Confirmation of the Involvement of Protein Domain Movement during the Catalytic Cycle of the Cytochrome bc$_1$ Complex by the Formation of an Intersubunit Disulfide Bond between Cytochrome b and the Iron-Sulfur Protein

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To study the essentiality of head domain movement of the Rieske iron-sulfur protein (ISP) during bc$_1$ catalysis, *Rhodobacter sphaeroides* mutants expressing His-tagged cytochrome bc$_1$ complexes with three pairs of cysteines engineered (one cysteine each) on the interface between cytochrome b and ISP, A185C(cytb)/K70C(ISP), I326C(cytb)/G165C(ISP), and T386C(cytb)/K164C(ISP), were generated and characterized. Formation of an intersubunit disulfide bond between cytochrome b and ISP is detected in membrane (intracytoplasmic membrane and air-aged chromatophore), and purified bc$_1$ complex was prepared from the A185C(cytb)/K70C(ISP) mutant cells. Formation of the intersubunit disulfide bond in this cysteine pair mutant complex is concurrent with the loss of its bc$_1$ activity. Reduction of this disulfide bond by β-mercaptoethanol restores activity, indicating that mobility of the head domain of ISP is functionally important in the cytochrome bc$_1$ complex. The rate of intramolecular electron transfer, between 2Fe2S and heme c$_1$, in the A185C(cytb)/K70C(ISP) mutant complex is much lower than that in the wild type or in their respective single cysteine mutant complexes, indicating that formation of an intersubunit disulfide bond between cytochrome b and ISP arrests the head domain of ISP in the “fixed state” position, which is too far for electron transfer to heme c$_1$.

The cytochrome bc$_1$ complex (also known as ubiquinol-cytochrome c reductase or complex III) is an essential segment of the energy-conserving electron transfer chains of mitochondria and many respiratory and photosynthetic bacteria (1). This complex catalyzes electron transfer from ubiquinol to cytochrome c and concomitantly translocates protons across the membrane to generate a membrane potential and pH gradient for ATP synthesis. Recently the cytochrome bc$_1$ complexes from bovine (2, 3) and chicken (4) heart mitochondria, which contain 11 nonidentical protein subunits, were crystallized, and their structures were solved at 2.9 Å resolution. The structural information obtained not only answered a number of questions concerning the arrangement of the redox centers, transmembrane helices, and inhibitor binding sites but also suggested movement of the head domain of the iron-sulfur protein (ISP)$^1$ during bc$_1$ catalysis. This suggests arose from observation of a particularly low electron density area in the intermembrane space portion of the complex, where the extramembrane domains of ISP and cytochrome c$_1$ reside (2). This movement hypothesis is further supported by observation of various positions for 2Fe2S in the different crystal forms (3, 4) and in complexes loaded with different inhibitors (4, 5).

In tetragonal I4$_2$2$_2$ crystals of native oxidized bovine cytochrome bc$_1$ complex, the position of the 2Fe2S cluster is 27 Å from heme b$_2$, and 31 Å from heme c$_1$ (the “fixed state” position) (2, 5). Binding of stigmatellin or 5-n-undecyl-6-hydroxy-4,7-dioxobenzothiazole enhances the electron density of the anomalous scattering peak of 2Fe2S, suggesting that these inhibitors arrest the mobility of ISP in the fixed state position (5). Conversely, binding of (E)-β-methoxyacrylate-stilbene or myxothiazol to the complex abolishes the electron density of the anomalous scattering peak of 2Fe2S, suggesting that these inhibitors increase the mobility of ISP in the crystal and that 2Fe2S has no predominant position (referred to as the “released” or “loose” position) in this inhibited state (5). In orthorhombic crystals (P2$_1$2$_1$2$_1$) of the chicken enzyme, binding of stigmatellin shifts 2Fe2S from the so-called “distal or c$_1$ position” to the “proximal or b position” (4). The b position in the P2$_1$2$_1$2$_1$ crystal is believed to be the same as the fixed state position observed in I4$_2$2$_2$ crystals. In bovine P6$_5$ crystals 2Fe2S is located between the b state and c$_1$ state positions (the “intermediate” position) (3). The observation of more than two positions (intermediate and c$_1$ positions) of 2Fe2S in P6$_5$ crystals (3) supports the idea of one fixed position and other released (loose) positions, suggested by the I4$_2$2$_2$ structure.

If movement of the head domain of ISP is required for bc$_1$ catalysis, locking the head domain of ISP in a given position should abolish the bc$_1$ complex activity. One way to lock the 2Fe2S cluster of ISP in a fixed position is to form a disulfide bond (disulfide bridge) between a pair of genetically engineered cysteines on the interface between the head domain of ISP and cytochrome b. However, genetic manipulation of bovine heart mitochondria is not practical. *Rhodobacter sphaeroides* is an ideal system for studying the intersubunit disulfide bond formation by molecular genetics. The four-subunit bacterial complex is functionally analogous to the mitochondrial enzyme; the largest three subunits (cytochrome b, cytochrome c$_1$, and ISP) are homologous to their mitochondrial counterparts, and this system is readily manipulated genetically. In addition, *R. spha-
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erochemicals expressing His$_6$-tagged cytochrome bc$_1$ complex has been prepared (6, 7). This greatly speeds up the isolation of the bc$_1$ complex from wild type or mutant cells.

In fact, the study of the neck region of ISP using this system (6, 8) provided the first functional evidence for movement of the head domain of ISP during bc$_1$ catalysis. The molecule of ISP can be divided into three domains: head, tail, and neck, with the 2Fe2S cluster located at the tip of the head (9, 10). Because the three-dimensional structures of the head and tail domains are rigid and are the same in the fixed and released states, a bending of the neck is required for movement of the head domain. For the neck region to bend, some flexibility is imperative. Mutants with increased neck rigidity, generated by deletion or double- or triple-proline substitution, have greatly reduced electron transfer activity with an increased activation energy (6). Formation of a disulfide bond between two engineered cysteines, having only one amino acid residue between them, in the neck region near the transmembrane helix, also drastically reduces electron transfer activity (8), presumably because of increased neck rigidity. Cleavage of the disulfide bond by reduction or alkalization restores activity to that of the wild type enzyme (8). These results clearly demonstrate a need for neck flexibility in catalysis.

To further establish that movement of the head domain of ISP is essential for the bc$_1$ complex, we generated mutants expressing His$_6$-tagged bc$_1$ complex with pairs of cysteine substituted (one cysteine each) at the interface between cytochrome $b$ and the head domain of ISP. We predicted that formation of an intersubunit disulfide bond between the engineered cysteine pair would arrest the mobility of ISP to the fixed state and decrease electron transfer activity. Herein we report procedures for generating three cysteine pair mutants with one each on cytochrome $b$ and ISP in close proximity (interface) to each other. Mutants with single cysteine substitutions at indicated positions were also generated and characterized to confirm that the generated cysteine pair mutants are not at critical positions in the bc$_1$ complex and, hence, are suitable for this study. The photosynthetic growth behavior, cytochrome bc$_1$ complex activity, SDS-PAGE patterns, and EPR characteristics of the 2Fe2S cluster in purified complexes from wild type and mutant strains were examined and compared as were the rates of pH induced intramolecular electron transfer between 2Fe2S and heme c$_1$.

**EXPERIMENTAL PROCEDURES**

**Materials—**Cytochrome c (horse heart, type III) was purchased from Sigma. N-Dodecyl-$\beta$-D-maltoside and N-octyl-$\beta$-D-glucoside were from Anatrace. 2,3-Dimethoxy-5-methyl-6-geranyl-1,4-benzoquinol (Q$_h$H$_2$) was prepared in our laboratory as previously reported (11). All other chemicals were of the highest purity commercially available.

**Generation of R. sphaeroides Strains Expressing the bc Complexes with Single or Pairs of Cysteine Substitutions on Cytochrome b and ISP—**Mutations were constructed by site-directed mutagenesis using the Altered Sites system from Promega. The oligonucleotides used for mutagenesis were amplified (one cysteine each) at the interface between cytochrome $b$ and the head domain of ISP. We predicted that formation of an intersubunit disulfide bond between the engineered cysteine pair would arrest the mobility of ISP to the fixed state and decrease electron transfer activity. Herein we report procedures for generating three cysteine pair mutants with one each on cytochrome $b$ and ISP in close proximity (interface) to each other. Mutants with single cysteine substitutions at indicated positions were also generated and characterized to confirm that the generated cysteine pair mutants are not at critical positions in the bc$_1$ complex and, hence, are suitable for this study. The photosynthetic growth behavior, cytochrome bc$_1$ complex activity, SDS-PAGE patterns, and EPR characteristics of the 2Fe2S cluster in purified complexes from wild type and mutant strains were examined and compared as were the rates of pH induced intramolecular electron transfer between 2Fe2S and heme c$_1$.

**Using the Qiagen Plasmid Mini Prep kit. Because R. sphaeroides cells contain four types of endogenous plasmids, the isolated plasmids lack the purity and concentration needed for direct sequencing. Therefore, a 2.5-kilobase pair DNA segment containing the mutation sequence was amplified from the isolated plasmids by the polymerase chain reaction and purified with 1% agarose gel electrophoresis. The 2.5-kilobase pair polymerase chain reaction product was recovered from the gel with an QIAquick PCR Purification kit from Qiagen. The plasmid was sequenced for the full length of the gene. The oligonucleotides for the mutagenesis were prepared by the Recombinant DNA/Protein Core Facility at the Oklahoma State University.

**Growth of Bacteria—**E. coli cells were grown at 37 °C in LB medium. For photosynthetic growth of the plasmid-bearing R. sphaeroides BC17 cells, an enriched Sistrom’s medium containing 5 mM glutamate and 1 mM taurine was used (15). Photosynthetic growth conditions for R. sphaeroides were essentially as described previously (6). Cells harboring mutated pbc genes on the pRRK/pBCFbQ plasmid were grown photosynthetically for one or two serial passages to minimize any pressure for reversion. For semi-aerobic growth of R. sphaeroides, an enriched Sistrom’s medium supplemented with 20 amino acids and extra rich vitamins was used. These semi-aerobic cultures were grown in 500 ml of enriched Sistrom’s medium in 2-liter Belco flasks with vigorous shaking (220 rpm) for 24 h at 30 °C. The inoculation volumes used for both photosynthetic and semi-aerobic cultures were at least 5% of the total volume. Antibiotics were added to the following concentrations: ampicillin (125 µg/ml), kanamycin sulfate (30 µg/ml), tetracycline (10 µg/ml for E. coli and 1 µg/ml for R. sphaeroides), and trimethoprim (100 µg/ml for E. coli and 30 µg/ml for R. sphaeroides).

**Enzyme Preparations and Activity Assay—**Chromatophore and intracellular membrane (ICM) were prepared as described previously (6) and stored at −80 °C in the presence of 20% glycerol until use. The His$_6$-tagged cytochrome bc$_1$ complexes were purified from frozen chromatophores by the method of Tian et al. (6). Purified cytochrome bc$_1$ complexes were stored at −80 °C in the presence of 20% glycerol. To assay ubiquinol-cytochrome c reductase activity, chromatophores, ICM, or purified cytochrome bc$_1$ complexes were diluted with 50 mM Tris-Cl, pH 8.0, containing 200 mM NaCl to a final concentration of cytochrome $b$ of 5 µM. Five µl of the diluted samples were added to 1 ml of assay mixture containing 100 mM of Na/K phosphate buffer, pH 7.4, 0.3 mM of EDTA, 100 µM of cytochrome c, and 25 µM of Q$_h$H$_2$. Activities were determined by measuring the reduction of cytochrome c (the increase of the absorbance at wavelength 550 nm) in a Shimadzu UV 2101 PC spectrophotometer at 23 °C, using a millimolar extinction coefficient of 18.5 for calculation. The nonenzymatic oxidation of Q$_h$H$_2$, determined under the same conditions, in the absence of enzyme, was subtracted during specific activity calculations.

**Determination of pH-induced Reduction and Oxidation of ISP and Cytochrome $c_1$ in the Partially Reduced Wild Type and Mutant bc$_1$ Complexes—**The wild type or mutant bc$_1$ complex was diluted in 3 ml of 20 mM Tris-Cl buffer, pH 8.0, containing 200 mM NaCl and 0.01% deoxydiamylolactone. The concentration of cytochrome c$_1$ was adjusted to about 10 µM. Different amounts of NaOH or HCl were added to give the indicated pH levels. Fully oxidized or reduced cytochrome c$_1$ and ISP was obtained by addition of K$_3$Fe(CN)$_6$ or sodium ascorbate. Reduction of cytochrome c$_1$ was followed by measuring the increase of the a$_1$-absorption (553–545 nm) in a Shimadzu UV 2101 PC spectrophotometer. Reduction of ISP was followed by measuring the negative CD peak, at 500 nm, of partially reduced ISP minus fully oxidized complex in a JASCO J-715 spectropolarimeter (13–15). The same samples were used for the absorption and CD measurements. Instrument settings for the spectropolarimeter were: scan speed, 100 nm/min; step resolution, 1 nm; accumulation, 10 traces for averaging; response, 1 s; bandwidth, 1.0 nm; sensitivity, 10 mdeg; and slit width, 500 µm.

**Determination of Reduction Potentials of the 2Fe2S Cluster in Wild Type and Mutant bc$_1$ Complexes—**The redox status of heme c$_1$ or the 2Fe2S cluster was determined as described above. The cytochrome c$_1$ partially reduced wild type and mutant bc$_1$ complexes were prepared and used for the absorption and CD measurements. The redox potentials of ISP were calculated from the redox statuses of heme c$_1$ and 2Fe2S, at pH 8.0, using 230 mV for the redox potential of heme c$_1$ (16).

**Determination of Electron Transfer Rates between the 2Fe2S Cluster and Heme c$_1$ in Wild Type and Mutant bc$_1$ Complexes—**The method used is essentially the same as that previously reported (15). The His$_6$-tagged, cytochrome c$_1$ half-reduced cytochrome bc$_1$ complex was prepared by reduction of the head domain of ISP (6). Purified cytochrome bc$_1$ complexes were stored at −80 °C in the presence of 20% glycerol until use. The His$_6$-tagged, cytochrome c$_1$ half-reduced cytochrome bc$_1$ complex was prepared by reduction of the head domain of ISP (6). Purified cytochrome bc$_1$ complexes were stored at −80 °C in the presence of 20% glycerol until use.
RESULTS AND DISCUSSION

Photosynthetic Growth Behaviors of Mutants Carrying Cysteine Substitutions in the Interface between Cytochrome b and the Head Domain of ISP—Three pairs of amino acid residues: Ala185(cytb)/Lys70(ISP), Ile326(cytb)/Gly165(ISP), and Thr386(cytb)/Lys164(ISP) were selected for mutation to cysteines. These choices were based on the three-dimensional structural model of the four-subunit cytochrome bc1 complex of R. sphaeroides (Fig. 1A) constructed with coordinates from bovine cytochromes b and c1, ISP, and subunit XII (21). The distances between these three cysteine pairs are 6.1, 6.1, and 7.5 Å, respectively, in the bacterial complex (Fig. 1B). They are 6.5, 6.8, and 6.4 Å (Table I), respectively, when calculations are based on corresponding residues in the bovine enzyme. Mutants with a single cysteine substitution, at positions Ala185(cytb), Ile326(cytb), Thr386(cytb), Lys70(ISP), Gly165(ISP), or Lys164(ISP), were also generated and used as controls.

For a cysteine pair mutant to be useful in this study, the engineered cysteine positions must not be critical for cytochrome bc1 complex activity. Because the bc1 complex is absolutely required for the photosynthetic growth of R. sphaeroides, whether or not the engineered cysteine positions are critical to the complex can be determined by assaying photosynthetic growth. Mutants with cysteine substitutions at critical positions in the complex will not grow photosynthetically, whereas mutants with substitutions at noncritical positions will grow.

When mid-log phase, aerobically dark grown wild type and mutant cells were inoculated into enriched Sistrom’s medium and subjected to anaerobic photosynthetic growth conditions, the A185C(cytb), K70C(ISP), A185C(cytb)/K70C(ISP), I326C(cytb), and T386C(cytb) mutants grew at rates comparable with the A185C(cytb)/K70C(ISP) and K164C(ISP)/T386C(cytb)/I326C(ISP) mutants did not grow photosynthetically (Table I).

Because Gly165 of ISP is a critical position, mutant I326C(cytb)/G165C(ISP) does not support photosynthetic growth and cannot be used to study the effect of disulfide bond formation on the bc complex. The structural importance of Gly165 was further investigated by substituting alanine or threonine at this position. The ISP-G165A or G165T substitution also results in cells that do not grow photosynthetically, indicating that the size of the amino acid side chain at position 165 of ISP is critical. A similar size-activity relationship was previously observed for Gly156 of cytochrome b in Rhodobacter capsulatus (22) and Ser155 of cytochrome b in R. sphaeroides (23).

Fig. 1. Location of engineered cysteines and free endogenous cysteines in the structural model of the R. sphaeroides bc1 complex. A, the left monomer of cytochrome b is shown with purple ribbon, ISP (from the other monomer) is in green, and cytochrome c1 is in pink. Subunit IV and the other monomer are shown in gray. The ISP head domain was replaced, in ball and stick format, in the same color as the appropriate subunit. The free endogenous cysteines are shown as the wild type amino acid being replaced, in ball and stick format, in the same color as the appropriate subunit. The distances between the various engineered and endogenous free cysteines. The colors are the same as in A.

On the other hand, mutants A185C(cytb)/K70C(ISP) and T386C(cytb)/K164C(ISP) support photosynthetic growth, indicating that the engineered cysteine positions are noncritical to the complex. Therefore, formation of an intersubunit disulfide bond between cytochrome b and the ISP head domain was examined with these two cysteine pair mutants.

Formation of a Disulfide Bond between Cytochrome b and
ISP in the A185C(cytb)/K70C(ISP) Mutant bc₁ Complex—

Chromatophores freshly prepared from the A185C(cytb), K70C(ISP), A185C(cytb)/K70C(ISP), T386C(cytb), K164C(ISP), and T386C(cytb)/K164C(ISP) mutant cells have, respectively, 100, 57, 57, 39, 43, and 35% of the bc₁ activity found in wild type chromatophores (Table I). When these chromatophore preparations were subjected to Western blot analysis using antibodies against R. sphaeroides cytochrome b and ISP, no protein band corresponding to the adduct of cytochrome b and ISP was observed, indicating that no disulfide bond is formed between the two engineered cysteine in mutants A185C(cytb)/K70C(ISP) and T386C(cytb)/K164C(ISP) during anaerobic photosynthetic growth. The lack of disulfide bond formation is expected because photosynthetic growth is under strict anaerobic conditions, whereas disulfide bond formation is an oxidative process. Without oxygen no disulfide bond can be formed even if the two cysteines are in favorable positions.

When the His₆-tagged bc₁ complexes were purified from these six freshly prepared cysteine mutant chromatophores, all but the A185C(cytb)/K70C(ISP) mutant complex have the same bc₁ activity found in their respective chromatophores (Table I), based on cytochrome b content. The A185C(cytb)/K70C(ISP) mutant complex, when assayed immediately after preparation, has about 23% of the bc₁ activity complex found in its freshly prepared chromatophores. Activity in this cytochrome pair mutant complex decreased during storage at 0 °C. About 7% of the activity remained after 24 h. Under identical conditions, no activity loss was observed for wild type and mutant complexes of A185C(cytb), K70C(ISP), T386C(cytb), K164C(ISP), and T386C(cytb)/K164C(ISP).

To see whether or not the loss of bc₁ complex activity observed for mutant A185C(cytb)/K70C(ISP) results from disulfide bond formation during purification, SDS-PAGE patterns of these purified mutant complexes, with and without β-ME treatment, were examined (Fig. 2). When purified complexes were treated with SDS at 37 °C for 2 h and subjected to electrophoresis in the absence of β-ME (Fig. 2, lanes marked with −), the A185C(cytb), K70C(ISP), T386C(cytb), and K164C(ISP) mutants have the same electrophoretic pattern as that of the wild type complex. They all contain five protein bands with apparent molecular masses of 41, 33, 31, 23, and 14 kDa.

Western blot analysis with antibodies against R. sphaeroides cytochrome b, cytochrome c₁, ISP, and subunit IV identified these five protein bands, with decreasing molecular masses, as band I, cytochrome b; bands II and III, cytochrome c₁; band III, ISP; and band IV, subunit IV. The lack of any protein band with an apparent molecular mass of 64 kDa, the size of an adduct band of cytochrome b and ISP, indicates that no intersubunit disulfide bond is formed between an engineered cysteine in cytochrome b (A185C or T386C) or ISP (K70C or K164C) and an endogenous cysteine in ISP or cytochrome b, respectively.

This lack of intersubunit disulfide bond formation between an engineered cysteine in cytochrome b or ISP and an endogenous cysteine in ISP or cytochrome b is as expected. The R. sphaeroides cytochrome bc₁ complex has nine endogenous cysteine residues: one in cytochrome b, four in cytochrome c₁, and four in ISP. It has been established that two cysteines (Cys₁₂⁹ and Cys₁₄⁹) in the ISP serve as ligands for the 2Fe₂S center. The distances were measured from C-β to C-β (except with glycine, which is to C-α).

\[ \text{Distance (Å)} = \sqrt{(x_{\text{ISP}} - x_{\text{cyt b}})^2 + (y_{\text{ISP}} - y_{\text{cyt b}})^2 + (z_{\text{ISP}} - z_{\text{cyt b}})^2} \]

- \( x \): Corresponding residues in bovine, Cys₁₂⁹ and Cys₁₄⁹ in the ISP.
- \( y \): Location in bovine, Cys₁₂⁹ and Cys₁₄⁹ in the ISP.
- \( z \): Distance between the two engineered cysteines in mutants A185C(cytb)/K70C(ISP), K164C(ISP) and T386C(cytb)/K164C(ISP).

| Mutants | Single mutation | Corresponding residues in bovine | Location | Distance (Å) |
|---------|----------------|---------------------------------|---------|-------------|
| Wild type | | | | |
| A185C(cytb) | | Ser⁴⁶⁹ | cd2 | 6.5 |
| K70C(ISP) | | Lys⁷⁵ | β3 | 6.1 |
| A185C(cytb)/K70C(ISP) | | | | |
| I386C(cytb) | | Ile⁶⁴⁴ | ef loop | 0.8 |
| K164C(ISP) | | Gly¹⁷⁴ | Pro loop | 1.3 |
| I386C(cytb)/K164C(ISP) | | | | |
| T386C(cytb) | | Glu³⁴⁴ | gβ loop | 0.9 |
| K164C(ISP) | | Lys₁⁷³ | Pro loop | 1.0 |
| T386C(cytb)/K164C(ISP) | | | | 0.8 |

The lack of intersubunit disulfide bond formation between an engineered cysteine in cytochrome b or ISP and an endogenous cysteine in ISP or cytochrome b is as expected. The R. sphaeroides cytochrome bc₁ complex has nine endogenous cysteine residues: one in cytochrome b, four in cytochrome c₁, and four in ISP. It has been established that two cysteines (Cys₁₂⁹ and Cys₁₄⁹) in the ISP serve as ligands for the 2Fe₂S center. The distances were measured from C-β to C-β (except with glycine, which is to C-α).
been dissociated with SDS. The cytochrome $b_1$ complex, in its native state, with or without $\beta$-ME treatment, has the same electron transfer activity. Moreover, the distance between Cys$^{169}$ and Cys$^{145}$ of cytochrome c$_1$ in the structural model of this bacterial complex is 23.5 Å (Fig. 1B), too large for disulfide bond formation.

The electrophoretic pattern of the T386C(cytb)/K164C(ISP) mutant complex in the absence of $\beta$-ME (Fig. 2, lane 11) shows a faint band with an apparent molecular mass of 64 kDa that disappears in the presence of $\beta$-ME (Fig. 2, lane 10). The failure of this 64-kDa protein to react with antibodies against cytochrome $b$ and ISP and the lack of $\beta$-ME effect on cytochrome $b_1$ activity of this mutant complex lead us to assign this protein as a contaminant rather than an adduct of ISP and cytochrome $b$. Thus, no intersubunit disulfide bond is formed between cytochrome $b$ and ISP in the T386C(cytb)/K164C(ISP) mutant complex.

The electrophoretic pattern of the A185C(cytb)/K70C(ISP) mutant complex (Fig. 2, lane 5), in the absence of $\beta$-ME, differs from those of wild type and mutant complexes of A185C(cytb), K70C(ISP), T386C(cytb), K164C(ISP), and T386C(cytb)/K164C(ISP) in two aspects: the appearance of a protein band with an apparent molecular mass of 64 kDa and a decrease in the band intensities of cytochrome $b$ and ISP. The 64-kDa protein band is established as an adduct of cytochrome $b$ and ISP, resulting from intersubunit disulfide bond formation between these two subunits, by the following experimental results. First, the 64-kDa protein band reacts with antibodies against cytochrome $b$ and ISP (data not shown); second, when the mutant complex is treated with SDS and $\beta$-ME and then subjected to SDS-PAGE in the presence of $\beta$-ME, the 64-kDa protein band disappears, and the band intensities of cytochrome $b$ and ISP increase (Fig. 2, lane 4); and third, the protein isolated from the 64-kDa gel slice, after treatment with $\beta$-ME, can be resolved into cytochrome $b$ and ISP (data not shown).

Although the 64-kDa protein band that reacts with antibodies against cytochrome $b$ and ISP is absent from freshly prepared chromatophores from A185C(cytb)/K70C(ISP) mutant cells, it appears in ICM preparations obtained from cells grown semiaerobically. The A185C(cytb)/K70C(ISP) mutant ICM has no cytochrome $b_1$ complex activity (Table II). Also, when freshly prepared mutant chromatophores are incubated at 0 °C under air, the 64-kDa protein band intensity increases as the $b_1$ activity decreases. It usually takes more than a week for the loss in activity to reach 50%. These results confirm that formation of a disulfide bond from the two engineered cysteines on cytochrome $b$ and ISP in the A185C(cytb)/K70C(ISP) mutant complex is promoted by oxygen and is directly related to the loss of $b_1$ activity in this mutant complex.

**Table II**

| Mutants     | Chromatophore | ICM           | Purified $b_1$ |
|-------------|---------------|---------------|---------------|
|             | $\beta$-ME   | $\beta$-ME    | $\beta$-ME    |
| Wild type   | 2.3           | 2.3           | 2.5           |
| A185C       | 2.3           | 2.3           | 2.5           |
| K70C        | 1.3           | 1.3           | 1.4           |
| A185C/K70C  | 1.3           | 1.3           | 0             |

$^a$ Enzymatic activity is expressed as μmol of cytochrome $c$ reduced/min/mmol cytochrome $b$ at room temperature.
$^b$ $\pm$ samples were prepared with buffers containing 100 mM $\beta$-ME.
$^c$ The $b_1$ activity was measured after the sample had been stored on ice for 1 day.

Effect of $\beta$-ME on the Disulfide Bond Formation and $b_1$ Activity—To further confirm that the formation of a disulfide bond between cytochrome $b$ and ISP causes the A185C(cytb)/K70C(ISP) mutant complex to lose $b_1$ complex activity, the effect of $\beta$-ME on $b_1$ complex activity and disulfide bond formation was examined. When purified A185C(cytb)/K70C(ISP) mutant complex, which has been incubated at 0 °C for 24 h, was treated with $\beta$-ME, the activity was restored to the same level as that in freshly prepared chromatophores (Table II). No adduct of cytochrome $b$ and ISP was detected in the $\beta$-ME-treated A185C(cytb)/K70C(ISP) mutant complex. When this complex was purified from the freshly prepared chromatophores in the presence of 100 mM $\beta$-ME, it had the same activity as that found in the chromatophores, and no cytochrome $b$-ISP adduct was detected. A similar $\beta$-ME effect is observed for the cytochrome $b_1$ complex in mutant ICM (Table II). It should be emphasized that the observed activity restoration or preservation is not due to nonenzymatic reduction of cytochrome $b$ by $\beta$-ME, because the only antimycin-sensitive cytochrome $c$ reduction is used for activity calculations. Under identical conditions, $\beta$-ME has no effect on the $b_1$ activities in the A185C(cytb), K70C(ISP), T386C(cytb), K164C(ISP), and T386C(cytb)/K70C(ISP) mutant complexes.

Effect of Disulfide Bond Formation between Cytochrome $b$ and ISP on EPR Characteristics and Redox Potential of the 2Fe2S Cluster—Although evidence presented above clearly...
demonstrates that the loss of bc1 complex activity in the A185C(cytb)/K70C(ISP) mutant correlates with the formation of a disulfide bond between the two engineered cysteines in cytochrome b and ISP, it is unknown how disulfide bond formation causes the activity loss. Because this disulfide bond is formed in the interface between cytochrome b and ISP, it is possible that the microenvironments or the redox potential of the ISP cluster are altered, thus leading to activity loss. To test this possibility, EPR characteristics and redox potentials of the 2Fe2S cluster in the A185C(cytb)/K70C(ISP), with and without β-ME treatment, are compared.

As shown in Fig. 3, the 2Fe2S in the A185C(cytb)/K70C(ISP) mutant complex, with or without β-ME treatment, has the same EPR spectrum, with the $g_x$ signal at 1.775, $g_y$ at 1.900, and $g_z$ at 2.020. This result indicates that the microenvironments of the ISP cluster in the A185C(cytb)/K70C(ISP) mutant complex are not affected by disulfide bond formation between the two engineered cysteines in cytochrome b and ISP. Therefore, the loss of bc1 activity is not due to a change of microenvironments in the ISP.

However, it should be noted that replacing the K70 of ISP with cysteine in the A185C(cytb)/K70C(ISP) mutant complex shifts the $g_x$ signal of the 2Fe2S cluster from 1.800 to 1.775. This is deduced from the observation that the 2Fe2S cluster in the A185C(cytb) mutant complex has an EPR spectrum identical to that observed in the wild type complex, with resonance of $g_x = 1.800$, $g_y = 1.900$, and $g_z = 2.020$ (Fig. 3), whereas the 2Fe2S in the K70C(ISP) mutant complex has a broadened $g_x$ signal that is shifted to 1.768. The $g_x$ signal of 2Fe2S in the A185C(cytb)/K70C(ISP) mutant complex is sharper than that in the K70C(ISP) bc1 complex but broader than that in the wild type or A185(cytb) mutant complex. As expected, the effect on the bc1 complex is small because the K70C(ISP) and β-ME-treated A185C(cytb)/K70C(ISP) mutant complexes retain more than 50% of the bc1 activity found in the wild type.

The line shape of the $g_x$ signal of the 2Fe2S cluster is thought to be mediated by the oxidation state of ubiquinone present in the Q$_{s}$ site (26–30). The $g_x$ of bc1 from R. sphaeroides is at $g = 1.800$ when ubiquinone is present but shifts to 1.750 and broadens when ubiquinol is present. When ubiquinone is extracted from chromatophore membranes, the $g_x$ signal of the “depleted state” is at $g = 1.765$ and is considerably broader than those seen in the presence of either ubiquinone or ubiquinol. The change in the $g_x$ signal because of oxidation-reduction state of Q in the Q$_{s}$ site of the bc1 complex is similar to that observed for the substitution of Leu for Phe$^{144}$ (F144L) in the cytochrome b from R. capsulatus (29). The F144L bc1 complex in R. capsulatus chromatophores was reported to have a very low turnover rate with a broadened, redox state-insensitive, $g_x$ value at 1.765. It was suggested that these properties of the F144L complex resulted from a reduced affinity for quinone and quinol exhibited by the Q$_{s}$ center of the mutated complex. Because the Lys$^{70}$ of ISP is in the vicinity of the putative Q$_{s}$ pocket of cytochrome b, perhaps substitution of Lys$^{70}$ with cysteine, as in the K70C and β-ME-treated A185C/K70C mutant complexes, reduces the affinity of the Q$_{s}$ site of cytochrome b for quinone and quinol and thus decreases activity.

The redox potentials of the 2Fe2S clusters in the complexes of wild type, A185C(cytb), K70C(ISP), and A185C(cytb)/K70C(ISP) are 231, 228, 234, and 232 mV, respectively. Because the redox potentials are similar, the loss of activity in the cysteine pair mutant cannot be attributed to a change of the redox potential of the 2Fe2S cluster of ISP.

Effect of the Disulfide Bond Formation between Cytochrome b and ISP on the Rate of Intramolecular Electron Transfer between 2Fe2S and Heme c1—One way to unambiguously establish that formation of an intersubunit disulfide bond between cytochrome b and ISP arrests the movement of the head domain of ISP to the fixed state is to compare the rate of intramolecular electron transfer between 2Fe2S and heme c1 in wild type and cysteine pair mutant complexes. It has been reported that intramolecular electron transfer between heme c1 and the 2Fe2S cluster in the bovine complex can be induced by changing the pH of the enzyme solution (15). This is based on the fact that the redox potential of heme c1 is independent of pH, whereas the redox potential of 2Fe2S is pH-dependent (higher the pH lower the redox potential). At pH 8.0 heme c1 and 2Fe2S have the same redox potentials. Thus, when the pH of the enzyme solution is raised above 8.0, the...
2Fe2S becomes less reduced than heme c1 if the preparation is 50% reduced at pH 8.0. However, when the pH is adjusted to a lower value than 8.0, the 2Fe2S becomes more reduced than heme c1. Electron shuffling between 2Fe2S and heme c1, in the partially reduced complex, is pH-dependent.

To be sure that the electron transfer between heme c1 and 2Fe2S in the R. sphaeroides bc1 complex can also be induced by a change of pH, the redox status of heme c1 and 2Fe2S in a cytochrome c1 half-reduced wild type bc1 complex was monitored at various pHs (Fig. 4). Similar to results obtained with the bovine complex, at pH 8.1, heme c1 has the same redox potential as the 2Fe2S cluster in the R. sphaeroides bc1 complex. When the pH is increased, 2Fe2S becomes less reduced, and when it is lowered 2Fe2S becomes more reduced; heme c1 changes in opposite directions.

Fig. 5 shows time trace of acidification and alkalization-induced intramolecular electron transfer between 2Fe2S and heme c1 in wild type and A185C(cytb)/K70C(ISP) mutant complexes. The rates of electron transfer from heme c1 to 2Fe2S induced by acidification (reverse) and from 2Fe2S to heme c1 (forward) induced by alkalization of wild type bacterial complex both have a reaction half-life (t1/2) of about 1–2 ms (Fig. 5, A and C). The rates of pH-induced electron transfer between 2Fe2S and heme c1 in the A185C(cytb)/K70C(ISP) mutant complex are very slow; the t1/2 for the forward and backward reactions are 100 and 10 s, respectively (Fig. 5, D and B). The rates of electron transfer between 2Fe2S and heme c1 in mutant complexes of A185C(cytb) and K70C(ISP) are the same as those observed in the wild type complex (from ubiquinol to cytochrome bc1), too far for electron transfer.

The observation that the rates of electron transfer between 2Fe2S and heme c1 in mutant complexes of A185C(cytb) and K70C(ISP), which have, respectively, 100 and 53% of the ubiquinol-cytochrome c reductase activity found in the wild type complex, are the same as that observed in the wild type complex suggests that electron transfer between 2Fe2S and heme c1 is not the rate-limiting step in the electron transfer reaction catalyzed by the bc1 complex (from ubiquinol to cytochrome c1). This suggestion is also consistent with the observation that the rate of electron transfer from 2Fe2S to heme c1 in the wild type bacterial complex is comparable with that in the bovine complex (15), even though the rate of electron transfer from ubiquinol to cytochrome c catalyzed by the bacterial complex is only one-tenth that of the bovine complex.

The fact that formation of an intersubunit disulfide bond between the engineered cysteines at the position 185 of cytochrome b and 70 of ISP results in loss of bc1 activity suggests that movement of the head domain of ISP is independent from cytochrome b, at least in the cd2 region where the A185C is located. However, some regions of cytochrome b may have a synchronous movement with the head domain of ISP and may even provide the driving force for the movement of the ISP head. Formation of an intersubunit disulfide bond between such a region and the head domain of ISP should not have an adverse effect on electron transfer activity.

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Confirmation of the Involvement of Protein Domain Movement during the Catalytic Cycle of the Cytochrome \( bc_1 \) Complex by the Formation of an Intersubunit Disulfide Bond between Cytochrome \( b \) and the Iron-Sulfur Protein

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