Flexibility of the Neck Region of the Rieske Iron-Sulfur Protein Is Functionally Important in the Cytochrome $bc_1$ Complex

(Received for publication, June 18, 1998, and in revised form, July 28, 1998)

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The crystal structure of the mitochondrial cytochrome $bc_1$ complex suggests that movement of the extramembrane (head) domain of the Rieske iron-sulfur protein (ISP) is involved in electron transfer. Such movement requires flexibility in the neck region of ISP. To test this hypothesis, *Rhodobacter sphaeroides* mutants expressing His-tagged cytochrome $bc_1$ complexes with altered ISP necks (residues 39–48) were generated and characterized. Mutants with increased rigidity of the neck, generated by a double-proline substitution at Ala-46 and Ala-48 (ALA-PLP) or by a triple-proline substitution of ADV at residues 42–44 (ADV-PPP), have reactions comparable to that of complement cells, indicating that the length of the ISP neck is less critical than its flexibility in support of photosynthetic growth. The ADV and ALA-PLP mutant membranes have 10 and 30% of the cytochrome $bc_1$ complex activity found in the complement membrane, respectively, whereas the ADV-PPP mutant membrane contains no cytochrome $bc_1$ complex activity. The loss of cytochrome $bc_1$ complex activity in the ADV membrane is attributed to improper docking of the head domain of ISP on cytochrome $b$, as indicated by a drastic change in the EPR characteristics of the Rieske iron-sulfur cluster. The loss of cytochrome $bc_1$ complex activity in the ALA-PLP and ADV-PPP mutant membranes results from the decreased mobility of the ISP head domain due to the increased rigidity of the ISP neck. The ALA-PLP mutant complex has a larger activation energy than the wild-type complex, suggesting that movement of the head domain decreases the activation energy barrier of the cytochrome $bc_1$ complex. Using the conditions developed for the isolation of the His-tagged complement cytochrome $bc_1$ complex, a two-subunit complex (cytochromes $b$ and $c_1$) was obtained from the ADV and ADV-PPP mutants, indicating that mutations at the neck region of ISP weaken the interactions among cytochrome $b$, ISP, and subunit IV.

1 The abbreviations used are: ISP, Rieske iron-sulfur protein; Q, ubiquinone; $Q_0$, ubiquinol oxidation site; $Q_1$, ubiquinone reduction site; $b_{h_1}$, low potential cytochrome $b$; $b_{h_2}$, high potential cytochrome $b$; DM, dodecyl maltoside; NTA, nitrilotriacetic acid; $Q_{10}$, 2,3-dimethoxy-5-methyl-6-(10-bromodecyl)-1,4-benzoquinol; kb, kilobase pair; ICM, intracytoplasmic membrane.
heme $c_1$ is difficult to understand in view of the rapid electron transfer rate observed for these two redox centers (10, 11). Movement of the extramembrane domain of ISP, as described below, offers an explanation for this paradox. The [2Fe-2S] cluster is reduced by the first electron of ubiquinol at a position 27 Å from heme $b_h$ and 31 Å from cytochrome $c_1$. The reduced [2Fe-2S] cluster cannot donate an electron to cytochrome $c_1$ before the second electron of ubiquinol is transferred to heme $b_h$. It was speculated that either the change of the ubisemiquinone binding position before the reduction of heme $b_h$ or the electron transfer from heme $b_h$ to $b_L$ causes a conformational change in cytochrome $b$ that forces or allows reduced [2Fe-2S] to move close enough to heme $c_1$ for fast electron transfer (6, 8).

The ISP structure of beef heart mitochondria has three domains: the membrane-spanning N-terminal domain consisting of residues 1–62 (tail), the soluble C-terminal extramembrane domain consisting of residues 73–196 (head), and the flexible linking domain comprising residues 63–72 (neck). ISP is associated with the complex via the membrane-spanning N-terminal domain (4, 7, 9). The [2Fe-2S] cluster is located in the rigid head domain as shown in the high resolution structure of the water-soluble fragment of ISP (12, 13). The electron density in the neck region is low; the structure was deduced by connecting the C-terminal end of the transmembrane helix to the N terminus of the head domain. No ordered secondary structure was reported in the neck region (4, 7).

Although the position of the iron-sulfur cluster changed from a fixed state to a released state upon methoxyacetylate stilbene binding (6), the three-dimensional structures of the head and tail domains of ISP remain the same in these two states, suggesting that a bending of the neck is required for movement of the head domain (7). For the neck region to bend, some flexibility is imperative. The beef ISP neck has 10 amino acid residues with a sequence of SASAVLMS. This region is highly conserved in all iron-sulfur proteins (Fig. 1). The well conserved alanine residues may provide the needed flexibility. In the crystal structure, the neck region is in close contact with the cd1 helix of cytochrome $b$, but is not involved in compact docking between subunits, thus leaving enough space for bending (Fig. 2). The neck region is exposed to solvent as indicated by its susceptibility to several proteases, including thermolysin and trypsin (13). If movement of the head domain of ISP is required for $b_{c1}$ catalysis and the neck region of ISP confers the necessary mobility, changing the flexibility of the neck region of ISP should drastically affect the catalytic activity of the $b_{c1}$ complex. One way to prove this suggestion is to prepare recombinant mutant ISP, with increased rigidity in the neck, by site-directed mutagenesis followed by in vitro reconstitution of mutant ISP to an ISP-depleted $b_{c1}$ complex. Biochemical and biophysical characterizations of the reconstituted $b_{c1}$ complexes should reveal the essentiality of neck flexibility. Although the beef cDNA for ISP has been cloned and sequenced (14), the unavailability of reconstitutively active recombinant ISP and the difficulty in preparing fully reconstitutively active ISP-depleted $b_{c1}$ complex (15) have prevented us from taking this approach. The four-subunit cytochrome $bc_1$ complex from the photosynthetic bacterium *Rhodobacter sphaeroides* is functionally analogous to the mitochondrial $bc_1$ complex. Since the largest three subunits are homologous to their mitochondrial counterparts and are readily manipulated genetically, this organism is ideal for studying the neck region of ISP by site-directed mutagenesis.

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In this report, we characterized the complex of three *R. sphaeroides* mutants expressing His$_6$-tagged cytochrome $b_{c1}$ complexes with altered ISP neck regions. The length of the neck was shortened by deletion, and its rigidity was increased by proline substitution at various positions. The photosynthetic growth behavior, EPR characteristics of the Rieske [2Fe-2S] cluster, activation energy, and the cytochrome $bc_1$ complex activity in membranes and the purified state from the complement and mutant strains were examined and compared. The effect of the neck region of ISP on the interaction between cytochrome $b$ and ISP or subunit IV was determined.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dodecyl maltoside (DM) and octyl glucoside were purchased from Anstracite. Stigmatellin was from Fluka. All other chemicals were of the highest purity commercially available. The Ni$_2$NTA resin used for purification of the His$_6$-tagged cytochrome $b_{c1}$ complex was purchased from Qiagen Inc. Restriction endonucleases and other DNA-modifying enzymes were purchased from Promega, Life Technologies, Inc., and New England Biolabs, Inc. pSELECT-1 plasmid, R408 helper phage, and BMBH71-18 mutS and D109 Escherichia coli strains used in mutagenesis were from Promega. pSELNB3503 and pRK695/cFBCQ were constructed in our laboratory (16). E. coli S17 (17) and *R. sphaeroides* BC17 (18) were generously provided by Dr. R. B. Gennis (University of Illinois), 2,3-Dimethoxy-5-methyl-6-(10-bromodecyl)-1,4-benzoquinol (Q$_1$C$_{10}$BrH$_2$) was synthesized as described previously (19).

**Growth of Bacteria**—*E. coli* cells were grown at 37 °C on LB medium. Extra-rich medium (TVP) was used in procedures for the rescue of single-stranded DNA or the purification of low copy number plasmids (20). *R. sphaeroides* cells were grown at 30 °C on enriched Sistrom's medium either semi-aerobically or photoheterotrophically (photosynthetic growth) as reported (21). Antibiotics were added at the following concentrations: ampicillin, 100–125 μg/ml; tetracycline, 10–15 mg/liter for *E. coli* and 1 mg/liter for *R. sphaeroides*; kanamycin sulfate, 30–50 mg/liter for *E. coli* and 20 mg/liter for *R. sphaeroides*; and trimethoprim, 85–100 mg/liter for *E. coli* and 25 mg/liter for *R. sphaeroides*.

Construction of an R. sphaeroides Strain Expressing the His$_6$-tagged Cytochrome $b_{c1}$ Complex—A 1.2-kb XbaI-HindIII fragment containing the $fbcC$ and $fbcQ$ genes from pRK695/cFBCQ, containing $fbcF$, $fbcB$, $fbcC$, and $fbcQ$, was inserted into a modified pSELECT-1 vector in which the unique Acc65I site was eliminated. The resulting pSIEL/cFBCQ was used as template for site-directed mutagenesis to introduce an Acc65I recognition site right before the stop codon of the $fbcC$ gene. The Altered Sites in Vitro Mutagenesis System from Promega (22) was used for all the site-directed mutagenesis constructions. Two complementary oligonucleotides with His$_{65}$ tag coding sequence and the Acc65I overhang

**FIG. 1.** Sequence alignment of amino-terminal portions of Rieske iron-sulfur proteins from different species. The neck regions are shaded. Boldface indicates the thermolysin cleavage site that Link et al. used to generate the head domain for structure analysis of the bovine protein. The ISP's are from bovine heart (Bo), R. sphaeroides (Rs), *R. capsulatus* (Rc), *S. cerevisiae* (Sc), *Paracoccus denitrificans* (Pd), and *Neurospora crassa* (Pd).
attached at the 5′-ends (5′-GTACGGGC CATCAC CAC CATCAC CATCAC TAA-3′ and 3′-CCCG GTA GTG GTG GTA GTG ATTCATG-5′) were synthesized, annealed together by heating up to 70 °C and cooling slowly to room temperature, and ligated into the Acc65I site of pSEL-fbcCHQ to generate pSEL-fbcC1Q. The 6-histidine insertion was confirmed by DNA sequencing. A 1.2-kb XbaI-HindIII fragment containing fbcC1Q from pSEL-fbcC1Q was subcloned into an expression vector (pRKD418) containing the fbcFBCQ genes to generate pRKF/fbcFBCQ, which was then mobilized into R. sphaeroides BC17 by parental conjugation.

Generation of R. sphaeroides Strains Expressing the bc1 Complexes with Altered ISP—Mutations were constructed by site-directed mutagenesis using the Altered Sites system. Oligonucleotides were synthesized at the Oklahoma State University Recombinant DNA/Protein Core Facility. The oligonucleotides used are GCTGATCAACCAAATGAATCCGTCGCCGCCGCCGTCAATCGTACGGGC CATCAC CAC CATCAC CATCAC TAA-3′ and 3′-CCCG GTA GTG GTG GTA GTG ATTCATG-5′. The side chains of conserved amino acid residues that were mutated are displayed as green sticks. Cytochrome b is a green ribbon, with the cd helices highlighted in blue. Stigmatellin (purple stick) is within the Qo pocket.

Enzyme Preparations and Activity Assay—Chromatophore membranes were prepared from BC17 cells harboring complement (sequence is the same as the wild type) or mutant pRKF/fbcFBCQ as described previously (23) and stored at very high concentration in the presence of 20% glycerol at −80 °C. To purify the His6-tagged cytochrome bc1 complex, the chromatophore suspensions were thawed and adjusted to a concentration of 25 μM Q10BrH2. The 6-histidine insertion was confirmed twice by DNA sequencing before and after photosynthetic or semi-aerobic growth of the cells. The activation energy was calculated from an Arrhenius plot.

Determination of the Activation Energy of the Cytochrome bc1 Complex—The activation energy of the bc1 complex was determined in both chromatophore membrane and purified preparations. This is essentially assaying steady-state enzyme activity at various temperatures. The temperatures of the assay mixture were controlled (±0.1 °C) by a Shimadzu TCC controller installed on a Shimadzu UV2101PC spectrophotometer. Activity was measured from 9 to 30 °C at 3 °C intervals. The activation energy was calculated from an Arrhenius plot.

Other Biochemical and Biophysical Techniques—Protein concentration was measured by the method of Lowry et al. (25). Cytochrome b (26) and cytochrome c1 (27) were determined according to published methods. SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (28) using a Bio-Rad Mini-Protein dual-slub vertical cell. Western blotting was performed using rabbit polyclonal antibodies against cytochrome b and cytochrome c1.
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**RESULTS AND DISCUSSION**

**Characterization of the His$_s$-tagged Cytochrome bc$_1$ Complex**—The cytochrome bc$_1$ complex, which was overexpressed in *R. sphaeroides* BC17 cells by a low copy number expression vector (pRKD418) containing the fbcFBCQ genes, was routinely purified from chromatophore preparations by DM solubilization followed by DEAE-Bio-Gel A and DEAE-Sepharose CL-6B column chromatography. Although this purification scheme produces an enzyme complex of high purity and activity, it is time-consuming and gives low yields. This procedure requires extensive washing of the DEAE-Bio-Gel A column (usually 20 column volumes) to remove contaminating proteins, chlorophyll, and other pigments from the absorbed bc$_1$ complex. This step takes at least 8 h.

To speed up the preparation of the bc$_1$ complex from complement and mutant cells, a His$_s$ tag was genetically engineered into the C terminus of the cytochrome c$_1$ subunit to allow the use of Ni$_2^+$/NTA affinity agarose in a one-step purification. This construction was achieved by ligating annealed His$_s$-tag coding oligonucleotides, with the Acc651 overhang attached at the 5’-ends, into the Acc651 site created right before the stop codon of the fbcC gene in pSEL/bcCQ plasmid to generate pSEL/bcCQ$_Q$. The fragment containing the fbcCQ$_Q$ genes in pRKDbFBCQ$_Q$ was replaced with the fragment containing the fbcC$_Q$ genes from pSEL/bcC$_Q$ to generate pRKDbFBCQ$_Q$. *R. sphaeroides* BC17 cells harboring pRKDbFBCQ$_Q$ have photosynthetic and respiratory growth behaviors similar to those of untagged cells. This construction adds 9 amino acid residues (GTGHHHHHH) to the C terminus of cytochrome c$_1$ in the expressed bc$_1$ complex. The spectral properties and bc$_1$ complex activity in the His$_s$-tagged chromatophores are similar to those in untagged chromatophores. The effectiveness of DM in the solubilization of tagged and untagged cytochrome bc$_1$ complexes from their respective chromatophores is comparable; 95% of the cytochrome b present in chromatophores was solubilized when 0.56 mg of DM/nmol of cytochrome b was used. About 50% of the cytochrome bc$_1$ complex in chromatophores was recovered from the Ni$_2^+$/NTA column. The His$_s$-tagged bc$_1$ complex has purity, activity, and cytochrome content similar to those of the untagged enzyme complex. The yield of the purified His$_s$-tagged bc$_1$ complex was twice that of untagged bc$_1$ preparations. The total time for purification of the His$_s$-tagged complex was ~6 h as compared with 2 days for the conventional purification. The molecular mass of the His$_s$-tagged bc$_1$ complex, determined by sedimentation velocity or sedimentation equilibrium, is 220 kDa, indicating that the isolated complex is in a dimeric state. The EPR characteristics of the [2Fe-2S] cluster in the purified His$_s$-tagged bc$_1$ complex are the same as those in the untagged complex (data not shown).

**Characterizations of the Cytochrome bc$_1$, Complexes Containing an Altered ISP Neck**—To establish that flexibility of the neck region of ISP is essential for the head domain movement required for bc$_1$ catalysis, mutants expressing bc$_1$ complexes with increased ISP neck rigidity were generated and characterized. The *R. sphaeroides* ISP neck is composed of residues 39–48 with the sequence NPNSADVQALa-Ala-Asp-Val-Ala-Val, and Ala-48 are the conserved amino acid residues. We focused our mutational studies on these 5 residues. The flexibility of the ISP neck is expected to decrease when proline residues are introduced because proline has a conformational constraint due to the cyclic nature of its pyrrolidine side chain. Since the ADV residues are located in what appears to be the most flexible part of the neck, deletion of these residues is expected to affect the movement or the positioning of the head of ISP.

Three *R. sphaeroides* mutant strains were generated: ΔADV, in which the ADV residues (residues 42–44) are deleted; ALA-PLP, in which Ala-46 and Ala-48 are substituted with prolines; and ADV-PPP, in which Ala-42, Asp-43, and Val-44 are substituted with prolines. All mutations were constructed by site-directed mutagenesis using a 3.5-kb EcoRI-HindIII *R. sphaeroides* DNA carrying the fbcFBCQ genes in pSELECT-1 plasmid (pSELNB3503) as template. A 2.5-kb EcoRI-PinAI fragment containing the kanamycin-resistant gene in pRKDbFBCQ$_Q$ was replaced with a 1.7-kb EcoRI-PinAI fragment containing the mutated ISP gene from pSELNB3503 to generate pRKDbFBCQ$_Q$, which was then mobilized into *R. sphaeroides* BC17 cells. Mutations in pRKDbFBCQ$_Q$ harbored in *E. coli* S17-1 (before conjugation) and photosynthetically or semi-aerobically dark-grown *R. sphaeroides* cells (after conjugation) were confirmed by DNA sequencing after the targeted DNA fragment was amplified by polymerase chain reaction.

The ΔADV mutant cells grew photosynthetically at a rate similar to that of complement cells. The ALA-PLP mutant was also capable of photosynthetic growth, but at a maximal doubling rate of ~50% that of the complement strain. The ADV-PPP mutant was unable to grow photosynthetically (Table I, first column), but could grow under semi-aerobic conditions. These results indicate that the ISP neck flexibility is more critical than its length in supporting photosynthetic growth.

To investigate whether mutations at the neck region of ISP affect cytochrome bc$_1$ complex activity, ubiquinol-cytochrome c reductase activity in chromatophores from the ΔADV and ALA-PLP strains and in the intracytoplasmic membrane (ICM) of the ADV-PPP strain was assayed and compared with that of the complement strain. To study subunit association of cytochrome bc$_1$ complexes in the membrane, Western blot analyses of chromatophores and ICM of mutants were performed and

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**Table I**

| Mutations       | Photosynthetic Growth | Enzyme activity$^a$ | Subunit composition |
|-----------------|-----------------------|---------------------|---------------------|
|                 |                       | Chromophore or ICM  | Purified complex    |
| Complement      | ++                    | 2.0                 | FBCQ                |
| ΔADV            | ++                    | 0.2                 | FBCQ                |
| ALA-PLP         | ++                    | 0.7                 | FBCQ                |
| ADV-PPP         | --                    | 0.7                 | FBCQ                |

$^a$ Enzyme activity is expressed as micromoles of cytochrome c reduced per min/nmol of cytochrome b.

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PPP mutants; lane 9 of the membrane. However, we observed an apparently elevated level of cytochrome membrane. These results indicate that these mutants used to detect these three subunits. The antibody’s titer for cytochrome ples containing 75 pmol of cytochrome

Membrane samples were prepared electrophoretically to a polyvinylidene difluoride membrane. Polyclonal antibodies raised against subunits of R. sphaeroides bc\textsubscript{1} complex (cytochrome c\textsubscript{1} (Cyt c\textsubscript{1}), ISP, and subunit IV (Sub IV)) were used to detect these three subunits. The antibody’s titer for cytochrome c\textsubscript{1} is much higher than those for subunit IV and ISP. To reduce the nonspecific background reaction, the membrane was cut into two pieces so that the upper part, containing proteins with a molecular mass $>$23 kDa, including cytochrome c\textsubscript{1}, was developed with an alkaline phosphatase system. Lane 1, prestained molecular mass standards; lane 2, photosynthetic growth wild-type complement chromatophores; lane 3, semi-aerobic wild-type complement ICM; lane 4, ΔADV mutant chromatophores; lane 5, ALA-PPP mutant chromatophores; lane 6, ADV-PPP mutant ICM; lanes 7 and 8, effluent from the Ni\textsuperscript{2+}-NTA column, which is the unbound portion from the detergent extraction mixture for the ΔADV and ADV-PPP mutants; lane 9, purified His\textsubscript{6}-tagged complement bc\textsubscript{1} complex.

compared with those of the complement strain. Chromatophores from the ΔADV and ALA-PLP mutant cells had 10 and 30% of the ubiqunol-cytochrome c reductase activity found in the complement chromatophores, respectively. As expected, ICMs of the ADV-PPP mutant had no ubiqunol-cytochrome c reductase activity because the cytochrome bc\textsubscript{1} complex is an obligatory enzyme complex for photosynthetic growth, and this mutant was unable to grow photosynthetically. These results indicate that shortening the length or decreasing the flexibility of the ISP neck drastically decreases the cytochrome bc\textsubscript{1} complex activity in membranes. It should be noted that the cytochrome bc\textsubscript{1} complex activities in the photosynthetic chromatophore membrane and in semi-aerobic ICM from complement cells are the same.

When membranes from these three mutants and the complement cells were subjected to Western blot analysis with antibodies against R. sphaeroides cytochrome c\textsubscript{1}, ISP, and subunit IV, stoichiometric amounts of these three subunits were detected in all three mutant membranes (Fig. 3). Absorption spectral analysis also revealed that the ratio of cytochrome b to c\textsubscript{1}c\textsubscript{2} in all these mutant membranes was similar to that in the complement membrane. These results indicate that these mutations did not affect the assembly of ISP protein into the membrane. However, we observed an apparently elevated level of the bc\textsubscript{1} complex in the ΔADV mutant membrane, as indicated by the elevated level of cytochrome b (19 nmol/mg of protein), relative to that found in membranes from complement cells (16 nmol/mg of protein). It should be noted that membranes from complement cells already possess three times the amount of cytochrome b found in wild-type R. sphaeroides, presumably due to a gene dosage effect. Therefore, the increased level of expression could be a regulatory response compensating the lowered electron transfer activity in the ΔADV mutant. This explains why 10% bc\textsubscript{1} (specific) activity observed in the mutant complex is sufficient to support the photosynthetic growth.

Effect of Mutation on the Rieske Iron-Sulfur Cluster and on Assembly of ISP into the Cytochrome bc\textsubscript{1} Complex—Since the bc\textsubscript{1} complex activity decreased by 90, 70, and 100% in the ΔADV, ALA-PLP, and ADV-PPP mutant membranes, respectively, with no decrease in the amount of ISP, it is important to determine whether the activity loss resulted from improper assembly of ISP into the complex or from the fact that the ISP head domain is less mobile. We addressed this question by comparing EPR characteristics of the [2Fe-2S] cluster in complement and mutant membranes because EPR signals from the iron-sulfur protein, especially the g\textsubscript{s} signature, are very sensitive to changes in its microenvironments. Also, we compared the subunit stoichiometry in the purified His\textsubscript{6}-tagged mutant complexes with that found in the complement complex.

When the [2Fe-2S] cluster was reduced by a small excess of ascorbate, the complement chromatophore or ICM had a spectrum that was essentially the same as that previously reported for the chromatophores from wild-type R. sphaeroides, with resonance at $g_{s} = 2.02$ and $g_{s} = 1.9$ (Fig. 4, spectra A and D). The $g_{s} = 2.02$ signal of the [2Fe-2S] cluster could not be resolved in membrane preparations because it was shielded by many other signals.

The [2Fe-2S] cluster in ΔADV chromatophore membranes showed no detectable g\textsubscript{s} signal (totally broadened) and a very small g\textsubscript{s} signal (Fig. 4, spectrum B), indicating that the microenvironments of the iron-sulfur cluster have been drastically altered in this deletion mutant. In the bc\textsubscript{1} crystal structure, the iron-sulfur cluster sits at the tip of the head domain of ISP, and this tip of ISP fits into the concave hydrophobic surface of the Q\textsubscript{b} pocket located in the cytochrome b subunit (4). Changing the microenvironments of the [2Fe-2S] cluster by mutation of residues in the docking interface of ISP and cytochrome b, such as Leu-132 (29) and Gly-133 (30) in ISP and Ile-292 (31) in cytochrome b, resulted in a loss of the $g_{s}$ signal of the [2Fe-2S] cluster and a decrease in electron transfer activity. Since the neck region is spatially separated from the docking interface of ISP and cytochrome b, the change of microenvironments of the [2Fe-2S] cluster in the ΔADV mutant complex, indicated by the $g_{s}/g_{s}$ signal change, is probably due to the improper docking of the head domain of ISP on cytochrome b as a result of the shortened neck. Loss of cytochrome bc\textsubscript{1} complex activity (90%) in the ΔADV membrane is therefore attributed to improper assembly of ISP into the complex. It should be noted that the
drastic decrease in the amplitude of the $g_s$ signal in the ΔADV mutant chromatophores is not due to a destabilizing effect on the oxidized [2Fe-2S] cluster as reported for the T134R, T134H, or T134G mutation in ISP of Rhodobacter capsulatus (29) since the signal was not increased in EPR measurements of ΔADV chromatophores prepared by including 20 mM ascorbate to keep the [2Fe-2S] cluster in the reduced state. Such a decrease in the EPR signal is not caused by a labile [2Fe-2S] cluster as the result of ADV deletion because when the membrane was prepared by a gentler method, such as treating freshly grown cells with lysozyme and an appropriate amount of detergent to disrupt the membrane, no increase in EPR signals and the membrane $b_6$ activity was observed. However, when the mutant membrane was incubated with 100 μM stigmatellin, a small but distinctive $g_s = 1.78$ signal showed up, similar to that of the stigmatellin-treated complement membrane (data not shown). No $b_6$ activity was detected in this inhibitor-treated ΔADV membrane, which further confirmed the typical response of $g_s$ signal to Q$_o$ site inhibitor. Therefore, the decrease in the EPR signal in the mutant membrane is not due to the destruction of the [2Fe-2S] cluster during the preparation of the membrane, but rather an intrinsic property of the mutant. Similar results were observed when cell pastes were used in EPR analysis (data not shown).

If ISP is indeed improperly assembled into the $b_6$ complex in the ΔADV mutant chromatophore, the mutant complex is expected to be less stable than the wild type. In other words, the binding affinity of ISP for other subunits in the ΔADV mutant complex is probably weaker than that in the wild-type complex. To confirm this speculation, chromatophore membranes from the ΔADV and complement cells were treated with dodecyl maltoside at 0.55 mg/nmol of cytochrome $b$. Although the amounts of cytochromes $b$ and $c_1$, ISP, and subunit IV solubilized from these two chromatophore membranes were the same, no $b_6$ complex activity was detected in the detergent-solubilized membrane fraction from ΔADV. When dodecyl maltoside-solubilized chromatophore membranes from ΔADV and complement cells were individually applied to a Ni$^{2+}$-NTA affinity column and analyzed for subunit composition in the histidine-eluted fractions, the His$_6$-tagged ΔADV complex was found to contain only cytochromes $b$ and $c_1$ (Fig. 5, lane 5). ISP and subunit IV were detected in the unbound fraction by Western blot analysis (Fig. 3, lane 7), whereas the complex complement contained cytochromes $b$ and $c_1$, ISP, and subunit IV (Fig. 5, lanes 2 and 3). Although the lack of ISP in the ΔADV complex was expected, the lack of subunit IV in the complex was rather surprising. Perhaps residues ADV of the ISP neck are involved in the packing of ISP and subunit IV with cytochrome $b$ and $c$ in the complex.

EPR characteristics of the Rieske [2Fe-2S] cluster in the ΔADV-PPP membrane (ICM) (Fig. 4, spectrum E) are the same as those of the [2Fe-2S] cluster in complement chromatophores, and binding of the Q$_o$ site inhibitor stigmatellin induces an upfield shift in the $g_s$ signal that is similar to that observed with complement chromatophores (data not shown). These results indicate that the environments of the iron-sulfur cluster are not changed by this mutation (ΔADV-PPP). Although the increased rigidity of the ISP neck did not affect the docking of the ISP head domain on the cytochrome $b$ protein, the complex was unstable as evident from the dissociation of ISP and subunit IV from cytochromes $b$ and $c_1$ when this mutant complex was purified from the DM-solubilized membrane (Fig. 5, lane 6). The complete lack of cytochrome $b_6$ complex activity in the ΔADV-PPP membrane may be attributed to weak binding of ISP and subunit IV with cytochromes $b$ and $c_1$ as well as a lesser head domain mobility of ISP during $b_6$ catalysis.

EPR characteristics of the Rieske [2Fe-2S] cluster in the ΔADV complex were as reported for the T134R, T134H, or T134G mutation in ISP of Rhodobacter capsulatus (29) since the signal was not increased in EPR measurements of ΔADV chromatophores prepared by including 20 mM ascorbate to keep the [2Fe-2S] cluster in the reduced state. Such a decrease in the EPR signal is not caused by a labile [2Fe-2S] cluster as the result of ADV deletion because when the membrane was prepared by a gentler method, such as treating freshly grown cells with lysozyme and an appropriate amount of detergent to disrupt the membrane, no increase in EPR signals and the membrane $b_6$ activity was observed. However, when the mutant membrane was incubated with 100 μM stigmatellin, a small but distinctive $g_s = 1.78$ signal showed up, similar to that of the stigmatellin-treated complement membrane (data not shown). No $b_6$ activity was detected in this inhibitor-treated ΔADV membrane, which further confirmed the typical response of $g_s$ signal to Q$_o$ site inhibitor. Therefore, the decrease in the EPR signal in the mutant membrane is not due to the destruction of the [2Fe-2S] cluster during the preparation of the membrane, but rather an intrinsic property of the mutant. Similar results were observed when cell pastes were used in EPR analysis (data not shown).
required for bc1 complex activity.

Effect of Mutation on the Activation Energy of the Cytochrome bc1 Complex—If the head domain movement of ISP is required for bc1 activity, it is important to know whether this step contributes to the activation energy barrier of the bc1-catalyzed reaction. This question can be addressed by comparing the activation energies of the cytochrome bc1 complexes, in chromatophores or in the purified state, of ALA-PLP mutant and complement cells. An increase in activation energy for the ALA-PLP complex would indicate that the head domain movement of ISP contributes to the activation energy barrier. This deduction is based on the fact that the only difference between the ALA-PLP mutant complex and the complement complex is the increased ISP head mobility of the former. Fig. 6 shows Arrhenius plots of bc1 complex activity in ALA-PLP mutant and complement chromatophores. Since the concentrations of electron donor (ubiquinol) and electron acceptor (cytochrome c) used in the bc1 activity assay mixture were at the saturation level, diffusion limitation of cytochrome c was avoided, and no production inhibition was observed. An activation energy of 24.7 kJ/mol was obtained for the bc1-catalyzed electron transfer from ubiquinol to cytochrome c. Ubiquinol-cytochrome c reductase activity decreases as the potential of ISP declines from approximately +280 to +100 mV, which confirms that oxidation of ubiquinol by ISP is the rate-limiting partial reaction in the bc1 complex and that the rate of this reaction is extensively influenced by the midpoint potential of the [2Fe-2S] cluster. These investigators suggested that deprotonation of ubiquinol is not the rate-limiting step in the ubiquinol oxidation catalyzed by the cytochrome bc1 complex.

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

REFERENCES

1. Trumpower, B. L., and Gennis, R. B. (1994) Annu. Rev. Biochem. 63, 675–716
2. Mitchell, P. (1976) J. Theor. Biol. 62, 327–367
3. Yu, C.-A., Kachrin, M. A., Yu, L., Xia, D., Kim, H., and Deisenhofer, J. (1996) Biochemistry 35, 47–53
4. Xia, D., Yu, C.-A., Kim, H., Xia, J. Z., Kachurin, A. M., Zhang, L., Yu, L., and Deisenhofer, J. (1997) Science 277, 60–66
5. Kim, H., Xia, D., Deisenhofer, J., Yu, C.-A., Kachurin, A., Zhang, L., and Yu, L. (1997) FEBS Lett. 411, 1084–1088
6. Kim, H., Xia, D., Yu, C.-A., Kachurin, A., Zhang, L., Yu, L., and Deisenhofer, J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 8026–8033
7. Zhang, Z., Huang, L.-S., Schulte, V. M., Chi, Y.-L., Kim, K. K., Huang, L.-W., Crofts, A. R., Berry, E. A., and Kim, S.-H. (1998) Nature 392, 677–684
8. Yu, C.-A., Xia, D., Kim, H., Deisenhofer, J., Zhang, L., Kachurin, A. M., and Yu, L. (1998) Biochim. Biophys. Acta 1365, 151–158
9. Iwata, S., Lee, J. W., Okada, K., Lee, J. K., Iwata, M., Rasmussen, B., Link, T. A., Rasmasswamy, S., and Jap, B. K. (1998) Science 281, 64–71
10. Crofts, A. R., and Meinhardt, S. W. (1982) Biochim. Biophys. Acta 710, 201–203
11. Tian, H., Ohnishi, T., and Palmer, G. (1987) J. Biol. Chem. 262, 9877–8684
12. Iwata, S., Saynovits, M., Link, T. A., and Michel, H. (1996) Structure 4, 567–579
13. Link, T. A., Saynovits, M., Assemann, C., Iwata, S., Ohnishi, T., and Von Jagow, G. (1996) Eur. J. Biochem. 237, 71–75
14. Usui, S., Yu, L., and Yu, C.-A. (1990) Biochim. Biophys. Res. Commun. 167, 575–579
15. Engel, W. D., Michalski, C., and Von Jagow, G. (1983) Eur. J. Biochem. 132, 395–402
16. Mather, M. W., Yu, L., and Yu, C.-A. (1995) J. Biol. Chem. 270, 28668–28675
17. Crofts, A. R., and Prieur, G. F. (1983) Biol. Technology 1, 784–791
18. Yun, C. H., Beci, R., Crofts, A. S., Kaplan, S., and Gennis, R. B. (1990) Eur. J. Biochem. 194, 399–411
19. Yu, C.-A., and Yu, L. (1982) Biochim. Biophys. Acta 710, 4069–4101
20. Khosravi, M., Ryan, W., Webager, D. A., and Stark, B. C. (1990) Plasmid 23, 138–143
21. Daldal, F., Tokito, M., Davidson, E., and Faham, M. (1989) EMBO J. 8, 3951–3961
22. Kurchin, B. E. (1986) Gene 42, 139–148
23. Kurchin, B. E. (1987) Biochem. Soc. Trans. 15, 201–203
24. Tian, H., Yu, L., Mather, M. W., and Yu, C.-A. (1997) J. Biol. Chem. 272, 23722–23728
25. Lowry, O. H., Roseborough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
26. Berden, J. A., and Slater, E. C. (1970) Biochim. Biophys. Acta 216, 237–249
27. Yu, L., Dong, J. H., and Yu, C.-A. (1986) Biochim. Biophys. Acta 852, 203–211
28. Simonet, V. K. (1970) Nature 227, 689–692
29. Liebl, U., Sled, V., Brasseur, G., Ohnishi, T., and Daldal, F. (1997) Biochim. Biophys. Acta 1365, 11996–11998
30. Van Doren, S. R., Gennis, R. B., Barquera, B., and Crofts, A. R. (1995) Biochemistry 34, 8083–8091
31. Crofts, A. R., Barquera, B., Bechmann, G., Guerova, M., Salcedo-Hernandes, R., Hacker, B., Hong, S., and Gennis, R. B. (1995) in Photosynthesis: From Light to Biosphere (Mathis, P., ed) Vol II, pp. 493–500, Kluwer Academic Publishers, Dordrecht, The Netherlands
32. DeGiorgi, P., and Lenaz, G. (1991) Arch. Biochim. Biophys. 289, 303–312
33. Zhang, L., Yu, L., and Yu, C.-A. (1998) Biophys. J. 74, 186 (abstr.)
34. Crofts, A. R., and Wang, Z. (1989) Photosynth. Res. 22, 69–87
35. de Vries, S., Albracht, S. P. J., Berden, J. A., and Slater, E. C. (1981) J. Biol. Chem. 256, 11996–11998
36. Link, T. A. (1997) FEBS Lett. 412, 257–264
37. Brandt, U., and Okun, J. U. (1997) Biochim. Biophys. Acta 1365, 11234–11240
38. Denke, E., Merhitz-Zahrndrich, T., Hatzfeld, O. M., Snyder, C. H., Link, T. A., and Trumpower, B. L. (1998) J. Biol. Chem. 273, 9085–9093