The Role of Extra Fragment at the C-terminal of Cytochrome b (Residues 421–445) in the Cytochrome bc\textsubscript{1} Complex from \textit{Rhodobacter sphaeroides}\textsuperscript{*}

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Sequence alignment of cytochrome b of the cytochrome bc\textsubscript{1} complex from various sources reveals that bacterial cytochrome b contain an extra fragment at the C terminus. To study the role of this fragment in bacterial cytochrome bc\textsubscript{1} complex, \textit{Rhodobacter sphaeroides} mutants expressing His-tagged cytochrome bc\textsubscript{1} complexes with progressive deletion from this fragment (residues 421–445) were generated and characterized. The cytbΔ(433–445) bc\textsubscript{1} complex, in which 13 residues from the C-terminal end of this fragment are deleted, has electron transfer activity, subunit composition, and physical properties similar to those of the complement complex, indicating that this region of the extra fragment is not essential. In contrast, the electron transfer activity, binding of cytochrome b, ISP, and subunit IV to cytochrome c\textsubscript{1}, redox potentials of cytochromes and c\textsubscript{1} in the cytbΔ(427–445), cytbΔ(425–445), and cytbΔ(421–445) mutant complexes, in which 19, 21, or all residues of this fragment are deleted, decrease progressively. EPR spectra of the [2Fe-2S] cluster and the cytochromes b in these three deletion mutant bc\textsubscript{1} complexes are also altered; the extent of spectral alteration increases as this extra fragment is shortened. These results indicate that the first 12 residues (residues 421–432) from the N-terminal end of the C-terminal extra fragment of cytochrome b are essential for maintaining structural integrity of the bc\textsubscript{1} complex.

The cytochrome bc\textsubscript{1} complex is an essential energy transduction electron transfer complex in mitochondria and many aerobic and photosynthetic bacteria (1). The complex catalyzes electron transfer from ubiquinol to cytochrome c\textsubscript{1} with concomitant translocation of protons across the membrane to generate a membrane potential and proton gradient for ATP synthesis. All the cytochrome bc\textsubscript{1} complexes contain three core subunits, cytochrome b, cytochrome c\textsubscript{1}, and Rieske iron-sulfur protein (ISP),\textsuperscript{1} which house two b-type hemes (b\textsubscript{1} and b\textsubscript{1H}), one c-type heme (heme c\textsubscript{1}), and a high potential [2Fe-2S] cluster, respectively. In addition to these three core subunits, the cytochrome bc\textsubscript{1} complex also contains varying numbers (one to eight) of non-redox containing subunits, known as supernumerary subunits (2, 3).

Because the bacterial complexes contain no (or one) supernumerary subunit, it is unlikely that the structures of the core subunits in these complexes are, as suggested for the mitochondrial complex (4), stabilized through interactions between core subunits and their neighboring supernumerary subunits. Perhaps interactions between a part of a core subunit and another part of the same subunit or another core subunit contribute to the stability of a core subunit in the bacterial complex. This speculation finds some support from the fact that core subunits in bacterial complexes are generally bigger than their counterparts in the mitochondrial complex.

Sequence alignment of cytochrome b, cytochrome c\textsubscript{1}, and ISP in bacterial complexes with their counterparts in mitochondrial complexes reveals four extra fragments in bacterial cytochrome b and one each in bacterial cytochrome c\textsubscript{1} and ISP (5). These extra fragments are modeled into the structure of the \textit{Rhodobacter sphaeroides} bc\textsubscript{1} complex by using coordinates of mitochondrial supernumerary subunits (5). These findings encouraged us to suggest that these extra fragments may possess mitochondrial supernumerary subunit function in stabilizing the structure of the core subunits in the bacterial complex. This suggestion is further supported by the recent finding that ISP is lost from the \textit{R. sphaeroides} bc\textsubscript{1} complex if the extra fragment of ISP is deleted or substituted with alanine (6). Of course, confirmation of this suggestion will have to wait until the function of extra fragments in cytochrome b and c\textsubscript{1} is established.

Cytochrome b holds a central role in the cytochrome bc\textsubscript{1} complex because it houses two ubiquinone binding sites, Q\textsubscript{L} and Q\textsubscript{b}, and two redox centers, heme b\textsubscript{L} and heme b\textsubscript{H}. According to the Q-cycle mechanism (7), electrons from ubiquinol are bifurcated at the Q\textsubscript{b} site. The first electron of ubiquinol is transferred through the so-called “high potential” chain consisting of [2Fe-2S] and heme c\textsubscript{1}. The second electron of ubiquinol is passed through the “low potential” chain consisting of hemes b\textsubscript{L} and b\textsubscript{H}. Thus, maintaining the structural stability of cytochrome b in the bc\textsubscript{1} complex is crucial for the electron and proton transfer functions of this complex.

In the structure model of \textit{R. sphaeroides} cytochrome bc\textsubscript{1} complex (5), the four extra fragments of cytochrome b are located at the N terminus (residues 2 to 12), the connecting loop between helices D and E (residues 232 to 239), the connecting loop between helices E and F (residues 309 to 326), and the C terminus (residues 421–445) (see Fig. 1). We started functional studies of these cytochrome b extra fragments with the one at the C terminus because this extra fragment is in close proximity to subunit IV and ISP. Herein we report generation of

\textsuperscript{1} The abbreviations used are: ISP, Rieske iron-sulfur protein; cyt., cytochrome; Q\textsubscript{L}, Br\textsubscript{H\textsubscript{2}}; 2,3-dimethoxy-5-methyl-6-[10-hydroxymethyl]-1,4-benzoquinol; [2Fe-2S], Rieske iron-sulfur cluster; Ni-NTA, nickel-nitrilotriacetic acid; DM, dodecyl-β-D-maltoside; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

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Bacterial Cytochrome bc₃ Complex

**Materials**—Cytochrome c (horse heart, Type III) was from Sigma. N-Dodecyl-β-D-maltoside and N-dodecyl-β-D-glucoside were from Anatrace. Ni-NTA gel and Qiaprep Spin Miniprep kit were from Qiagen.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cytochrome c (horse heart, Type III) was from Sigma. N-Dodecyl-β-D-maltoside and N-dodecyl-β-D-glucoside were from Anatrace. Ni-NTA gel and Qiaprep Spin Miniprep kit were from Qiagen. Pyocyanine was purchased from Color Your Enzyme, Canada. 2,3-Dimethoxy-5-methyl-6-(10-bromodecyl)-1,4-benzoquinol (Q₀C₁₀BrH₂) was prepared in our laboratory as previously reported (8). All other chemicals were of the highest purity commercially available.

**Growth of Bacteria**—Escherichia coli cells were grown at 37 °C in LB medium. Photosynthetic growth conditions for Rhodobacter sphaeroides were as previously reported (10). DNA sequencing and primer synthesis were performed by the Recombinant DNA/Protein Core Facility at Oklahoma State University.

**Enzyme Preparations and Activity Assay**—Chromatophores were prepared from frozen cell paste, and cytochrome bc₃ complexes with a His tag placed at the C terminus of cytochrome c were purified from chromatophores as previously reported (11). To assay the cytochrome bc₃ complex activity, chromatophores or purified cytochrome bc₃ complexes were diluted with 50 mM Tris-Cl, pH 8.0, containing 200 mM NaCl and 0.01% N-dodecyl-β-D-maltoside (DM) to a final concentration cytochrome c of 3 μM, using dodecylmaltoside treatment. Appropriate amounts of the diluted samples were added to 1 ml of assay mixture containing 100 mM Na⁺/K⁺ phosphate buffer, pH 7.4, 1 mM EDTA, 100 μM cytochrome c, and 25 μM Q₀C₁₀BrH₂. Activity was determined by measuring the reduction of cytochrome c (the increase of absorbance at 550 nm) in a Shimadzu UV 2101 PC spectrophotometer at 25 °C, using a millimolar extinction coefficient of 18.5 for calculation. The nonenzymatic oxidation of Q₀C₁₀BrH₂, determined under the same conditions, in the absence of enzyme, was subtracted. Although the chemical properties of Q₀C₁₀BrH₂ are comparable with those of Q₀C₁₀H₂, it is a better substrate for the cytochrome bc₃ complex (8). Specific activity is defined in the legend to Fig. 3.

**Potentiometric Titrations of the Cytochromes b and c₁ in Complement and Mutant Cytochrome bc₃ Complexes**—Redox titrations of cytochromes b and c₁ in complement and mutant bc₃ complexes were essentially as according to the previously published method (12, 13). 3-ml aliquots of the bc₃ complex (2 μM cytochrome b or 2 μM cytochrome c₁) in 0.1 M Na⁺/K⁺ phosphate buffer, pH 7.0, containing 20 μM phenazine methosulfate (midpoint redox potential (Eₘ') = +80 mV), 20 μM phenazine ethosulfate (Eₘ' = +55 mV), 20 μM phenazine ethosulfate (Eₘ' = +120 mV), 20 μM pyocyanine (Eₘ' = −34 mV), 25 μM 1,4-benzoquinone (Eₘ' = +292 mV), 25 μM 1,2-naphthoquinone (Eₘ' = +143 mV), 25 μM 1,4-naphthoquinone (Eₘ' = +36 mV), 50 μM duroquinone (Eₘ' = +5 mV), 70 μM 2,3,5,6-tetramethyl-p-phenylenediamine (Eₘ' = +260 mV), and 15 μM 2-hydroxy-1,4-naphthoquinone (Eₘ' = −145 mV), were used. Reductive titrations were carried out by addition of sodium dithionite solution to the ferricyanide-oxidized sample; oxidative titrations were carried out by addition of ferricyanide solution to the dithionite-reduced sample. At indicated Eₛ values, the optical density at 562 nm, minus that at 575 nm, is used for cytochrome b and that at 552 minus 540 nm for cytochrome c₁. The midpoint potentials of cytochrome b₃ and b₄ were calculated by fitting the redox titration data, obtained for cytochrome b, the Nernst equation for a one-electron carrier (n = 1) with two components using Kaleidagraph, and that of cytochrome c₁ was fitted for a one-electron carrier with one component.

**Other Biochemical and Biophysical Techniques**—The contents of cytochrome b (14) and cytochrome c₁ (15) were determined according to published methods. SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (16) using a Bio-Rad Mini-Protean® 3 cell. Western blotting used rabbit polyclonal antibodies raised against R. sphaeroides ISP and subunit IV. The polypeptides separated in the SDS-PAGE gel were transferred to a 0.22-μm nitrocellulose membrane for immunoblotting. Protein A conjugated to horseradish peroxidase was used as the secondary antibody. EPR spectra were recorded with a Bruker EMX spectrometer, equipped with a liquid helium flow cryostat, at 7 K. Instrument settings are detailed in the legends of relevant figures.

The mass spectrometry determination of molecular weights of cytochrome b in the complement and mutant bc₃ complexes was performed with an Applied Biosystems DE-PRO MALDI-TOF mass spectrometer operated in delayed-extraction positive-ion linear mode according to the method of Ghaem et al. (17) with modifications. Samples (∼30 μl) were loaded with 10 volumes of 10% trichloroacetic acid, chilled for 5 min on ice, centrifuged, and the precipitate was rinsed briefly with 95% ethanol to remove salts. The resulting pellet was dissolved in 30 μl of 99% formic acid and a 1:1 dilution with 99% formic acid to a final volume of 60 μl, which gave the best crystals and signal. As matrix for MALDI-TOF analyses, 1% of 2,5-dihydroxybenzoic acid in 30% acetonitrile, 0.1% trifluoroacetic acid and 1% 5-methoxysalicilic acid in 30% acetonitrile, 0.1% trifluoroacetic acid were mixed 9:1 (v/v). This mixture solution was then used to screen for recombinant plasmids containing the mutant cytochrome b gene.

The pRK418/fbcFB_C₆₋₁₇_O plasmid in E. coli S17-1 cells was mobilized into R. sphaeroides BC-17 cells by a plate-mating procedure (10). The presence of engineered mutations was confirmed twice by DNA sequencing of the 962-base pair BatEII-XbaI fragment before and after photosynthetic growth as previously reported (10). DNA sequencing and primer synthesis were performed by the Recombinant DNA/Protein Core Facility at Oklahoma State University.

**Data Availability**—This article contains Supplementary Material available at http://www.jbc.org/

**Fig. 1**. Location of the C-terminal extra fragment of cytochrome b in the proposed structural model of R. sphaeroides cytochrome bc₃ complex. One monomer (left) is displayed in solid ribbons, and the symmetric monomer (right) is displayed in three-treadline ribbons. Cytochrome c₁ is rust; ISP is brown; subunit IV is silvery; cytochrome b is green; and the C-terminal extra fragment is black.
RESULTS AND DISCUSSION

Comparison of Electron Transfer Activity, Subunit Composition, and Detergent Lability of Cytochrome bc₁ Complexes in Chromatophore Membranes from Complement and C-terminal Truncated Cytochrome b Mutants—The C-terminal extra fragment of R. sphaeroides cytochrome b corresponds to residues 421–445 with a sequence of PATIEEDFNAHYSPATGGTKTVVAE (see Fig. 2). To probe the role of this fragment, PATIEEDFNAHYSPATGGTKTVVAE...

Fig. 2. Partial sequence comparison in the C-terminal extra fragment of various cytochrome b. The abbreviations used are: Rs, R. sphaeroides; Rc, R. capsulatus; Pd, P. denitrificans; Bf, beef; Ye, yeast; Ck, chicken.

Fig. 3. Effect of DM concentration on bc₁ activity and solubilization of cytochrome b from complement and mutant chromatophores. 1-ml aliquots of chromatophore preparations from complement (○), and mutants cyt bhΔ-(433–445) (●), cyt bhΔ-(427–445) (×), cyt bhΔ-(425–445) (□), and cyt bhΔ-(421–445) (■), containing 50 μg of cytochrome b, were added at the indicated amounts of DM. After incubating at 0 °C for 1 h, appropriate aliquots were withdrawn from each sample and assayed for ubiquinol-cytochrome c reductase activity using a 3-ml assay mixture (A). The rest of samples were subjected to centrifugation at 100,000 × g for 90 min. The cytochrome b content in supernatant fractions was determined (B). The 100% cytochrome b content refers to that in the DM-treated sample before centrifugation. The unit of specific activity is μmol of cytochrome c reduced/min/nmol of cytochrome b.
Bacterial Cytochrome bc₁ Complex

Characterization of mutants having deletions on the C-terminal extra fragment of cytochrome b

TABLE I
Characterization of mutants having deletions on the C-terminal extra fragment of cytochrome b

| Strains | Ps³ | Chromatophore | Purified complex |
|---------|-----|---------------|-----------------|
|         |     | Specific activity | Cyt b/cyt c₁ + c₂ | Subunit composition | Specific activity | Cyt b/cyt c₁ | Subunit composition |
| Complement | +++⁺⁺⁺⁺ | 2.24 | 1.26 | FBCQ² | 3.20 | 1.51 | FBCQ |
| cytβΔ-(433–45) | +++⁺⁺⁺⁺⁺⁺⁺ | 2.23 | 1.30 | FBCQ | 2.91 | 1.45 | FBCQ |
| cytβΔ-(427–45) | +++⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺ | 1.87 | 1.35 | FBCQ | 2.88 | 0.81 | FBCQ |
| cytβΔ-(425–45) | +++⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺ | 1.52 | 1.35 | FBCQ | 1.69 | 0.72 | FBCQ |
| cytβΔ-(421–45) | +++⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺⁺ | 1.32 | 1.23 | FBCQ | 0 | 0.29 | FB |

Whereas the complement chromatophores treated with 0.35 mg of DM/nmol of cytochrome b show maximum bc₁ activity, mutant chromatophores of cytβΔ-(433–445), cytβΔ-(427–445), cytβΔ-(425–445), and cytβΔ-(421–445) treated with the same concentration of DM lost, respectively, 0, 22, 35, and 81% of their maximum bc₁ activity, indicating that the lability of the bc₁ complex toward detergent treatment increases as the size of the C-terminal extra fragment decreases. The increase in lability of the bc₁ complex to detergent treatment should be indicative of a decrease in structural stability of the complex. Thus, the structural stability of the bc₁ complex decreases as the C-terminal extra fragment of cytochrome b shortens.

It should be noted that the effectiveness of DM in solubilizing the bc₁ complex from these deletion mutant chromatophores is comparable with that for the complement chromatophores (Fig. 3B). When complement and mutant chromatophores of cytβΔ-(433–445), cytβΔ-(427–445), cytβΔ-(425–445), and cytβΔ-(421–445) treated with various concentrations of DM were centrifuged at 100,000 × g for 90 min, the contents of cytochrome b, determined spectrophotometrically (see Fig. 3B), ISP, and subunit IV determined by Western blotting (data not shown), in the supernatant fractions, at a given DM concentration, are about the same. This suggests that the loss of bc₁ activity in the
cyt\(b\)-(421–445) mutant chromatophores treated with 0.35 mg of DM/nmol of cytochrome \(b\) is not because of a decrease of binding affinity of the \(bc_1\) complex to the membrane, but to a lesser structural integrity of the mutant complex.

Effect of the C-terminal Extra Fragment of Cytochrome \(b\) on the Binding of Cytochrome \(b\), ISP, and Subunit IV to Cytochrome \(c_1\).—To further confirm that the structural integrity of the \(bc_1\) complex decreases as the C-terminal extra fragment of cytochrome \(b\) decreases, binding affinities of protein subunits in mutant complexes of cyt\(b\)-(433–445), cyt\(b\)-(427–445), cyt\(b\)-(425–445), and cyt\(b\)-(421–445) were determined and compared with those of the complement complex. Because the expressed \(R.\) \(sphaeroides\) \(bc_1\) complex has a His\(_4\) tag at the C-terminus of cytochrome \(c_1\), one can determine binding affinities of cytochrome \(b\), ISP, and subunit IV to cytochrome \(c_1\) in complement and mutant complexes using a Ni-NTA column.

When various chromatophores were treated with 1.2\% DM and centrifuged at 200,000 \(\times\) \(g\) for 90 min, about 80\% of cytochrome \(b\), cytochrome \(c_1\), ISP, and subunit IV in the complement and mutant chromatophores was recovered in the supernatant fractions. When these supernatant fractions were applied to Ni-NTA columns, most of cytochrome \(c_1\) in all of these supernatant fractions were absorbed on the Ni-NTA gel, and were recovered in column eluates using an eluting buffer containing 200 mM histidine. This is as expected because the His\(_4\) tag is placed at the C-terminus of cytochrome \(c_1\). The \(b/c_1\) ratios in column eluates of the complement and mutants cyt\(b\)-(433–445), cyt\(b\)-(427–445), cyt\(b\)-(425–445), and cyt\(b\)-(421–445) are 1.51, 1.45, 0.81, 0.72, and 0.29, respectively (see Table I), indicating that the binding affinity of cytochrome \(b\) to cytochrome \(c_1\) decreases as the C-terminal extra fragment of cytochrome \(b\) shortens. The amount of mutant cytochrome \(b\) of cyt\(b\)-(433–445), cyt\(b\)-(427–445), cyt\(b\)-(425–445), and cyt\(b\)-(421–445) associated with cytochrome \(c_1\), as compared with the amount of wild-type cytochrome \(b\) associated with cytochrome \(c_1\), decreases by 4, 46, 52, and 80\%, respectively.

Fig. 4 shows Western blot analysis of ISP and subunit IV recovered in the Ni-NTA column eluate (purified \(bc_1\) complex) and effluent fractions from DM-solubilized complement and mutant chromatophores. The purified cyt\(b\)-(421–445) mutant complex contains no detectable ISP or subunit IV, however, purified mutant complexes of cyt\(b\)-(425–445), cyt\(b\)-(427–445), and cyt\(b\)-(433–445) have 50, 80, and 100\%, respectively, of the amount of ISP or subunit IV found in the complement complex (see Fig. 4A). These results are consistent with the presence of increasing amounts of ISP and subunit IV in the Ni-NTA column eluents of cyt\(b\)-(433–445), cyt\(b\)-(427–445), cyt\(b\)-(425–445), and cyt\(b\)-(421–445) (see Fig. 4B). It should be noted that subunit IV of the \(bc_1\) complex produced by \(R.\) \(sphaeroides\) BC-17 cells carrying the pKD418/\(fbc\)BCQ plasmid includes chromosomal and plasmid copies, whereas cytochrome \(b\), \(c_1\), and ISP have only the plasmid copy. These results indicate that the binding affinities of ISP and subunit IV to the complex are affected by the first 12 residues (residues 421–432) at the N-terminal end of the C-terminal extra fragment of cytochrome \(b\); binding affinity decreases as the fragment size decreases.

The finding that the binding of ISP and subunit IV to cytochrome \(c_1\) is affected by the C-terminal extra fragment of cytochrome \(b\) is rather surprising, because they are not in the same subunit as cytochrome \(b\). Perhaps interactions between the C-terminal extra fragment of cytochrome \(b\) and the N-terminal portion of ISP or subunit IV, located on the cytoplasmic side of the chromatophore membrane, are required for maintaining the structures of ISP and subunit IV for binding to cytochrome \(c_1\). Thus, the mutations on the C-terminal extra fragment of cytochrome \(b\) induce conformational changes on ISP and subunit IV that weaken their binding affinities for cytochrome \(c_1\). Alternatively, the binding of ISP or subunit IV to cytochrome \(c_1\) is through cytochrome \(b\) and mutation of the extra fragment not only decreases the binding affinity of cytochrome \(b\) to cytochrome \(c_1\), but also to ISP and subunit IV.

The Abnormality of Electrophoretic Mobilities of C-terminal Truncated Cytochrome \(b\) Mutants—Fig. 5 shows electrophoretic mobility of wild-type and C-terminal-truncated cytochrome \(b\) in separation gels containing two concentrations of acrylamide (T) and bisacrylamide (C). In a separating gel having T = 12.5\% and C = 3\%, a system used routinely for SDS-PAGE analysis of the \(R.\) \(sphaeroides\) \(bc_1\) complex, wild-type and mutant \(bc_1\) complexes of cytochrome \(b\) of cyt\(b\)-(433–445), cyt\(b\)-(427–445), cyt\(b\)-(425–445), and cyt\(b\)-(421–445) are 1.51, 1.45, 0.81, 0.72, and 0.29, respectively (see Table I), indicating that the binding affinity of cytochrome \(b\) to cytochrome \(c_1\) decreases as the C-terminal extra fragment of cytochrome \(b\) shortens. The amount of mutant cytochrome \(b\) of cyt\(b\)-(433–445), cyt\(b\)-(427–445), cyt\(b\)-(425–445), and cyt\(b\)-(421–445) associated with cytochrome \(c_1\), as compared with the amount of wild-type cytochrome \(b\) associated with cytochrome \(c_1\), decreases by 4, 46, 52, and 80\%, respectively.
cyt\(b\text{-H}_9004\)-(427–445), cyt\(b\text{-H}_9004\)-(425–445), and cyt\(b\text{-H}_9004\)-(421–445) have \(R_f\) values of 16.2, 17.5, 18.5, 19, and 19 mm, respectively (see Fig. 5 \(A\)). As expected, the \(R_f\) values of cytochrome \(c_1\), ISP, and subunit IV in mutant \(bc_1\) complexes are the same as those in the wild-type complex, because shortening the C-terminal extra fragment of cytochrome \(b\) should not affect the molecular masses of other subunits in the complex. The \(R_f\) values of cytochrome \(b\) obtained in these mutant complexes are larger than the calculated values, as the molecular mass of cytochrome \(b\) is decreased by 1200, 1947, 2206, 2588, respectively, from that of wild-type cytochrome \(b\). It should be noted that the larger than calculated \(R_f\) values observed are not because of reduction of the molecular mass of mutant \(b\) proteins by proteolytic enzyme digestion, because the molecular mass of cytochrome \(b\) in the cyt\(b\Delta\text{-H}_9004\)-(421–445) mutant complex, determined by MALDI-TOF mass analysis, is 47,264, which corresponds to the calculated value.

To further confirm that C-terminal-truncated cytochrome \(b\) have abnormal electrophoretic mobility in SDS-PAGE, purified wild-type and mutant cytochrome \(bc_1\) complexes were subjected to SDS-PAGE using a separating gel having T = 16% and C = 4% (see Fig. 5\(B\)). The electrophoretic mobility of

\[\text{Eh(mV)}\]

\[\begin{align*}
\text{complement} & : -87 \, \text{mV} \\
\text{cyt}b\Delta\text{-H}_9004\text{(433–445)} & : -73 \, \text{mV} \\
\text{cyt}b\Delta\text{-H}_9004\text{(427–445)} & : -115 \, \text{mV} \\
\text{cyt}b\Delta\text{-H}_9004\text{(425–445)} & : -145 \, \text{mV} \\
\text{cyt}b\Delta\text{-H}_9004\text{(421–445)} & : -160 \, \text{mV} \\
\end{align*}\]

\[\text{Reduction(%) } \begin{align*}
\text{complement} & : 41 \, \text{mV} \\
\text{cyt}b\Delta\text{-H}_9004\text{(433–445)} & : 49 \, \text{mV} \\
\text{cyt}b\Delta\text{-H}_9004\text{(427–445)} & : 25 \, \text{mV} \\
\text{cyt}b\Delta\text{-H}_9004\text{(425–445)} & : -41 \, \text{mV} \\
\text{cyt}b\Delta\text{-H}_9004\text{(421–445)} & : 70 \, \text{mV} \\
\end{align*}\]
wild-type *R. sphaeroides* cytochrome *b*, relative to cytochrome *c*<sub>1</sub>, is decreased when the concentrations of *T* and *C* in a separation gel is increased. This increased distance between wild-type cytochrome *b* and *c*<sub>1</sub> enables us to obtain the *R<sub>c</sub>* values for the cytochrome *b* mutants, relative to cytochrome *c*<sub>1</sub>. The values are: 0.70, 0.76, 0.81, 0.84, and 0.86, respectively. These values are larger than those calculated. Thus, cytochrome *b* with decreasing lengths of the C-terminal extra fragment exhibits abnormal electrophoretic mobility in SDS-PAGE. Perhaps removal of the C-terminal extra fragment in cytochrome *b* makes cytochrome *b* protein assume a molecular shape more globular than the wild-type protein, in the presence of SDS, which moves faster in SDS-PAGE than expected.

**Effect of Mutations on the Rieske Iron-Sulfur Cluster**—Western blotting and SDS-PAGE analysis indicate that the amount of ISP in the bc<sub>1</sub> complex decreases as the C-terminal extra fragment of cytochrome *b* decreases; no ISP is detected in the complex with cytochrome *b* lacking this extra fragment. To see whether or not the mutations on the C-terminal extra fragment of cytochrome *b* also affect the microenvironments of the iron-sulfur cluster, EPR spectra of the [2Fe-2S] cluster in complement and mutant bc<sub>1</sub> complexes were determined and compared (see Fig. 6).

When complement and mutant complexes of cyt<sub>b</sub>Δ-(433–445), cyt<sub>b</sub>Δ-(427–445), cyt<sub>b</sub>Δ-(425–445), and cyt<sub>b</sub>Δ-(421–445), at a cytochrome *b* concentration of 230 μM, were reduced by a small excess of ascorbate, the EPR signal of the Rieske iron-sulfur cluster in the complement complex is essentially the same as that previously reported for the wild-type *R. sphaeroides* bc<sub>1</sub> complex (18, 19), with resonance at *g<sub>x</sub>* = 1.80, *g<sub>y</sub>* = 1.90, and *g<sub>z</sub>* = 2.02 (see Fig. 6A). The signatures of *g<sub>x</sub>* and *g<sub>y</sub>* of [2Fe-2S] cluster in the cyt<sub>b</sub>Δ-(433–445), cyt<sub>b</sub>Δ-(427–445), and cyt<sub>b</sub>Δ-(425–445) mutant complexes are the same as those detected in the complement complex, but with signal amplitudes decreasing as the C-terminal extra fragment of cytochrome *b* shortens (see Fig. 6A). No EPR spectrum of the [2Fe-2S] cluster is detected in the cyt<sub>b</sub>Δ-(421–445) mutant complex. These results are consistent with Western blot results showing that the amount of ISP in the bc<sub>1</sub> complex decreases as the C-terminal extra fragment of cytochrome *b* decreases and no ISP is detected in the complex that has cytochrome *b* lacking the entire C-terminal extra fragment.

In contrast to *g<sub>x</sub>* and *g<sub>y</sub>*, the *g<sub>z</sub>* signal of [2Fe-2S] in mutant complexes of cyt<sub>b</sub>Δ-(433–445), cyt<sub>b</sub>Δ-(427–445), and cyt<sub>b</sub>Δ-(425–445) mutant complexes changes progressively from a relatively sharp peak with *g<sub>z</sub>* = 1.80 to a broadened peak with *g<sub>z</sub>* = 1.76 (see Fig. 6B). Whereas the *g<sub>z</sub>* signal from the cyt<sub>b</sub>Δ-(433–445) mutant complex is quite sharp with *g<sub>z</sub>* = 1.80 peak, that from the cyt<sub>b</sub>Δ-(425–445) mutant complex is broader with *g<sub>z</sub>* = 1.76 peak. These results indicate that the alteration of the microenvironments of the [2Fe-2S] cluster increases as the C-terminal extra fragment of cytochrome *b* decreases. This finding is somewhat surprising, because in the model structure of *R. sphaeroides* cytochrome *b*<sub>1</sub> complex (5), constructed by using the coordinates of subunits from beef heart mitochondrial bc<sub>1</sub> complex, the Rieske iron-sulfur cluster is located at the head domain of ISP on the periplasmic side of the chromatophore membrane (positive side), whereas the C-terminal extra fragment of cytochrome *b* is located at the cytoplasmic side of this chromatophore membrane. Thus, the effect of shortening the C-terminal extra fragment of cytochrome *b* on the EPR signature of the Rieske *2Fe-2S* center would appear to be long range.

The line shape of the *g<sub>x</sub>* signature of [2Fe-2S] clusters is thought to be sensitive to the redox state of ubiquinone present in the *Q<sub>a</sub>* center (18–23). The *g<sub>x</sub>* of bc<sub>1</sub> from wild-type *R. sphaeroides* is at *g<sub>x</sub>* = 1.80 when oxidized ubiquinone is present but shifts to 1.76 and becomes much broader when ubiquinol is present. In a study of the effect of extraction of ubiquinone, from chromatophore membranes, on the iron-sulfur cluster, Ding et al. (23) found that the *g<sub>x</sub>* signal became very broad and was located at ~1.765 upon deletion of ubiquinone from the *R. capsulatus* chromatophore membrane. Although the broadened, *g<sub>x</sub>* = 1.76 resonance observed in the cyt<sub>b</sub>Δ-(427–445) and cyt<sub>b</sub>Δ-(425–445) mutant complexes resembles the “reduced state” or the “depleted state” spectrum, it is not because of changes in the redox state of Q or a decrease in Q in the mutant complex, because no EPR spectrum of [2Fe-2S] cluster is detected in these two mutant complexes without treatment with ascorbate and the amount of Q in these two mutant complexes is the same as that in the complement complex. It should also be noted that upon complete reduction by addition of dithionite, the broadened, *g<sub>x</sub>* = 1.76 signal observed in the mutant complexes remains unchanged, whereas the *g<sub>x</sub>* = 1.80 signals becomes broadened and shifts to a *g<sub>x</sub>* = 1.76 signal.
similar to the changes observed in the complement complex (18, 19).

The broadened, \( g_z = 1.76 \) EPR signal observed in the cyt\( \Delta (427-445) \) and cyt\( \Delta (425-445) \) mutant complexes is similar to the \( g_z \) signal observed for the substitution of Leu for Phe-144 (F144L) in the cytochrome \( b \) from \( R. \) capsulatus (22) and of serine for Thr-160 in cytochrome \( b \) from \( R. \) sphaeroides (10).

The F144L bc1 complex in \( R. \) capsulatus and the T160S mutant complex in \( R. \) sphaeroides chromatophores were reported to have a decreased turnover rate with a broadened, redox state-insensitive \( g_z \) value at 1.765. It was suggested that these properties of the F144L and T160S complexes resulted from a reduced affinity for quinone and quinol at the Qo site of the mutated complex. One possibility, which could account for the decreased turnover rate of the cyt\( \Delta (427-445) \) and cyt\( \Delta (425-445) \) mutant complexes and their reduced state or the high field shift of the \( g_z \) EPR signal, is that shortening the C-terminal extra fragment to less than 6 residues induces conformational changes at the Qo site, which raise the effective redox potential of bound ubiquinol beyond the optimal range for transfer to the \([2Fe-2S]\) cluster.

Effect of the Mutations on Redox potentials and EPR Characteristics of Cytochrome b in the bc1 Complex—

**Fig. 9.** Potentiometric titration of cytochrome c₁ in purified complement and mutant bc₁ complexes. The experimental conditions were as described under “Experimental Procedures” and in the legend to Fig. 7. The filled circles are reductive titration data and open circles are oxidative titration data. The solid lines represent the calculated \( n = 1 \) redox titrations with the indicated midpoint redox potentials.
complement complex are −87 and 41 mV, respectively, similar to previously reported values (19). The redox potentials of bL and bH in the cyt bΔ(433–445) mutant complex are −73 and 49 mV, respectively, indicating that deleting 13 residues has little effect on redox potentials of cytochrome b. In contrast, the redox potential of bL in mutant complexes of cyt bΔ(427–445), cyt bΔ(425–445), and cyt bΔ(421–445) decreases by 28, 58, and 73 mV, respectively, and that of bH decreases by 16, 82, and 111 mV, respectively, compared with counterparts in the complement complex. Thus residues 421–432 in the C-terminal extra fragment are essential for maintaining redox potentials of cytochromes. The effect is larger in the fragment are essential for maintaining redox potentials of the cyt c1, o rI S P cytochrome R. sphaeroides minus of cytochrome R. sphaeroides to previously reported values (19). The redox potentials of cytochrome R. sphaeroides bc1 complex, the C-terminal extra fragment, and mutant complexes, taken after the samples each trial of reductive and oxidative titrations.

It should be noted again that the C-terminal extra fragment of cytochrome b is located on the cytoplasmic side of the chromatophore membrane, yet its deletion affects redox components, such as heme bH, iron-sulfur cluster, and heme cL, located on the periplasmic (opposite) side of the chromatophore membrane. Therefore, these effects cannot be explained by direct interaction between the extra fragment of cytochrome b and the redox components or their ligands or their vicinity peptides. These long range effects are probably because of globular changes in the deleted mutant complex. That deleted cytochrome b proteins have electrophoretic mobilities greater than those of comparable molecular mass, in SDS-PAGE, suggests that the deleted cytochrome b proteins are more globular than the unaltered protein.

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The Role of Extra Fragment at the C-terminal of Cytochrome b (Residues 421–445) in the Cytochrome bc1 Complex from *Rhodobacter sphaeroides*

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