Resolution and Reconstitution of Succinate-Ubiquinone Reductase from Escherichia coli

(Received for publication, December 13, 1996, and in revised form, February 4, 1997)

Xudong Yang, Linda Yu, and Chang-An Yu

From the Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, Oklahoma 74078

A modified procedure is developed for isolation of highly purified succinate-ubiquinone reductase from Escherichia coli NM256 containing a cloned sdh operon in a multicopy plasmid. Succinate-ubiquinone reductase is solubilized from the membrane by polyoxyethylene-9-lauryl ether and purified by DEAE-Sepharose CL-6B column chromatography. The isolated reductase is resolved into a reconstitutively active, two-subunit succinate dehydrogenase and a two-subunit membrane anchoring protein fraction (the SdhC-SdhD fraction) by alkaline (pH 10.2) treatment of the reductase in the presence of 1 M urea, followed by DEAE-Sepharose CL-6B column chromatography under anaerobic conditions. Isolated succinate dehydrogenase and the SdhC-SdhD fraction alone show no succinate-ubiquinone reductase activity. However, when a given amount of the SdhC-SdhD fraction is mixed with varying amounts of succinate dehydrogenase or vice versa succinate-ubiquinone reductase activity increases as the amount of succinate dehydrogenase or the SdhC-SdhD fraction added increases. Maximum reconstitution is obtained when the weight ratio of succinate dehydrogenase to the SdhC-SdhD fraction reaches 5:26. This ratio is slightly higher than the calculated value of 3.37, obtained by assuming 1 mol of succinate dehydrogenase reacts with 1 mol of SdhC and SdhD. The isolated SdhC-SdhD fraction contains 35 nmol cytochrome b\textsubscript{556}/mg protein. Unlike mitochondrial cytochrome b\textsubscript{556}, the cytochrome b\textsubscript{556} is reducible by succinate in the isolated and complex forms. Furthermore, cytochrome b\textsubscript{556} in the isolated SdhC-SdhD fraction has absorption properties, carbon monoxide reactivity, and EPR characteristics similar to those of cytochrome b\textsubscript{556} in intact succinate-ubiquinone reductase, indicating that its heme environments are not affected by the presence of succinate dehydrogenase. However, the redox potential of cytochrome b\textsubscript{556} in the SdhC-SdhD fraction (22 mV) increases slightly when complexed with succinate dehydrogenase (34 mV). No hybrid succinate-ubiquinone reductase is formed from mitochondrial QPs (the membrane-anchoring protein fraction of bovine heart mitochondrial succinate-ubiquinone reductase) and E. coli succinate dehydrogenase or vice versa. However, the cytochrome b\textsubscript{556} in E. coli SdhC-SdhD fraction is reducible by succinate in the presence of mitochondrial succinate dehydrogenase, and the rate of cytochrome b\textsubscript{556} reduction correlates with the reconstitutive activity of the mitochondrial succinate dehydrogenase.

The Escherichia coli succinate-ubiquinone reductase catalyzes the succinate-dependent reduction of ubiquinone (Q) during aerobic respiration (1). The purified complex contains four protein subunits with apparent molecular weights of 64,000, 28,000, 19,500, and 17,500, as estimated by SDS-PAGE (2) and has five redox prosthetic groups: one covalent bound FAD, three iron-sulfur clusters (4Fe-4S, 2Fe-2S, and 3Fe-4S), and one protoheme IX, b\textsubscript{556}. The larger two subunits are succinate dehydrogenase. The 64-kDa subunit (Fp) houses FAD and the 28-kDa subunit (Ip) houses three iron-sulfur clusters. The two smaller subunits are membrane-anchoring proteins that house cytochrome b\textsubscript{556}. The ligand for cytochrome b\textsubscript{556} is a bis-histidine (3). Both membrane-anchoring subunits are thought to be required for cytochrome b\textsubscript{556} heme ligation (4), are essential for attachment of soluble succinate dehydrogenase to the inner surface of the cytoplasmic membrane, and are required for the reduction of ubiquinone. The membrane-anchoring protein fraction is named QPs (Q-binding protein in succinate-ubiquinone reductase) in the mitochondrial system (5).

In E. coli, the genes for the Fp and Ip subunits and for the membrane anchoring proteins are located in one operon (sdh), which is transcribed in the following sequence: sdhC, sdhD, sdhA (Fp), sdhB (Ip) (6, 7). This operon has been cloned and sequenced (6, 7). The amino acid sequences of the Fp and Ip subunits of succinate dehydrogenase are highly conserved when compared with enzymes from different species. For example, the Ip subunits from beef heart mitochondria and from E. coli are approximately 50% identical (6). On the other hand, amino acid sequences of the membrane anchoring subunits differ significantly among species (8). Of the available amino acid sequences of membrane anchoring subunits, QPs2 from beef heart mitochondria has higher sequence similarity to SdhC of E. coli than to SdhC of other bacteria (9). However, QPs3 from beef heart mitochondria has no sequence similarity to E. coli SdhD (10).

Although E. coli succinate-ubiquinone reductase is structurally and functionally similar to the mitochondrial enzyme, cytochrome b\textsubscript{556} in the E. coli enzyme is fully reducible by succinate (2), whereas that in mitochondria is not (11), indicating that the role of cytochrome b may differ in these two enzyme systems. In E. coli, cytochrome b\textsubscript{556} may be involved in electron transfer, whereas in mitochondria cytochrome b\textsubscript{556} plays a structural role. The latter was established by the restoration of the absorption properties, redox potential, and EPR characteristics of cytochrome b\textsubscript{556} during formation of a thenoyltrifluoroacetone (TTFA)-sensitive succinate-ubiquinone reductase from isolated QPs and succinate dehydrogenase (11).

Although intensive study of mitochondrial succinate-ubiqui-
none reductase has generated a wealth of information (8, 12, 13), the electron transfer mechanisms in this region of the chain are not understood. Bovine heart mitochondrial succinate-ubiquinone reductase has been resolved into reconstitutively active succinate dehydrogenase and QPs. High resolution SDS-PAGE of QPs reveals three protein bands, QPs1, QPs2, and QPs3, respectively (14). Although the cDNAs encoding QPs1 and QPs3 have been cloned and sequenced (10, 14), the use of a genetic approach to elucidate the structure-function of QPs, especially to identify the amino acid residues involved in ubiquinone-binding and succinate dehydrogenase docking, must await the availability of active recombinant QPs subunits.

The facts that E. coli sdh operon has been cloned and sequenced and the enzyme from this source has been purified make this an attractive model system for structure-function studies of mitochondrial succinate-ubiquinone reductase. However, the difference in succinate reducibility of cytochrome b in these two reductases, together with the fact that E. coli SdhD bears no sequence similarity with QPs3 of beef mitochondria, raises the possibility that interactions between succinate dehydrogenase and membrane anchoring subunits in these two systems are different. Recently we developed a method to resolve E. coli succinate-ubiquinone reductase into reconstitutively active succinate dehydrogenase and membrane-anchoring protein fraction (the SdhC-SdhD fraction). This, together with the availability of reconstitutively active succinate dehydrogenase and QPs from beef heart mitochondria, enables us to compare the mode of interaction between succinate dehydrogenase and membrane anchoring subunits in these two systems.

Herein we report the preparation of reconstitutively active succinate dehydrogenase and the SdhC-SdhD fraction from E. coli succinate-ubiquinone reductase. The physical properties and reconstitutive activity of purified succinate dehydrogenase and the SdhC-SdhD fraction from E. coli were measured and compared with their mitochondrial counterparts. Formation of hybrid succinate-ubiquinone reductase was tested with mitochondrial succinate dehydrogenase and E. coli SdhC-SdhD fraction and vice versa.

EXPERIMENTAL PROCEDURES

Materials—TTF, dichlorphenol-indophenol (DCPIP), polyoxymethylene-9-lauryl ether (E9C12), fumaric acid, and succinic acid were obtained from Sigma. DEAE-Sepharose CL-6B was from Pharmacia Bio-Tech Inc. Oxalic acid was from Aldrich. Other chemicals were the highest purity commercially available.

2,3-Dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone (Q2) was synthesized in our laboratory as previously reported (15). Calcium phosphate was prepared according to Jenner (16) and mixed at a 3:1 ratio with cellulose powder prior to use in chromatography. Bovine heart mitochondrial succinate-ubiquinone reductase (17), succinate dehydrogenase (18), and QPs (11) were prepared by the reported methods. E. coli K12 strain NM256/pGIS133 (6), which has plasmid containing a HI fragment (19), was used in this study. E. coli strain containing the sdh operon on a pGS133 plasmid, the detergent used in this method, Lubrol PX, is no longer commercially available. In order for this simple purification procedure to be effective, a detergent that solubilized and redissolved in 30 mM Tris Cl, pH 7.5. The physical properties and reconstitutive activity of purified succinate dehydrogenase and the SdhC-SdhD fraction from E. coli were measured and compared with their mitochondrial counterparts. Formation of hybrid succinate-ubiquinone reductase was tested with mitochondrial succinate dehydrogenase and E. coli SdhC-SdhD fraction and vice versa.

Isolation and Purification of Succinate-Ubiquinone Reductase from the Membrane Preparation of E. coli/NM256/pGIS133—28 ml of frozen membrane preparation, 10 mg/ml in 10 mM Tris Cl, pH 7.5, containing 10% glycerol and 0.1% EDTA were thawed and freshly prepared E9C12 (25% stock solution in H2O) was added to a final concentration of 4%. The membrane was incubated at 0 °C with constant stirring for 1 h, 24 ml of 50 mM potassium phosphate buffer, pH 7.5, was added, and the mixture was centrifuged at 41,000 × g for 1 h. The supernatant obtained was applied to a DEAE-Sepharose CL-6B column (3.5 × 5 cm) equilibrated with 20 mM Tris-Cl, pH 7.5, containing 1% E9C12 (w/v), and 1 mM phenylmethylsulfonyl fluoride. The column was washed, in sequence, with 100 ml of 20 mM Tris-Cl, pH 7.5, containing 1% E9C12 (w/v), and 1 mM phenylmethylsulfonyl fluoride, 100 ml of the same buffer containing 0.1 mM NaCl, and a linear gradient formed from 500 ml of the same buffer containing 0.1 mM NaCl and 500 ml of the same buffer containing 0.25 mM NaCl. The fractions containing succinate-ubiquinone reductase were collected and combined, and succinate was added to a final concentration of 10 mM. The reductase was concentrated by precipitation with 35% ammonium sulfate saturation. The floating precipitate obtained was dissolved in 50 mM Tris-succinate buffer, pH 7.4, to a protein concentration of 10 mg/ml and used for preparation of succinate dehydrogenase and the SdhC-SdhD fraction.

Preparation of Succinate-Ubiquinone Reductase from E. coli Succinate-Ubiquinone Reductase—8 ml of the isolated succinate-ubiquinone reductase, 10 mg/ml, in 50 mM Tris-succinate buffer, pH 7.4, was dialyzed against 800 ml of 30 mM Tris-Cl buffer, pH 7.5, containing 10 mM sodium succinate, for 4 h with one change of buffer. The dialyzed preparation was adjusted to a protein concentration of 6 mg/ml with 30 mM Tris-Cl buffer, pH 7.5, containing 10 mM sodium succinate and flushed with argon for 30 min with stirring. All subsequent steps were carried out on ice or in the cold room (4 °C) under argon atmospheres. All solutions used were bubbled with argon gas for at least 10 min before use. Urea (8 mM) solution was slowly added to the enzyme solution to a final concentration of 1 M. The mixture was then brought to pH 10.2 by adding 1 N NaOH dropwise. After incubation for 10 min the sample was loaded onto a DEAE-Sepharose column (1.6 × 3 cm) equilibrated with 30 mM Tris-Cl, pH 10.0, containing 0.5 M urea and 10 mM sodium succinate. Succinate dehydrogenase was adsorbed to the column, while the SdhC-SdhD fraction was recovered in the effluent.

The column effluent was immediately adjusted to pH 7.0 with 1 mM Tris·Cl, pH 6.5, and dialyzed against 30 mM Tris-Cl, pH 7.5. The dialyzed E. coli SdhC-SdhD fraction was subjected to ammonium sulfate fractionation. The purified SdhD-SdhD fraction was recovered as a floating precipitate between 10 and 35% ammonium sulfate saturation and redisolved in 30 mM Tris-Cl, pH 7.5.

The column containing adsorbed succinate dehydrogenase was washed with 2 column volumes of 30 mM Tris-Cl, pH 10.0, containing 0.5 M urea and 10 mM sodium succinate so that 30 mM Tris-Cl, pH 7.5, containing 10 mM sodium succinate until the pH of the eluate was neutral. This succinate dehydrogenase was eluted from the column with 30 mM Tris-Cl, pH 7.5, containing 250 mM NaCl and 10 mM sodium succinate. The succinate dehydrogenase fractions were combined and treated with ammonium sulfate to 50% saturation. The precipitate recovered was dissolved in a small volume of 50 mM sodium-potassium phosphate buffer, pH 7.8 containing 20 mM succinate. This solution was divided into 100-μl portions and stored under argon at ~80 °C until use.

Succinate-ubiquinone reductase activity was assayed as previously reported (17). An appropriate amount of enzyme complex was added to an assay mixture (1 ml) containing 50 mM sodium-potassium phosphate, pH 7.0, 50 μM DCPIP, 20 mM succinate, 1 mM EDTA, 0.01% Triton X-100, and 25 μM of Q2. Activity was determined by measuring the reduction of DCPIP (the decrease in absorbance at 600 nm). A millimolar extinction coefficient of 21 was used to calculate activity.

Absorption spectra were recorded with a Shimadzu spectrophotometer. The content of cytochrome b562 was determined from the reduced-minus-oxidized spectrum using the extinction coefficient of 22.8 cm−1 M−1 for the wavelength pair 558–575 nm (2). SDS-PAGE was carried out by the method of Kita et al. (20) or Schagger et al. (21) in which the 10% glycerol in the separating gel is replaced by 8% urea (14).

Potentiometric titrations of cytochrome b562 in the isolated SdhD-SdhD fraction and in intact and reconstituted succinate-ubiquinone reductases were carried out anaerobically using the ferri-ferro-oxalate system, at pH 7.0 (midpoint redox potential = 0 mV), as described previously (22). EPR measurements were performed using a Bruker ER 200D spectrometer at liquid helium temperature. The instrument settings are given in the relevant figure legends.

RESULTS AND DISCUSSION

Isolation and Properties of E. coli Succinate-Ubiquinone Reductase—Although a one-step purification procedure has been reported (2) for isolation of succinate-ubiquinone reductase from an E. coli strain containing the sdh operon on a pGS133 plasmid, the detergent used in this method, Lubrol PX, is no longer commercially available. In order for this simple purifi-
Reconstitution of Succinate-Ubiquinone Reductase from E. coli

Resolution of Succinate-Ubiquinone Reductase into Succinate Dehydrogenase and the Membrane-anchoring Protein Fraction (the SdhC-SdhD Fraction)—Succinate-ubiquinone reductase is resolved into succinate dehydrogenase and the SdhC-SdhD fraction by a procedure involving alkaline (pH 10.2) treatment in the presence of 1 mM urea, followed by DEAE-Sepharose CL-6B column chromatography. All steps are carried out under anaerobic conditions (argon atmosphere), and all reagents are bubbled with argon gas for at least 10 min before use. The alkaline pH, together with urea, splits succinate dehydrogenase from the SdhC-SdhD fraction, and these two fractions are separated on a DEAE-Sepharose CL-6B column. The dark green-colored succinate dehydrogenase is tightly absorbed to the column, whereas the reddish-colored SdhC-SdhD fraction is recovered in the effluent. The absorbed succinate dehydrogenase was eluted with 250 mM NaCl. The greenish-colored material remaining on the column could not be eluted even with 1 M salt and was identified as denatured succinate dehydrogenase. The denaturation of succinate dehydrogenase does not occur in the column chromatographic step because when DEAE-Sepharose CL-6B gel is used in a batchwise eluting manner, instead of in a column, denatured succinate dehydrogenase is also observed. Prolonging the column chromatographic step increases the amount of succinate dehydrogenase denatured. In general, for a 1.5-h-long column chromatographic step, the recovery of succinate dehydrogenase is about 60–70%, whereas the recovery of the SdhC-SdhD fraction is nearly 100%.

The pH of SdhC-SdhD-containing effluent from the DEAE-Sepharose CL-6B column must be neutralized immediately. Urea in the effluent is removed by dialysis. Because dialysis does not remove E9C12, a relatively high concentration of detergent (2%) is present in the SdhC-SdhD preparation. High detergent concentrations destabilize cytochrome b$_{556}$ and make it very difficult to concentrate samples for use in experiments needing high protein concentrations, such as EPR. Ammonium sulfate fractionation of the dialyzed SdhC-SdhD fraction removes the excess E9C12 from the preparation as a thick white waxy floating precipitate formed between 5 and 20% ammonium sulfate saturation. The pure SdhC-SdhD fraction is recovered as a floating red precipitate formed between 20 and 35% ammonium sulfate saturation and dissolved in a small volume of buffer to give a protein concentration of 10–20 mg/ml. About 60% of the SdhC-SdhD in succinate-ubiquinone reductase is recovered in this final step. The SdhC-SdhD fraction thus obtained is ready for use in EPR experiments and can be stored at −80°C for months without loss of reconstitutive activity.

Purity and Reconstitutive Activity of E. coli Succinate Dehydrogenase and Membrane-anchoring Protein Fraction—Isolated succinate dehydrogenase shows two protein bands on SDS-PAGE, with apparent molecular masses of 64 and 28 kDa (lane 3 of Fig. 1), corresponding to SdhA and SdhB in succinate-ubiquinone reductase. The isolated membrane-anchoring fraction shows two protein bands with apparent molecular masses of 14 and 13 kDa (lane 4 of Fig. 1), corresponding to SdhC and
SdhD. The purity of isolated succinate dehydrogenase and membrane-anchoring protein fraction, judging from SDS-PAGE, is more than 90%.

Fig. 2 shows the reconstitution of succinate-ubiquinone reductase from succinate dehydrogenase and the SdhC-SdhD fraction. Isolated succinate dehydrogenase or the SdhC-SdhD fraction alone has no succinate-ubiquinone reductase activity. However, when a given amount of succinate dehydrogenase is mixed with varying amounts of the SdhC-SdhD fraction, the succinate-ubiquinone reductase activity increases as the amount of the SdhC-SdhD fraction used is increased (Fig. 2A). Maximal reconstitution is obtained when the weight ratio of succinate dehydrogenase to the SdhC-SdhD fraction reaches 5.26. The maximum reconstituted activity is 3.1 μmol succinate oxidized/min/nmole cytochrome b_{556} at 23 °C. The addition of 3 mM TTFA in the assay mixture inhibits 50% of the reconstituted activity. Because the maximum reconstituted activity obtained and the degree of TTFA sensitivity are the same as those observed for intact succinate-ubiquinone reductase, the resolved succinate dehydrogenase and SdhC-SdhD fraction are reconstitutively active.

The weight ratio of 5.26 for maximum activity is also obtained when the reconstitution is performed with a given amount of the SdhC-SdhD fraction and varying amounts of succinate dehydrogenase (Fig. 2B). If intact succinate-ubiquinone reductase contains four subunits, with molecular weights of 64,268, 26,637, 14,167, and 12,792, in equal molar ratio, the weight ratio between succinate dehydrogenase and the SdhC-SdhD fraction should be 3.37. That the experimental value is higher than the calculated value is probably due to slight denaturation of the soluble succinate dehydrogenase used, because it is known to be labile in the soluble form.

Absorption Spectra, Redox Potential, Carbon Monoxide Reactivity, and EPR Characteristics of Cytochrome b_{556}—The isolated E. coli SdhC-SdhD fraction contains 35 nmol cytochrome b_{556} mg protein. The oxidized cytochrome shows broad absorption at the α- and β-regions and EPR Characteristics of Cytochrome b_{556}—The oxidized cytochrome shows broad absorption at the α- and β-regions with Soret absorption at 412 nm (see Fig. 3). When the sample is reduced with dithionite, an α-absorption at 558 nm, a broad β-absorption peak at between 526 and 528 nm, and the Soret absorption at 424 nm are observed (Fig. 3). These absorption characteristics are identical to those observed for cytochrome b_{556} in intact or reconstituted E. coli succinate-ubiquinone reductase, indicating that the absorption characteristics of cytochrome b_{556} are not affected by the presence of succinate dehydrogenase. This is in contrast to the beef heart mitochondrial system in which the spectral characteristics of cytochrome b_{556} in isolated QPs differ from those of cytochrome b_{556} in the reductase. The dithionite-reduced cytochrome b_{556} in isolated mitochondrial QPs has a symmetrical α-absorption peak at 560 nm, whereas in succinate-ubiquinone reductase it exhibits an α-absorption maximum at 560.5 nm with a discernible shoulder at 553 nm. The addition of succinate dehydrogenase to QPs to form succinate-ubiquinone reductase regenerates the 553 nm shoulder peak, indicating that the heme environments of cytochrome b_{556} are affected by the presence of succinate dehydrogenase.

Cytochrome b_{556} in isolated succinate-ubiquinone reductase is reducible by succinate. Cytochrome b_{556} in the isolated SdhC-SdhD fraction is also reducible by succinate in the presence of succinate dehydrogenase under anaerobic conditions. The succinate reduced spectrum of cytochrome b_{556}, either in the isolated SdhC-SdhD fraction or in succinate-ubiquinone reductase, is the same as that obtained with dithionite. On the
Reconstitution of Succinate-Ubiquinone Reductase from E. coli

other hand, cytochrome $b_{556}$ in intact mitochondrial succinate-ubiquinone reductase or isolated QPs is not reducible by succinate.

Reduced cytochrome $b_{556}$ and cytochrome $b_{560}$ in isolated succinate-ubiquinone reductases from E. coli and beef heart mitochondria, respectively, do not react with carbon monoxide. Reduced cytochrome $b_{556}$ in the isolated E. coli SdhC-SdhD fraction does not react with carbon monoxide, whereas cytochrome $b_{560}$ in isolated mitochondrial QPs in its reduced form is completely reactive with carbon monoxide. The addition of mitochondrial succinate dehydrogenase to mitochondrial QPs converts carbon monoxide reactive cytochrome $b_{556}$ in QPs into a carbon monoxide nonreactive form. As expected, after the addition of E. coli succinate dehydrogenase to the E. coli SdhC-SdhD fraction, cytochrome $b_{556}$ in the SdhC-SdhD fraction remains totally nonreactive toward carbon monoxide. These results suggest that the environments of the fifth and sixth ligands of mitochondrial cytochrome $b_{560}$ differ from those of E. coli cytochrome $b_{556}$, even though the ligands for both cytochromes are bis-histidines (3, 23).

The midpoint potential of cytochrome $b_{556}$ in the isolated E. coli SdhC-SdhD fraction is estimated to be +22 mV, about 12 mV lower than that of cytochrome $b_{556}$ in intact (34 mV) succinate-ubiquinone reductases. The addition of succinate dehydrogenase to the SdhC-SdhD fraction increases the midpoint potential of cytochrome $b_{556}$ to 33 mV with concurrent reconstitution of succinate-ubiquinone reductase. The redox potential of E. coli cytochrome $b_{556}$ in the isolated SdhC-SdhD fraction is 166 mV higher than that of cytochrome $b_{560}$ in isolated mitochondrial QPs (−144 mV); and the redox potential of cytochrome $b_{556}$ in intact E. coli succinate-ubiquinone reductase is 219 mV higher than that of cytochrome $b_{560}$ in intact mitochondrial succinate-ubiquinone reductase (−185 mV). These values correlate with the fact that mitochondrial cytochrome $b_{560}$ is not reducible by succinate in either the isolated or complex form, whereas E. coli cytochrome $b_{556}$ is reducible by succinate in both forms. Like mitochondrial cytochrome $b_{560}$, the redox potential of E. coli cytochrome $b_{556}$ is affected by the interaction of SdhC-SdhD with succinate dehydrogenase.

Fig. 4 compares EPR characteristics of cytochromes $b$ in isolated membrane-anchoring fraction and intact and reconstituted succinate-ubiquinone reductases from E. coli and mitochondrial systems. Cytochrome $b_{556}$ in the E. coli SdhC-SdhD fraction shows an EPR signal at $g = 3.65$ (Fig. 4A). This signal is identical to that of cytochrome $b_{556}$ in intact and reconstituted E. coli succinate-ubiquinone reductases, indicating that removal of succinate dehydrogenase did not affect the heme environments of cytochrome $b_{556}$. This is different from that observed for mitochondrial cytochrome $b_{560}$. Cytochrome $b_{560}$ in intact succinate-ubiquinone reductase exhibits a EPR signal at $g = 3.46$, whereas it shows two EPR signals at $g = 3.07$ and 2.92 in isolated QPs (Fig. 4B). The addition of succinate dehydrogenase to QPs converts the $g = 3.07$ EPR signal of cytochrome $b_{560}$ in QPs to $g = 3.46$, indicating that the heme environment of cytochrome $b_{560}$ is affected by the interaction of heart mitochondrial QPs in the presence and the absence of succinate dehydrogenases from E. coli and beef heart mitochondria. To 0.2-ml aliquots of QPs were added 0.2 ml of 50 mM sodium-potassium phosphate buffer, pH 7.4, containing no additions, 25 mg of E. coli succinate dehydrogenase, and 27 mg of beef heart mitochondrial succinate dehydrogenase. The mixtures were incubated at 0 °C for 1 h and assayed for succinate-ubiquinone reductase activity before being placed in EPR tubes and frozen at −80 °C. The EPR instrument settings were: modulation frequency, 100 KHz; modulation amplitude, 20 G; time constant, 0.5 s; microwave frequency, 9.42 GHz; microwave power, 20 milliwatts; scan rate, 200 s; and temperature, 11 K.

![Graph](image-url)

**Fig. 4.** Effect of succinate dehydrogenase on EPR spectra of cytochrome $b_{556}$ in QPs from beef heart mitochondria and of cytochrome $b_{560}$ in the SdhC-SdhD fraction from E. coli. **A**, EPR spectra of cytochrome $b_{556}$ in E. coli SdhC-SdhD fraction in the presence and the absence of succinate dehydrogenases from E. coli and beef heart mitochondria. 0.2-ml aliquots of the E. coli SdhC-SdhD fraction, 280 μM cytochrome $b_{556}$ in 50 mM sodium-potassium phosphate buffer, pH 7.4, were mixed with 0.2 ml of 50 mM sodium-potassium phosphate buffer, pH 7.8, containing no additions, 27 mg of succinate dehydrogenase from beef heart mitochondria, and 28 mg of succinate dehydrogenase from E. coli. The mixtures were incubated at 0 °C for 1 h and assayed for succinate-ubiquinone reductase activity before being placed in the EPR tubes and frozen at −80 °C. **B**, EPR spectra of cytochrome $b_{560}$ in beef heart mitochondrial QPs in the presence and the absence of succinate dehydrogenases from E. coli and beef heart mitochondria. To 0.2-ml aliquots of QPs were added 0.2 ml of 50 mM sodium-potassium phosphate buffer, pH 7.4, containing no additions, 25 mg of E. coli succinate dehydrogenase, and 27 mg of beef heart mitochondrial succinate dehydrogenase. The mixtures were incubated at 0 °C for 1 h and assayed for succinate-ubiquinone reductase activity before being placed in EPR tubes and frozen at −80 °C. The EPR instrument settings were: modulation frequency, 100 KHz; modulation amplitude, 20 G; time constant, 0.5 s; microwave frequency, 9.42 GHz; microwave power, 20 milliwatts; scan rate, 200 s; and temperature, 11 K.
Reconstitution of Succinate-Ubiquinone Reductase from E. coli

The availability of isolated, reconstitutively active membrane-anchoring fraction and mitochondrial succinate dehydrogenase and vice versa—The availability of isolated, reconstitutively active membrane-anchoring fraction and succinate dehydrogenase from the E. coli and bovine mitochondrial systems enables us to study the formation of a hybrid succinate-ubiquinone reductase from the E. coli membrane-anchoring protein fraction and mitochondrial succinate dehydrogenase and vice versa. When a given amount of E. coli succinate dehydrogenase is mixed with increasing amounts of mitochondrial QPs, no succinate-ubiquinone reductase activity is detected (see Fig. 2A, cross symbols). Failure to reconstitute is also observed when the E. coli SdhC-SdhD fraction is mixed with increasing amounts of mitochondrial succinate dehydrogenase (see Fig. 2B, cross symbols). Because no hybrid succinate-ubiquinone reductases are formed, it is of interest to see whether or not this is due to a lack of interaction between succinate dehydrogenase and the membrane-anchoring protein fraction from these two systems. Reduction of cytochrome b_{556} in the isolated E. coli SdhC-SdhD fraction in the presence of mitochondrial succinate dehydrogenase or a change of EPR characteristics of cytochrome b_{556} in isolated mitochondrial QPs upon the addition of E. coli succinate dehydrogenase would indicate interaction. The first indication is based on the fact that cytochrome b_{556} in the isolated E. coli SdhC-SdhD fraction is reducible by succinate upon the addition of E. coli succinate dehydrogenase. The second indication is based on the observation that one of the EPR signals (g = 3.07) of cytochrome b_{556} in isolated mitochondrial QPs converts to a g = 3.46 signal upon the addition of mitochondrial succinate dehydrogenase.

Table I shows that the cytochrome b_{556} in E. coli SdhC-SdhD fraction is reducible by succinate in the presence of reconstitutively active mitochondrial or E. coli succinate dehydrogenase. The rate of reduction correlates with the reconstitutive activity of the added succinate dehydrogenase. When the isolated E. coli SdhC-SdhD fraction is mixed with mitochondrial succinate dehydrogenases having decreasing low K_m ferricyanide reductase activity, the rate of cytochrome b_{556} reduction by succinate decreases correspondingly. No cytochrome b_{556} reduction is observed when a mitochondrial succinate dehydrogenase possessing no low K_m ferricyanide reductase activity is added to the E. coli SdhC-SdhD fraction. Because the low K_m ferricyanide reductase activity in soluble succinate dehydrogenase correlates with its reconstitutive activity, the rate of cytochrome b_{556} reduction by succinate in the E. coli SdhC-SdhD fraction correlates with the reconstitutive activity of succinate dehydrogenase. Succinate dehydrogenases with decreasing low K_m ferricyanide reductase activity are obtained by exposing fully reconstitutively active soluble succinate dehydrogenase to air for increasing lengths of time. The rate of cytochrome b_{556} reduction by succinate is faster with mitochondrial succinate dehydrogenase than with the E. coli enzyme at the same protein concentration. This is in line with the fact that isolated succinate dehydrogenase from beef heart mitochondria has a higher low K_m ferricyanide activity than does succinate dehydrogenase from E. coli. As expected, no reduction of cytochrome b_{556} by succinate is observed when mitochondrial succinate-ubiquinone reductase or succinate-cytochrome c reductase is added to the E. coli SdhC-SdhD fraction because the succinate dehydrogenase in these reductase complexes contains no low K_m ferricyanide reductase activity (or reconstitutive activity).

Because cytochrome b_{556} is not reducible by succinate in the presence of reconstitutively inactive succinate dehydrogenase or intact succinate-ubiquinone reductase from beef heart mitochondria, the electron for the reduction of cytochrome b_{556} in E. coli SdhC-SdhD fraction must come from the low K_m ferricyanide reductase site of succinate dehydrogenase. These results indicate that succinate dehydrogenase from beef heart mitochondria can interact with the E. coli SdhC-SdhD fraction and reduce cytochrome b_{556} at the expense of succinate. However, this reduced cytochrome b_{556} is unable to reduce Q, and thus no succinate-ubiquinone reductase is formed. The inability of reduced cytochrome b_{556} to reduce Q may indicate that cytochrome b_{556} in E. coli succinate-ubiquinone reductase is not the donor of the first electron for Q. Possibly, the electron acceptor for cytochrome b_{556} is a ubisemiquinone radical and the donor of the first electron for Q is E. coli succinate dehydrogenase. In the hybrid system, the mitochondrial succinate dehydrogenase is unable to provide the first electron to reduce Q to ubisemiquinone. Alternatively, the reduction of cytochrome b_{556} is not required for succinate-ubiquinone reductase activity in the E. coli system.

Interaction between mitochondrial QPs and E. coli succinate dehydrogenase is suggested by the decrease in the signal height of the g = 3.07 EPR signal of cytochrome b_{556} in QPs upon the addition of E. coli succinate dehydrogenase (see Fig. 4B). However, this interaction differs from that observed in the mitochondrial reconstituting system in which the g = 3.07 EPR signal of cytochrome b_{556} in QPs is converted to a g = 3.46 signal. Because the addition of E. coli succinate dehydrogenase to mitochondrial QPs causes a decrease in the g = 3.07 signal height of cytochrome b_{556} without producing the g = 3.46 signal, no succinate-ubiquinone reductase activity is reconstituted. This suggests that E. coli succinate dehydrogenase interacts with mitochondrial QPs to some degree but not enough to form an active reductase.

Acknowledgment—We thank Dr. Roger Koppel for the critical review of this manuscript.

REFERENCES
1. Spencer, M. E., and Guest, J. R. (1974) J. Bacteriol. 117, 947–953
2. Rito, K., Vihat, C. R. T., Meinhardt, S., Guest, J. R., and Gennis, R. B. (1989) J. Biol. Chem. 264, 2672–2677
3. Peterson, J., Bibat, C., and Gennis, R. B. (1994) Febs Lett. 353, 155–156
4. Nakamura, K., Yamaki, M., Sarada, M., Nakayama, S., Vihat, C. R. T., Gennis, R. B., Nakayashiki, T., Inokuchi, H., Kojima, S., and Rito, K. (1996) J. Biol. Chem. 271, 521–527
5. Yu, C. A., and Yu, L. (1980) Biochemistry 19, 3579–3585
6. Wood, D., Darlison, M. G., Wilde, R. J., and Guest, J. R. (1984) Biochem. J. 222, 519–534
7. Darlison, M. G., and Guest, J. R. (1984) Biochem. J. 223, 507–517
8. Hagerhall, C., and Hederstedt, L. (1996) Febs Lett. 396, 25–31
9. Yu, L., Wei, Y.-Y., Usui, S., and Yu, C.-A. (1992) J. Biol. Chem. 267, 24508–24515
10. Shenoy, S. K., Yu, L., and Yu, C. A. (1996) FASEB J. 10, 2837
11. Yu, L., Xu, J.-X., Haley, P. E., and Yu, C.-A. (1997) J. Biol. Chem. 262, 1137–1143
12. Hederstedt, L., and Ohnishi, T. (1992) in Molecular Mechanisms in Bioenergetics (Rinne, L., ed) pp. 163–198, Elsevier Science Co.
13. Ackrell, B. A. C., Johnson, M., Gunsalus, R. P., and Cecchini, G. (1992) in Chemistry and Biochemistry of Flavoenzymes (Muller, F., ed) Vol. III, pp. 229–297, CRC Press, London
14. Lee, G. Y., He, D.-Y., Yu, L., and Yu, C.-A. (1995) J. Biol. Chem. 270, 6193–6198
15. Yu, C. A., and Yu, L. (1993) J. Bioenerg. Biomembr. 23, 259–273
16. Jenner, E. L. (June 5, 1973) U. S. Patent 3,737,516
17. Yu, L., and Yu, C. A. (1982) J. Biol. Chem. 257, 2016–2021
18. Yu, C. A., and Yu, L. (1980) Biochim. Biophys. Acta 691, 409–420
19. Koland, J. G., Miller, M. J., and Gennis, R. B. (1984) Biochemistry 23, 445–453
20. Kita, K., Konishi, K., and Anraku, Y. (1984) J. Biol. Chem. 259, 3368–3374
21. Schägger, H., Link, T. A., Engel, W. D., and von Jagow, G. (1986) Methods Enzymol. 126, 224–257
22. Kita, K., Yamato, I., and Anraku, Y. (1978) J. Biol. Chem. 253, 8910–8915
23. Crouse, B. R., Yu, C. A., Yu, L., and Johnson, M. K. (1995) FEBS Lett. 367, 1–4
