the soluble cytochrome b–c_1 complex was recovered in the final purification step.

Table I: Purification Data for QPs

treatment	vol (mL)	protein (mg/mL)	act. sp [μmol of succinate/(min mg)]	act. recovery (%)
	Method I			
dialyzed b-c_1 complex	8	12	2.1	
TUT-treated b-c_1 complex	8.4	11	4.1	100
calcium phosphate column, effluent	14.3	3.2	7.0	84
$(NH_4)_2SO_4$ fractionation (25–35%)	2.3	1.6	17.0	16
	Method II			
DOC-solubilized b-c_1 complex	31	15	4.2	100
ammonium acetate fractionation (5–10%)	11	10	16.1	91
TUS-$(NH_4)_2SO_4$ (17–23%)	6.9	5	43	76
PUS plus 0.3% βME and $(NH_4)_2SO_4$ (19–32%)	8.5	2	54	47
supernatant soln of 10 h, 53000g centrifugation	8.2	1.2	79	40

(*b*) *Method II.* Although a purified QPs is obtained by method I, the recovery of activity is low. To improve the isolation procedure and at the same time save the ubiquinol–cytochrome c reductase, we developed a new method for the isolation of QPs also using the soluble b–c_1 complex as the starting material. The dialyzed soluble b–c_1 complex was first precipitated with 2/3 volume of neutralized, saturated ammonium sulfate solution. The precipitate was collected by centrifugation and resuspended in a 50 mM Tris-HCl buffer, pH 8.0, containing 0.67 M sucrose, to a protein concentration of ~15 mg/mL. The protein was then solubilized with potassium deoxycholate, 0.4 mg/mg of protein. The insoluble material, if any, was removed by centrifugation, and the clear solution was subjected to ammonium acetate fractionation. A 50% saturated ammonium acetate solution was used, which was prepared by dissolving 454 g of ammonium acetate into 613 mL of water according to Hatefi & Rieske (1967). Crude QPs which contained more than 90% of the total QPs present in the b–c_1 complex was collected in the precipitate formed between 5 and 10% ammonium acetate saturation. The purified b–c_1–III (ubiquinol–cytochrome c reductase) was precipitated between 10 and 18.5% ammonium acetate saturation. The crude QPs thus obtained was dissolved in 50 mM Tris-HCl buffer, pH 8.0, containing 2 M urea and 0.67 M sucrose (TUS), to a protein concentration of ~10 mg/mL, and the solution was subjected to ammonium sulfate fractionation with neutralized, saturated ammonium sulfate solution. QPs was precipitated between 17 and 23% ammonium sulfate saturation. The precipitate was collected by centrifugation at 20000 rpm for 20 min and dissolved in 50 mM phosphate buffer, pH 7.4, containing 2 M urea, 0.25 M sucrose (PUS), and 0.3% β-mercaptoethanol (βME) to a protein concentration of ~5 mg/mL. The solution was then incubated at 0 °C for 1 h and

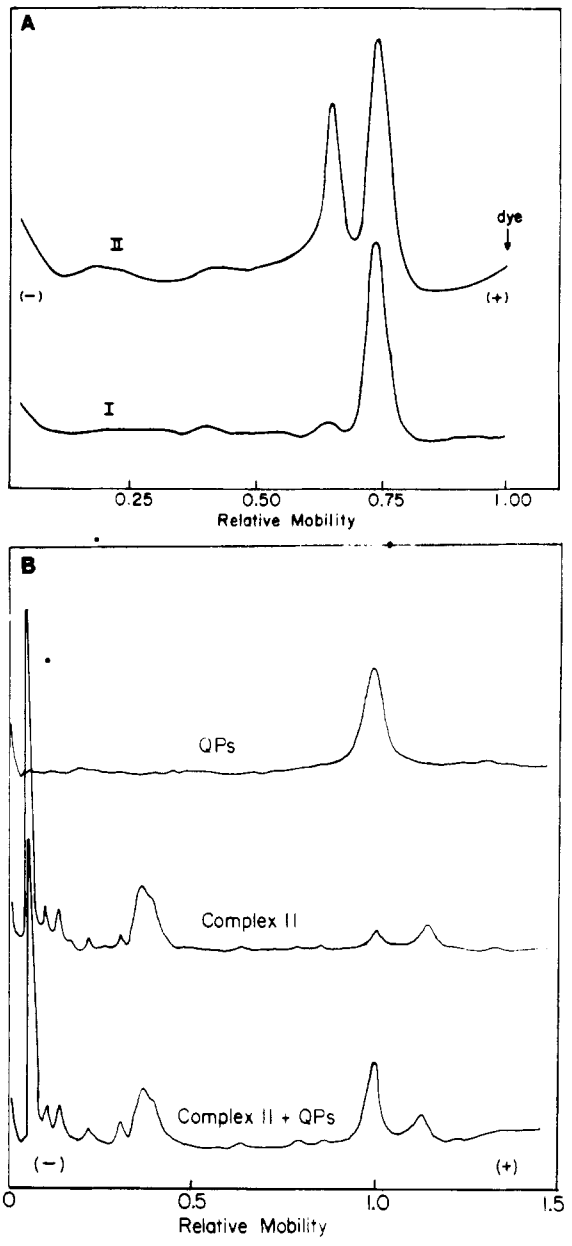

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoretic patterns of QPs. (A) In the Weber–Osborn gel system; the upper curve represents densitometric tracing (600 nm) of $NaDodSO_4$–polyacrylamide gel electrophoretic column of QPs, 20 μg, prepared by method II and the lower curve represents 10 μg of QPs prepared by method I. The relative mobility is expressed in related to the mobility of tracking dye. (B) In the Swank–Munkres gel system; the upper curve represents 10 μg of QPs prepared by method I, the middle curve is 20 μg of complex II, and the bottom curve is the mixture of 20 μg of complex II and 4 μg of QPs prepared by method I. The relative mobility is expressed relative to that of cytochrome *c*.

fractionated with ammonium sulfate. The precipitate formed between 19 and 32% ammonium sulfate saturation was collected and redissolved in 50 mM sodium potassium phosphate buffer, pH 7.4, containing 2 M urea, to a protein concentration of ~2 mg/mL. The solution was centrifuged at 53 000 rpm for 10 h, and purified QPs was recovered in the supernatant solution which was then concentrated with ammonium sulfate precipitation.

Although this isolation procedure has a higher recovery of QPs activity than method I and produces, as well, a purified b–c_1–III (ubiquinol–cytochrome *c* reductase), it suffers from a slight contamination by cytochrome *b* in the QPs preparation. Through studies of the spectral properties and redox behavior

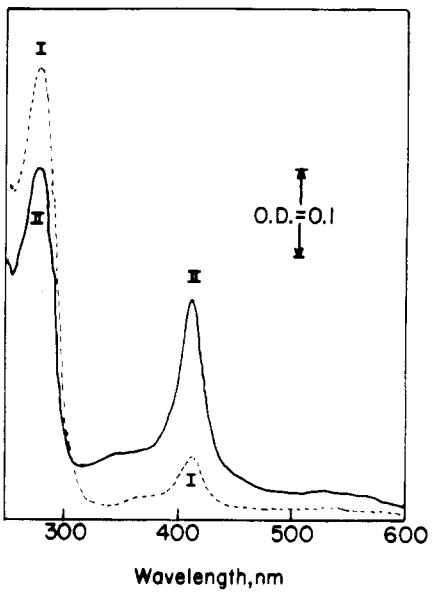

FIGURE 2: Absorption spectra of QPs prepared by methods I and II: QPs prepared by method I, 0.25 mg/mL in 50 mM Tris–acetate buffer, pH 6.5, and QPs prepared by method II, 0.2 mg/mL in 50 mM Tris-HCl buffer, pH 8.0, containing 0.67 M sucrose. The spectra were taken in a Cary spectrophotometer, Model 14, at room temperature, using 1-cm light path cuvette.

of cytochrome *b* in the QPs preparation, we found that the contaminated cytochrome *b* is denatured and can be reduced only by sodium dithionite. These purification data are also given in Table I.

Purity of the Isolated QPs. The purified protein obtained from method I shows only one protein band in the $NaDodSO_4$–polyacrylamide gel electrophoretic columns of both the Weber & Osborn (1969) and the Swank & Munkres (1971) systems, indicating that the protein is over 90% pure. Figure 1A shows the densitometric tracing of QPs obtained by methods I and II in the $NaDodSO_4$–polyacrylamide gel electrophoretic column of the Weber–Osborn system. Figure 1B shows the densitometric tracing of QPs, complex II, and a mixture of QPs and complex II in the Swank–Munkres system of the $NaDodSO_4$–polyacrylamide gel electrophoretic column. The single protein peak of QPs obtained by method I in these systems indicates that the protein is composed of one polypeptide, which is identical with the second fast running band of complex II. When the electromobility of QPs is compared with complex II in the Weber–Osborn system of the $NaDodSO_4$–polyacrylamide gel, QPs had the same mobility as the first running band of Complex II (Yu et al., 1977a). These results suggest that the two fast moving bands of complex II are reversed in order in these two systems of $NaDodSO_4$–polyacrylamide gel. This finding is similar to that observed in complex III (Capaldi et al., 1977).

Although QPs obtained by method I shows a single protein band in the analytical $NaDodSO_4$–polyacrylamide gel electrophoretic pattern, at high protein concentration, a trace amount of cytochrome *b* (<1 nmol of *b*/mg of protein) can be detected. No other components, beside Q and a trace amount of cytochrome *b*, can be detected spectrally or chemically in the isolated protein. The absorption spectra of QPs prepared by method I is given Figure 2. Although the purity of QPs obtained by method I is higher than that obtained by method II, the specific activity of QPs from method I is rather low, indicating that some denatured protein is present in the final product. As can be seen from the purification data in Table I, the last step of ammonium sulfate precipitation has

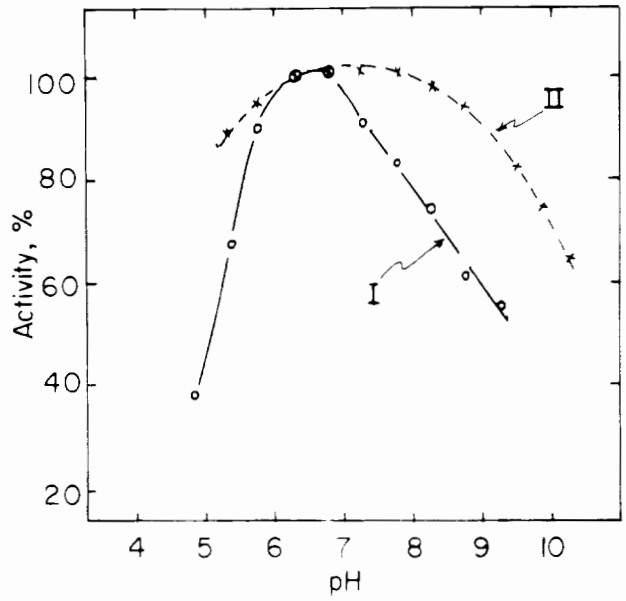

FIGURE 3: Effect of pH on the stability of QPs. Purified QPs prepared by methods I (○) and II (×) at a protein concentration of 0.3 mg/mL were incubated in 0.1 M Tris–acetate buffer at various pH values for 12 h at 0 °C. At the end of incubation, the solutions were neutralized to pH 7.4, before reconstitution with excess succinate dehydrogenase. The reconstitution experiments were carried out as described under Experimental Section. The activity at pH 7.0 was used as 100%.

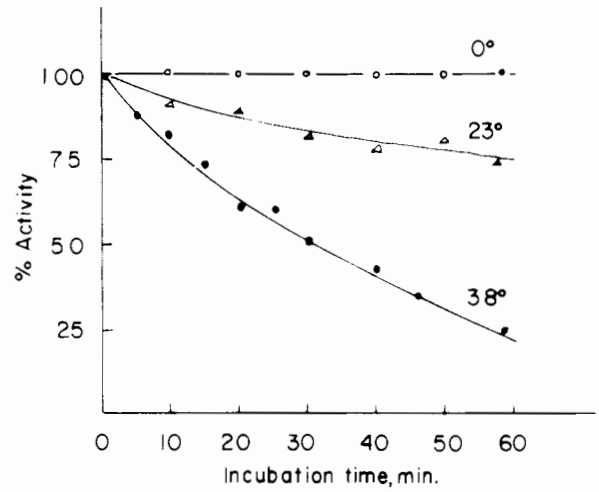

FIGURE 4: Effect of incubation temperature on the stability of QPs. QPs prepared by method II was used in this study. Samples of QPs at a protein concentration of 2 mg/mL in Tris-HCl buffer, pH 8.0, containing 0.67 M sucrose were incubated at the indicated temperatures. Aliquots were withdrawn for reconstitution with excess succinate dehydrogenase, and the reconstituted succinate–Q oxidoreductase activity was determined. Activity at zero-time incubation was used as 100%.

seriously inactivated the enzyme. Attempts to protect the enzyme from inactivation at this step have thus far not been successful.

QPs obtained by method II, on the other hand, has a high specific activity but less purity. The protein is estimated by $NaDodSO_4$–polyacrylamide gel electrophoresis to be ~70% pure. The major contaminatant is cytochrome *b*[2] with a molecular weight of 17 000 (Yu et al., 1975). The contamination by cytochrome *b* of the QPs preparation is evident from the presence of the Soret absorption at 418 nm (Figure 2). The $NaDodSO_4$–polyacrylamide gel electrophoretic pattern and absorption spectra of QPs obtained by method II are given in Figures 1A and 2, respectively. There are two protein bands in the $NaDodSO_4$–polyacrylamide gel electrophoretic column which correspond to molecular weights of 17 000 and 15 000. The band with high intensity (15 000) is attributed to QPs, and the band with less intensity is attributed (17 000) to cytochrome *b*. Although the possibility exists that the presence of cytochrome *b* protein may have a stabilizing effect or even enhance QPs activity, cytochrome *b* alone is absolutely inactive in a reconstitution with soluble SDH to form succinate–Q oxidoreductase. Moreover, cytochrome *b* in QPs can be further denatured by the treatment of thiol compounds such as βME without altering the activity of QPs. The further denaturation of the contaminating cytochrome *b* is confirmed by the change of its spectral properties.

Stability of QPs. QPs obtained by either method is very stable in the neutral pH range at 0 °C. The stability decreases as the pH of the solution becomes either alkaline or acidic. Figure 3 show the pH profile of QPs obtained by both methods. QPs prepared by method II is more stable at alkaline pH than that obtained by method I. One possible explanation for this difference is the presence of Triton X-100 in QPs obtained from method I.

The enzyme is stable at 0 °C or lower; it can be stored in a deep freezer (−70 °C) for months without any loss of reconstitutive activity. Figure 4 shows the stability of QPs at various incubating temperatures. QPs loses about one-fourth of its activity during 1 h of incubation at 23 °C, while at 38 °C, the protein has a half-life of 30 min. Commonly used enzyme stabilizing agents such as thiol compounds, glycerol, and other polyglycols do not show any protecting effect on this protein against heat denaturation. No difference in stability was observed between preparations stored anaerobically and those stored aerobically.

Solubility of QPs. QPs as isolated is very soluble even after prolonged dialysis against a detergent-free buffer system, indicating that the protein has a very strong association with detergents used during purification. The bound detergent can be removed only by passage through a high exclusion volume gel column filtration such as Bio-Gel A-0.5m. After removal of detergents by this treatment, QPs becomes aggregated but can be resolubilized by Triton X-100 or deoxycholate.

Molecular Weight of QPs. Judging from its relative mobility in a $NaDodSO_4$–polyacrylamide gel electrophoretic column, the molecular weight (subunit weight) of QPs should be ~15 000, showing a slightly slower mobility than that of cytochrome *c*. The actual molecular weight, however, has not yet been precisely determined by other methods because of the difficulties resulting from its strong association with detergent in the isolated form. In the absence of strong detergents, such as $NaDodSO_4$, the isolated QPs is in a highly aggregated form which has a molecular weight ~1 000 000 as shown from its eluting volume from Bio-Gel A-5m column filtration. A partial deaggregation of QPs was observed upon reconstitution with purified SDH in the absence of added detergents. In the presence of 0.2% Triton X-100, the reconstituted complex is dispersed to the monomeric state with a molecular weight of ~120 000.

Chemical Composition of QPs. Table II shows the chemical composition of QPs obtained by both methods. QPs obtained by method I contains less Q and phospholipid than that ob-

[2] Cytochrome *b* present in QPs preparation obtained by method II may be identical with the cytochrome *b* observed in complex II, as complex II also has a major protein component with a molecular weight of 17 000. According to Davis et al. (1973) this cytochrome *b* is different from the *b* cytochromes present in complex III.

Table II: Chemical Composition of QPs

components	method I	method II
ubiquinone (nmol/mg of protein)	10	17–25
flavin (nmol/mg of protein)	0	0
non-heme iron (nmol/mg of protein)	0	
cytochrome *b* (nmol/mg of protein)	1	8
phospholipids (%)	30	52
distribution of major phospholipids (mol %)		
phosphatidylcholine	85	52
phosphatidylethanolamine		33
diphosphatidylglycerol		13
others	15	2

tained by method II. The major phospholipid components also differ in the two preparations, although both have phosphatidylcholine as the major phospholipid. The difference in phospholipid composition between the two QPs preparations is expected as QPs obtained by method II contains cytochrome *b* which may have a phospholipid affinity different from that of QPs itself. The phospholipid composition of QPs obtained by method II is very similar to that of complex II. The isolated QPs contains no prosthetic groups other than Q and denatured cytochrome *b*.

Ubiquinone Content in the Isolated QPs. As can be seen in Table II, QPs isolated by method I contains less than 10 nmol of Q/mg of protein whereas QPs obtained from method II contains more than 20 nmol of Q/mg of protein. The higher content of Q in QPs by method II agrees with the higher specific activity. The rather low specific activity in QPs obtained by method I may result from the loss of Q and subsequent aggregation or even denaturation of QPs because addition of Q to the protein does indeed increase the activity to a certain degree. Even this stimulated activity, however, is well below that of the QPs obtained by method II. Direct determination of Q in QPs of method I is very difficult due to the presence of Triton X-100 in the preparation. The binding kinetics of Q with QPs and the effect of different treatments on these kinetics are currently under investigation. The high hydrophobicity of QPs and Q has made the binding studies rather difficult.

Sulfhydryl Groups of QPs. It has been shown that the most readily alkylated half of the sulfhydryl groups in the soluble b–c_1 complex is essential for the complex to reconstitute with soluble SDH to form succinate–Q oxidoreductase activity (Yu et al., 1974). It is, therefore, of interest to see how important the sulfhydryl groups of QPs are for reconstitution with soluble SDH. When QPs was treated with various amounts of *p*-(hydroxymercuri)benzoate (PHMB) and then reconstituted with excess SDH, it was observed that QPs lost all ability to convert SDH into succinate–Q reductase when the protein (0.13 mg/mL) was incubated with 30 μM of PHMB. The total PHMB titratable groups in QPs and their kinetic behavior toward the alkylating reagent as well as their role in enzymatic function remain to be investigated.

Amino Acid Composition of QPs. Table III shows the amino acid composition of the pure QPs obtained by method I. No striking difference was observed between the hydrophobicity of amino acid composition of QPs and that of other membrane proteins. The amino acid compositions of the 15 000 molecular weight subunit obtained from purified cytochrome c_1 and from the b–c_1 complex are included for comparison. The difference among these subunits is very obvious and was expected. This result supports the claim that multiple polypeptides are present in the 15 000 subunit of the soluble b–c_1 complex and that QPs is just one of these.

Table III: Amino Acid Composition of QPs

amino acids	concn (mol/mol of protein) QPs	c_1 S[a]	subunit VII[b]
Lys	8.2	6.8	8.5
His	3.4	3.3	2.4
Arg	8.4	6.7	4.1
Asp	9.8	8.1	9.1
Thr	4.6	5.2	3.6
Ser	6.3	4.6	6.3
Glu	11.9	19.8	14.9
Pro	7.7	3.9	4.8
Gly	8.7	3.7	6.5
Ala	10.2	7.0	7.8
Val	5.4	6.2	5.2
Ile	5.8	2.5	4.7
Leu	12.7	12.3	10.9
Tyr	4.7	2.2	2.5
Phe	3.9	4.1	2.6
Met		0.5	2.9
Cys		2.1	
Try		1.1	

[a] Taken from Chaing (1976). [b] Taken from Yu et al. (1977).

Effect of Urea, Sodium Dodecyl Sulfate, and Other Detergents on QPs. During the course of isolation of QPs, it was noted that the protein binds detergents such as deoxycholate more strongly than do other proteins in the b–c_1 complex, yet the detergents show no harmful effect on activity. Therefore, it is of interest to see the effect of stronger detergents such as $NaDodSO_4$ and cetyltrimethylammonium bromide (CTMAB) on the reconstitutive activity of QPs. The protein was incubated with various amounts of $NaDodSO_4$ at 0 °C for 30 min before being reconstituted with soluble SDH to form succinate–Q oxidoreductase. QPs suffered little loss of activity when $NaDodSO_4$ concentration is less than 1 mg/mg of protein. A similar result was obtained when QPs was treated with 1% CTMAB at 0 °C. The purified QPs is also very stable toward treatment with urea and other nonionic detergents such as Triton X-100 and Tween-80 when they are used alone. For example, no significant loss of activity was observed when QPs (6 mg/mL) was incubated with urea at the concentration as high as 4 M. However, when QPs was incubated with 2 M urea and 2% Triton X-100, ~30% inactivation of the reconstitutive activity was observed.

Effect of Phospholipase A_2 on QPs. Isolated QPs, as indicated in Table II, contains phosphatidylcholine as the major phospholipid. The role of phospholipids in QPs as well as in complex II, is however, not yet established as the delipidated succinate–cytochrome *c* reductase retains most of the succinate–Q reductase activity. Therefore, it is of interest to see the effect of phospholipase A_2 on these enzyme complexes. When QPs, 1 mg/mL, was treated with purified phospholipase A_2 in the concentration of 10 μg/mg of QPs protein, at room temperature, no loss of reconstitutive activity besides that resulting solely from the incubation was observed over several hours. Under the same conditions, complex II also suffered no loss of succinate–Q reductase activity. This results suggest that phospholipids play no direct role in the catalytic activity of QPs and complex II.

Discussion

The existence of QPs, which is responsible for converting soluble SDH to succinate–Q oxidoreductase, is now physically established through the successful isolation of this protein and its reconstitution with SDH.

Method I suffers seriously from loss of activity at the last step of the purification procedure whereas method II produces a less pure preparation (70–80% pure) of QPs. Fortunately,

these two methods are complementary, since method I gives a QPs which shows only one protein band in the NaDodSO$_4$–polyacrylamide gel electrophoretic pattern of both the Weber–Osborn and the Swank–Munkres systems, allowing us to identify which subunit in the cytochrome b–c_1–III complex or complex II is responsible for QPs, and QPs prepared by method II has a higher reconstitutive activity and is suitable for physical studies. The contamination by cytochrome b in QPs does not pose any problem for activity as it can be further denatured by treatment with thiol compounds and Zwittergent 314. When QPs prepared by method II was treated with 1% Zwittergent in the presence of 0.1% βME, followed by dialysis against 50 mM phosphate buffer, pH 7.4, containing 0.25 M sucrose, overnight, the Soret absorption of the contaminated cytochrome b decreased greatly, and little effect on the reconstitutive activity of QPs was observed. This observation supports the claim that cytochrome b is not involved in succinate–Q oxidoreductase reaction. However, it is not clear at the present time whether the presence of cytochrome b protein in QPs preparation has some stabilizing effect on the activity or not. Attempts to completely remove the denatured cytochrome b protein from QPs prepared by method II have so far been unsuccessful. This leaves room for the possibility that the lowered activity of QPs as prepared by method I is due to the removal of cytochrome b protein rather than to denaturation during isolation. It is obvious that the catalytic role of the cytochrome b protein, which is present in QPs prepared by method II, cannot by completely ruled out until a fully active, single polypeptide QPs is obtained. However, judging from the difference in stability and activity between the two QPs preparations, it seems probable that cytochrome b protein may have some stabilizing effect or structural role in QPs.

As shown under Results, the purified QPs is very stable. It can sustain treatment with high concentration of either urea (up to 4 M) or detergents (up to 2%) alone at 0 °C. Surprisingly, QPs in the cytochrome b–c_1 complex is more labile to detergent than isolated QPs. For example, treatment of the b–c_1 complex with 1% of a cationic detergent such as CTMAB destroyed the reconstitutive activity of the b–c_1 complex completely whereas under the same conditions the purified QPs suffered no loss of reconstitutive activity. Similar results were observed when anionic detergents such as sodium dodecyl sulfate were used. One possible explanation for this unusual phenomenon is that the isolated QPs is in a highly aggregated form and the detergent binding sites have been covered up either by phospholipids or by detergents such as the Triton X-100 or deoxycholate used during the isolation. The isolated QPs possesses a very strong binding affinity to detergents such as deoxycholate used in the isolation, and removal of these detergents is very difficult, even by prolonged dialysis.

As can be seen in Table II, the isolated QPs contains ~1 μmol of phospholipids/mg of protein. Addition of phospholipids, asolectin, or phospholipids prepared from mitochondria does not enhance the activity. The requirement and the actual role of phospholipids in this particular region of the electron transport chain has not yet been established. Evidence for (Bruni & Racker, 1968; McPhail & Cunningham, 1975) and evidence against (Yu et al., 1973; Tyler & Estabrook, 1966) the requirement of phospholipids for succinate–Q oxidoreductase activity are both available. In our previous report (Yu et al., 1973) we showed that in the phospholipid-depleted succinate–cytochrome c reductase preparation (~90% phospholipids removed), ~60% of succinate–Q oxidoreductase activity was retained but all the ubiquinol–cytochrome c reductase activity was lost, indicating that phospholipids may not be required in the succinate–Q oxidoreductase region. The present result, ineffectiveness of phospholipase A_2 on QPs, have further supported this deduction. A remote possibility, however, exists that phospholipids may be structurally important if they are associated specifically with QPs. The presence of a significant amount of phospholipids in the isolated QPs is worth further investigation. Since the reaction with phospholipase A_2 produces lysophospholipids, which have properties similar to detergents, perhaps it is safe to say at this point that succinate–Q reductase requires no specific phospholipids for activity but needs a fluid environment, as provided by detergent or some similar substance, to catalyze the reaction.

Since the electron acceptor of succinate–Q oxidoreductase is the b–c_1 complex which has been shown to be embedded in the membrane (Gellerfors & Nelson, 1977), part of the QPs molecule would probably also be embedded in the membrane in order to provide better contact with the b–c_1 complex. Therefore, it is possible that the role of PL in QPs is to sit QPs in the membrane, similar to the behavior of the hydrophobic portion of cytochrome b_5 (Strittmatter et al., 1972). The electron transfer between succinate and Q may actually occur in the region of the protein molecules where phospholipids are not involved (Tyler & Estabrook, 1966). This deduction would explain why in a more complex system, such as the delipidated preparation of succinate–cytochrome c reductase, phospholipids are not needed for succinate–Q oxidoreductase activity, as the hydrophobic region of QPs interacts with the hydrophobic part of another protein. When QPs is solubilized by detergent, its hydrophobic region becomes exposed and may then be bound to phospholipids or to the hydrophobic portion of another QPs molecule or detergents. The observation of a highly aggregated QPs and high content of phospholipids supports this deduction.

The results of amino acid composition show that QPs is not very hydrophobic, indicating that a specific localization of hydrophobic amino acid residues in a so-called hydrophobic patch must exist in QPs. The interaction between a hydrophobic patch and phospholipids becomes more prominent when QPs is detached from the b–c_1 complex. Elucidation of the interaction between QPs and phospholipids will require more experimentation.

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References

Ames, B. N., & Dubin, D. T. (1960) *J. Biol. Chem. 235*, 767–775.

Boyer, P. D. (1964) *J. Am. Chem. Soc. 76*, 4331–4337.

Bruni, A., & Racker, E. (1968) *J. Biol. Chem. 243*, 962–871.

Capaldi, R. A., Bell, R. L., & Branchek, T. (1977) *Biochem. Biophys. Res. Commun. 74*, 425–433.

Chaing, Y. L. (1976) Ph.D. Thesis, State University of New York at Albany, Albany, NY.

Davis, K. A. Hatefi, Y., Poff, K. L., & Butler, W. L. (1973) *Biochim. Biophys. Acta 325*, 341–356.

Gellerfors, P., & Nelson, D. (1977) *Eur. J. Biochem. 80*, 275–282.

Gornall, A. G., Bardawell, C. J., & David, M. M. (1949) *J. Biol. Chem. 177*, 751–766.

Green, D. E. (1962) *Comp. Biochem. Physiol. 4*, 81–121.

Hatefi, Y., & Rieske, J. S. (1967) *Methods Enzymol. 10*, 225–230.

Hatefi, Y., Haavik, A. G., & Griffiths, D. E. (1962) *J. Biol. Chem. 237*, 1681–1685.

King, T. E. (1961) *J. Biol. Chem. 236*, 2342–2346.
Kröger, A., & Klingenberg, M. (1967) *Curr. Top. Bioenerg. 2*, 151–171.
McPhail, L., & Cunningham, C. (1975) *Biochemistry 14*, 1122–1131.
Moore, S., & Stein, W. H. (1963) *Methods Enzymol. 6*, 819–840.
Oberlin, M. (1925) *Arch. Pharm. Ber. Dtsch. Pharm. Ges. 263*, 641–668.
Redfearn, E. R. (1967) *Methods Enzymol. 10*, 381–384.
Rouser, G., & Fleischer, S. (1967) *Methods Enzymol. 10*, 385–460.
Shunk, C. H., Linn, B. O., Wong, E. L., Wittereich, P. E., Robinson, F. M., & Folkers, K. (1958) *J. Am. Chem. Soc. 80*, 4753.
Strittmatter, P., Rogers, M. J., & Spaty, L. (1972) *J. Biol. Chem. 247*, 7188–7194.
Swank, R. T., & Munkres, K. D. (1971) *Anal. Biochem. 39*, 462–477.
Takemori, S., & King, T. E. (1964) *J. Biol. Chem. 239*, 3546–3558.
Tyler, D. D., & Estabrook, R. W. (1966) *J. Biol. Chem. 241*, 1672–1680.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.
Yu, C. A., & Yu, L. (1980) *Biochim. Biophys. Acta* (in press).
Yu, C. A., Yu, L., & King, T. E. (1974) *J. Biol. Chem. 249*, 4905–4910.
Yu, C. A., Yu, L., & King, T. E. (1975) *Biochem. Biophys. Res. Commun. 66*, 1194–1200.
Yu, C. A., Yu, L., & King, T. E. (1977a) *Biochem. Biophys. Res. Commun. 78*, 259–265.
Yu, C. A., Yu, L., & King, T. E. (1977b) *Biochem. Biophys. Res. Commun. 79*, 939–946.
Yu, C. A., Yu, L., & King, T. E. (1977c) *Biochim. Biophys. Acta 495*, 232–247.
Yu, C. A., Nagaoka, S., Yu, L., & King, T. E. (1978) *Biochem. Biophys. Res. Commun. 82*, 1070–1078.
Yu, L., Yu, C. A., & King, T. E. (1973) *Biochemistry 12*, 540–546.
Ziegler, D. M., & Doeg, K. A. (1962) *Arch. Biochem. Biophys. 97*, 41–50.

Quantitative Measurements of Membrane Potential in *Escherichia coli*†

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ABSTRACT: By use of giant cells of *Escherichia coli* induced by growth in the presence of 6-amidinopenicillanic acid, membrane potentials have been measured by two completely independent techniques: *directly* with intracellular microelectrodes and *indirectly* from the steady-state distribution of [^{3}H]tetraphenylphosphonium. Under a variety of conditions, the two methods yield values that agree very closely. Thus, with both techniques, the membrane potential approximates −85 mV (interior negative) at pH 5.0 and −142 mV at pH 8.0, with an average slope of −22 mV/pH unit over the range pH 5.0–7.0. A parallel study of membrane vesicles prepared from giant cells was undertaken using tetraphenylphosphonium distribution alone as a measure of membrane potential. The vesicles were found to exhibit a much smaller slope of membrane potential vs. extracellular pH (about −6 mV/pH unit) than intact giant cells. The results indicate that distribution studies with these lipophilic cations provide an excellent measure of membrane potential and are discussed in relation to calculations of H^+/substrate stoichiometry for proton-symport systems in *E. coli*.

According to the generalized chemiosmotic hypothesis (Mitchell, 1961, 1966, 1968, 1973, 1979), energy derived from respiration, photochemical reactions, or ATP[1] hydrolysis is transformed into a transmembrane difference of electrochemical potential for protons ($\Delta\bar{\mu}_{H^+}$) that is the immediate driving force for a wide range of processes in energy-transducing cells and organelles. Since both the chemical concentration of protons and the electric field acting on them enter into the electrochemical potential, it is obvious that demonstration of the validity of the chemiosmotic hypothesis depends upon quantitative measurement of the membrane potential ($\Delta\psi$), as well as the transmembrane pH difference (ΔpH). The formal relationship is

$$\Delta\bar{\mu}_{H^+} = (\psi_i - \psi_0) + \frac{RT}{F} \ln ([H^+]_i/[H^+]_0) = \Delta\psi - \frac{2.3RT}{F}\Delta\text{pH} \quad (1)$$

where both differences ($\Delta\psi$ and ΔpH) are taken in the same direction, cytoplasm minus cell exterior.

Although large quantities of data have been reported which support the generalized chemiosmotic hypothesis, few energy-transducing membranes can be subjected to direct electrophysiological study, and most estimates of both $\Delta\psi$ and ΔpH have relied on the use of permeant ions and permeant weak acids or bases as distribution indicators. As far as *Es-*

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[1] Abbreviations used: $\Delta\bar{\mu}_{H^+}$, the proton electrochemical gradient; V_m, the measured potential difference across the cell membrane; $\Delta\psi$, actual membrane potential; ΔpH, pH difference across the membrane; TPP^+, tetraphenylphosphonium (bromide salt); $TPMP^+$, triphenylmethylphosphonium (bromide salt); EDTA, ethylenediaminetetraacetic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; ATP, adenosine 5′-triphosphate.

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