The Iron-Sulfur Cluster of the Rieske Iron-Sulfur Protein Functions as a Proton-exiting Gate in the Cytochrome \( bc_1 \) Complex*

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Buddha Gurung‡, Linda Yu‡, Di Xia§, and Chang-An Yu‡¶

From the ‡Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, Oklahoma 74078 and the §Laboratory of Cell Biology Center, NCI, National Institutes of Health, Bethesda, Maryland 20892

The destruction of the Rieske iron-sulfur cluster ([2Fe-2S]) in the \( bc_1 \) complex by hematoporphyrin-promoted photoinactivation resulted in the complex becoming proton-permeable (Miki, T., Yu, L., and Yu, C.-A. (1991) Biochemistry 30, 230–238). To study further the role of this [2Fe-2S] cluster in proton translocation of the \( bc_1 \) complex, Rhodobacter sphaeroides mutants expressing His-tagged cytochrome \( bc_1 \) complexes with mutations at the histidine ligands of the [2Fe-2S] cluster were generated and characterized. These mutants lacked the [2Fe-2S] cluster and possessed no \( bc_1 \) activity. When the mutant complex was co-inlaid in phospholipid vesicles with intact bovine mitochondrial \( bc_1 \) complex, or cytochrome \( c \) oxidase, the proton ejection, normally observed in intact reductase or oxidase vesicles during the oxidation of their corresponding substrates, disappeared. This indicated the creation of a proton-leaking channel in the mutant complex, whose [2Fe-2S] cluster was lacking. Insertion of the \( bc_1 \) complex lacking the head domain of the Rieske iron-sulfur protein, removed by thermolysin digestion, into PL vesicles together with mitochondrial \( bc_1 \) complex also rendered the vesicles proton-permeable. Addition of the excess purified head domain of the Rieske iron-sulfur protein partially restored the proton-pumping activity. These results indicated that elimination of the [2Fe-2S] cluster in mutant \( bc_1 \) complexes opened up an otherwise closed proton channel within the \( bc_1 \) complex. It was speculated that in the normal catalytic cycle of the \( bc_1 \) complex, the [2Fe-2S] cluster may function as a proton-exiting gate.

The cytochrome \( bc_1 \) complex, also known as ubiquinol-cytochrome \( c \) reductase or complex III, is the central segment of the energy-conserving, electron transfer chain of the mitochondria and many respiratory and photosynthetic bacteria (1). This enzyme complex catalyzes electron transfer from ubiquinol to cytochrome \( c \) (c\(_{12}\) in bacteria) with concomitant translocation of protons across the membrane to generate a proton electrochemical gradient required for ATP synthesis by ATP synthase. The cytochrome \( bc_1 \) complex from all species contain three core subunits, cytochrome \( b \), cytochrome \( c_1 \), and Rieske iron-sulfur protein (ISP);\(^1\) that house two \( b \)-type cytochromes (\( b_{562} \) and \( b_{566} \)), one \( c \)-type cytochrome (\( c_1 \)), and a high potential Rieske [2Fe-2S] cluster, respectively. However, the number of non-redox group containing subunits, also called supernumerary subunits, in the complex varies from species to species. Recently, the three-dimensional crystal structures of mitochondrial \( bc_1 \) complexes from bovine (2, 3), chicken (4), and yeast (5), which contain seven to eight supernumerary subunits in addition to the three core subunits, have been obtained. The structures of the cytochrome \( b_{56}\) complex, a complex analogous to the cytochrome \( bc_1 \) complex that provides an electronic connection between photosystems I and II, have also been established for the thermophilic cyanobacterium Mastigocladus laminosus (6) and in the algae Chlamydomonas reinhardtii (7), respectively. The three-dimensional structural information for the mitochondrial \( bc_1 \) complex establishes the location of the redox centers, the number of transmembrane helices, quinone binding at the Qi site, and inhibitor binding at both the Qo and Qi sites (2–4, 8, 9). Moreover, it suggests mobility of the extrinsic head domain of ISP during \( bc_1 \) catalysis. Strong evidence in support of this movement has been reported (4, 10–13, 15–19).

The proton-motive Q-cycle model (20, 21) has been favored for describing electron and proton translocation in the cytochrome \( bc_1 \) complex. The key feature of this model is the presence of two separate ubiquinol/ubiquinone-binding sites as follows: a ubiquinol oxidation site (Qo) near the P side (intermembranes space) of the mitochondrial inner membrane, and a ubiquinone reduction site (Qi) near the N side (matrix). Because of a lack of information on the binding of ubiquinol at the putative Qo site from the three-dimensional structures, the detailed bifurcation of ubiquinol oxidation, the key step in the Q-cycle mechanism is difficult to establish. Several models of the bifurcated oxidation of ubiquinol at the Qo site have been proposed (22–28).

It has been established that for every electron transferred through the \( bc_1 \) complex, two protons are translocated across the membrane. This 2\( H^+ / e^- \) stoichiometry has been verified in a wide variety of species, \textit{in vitro}, by studying \( bc_1 \) complexes in phospholipid (PL) vesicles. Proton pumping by the \( bc_1 \) complex inlaid PL vesicles requires an intact membrane that prevents nonspecific proton leakage. Any compromise of membrane integrity results in loss of the proton electrochemical gradient, as is observed when an uncoupler like carbonyl cyanide \( m \)-chlorophenylhydrazone (CCCP) is added to either intact mitochondria or PL vesicles harboring electron transfer complexes.

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\(^{1}\) To whom correspondence should be addressed: 255 NRC, Oklahoma State University, Stillwater, OK 74078. Tel.: 405-744-6612; Fax: 405-744-7799; E-mail: cayuq@okstate.edu.

\(^{2}\) The abbreviations used are: ISP, Rieske iron-sulfur protein; [2Fe-2S], Rieske iron-sulfur cluster; PL, phospholipid; CCCP, carbonyl cyanide \( m \)-chlorophenylhydrazone; ISF, Rieske iron-sulfur fragment; CoO, cytochrome \( c \) oxidase; Q\(_{1}\)C\(_{1}\)-BeH\(_{4}\), 2,3-dimethoxy-5-methyl-4-bromodicyclo-1,4-benzoquinol; ICM, internaloplastmic membrane; Ni-NTA, nickel-nitrilotriacetic acid; DM, \( n \)-dodecyl \( \beta \)-d-maltopyranoside; TM, transmembrane; MOPS, 4-morpholinoepanesulfonic acid.
integrity of the membrane is maintained by its protein and lipid components.

Although the proton translocation pathway in the cytochrome bc₁ complex is not fully understood, the involvement of the [2Fe-2S] cluster of ISP has been suggested. When bovine mitochondrial bc₁ complex is illuminated with a projector light in the presence of hemoporphyrin under aerobic conditions, the complex becomes inactivated as the [2Fe-2S] cluster in the treated complex is destroyed (29). When the photo-inactivated bc₁ complex is co-inlaid in PL vesicles with intact bovine bc complex, no proton ejection is detected during ubiquinol oxidation. These findings suggest that a proton-permeable channel is created in the photo-inactivated complex. However, because the hemoporphyrin-promoted photo-inactivation site in the photo-inactivated complex. However, because the hemoporphyrin-promoted photo-inactivation site in the photo-inactivated complex.

The HI1001 RI-XbaI fragment from the pGEM7Zf(fbc)-parentheses denote forward and reverse primers, respectively. The EcoCGC; H152C(F), GTTCTGCCCCTGCTGCGGATCGCACTACG, and acid residues in the specific, the formation of a proton-leaking channel in the photo-inactivated complex is not understood, the involvement of the [2Fe-2S] cluster.

Here we report the generation and characterization of Rhodobacter sphaeroides strains expressing cytochrome bc₁ complexes with substitutions at one or both the histidine residues His-131 and His-152 of the ISP, which serve as ligands to the [2Fe-2S] cluster.

We determined by measuring the reduction of cytochrome c, by following the increase in the absorbance at 550 nm, in a Shimadzu UV-2101 PC spectrophotometer at 23 °C using a millimolar extinction coefficient of 18.5 mM⁻¹ cm⁻¹ for calculations. The non-enzymatic oxidation of cytochrome c, determined under similar conditions, was subtracted during calculations for the specific activity. Potassium cyanide was added to a final concentration of 30 μM to the assay mixture to inhibit cytochrome c oxide (Co) when determining the bc₁ activity in ICMS or chromatophores.

Preparation of Electron Transfer Complex Inlaid PL Vesicles—Protein-PL vesicles were prepared by the cholate dialysis method of Kogoma and Racker (30). Bovine heart ubiquinol-cytochrome c reductase or CoE, either singly or in combination with R. sphaeroides wild-type or mutant bc₁ complex, was mixed with 1 ml of asolectin micellar solution to give an asolectin (mg)/protein (mg) ratio of 40. The asolectin micellar solution was prepared by sonicating 200 μM of acetone-washed asolectin in 4 ml of 50 mM sodium phosphate buffer, pH 7.4, containing 1 mM EDTA. The dialysis dilute samples were added to 1 ml of assay mixture containing 100 mM of sodium phosphate buffer, pH 7.4, 0.3 mM EDTA, 50 μM ferricytochrome c, and 25 μM Q₁/C₁Br₂H₄. Activities were determined by measuring the redox of cytochrome c by following the increase in the absorbance at 550 nm, in a Shimadzu UV-2101 PC spectrophotometer at 23 °C using a millimolar extinction coefficient of 18.5 mM⁻¹ cm⁻¹ for calculations. The non-enzymatic oxidation of cytochrome c, determined under similar conditions, was subtracted during calculations for the specific activity. Potassium cyanide was added to a final concentration of 30 μM to the assay mixture to inhibit cytochrome c oxide (Co) when determining the bc₁ activity in ICMS or chromatophores.

Determination of Proton Translocating Activity of bc₁ Complex—BC-17 cells were grown semi-aerobically in 800 ml of enriched Sistrom’s medium in 2-liter Bellco flasks with vigorous shaking (150 rpm) for about 26 h at 30 °C in the dark. The inoculation volumes used for semi-aerobic growth were at least 5% of the total volume. Antibiotics were added to the following concentrations: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; and trimethoprim (100 μg/ml for E. coli and 1 μg/ml for R. sphaeroides), and tetracycline (10 μg/ml for E. coli and 30 μg/ml for R. sphaeroides).

Enzyme Preparations and Activity Assay—Chromatophores and intra-plastidic membranes (ICMs) were prepared as described previously (10) and stored at −80 °C in the presence of 20% glycerol. The GfB complex cytochrome bc₁ complexes were purified from those ICMS or chromatophores by the method of Tian et al. (10). Purified cytochrome bc₁ complexes were stored at −80 °C in the presence of 10% glycerol. To assay ubiquinol-cytochrome c reductase activity, membrane preparations or purified cytochrome bc₁ complexes were diluted with 50 mM Tris-Cl, pH 8.0, containing 200 mM NaCl and 0.01% n-dodecyl β-D-maltopyranoside (DM) to a final cytochrome bc₁ concentration of 1 μM. The diluted samples were added to 1 ml of assay mixture containing 100 mM of sodium phosphate buffer, pH 7.4, 0.3 mM EDTA, 50 μM ferricytochrome c, and 25 μM Q₁/C₁Br₂H₄. Activities were determined by measuring the redox of cytochrome c by following the increase in the absorbance at 550 nm, in a Shimadzu UV-2101 PC spectrophotometer at 23 °C using a millimolar extinction coefficient of 18.5 mM⁻¹ cm⁻¹ for calculations. The non-enzymatic oxidation of cytochrome c, determined under similar conditions, was subtracted during calculations for the specific activity. Potassium cyanide was added to a final concentration of 30 μM to the assay mixture to inhibit cytochrome c oxide (Co) when determining the bc₁ activity in ICMS or chromatophores.

Generation of R. sphaeroides Strains Expressing the His₆-tagged bc₁ Complex—Estelle was obtained from Associate Concentrate and purified according to the method of Kogoma and Racker (30). n-Dodecyl β-D-maltoside and n-dodecyl β-D-glucoside were from Anatrace. Ni-NTA gel and Qiagen Spin Prep Kits were obtained from Qiagen. 3-Dimercaptosuccinic acid (DMSA) and 1,4-benzoquinol (Q₀C₁₀Br₂H₂) was prepared in our laboratory as commercially available.
Cytochrome bc\textsubscript{1} Complex

Characterizations of mutants lacking the Rieske (2Fe-2S) cluster

| Strains | Cytochrome bc\textsubscript{1} complex | Purified complex |
|---------|---------------------------------------|-----------------|
|         | Cyt b/(cyt c\textsubscript{1} + cyt c\textsubscript{1}) | ISP content\textsuperscript{a} | Cyt b/(cyt c\textsubscript{1}) | ISP content\textsuperscript{b} |
| Complement | 1.20 | 1.00 | 1.31 | 1.00 |
| H131N | 1.16 | 0.09 | 0.46 | 0.00 |
| H152N | 1.20 | 0.58 | 0.50 | 0.40 |
| H131C | 1.21 | 0.64 | 0.80 | 0.55 |
| H131C/H152C | 1.20 | 0.98 | 1.10 | 0.82 |

\textsuperscript{a} A\textsubscript{max} = 551 nm for cytochrome c\textsubscript{1} and A\textsubscript{max} = 560 nm for cytochrome b.

\textsuperscript{b} ISP content was quantified using a Bio-Rad GS-700 imaging densitometer. It is expressed as a fraction of the amount present in the complement wild-type complex.

RESULTS AND DISCUSSION

Characterizations of mutants Carrying Mutations at the Histidine Ligands of [2Fe-2S] Cluster in ISP—Four R. sphaeroides mutants expressing His\textsubscript{g}-tagged bc\textsubscript{1} complexes with mutations at the histidine ligands of [2Fe-2S] cluster were generated for this study. They are three single mutants, H131N, H152N, and H131C, and a double mutant, H131C/H152C. For mutants to be useful for this study, the resulting mutant complexes must lack the [2Fe-2S] cluster but have protein and redox components similar to those in the wild-type complex.

When aerobically grown wild-type and mutant cells were inoculated into enriched Sistrom's medium at mid-log phase and subjected to anaerobic photosynthetic growth conditions, all four mutants did not grow. However, these mutants grew aerobically and semi-aerobically at rates comparable with that of wild-type cells. These results indicated that the four mutants have a defective cytochrome bc\textsubscript{1} complex, because this complex is absolutely required for photosynthetic growth of this bacterium.

When ICMs were prepared from semi-aerobically grown mutant cells and assayed for ubiquinol-cytochrome c\textsubscript{1} reductase activity, none was detected in the four mutant membranes. Absorption spectral analysis revealed that the content and absorption spectral properties of cytochromes b and cytochromes (c\textsubscript{1} + c\textsubscript{1}) in all these mutant membranes were similar to those in the complement chromatophores or ICM (see Table I), indicating that the mutation did not affect the assembly of cytochrome b and c\textsubscript{1} into the membrane. EPR analysis revealed that these four mutant membranes contained no [2Fe-2S] cluster of ISP, indicating that the mutation resulted in an inability of the [2Fe-2S] cluster to be ligated to apo-ISP, thereby leading to loss of bc\textsubscript{1} activity. Western blot analysis using antibodies against R. sphaeroides ISP showed that H131N, H152N, H131C, and H131C/H151C mutant ICMs had 9, 58, 64, and 98% of the apo-ISP in the wild-type chromatophores, respectively (see Table I). Apparently, the stability of apo-ISP was affected by the type of amino acid substituting for the histidine ligand and whether the alteration was at His-131 or His-152.

The finding that the H152N mutant ICM had more apo-ISP than did the H131N mutant membrane was similar to observations in yeast mutants of H161R and H181R (44). However, it differed from reports on mutation at histidine ligands of the bc\textsubscript{1} complex in all these mutant membranes were similar to those in the complement wild-type complex.

Other Biochemical and Biophysical Techniques—Protein concentration was determined by the method of Lowry et al. (38). Cytochrome b (39) and cytochrome c\textsubscript{1} (40) concentrations were determined according to published methods. SDS-PAGE was performed according to Laemmlli (41) using a Bio-Rad Mini-protein dual slab vertical cell. The polypeptides separated in the SDS-polyacrylamide gel were transferred electrophoretically to a 22-μm nitrocellulose membrane for Western blotting. Polyclonal antibodies generated against ISP of the R. sphaeroides bc\textsubscript{1} complex were used as the primary antibody to detect their respective antigens (10). Protein A-conjugated to horseradish peroxidase, from Bio-Rad, was used as the second antibody. The [2Fe-2S] cluster of ISP was determined by EPR, using a Bruker EMX spectrometer equipped with an Air Products flow cryostat, and by circular dichroism (42, 43), using a Jasco J-715 spectropolarimeter. Instrument settings are detailed in the legends of the relevant figures.

http://www.jbc.org/content/248/9/4979.full.pdf
Cytochrome \( bc_1 \) Complex

FIG. 1. EPR and CD characterization of the [2Fe-2S] cluster in the cytochrome \( bc_1 \) complexes of complement and mutant complexes. EPR spectra of purified \( bc_1 \) complexes of wild-type (A), H131C (B), and H131C/H152C (C) are shown in the left-hand panels. The \( bc_1 \) complexes were incubated with 1.5 mM ascorbate on ice for 30 min and frozen in liquid nitrogen. EPR spectra were recorded at 8 K with the following instrument settings: microwave frequency, 9.3 GHz; modulation frequency, 100 kHz; modulation amplitude, 6.3 G; time constant, 655.4 ms; and sweep time, 167.8 s. CD spectra for purified \( bc_1 \) complexes of complement (A), H131C (B), and H131C/H152C (C) are shown in the right-hand panels. The \( bc_1 \) complexes were diluted to a final concentration of 5.5 \( \mu \)M in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.01% DM. The experimental conditions were as follows: bandwidth, 1.0 nm; step resolution, 1.0 nm; cell length, 1 cm; and scan speed, 50 nm/min. Each trace represents an average of four data sets. Reduced (solid lines) and oxidized (dotted lines) \( bc_1 \) complexes were prepared by the addition of ascorbate and ferricyanide, respectively.

![EPR spectra of purified bc1 complexes](image)

FIG. 2. Absorption spectra of purified \( bc_1 \) complexes of the complement wild-type (A) and mutants lacking the [2Fe-2S] cluster, H131C/H152C (B), H131C (C), H131C (D), and H152N (E). Each optical spectrum is a calculated difference spectrum of the dithionite-reduced minus ferricyanide-oxidized cytochromes (\( A_{\text{max}} = 551 \text{ nm for cytochrome } c_1; A_{\text{max}} = 560 \text{ nm for cytochrome } b_c \)).

![Absorption spectra of purified bc1 complexes](image)

1.10 respectively, indicating that the binding affinity between cytochrome \( b \) and cytochrome \( c_1 \) was affected by the nature and position of the substituting amino acids. A decrease in the \( b/c_1 \) ratio indicates a decrease in binding affinity between the two cytochromes. Because the expressed \( R. \text{sphaeroides} bc_1 \) complex was His\(_6\)-tagged at the C terminus of cytochrome \( c_1 \), all cytochrome \( c_1 \) from the dodecyl maltoside-solubilized membrane, regardless of whether or not it was associated with cytochrome \( b \), was absorbed to the Ni-NTA column used in the one-step purification of the complex. Cytochrome \( b_c \), which was not tightly associated with cytochrome \( c_1 \), will appear in the effluent. Consequently, the eluted complex would have a lower \( b/c_1 \) ratio as compared with the wild-type complex. The possibility that the lower cytochrome \( b/c_1 \) ratio observed in the mutant complexes resulted from a difference in the effectiveness of DM solubilization of the mutant complex from the membrane has been ruled out. DM solubilization of the \( bc_1 \) complexes from the mutant membranes was comparable with that of the complement (wild-type) chromatophores. It should be mentioned that EPR signals for both \( b/c_1 \) and \( b_c/H \) were detected in the H131N mutant complex, indicating that this mutation did not affect the heme environments of cytochrome \( b \), despite the loss of more than 65% of cytochrome \( b \) from the DM-solubilized membrane during purification by an Ni-NTA column. The EPR signals for \( b/c_1 \) and \( b_c/H \) were observed in all of the [2Fe-2S] clusters lacking mutants (Fig. 3).

The purified H131N mutant complex contained no detectable apo-ISP or subunit IV, purified mutant complexes of H152N, H131C, and H131C/H152C have 40, 55, and 82%, respectively, of the amount of apo-ISP or subunit IV found in the complement complex. When the latter three mutant complexes were subjected to SDS-PAGE analysis, four major protein bands corresponding to cytochrome \( b \), cytochrome \( c_1 \), ISP, and subunit IV were observed, as in the complement wild-type complex (see Fig. 4). Thus, a significant amount of ISP, 40–82%, was still present in the [2Fe-2S] cluster-lacking mutant complexes but not in H131N. It has been reported that a yeast mutant strain whose [2Fe-2S] cluster is lacking has about 83% of the ISP, relative to cytochrome \( c_1 \), in the purified complex, when compared with its wild-type counterpart (46).

Clearly, the assembly of the \( bc_1 \) complex depends critically on the structural integrity of the head domain of ISP, particularly at the tip of the ISP where interaction between ISP and cytochrome \( b \) takes place. A unique feature of the ISP head domain structure is the tip area where the [2Fe-2S] cluster is located; the surface topology of the tip is strikingly smooth with only main chain atoms facing the solvent environment. Substitutions of H131C and H152C by molecular modeling using the bovine ISP structure as a template (Fig. 5) demonstrated the formation of two possible disulfide bonds, between Cys-141 and...
Cys-161 for one pair and between Cys-139 and Cys-158 for the other pair, in the absence of the [2Fe-2S] cluster. Together with the disulfide pair in the native structure between Cys-144 and Cys-160, the three pairs of disulfide bridges in the mutant would stabilize the structure of ISP at the tip region and, more importantly, perhaps maintain the smooth surface topology of the wild-type ISP, whereas any of the other single amino acid substitutions would inevitably destroy this important feature to a different extent. The H131N and H152N mutations were substitutions would inevitably destroy this important feature to a different extent. The H131N and H152N mutations were particularly unfavorable due to exposed large side chains.

Among the four [2Fe-2S] cluster-lacking mutant bc₁ complexes, H131C and H131C/H152C are suitable for probing the function of the [2Fe-2S] cluster in proton translocation, because they differ from the wild-type complex only in the lack of the [2Fe-2S] cluster. The contents of the subunits and spectral properties of cytochromes b and c₁ in these two mutant complexes are similar to those in the complement complex. Moreover, the amount of ISP in the purified complexes was over 50% compared with the complement (wild type) complex. Therefore, any changes in proton translocation activity detected in these two mutant complexes can be unambiguously attributed to the lack of a [2Fe-2S] cluster.

Proton Translocation Activity of Intact Mitochondrial bc₁ Complex Co-inlaid in PL Vesicles with Wild-Type and H131C/H152C Mutant of R. sphaeroides ISP. The horseradish peroxidase system was used to develop the membrane (B). Lane designations are the same as above. Sub.IV, subunit IV.

The model is represented with the bovine ISP structure, and the double mutation in ISP of R. sphaeroides bc₁ corresponds to H141C/H161C of the bovine sequence. The tip area of the ISP is highlighted with a red circle. The three pairs of disulfide bonds are depicted with the ball-and-stick model, with carbon atoms in black and sulfur atom in yellow, and are as labeled.
(H\(^{+}/e\)) in protein-PL vesicles containing intact mitochondrial bc\(_1\) and the H131C/H152C mutant complexes decreased as the relative amount of mutant complex in the vesicle increased. A complete loss of proton-pumping activity was observed when the mutant complex concentration was 3-fold that of the mitochondrial bc\(_1\) complex. An excess of the [2Fe-2S] cluster-lacking mutant complex, H131C/H152C (arrow 1), 3 \(\mu\)M CCCP (arrow 2), 6 \(\mu\)M of ferricyanide (arrow 3), and 5 \(\mu\)M of HC3 (arrow 4). Note: the proton-pumping ratio (H\(^{+}/e\)) = z/y.

**Table II**

| Vesicle type | H\(^{+}/e\) \(^{a}\) |
|-------------|---------------------|
| Mitochondrial bc\(_1\) | 1.9 |
| Wild type R. sphaeroides bc\(_1\) | 1.5 |
| Mitochondrial bc\(_1\) + wild type | 1.7 |
| Mitochondrial bc\(_1\) + H131C/H152C | 1.0 |
| Mitochondrial bc\(_1\) + thermolysin-digested bacterial bc\(_1\) | 1.0 |
| Mitochondrial bc\(_1\) + thermolysin-digested bacterial bc\(_1\) + ISF | 1.3 |

\(^{a}\) H\(^{+}/e\) is defined under "Experimental Procedures." For the experimental conditions, see legend to Fig. 6.

**Proton Translocation Activity of Intact Mitochondrial Cytochrome c Oxidase Co-inlaid in PL Vesicles with Wild-type and Mutant Bacterial Complexes**—The ability of the H131C/H152C mutant bc\(_1\) complexes to form proton-leaking channels was further demonstrated by co-embedding with bovine mitochondrial CcO. Like the cytochrome bc\(_1\) complex, electron transfer through CcO is coupled to proton translocation across the membrane in which the complex is housed. When purified bovine CcO was embedded in PL vesicles, the accumulation of vectorially translocated protons was detected during the oxidation of reduced cytochrome c as indicated by acidification of the external medium (Fig. 7). However, when CcO was co-inlaid in PL vesicles with a 5-fold or higher molar excess of the H131C/H152C mutant bc\(_1\) complex, the vesicles produced an instant alkalization phase during the oxidation of reduced cytochrome c. The pattern of pH increase was similar to that observed in CCCP-treated PL vesicles embedding CcO, thereby further indicating the presence of a proton-leaking channel in the bc\(_1\) complex lacking the [2Fe-2S] cluster. It should be noted that the proton translocation activity of CcO remained unchanged when co-inlaid in PL vesicles with a 5-fold molar excess of wild-type bc\(_1\) complex.

**Restoration of Proton Translocation Activity to Protein-PL Vesicles Containing Intact Mitochondrial bc\(_1\) and the Thermolysin-digested Bacterial Complex with the Head Domain of ISP**—Because the cytochrome bc\(_1\) complex lacking the [2Fe-2S] cluster had a proton-leaking channel, the cluster may function as a proton-exiting gate regulating the controlled, vectorial extrusion of protons across the bc\(_1\) complex. If removal of the [2Fe-2S] cluster of ISP made the bc\(_1\) complex proton-permeable, a bc\(_1\) complex with the ISP head domain removed should be similar, because the [2Fe-2S] cluster is located in the head domain of ISP. To confirm this prediction, a bacterial bc\(_1\) complex lacking the ISP head domain was prepared by thermolysin digestion and co-inlaid into PL-vesicles with intact mitochondrial bc\(_1\) complex. The proton-pumping activity of the resulting protein-PL vesicles was measured. No proton pumping was observed in protein-PL vesicles containing thermolysin-digested bacterial bc\(_1\) complex and mitochondrial bc\(_1\) complex at an 8:1 molar ratio. The requirement of a large excess of thermolysin-digested complex to abolish completely the proton transfer activity may be due to incomplete removal of the head domain of ISP by thermolysin. To examine this possibility, the digested complex, which has no ubiquinol-cytochrome c reductase activity, was subjected to SDS-PAGE and Western blot analysis. About 20% of the ISP in the complex was resistant to thermolysin digestion (see Fig. 8). Also, the extent of Rieske ISP cleaved by thermolysin in the H131C/H152C mutant complex was similar to that observed for the wild-type complex (Fig. 8). This indicated that the thermolysin cleavage site in the Rieske ISP was equally accessible to the protease in both the H131C/H152C mutant and wild-type complexes. Thus, the loss of the [2Fe-2S] cluster did not result in a conformational change of the Rieske ISP. Similar observations of incomplete ISP digestion are made with R. capsulatus, where incubation of the bc\(_1\) complex with thermolysin for prolonged periods, with fresh additions of protease at 4-h intervals, does not yield complete digestion (51).

Addition of excess purified ISF to proton-leaking PL vesicles co-embedding mitochondrial bc\(_1\) and thermolysin-digested bacterial bc\(_1\) complex partially restored the proton-pumping capability with H\(^{+}/e\) ratios ranging from 1.35 to 1.40. The purified ISF probably docks at the vacant position once occupied by the cleaved head domain of the ISP of the bacterial complex. Re-
introduction of the [2Fe-2S] cluster containing the head domain seals the proton channel across the $bc_1$ complex, preventing the uncontrolled and unimpeded proton flow across the protein-PL vesicles. As expected, the addition of excess ISP to PL vesicles embedding the mitochondrial $bc_1$ complex and the mutant complex, H131C/H152C, did not restore the proton-pumping activity because the mutant complex lacked only the [2Fe-2S] cluster, not the entire ISP, providing no room for added ISP to dock. Similarly, the addition of excess ISP to PL vesicles embedding the mitochondrial $bc_1$ complex and the 2-band $bc_1$ complex (a mutant $bc_1$ complex containing only the cytochrome $b$ and cytochrome $c_1$ subunits) did not restore the proton-pumping capability of such vesicles. The tail domain of ISP, which is present in the thermolysin-digested complex and absent in the 2-subunit $bc_1$ complex, may play a role in maintaining the conformation of the $bc_1$ complex required for docking of ISP.

**The Existence of Proton Pathway in the Cytochrome $bc_1$ Complex**—The observation of proton leakage in the $bc_1$ complex with damaged ISP suggested a passage in the complex, channeling proton backflow from the periplasmic to the cytoplasmic side (from the inter-membrane space to matrix in mitochondria). It is unlikely, although it cannot be ruled out, that such a channel exists in the trans-membrane (TM) region at the interfaces of different subunits, such as that between cytochromes $b$ and $c_1$, because the only damage needed to produce the proton leakage is the removal of the [2Fe-2S] cluster. It is conceivable that the culprit lies in the cytochrome $b$ subunit as it contributes most to the TM region of the complex. Based on the crystallographically refined structures (5, 8, 9), a solvent distribution around the subunit cytochrome $b$ was generated (Fig. 9), from which three principal solvent-accessible regions in cytochrome $b$ could be defined where water molecules penetrate deep into the subunit; they are the Qi site, the Qo site, and the dimer interface at the matrix side (Fig. 9). There is, however, a gap of roughly 17 Å in the mid-section of the TM domain where no ordered water molecules were found crystallographically.

Proton transfer pathways involving internal water molecules that provide hydrogen bonds and facilitate proton diffusion have been identified in other membrane proteins like bacteriorhodopsin (52) and CcO (53). Although the mechanisms for proton translocations are different for different proteins, the underlying principle for bringing water molecules or protons to the active sites is similar. Several pathways for proton leakage are possible. As indicated in Fig. 9, the proton entry for the leakage must be located at the Qo site where protons are ejected under normal circumstances. All other venues near the intermembrane space side are sealed. In the native cytochrome $bc_1$ complex, the [2Fe-2S] cluster undergoes redox state changes during the catalytic cycle. The ejection of protons must be controlled by the protonation and deprotonation of the histidine ligands of the [2Fe-2S] cluster. The histidine ligands uptake a proton from the substrate, ubiquinol, upon reduction of the [2Fe-2S] cluster and release them to the inter-membrane space when oxidized by cytochrome $c_1$, as was suggested recently (14). It is less clear how the second proton is pumped out, although Glu-271 of cytochrome $b$, as speculated recently (14). The absence of the histidine ligands, and thus the loss of the [2Fe-2S] cluster, explain well the loss of the proton-pumping capability in PL vesicles incorporating the mutant $bc_1$ complexes. The exact mechanism of how protons leak into the Qo site in the absence of the [2Fe-2S] cluster remains to be seen.

There are two likely exit pathways for the proton leakage; one is via the Qi site, and the other is through a hole at the dimer interface (Fig. 9). We have suggested earlier (9) that two residues in the Qi pocket may be involved in fetching protons from the matrix side; Lys-227 and His-201 undergo conformational changes that are coupled to ubiquinone reduction at the Qi site. The same residues could serve as an exit gate in the case of proton leakage, except that the gate is decoupled to ubiquinone reduction and is spontaneously open. Alternatively, the proton could exit from the hole at the dimer interface near the matrix side (Fig. 9). The hole is just outside His-201 and is filled with ordered water molecules.

The possibility of a direct proton translocation path going through the interior of cytochrome $b$ is slim because the overall structure is rather rigid as analyzed by the binding of various inhibitors (8). A likely pathway linking the entrance at the Qo site to the exit gate at the Qi site for spontaneous proton leakage is the large cavity formed between the two symmetry-related cytochrome $b$ subunits. Although this cavity is fairly hydrophobic, one could argue that the existence of the membrane potential (pH gradient) could overcome the thermodynamic barrier and facilitate proton movement from one side to the other.

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Buddha Gurung, Linda Yu, Di Xia and Chang-An Yu

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