Properties of Bovine Heart Mitochondrial Cytochrome $b_{560}$

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A large-scale preparation of the two-subunit protein complex (QPs) that converts succinate dehydrogenase into succinate-ubiquinone reductase from cytochrome $b_{5}-c$ particles is achieved by a procedure involving Triton X-100 solubilization and calcium phosphate column chromatography at different pH values. The isolated two-subunit QPs contains 25 nmol of cytochrome $b_{560}/mg$ of protein and is able to reconstitute with soluble succinate dehydrogenase to form a TTFA-sensitive succinate-ubiquinone reductase. The maximum reconstitutive activity is 100 mmol of succinate oxidized per min per mg of QPs protein at 23 °C. Although cytochrome $b_{560}$ in isolated QPs is not succinate reducible and its dithionite reduced form is reactive to carbon monoxide, cytochrome $b_{560}$ is shown to be physiologically associated with succinate dehydrogenase by the following observations. 1) The dithionite reduced form of cytochrome $b_{560}$ in isolated QPs has a symmetrical a-absorption peak, which upon reconstitution with succinate dehydrogenase becomes slightly broadened and shows a shoulder at around 553 nm, identical to that of cytochrome $b_{560}$ in succinate-ubiquinone reductase. 2) Upon addition of succinate dehydrogenase to QPs, about 50% of the reduced form of cytochrome $b_{560}$ in QPs becomes insensitive to carbon monoxide treatment. 3) The redox potential of cytochrome $b_{560}$ in QPs is $-144 \text{mV}$ which is higher than that of cytochrome $b_{560}$ in succinate-ubiquinone reductase ($-185 \text{mV}$). Upon addition of succinate dehydrogenase, the redox potential of about 46% of the cytochrome $b_{560}$ in QPs preparation becomes identical to that of cytochrome $b_{560}$ in succinate-ubiquinone reductase. 4) Cytochrome $b_{560}$ in the QPs preparation shows two epr signals, $g = 3.07$ and $g = 2.92$, whereas cytochrome $b_{560}$ in succinate-ubiquinone reductase exhibits only one epr signal at $g = 3.46$. When QPs is reconstituted with succinate dehydrogenase to form succinate-ubiquinone reductase, the $g = 3.46$ epr signal reappears at the expense of the $g = 3.07$ signal. Based on epr measurement at liquid helium temperature, about 18% of the total cytochrome $b$ in the isolated active succinate-cytochrome $c$ reductase is cytochrome $b_{560}$, indicating that cytochrome $b_{560}$ is indeed a unique cytochrome $b$ and not a denatured product of cytochrome $b_{560}$ or $b_{560}$.

The existence of a special type of $b$ cytochrome, cytochrome $b_{560}$, in the succinate-ubiquinone reductase region of the mitochondrial electron transfer chain was first reported by Davis et al. (1, 2). Although all the isolated succinate-ubiquinone reductase (3, 4) and Complex II (5-7) preparations contain cytochrome $b_{560}$, this cytochrome has often been regarded as a contaminant of the denatured cytochromes from ubiquinol-cytochrome $c$ reductase because it is not reducible by succinate, and the spectra of dithionite reduced cytochrome $b_{560}$ in these preparations resemble the spectra of a mixture of denatured cytochromes $b$ and $c_1$ of ubiquinol-cytochrome $c$ reductase. Thus, the importance of this particular cytochrome $b_{560}$ has been slighted in the mammalian system. The participation of cytochrome $b_{560}$ in succinate dehydrogenase of Bacillus subtilis (8) and Neurospora crassa (9), however, is well established. Recently, a substrate reducible cytochrome $b_{560}$ was detected in muscle mitochondrial succinate-ubiquinone reductase from Ascaris suum (10).

The isolation of a two-subunit protein preparation (QPs) which is capable of converting succinate dehydrogenase into succinate-ubiquinone reductase has been reported by many laboratories (11-13). These preparations have a cytochrome $b$ content ranging from 2 to 14 nmol/mg of protein. The molecular masses reported for these two protein subunits, which differ slightly between laboratories, are about 15 and 13 kDa. The $M_f = 13,000$ protein (11, 14) was thought to be the ubiquinone reactivity conferring protein, because a highly purified QPs preparation containing only trace amounts of cytochrome $b_{560}$ had partial activity in reconstituting succinate dehydrogenase to succinate-ubiquinone reductase. This observation plus the fact that cytochrome $b_{560}$ in isolated succinate-ubiquinone reductase does not undergo a redox state change during catalysis and is present in a substoichiometric amount with respect to flavin casts doubt on the essentiality of this cytochrome in electron transfer from succinate to ubiquinone. On the other hand, when succinate-ubiquinone reductase was photolyzed with the $^3$Harylazido-Q derivative, both $M_f = 15,000$ and $M_f = 13,000$ proteins were heavily labeled (3), and the fact that cytochrome $b_{560}$ containing QPs has higher reconstitutive activity than does QPs containing no cytochrome $b_{560}$ suggests that this cytochrome may play an important structural role in this region of electron transfer chain (11).

Recently we developed a relatively simple method to prepare the two-subunit QPs from the cytochrome $b_{5}-c$ particles. This preparation has a cytochrome $b_{560}$ content and reconstitutive activity higher than those of previously reported preparations (11-13) and is easier to obtain in large quantity. This method involves the use of detergent present in the preparation and the electrophoresis system used. The molecular weights of these two subunits were previously reported to be 17,000 and 15,000 (11).
enables us to systematically study cytochrome b₆₀₆₀, especially for those which require high concentration of sample, such as epr characterization. Epr studies on cytochrome b in ubiquinol-cytochrome c reductase have been reported by several laboratories (15-17).

Herein, we report the large-scale preparation and properties of the two-subunit QPs. The involvement of cytochrome b₆₀₆₀ in binding of succinate dehydrogenase to QPs is demonstrated by the restoration of the absorption properties, redox potential, and epr characteristics of cytochrome b₆₀₆₀ during formation of a TTFAsensitive succinate-ubiquinone reductase from isolated QPs and ubiquinone dehydrogenase.

**EXPERIMENTAL PROCEDURES**

**Materials**—Triton X-100, 2,6-dichlorophenolindophenol, and 2-thienyltrifluoroacetone (TTFA) were from Sigma. Bio-Beads™ SM-2, 20-50 mesh, were from Bio-Rad. 2,3-Dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone was synthesized in our laboratory (18). Calcium phosphate was prepared according to Jenner (19). This preparation was aged in a cold room for 1 month before mixing with cellulose powder and used for column chromatography. Other chemicals were of the highest purity commercially available.

**Methods**—Succinate-cytochrome c reductase (3), the cytochrome b₅₆₀, particles (20), succinate dehydrogenase (20), succinate-ubiquinone reductase (3), and type II NADH dehydrogenase (21) were prepared and assayed according to methods reported previously. Cytochrome b content in QPs and succinate-ubiquinone reductase was determined by pyridine hemochromogen spectra using the millimolar extinction coefficient of 34.8 for reduced absorbance at 557 nm minus 600 nm (22). Cytochrome b content in succinate-cytochrome c reductase and ubiquinol-cytochrome c reductase was determined by using the millimolar extinction coefficient of 28.5 for reduced minus oxidized absorbance at 562 nm minus 575 nm (23). Protein was determined by the Lowry method (24) in the presence of 1% sodium dodecyl sulfate (SDS). Absorption spectra were measured in a Cary spectrophotometer, model 14 or 219. All enzymatic activities were assayed at room temperature. The SDS-PAGE was performed by the method of Weber and Osborn (25) in the presence of 4 M urea. The epr measurements were made with a Bruker ER-200D at liquid helium temperature. The epr settings are detailed in the legends of the figures. The redox titration was performed according to the method described by Dutton et al. (26).

**Isolation of Two-subunit QPs from Cytochrome b₅₆₀.** The cytochrome b₅₆₀ particles devoid of succinate dehydrogenase were prepared from succinate-cytochrome c reductase by twice alkaline extraction (pH 10) under anaerobic conditions. These particles were suspended in 50 mM Tris-C1, pH 7.8, containing 0.67 mM succinate, pH 7.0, containing 0.25 mM succinate and frozen at -50°C until use. Alternatively, Triton-free QPs can be obtained from the second calcium phosphate column by precipitation with an equal volume of acetone (-20°C) while maintaining sample temperature at ±1°C during acetone addition.

**RESULTS AND DISCUSSION**

Isolation and Enzymatic Activity of Two-subunit QPs—As indicated in Table I, about 64% of the reconstitutive activity in the cytochrome b₅₆₀ particles is recovered in the final preparation. The reconstitutive activity is measured by the ability to convert soluble succinate dehydrogenase to succinate-ubiquinone reductase. The first calcium phosphate column separates QPs from ubiquinol-cytochrome c reductase in the Triton X-100-solubilized cytochrome b₅₆₀. The second calcium phosphate column removes any contaminating succinate dehydrogenase, succinate-ubiquinone reductase, or unsplit cytochrome b₅₆₀ complex (adduct of ubiquinol-cytochrome c reductase and QPs). In some batches when separation on the first column is exceptional, the second column does not increase the specific activity or the purity of QPs. However, in our routine procedure the second column is included to ensure a constant purity of preparation since the enzyme is stable at conditions used (pH 6.0) and this step is not very time consuming.

The purity of QPs obtained by this method, judging from SDS-PAGE, is the same as that of previously reported preparations (11-13). The new method, however, has several advantages: 1) it is suitable for large-scale preparation because it is rather simple and highly reproducible; 2) it is economical; all components of succinate-cytochrome c reductase, succinate dehydrogenase, QPs, and ubiquinol-cytochrome c reductase are conserved; 3) the detergent-free QPs can be obtained at a high protein concentration suitable for epr study; and 4) the detergent-free QPs may be stored at -80°C for months without loss of activity and can be redispersed by detergents such as deoxycholate or Triton X-100. The QPs prepared by this method shows two protein bands on SDS-PAGE with apparent molecular masses of 15 and 13 kDa. It contains 25 nmol of cytochrome b/mg of protein. It should be mentioned that the content of cytochrome b₆₀₆₀ in QPs preparations varies slightly from one batch of submitochondrial particles to another; it ranges from 22 to 26 nmol/mg of protein, yet the purity of QPs, judged by SDS-PAGE, remains constant. QPs alone shows no enzymatic activity but combined with soluble succinate dehydrogenase forms TTFAsensitive succinate-ubiquinone reductase. Fig. 1 shows the titration of QPs with succinate dehydrogenase. The maximum reconstitutive activity of QPs, obtained when the weight ratio

**FIG. 1.** Formation of the TTFAsensitive succinate-ubiquinone reductase by QPs and succinate dehydrogenase (SDH). Aliquots of 0.2 ml of isolated QPs, 0.18 mg/ml, in 50 mM Tris-C1, pH 7.4, containing 30 mM succinate and 0.5% Triton X-100 were reconstituted with varying amounts of succinate dehydrogenase (1 mg/ml) in 20 mM Tris-succinate, pH 7.8. The final volumes were adjusted to 0.6 ml with 20 mM Tris-succinate, pH 7.8. After incubation at 0°C for 30 min, the succinate-ubiquinone reductase activity was assayed in the presence of 30 μM O₂ in the assay mixture with and without 1 mM TTFAsensitive succinate-ubiquinone reductase activity.
of QPs to succinic dehydrogenase reaches 4, is calculated to be around 100 μmol of succinate oxidized per mg of protein at 23°C. Assuming intact succinate-ubiquinone reductase contains 4 subunits, with molecular masses of 70, 26, 15, and 13 kDa, in equal molar ratio, the weight ratio between succinate dehydrogenase and two-subunit QPs should be 3.4. That the experimental value of 4 is only slightly higher than the calculated value can be explained by slight denaturation of the soluble succinate dehydrogenase used, since it is known to be labile in the soluble form. Another possibility is that both succinate dehydrogenase and QPs are partially denatured such that the weight ratio remains fairly close to the calculated value.

Absorption Properties of Cytochrome \( b_{560} \)—Fig. 2 shows the absorption spectra of cytochrome \( b_{560} \) in the isolated QPs. The oxidized cytochrome shows broad absorption at the α- and β-regions with Soret absorption at 412 nm. No change in the spectral characteristics is observed when the preparation is treated with succinate or ascorbate under aerobic or anaerobic conditions, indicating that cytochrome \( b_{560} \) is not reducible by either compound. However, when the sample is reduced with dithionite, an α-absorption at 560 nm, a broad β-absorption peak at between 526 and 528 nm, and Soret absorption at 424 nm are observed.

The millimolar extinction coefficient of cytochrome \( b_{560} \) in isolated QPs or succinate-ubiquinone reductase is 20.5 for reduced minus oxidized absorbance at 560 nm minus 575 nm. The millimolar extinction coefficient is determined by pyridine hemochromogen spectra using the millimolar extinction coefficient of 34.6 (22) for the reduced absorbance at 557 nm minus 600 nm. The millimolar extinction coefficient for the oxidized Soret absorbance of cytochrome \( b_{560} \) in QPs is estimated to be 158.

It was reported (12) that the dithionite reduced spectra of isolated cytochrome \( b_{560} \) from Complex II deteriorated quickly at room temperature. The dithionite reduced spectra of cytochrome \( b_{560} \) in QPs, prepared without removing most of the Triton X-100 with Bio-Beads, also deteriorates rather quickly; about 80% of the reduced spectra disappears after 1.5 h at room temperature. The addition of more dithionite fails to regenerate the reduced form of the spectra, indicating that the sample has been irreversibly denatured. It is likely that the denaturation occurs at the heme moiety because the alkaline pyridine hemochromogen spectra is also diminished. The Soret absorption ratio between the reduced and oxidized cytochrome \( b_{560} \) in partially detergent-free QPs is 1.3 (see Fig. 2). However, this ratio varies from 0.9 to 1.3 with isolated QPs preparations without removing most of the Triton X-100. The rate of destruction of the dithionite reduced absorption characteristics and the ratio of Soret absorptions between reduced and oxidized cytochrome \( b_{560} \) in isolated QPs are affected by the amount of Triton X-100 in the sample. When most of the Triton X-100 in QPs is removed, the dithionite reduced spectra of cytochrome \( b_{560} \) becomes more stable. The mechanism of the denaturation of cytochrome \( b_{560} \) in the presence of dithionite is not clear.

Cytochrome \( b_{560} \) in QPs is also reducible by NADH in the presence of type II NADH dehydrogenase under anaerobic conditions. This reduced spectrum, which is the same as that obtained by dithionite reduction, is stable under anaerobic conditions.

When the absorption spectra of cytochrome \( b_{560} \) in isolated QPs are compared with those of cytochrome \( b_{560} \) in succinate-ubiquinone reductase, they differ slightly in the α- and β-absorption regions. Reduced cytochrome \( b_{560} \) in succinate-ubiquinone reductase exhibits an α-absorption maximum at 560.5 nm with a discernible shoulder at 553 nm (Fig. 3A) whereas in isolated QPs it has a symmetrical α-absorption peak at 560 nm (Fig. 3B). Since the dithionite reduced form of denatured cytochrome \( c_1 \) also shows an α-absorption at 553 nm, it is important to determine whether this 553 nm α-absorption shoulder is an intrinsic property of cytochrome \( b_{560} \) in succinate-ubiquinone reductase or merely the result of contamination with a trace amount of denatured cytochrome \( c_1 \). Since isolated bovine heart mitochondrial succinate-ubiquinone reductase, prepared by the method developed in our laboratory (3), has no cytochrome \( c_1 \) protein band, \( M_w = 30,000 \).
In the SDS-PAGE, the shoulder must be an intrinsic property of cytochrome b in succinate-ubiquinone reductase. However, in a less pure preparation of succinate-ubiquinone reductase, such as Complex II (5-7) contaminated 15-20% with cytochrome b-c, complex (12), it is very likely that a shoulder upon reconstitution of succinate-ubiquinone reductase from isolated succinate-ubiquinone reductase resulting from a physical association of QPs with succinate dehydrogenase. Cytochrome b in isolated QPs or other preparations devoid of succinate dehydrogenase should not possess this absorption shoulder. Therefore, the absorption shoulder at 553 nm, observed in an isolated cytochrome b preparation (12) obtained from Complex II, was probably due to contamination with cytochrome c or ubiquinol-cytochrome c reductase. Contamination of Complex II preparation with cytochromes of Complex III is well known (12).

**Carbon Monoxide Reactivity of Cytochrome b**

Unlike cytochrome b in isolated succinate-ubiquinone reductase, in which the reduced form is not reactive toward carbon monoxide (2, 3), reduced cytochrome b in QPs is completely reactive. As shown in Fig. 4A, when carbon monoxide was added to dithionite reduced QPs, the spectral characteristics of cytochrome b are lost. Similar spectral behavior is observed under anaerobic conditions, suggesting that the loss of reduced spectra is not due to reoxidation. In dithionite reduced reconstituted succinate-ubiquinone reductase, only about 50% of the cytochrome b is reactive to carbon monoxide (see Fig. 4B). The nonreactive portion of cytochrome b in the reconstituted system has absorption properties identical to those of intact succinate-ubiquinone reductase, that is an absorption shoulder at 553 nm. This result indicates that the carbon monoxide reactivity of cytochrome b results from removal of its interacting partner. It should be emphasized that the conversion of carbon monoxide reactive cytochrome b to carbon monoxide nonreactive cytochrome b is not merely the result of the physical presence of succinate dehydrogenase. A specific interaction must occur because reconstitutively inactive succinate dehydrogenase is not able to convert cytochrome b into the carbon monoxide inactive form (see Fig. 4C).

**Fig. 3.** Difference spectra of cytochrome b in succinate-ubiquinone reductase, isolated QPs, and reconstituted succinate-ubiquinone reductase in the α- and β-regions. A, dithionite reduced succinate-ubiquinone reductase (1.8 μM cytochrome b in 50 mM Tris-Cl buffer, pH 7.2) against oxidized sample; B, dithionite reduced QPs (1.6 μM cytochrome b, in 50 mM Tris-Cl buffer, pH 7.2) against oxidized sample; C, dithionite reduced reconstituted succinate-ubiquinone reductase against the sum of dithionite reduced QPs and succinate dehydrogenase. A pair of dividing cuvettes was used. In the reference cuvette, 1 ml of QPs (3.2 μM cytochrome b in 50 mM Tris-Cl buffer, pH 7.2) and 1 ml of succinate dehydrogenase (2 mg/ml, in 30 mM Tris-succinate, pH 7.8) were placed separately in the two compartments. In the sample cuvette, a mixture of 1 ml of QPs (3.2 μM cytochrome b) and 1 ml of succinate dehydrogenase (2 mg/ml in 20 mM Tris-succinate, pH 7.8) was placed in both compartments. After the base line was recorded, aliquots of 10 μl of dithionite solution (20 mM) were added to each compartment of the cuvette and spectra again recorded. The QPs preparation was stirred with BioBeads for 3 h at 0 °C before use.

in the SDS-PAGE, the shoulder must be an intrinsic property of cytochrome b in succinate-ubiquinone reductase. However, in a less pure preparation of succinate-ubiquinone reductase, such as Complex II (5-7) contaminated 15-20% with cytochrome b-c, complex (12), it is very likely that a part of the 553-nm absorption observed results from denatured cytochrome c.

Convincing evidence that this 553-nm absorption shoulder is an intrinsic property of cytochrome b in succinate-ubiquinone reductase would be the regeneration of such a shoulder upon reconstitution of succinate-ubiquinone reductase from isolated succinate dehydrogenase and QPs. If this 553-nm absorption shoulder of cytochrome b is the result of a physical association of QPs with succinate dehydrogenase, one would expect to see the appearance of an absorption peak at 553 nm when a spectrum of dithionite reduced reconstituted succinate-ubiquinone reductase is compared with a spectrum of the sum of dithionite reduced QPs and dithionite reduced succinate dehydrogenase. When such a difference spectrum is measured, as shown in Fig. 3C, absorption peaks are observed at 553 and 562 nm. This indicates that the reconstitution of QPs with succinate dehydrogenase causes a slight change in the absorption properties of cytochrome b. Thus, the absorption of cytochrome b is broadened, displays a slight red shift (~0.5 nm), and exhibits a shoulder at 553 nm. Clearly the 553-nm absorption shoulder is an intrinsic property of cytochrome b in succinate-ubiquinone reductase resulting from a physical association of QPs with succinate dehydrogenase. Cytochrome b in isolated QPs or other preparations devoid of succinate dehydrogenase should not possess this absorption shoulder. Therefore, the absorption shoulder at 553 nm, observed in an isolated cytochrome b preparation (12) obtained from Complex II, was probably due to contamination with cytochrome c or ubiquinol-cytochrome c reductase. Contamination of Complex II preparation with cytochromes of Complex III is well known (12).

**Fig. 4.** Carbon monoxide reactivity of cytochrome b in QPs, in a mixture of QPs and reconstitutively active succinate dehydrogenase or reconstitutively inactive succinate dehydrogenase. A, 1 ml of QPs, 11 μM cytochrome b in 20 mM Tris-succinate, pH 7.0, containing 0.2% deoxycholate and 0.25% sucrose mixed with 1 ml of 30 mM Tris-succinate buffer, pH 7.4; B, 1 ml of QPs mixed with 1 ml of reconstitutively active succinate dehydrogenase (4.4 mg/ml) in 20 mM Tris-succinate, pH 7.4; C, 1 ml of QPs mixed with 1 ml of inactive succinate dehydrogenase (4.4 mg/ml). Reconstitutively inactive succinate dehydrogenase was obtained by incubating active succinate dehydrogenase at 0 °C for 2 h under aerobic conditions. The solid curve (- - -) represents the difference spectra of the dithionite reduced minus oxidized form, and the broken curves (----) are the difference spectra of the CO-treated dithionite reduced minus oxidized form.
Since only about 50% of the cytochrome b_{600} in isolated QPs can be converted to the carbon monoxide nonreactive form by reconstitution with succinate dehydrogenase, it is likely that only about 50% of the cytochrome b_{600} in the isolated QPs preparation is in the reconstitutively active form. This deduction is compatible with the fact that the specific activity (reconstitutive) of isolated QPs, based on cytochrome b_{600}, is only about 50% of that in succinate-ubiquinone reductase. Studies on the epr characteristics and redox potentials of cytochrome b_{600}, described below, also suggest that approximately 50% of the cytochrome b_{600} in isolated QPs is in the reconstitutively active form.

**Redox Potential of Cytochrome b_{600}**—Fig. 5 shows redox titration curves of cytochrome b_{600} in isolated QPs, intact, and reconstituted succinate-ubiquinone reductases at pH 7.0. The midpoint potential of cytochrome b_{600} in QPs is estimated to be -144 mV (Fig. 5A), which is higher than that of cytochrome b_{600} in intact succinate-ubiquinone reductase (-185 mV). When QPs is reconstituted with succinate dehydrogenase to form succinate-ubiquinone reductase, the redox potential of cytochrome b_{600} becomes -164 mV (Fig. 5B). The redox potential titration curve of cytochrome b_{600} in the reconstituted system can be resolved into two n = 1 components: one with midpoint potential of -185 mV (46%) and another of -144 mV (54%). Since the redox potential of about 46% of cytochrome b_{600} in QPs is changed to that of cytochrome b_{600} in isolated succinate-ubiquinone reductase, upon the addition of succinate dehydrogenase, it is likely that only that portion of cytochrome b_{600} in QPs is in a reconstitutively active form. The change of the redox potential of cytochrome b_{600} upon reconstitution suggests interaction between cytochrome b_{600} and succinate dehydrogenase. Since the change in the midpoint potential of cytochrome b_{600} upon dissociation from its interacting component is very similar to that of the iron sulfur center of S-2 of succinate dehydrogenase, it is likely that cytochrome b_{600} interacts closely with S-2 of succinate-dehydrogenase in succinate-ubiquinone reductase. The midpoint potential of S-2 in soluble succinate dehydrogenase is -400 mV; the midpoint potential of the corresponding iron sulfur cluster in succinate-ubiquinone reductase is -260 mV (27).

The low redox potentials of cytochrome b_{600} observed in both succinate-ubiquinone reductase, reconstituted succinate-ubiquinone reductase, and isolated QPs are in line with the fact that cytochrome b_{600} is not reducible by succinate either in the isolated or complexed form. This low potential of cytochrome b_{600} is the main reason for skepticism about its direct participation in the catalytic function of succinate-ubiquinone reductase. A midpoint potential of less than -80 mV was estimated for cytochrome b_{600} in Complex II by the putative reduction using a fumarate/succinate mixture (12). This estimation should be treated with caution because cytochrome b_{600} was shown (3) and reconfirmed by the present study to be not succinate reducible.

**Epr Characteristics of Cytochrome b_{600}**—Fig. 6 compares the epr spectra of cytochrome b_{600} in isolated QPs, the cytochrome b-c1 particle, intact succinate-ubiquinone, and reconstituted succinate-ubiquinone reductases. Cytochrome b_{600} in QPs preparation shows two epr signals, of roughly equal intensity, at g = 3.07 and g = 2.92. These two signals also appear in the cytochrome b-c1 particle (see spectra a of Fig. 6). The cytochrome b-c1 particles were prepared by treatment of succinate-cytochrome c reductase with alkaline pH (10.0) under anaerobic conditions. Functionally they are equal to succinate-cytochrome c reductase minus succinate dehydrogenase. In the cytochrome b-c1 particle preparation all three species of cytochromes b (b_{621}, b_{560}, and b_{600}) are epr detectable. Cytochrome b_{600} in isolated succinate-ubiquinone reductase exhibits mainly one epr signal at g = 3.46. This is different from those of cytochrome b_{600} (g = 3.43) and cytochrome b_{560} (g = 3.76) (15-17). A small second peak at g = 3.07 is also observed apparently due to the presence of a trace amount of denatured succinate dehydrogenase in the succinate-ubiquinone reductase preparation. However, when QPs was reconstituted with soluble succinate dehydrogenase to form succinate-ubiquinone reductase, a g = 3.46 signal appears, the g = 3.07 signal disappears, and the g = 2.92 signal remains unchanged. These results indicate that there are two forms of cytochrome b_{600} in the isolated QPs. One (g = 3.07) is reconstitutively active with succinate dehydrogenase; the other (g = 2.92) is reconstitutively inactive. The relative concentrations of cytochrome b_{600} with g = 3.07 and g = 2.92 in isolated QPs are estimated to be 47 and 53%, respectively, using the reported proportionality factors (16). This observation is consistent with the finding that only about 50% of the cytochrome b_{600} in QPs is in the reconstitutively active form, which is converted to carbon monoxide nonreactive cytochrome b_{600} and decreases its redox potential from -144 to -185 mV, by reconstitution with succinate dehydrogenase.

![Fig. 5. Potentiometric titration of cytochrome b_{600} in QPs, and in intact and in reconstituted succinate-ubiquinone reductases. A, 10-ml aliquot of QPs (4.2 μM cytochrome b_{600}) (C, □) and intact succinate-ubiquinone reductase (3.3 μM cytochrome b_{600}) (Δ, △); and B, reconstituted succinate-ubiquinone reductase (4 μM cytochrome b_{600}) (C, □) in 50 mM Tris-Cl buffer, pH 7.0, containing 10 μM 2-hydroxy-1,4-naphthaquinone, anthraquinone-2-sulfonic acid (sodium salt), pyocyanine, and duroquinone were used. Reductive titration curves (closed symbols) were by addition of sodium dithionite solution; oxidative curves (open symbols) were by addition of ferricyanide solution to the dithionite reduced sample. The reduction of cytochrome b_{600} was measured by taking the absorption spectra from 600 to 500 nm in an Aminco dual wavelength spectrophotometer. The solid lines in A represent the calculated n = 1 redox titrations with the midpoint potentials of -144 and -185 mV. The data (C, □) obtained from the redox titration of cytochrome b_{600} of reconstituted succinate-ubiquinone reductase (B) is resolved into two components with the redox potentials of -144 mV (C, □) and -185 mV (Δ, △). The solid line is drawn from the calculated data obtained from a system containing two n = 1 components of the said midpoint potentials (-144 and -185 mV) at relative concentrations of 53 and 46%, respectively.
Cytochrome b₅₆₀

Although the evidence presented here clearly indicates that the properties of cytochrome b₅₆₀ in QPs are changed upon binding to succinate dehydrogenase, whether these effects on the heme environment are due to the direct binding of succinate dehydrogenase to cytochrome b₅₆₀ or due to an indirect effect of the binding of succinate dehydrogenase to the M₉ ≈ 13,000 protein, or both, remains unclear because isolated QPs contains two subunits.

Detection of Cytochrome b₅₆₀ in Succinate-Cytochrome c Reductase—Although cytochrome b₅₆₀ of succinate-ubiquinone reductase exhibits an epr signal different from those of cytochrome b₅₆₂ and b₅₆₅ of ubiquinol-cytochrome c reductase (QCR), the detection of cytochrome b₅₆₀ in succinate-cytochrome c reductase is complicated by the abundance of cytochromes b₅₆₀ and b₅₆₅ and the relatively small amount of cytochrome b₅₆₀ in this preparation. Taking advantage of the fact that cytochrome b₅₆₀ of succinate-ubiquinone reductase is not reducible by succinate and the availability of highly concentrated succinate-cytochrome c reductase in our laboratory, we have been able to detect the cytochrome b₅₆₀ in the succinate reduced form of succinate-cytochrome c reductase. The succinate nonreducible cytochrome b is succinate cytochrome c reductase usually accounts for 25–30% of the total cytochrome b in the preparation. This includes all cytochrome b₅₆₀ and the portion of cytochrome b₅₆₀ of ubiquinol-cytochrome c reductase not reducible by succinate. The epr spectra of the succinate-reduced form of succinate-cytochrome c reductase is shown in spectra b of Fig. 6. The two epr signals (g = 3.46, g = 3.76) observed in succinate-reduced succinate-cytochrome c reductase correspond to the cytochrome b₅₆₀ signal (g = 3.76) of ubiquinol-cytochrome c reductase. From spectra a and (a − b) of Fig. 6, using the proportionality factors reported by DeVries and Albracht (16), the relative distribution of cytochromes b₅₆₂, b₅₆₅, and b₅₆₀ in succinate-cytochrome c reductase is estimated to be 38, 44, and 18%, respectively. Since isolated succinate-cytochrome c reductase contains 3.6 mol of cytochrome b₅₆₀/mol of flavin (3), the molar ratio of cytochrome b₅₆₀ to flavin is 0.65. The molecular ratio of cytochrome b₅₆₀ to flavin in isolated succinate-ubiquinone reductase preparations is between 0.3 and 0.5. Cytochrome b₅₆₀ content in succinate-ubiquinone reductase is estimated using a millimolar extinction coefficient of 20.5, instead of 28.5 (3), for reduced minus oxidized at 560 nm minus 575 nm, or by alkaline pyridine hemochromogen spectra using a millimolar extinction coefficient of 34.6.

Fig. 7 shows the power saturation behavior of cytochrome b₅₆₀, cytochrome b₅₆₂, and cytochrome b₅₆₅. Cytochromes b₅₆₀ and b₅₆₂ start to saturate at approximately 6 and 20 milliwatts, respectively, while cytochrome b₅₆₅, the easiest one of the three to power saturate, starts to saturate at lower than 1 milliwatt. The power saturation behaviors of cytochrome b₅₆₀ in succinate-ubiquinone reductase (g = 3.46) and of the epr signal (g = 3.46) of succinate reduced succinate-cytochrome c reductase are identical, suggesting that the latter signal does indeed belong to cytochrome b₅₆₀.

Possible Role of Cytochrome b₅₆₀ in the Reduction of Fumarate—From the evidence presented in previous sections, it is clear that there is a strong physical association between cytochrome b₅₆₀ and succinate dehydrogenase. However, because cytochrome b₅₆₀ in succinate-ubiquinone reductase is not reduced by succinate, even under anaerobic conditions, a catalytic role for this cytochrome in succinate oxidation seems unlikely. On the other hand, under anaerobic conditions di-thionite or NADH reduced cytochrome b₅₆₀ in succinate-ubiquinone reductase or QPs, in the presence of succinate.
dehydrogenase, is fully oxidized by fumarate, suggesting that cytochrome $b_{560}$ plays a role in fumarate reduction. The reduced form of cytochrome $b_{560}$ is not oxidized by fumarate in the absence of succinate dehydrogenase. In other words, cytochrome $b_{560}$ apparently serves as the electron entrance for fumarate reduction but not as the electron exit for succinate oxidation. In the isolated system reduction of fumarate can only be demonstrated under anaerobic conditions, whereas in the intact mitochondrial inner membrane the reduction of fumarate occurs under specific conditions, such as when the membrane is in a highly energized state. The well established phenomenon of energy-dependent reversed electron transfer is an example (28). Under anaerobic conditions, cytochrome $b_{560}$ in QPs or succinate-ubiquinone reductase is reducible by NADH in the presence of type II NADH dehydrogenase. This NADH reduced cytochrome is oxidizable by fumarate only after succinate dehydrogenase is added. In anaerobic organisms, fumarate serves as a terminal electron acceptor; in aerobic organisms no such function is needed. The reduction of fumarate at the expense of NADH might play a role in the regulation of electron transfer and oxidative phosphorylation.

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