Isolation and Characterization of a Two-Subunit Cytochrome \(b-c_1\) Subcomplex from \textit{Rhodobacter capsulatus} and Reconstitution of Its Ubihydroquinone Oxidation (Q\(_o\)) Site with Purified Fe-S Protein Subunit†

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ABSTRACT: The presence of a two-subunit cytochrome (cyt) \(b-c_1\) subcomplex in chromatophore membranes of \textit{Rhodobacter capsulatus} mutants lacking the Rieske iron–sulfur (Fe-S) protein has been described previously [Davidson, E., Ohnishi, T., Tokito, M., and Daldal, F. (1992) \textit{Biochemistry} 31, 3351–3358]. Here, this subcomplex was purified to homogeneity in large quantities, and its properties were characterized. As expected, it contained stoichiometric amounts of cyt \(b\) and cyt \(c_1\) subunits forming a stable entity devoid of the Fe-S protein subunit. The spectral and thermodynamic properties of its heme groups were largely similar to those of a wild-type \(bc_1\) complex, except that those of its cyt \(b_6\) heme were modified as revealed by EPR spectroscopy. Dark potentiometric titrations indicated that the redox midpoint potential (\(E_{m0}\)) values of cytochromes \(b_1\), \(b_6\), and \(c_1\) were very similar to those of a wild-type \(bc_1\) complex. The purified \(b-c_1\) subcomplex had a nonfunctional ubihydroquinone (UQH\(_2\)) oxidation (Q\(_o\)) site, but it contained an intact ubiquinone (UQ) reductase (Q\(_i\)) site as judged by its ability to bind the Q\(_i\) inhibitor antimycin A, and by the presence of antimycin A sensitive Q\(_i\) semiquinone. Interestingly, its Q\(_o\) site could be readily reconstituted by addition of purified Fe-S protein subunit. Reactivated complex exhibited myxothiazol, stigmatellin, and antimycin A sensitive cyt \(c\) reductase activity and an EPR \(g_s\) signal comparable to that observed with a \(bc_1\) complex when the Q\(_o\) site is partially occupied with UQ/UQH\(_2\). However, a mutant derivative of the Fe-S protein subunit lacking its first 43 amino acid residues was unable to reactivate the purified \(b-c_1\) subcomplex although it could bind to its Q\(_o\) site in the presence of stigmatellin. These findings demonstrated for the first time that the amino-terminal membrane-anchoring domain of the Fe-S protein subunit is necessary for UQH\(_2\) oxidation even though its carboxyl-terminal domain is sufficient to provide wild-type-like interactions with stigmatellin at the Q\(_o\) site of the \(bc_1\) complex.

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\text{The ubihydroquinone cytochrome (cyt)\(c\) oxidoreductase (\(bc_1\) complex) is an integral membrane protein complex, present in a wide range of organisms including bacteria, mitochondria, and chloroplasts (where it is known as the \(bc\) complex) (for recent reviews, see refs 1–5). It is located in the cytoplasmic membrane, and is a site where energy transduction is coupled to ATP synthesis. In the phototrophic bacterium \textit{Rhodobacter capsulatus}, the \(bc_1\) complex is a key component of both the photosynthetic and respiratory electron-transport chains (2, 5).}
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\text{\textit{R. capsulatus} \(bc_1\) complex consists of the cyt \(b\), cyt \(c_1\), and Rieske iron–sulfur (Fe-S) protein subunits, which are encoded by the petABC (fbcFBC) operon (6), and contains four metal centers. Cyt \(b\) has two noncovalently bound hemes, \(b_1\) and \(b_6\), named after their relatively high (+50 mV) and low (−90 mV) redox midpoint potentials (\(E_{m0}\)), respectively (7, 8). Cyt \(c_1\) and the Fe-S protein subunits are attached to the membrane via their carboxyl- and amino-terminal ends, respectively. The carboxyl-terminal domain of the latter subunit contains its [2Fe-2S] cluster (9), while the amino-terminal part of the former has a \(c\)-type heme, and both of these subunits are exposed to the periplasm. The \(bc_1\) complex also contains two ubihydroquinone (UQH\(_2\))/ubiquinone (UQ) binding domains named Q\(_i\) and Q\(_o\) sites, respectively. The Q\(_o\) site is located near the cytoplasmic side of the membrane and catalyzes reduction of UQ, while UQH\(_2\) oxidation takes place at the Q\(_i\) site located near the periplasmic side of the membrane (10). During recent years a great deal of structural and functional information has been gathered about cyt \(b\) and its UQ/UQH\(_2\) binding sites from}
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studies of inhibitor-resistant or nonfunctional mutations located in the bc₁ complex (11–13). These studies have revealed a low-resolution topology of these sites which is now largely confirmed by the recent determination of the three-dimensional structure of various mitochondrial bc₁ complexes (14, 15). The available structures have established the distances between the redox centers of the bc₁ complex and the overall organization of the subunits. Moreover, the different forms of crystals obtained using bc₁ complexes purified from different species revealed that while the distances between the cyt c₁ and cyt b heme groups remain constant those of the [2Fe-2S] cluster to cyt bₙ and cyt c₁ vary considerably (15). This finding suggested that the Fe-S protein may be mobile enough to yield different forms of crystals in which it occupies distinct positions with respect to the other subunits of the bc₁ complex. Whether the proposed mobility of the Fe-S protein subunit is an intrinsic component of Q₁₀ site catalysis remains to be seen.

Our earlier work has indicated that R. capsulatus mutants affecting the universally conserved cysteine and histidine ligands of the [2Fe-2S] cluster of the Fe-S protein subunit produce large amounts of cyt b and cyt c₁ while they completely lack the Fe-S protein subunit (16). In chromatophore membranes of such mutants, apparently cyt b and cyt c₁ recognize each other to form a subtwo-subunit complex with an intact Q₁ site and an inactive Q₈ site (17). Purification of this bc₁ subcomplex was therefore attempted in order to prove that these subunits indeed form a subcomplex in the absence of the Fe-S protein. Here we report the isolation and detailed characterization of a stable, two-subunit bc₁ subcomplex from R. capsulatus. We demonstrate for the first time for a bacterial bc₁ complex that the purified bc₁ subcomplex can regain its cyt c reductase activity upon addition of purified Fe-S protein subunit in vitro provided that the amino-terminal membrane anchor of this subunit is intact. Moreover, we show that in the presence of the Q₈ site inhibitor stigmatellin a soluble derivative of the Fe-S protein subunit lacking the membrane anchor can reconstitute a stigmatellin-inhibited Q₁₀ site, intact. The overall findings therefore establish that the amino-terminal membrane anchor of the Fe-S protein subunit is required for the formation of an active Q₈ site, even though its [2Fe-2S]-carrying carboxyl-terminal portion is sufficient to interact with cyt b appropriately in the presence of stigmatellin. The availability of a reconstitutively active bc₁ subcomplex coupled with an easily useful in dissecting the mechanism of UQH₂ oxidation at the Q₁₀ site of the bc₁ complex.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. The R. capsulatus strains pMTS1/MT-RBC1 overproducing the wild-type bc₁ complex (18), pF:H135L/MT-RBC1 lacking the Fe-S protein subunit and overproducing the cyt b–c₁ subcomplex (16), and pB:(T163F+G182S)/MT-RBC1 producing a soluble form of the Fe-S protein subunit (19) have been described previously. These strains were grown chemoheterotrophically in a “mixed medium” [1 volume of medium A (20 °C) + 1 volume of MpyE (6)] containing 0.625 μg/mL tetracycline [pF:H135L/MT-RBC1 and pB: (T163F+G182S)/MT-RBC1] or 10 μg/mL kanamycin (pMTS1/MT-RBC1) either in 2 L flasks using a rotary shaker at 150 rpm or in 20 L carboys under semiaerobic conditions. Under the conditions used here, the typical biomass yield was about 3–4 g of cells (wt weight) per liter of growth medium.

Purification of Wild-Type bc₁ Complex and bc₁ Subcomplex. The bc₁ complex and bc₁ subcomplex were isolated from chromatophore membranes by a variation of the procedure described in (8). Chromatophore membranes were obtained from frozen cell paste after two passages through a French pressure cell according to (21) and either used immediately or stored frozen at −80 °C. They were resuspended to a final protein concentration of 10 mg/mL in 50 mM Tris-HCl buffer, pH 8.0, containing 100 mM NaCl, 20% glycerol, 1 mM PMSE, and 1 mM EDTA. Dodecyl maltoside (DDM, 20% w/v stock) was added dropwise to this suspension to a final concentration of 1 mg/ml of total protein. The mixture was stirred for 45 min at 4 °C, then centrifuged at 12000g for 90 min. The supernatant was collected, adjusted to pH 8.0, and loaded onto a DEAE- BioGel A column (2.6 × 55 cm) previously equilibrated with 50 mM Tris-HCl, pH 8.0, 20% glycerol, and 100 mM NaCl (equilibration buffer). The column was washed first with 2–3 bed volumes of equilibration buffer containing 0.01% (w/v) dodecyl maltoside, followed by 5–6 bed volumes of the same buffer containing 150 mM NaCl. These washes removed large amounts of photosynthetic pigments as well as the cbb₃-type cyt c oxidase (22). The adsorbed wild-type bc₁ complex, or the bc₁ subcomplex, was eluted with 3 bed volumes of a linear 150–400 mM NaCl gradient. For the bc₁ complex, fractions containing the highest DBH₂:cyt c reductase activity were pooled. In the case of the bc₁ subcomplex, fractions were monitored for their absorption at 280 and 420 nm, and for their dithionite-reduced minus ferricyanide-oxidized optical difference spectra at 500–600 nm, and those containing the highest concentrations of cyt b and cyt c₁ were pooled. Under the conditions used, both the bc₁ complex and the bc₁ subcomplex were typically eluted between 230 and 270 mM NaCl concentrations. The pooled fractions were dialyzed against the equilibration buffer without glycerol, and concentrated using an Amicon Diaflo apparatus and a PM30 membrane. The concentrated sample (25–30 mL) was applied onto a DEAE-Fractogel (Toyopearl 650) column (2.6 × 17 cm) equilibrated with the equilibration buffer without glycerol but containing 0.01% (w/v) DDM. The column was washed with 3 bed volumes of equilibration buffer containing 150 mM NaCl, and eluted with 3 bed volumes of a linear 150–400 mM NaCl gradient in the same buffer. Fractions containing the bc₁ complex or the bc₁ subcomplex were pooled, concentrated as above using a PM30 membrane, and stored at −80 °C.

Purification of the Fe-S Protein Subunit from the Wild-Type bc₁ Complex and Its Reconstitution to the Purified bc₁ Subcomplex. Reconstitutively active R. capsulatus Fe-S protein was obtained from purified bc₁ complex using Phenyl Sepharose chromatography according to (23) for R. sphaeroides, except that in the case of R. capsulatus 2% sodium cholate was required for the dissociation of the Fe-S protein subunit from the bc₁ complex. The purified bc₁ subcomplex was reconstituted in vitro with purified Fe-S protein essentially as described by (24) with the following modifications. The R. capsulatus bc₁ subcomplex (1.12 nmol) was mixed with 275 μg of lecithin and varying amounts of...
purified Fe-S protein, and the final volume was brought to 150 μL with 40 mM potassium phosphate buffer, pH 7.4 containing 0.5 mM EDTA. This mixture was incubated for 1 h at 35 °C, then diluted 2-fold with 50 mM Tris-HCl, pH 8.0, buffer containing 100 mM NaCl and 0.01% (w/v) DDM, and centrifuged at 22000g for 4 h. This treatment, which pelleted the reconstituted bc1 complex and left the unbound Fe-S protein in the supernatant, was repeated twice and the pellet resuspended in the same buffer to a final concentration of 40–45 μM cyt c1. The DBH2:cyt c reductase activity was measured essentially as described earlier (21) but in the presence of 0.02% Tween 20. The assay mixture (2 mL total volume) contained 40 mM phosphate buffer, pH 7.4, 0.5 mM EDTA, 250 μg of lecithin, 0.02% Tween 20, and 50 μM horse heart cyt c. The DBH2 was added to a concentration of 40 μM from a DMSO stock solution, and non enzymatic reduction of cyt c was followed at 550 nm with a Hitachi U-3200 spectrophotometer. The reaction was initiated by adding the enzyme, and the rate of cyt c reduction monitored at 550 nm was determined using an extinction coefficient ε550 of 18.5 mM−1 cm−1 (25). The DBH2-dependent cyt c reductase activity sensitive to the bc1 complex inhibitors myxothiazol, stigmatellin, and antimycin A was taken as an indication of the reactivation of the b–c1 subcomplex.

For EPR analysis, the reconstitution assay was scaled up by about 10-fold so that 14 nmol of purified b–c1 subcomplex was incubated with 10 nmol of purified Fe-S protein and treated as above. Small aliquots of the reconstituted subcomplex were used for SDS–PAGE and DBH2:cyt c reductase activity analyses. The remainder of the sample was prepared for EPR spectroscopy and its EPR spectrum recorded as described earlier (26).

Reconstitution of the b–c1 Subcomplex with a Soluble Form of the Fe-S Protein. For this purpose, highly concentrated membrane-free chromatophore supernatants of the double mutant pb:(T163F+G182S)/MT-RBC1 producing a soluble form of the Fe-S protein subunit (19) were used. The concentrated supernatants (18 mg/mL) were divided into two 300 μL portions, to one of which stigmatellin was added to a final concentration of 40 μM. They were then mixed with the b–c1 subcomplex (14 nmol) and lecithin (275 μg) and incubated for 1 h at 35 °C. Small aliquots were taken to measure the DBH2:cyt c reductase activity (21), the remainder of the samples was reduced with 20 μM sodium ascorbate, and their EPR spectra were recorded as described previously (26).

Spectroscopy. Optical spectra were obtained using a Hitachi U-3200 spectrophotometer with a 1 cm light path cuvettes. Cyt c1 content was calculated from ascorbate-reduced minus ferricyanide-oxidized optical difference spectra using an extinction coefficient ε550–542 of 20 mM−1 cm−1 (27). Cyt b content was calculated from dithionite-reduced minus ascorbate-reduced optical difference spectra using an extinction coefficient ε560–574 of 28 mM−1 cm−1 (28). Dark potentiometric titrations of the b–c1 subcomplex, and the wild-type bc1 complex, were carried out according to (29), using a double-beam spectrophotometer (University of Pennsylvania Instrumentation Group) fitted with an anaerobic redox cuvette. Mediation between the electrodes, solution, and redox centers was achieved using a collection of redox mediator dyes chosen for their electrochemical potentials and their lack of optical interference in the 500–600 nm region. For these experiments, the total bc1 complex, or the b–c1 subcomplex, was adjusted to a protein concentration of 2–3 μM in 50 mM MOPS, 100 mM KCl buffer, pH 7.0, in the presence of the following mediators: N-methylidybenzopyrazine methosulfate (PMS), N-ethylidybenzopyrazine ethosulfate (PES), 1,4-naphthoquinone, 1,2-naphthoquinone, and 2-hydroxy-1,4-naphthoquinone at 25 μM each and 50 μM 2,3,5,6-tetramethylphenylenediamine (DAD). Spectra were taken at E0 intervals of 5–20 mV during a reductive titration, and the spectral data thus acquired were manipulated using the Labview software program (National Instruments) developed for this spectrophotometer by Dr. H. Ding. The optical spectra of the individual hemes bh, bl, and c1 were derived from the titration data using proper E0 cuts.

The antimycin A induced shift of the cyt b absorption spectrum was recorded with a Beckman DU640 spectrophotometer using 2.5 μM bc1 complex, or b–c1 subcomplex, resuspended in 50 mM Tris-HCl, pH 8.0, buffer containing 100 mM NaCl and 0.01% (w/v) DDM. Samples were first reduced with dithionite, and base lines were recorded; then antimycin A was added to a final concentration of 30 μM from a stock solution in DMSO, and the reduced plus antimycin A minus reduced spectra were recorded between 400 and 600 nm.

EPR spectra were obtained using a Bruker Model ESP-300 E EPR spectrometer, equipped with a helium cryostat. The experimental conditions were as described earlier (8, 26), and are given in the legends of the corresponding figures.

Other Assays and Analytical Measurements. Protein concentrations were determined by the method of Lowry et al. (30), using bovine serum albumin as a standard. One-dimensional SDS–PAGE was performed according to Laemmli (31) using a 15% linear gel. For immunoblot analyses, gels were blotted onto Immobilon-P membranes (Millipore) (32), and monoclonal antibodies raised against the subunits of the bc1 complex from R. capsulatus (21) were used as immunoprobes. Goat anti-mouse IgG conjugated to horseradish peroxidase was used as a secondary antibody, and detection of the antigen–antibody immunocomplex was enhanced using 3,3′-diaminobenzidine tetrachloride in the presence of 0.12% NiCl2.

The UQ content of the purified b–c1 subcomplex was determined as described by (33). Briefly, 36 nmol of purified b–c1 subcomplex in 50 mM Tris-HCl, pH 8.0, buffer, containing 100 mM NaCl and 0.01% (w/v) DDM, was extracted with a 10-fold excess of acetone/methanol (1/1, v/v) mixture. The mixture was shaken gently for 10 min, followed by the addition of an equal volume of petroleum ether. The latter solvent containing UQ was collected, washed with 95% (v/v) methanol, and evaporated. The dried extract was dissolved in ethanol and the UQ amount determined using borohydride reduced minus ferric chloride oxidized absorption difference spectra and an ε275 of 14 mM−1 cm−1.

Chemicals. DEAE-BioGel A and DEAE- Fractogel (Toyopearl-650) were obtained from BioRad and EM Separation Technology, respectively. Dodecyl maltoside was purchased from Anatrace Inc., and lecithin, Tween 20, horse heart cytochrome c, PMS, PES, and antimycin were obtained from Sigma. Myxothiazol was purchased from Boehringer-Mannheim Biochemicals, and 2-hydroxy-1,4-naphthoquinone
and 2,3,5,6-tetramethyl-1,4-phenylenediamine (DAD) were from Aldrich. All other chemicals were of reagent grade or of highest quality commercially available.

RESULTS

**Purification of the b–c₁ Subcomplex.** The b–c₁ subcomplex was purified essentially as described for the wild-type bc₁ complex (8). This procedure yielded about 30 mg of highly pure b–c₁ subcomplex starting with approximately 1.5 g of chromatophore membranes prepared using 165 g of cells (wet weight). The only contaminants in the final preparations of the b–c₁ subcomplex, and also the bc₁ complex, were about 5–10% of photosynthetic pigments associated with the light-harvesting complexes I and II. A comparison of the composition and enzymatic activity of purified b–c₁ subcomplex and bc₁ complex is presented in Table 1. Cyt b and cyt c₁ contents (15.8 and 11.0 nmol/mg of protein, respectively) of the b–c₁ subcomplex are about 30% less than those of the wild-type bc₁ complex (22 and 15 nmol/mg of protein, respectively) although the cyt b to cyt c₁ ratios (approximately 1.45 to 1.50) are similar in both cases. Assuming that the two preparations are of similar purity, this difference suggests that the b–c₁ subcomplex is more prone to loss of its prosthetic groups during purification. As expected, the purified b–c₁ subcomplex exhibited no detectable DBH₂-dependent antimycin A sensitive cyt c reductase activity while the purified bc₁ complex had high DBH₂:cyt c reductase activity [about 41 μmol of horse heart cyt c reduced (mg of protein)⁻¹ min⁻¹], which was completely inhibited by antimycin A (Table 1).

SDS–PAGE analysis of purified wild-type bc₁ complex revealed three major bands with molecular masses of 41, 31, and 24 kDa corresponding to cyt b, cyt c₁, and the Fe-S protein subunits, respectively (Figure 1A). In contrast, the purified b–c₁ subcomplex contained only two major bands with molecular masses of 41 and 31 kDa. Furthermore, immunoblot analysis using specific monoclonal antibodies revealed that the 24 kDa Fe-S protein subunit is completely absent in the b–c₁ subcomplex preparation (Figure 1B). The additional bands of higher molecular mass seen both with the bc₁ complex and with the b–c₁ subcomplex correspond to their aggregated forms as observed previously (8). The high purity of b–c₁ subcomplex prepared by the protocol used here was indicated by the high cytochrome content and SDs–PAGE patterns, as well as by a high absorption ratio (0.92) of the 411 to 280 nm peaks of the oxidized b–c₁ subcomplex (data not shown).

**Spectral Properties and Electrochemistry of the Redox Centers.** The b–c₁ subcomplex isolated from *R. capsulatus* displayed an optical spectrum characteristic of the bc₁ complexes isolated from other sources (34). Its oxidized spectrum has a Soret absorption peak at 411 nm, and the dithionite-reduced spectrum ν absorption bands at 553 and 558.5 nm, β absorption bands at 523.5 and 529 nm, and a Soret band at 426.5 nm (data not shown). These peak values were essentially the same as those observed with a wild-type bc₁ complex (8).

The effect of the absence of the Fe-S subunit protein on the spectral and thermodynamic properties of the redox centers b₃H, b₃L, and c₁ was tested by comparison of the b–c₁ subcomplex with the wild-type bc₁ complex. The redox midpoint potential (Eₘ⁰) values of the individual hemes centers were determined by dark equilibrium titration (Figure 2). In the b–c₁ subcomplex, cyt b₃L and b₃H titrated with Eₘ⁰ values of 35 and −130 mV while in the wild-type bc₁ complex they titrated with Eₘ⁰ values of 43 and −138 mV, respectively. Thus, within the limits of experimental errors (±15 mV), no significant difference in the Eₘ⁰ values of

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Table 1: Purification of *R. capsulatus* b–c₁ Subcomplex from a Mutant Lacking the Fe-S Protein and Its Comparison to the Wild-Type bc₁ Complex

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Cyt b (nmol)</th>
<th>Cyt c (nmol)</th>
<th>Yield (%)</th>
<th>Purification (x-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>chromatophores + DDM</td>
<td>1740</td>
<td>1.20</td>
<td>2088</td>
<td>1.25</td>
<td>2175</td>
</tr>
<tr>
<td>DDM extract</td>
<td>1582</td>
<td>1.26</td>
<td>2192</td>
<td>1.27</td>
<td>2009</td>
</tr>
<tr>
<td>DEAE-BioGel A pool</td>
<td>100</td>
<td>12.30</td>
<td>1230</td>
<td>8.90</td>
<td>890</td>
</tr>
<tr>
<td>concentrated pool applied on Fractogel</td>
<td>60</td>
<td>13.00</td>
<td>780</td>
<td>9.30</td>
<td>558</td>
</tr>
<tr>
<td>Fractogel pool</td>
<td>43</td>
<td>15.40</td>
<td>662</td>
<td>10.80</td>
<td>464</td>
</tr>
<tr>
<td>PM30K concentrate</td>
<td>30</td>
<td>15.80</td>
<td>474</td>
<td>11.00</td>
<td>330</td>
</tr>
</tbody>
</table>

(A) Purification of b–c₁ Subcomplex

(B) Comparison of b–c₁ Subcomplex to bc₁ Complex

<table>
<thead>
<tr>
<th></th>
<th>Cyt b content</th>
<th>Cyt c content</th>
<th>DBH₂: cyt c reductase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>b–c₁ subcomplex</td>
<td>15.8</td>
<td>11.0</td>
<td>0</td>
</tr>
<tr>
<td>bc₁ complex</td>
<td>22.0</td>
<td>15.0</td>
<td>41.0</td>
</tr>
</tbody>
</table>

a Nanomoles per milligram of protein. b Antimycin A sensitive activity (micromoles of cyt c reduced per milligram of protein per minute).
cyt bH and bL between the b–c1 subcomplex and the bc1 complex was observed. A third component referred to as cyt bc1s0 (18, 35, 36) was also detectable, and $E_{m7}$ values of 160 mV for the b–c1 subcomplex and 134 mV for the bc1 complex were used to obtain the best fit to the experimental data shown in Figure 2. Finally, for cyt c1 subunit, very similar $E_{m7}$ values for the b–c1 subcomplex (305 mV) and the bc1 complex (295 mV) were observed.

When the optical spectra of cytochromes bH, bL, and c1 were compared, no significant differences were detected in the absorption maxima of cyt bH and cyt c1 between the b–c1 subcomplex and the bc1 complex. However, the optical spectrum of the b–c1 subcomplex was clearly modified (Figure 3, panels A and B). While cyt bL of a wild-type bc1 complex had a typical split α peak spectrum (558 and 564.8 nm), that of the b–c1 subcomplex displayed a single broad peak with a maximum around 559.6 nm. Considering that in the chromatophore membranes (17), and also following the BioGel chromatography step (data not shown), cyt bL of the b–c1 subcomplex still exhibited its typical split α peak, this finding together with the lower content of total cyt b in the purified b–c1 subcomplex suggested that the conformation of cyt bL was modified, and part of it was lost, during the final steps of purification and storage.

EPR Characteristics of Cyt b Hemes of the Isolated b–c1 Subcomplex. The low-spin cyt b hemes of the bc1 complex exhibit unusual EPR spectra (35), and their $g_z$ values (3.78 and 3.45 for cyt bH and cyt bL, respectively) are known. Thus, the EPR characteristics of the cyt bH and bL of isolated b–c1 subcomplex and bc1 complex were compared to further assess the extent of the perturbation detected by optical spectroscopy. While the cyt b hemes of the purified b–c1 subcomplex, and the bc1 complex, have similar $g_z$ signals ($g_z = 3.79$ for cyt bH and 3.46 for cyt bL), on the other hand, the overall line shape of the EPR spectrum obtained using the b–c1 subcomplex was modified (Figure 4) by a signal with a $g_z$ value of 3.69. This EPR resonance, observed in the b–c1 subcomplex spectrum, indicates that the conformation of a large portion of the cyt bL heme was modified. Finally, a significant amount of an EPR signal at a $g = 6$ value corresponding to a high-spin heme was also detected in both the b–c1 subcomplex and the bc1 complex (data not shown), as previously reported for the bc1 complex from R. sphaeroides (37).

UQ Content and the Q$_i$ Site of Isolated b–c1 Subcomplex. UQ content of the purified b–c1 subcomplex was determined as described under Materials and Methods to assess the occupancy of its Q binding sites. It contained about 0.5 nmol of UQ/nmol of cyt b–c1 complex. The spectra were derived from the data obtained during the redox titrations shown in Figure 2 using appropriate $E_b$ values as follows for b–c1 subcomplex (panel A) and bc1 complex (panel B), respectively: cyt bH, 10 minus 200 mV and 2 minus 190 mV; cyt bL, −188 minus −24 mV and −187 minus −15 mV; cyt c1, 202 minus 401 mV and 200 minus 407 mV. Note the modified cyt b spectrum of the purified b–c1 subcomplex.
maxima at 560 nm (Figure 5A with inset). Thus, antimycin A binds to the isolated $b^{-c_1}$ subcomplex in a way similar to the wild-type $bc_1$ complex.

Finally, the functional intactness of the $Q_o$ site of the $b^{-c_1}$ subcomplex was also probed by monitoring its antimycin A sensitive, ubisemiquinone radical signal at $g = 2.005$ using EPR spectroscopy. Upon addition of this inhibitor to the purified $b^{-c_1}$ subcomplex, the antimycin A sensitive ubisemiquinone radical disappeared in a manner similar to that observed with the wild-type $bc_1$ complex (8) (Figure 5B). The data indicated that at least a fraction of the UQ detected in the purified $b^{-c_1}$ subcomplex was associated with its $Q_o$ site which was able to generate a stable $Q_i$ ubiquinone radical like the wild-type $bc_1$ complex.

Reconstitution of UQH$_2$ Oxidation Activity of the Isolated Inactive $b^{-c_1}$ Subcomplex with Purified Fe-S Protein Subunit. Incubation of purified $b^{-c_1}$ subcomplex and the Fe-S protein subunit at 35 °C as described under Materials and Methods led to the recovery of the DBH$_2$-dependent cyt $c$ reductase activity of the $b^{-c_1}$ subcomplex (Figure 6). The amount of recovered activity increased as a function of the amount of Fe-S protein subunit added. The highest activity was reached at a ratio of 1 mol of Fe-S protein subunit to 1.3 mol of purified $b^{-c_1}$ subcomplex. The recovered activity [approximately 0.32 μmol of cyt $c$ reduced min$^{-1}$ (mol of cyt $c_1$)$^{-1}$ or 10.2 μmol of cyt $c$ reduced min$^{-1}$ (mg of protein)$^{-1}$] amounted to approximately 25% of that of the wild-type $bc_1$ complex, and was inhibited completely by addition of antimycin A and myxothiazol (Figure 6, inset). Additional proof that the recovered DBH$_2$:cyt $c$ reductase activity was indeed due to reconstitution of the purified Fe-S protein subunit to the $b^{-c_1}$ subcomplex was obtained by SDS–PAGE and EPR analyses. The presence of the 24 kDa Fe-S polypeptide, which was undetectable previously in the purified $b^{-c_1}$ subcomplex (Figure 1), became evident upon reconstitution (Figure 7, panel A). Similarly, the EPR $g_x = 1.90$ and $g_y = 1.80$ signals which were absent in the EPR spectrum of the $b^{-c_1}$ subcomplex (Figure 7, panel B, trace B) became comparable to that of the $bc_1$ complex (trace A). The shift of the Fe-S protein subunit $g_z = 1.80$ signal from 1.765 to 1.800 in the reconstituted $b^{-c_1}$ subcomplex suggested that the $Q_o$ site of the reconstituted sample was competent to bind UQ/UQH$_2$ appropriately, although it was partially empty (33).

The Amino-Terminal Anchor Domain of the Fe-S Protein Subunit Is Required for the Reconstitution of the $Q_o$ Site of the $b^{-c_1}$ Subcomplex. Very recently, we have described a $R$. capsulatus mutant which produces a soluble form of the Fe-S protein subunit via proteolytic cleavage at its amino-terminal position 44 (19). This soluble derivative of the Fe-S protein subunit has an intact [2Fe-2S] cluster with an $E_m$ value similar to that of a wild-type complex even though it is no longer anchored to the membrane. The availability of this mutant provided us the opportunity to probe the role of the amino-terminal membrane anchor to the Fe-S protein subunit for its binding to the $Q_o$ site of the $bc_1$ complex. Thus, chromatophore membrane supernatants containing a soluble form of the Fe-S protein subunit prepared as described in (19) were used to reconstitute the $b^{-c_1}$ subcomplex. However, no DBH$_2$:cyt $c$ reductase activity was recovered under the same experimental conditions which fully restored the activity of $b^{-c_1}$ subcomplex when an intact Fe-S protein subunit was used. Furthermore, additional proof that the soluble derivative of the Fe-S protein could not reconstitute the $Q_o$ site of $b^{-c_1}$ subcomplex was obtained by EPR spectroscopy. Like the purified Fe-S protein, the soluble derivative also had no pronounced EPR $g_z = 1.80$ signal, and it did not respond to the addition of stigmatellin (Figure 8, traces A and B), as described previously for the bovine (40) and $R$. capsulatus (19) soluble Fe-S protein subunits. Unlike with the intact Fe-S subunit (Figure 7), this EPR $g_z$ signal was not restored when the $b^{-c_1}$ subcomplex was incubated with the soluble form of the Fe-S protein subunit (Figure 8, trace D). Thus, the amino-terminal anchor of the Fe-S protein is required for the reconstitution of a catalytically active $Q_o$ site of the $bc_1$ complex.

Next, considering that the carboxyl-terminal portion of the Fe-S protein subunit carrying the [2Fe-2S] cluster interacts closely with cyt $b$ at the $Q_o$ site, as revealed by the very recent genetic (41) and structural (15) studies, we postulated that stigmatellin would mediate binding of the soluble Fe-S protein subunit into the $Q_o$ site. Indeed, when the $b^{-c_1}$ subcomplex was incubated with the soluble form of the Fe-S protein subunit in the presence of stigmatellin, an EPR $g_z = 1.782$ signal identical to that observed with a wild-type $bc_1$ complex in the presence of this inhibitor was obtained (Figure 8, trace E). Thus, the soluble form of the Fe-S protein subunit can occupy the $Q_o$ site of the $b^{-c_1}$ subcomplex at a position similar to that of the intact Fe-S protein in the presence of stigmatellin. Therefore, the overall data revealed that for reconstitution of a catalytically active $Q_o$ site, at least two interaction points, one provided by the amino-terminal anchor and the other by the carboxyl-terminal domain of the Fe-S protein, are necessary.
In the present work, the *R. capsulatus* b–c\(_1\) subcomplex lacking the Fe-S protein subunit was purified to homogeneity in large quantities, and characterized in detail. The purified b–c\(_1\) subcomplex remained stable upon storage at -80 °C for over 1 year without significant loss of its reconstitutively active state. Although little is known about the interactions between the subunits of the bc\(_1\) complex, the ability to isolate it as a physical entity indicated that cyt b and cyt c\(_1\) can associate with each other to form a subcomplex in the absence of the Fe-S protein both in vivo (17) and in vitro.

The purified b–c\(_1\) subcomplex contained slightly less b- and c-type hemes in comparison to the purified bc\(_1\) complex, and removal of the Fe-S protein subunit modified slightly the spectral characteristics of its hemes b\(_L\), leaving the \(E_m\) values of its hemes b\(_H\), b\(_L\), and c\(_1\) similar to those of the b–c\(_1\) complex. It therefore appears that the Fe-S protein subunit...
does not tightly shield cyt $b_1$ from solvent exposure, an observation consistent with the three-dimensional structure of the bc$_1$ complex and with the proposed movement of the Fe-S protein subunit during Q$_o$ site catalysis (14, 15). Furthermore, as expected, the elimination of the Fe-S subunit does not affect the properties of the Qi site in terms of its interactions with antimycin A or its ability to stabilize a semiquinone intermediate.

The effect of in vivo removal of the Fe-S protein from the bc$_1$ complexes varies between different species. In R. capsulatus (17), removing the [2Fe-2S] cluster has little effect on the steady-state presence of the cyt $b$ and cyt c$_1$ subunits in chromatophore membranes. However, the Chlamydomonas reinhardtii mutant ac21 missing the Fe-S protein subunit does not affect the properties of the Q$_i$ site in terms of its interactions with antimycin A or its ability to stabilize a semiquinone intermediate.

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The most striking result obtained in this study is our ability to reconstitute the cyt $c$ reductase activity of the bc$_1$ complex with purified wild-type Fe-S protein (Figure 9). The reconstituted complex exhibits good activity (about 25% of that of a wild-type complex), and the assay developed here is simple and sensitive, and can be readily scaled up for physical measurements requiring large amounts of sample materials, such as an EPR spectroscopy. Reconstitution of the cyt $c$ reductase activity of a bc$_1$ complex depleted of its Fe-S protein subunit has been reported.
previously for the beef heart mitochondrial \(bc_1\) complex (24, 45) but not for any bacterial species. This was possible because of the overproduction of the \(bc_1\) complex and the availability of \(R.\) capsulatus mutants which facilitated its purification (17). In addition, the use of Tween 20, which inhibits reduction of cyt \(c\) by the Fe-S protein via the Q\(_o\) site independent oxidation of ubihydroquinone, possibly by acting as a quencher of superoxide and semiquinone radicals thus formed (1–5), rendered possible the detection of low, but specific, Q\(_o\) site dependent (i.e., sensitive to myxothiazol) cyt \(c\) reductase activity of the reconstituted complex.

The in vitro assay developed here was exploited to probe whether the amino-terminal anchor of the Fe-S protein subunit is essential for steady-state Q\(_o\) site catalysis. The very recent genetic (41) and structural (14, 15) data suggest that a specific segment composed of the amino acid residues (M\(_{130}\)NASADVK\(_{146}\) in \(R.\) capsulatus) located between the end of the transmembrane anchor and the beginning of the periplasmic portion of the Fe-S protein subunit may mediate its mobility proposed to be required for Q\(_o\) site catalysis. Since unlike the intact Fe-S protein subunit the soluble derivative of the Fe-S protein subunit produced by the \(R.\) capsulatus mutant pB:(T163F) (19) could not reanimate the Q\(_o\) site of the \(b-c_1\) subcomplex, it therefore appears that the first 44 amino acid residues of this subunit are required for steady-state Q\(_o\) site activity. Remarkably though, the same soluble derivative of the Fe-S protein can reassociate appropriately with the Q\(_o\) site of the \(b-c_1\) subcomplex provided that stigmatellin is present. This association leads to the appearance of the characteristic EPR \(g_s = 1.781\) signal observed only with a wild-type \(bc_1\) complex. The characteristics of this EPR \(g_s\) signal are tightly correlated with the immediate environment of the [2Fe-2S] cluster (47), and the \(g_s\) signal detected after reconstitution is very similar to that observed with a wild-type \(bc_1\) complex. It therefore appears that in the presence of stigmatellin the soluble form of the Fe-S protein subunit interacts with the Q\(_o\) domain of cyt \(b\) with a topology similar to that seen with the wild-type \(bc_1\) complex inhibited by stigmatellin (15).

These findings establish clearly that both the amino-terminal and the [2Fe-2S] cluster-containing carboxyl-terminal portions of the Fe-S protein need to be intact for a catalytically active Q\(_o\) site.

In summary, this work established that an inactive \(b-c_1\) subcomplex, containing only the cyt \(b\) and cyt \(c_1\) subunits, can be purified to homogeneity in large scale from chromatophore membranes of a \(R.\) capsulatus mutant lacking the Fe-S protein. The purified \(b-c_1\) subcomplex has properties similar, but not identical, to those of the purified \(bc_1\) complex, and can be reactivated for cyt \(c\) reductase activity upon addition of purified Fe-S protein subunit provided that the transmembrane anchor of the latter subunit is intact. The availability of a facile in vitro reconstitution assay for a simple bacterial \(bc_1\) complex is very promising for dissecting the series of events that take place during UQH\(_2\) oxidation at the Q\(_o\) site of the \(bc_1\) complex.

REFERENCES
