STUDIES OF EXTRA FRAGMENTS OF THE CYTOCHROME $BC_1$ COMPLEX FROM RHODOBACTER SPHAEROIDES AND THE INTERACTION BETWEEN CYTOCHROME $CAA_3$ AND $F_1F_0$-ATP SYNTHASE FROM ALKALIPHILIC BACILLUS PSEUDOFIRMUS OF$_4$

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STUDIES OF EXTRA FRAGMENTS OF THE CYTOCHROME $BC_1$ COMPLEX FROM RHODOBACTER SPHAEROIDES AND THE INTERACTION BETWEEN CYTOCHROME CAA$_3$ AND $F_1F_0$-ATP SYNTHASE FROM ALKALIPHILIC BACILLUS PSEUDOFIRMUS OF$_4$

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<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>cyt</td>
<td>cytochrome</td>
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<td>dam</td>
<td>DNA adenine methylation</td>
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<td>DM (LM)</td>
<td>Dodecyl β-maltoside</td>
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<td>DSC</td>
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<td>E. coli</td>
<td>Escherichia coli</td>
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<td>EPR</td>
<td>Electron paramagnetic resonance</td>
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<td>HRP</td>
<td>Horseradish peroxide</td>
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<td>ICM</td>
<td>Intra-cytoplasmic membrane</td>
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<td>ISP</td>
<td>Rieske iron-sulfur protein</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>kb</td>
<td>Kilo base pair</td>
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<tr>
<td>LB</td>
<td>Lennox L. Broth</td>
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<td>MALDI-TOF</td>
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<td>rpm</td>
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<td>R. sphaeroides</td>
<td>Rhodobacter sphaeroides</td>
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<td>Sodium dodecyl sulfide</td>
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<td>Tris</td>
<td>Tris (hydroxymethyl) aminomethane</td>
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<td>UHDBT</td>
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Chapter I

Introduction

Cytochrome $bc$ complexes in Respiration and Photosynthesis

Ubiquinol-cytochrome $c$ oxidoreductase, also known as complex III or cytochrome $bc_1$ complex, which belongs to the superfamily of ‘$bc$ complexes’, is present in the inner membrane of the mitochondria (Fig. 1) or in the cytoplasmic membrane of many bacteria (1-5). Cytochrome $bc_1$ complex, the central component of the energy-conserving electron transfer pathway for their respiration or photosynthesis, catalyzes electron transfer from ubiquinol to soluble $c$-type cytochromes ($cytc$ in mitochondria, $cytc_2$ in some bacteria) with concomitant generation of a proton gradient and membrane potential, which is referred to as proton motive force (pmf). The proton motive force is the driving force for synthesis of ATP by the complex V ($F_1F_0$-ATP synthase) (Fig. 1) in the inner mitochondria membrane.

All cytochrome $bc_1$ complexes contain three core subunits, cytochrome $b$, cytochrome $c_1$, and Reiske iron-sulfur protein (ISP), which house two $b$-type hemes ($b_L$ and $b_H$), one $c$-type heme (heme $c_1$), and a high potential [2Fe-2S] cluster (6), respectively.
Fig. 1. Mitochondrial electron transfer chain.
In mitochondria, the cytochrome $bc_1$ complex is a part of the respiratory electron transport chain. The electrons derived from glucose and fatty acids oxidation are transferred to the NAD$^+$ and FAD to form NADH and FADH$_2$ in the reactions catalyzed by the glycolytic enzymes, β-oxidation enzymes and the citric acid cycle enzymes (Fig.2.). The electrons, from reoxidation of NADH and FADH$_2$, pass through the electron-transport chain, composed of four complexes: NADH-ubiquinol reductase (complex I) (7) (Fig.1.), succinate-ubiquinol reductase (complex II), ubiquinol-cytochrome c reductase (complex III or cytochrome $bc_1$ complex), and cytochrome c oxidase (complex IV), to generate the pmf as mentioned above.

In addition to the respiratory system, the cytochrome $bc_1$ complex is also found in the electron transport system of purple photosynthetic bacterium. In the facultative photosynthetic purple nonsulfur bacterium *Rhodobacter sphaeroides*, the first steps of the conversion of light into chemical energy are accomplished by a cyclic electron transfer system, which generates a proton gradient across the chromatophore membrane. Photo-induced cyclic electron transfer involves two large transmembrane multiprotein complexes, the reaction center (RC) and the cytochrome $bc_1$ complex, functionally connected through redox reactions of quinone in the lipid phase and of a water soluble protein (cytochrome $c_2$) in the periplasmic phase (8). Absorption of one photon leads to photo-oxidation of the primary electron donor, P870 (a bacteriochlorophyll), of the RC, which delivers an electron through a bacteriopheophytin to the primary quinone acceptor $Q_A$. The electron is then transferred inside the reaction center to the secondary quinone acceptor $Q_B$, which, equilibrates with the Q-pool in the membrane following its full reduction and protonation to $QH_2$ after accepting two electrons. The $QH_2$ now serves as
Fig. 2. A summary of energy-generating metabolism in mitochondria(9).
the substrate for the cytochrome $bc_1$ complex reaction. Same as in the mitochondrial $bc_1$
complex, for every mole of $\text{QH}_2$ that is oxidized, two moles of cytochrome $c_2$ are
reduced, and four moles of proton are translocated across the membrane. The cycle is
completed by re-reduction of the photo-oxidized P870 by reduced cytochrome $c_2$ (10). In
many other purple bacteria, like *Rhodopseudomonas viridis*, a RC-bound tetraheme
cytochrome c, containing two high-potential and two low-potential hemes, acts as the
immediate electron donor to P870 (10) (Fig. 3). Therefore, in the cyclic pathway, there is
no exogenous electron donor or a final electron acceptor. Electrons continuously recycle
between the reaction center and the $bc_1$ complex with light energy serving as the driving
force, resulting protons pumped across the membrane to form the proton motive force.

The cytochrome $b_6f$ complex, plastoquinol-plastocyanin oxidoreductase, which also
belongs to superfamily of $bc$ complex, functions in the electron transport chains of
oxygenic photosynthesis carried out by algae, *Chlamydomonas reinhardtii* (11),
thermophilic cyanobacteria, *Mastigocladus laminosus* (12) and higher plants. The
multisubunit $b_6f$ membrane protein complex mediates electron transfer between the
photosystem II, in which $\text{H}_2\text{O}$ is the electron donor, and the photosystem I. Consistent
with a basic paradigm of membrane energy transduction (13), electron transfer through
the $b_6f$ complex is coupled to proton translocation across the membrane. The transfer of
protons across the $b_6f$ complex, utilizing oxidation and reduction of lipophilic quinone,
establishes a proton electrochemical potential gradient across the membrane that is
negative on the stromal side and positive on the thylakoid luminal side (14-18). The
chloroplast $b_6f$ complex is a dimer and comprised of 8–9 subunits (4–5 large and 3–4
small subunits) (20), with its four “large” subunit being cytochrome $f$, which contains
Fig. 3. Photosynthetic electron-transport system of purple photosynthetic bacteria. (a) A schematic diagram indicating the arrangement of the system components in the bacterial plasma membrane and the flows of electrons (black arrows) and protons (blue arrows) that photon ($h\nu$) absorption promotes through them. (b) The approximate standard reduction potentials of the photosynthetic electron-transport system’s various components (19).
one c-type heme, cytochrome $b_6$, which contains two $b$-type hemes, subunit IV, and a Rieske iron-sulfur protein which contains one [2Fe-2S] cluster. The cytochrome $b$ of mitochondrial complex III is a fusion of chloroplast cytochrome $b_6$ and subunit IV. The cytochrome $b$ subunit of the cytochrome $b_6f$ complex corresponds to the N-terminal heme-bearing part of $bc_1$ complex’s cytochrome $b$ subunit, while a sequence similar to the C-terminal part presents in subunit IV (21,22). The Rieske iron-sulfur proteins in the two complexes are homologous and structurally similar. However, cytochrome $f$ is unrelated to its counterpart in complex III, cytochrome $c_1$ although both are $c$-type cytochromes (19). Moreover, there are at least three small subunits in higher plant cytochrome $b_6f$, called PetA, PetM and PetL subunit (23-26).

The photosynthetic pathway occurring in chloroplast with cytochrome $b_6f$ complexes is very complicated. There are two photosynthetic systems in this pathway, photosystem I (PSI) and photosystem II (PSII), the latter is equivalent to the reaction center in $R. sphaeroides$. In chloroplast, water is the electron donor for the photosynthesis. Electrons are released from a water splitting reaction mediated by a Mn-containing protein complex, and $O_2$ is also generated in this reaction. Plastoquinol (PQ), equivalent to ubiquinol, which is reduced in photosystem II, transfers an electron through the cytochrome $b_6f$ complex to plastocyanin (PC), functional analog of cytochrome $c$. The reduce PC then transfers the electron to photosystem I, whose redox potential is low enough to reduce NADP$^+$. After being excited by light energy, photosystem I transfers the electron to NADP$^-$ and produces NADPH via an NADP reductase. Therefore, a strong electron donor (NADPH) is produced by this pathway in addition to generating the proton motive force. Alternatively, the electron may be returned to the cytochrome $b_6f$
complex in a cyclic process that only translocated protons into the thylakoid lumen (Fig.4.).

Proton-motive Q cycle

The proton-motive Q-cycle model (27, 28), which was first proposed by Mitchell (27) and modified later (7, 29, 30), has been favored for describing electron transfer, the proton translocation, the 2:1 \( H^+ / e^- \) ratio and the “oxidant-induced reduction of cytochrome \( b \)” in the cytochrome \( bc_1 \) complex. There are two features for this Q-cycle mechanism (7, 29, 31, 32) (Fig.5.). (1) The presence of two separate ubiquinol/ubiquinone binding sites; an ubiquinol oxidation site (Qo) near the P (intermembranes space) side of the mitochondrial inner membrane, and a ubiquinone reduction site (Qi) near the N (matrix) side. (2) The bifurcated electron transfer at the Qo site. The mechanism of ubiquinol oxidation at Qo site involves a divergent oxidation in which the ubiquinol at the Qo site is oxidized by ISP and cytochrome \( b_L \) (4, 33) either in a concerted mechanism (34-37) or in a sequential mechanism (38-42). In the concerted mechanism, oxidation of ubiquinone requires that both the iron-sulfur protein and cytochrome \( b_L \) be oxidized and that cytochrome \( b_L \) reduction precedes iron-sulfur protein reduction of cytochrome \( c_1 \) (36). In the sequential mechanism, the iron-sulfur protein oxidizes the quinol to semiquinone, which then reduces cytochrome \( b_L \) (Fig.5B). However, the presence of such semiquinone has not been detected (35, 43).

In both mechanisms, two protons are released into the positive side of the membrane when the quinol is oxidized. The first electron is transferred to cytochrome \( c \)
Fig. 4. Detailed diagram of the photosynthesis. OEC, oxygen evolving complex; FQR, ferredoxin-plastoquinone reductase; FNR is ferredoxin-NADP+ reductase (44).
Fig. 5. The proton motive Q cycle with (A) concerted and (B) sequential bifurcated reaction at Qo site.
through the high potential chain, which has been transferred to ISP, heme $c_1$ and then to heme $c$ ($c_2$ in bacteria). The other electron is transferred through the low potential chain, which has been transferred to heme $b_L$ and heme $b_H$. At the Qi site, a ubiquinone (Q) accepts an electron from the $b_H$ heme to form a relatively stable ubisemiquinone ($Q^{+p}$). At this point, the reaction is only half complete, with only one of the two electrons from the ubiquinol being transferred to cytochrome $c$. In the second half of the Q cycle, all steps are repeated: one ubiquinol is oxidized, one cytochrome $c$ is reduced, two protons are deposited into the positive side of the membrane, and the $b_H$ heme is reduced via the $b_L$ heme. At the Qi site, the ubisemiquinone, which is generated in the first half of the Q cycle, accepts another electron from the $b_H$ heme and uptakes two protons from the negative side of the membrane to form ubiquinol ($QH_2$) and complete one Q cycle. The net result of one complete Q cycle generates one molecule of oxidized ubiquinone, two molecules of reduced cytochrome $c$, uptake two protons from the negative side of the membrane, and deposits four protons to the positive side of the membrane,

$$QH_2 + 2 \text{ cyt } c^{3+} + 2H^+_{(N-side)} \rightarrow Q + 2 \text{ cyt } c^{2+} + 4H^+_{(P-side)}$$

The proton motive Q-cycle mechanism is supported by many biophysical and biochemical experimental results:

a) It accounts for the oxidant-induced cytochrome $b$ reduction phenomenon.

A pulse of oxygen induces oxidation of cytochrome $c$ and $c_1$, which is accompanied by a transient reduction of cytochrome $b$. Rapid oxidation of the $c$-type cytochromes and ISP accelerated ubiquinol oxidation, resulting in a transient increased reduction of cytochrome $b$. Antimycin blocks reoxidation of $b_H$, enhancing the oxidant-induced reduction (45). Therefore, in the presence of
ferricyanide, not only cytochrome $c_1$ becomes oxidized as expected but cytochrome $b$ is reduced (46).

b) There are two groups of inhibitors that bind to Qi and Qo sites. Qo site inhibitors block the oxidation of ubiquinol, whereas the Qi site inhibitors block the electron transfer from heme $b_H$ to quinone or semiquinone (4, 7, 47-49). Class I inhibitors are further divided into three sub-classes (Ia, Ib, and Ic) based on chemical characteristics of the inhibitors, and on spectroscopic and biophysical effects of the $b_L$ heme and the iron-sulfur cluster of ISP upon binding of the inhibitors. Class Ia inhibitors typically contain a $\beta$-methoxyacrylate (MOA) group (or a close chemical analogue) as a characteristic structural element and are referred to as the MOA inhibitors; they presumably block the electron transfer from quinol to the ISP, accompanying a red shift in the $\alpha$ and $\beta$-bands of the reduced heme $b_L$ spectrum. Examples of the class Ia inhibitors are MOAS and myxothiazol. Class Ib inhibitors possess a chromone ring system and are believed to inhibit electron transfer from ISP to cyt $c_1$; they generate a pronounced increase in redox potential of the ISP and, like class Ia inhibitors, also cause a red shift of the reduced heme $b_L$ spectrum. Stigmatellin is a representative of class Ib inhibitors. Class Ic inhibitors are 2-hydroxy quinone analogues such as 5-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT); they block the electron transfer in a similar way to the chromone inhibitors, but cause a smaller positive redox potential shift of the ISP and have no effect on the spectrum of the $b_L$ heme. Azoxystrobin (56) and famoxadone (51) also belong to Class I inhibitors. Antimycin belongs to class II inhibitors. NQNO is a dual site inhibitor (51,52) (Fig.6).
Fig. 6. Chemical structures of $bc_1$ inhibitors.
c) Two protons being translocated across the membrane per one electron was observed during \(bc_1\) complex catalysis (53).

d) A stable ubisemiquinone radical was detected in isolated mitochondrial \(bc_1\) complex and the EPR signal from this semiquinone was eliminated upon addition of antimycin A (54), an inhibitor with higher affinity for Qi site than ubiquinone or ubisemiquinone.

Three-Dimensional Crystal Structure of Cytochrome \(bc_1\) Complex

On the basis of x-ray diffraction data to a resolution of 2.9Å, atomic model of the \(bc_1\) complex from bovine heart mitochondria was first built by Chang-an Yu’s group in collaboration with Deisenhofer’s group in 1997 (55). Crystallographic structures of mitochondrial \(bc_1\) complex from other sources such as chicken and yeast were available from other groups (56-58), and later the resolutions were improved to 2.4Å in the bovine \(bc_1\) complex structure (52).

a) Structure overview:

An overall view of the cytochrome \(bc_1\) complex viewed parallel to the membrane is presented in Fig.7. Two \(bc_1\) complex monomers interact in the crystal to form a dimer around a crystallographic twofold symmetry axis. The dimeric \(bc_1\) complex is pear-shaped with a maximal diameter of 130 Å and a height of 155 Å (55). The cytochrome \(bc_1\) complex can be divided into three regions, the intermembrane space region, the transmembrane helix region and the matrix region. The intermembrane space region is composed of the functional domain of cytochrome \(c_1\), ISP and subunit 8
Fig. 7. Ribbon model of the bovine dimeric cytochrome bc₁ complex (59) from bovine heat mitochondria. The molecule can be divided into three regions from the top to bottom: the intermembrane space region, membrane-spanning region and the matrix region. The dimensions for each region of the molecule and the color code for each subunit are indicated.
extending 38Å into the cytoplasm from the membrane surface. The transmembrane helix region is about 42Å thick with thirteen transmembrane helices in each monomer. Most parts of cytochrome \( b \) are located within the membrane. It has eight transmembrane helices, and the transmembrane region of cytochrome \( b \) houses heme \( b_L \), \( b_H \), and Qo, Qi pocket. Subunit 7, 10, and 11 each has a transmembrane helix. ISP and cytochrome \( c_1 \) each has a transmembrane anchor in their N-terminal and C-terminal, respectively. Two symmetry related large cavities are found in the membrane spanning region, which are made of the transmembrane helices D, C, F and H in one monomer and helices D’ and E’ from the other monomer of cytochrome \( b \), and the transmembrane helices of cytochrome \( c_1 \), ISP subunit 10 and 11 (Fig.14A). More than half of the molecular mass is located in the matrix region of the molecule, extending from the trans-membrane helix region by 75Å, which consists of core 1, core 2, subunit 6, and part of subunit 7 and subunit 9. Subunit core II and cytochrome \( b \) contribute major dimer interactions across the two-fold symmetry axis.

b) Cytochrome \( b \) subunit:

The cytochrome \( b \) subunit of the bovine \( bc_1 \) complex has 379 amino acid residues and consists of eight membrane spanning helices named sequentially from A to H with both the N and C-terminal of cytochrome \( b \) subunit located in the mitochondrial matrix. The eight transmembrane helices of cytochrome \( b \) are arranged in two helical bundles: bundle I consists of the first five helices that incorporates two \( b \)-type hemes (\( b_L \) and \( b_H \)); bundle II is made of the rest of the helices. The two helical bundles contact each other at the matrix site of the membrane but separate from each other at the intermembrane space, thus creating the so-called Qo pocket between the two bundles near the intermembrane
space side. The CD loop contains two helices cd1 and cd2 of 15 and 9 residues, respectively, arranged in a hairpin, providing a “lid” for the Qo site and contributing residues to the docking site (the ISP crater) for interaction with ISP. The EF loop bridges between the two helical bundles as well as takes part in the formation of the ISP interaction site. Toward the end of the EF loop, there is a 12-residue helix, named ef, situated in a central position inside the Qo pocket. The PEWY motif, conserved in all organisms, is found at the beginning of the helix (50, 51) (Fig.8).

The Qi pocket (52) is located near the matrix side of the membrane with its entrance open to the center of the membrane bilayer. From the entrance, the cavity dives nearly vertically toward the matrix side of the membrane and is surrounded by residues from transmembrane helices A (Trp$^{31}$, Asn$^{32}$, Gly$^{34}$, Ser$^{35}$), D (Ala$^{193}$, Met$^{194}$, Leu$^{197}$, His$^{201}$), and E (Tyr$^{224}$, Lys$^{227}$, Asp$^{228}$); the amphipathic surface helix a (Phe$^{18}$); the A loop (Ile$^{27}$); the DE loop (Ser$^{205}$, Phe$^{220}$); and atoms from the high-potential heme $b_h$. These residues are identified by their interactions with the bound antimycin A (Fig.9A), NQNO (Fig.9B), and substrate ubiquinone (Fig.9C) in the cyt$b$ subunit. Among these residues, Ser$^{35}$, His$^{201}$, Lys$^{227}$, Asp$^{228}$, and Ser$^{205}$ are highly conserved (Fig.10) and located near the bottom of the pocket. The Qi pocket has a hydrophobic entrance but turns progressively hydrophilic inward; it has the dimensions of roughly 7 x 10 x 13Å and is potentially capable of accessing the mitochondrial matrix through the conserved residues His$^{201}$ and Lys$^{227}$.

The Qo pocket of the native $bc_1$ complex (50) has the shape of a saddle. With a relatively wide opening at one end, it becomes increasingly narrower moving inwards; the pocket gets roomy again after the narrow, flat constriction and is sealed at the other end.
Fig. 8. Ribbon diagram of the dimeric cyt*b and ISP in the $bc_1$ complex. Two cyt*b subunits (labeled cyt*b in light blue and cyt*b-sym in green, respectively) and two ISP subunits (labeled ISP in yellow and ISP-sym in red, respectively) related by a molecular 2-fold symmetry are shown. The eight transmembrane helices of cyt*b are named sequentially from A to H. The two $b$-type hemes labeled $b_L$ and $b_H$ are shown as the ball-and-stick models. Two active sites in the cyt*b are labeled: one is Qi site, the other is Qo site. The surface depression in cyt*b on the inter-membrane space side is labeled as the ISP-docking crater for interacting with the ISP.
Fig. 9. Interactions of the protein environment at the Qi site of the cyt b subunit with bound inhibitors and substrate. Secondary structure elements surrounding the Qi pocket, including portions of the N-terminal helix a, transmembrane helices A, D, and E, and extramembrane loops A and DE, are shown and labeled. Residues interacting with bound inhibitors or substrate and the $b_{11}$ heme are drawn in stick models and are labeled with carbon atoms in yellow, nitrogen in blue, oxygen in red, and iron in orange. H-bonds are indicated with the pinkish dotted lines. Water molecules are shown as the isolated red balls. Inhibitors and the substrate ubiquinone are drawn as the ball-and-stick models with carbon atoms in black, nitrogen in light blue, and oxygen in red. (A) Qi pocket with the bound antimycin A, (B) Qi pocket with the bound NQNO, and (C) Qi pocket with the bound ubiquinone with two isoprenoid units visible in electron density.
Fig. 10. Sequence alignments of Qi pocket residues of cytb subunits from different species: BT (B. taurus), SC (S. cerevisiae), SP (S. pombe), LT (L. tarentolae), RC (R. capsulatus), and RR (R. rubrum). The helical secondary structure elements are indicated with the sinuous curves in brown and as labeled. Amino acid positions in the bovine sequence are numbered above the sequences. Absolutely conserved residues in all sequences are colored red, those with a single conserved change are in magenta and those with two or more conserved changes are in green.
end (Fig.11). The Qo pocket is highly hydrophobic and lined with six prominent aromatic residues in addition to a number of aliphatic residues. The pocket is delimited against the membrane by three transmembrane helices (B, C and E) and by the buried ef helix, and capped by the cd1 and cd2 helices isolating the Qo site from the intermembrane space. Moreover, His161 of the ISP, ligand to one of the iron atoms in the 2Fe-2S cluster, may also contribute to the Qo site when the ISP is bound at the Qo site. The Qo pocket is exceptionally rich in highly conserved residues (Fig.12).

c) Iron-sulfur protein (ISP)

The structure of Rieske iron-sulfur protein (ISP) can be divided into three domains: the N-terminal domain, tail domain, (residues 1-62); the flexible linking domain, neck region, (residues 63-72); the soluble C-terminal domain, head domain, (residues 73-196), which is located in the intermembrane space region. The Rieske iron sulfur subunit is anchored to the membrane by the tail domain, and no ordered secondary structure was reported in the neck region (55, 56). In the bovine heart mitochondrial cytochrome $bc_1$ complex, the [2Fe-2S] cluster is coordinated by two cysteine and two histidine residues (Cys$^{139}$ and His$^{141}$; Cys$^{158}$ and His$^{161}$) (60) (Fig.12.) within a C-terminal water soluble domain which is bound to the rest of the $bc_1$ complex through this N-terminal membrane anchor (61, 62). In ISP, the [2Fe-2S] cluster is located at the tip of the head domain. Moreover, ISPs extend across the interface between the two monomers, with the transmembrane helix in one monomer and the head domain within the other, thus forming the intertwining dimer (59).

d) Cytochrome $c_1$ subunit.

Cytochrome $c_1$ both structurally and functionally links the cytochrome $bc_1$ complex
Fig. 11. Structural environment of the native Qo site. The Qo pocket is depicted as a GRASP (63) surface with the surrounding secondary structure elements. Helices cd1, cd2 and ef as well as parts of B, C, E and F helices are shown and labeled. The heme $b_L$ of cyt$b$, 2Fe-2S cluster of the ISP, and the conserved residues are also shown in the ball-and-stick form. Carbon atoms are colored yellow, oxygen red, nitrogen blue and sulfur green.
Fig. 12. Sequence alignment of the Qo pocket residues. The Qo site is composed of residues from two sequence segments: one from residue 120 to 182 and another from residue 250 to 300. The sequence shown include BT (B. Taurus), SC (S. cerevisiae), SP (S. pombe), RC (R. capsulatus), RR (R. rubrum), SU (Sea Urchin, S. purpuratus), ST (S. tenacellus) and WT (wheat cyt b6, T. aestivum). Helical elements are indicated as green boxes and labeled. Absolutely conserved residues in all sequences are colored red; highly conserved residues with one conserved change are colored blue; highly conserved residues with more than one conserved change are colored magenta.
Fig. 13. Structure of the “Rieske” [2Fe-2S] cluster.
with its physiological redox partners, soluble or membrane-attached cytochromes c or head domain of high-potential iron-sulfur proteins. Cytochrome c\(_1\) provides a docking site for these proteins on its surface at the membrane-aqueous interface. Cytochrome c\(_1\) is oxidized by cytochrome c (in bacteria, cytochrome c\(_2\)), in turn, the oxidized cytochrome c\(_1\) acts to oxidize the [2Fe2S] cluster, which then moves to the Qo site. Cytochrome c\(_1\) is attached to the membrane by a transmembrane helix located at the C-terminal end of the protein. The hydrophilic extramembrane domain including the heme is located in the intermembrane space region. The extrinsic domain of cytochrome c\(_1\) contains the consensus heme-binding motif (CXXCH) located close to the N-terminus of the polypeptide that provides sites for covalent attachment of the heme group (cysteines) and fifth axial ligation to the heme iron (histidine). It also contains the invariant methionine residue present near the C-terminus that acts as the sixth axial ligand to the heme iron (56, 57, 64). The carboxy group of one of the propionates of the heme c\(_1\) forms a salt bridge with Arg\(^{120}\), while the other propionate of the heme c\(_1\) extends toward the ISP. A methyl group on the porphyrin ring is solvent-exposed, likely near the binding site for cytochrome c (6). The x-ray crystal structures of beef and chicken cytochromes bc\(_1\) have revealed that the exposed heme CD edge of cytochrome c\(_1\) on the cytoplasmic surface of the membrane is surrounded by acidic residues that could form a docking site for cytochrome c (55-57). The acidic residues (Glu\(^{74}\), Glu\(^{101}\), Asp\(^{102}\), Glu\(^{104}\), Asp\(^{109}\), Glu\(^{162}\), Glu\(^{163}\), and Glu\(^{168}\)) on the surface of cytochrome c\(_1\) of R. sphaeroides cytochrome bc\(_1\) complex are involved in binding positively charged cytochrome c. These acidic residues on opposite sides of the heme crevice of cytochrome c\(_1\) direct the diffusion and binding of cytochrome c from the intramembrane space (65).
Functional Indication from Structural Analysis of Cytochrome \(bc_1\) Complex

a) Head domain movement of ISP

Mobility of ISP head domain in the \(bc_1\) crystal was first suggested by observation of a particularly low electron density of its extramembrane head domain (55). The mobility of the head domain of ISP was further substantiated by the anomalous light scattering signals of the [2Fe-2S] cluster observed in native and co-crystals with various Qo site inhibitors. In a native bovine heart mitochondrial \(bc_1\) crystal, a much weaker anomalous light scattering signal was observed for the [2Fe-2S] cluster compared with that for the heme iron, \(b_{H}\) or \(b_{L}\), despite the presence of two irons in the cluster. Furthermore, the electron density of the anomalous light scattering signal of [2Fe-2S] cluster was strongly enhanced in a co-crystal with stigmatellin or UHDBT, indicating that these inhibitors arrest the mobility of ISP in the fixed state position (66), and ISP head domain is on the surface of cytochrome \(b\) (\(b\) position). Conversely, the electron density of the anomalous scattering peak of [2Fe-2S] is abolished in a co-crystal with MOA-stilbene or myxothiazol, indicating that these inhibitors increase the mobility of ISP in the crystal and [2Fe-2S] has no predominant position, the ISP head domain is in a released state and found at the \(c\)-interface (\(c_1\) position) (56, 67).

The hypothesis of the mobility of the ISP head domain not only provides an explanation for the unexpected findings in those crystal structures, but it also provides an explanation for the bifurcated reaction at the Qo site. The bifurcated quinol oxidation at the Qo site is the key step in the Q cycle mechanism as mentioned in the previous section. The major problem for the obligatory bifurcated reaction is that one of the two electrons
from quinol has to be transferred to the unfavorable low potential chain (cytochrome $b_L$ and $b_H$). It’s difficult to explain why both electrons are not moved to the thermodynamically favorable high potential chain (ISP and cytochrome $c_1$). On the other hand, in the bovine heart mitochondrial $bc_1$ crystal, the distances between heme $b_L$ and the [2Fe-2S] cluster and between the [2Fe-2S] cluster and heme $c_1$, are 27 and 31 Å, respectively. Although the distance of 27 Å between heme $b_L$ and the [2Fe-2S] cluster accommodates well the observed fast electron transfer between these two redox centers, the 31 Å distance between the [2Fe-2S] cluster and heme $c_1$ is difficult to understand in view of the rapid electron transfer rate observed for these two redox centers (32,68).

Movement of the extramembrane domain of ISP offers an explanation for these paradoxes. The [2Fe-2S] cluster is reduced by the first electron of ubiquinol at a position 27 Å from heme $b_L$ 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 $b_L$. It was speculated that the electron transfer from heme $b_L$ to $b_H$ 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, 56). This model also suggests that the re-oxidized ISP is unable to return to the $b$-position to be re-reduced, before the second electron is transferred to the low potential chain (40).

To study the movement of the head domain of ISP is required for $bc_1$ catalysis and the neck region of ISP confers the necessary mobility, molecular biology, biochemical and biophysical methods were used. Tian et al. (69) demonstrated that increasing the rigidity of the ISP neck region of the $R. sphaeroides bc_1$ complex by a double proline substitution at Ala$^{46}$ and Ala$^{48}$ or a triple proline substitution at residues
42-44 decreased the activity and increased the activation energy because the neck rigidity is increased in the mutant bc1 complexes. In a continuous work, Tian et al (70) constructed mutants with cysteines pairs in the ISP neck region, Ala42 and Val44; Pro40 and Ala42. Oxidation of these two cystines pair to form a disulfide bond resulted in restricting the flexibility of the ISP neck region, in turn, decreased the mobility of the head domain, thus decreased activity of the bc1 complex, however, the activity can be restored by reducing the disulfide bond with β-mercaptoethanol. The restored activity was diminished upon removal of β-mercaptoethanol but is retained if the β-mercaptoethanol-treated membrane was treated with the sulfhydryl reagent N-ethylmaleimide or p-chloromercuribenzoic acid. To further establish the essentiality of the movement of the head domain of ISP, R. sphaeroides bc1 mutants with a pair of cysteines engineered on the interface between ISP and cytochrome b, A185C (cyt b)/K70C (ISP) was generated and characterized (71). Formation of the intersubunit disulfide bond in this cysteine pair mutant complex arrests the head domain of ISP in the “fixed state” position that is too far for electron transfer to heme c1, resulting in the loss of the bc1 activity, reduction of this disulfide bond by β-mercaptoethanol restores the activity.

b) The intertwined dimeric structure of cytochrome bc1 complex

The dimeric association of cytochrome bc1 complex has been reported extensively throughout the literature (72-74). Recently, three dimensional structures of mitochondrial cytochrome bc1 complexes from beef (55, 57), chicken (56), and yeast (58) were determined. The structural information suggests the complex functioning as a dimer. The functional dimer hypothesis is supported by the following structural evidences:

(1) In the crystals of native oxidized bovine cytochrome bc1 complex, the [2Fe-2S]
cluster in one monomer is 27 Å from heme $b_L$ of the other monomer and 40 Å from the heme $b_L$ of the same monomer (55). This structural arrangement suggests that mitochondrial $bc_1$ complex functions as a dimer, because the distance between the [2Fe-2S] cluster of ISP of one monomer and heme $b_L$ of the other monomer is less than that between these groups in the same monomer. The shorter distance accommodates fast electron transfer from QH$_2$ to ISP and $b_L$. Xiao et al (59) demonstrated that the structure of the dimeric cytochrome $bc_1$ complex observed in the crystal also exists in solution. They constructed and characterized two pairs of cysteine substitutions, one in the interface between the head domain of ISP and cytochrome $b$ and the other between the tail domain of ISP and cytochrome $b$. An adduct protein containing two cytochrome $b$ and two ISP proteins is detected in the mutant complexes, confirming that the $bc_1$ complex exists as a dimer which the intertwining of ISP in one monomer is physically close to and interacting with the cytochrome $b$ and cytochrome $c_1$ in the 2-fold symmetry-related to the other monomer (Fig.7).

(2) The presence of two apparently non-communicating cavities in the dimeric complex, each connecting the Qo pocket of one monomer to the Qi pocket of the other. Interestingly the two quinone binding sites from the same monomer do not connect each other. The membrane spanning cavity may help the transfer of quinone between Qo and Qi sites located on different monomers. As a result of that, quinone reduced at Qi site of one monomer can be oxidized at the nearby Qo site of the other monomer without leaving the $bc_1$ complex (Fig.14A).

(3) The distance between the Fe atoms of the two hemes $b_L$ is only 21 Å, which is
Fig. 14. Cytochrome $bc_1$ exists as a functional and structural dimer. (A) Q binding cavity between cytb dimer. (B) Distance between redox centers in bovine dimeric cytochrome $bc_1$ complex (75).
approximately the same as that between heme $b_L$ and $b_H$ in one monomer (Fig.14B). Because of the short distance between the two hemes $b_L$, the electron transfer or equilibrating between inter-monomer hemes $b_L-b_L$ may occur during $bc_1$ catalysis (37,55,76), and such electron transfer may be facilitated by the aromatic pairs present between the two hemes $b_L$ in the two symmetry-related monomers (Fig.15). To test this hypothesis, Gong et al (75) constructed and characterized $R. sphaeroides$ mutants $bc_1$ complexes with mutations at three aromatic residues ($\text{Phe}^{195}$, $\text{Tyr}^{199}$, and $\text{Phe}^{203}$).

Replacing only $\text{Phe}^{195}$ to Ala decreases slightly the ubiquinol-cytochrome c reductase activity and increases the production of superoxide several fold, not only suggesting that this mutation interfered with electron transfer between two monomers, but also supporting the idea that the interruption of the electron transfer between the two $b_L$ hemes enhance the electron leakage to oxygen and thus decreasing the activity. On the other hand, replacing the $\text{Phe}^{195}$ with Tyr, His, or Trp results in mutant complexes having the same ubiquinol-cytochrome c reductase activity as the wild-type, indicating that the aromatic group at the position 195 is involved in electron transfer reaction.

Supernumerary Subunits of Cytochrome $bc_1$ Complex

There are two kinds of protein subunits in the cytochrome $bc_1$ complex, one are those contain a redox prosthetic group, such as heme $b_L$, $b_H$, $c_1$, and iron-sulfur cluster, called the core subunits, the other are those that do not, called the supernumerary subunits (77). All the $bc_1$ complexes contain three core subunits, cytochrome $b$, cytochrome $c_1$, and Rieske ISP, which are required for enzymatic activity of the complex
Fig. 15. Location of aromatic residues in the structural model of the *R. sphaeroides* bc₁ complex. (A) cyt*b* is shown in blue ribbon in one monomer and green in the symmetric monomer, ISP (from the symmetric monomer) is in yellow, and the cytc₁ form the same monomer is in pink. Both subunit IVs, ISP (from one monomer) and cytc₁ (from the symmetric monomer) are shown in turquoise. All hemes are indicated by red sticks. (B) shows the aromatic residues located on the interface of two *b₅* hemes from different cyt*bs*. The nearest edge to edge distances between aromatic residues from different monomers are indicated. Some peptide sequences have been omitted for clarity (75).
(77). However, $bc_1$ complexes from different sources contain varied numbers of supernumerary subunits (4,77). Cytochrome $bc_1$ complexes from some bacteria contain only three core subunits, this is the case with the enzyme from Paracoccus (78), Rhodospirillum rubrum (85), and Rhodobacter capsulatus (79). Whereas the $bc_1$ complex from bovine heart mitochondria has eight supernumerary subunits (Fig.7) (81), that from yeast (82) has seven. Rhodobacter sphaeroides has only one, subunit IV (Fig.16A).

Although the function of supernumerary subunits are not fully understand, the complexes without supernumerary subunits are less stable and have lower activity than those with supernumerary subunits (82). It has been reported that the activation of cytochrome $bc_1$ complexes purified from the bovine mitochondria, yeast, and from the chromatophores of R. sphaeroides and R. capsulatus, by a single chromatographic procedure are: 1152, 219, 128, and 64s$^{-1}$ (82). The increased activity in mitochondria complex may result from interactions of the core subunits with supernumerary subunits.

Some groups investigated the function of the supernumerary subunits of the mitochondrial cytochrome $bc_1$ complex. It’s shown that subunit I and II of yeast $bc_1$ complex is essential for maintaining the proper conformation of apocytochrome $b$ to aid in the addition of heme (83,84); subunit VI of yeast $bc_1$ complex is involved in manipulating dimer/monomer transition (85,86); subunit VII and VIII of yeast $bc_1$ complex are essential for assembly of the complex (87); subunit IX of yeast $bc_1$ complex interacts with the iron-sulfur protein, cytochrome $b$ and cytochrome $c_1$ (88); subunit I and II of plant or bovine $bc_1$ complex have the mitochondrial processing peptidase activity (89,90).
The gene of supernumerary subunit of *R. sphaeroides*, subunit IV (*fbcQ*), was cloned and sequenced (91). The molecular weight, deduced from the nucleotide sequence, is 14,348 Da (92). It is a relatively hydrophilic protein of 124 residues, with a transmembrane helix predicted at a hydrophobic stretch between residues 86 and 109 based on the hydropathy analysis (91), the C-terminal end on the periplasmic side and the N-terminal end on the cytoplasmic side (93). The interacting regions of subunit IV with cytochrome *b* in the core complex were identified as residues 41-53 and 77-85, which are on the cytoplasmic side of the chromatophore membrane and close to the DE loop and helix G of cytochrome *b*, respectively (94). Moreover, subunit IV is involved in the structural role of the complex indicated by mutational effects with a delay in photosynthetic growth and decreasing in the cytochrome *bc*₁ complex activity in chromatophores upon detergent treatment (95). The ubiquinone-binding functions of subunit IV is supported by photoaffinity labeling using azido-Q derivatives (92) and an increasing in the apparent Km for 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinol (QH₂) of the complex. By using site-directed mutagenesis on the subunit IV gene followed by *in vitro* reconstitution, residues 6-11 are essential for structural function of subunit IV. Trp79 is essential for the structural and ubiquinone binding functions of subunit IV (95). Subunit IV was over-expressed in *E. coli* as a glutathione S-transferase fusion protein to solve the difficulty presented by mutagenesis studies by the *in vivo* reconstitution. Subunit IV can then be released from the fusion protein by proteolytic cleavage with thrombin. The recombinant subunit IV is functionally active and can be properly assembled into the active cytochrome *bc*₁ complex without subunit IV (96). By using site-directed mutagenesis coupled with *in vitro* reconstitution, it’s possible to
further study the function of subunit IV.

Photosynthetic Bacteria: *Rhodobacter sphaeroides*

Our group uses *R. sphaeroides* bc₁ complex as a model system to study successfully the more elaborate mitochondrial cytochrome bc₁ complexes (6, 59, 69-71, 75, 94, 96). *R. sphaeroides*, an anoxygenic, non-sulfur, purple facultative photosynthetic bacterium, belongs to gram-negative bacterium of the proteobacteria group. Unlike plants and algae, which carry out photosynthesis in an aerobic environment and produce oxygen as a product of the water splitting reaction, *R. sphaeroides* carries out photosynthesis in an anaerobic condition, and does not produce oxygen. Moreover, *Rhodobacter sphaeroides* is metabolically highly diverse. It can grow photosynthetically or heterotrophically via aerobic or anaerobic respiration (97). It prefers to grow photoheterotrophically, under anaerobic conditions in light with various organic substrates. Therefore, the ecological niches of *R. sphaeroides* are anoxic parts of water and sediments, which receive light of sufficient quantity and quality to allow phototrophic development (98). Under aerobic conditions, it can grow well in the dark, as the synthesis of some photosynthetic pigments, for example, bacteriochlorophyll, in *R. sphaeroides* is repressed by oxygen (98), the culture becomes pink colored rather than brownish green as seen in photosynthetic cultures.

A very important feature of *R. sphaeroides* as a material for bc₁ complex study is that heterotrophic growth allows the isolation of mutants bc₁ complex impaired in
photosynthesis, since electron from quinol can be transferred to oxygen via a quinol oxidase as an alternative electron transfer pathway. Therefore, *R. sphaeroides* mutants with severely defective *bc*$_{1}$ complex are still able to grow in large quantities under aerobic conditions. The bacteria growing under “semi-aerobic dark” conditions develop intra-cytoplasmic membrane (ICM) that most of the photosynthetic machinery of *R. sphaeroides* is located (98-100), intra-cytoplasmic membrane with defective *bc*$_{1}$ complex can be prepared for biochemical and biophysical studies to assess the reasons of the defect.

In addition, the *bc*$_{1}$ complex from *R. sphaeroides* contains only four subunits (cytochrome *b*, cytochrome *c*$_{1}$, ISP and subunit IV) with the three catalytic subunits (cytochrome *b*, cytochrome *c*$_{1}$, ISP) homologous to their mitochondrial counterparts. It is much simpler than the mitochondrial complexes thus making it much easier for us to study structure-based functions of the *bc*$_{1}$ complex. Furthermore, efficient molecular engineering protocols for *R. sphaeroides* *bc*$_{1}$ complex are well established (6). (His)$_{6}$-tagged (69) on cytochrome *c*$_{1}$ of the cytochrome *bc*$_{1}$ complex is readily overexpressed in chromosomal deletion strain BC17 (lacking *fbc*FBC operon) by a plasmid carrying the genes complementing them in trans, which greatly facilitates the isolation of the *bc*$_{1}$ complex from cells using the nickel-nitrilotriacetic acid (Ni-NTA) agarose column. The purity, activity and cytochrome content of these histidine-tagged *bc*$_{1}$ complexes are similar to those of the un-tagged ones. Such genetic system has been used extensively to study site-directed mutants and proven to be extremely valuable for our knowledge of the cytochrome *bc*$_{1}$ complexes (6, 59, 65, 69-71, 75, 101-107).
The \textit{R. sphaeroides} \textit{bc}\textsubscript{1} complex is functionally homologous to the mitochondrial complex. However, since the \textit{R. sphaeroides} \textit{bc}\textsubscript{1} complex contains only one supernumerary subunit, it’s unlikely that the structures of the core subunits in the complex is stabilized through interactions between core subunits and their neighboring supernumerary subunits, as suggested for the mitochondrial complex (108). It’s possible that interactions between part of a core subunit and another part of the same subunit or another core subunit contribute to the stability of a core subunit in \textit{R. sphaeroides} \textit{bc}\textsubscript{1} complex. This hypothesis finds some support from the fact that core subunits in \textit{R. sphaeroides} \textit{bc}\textsubscript{1} complex are generally bigger than their counterparts in the mitochondrial complex due to specific insertions that are uniquely present in the core subunits.

Since the crystal structure of \textit{R. sphaeroides} \textit{bc}\textsubscript{1} is unavailable at this time, the homology modeling is a practical way to predict its three dimensional structure based on the assumption that sequence conservation is directly proportional to structural conservation, a structure model of the \textit{R. sphaeroides} \textit{bc}\textsubscript{1} complex was constructed using coordinates of subunits from bovine complex (6) (Fig.16A.). Amino acid sequence alignment of \textit{R. sphaeroides} cytochrome \textit{b}, cytochrome \textit{c}\textsubscript{1}, and ISP with their counterparts in the bovine mitochondrial \textit{bc}\textsubscript{1} complex reveals high sequence homology, except for several extra fragments in \textit{R. sphaeroides} \textit{bc}\textsubscript{1} complex. There are one extra fragment in ISP, two in cytochrome \textit{c}\textsubscript{1}, four in cytochrome \textit{b}. By swapping coordinates from template to model in all regions where there is a corresponding match in the
sequence alignment, the majority of cytochrome b, cytochrome c₁, and ISP were modeled. Subunit IV and the extra fragments of cytochrome b, cytochrome c₁, and ISP were modeled with the coordinates from the corresponding supernumerary subunits in the bovine bc₁ complex. In the structure model of R. sphaeroides bc₁ complex (6), the four extra fragments of cytochrome b are located at the N terminus (residues 2-12), the connecting loop between helices D and E (residues 232-239), the connecting loop between helices E and F (residues 309-326), and the C-terminus (residues 421-445); the two extra fragment of cytochrome c₁ are located between helix α2 and helix α3 (residues 109-125), and between helix α4 and helix α5 (residues 161-179), respectively; the one extra fragment of ISP (residues 96-107) is located near the middle portion of the ISP. The first two extra fragments of cytochrome b (residues 2-12, 232-239) are located on the cytoplasmic side of the chromatophore membrane; the third one (residues 309-326) is located near to heme b₅, iron-sulfur cluster, and the Qo site; the forth one (residues 421-445) is in close proximity to subunit IV and ISP, and also located on the cytoplasmic side of the chromatophore membrane (106). The extra fragment of ISP shows an α-helical structure in the structure model (105) (Fig.16B.). It was shown that the first two extra fragments of cytochrome b (residues 2-12, 232-239) contribute little effect on structural integrity of the bc₁ complex. Chapter III and IV of this thesis will present evidence for the essentiality of the extra fragments of R. sphaeroides cytochrome b (residues 421-445) and ISP (residues 96-107) for maintaining structural integrity of the bc₁ complex. These findings support the idea that the extra fragments possess the supernumerary subunit function in stabilizing the structure of the bacterial complex. The studies of other extra fragments from R. sphaeroides bc₁ complex are the ongoing projects in our lab.
Fig. 16. (A) Theoretical ribbon model of the R. sphaeroides dimeric cytochrome bc1 complex. ISP is red, cytb is green, cyt_c1 is silver, subunit IV is rust. (B) Location of the extra fragments of ISP, cytochrome b, and cytochrome c1 in the proposed structural model of R. sphaeroides cytochrome bc1 complex. Cyt_c1 is shown in both monomers, other subunits of the right monomer is grey for clarity, the extra fragments are labeled on the figure.
Cytochrome c oxidase and ATP-synthase

Cytochrome c oxidase

Cytochrome c oxidase (or Complex IV) is the terminal oxidase of cell respiration, reducing molecular oxygen (O\textsubscript{2}) to water via a mechanism coupled with a proton pumping process. The proton motive force produced by the proton pumping process and O\textsubscript{2} reduction is energetically harnessed for production of ATP by ATP synthase (109). The mammalian cytochrome c oxidase enzyme is a large multicomponent membrane protein comprising 13 different subunits, four redox-active metal sites (hemes \textit{a} and \textit{a}\textsubscript{3}, Cu\textsubscript{A} and Cu\textsubscript{B}), three redox-inactive metal sites (Mg\textsuperscript{2+}, Zn\textsuperscript{2+} and Na\textsuperscript{+}) and several different classes of phospholipids(110). Except for the redox-active metal sites, the roles of these components are essentially unknown. The O\textsubscript{2} reduction site comprises heme \textit{a}\textsubscript{3} (in high-spin state in the oxidized state) and Cu\textsubscript{B} (109). Electrons from cytochrome c are accepted by Cu\textsubscript{A} and transferred to the O\textsubscript{2} reduction site via heme \textit{a} (in six-coordinated low-spin state in both oxidation states (111)). Although cytochrome c oxidases of bacteria are less complex, they do include the three largest subunits (subunits I, II, and III) that are common to those of the mammalian enzymes. X-ray structures of bovine and bacterial enzymes indicate strong homology among the three-dimensional structures of these redox-active metal sites, providing convincing evidence for the existence of a conserved reaction mechanism (112).

It is believed that the protons are transferred through the D- and K-pathways in cytochrome c oxidase (113). No amino acid residues in the pathway are completely conserved. It has been claimed that completely different sets of amino acid residues
could function in a physiologically identical process (113). At present, X-ray structures of three bacterial oxidases have been solved at resolutions better than 2.8 Å (114-116). Two bacterial $aa_3$-type cytochrome $c$ oxidases have a glycine at the point corresponding to the site of Asp51 in bovine cytochrome $c$ oxidase (114,116). A small cavity capable of trapping a water molecule is located between the glycine and the peptide bond in the hydrogen-bond network. The glycine–water combination is expected to be capable of transferring protons from the hydrogen-bond network to the periplasmic phase with a conformational change controlling the accessibility of the water molecule to the aqueous phase. On the other hand, the $ba_3$-type cytochrome oxidase of an extreme thermophilic bacterium does not have a pathway homologous to the H pathway, but it does contain another hydrogen-bond network which extends across the enzyme molecule (116). These X-ray structural results also suggest variety in the structure of proton transfer pathways.

ATP-synthase

Mitochondrial ATP synthase is the terminal enzyme in oxidative phosphorylation and produces the energy ATP for the cell. It consists of two major units, $F_1$ in the matrix, which binds ADP/ATP and catalyzes ATP synthesis and hydrolysis, and $F_0$, which spans the inner mitochondrial membrane and directs protons to the $F_1$ moiety. As a result of this composition this enzyme is often referred to as $F_1F_0$ ATP synthase. $F_1$ is made up of the three $\alpha$ subunits, three $\beta$ subunits, and one subunit each of $\gamma$, $\delta$, and $\varepsilon$. $F_0$ also contains multiple subunits including an oligomycin sensitivity conferring protein (OSCP), 10-12 $c$ subunits involved in gating of the proton channel, an $\alpha$ subunit that facilitates transfer of protons from the inter membrane space to the $c$ subunits, two $b$ subunits that are thought
to function as stators between F₁ and F₀ and two F₆ subunits that also mediate sensitivity to oligomycin (117). Also present in F₀ are d, e, f, and g subunits that are found in close association with the OSCP subunit (118).

F₁F₀ ATPase is able to catalyze both ATP synthesis and hydrolysis. The switch between these two activities is dependent on the proton gradient between the matrix and the inter membrane space. ATPase can use the energy from ATP hydrolysis to pump proton into the intermembrane space or if a large enough proton gradient exists, use the proton flow through the complex (from inter membrane space to matrix) to drive ATP synthesis.

The 10-12 c subunits form a ring at the base of the γ subunit and the pumping of protons by the a subunit to the c ring and is thought to result in the turning of both the c ring and the entire F₁ complex. This rotation is believed to provide the energy for the synthesis of ATP by the F₁ complex. Tsunoda et al. showed that the ε subunit can exist in two conformations in the F₁ complex (119). When the ε subunit is perpendicular to the inner membrane, ATP hydrolysis is inhibited, proton pumping is inhibited, but ATP synthesis is not. On the other hand, when the ε subunit is parallel to the inner membrane, ATP hydrolysis is activated and proton pumping functions normally, but there is no effect on ATP synthesis. This data indicates that the ε subunit may be important in the regulation of switching between ATP synthesis and ATP hydrolysis.

Oligomycin is a specific inhibitor of ATP synthase. It binds to the membrane portion of the F₀ complex of the enzyme and inhibits proton translocation. Sensitivity of ATP synthase to oligomycin appears to be mediated not only by the OSCP but also by the F₆ subunit and the b subunits. OSCP is found in close association with the α and β
subunits of F₁, with which it cross-links, and is nearby some F₀ components of the stalk (120).
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Chapter II

Material and Methods

Growth of Bacteria: *Rhodobacter sphaeroides* and *Escherichia coli*

*Rhodobacter sphaeroides*

*R. sphaeroides* cells, wild-type and mutant were grown photosynthetically at 30°C in an enriched Sistrom medium containing 0.2% casamino acids and 5 mM glutamate (1, 2), the pH of the medium was adjusted to 7.1 with the mixture of 6N NaOH and 2N KOH to increase the sodium and potassium ion content of the medium to a more optimal level (1). When the cells reached the end of log growth phase, i.e., the density OD$_{600}$ of the cell culture reached about 2.0, cells were harvested by centrifugation at 3,300 x g for 45 minutes using Beckman J6-HC centrifuge and JS4.2 rotor. The cells were suspended with buffer containing 1mM EDTA, 20 mM Tris-succcinate, pH 7.5 at 4°C. The suspended cells were centrifuged at 22,000 x g for 25 using Beckman J2-HS centrifuge and JA14 rotor to wash off the medium. The supernatant was discarded and the celles were weighed, and stored at -20°C. About 5-6 grams of cells were routinely obtained from 1 liter of culture.

The concentrations and antibiotics used were: kanamycin sulfate, 30 µg/ml;
tetracycline, 1 µg/ml; and trimethoprim, 30 µg/ml.

*Escherichia coli*

The cells were grown aerobically at 37°C in LB medium. Antibiotics, when needed, were added at the following concentrations: ampicillin, 125 µg/ml; kanamycin sulfate, 30 µg/ml; tetracycline, 10 µg/ml; and trimethoprim, 100 µg/ml.

Construction of Expression Plasmid and Site-Directed Mutagenesis

Construction of an *R. sphaeroides* Strain Expressing the His$_6$-Tagged Cytochrome $bc_1$ Complex

The expression vector for His-tagged four-subunit cytochrome $bc_1$ complex (pRK418$^{fbc}$FB$_{KmBP}$C$_{6H}$Q) was constructed.

1. Construction of pRKD$^{fbcfB}_{KmBP}CQ$ (pRKDNB35KmBP) (2).

   The gene encoding a trimethoprim-resistant dihydrofolate reductase from R388 (3) (excised from pSup5Tp) and the engineered $fbc$-containing insert from pSELNB3503 (2) were combined into the multiple cloning site polylinker of pSL1180 by a series of in vitro manipulations. The resulting 4,400-base pair fragment containing the dihydrofolate reductase gene and the *R. sphaeroides fbc* operon were subcloned together into the HindIII and EcoRI sites of the vector pRK415 (4), producing pRKDNB3503. For the purpose of subcloning the 200-base pair *Bst*EII-$Pin$AI fragments from pSELNB3503
following mutagenesis, pRKDNB35KmBP was constructed by inserting the kanamycin resistance cassette from pUC4K between the BstEII and PinAI sites of pRKDNB3503. Using pRKDNB3503KmBP to receive the mutated BstEII-PinAI fragments eliminates the possibility of retaining or recloning the wild-type fragment when attempting to subclone the mutated fragments into the expression vector (5). Loss of kanamycin resistance was then used to screen for recombinant plasmids.

(2) Construction of pRKDfbcFBC$_{6H}Q$ (6).

A 1,200-base pair XbaI-HindIII fragment containing the fbcC and fbcQ genes from pRKDfbcFBCQ (2), containing fbcF, fbcB, fbcC, and fbcQ, was inserted into a modified pSELECT-1 vector in which the unique Acc65I site was eliminated. The resulting pSELfbcCQ was used as template for site-directed mutagenesis to introduce an Acc65I recognition site right before the stop codon of the fbcC gene. Two complementary oligonucleotides with His$_6$ tag coding sequence and the Acc65I overhang attached at the 5’-ends (5’-GTACGGGCC CAT CAC CAC CAC CAT CAC TAA-3’ and 3’-CCCG GTA GTG 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 pSELfbcCQ to generate pSELfbcC$_{6H}Q$. The 6-histidine insertion was confirmed by DNA sequencing. A 1,200-base pair XbaI-HindIII fragment containing fbcC$_{6H}Q$ from pSELfbcC$_{6H}Q$ was subcloned into an expression vector (pRKDNB3503) containing the fbcFBCQ genes to generate pRKDfbcFBC$_{6H}Q$. pRKDfbcFBC$_{6H}Q$ was transformed into E. coli S-17, and then mobilized into R. sphaeroides BC17 by using a plate-mating procedure (2). R. sphaeroides BC17 is a strain from which most of the fbcFBC operon has been deleted (7).
(3) Construction of pRKD418/fbcFBKmBrC\textsubscript{6H}Q.

A 4,887 base pair EcoRI-HindIII fragment containing \textit{fbc} F, \textit{fbc} B, \textit{fbc} C, and \textit{fbc} Q, was inserted into pRKD418 vector which contains an additional resistance gene and an enlarged multiple cloning site region (8). The resulting 16,587 base pair pRKD418 \textit{fbc} FBC\textsubscript{6H}Q plasmid was digested by EcoRI and XbaI to get 13,207 base pair fragment containing \textit{fbc} C with 6-Histidine at the C-terminal and \textit{fbc} Q. The plasmid pRKD/fbcFBKmBrCQ was also digested with EcoRI and XbaI to get the 4,632 base pair fragment containing \textit{fbc} F, \textit{fbc} B with kanamycin resistance cassette inserted between the BstEII and PinAI sites. Then a 13,207 base pair fragment was ligated with a 4,632 base pair fragment to get pRKD418/fbcFBKmBrC\textsubscript{6H}Q plasmid. Loss of kanamycin resistance was then used to screen for recombinant plasmids containing the mutant cytochrome \textit{b} gene.

Site-Directed Mutagenesis on Cytochrome \textit{b} with QuickChange

A 3,380 base pair EcoRI-XbaI fragment containing the \textit{fbc} F and \textit{fbc} B genes from pRKD/fbcFBCQ, containing \textit{fbc} F, \textit{fbc} B, \textit{fbc} C, and \textit{fbc} Q, was inserted into a pGEM7Zf(+) vector from Promega. The resulting pGEM7Zf(+)fbcFB was used as the template and two synthetic oligonucleotides containing desired mutation were used as primers for site-directed mutagenesis. All template and primers are listed in Table 1.

The mutagenic oligonucleotides primers used with QuickChange system must be designed to meet the following considerations (9):
<table>
<thead>
<tr>
<th>Mutams</th>
<th>Mutagenesis method</th>
<th>Template</th>
<th>primer</th>
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| cyt$b\Delta$(421–445)  | QuickChange        | pGEM$^a$| f:$^{b}$CGAGAAGCCGGTGCTCCGT$^{a}A$
|                         |                    |          | AGCGACCATCGAAGAAG$^c$
|                         |                    |          | r:$^{d}$CTTCTTCTGATGGTCGCTTTACG
|                         |                    |          | GAGCCACCGGCTTCTCG |
| cyt$b\Delta$(425–445)  | QuickChange        | pGEM     | f:$^{b}$CCGCCCGCGACCATCT$^{a}TAA$
|                         |                    |          | GAAGACTTCAAC       |
|                         |                    |          | r:$^{d}$GTTGAAGTCTTTCTT$^{a}TAGAT$
|                         |                    |          | GGTCGCGGGCGG       |
| cyt$b\Delta$(427–445)  | QuickChange        | pGEM     | f:$^{b}$CCCACGCCACCACCAGT$^{a}AA$
|                         |                    |          | TTCAACGCCCACCTACTCG|
|                         |                    |          | r:$^{d}$CGAGTAGTGGGCGTTGAATT$^{a}AT$
|                         |                    |          | TCTTCGATGGTCGCGGG  |
| cyt$b\Delta$(433–445)  | QuickChange        | pGEM     | f:$^{b}$CAACGCCCGACCTAC$^{a}TAA$
|                         |                    |          | CCCGCGACCAGGGGCGG |
|                         |                    |          | r:$^{d}$CGCCCGTCCGCCTGTT$^{a}TA$
|                         |                    |          | GTAGTGGGGCTTTG     |

$^a$ pGEM7Z(+)/bcFB
$^b$ forward primer
$^c$ all primers are listed from 5’ end to 3’ end
$^d$ reverse primer
1. Both mutagenic primers must contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid.

2. Primers should be between 25 and 45 bases in length, and the melting temperature (Tm) of the primers should be greater than or equal to 78°C. The following formula is commonly used for estimating the Tm of primers:

\[
Tm = 81.5 + 0.41 \times (\%GC) - 675/N - \%mismatch \quad (N \text{ is the primer length in bases}).
\]

3. The desired mutation should be in the middle of the primer with ~10-15 bases of correct sequence on both sides.

4. The primers optimally should have a minimum GC content of 40% and should terminate in one or more C or G bases.

The sample reactions were prepared as indicated below:

- 5 µl of 10 x reaction buffer
- 20 ng of double strand DNA template
- 125 ng of oligonucleotide primer #1
- 125 ng of oligonucleotide primer #2
- 1 µl of dNTP mix (10mM, from Promega)
- ddH₂O to a final volume of 50 µl

Then add 1 µl of *PfuTurbo* DNA polymerase (2.5 U/µl, from Stratagene®)

Then overlay each reaction with ~30 µl of mineral oil.

The thermal cycle was set up in a MiniCycler™ from M. J. Research as follows:

- Step 1: 95°C 2 minutes for initiation;
- Step 2: 95°C 50 seconds for denaturation;
- Step 3: 63°C 1 minute for annealing reaction;
Step 4: 72°C 7 minutes for extension reaction;

Step 5: Step 2 to Step 4 were repeated 18 times

Step 6: 72°C 7 minutes

The amplification can be checked by electrophoresis of 10 µl of the product on a 1% agarose gel. A ~6,000 base pair band should be clearly visualized at this stage.

Following the thermal cycle, the amplification product was treated with the restriction enzyme *Dpn*I (from Promega, 1 µl each time, two times). The *Dpn*I endonuclease is specific for hemimethylated and methylated DNA, therefore, this digestion is used to select mutation-containing synthesized DNA, since DNA isolated from most of *E.coli* stains are *dam* methylated at adenine residues in the sequence 5’…G<sup>m</sup>ATC…3’ and susceptible to *Dpn*I digestion, while the in vitro synthesized DNA is not. The *Dpn*I treated product was then transformed into XL1-Blue competent cells. Plasmid purified from a single XL1-Blue colony was digested with *Eco*RI and *Xba*I, ran the 1% agarose gel to check the size, if the size of the plasmid was correct, this plasmid was sequenced to confirm the mutation.

The pGEM7Z(+)fbcFB plasmid with the mutation (pGEM7Z(+)fbcFBm) was digested with *Bst*EII and *Xba*I (*Bst*EII / *Xba*I) or *Eco*RI and *Xba*I (*Eco*RI / *Xba*I) to get 961bp or 3379bp insert, respectively. The pRKD418fbcFB<sub>Km</sub>BP<sub>C 6H</sub>Q plasmid also digested with *Bst*EII and *Xba*I (*Bst*EII / *Xba*I) or *Eco*RI and *Xba*I (*Eco*RI / *Xba*I) to get 15,626 base pair or 13,208 base pair vector. Then the insert was ligated into the respective vector to get the pRKD418fbcFBmC<sub>6H</sub>Q plasmid. Loss of kanamycin resistance was used to screen for recombinant plasmids containing the mutant cytochrome *b* gene.
The pRKD418/fbcFBmCHQ plasmid in *E. coli* S17-1 cells was mobilized into *R. sphaeroides* BC-17 cells by a plate-mating procedure (2). The presence of engineered mutations was confirmed twice by DNA sequencing of the 962 base pair *BstEII-XbaI* fragment before and after photosynthetic growth.

**Protein Purification of Histidine-Tagged Cytochrome bc\(_1\) Complex**

Frozen *R. sphaeroides* cell (wild-type or mutated) was suspended in 20 mM Tris-succinate, 1 mM EDTA (3 ml/g of cells) and passed through French Press with at about 1000 psig with 1” diameter piston to break the cell. Protease inhibitor phenylmethanesulfonyl fluoride (PMSF), which was prepared routinely as 1 M in dimethyl sulfoxide (DMSO), was added to the cell suspension to a final concentration of 1mM before passing through the French Press, and was added to the same concentration one more time after the first time passage through the French Press.

The broken cells were centrifuged at 40,000 x g (19,000 rpm) with the Beckman J2-HS centrifuge and JA20 rotor for 20 minutes to get rid of the unbroken cell and cell debris. The supernatant was then subjected to centrifugation at 220,000 x g (54,500 rpm with Ti70 rotor) for 150 minutes to separate the chromatophore fraction from the soluble protein fraction. The precipitate obtained was suspended with 50 mM Tris-Cl, pH 7.5 (room temperature), 1 mM MgSO\(_4\) containing 1 mM PMSF, and then homogenized. The homogenate was then subjected to centrifugation at 220,000 x g (54,500 rpm with Ti70 rotor) for 90 minutes to recover chromatophore in precipitate. The resulting chromatophore was suspended in 50 mM Tris-Cl, pH 7.5 (room temperature), 1 mM
MgSO$_4$, 20% glycerol with final volume about 1 ml per 1g cell, and stored at -80°C.

To purify the His-tagged cytochrome $bc_1$ complex, the frozen chromatophore was thawed and adjusted to a cytochrome $b$ concentration of 25 µM with 50 mM Tris-Cl, pH 7.5 (room temperature), 1 mM MgSO$_4$. 10% of dodecylmaltoside (DM) solution (w/v) was added drop by drop to the chromatophore suspension to 0.56 mg/nmol of cytochrome $b$, 4 M NaCl was also added to the final concentration of 100 mM. Then the mixture was stirred at 4°C for 1 hour and then centrifuged at 220,000 x g (54,500 rpm with Ti70 rotor) for 90 minutes. The supernatant was collected and passed through a Ni-NTA agarose column (about 1ml bed volume for every 100nmol of cytochrome $b$ in the chromatophore suspension), equilibrated with 50 mM Tris-Cl, pH7.5 (room temperature) containing 1 mM MgSO$_4$. The column, absorbed with $bc_1$ complexes, was then subjected to a sequence of washing with (1) 4 volume of 50 mM Tris-Cl pH8.0 (4°C), 200 mM NaCl, 0.01%DM; (2) 4 volume of 50 mM Tris-Cl pH8.0 (4°C), 200mM NaCl, 5mM Histidine, 0.01%DM; (3) 2 volume of 50mM Tris-Cl pH8.0 (4°C), 200mM NaCl, 0.5% octyl glucoside (OG). The pure cytochrome $bc_1$ complex was eluted with 50 mM Tris-Cl pH8.0 (4°C), 200 mM NaCl, 200 mM Histidine, 0.5% OG, and concentrated to a final concentration of 300 µM or higher by using a Centriprep concentrator with a 30 KDa or 50 kDa cut-off. The purified complex was stored at -80°C in the presence of 20% glycerol.

**Determination of Cytochrome Content**

Absorption spectra were recorded at room temperature in a Shimadzu UV2101 PC spectrophotometer at 23 °. Samples were fully oxidized by the addition of aliquots of
10 mM potassium ferricyanide. The cytochrome $c_1$ was reduced by adding a few crystals of sodium ascorbate, and the content of cytochrome $c_1$ was determined from the sodium ascorbate reduced minus potassium ferricyanide difference spectrum using the extinction coefficient of 17.5 cm$^{-1}$mM$^{-1}$ for wavelength pair 552 and 540 nm (10). The cytochrome $b$ was reduced by adding a few crystals of sodium dithionite. The content of cytochrome $b$ was determined from the sodium dithionite reduced minus potassium ferricyanide difference spectrum using the extinction coefficient of 28.5 cm$^{-1}$mM$^{-1}$ for wavelength pair 560 and 576 nm (11).

**Enzymatic Activity Assay**

Chromatophores or purified cytochrome $bc_1$ complexes were diluted with 50 mM Tris-Cl, pH 8.0 (4°C), containing 200 mM NaCl and 0.01% dodecyl-maltoside to a final concentration of cytochrome $b$ of 3 µM. Appropriate amounts of the diluted samples were added to 1 ml of assay mixture containing 100 mM Na$^+/K^+$ phosphate buffer, pH 7.4, 1 mM EDTA, 100 µM cytochrome $c$ (from horse heart, Sigma), and 25 µM Q$_0$C$_{10}$BrH$_2$. Ubiquinol cytochrome $c$ reductase activity of *R. sphaeroides bc$_1$* complex was determined by measuring the reduction of cytochrome $c$ by following the increase of absorbance at 550 nm in a Shimadzu UV 2101 PC spectrophotometer at 23 °C, using a millimolar extinction coefficient of 18.5 cm$^{-1}$ for calculation. The nonenzymatic oxidation of Q$_0$C$_{10}$BrH$_2$, determined under the same conditions, in the absence of enzyme, was subtracted. Although the chemical properties of Q$_0$C$_{10}$BrH$_2$ are comparable with those of Q$_0$C$_{10}$H$_2$, it is a better substrate for the cytochrome $bc_1$ complex (12).
measure $bc_1$ activity in chromatophore, 30 µM potassium cyanide was added to the assay mixture to inhibit the cytochrome $c$ oxidase activity.

## Detergent Titration

Different amounts of dodecyl maltoside were added into 1 ml aliquots of chromatophore prepared from wild type and mutants cells containing 50 µM cytochrome $b$ in 50 mM Tris-Cl, pH 8.0 (4°C), 1 mM MgSO$_4$. After incubating at 0 °C for 1 h, appropriate aliquots were withdrawn from each sample and assayed for ubiquinol-cytochrome $c$ reductase activity using a 3 ml assay mixture containing 100 mM Na$^+$/K$^+$ phosphate buffer, pH 7.4, 1 mM EDTA, 100 µM cytochrome $c$ (from horse heart, Sigma), and 25 µM Q$_0$C$_{10}$BrH$_2$. The assay method is the same as the one described in Enzymatic Activity Assay section. The rest of the samples were subjected to centrifugation at 20,000 x g (75,000 rpm) in Beckman Optima™ TL-100 Ultracentrifuge with TLA 100.4 rotor for 90 min. The cytochrome $b$ content in supernatant fractions was determined as mentioned above. The 100% cytochrome $b$ content refers to that in the dodecyl maltoside-treated sample before centrifugation. The unit of specific activity is µmol of cytochrome $c$ reduced/min/nmol of cytochrome $b$.

## SDS-Polyacrylamide Gel Electrophoresis

Analytical SDS-polyacrylamide gel electrophoresis was performed using a Bio-Rad Mini-Protean® 3 Cell using Laemmli system (13). Gel preparation was according to
Table 2. Purified wild type and mutants $bc_1$ complex (50 pmol of cytochrome $c_1$) were digested in the sample buffer (0.0635 M Tris-CL, pH6.8, 2% SDS, 5% $\beta$-mercaptoethanol, 0.002% Bromophenol Blue and 10% glycerol) at 37°C for 30 minutes. Then the samples were subjected to SDS-PAGE under different conditions (Table 2).

Western blot

After SDS-PAGE was done, the gel was equilibrated with transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol) at room temperature for 20 min with gentle shaking. This procedure was repeated three times. The polypeptides separated in the SDS-PAGE gel were transferred to a 0.22 $\mu$m nitrocellulose membrane by using Trans-Blot SD Semi-Dry Transfer cell from BIO-RAD for 30 minutes, and the voltage used was 15V. The membrane was now ready for the immunoblotting assay.

The procedure of immunoblotting assay was as the following:

1) After electrophoretic blotting was done, the membrane was immersed in TBS buffer (20 mM Tris, 500 mM NaCl, pH7.5) for 10 minutes, face up;

2) The TBS solution was discarded and replaced with blocking solution (3% gelatin, 20 mM Tris, 500 mM NaCl, pH7.5), and gently agitated for one hour at room temperature;

3) The blocking solution was discarded and replaced with TTBS buffer (0.05% Tween-20, 20 mM Tris, 500 mM NaCl), and gently agitated at room temperature for 5 minutes. The membrane was washed using TTBS buffer one more time;
4) The TTBS solution was discarded and replaced with the first antibody solution (1% gelatin, 20 mM Tris, 500 mM NaCl, pH7.5, and the diluted rabbit polyclonal antibodies raised against purified ISP and subunit IV), and gently agitated overnight at room temperature.

5) The first antibody solution was discarded and replaced with TTBS, and gently agitated for 5 minutes at room temperature. The membrane was washed using TTBS buffer one more time;

6) The TTBS solution was discarded and replaced with Protein A horseradish peroxidase conjugate from Bio-rad (second antibody), and gently agitated for one hour at room temperature;

7) The second antibody solution was discarded and replaced with TTBS, and gently agitated for 5 minutes. The membrane was washed using TTBS buffer one more time;

8) The TTBS solution was discarded and replaced with TBS solution, gently agitated for 5 minutes at room temperature. The membrane was washed using TBS buffer one more time;

9) The TBS solution was discarded and added HRP color development solution prepared freshly ([a]. Dissolve 30 mg HRP Color Development Reagent from Bio-rad in 10 ml ice cold methanol, [b]. Immediately prior to use, add 30 µl of ice cold 30% H₂O₂ to 50 ml room temperature TBS, [c]. Mix [a] with [b] right before use.). During color development, the chamber which the membrane was soaked in the solution should be wrapped with aluminum foil. Gently agitate until color shows;
Table 2. SDS-PAGE gel preparation and running condition

<table>
<thead>
<tr>
<th></th>
<th>Staking gel</th>
<th>Separating gel</th>
<th>Running conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Regular</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T=12.5% C=3%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5M Tris-Cl, pH 8.0</td>
<td>1 ml</td>
<td>2.5 ml</td>
<td>Running buffer: 3.028% (w/v) Tris, 14.412% (w/v) glycine, 1% (w/v) SDS; 200V for 45 minutes</td>
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<tr>
<td>deionized H₂O</td>
<td>2.3 ml</td>
<td>3.2 ml</td>
<td></td>
</tr>
<tr>
<td>30% acrylamide:Bis</td>
<td>0.63 ml</td>
<td>4.17 ml</td>
<td></td>
</tr>
<tr>
<td>10% SDS</td>
<td>40 µl</td>
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</tr>
<tr>
<td>10% APS</td>
<td>50 µl</td>
<td>50 µl</td>
<td></td>
</tr>
<tr>
<td>TEMED</td>
<td>5 µl</td>
<td>5 µl</td>
<td></td>
</tr>
<tr>
<td><strong>High Resolution</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>T=16.5% C=4%</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Acrylamide</td>
<td>0.24 g</td>
<td>1.6 g</td>
<td>Running buffer: anode buffer: 0.2 M Tris, pH 8.9; cathode buffer: 0.1 M Tris, 0.1M Tricine, 0.1% SDS; 20V for 2 hours 30V overnight</td>
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<tr>
<td>Bis-acrylamide</td>
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<td>0.07 g</td>
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<tr>
<td>Gel buffer</td>
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<tr>
<td>Urea</td>
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</tr>
<tr>
<td>Total volume</td>
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<td>10 ml</td>
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</tr>
<tr>
<td>10% APS</td>
<td>50 µl</td>
<td>50 µl</td>
<td></td>
</tr>
<tr>
<td>TEMED</td>
<td>5 µl</td>
<td>5 µl</td>
<td></td>
</tr>
</tbody>
</table>

APS: Ammonium persulfate; Gel buffer: 3 M Tris, 1 M HCl, 0.3 % SDS; T: the total concentration of acrylamide and bisacrylamide; C: cross-linker concentration
10) The HRP color development solution was discarded and replaced with distilled water, gently agitated for 10 minutes and changed to the deionized water at least once.

11) The membrane was stored at dark.

Potentiometric Titration

Redox titrations of cytochromes b and c₁ in complement and mutant bc₁ complexes were essentially according to the previously published method (14,15). 3 ml of aliquots of the bc₁ complex (2 µM cytochrome b or 2 µM cytochrome c₁) in 0.1 M Na⁺/K⁺ phosphate buffer, pH 7.0, containing 20 µM phenazine methosulfate (midpoint redox potential (Em +80 mV), 20 µM phenazine ethosulfate (Em +55 mV), 20 µM phenazine (Em -120 mV), 20 µM pyocyanine (Em -34 mV), 25 µM 1,4-benzoquinone (Em +293 mV), 25 µM 1,2-naphthoquinone (Em +143 mV), 25 µM 1,4-naphthoquinone (Em +36 mV), 50 µM duroquineone (Em +5 mV), 70 µM 2,3,5,6-tetramethyl-p-phenylenediamine (Em +260 mV), and 15 µM 2-hydroxy-1,4-naphthoquinone (Em -145 mV), were used. Reductive titrations were carried out by addition of sodium dithionite solution to the ferricyanide-oxidized sample; oxidative titrations were carried out by addition of ferricyanide solution to the dithionite-reduced sample. At indicated $E_h$ values during the redox titration, absorption spectra from 600 to 500 nm were taken with a Shimadzu model UV-2100 spectrophotometer. The absorbance at 562 nm, minus that at 575 nm, is used for cytochrome b and that at 552 minus 540 nm for cytochrome c₁. The
midpoint potentials of cytochrome $b_L$ and $b_H$ were calculated by fitting the redox titration
data, obtained for cytochrome $b$, the Nernst equation for a one-electron carrier
($n = 1$) with two components using the KaleidaGraph 3.5 program, and that of cytochrome
$c_1$ was fitted for a one-electron carrier with one component.

**Electron Paramagnetic Resonance Spectrometry**

EPR spectra were recorded with a Bruker EMX spectrometer, equipped with a
liquid helium flow cryostat, at 7 K. Purified wild-type and mutant $bc_1$ complexes (230
$\mu$M cytochrome $b$) were treated with a small excess of ascorbate solution to fully reduce
cytochrome $c_1$ and frozen in liquid nitrogen immediately. EPR spectra of the [2Fe-2S]
cluster of the Rieske iron-sulfur protein were obtained from samples scanning once
from 3200 to 4000 G magnetic field. In order to get the $g_Y$ signal amplitude in mutant
complexes about the same as that in the wild type complex, different scanning times were
applied. EPR spectra were recorded with the following instrument settings: microwave
frequency, 9.3 GHz; microwave power, 2.2 milliwatts; modulation amplitudes, 6.3 G;
modulation frequency, 100 kHz; time constant, 665.4 ms; sweep time: 167.8 s;
conversion time, 163.8 ms. Sample preparation and instrument settings of EPR spectra of
cytochrome $b_L$ and $b_H$ were the same as those for the [2Fe-2S] cluster of the Rieske iron-
sulfur protein, except that the microwave power used was 108.1 milliwatts and
modulation amplitude was 20 G.
Saturation Transfer EPR (ST EPR) Measurement

EPR measurements were made with a Bruker EMX EPR spectrometer, using an aqueous quartz flat cell. The temperature of the microwave cavity was controlled by circulation of cooled nitrogen gas from a modified variable temperature housing assembly equipped with an electric temperature sensor. The instrument settings were as follows: field modulation frequency, 100kHz; microwave power, 107.8 mW; time constant, 1310.72 ms, modulation amplitude, 8 G; microwave frequency, 9.757 GHz, which were employed with phase-sensitive detection at 100 Hz (second harmonic) 90° out of phase. The phase was adjusted to minimize the second harmonic signal. Approximate rotational correlation time (τ2) was obtained from the ratio of the two field lines (L''/L).

Mass Spectrometry Determination

The mass spectrometry determination of molecular weights of cytochrome b in the wild type and mutant bc1 complexes was performed with an Applied Biosystems DE-PRO MALDI-TOF mass spectrometer operated in delayed-extraction positive-ion liner mode according to the method of Ghaim et al. (16) with modifications. Samples (~30 µl) were mixed with 6 volumes of 10% trichloroacetic acid, chilled for 5 min on ice, centrifuged, and the precipitate was rinsed briefly with 95% ethanol to remove salts. The resulting pellet was redissolved in 30 µl of 99% formic acid and a 1:1 dilution with 99%
formic acid to a final volume of 60 µl, which gave the best crystals and signal. As matrix for MALDITOF analyses, 1% of 2,5-dihydroxybenzoic acid in 30% acetonitrile, 0.1% trifluoroacetic acid and 1% 5-methoxysalicilic acid in 30% acetonitrile, 0.1% trifluoroacetic acid were mixed 9:1 (v/v). This matrix solution was mixed 1:1 (v/v) with protein samples. Matrix and sample were spotted onto the sample plate, and were cocrystallized by evaporation in a covered Petri dish under ambient temperature and pressure (16).

Differential Scanning Calorimetry (DSC)

Differential scanning calorimetric measurements were performed with a CSC 6100 NanoII DSC instrument. The $bc_1$ sample was diluted in phosphate buffer (Na/K Pi 100mM, pH 7.4) instead of Tris buffer to avoid the large pH change during the heating. Prior to being loaded in the “sample cell” of the machine, 0.50 ml sample of $bc_1$ solution, 2 mg/ml, in 50 mM K$^+/Na^+$ phosphate buffer, pH7.4, containing 100mM KCl and 0.5% octyl glucoside was degassed for 5 min to remove dissolved air, which may interfere with the DSC data during the heating process. The same volume of buffer was loaded into the “reference cell” of the instrument, after being degassed. During the scanning, both the sample and the buffer were heated from 10°C to 90°C, cooled back to 10°C and heated again to 90°C, with the scanning rate of 1°C/min for both heating and cooling. The cells were equilibrated at 10°C and 90°C for 10 min before heating and cooling. The difference of power input between the “sample cell” and the “reference cell” was recorded in µW, with one data point taken every 10 seconds, as heating and cooling of the
cells were done at a steady rate of 1°C per minute. The second heating scan was used as
the baseline. Melting temperature (Tm) and enthalpy change (ΔH) of the sample were
calculated by using the program known as “CpCalc” from the Nano DSC program group.
References

Chapter III

The Role of Extra Fragment at the C-terminal of Cytochrome \( b \) (Residues 421–445) in the Cytochrome \( bc_1 \) Complex from \( Rhodobacter sphaeroides \)

Introduction

The cytochrome \( bc_1 \) complex is an essential energy transduction electron transfer complex in mitochondria and many aerobic and photosynthetic bacteria (1). The complex catalyzes electron transfer from ubiquinol to cytochrome \( c_1 \) with concomitant translocation of protons across the membrane to generate a membrane potential and proton gradient for ATP synthesis. All the cytochrome \( bc_1 \) complexes contain three core subunits, cytochrome \( b \), cytochrome \( c_1 \), and Rieske iron-sulfur protein (ISP), which house two \( b \)-type hemes (\( b_L \) and \( b_H \)), one \( c \)-type heme (heme \( c_1 \)), and a high potential [2Fe-2S] cluster, respectively. In addition to these three core subunits, the cytochrome \( bc_1 \) complex also contains varying numbers (one to eight) of non-redox containing subunits, known as supernumerary subunits (2,3).

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

Sequence alignment of cytochrome b, cytochrome c₁, and ISP in bacterial complexes with their counterparts in mitochondrial complexes reveals four extra fragments in bacterial cytochrome b and one each in bacterial cytochrome c₁ and ISP (5). These extra fragments are modeled into the structure of the *Rhodobacter sphaeroides bc₁* complex by using coordinates of mitochondrial supernumerary subunits (5). These findings encouraged us to suggest that these extra fragments may possess mitochondrial supernumerary subunit function in stabilizing the structure of the core subunits in the bacterial complex. This suggestion is further supported by the recent finding that ISP is lost from the *R. sphaeroides bc₁* complex if the extra fragment of ISP is deleted or substituted with alanine (6). Of course, confirmation of this suggestion will have to wait until the function of extra fragments in cytochrome b and c₁ are established.

Cytochrome b holds a central role in the cytochrome bc₁ complex because it houses two ubiquinone binding sites, Qo and Qi, and two redox centers, heme bₗ and heme bₜ. According to the Q-cycle mechanism (7), electrons from ubiquinol are bifurcated at the Qo site. The first electron of ubiquinol is transferred through the so-called “high potential” chain consisting of [2Fe-2S] and heme c₁. The second electron of ubiquinol is passed through the “low potential” chain consisting of hemes bₗ and bₜ. Thus, maintaining the structural stability of cytochrome b in the bc₁ complex is crucial for the electron and proton transfer functions of this complex.
In the structure model of *R. sphaeroides* cytochrome *bc*₁ complex (5), the four extra fragments of cytochrome *b* are located at the N terminus (residues 2 to 12), the connecting loop between helices D and E (residues 232 to 239), the connecting loop between helices E and F (residues 309 to 326), and the C terminus (residues 421–445) (see Fig. 1). We started functional studies of these cytochrome *b* extra fragments with the one at the C terminus because this extra fragment is in close proximity to subunit IV and ISP. Herein we report generation of *R. sphaeroides* mutants expressing His-tagged cytochrome *bc*₁ complexes with progressive deletion of the C-terminal extra fragment of cytochrome *b*. The photosynthetic growth behavior, the *bc*₁ activity, and the amount of cytochrome *b*, cytochrome *c*₁, ISP, and subunit IV, in the chromatophore membrane and the purified state, of the complement and mutants, were determined and compared. The lability of mutant *bc*₁ complex, in the chromatophore membranes, toward dodecylmaltoside treatment, was investigated. The effect of mutations on EPR spectra of the cytochromes *b* and ISP and on redox potentials of the cytochromes *b* and *c*₁, in complement and mutant complexes, is also reported.

**Results and Discussion**

Comparison of Electron Transfer Activity, Subunit Composition, and Detergent Lability of Cytochrome *bc*₁ Complexes in Chromatophore Membranes from Complement and C-terminal Truncated Cytochrome *b* Mutants

The C-terminal extra fragment of *R. sphaeroides* cytochrome *b* corresponds to residues 421–445 with a sequence of PATIEEDFNHYSPATGGTKTVVAE (see Fig.
Fig. 17. **Location of the C-terminal extra fragment of cytochrome b in the proposed structural model of *R. sphaeroides* cytochrome bc1 complex.** One monomer (left) is displayed in **solid ribbons**, and the symmetric monomer (right) is displayed in **three-treadline ribbons**. Cytochrome c1 is **rust**; ISP is **brown**; subunit IV is **silver**; cytochrome b is **green**; and the C-terminal extra fragment is **black**.
Fig. 18. Partial sequence comparison in the C-terminal extra fragment of various cytochrome $b$. The abbreviations used are: $Rs$, $R. sphaeroides$; $Rc$, $R. capsulatus$; $Pd$, $P. denitrificans$; $Bf$, bovine; $Yt$, $S. cerevisiae$; $Ck$, chicken.
18). To probe the role of this fragment, *R. sphaeroides* mutants with progressive deletion of amino acid residues from the C terminus of cytochrome *b* were generated and characterized. These are: cyt*b*Δ(433–445), cyt*b*Δ(427–445), cyt*b*Δ(425–445), and cyt*b*Δ(421–445) with deletion of 13, 19, 21, and all residues from this C-terminal extra fragment of cytochrome *b*, respectively.

When these four C-terminal-truncated cytochrome *b* mutants were subjected to anaerobic photosynthetic growth conditions, all of them grew at a rate comparable with that of the complement cells. Chromatophores prepared from these mutant cells have the content and absorption spectral properties of cytochrome *b* and cytochrome *c*₁ + *c*₂ similar to those in the complement chromatophores (data not shown). The amounts of ISP and subunit IV in these mutant chromatophores, determined by Western blotting using antibodies against *R. sphaeroides* ISP and subunit IV, respectively, are also comparable with those in complement chromatophores. These results indicate that the C-terminal extra fragment of cytochrome *b* is not required for assembly of the cytochrome *bc*₁ complex protein subunits (cytochromes *b* and *c*₁, ISP, and subunit IV) into the chromatophore membrane.

Fig. 19A shows the effect of DM concentration on *bc*₁ activity in the complement and four mutant chromatophores. When chromatophores from the complement and four mutants, at a cytochrome *b* concentration of 25 µM, were treated with varying concentrations of DM (up to 0.64 mg of DM per nmol of cytochrome *b*), *bc*₁ activities in all preparations increase at lower and decrease at higher detergent concentrations. However, the detergent concentrations required for maximum activity increasing and decreasing differ significantly. Also, the activity increase or decrease in a given
Fig. 19. Effect of DM concentration on $bc_1$ activity and solubilization of cytochrome $b$ from complement and mutant chromatophores. 1-ml aliquots of chromatophore preparations from complement (o), and mutants cyt$b$Δ-(433-445) (x), cyt$b$Δ-(427-445) (Δ), cyt$b$Δ-(425-445) (□), and cyt$b$Δ-(421-445) (●), containing 50 μM cytochrome $b$, were added at the indicated amounts of DM. After incubating at 0°C for 1 hr, appropriate aliquots were withdrawn from each sample and assayed for ubiquinol-cytochrome $c$ reductase activity using a 3-ml assay mixture (A). The rest of samples were subjected to centrifugation at 100,000 × g for 90 min. The cytochrome $b$ content in supernatant fractions was determined (B). The 100% cytochrome $b$ content refers to that in the DM-treated sample before centrifugation. The unit of specific activity is μmol of cytochrome $c$ reduced/min/nmol of cytochrome $b$. 

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detergent concentration varies greatly among these chromatophores.

Mutant chromatophores of cyt\(b\Delta(433–445)\), cyt\(b\Delta(427–445)\), cyt\(b\Delta(425–445)\)
and cyt\(b\Delta(421–445)\), without DM treatment, have, respectively, 100, 82, 70, and 62% of
the \(bc_1\) activity found in untreated complement chromatophores. Maximum \(bc_1\) activities
are obtained for mutant chromatophores of cyt\(b\Delta(433–445)\), cyt\(b\Delta(427–445)\),
cyt\(b\Delta(425–445)\), and cyt\(b\Delta(421–445)\) at DM concentrations of 0.35, 0.25, 0.15, and 0.08
mg/nmol of cytochrome \(b\), respectively. These correspond, respectively, to 95, 83, 77, and 56%
of maximum \(bc_1\) activity of the complement chromatophores treated with 0.35
mg of DM per nmol of cytochrome \(b\) (see Fig. 19A). Thus the \(bc_1\) complexes with
decreasing lengths of the C-terminal extra fragment of cytochrome \(b\) require decreasing
amounts of DM to obtain maximum activity. Furthermore, maximum \(bc_1\) activity in the
chromatophore membrane decreases as the C-terminal extra fragment of cytochrome \(b\)
shortens.

Whereas the complement chromatophores treated with 0.35 mg of DM/nmol of
cytochrome \(b\) shows maximum \(bc_1\) activity, mutant chromatophores of cyt\(b\Delta(433–445)\),
cyt\(b\Delta(427–445)\), cyt\(b\Delta(425–445)\), and cyt\(b\Delta(421–445)\) treated with the same
concentration of DM lost, respectively, 0, 22, 35, and 81% of their maximum \(bc_1\) activity,
indicating that the lability of the \(bc_1\) complex toward detergent treatment increases as the
size of the C-terminal extra fragment decreases. The increase in lability of the \(bc_1\)
complex to detergent treatment should be indicative of a decrease in structural stability of
the complex. Thus, the structural stability of the \(bc_1\) complex decreases as the C-terminal
extra fragment of cytochrome \(b\) decreases. Residues 433–445 make little contribution to
the structural stability of the \(bc_1\) complex, as the electron transfer activity and detergent
lability of the cyt$b\Delta$(433–445) $bc_1$ are similar to those of the complement complex.

It should be noted that the effectiveness of DM in solubilizing the $bc_1$ complex from these deletion mutant chromatophores is comparable with that for the complement chromatophores (Fig. 19B). When complement and mutant chromatophores of cyt$b\Delta$(433–445), cyt$b\Delta$(427–445), cyt$b\Delta$(425–445), and cyt$b\Delta$(421–445) treated with various concentrations of DM were centrifuged at 200,000 x $g$ for 90 min, the contents of cytochrome $b$, determined spectrophotometrically (see Fig. 19B), ISP, and subunit IV determined by Western blotting (data not shown), in the supernatant fractions, at a given DM concentration, are about the same. This suggests that the loss of $bc_1$ activity in the cyt$b\Delta$(421–445) mutant chromatophores treated with 0.35 mg of DM/nmol of cytochrome $b$ is not because of a decrease of binding affinity of the $bc_1$ complex to the membrane, but to a lesser structural integrity of the mutant complex.

Effect of the C-terminal Extra Fragment of Cytochrome $b$ on the Binding of Cytochrome $b$, ISP, and Subunit IV to Cytochrome $c_1$

To further confirm that the structural integrity of the $bc_1$ complex decreases as the C-terminal extra fragment of cytochrome $b$ decreases, binding affinities of protein subunits in mutant complexes of cyt$b\Delta$(433–445), cyt$b\Delta$(427–445), cyt$b\Delta$(425–445), and cyt$b\Delta$(421–445) were determined and compared with those of the complement complex. Because the expressed $R. sphaeroides bc_1$ complex has a His$_6$ tag at the C terminus of cytochrome $c_1$, one can determine binding affinities of cytochrome $b$, ISP, and subunit IV
to cytochrome $c_1$ in complement and mutant complexes using a Ni-NTA column.

When various chromatophores were treated with 1.2% DM and centrifuged at 200,000 x g for 90 min, about 80% of cytochrome $b$, cytochrome $c_1$, ISP, and subunit IV in the complement and mutant chromatophores was recovered in the supernatant fractions. When these supernatant fractions were applied to Ni-NTA columns, most of cytochrome $c_1$ in all of these supernatant fractions as absorbed on the Ni-NTA gel, and as recovered in column eluates using an eluting buffer containing 200 mM histidine. This is as expected because the His$_6$ tag is placed at the C terminus of cytochrome $c_1$. The $b/c_1$ ratios in column eluates of the complement and mutants cyt$b\Delta$(433–445), cyt$b\Delta$(427–445), cyt$b\Delta$(425–445), and cyt$b\Delta$(421–445) are 1.51, 1.45, 0.81, 0.72, and 0.29, respectively (see Table 3), indicating that the binding affinity of cytochrome $b$ to cytochrome $c_1$ decreases as the C-terminal extra fragment of cytochrome $b$ shortens. The amount of mutant cytochrome $b$ of cyt$b\Delta$(433–445), cyt$b\Delta$(427–445), cyt$b\Delta$(425–445), and cyt$b\Delta$(421–445) associated with cytochrome $c_1$, as compared with the amount of wild-type cytochrome $b$ associated with cytochrome $c_1$, decreases by 4, 46, 52, and 80%, respectively.

Fig.20. shows Western blot analysis of ISP and subunit IV recovered in the Ni-NTA column eluate (purified $bc_1$ complex) and effluent fractions from DM-solubilized complement and mutant chromatophores. The purified cyt$b\Delta$(421–445) mutant complex contains no detectable ISP or subunit IV, however, purified mutant complexes of cyt$b\Delta$(425–445), cyt$b\Delta$(427–445), and cyt$b\Delta$(433–445) have 50, 80, and 100%, respectively, of the amount of ISP or subunit IV found in the complement complex (see
Table 3. Characterization of mutants having deletions on the C-terminal extra fragment of cytochrome b

<table>
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<tr>
<th>Strains</th>
<th>Ps(^a)</th>
<th>$\text{specific activity}^b$</th>
<th>$\text{cyt}b/\text{cyt}c_1+c_2$ subunit composition</th>
<th>Purified complex</th>
<th>$\text{specific activity}^b$</th>
<th>$\text{cyt}b/\text{cyt}c_1$ subunit composition</th>
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<tr>
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<td>1.30</td>
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</tr>
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<td>$\text{cyt}b\Delta(427-45)$</td>
<td>+++</td>
<td>1.87</td>
<td>1.39</td>
<td>FBCQ</td>
<td>2.88</td>
<td>0.81</td>
</tr>
<tr>
<td>$\text{cyt}b\Delta(425-45)$</td>
<td>+++</td>
<td>1.52</td>
<td>1.35</td>
<td>FBCQ</td>
<td>1.69</td>
<td>0.72</td>
</tr>
<tr>
<td>$\text{cyt}b\Delta(421-45)$</td>
<td>+++</td>
<td>1.32</td>
<td>1.23</td>
<td>FBCQ</td>
<td>0</td>
<td>0.29</td>
</tr>
</tbody>
</table>

\(^a\)Ps: photosynthetic growth. \(^b\)enzyme activity is expressed as µmol cytochrome c reduced/min/nmol cytochrome b. \(^c\)+++ indicates the cells can grow photosynthetically and the growth rate was essentially the same as that of the complement cells. \(^d\)FBCQ indicates gene products of the $fbcF$ (ISP) (F), $fbcB$ (cytochrome b) (B), $fbcC$ (cytochrome $c_1$) (C), and $fbcQ$ (subunit IV) (Q), respectively.
Fig. 20. Western blot analysis of ISP and subunit IV in the Ni-NTA column effluent and eluate fractions from DM-solubilized complement and mutant chromatophores. 2-ml of DM-solubilized complement (lane 2) and mutant chromatophores of cyt bΔ(433–445) (lane 3), cyt bΔ(427–445) (lane 4), cyt bΔ(425–445) (lane 5), and cyt bΔ(421–445) (lane 6) were applied to Ni-NTA columns (500-µl bed volume) equilibrated with 50 mM Tris-Cl, pH 8.0, containing 1 mM MgCl₂. The column was washed with 1 ml of 50 mM Tris-Cl, pH 8.0, containing 200 mM NaCl and 0.01% DM, and then eluted with 1 ml of 50 mM Tris-Cl, pH 8.0, containing 200 mM NaCl, 200 mM histidine, and 0.5% octyl glucoside. The volume of column eluate was adjusted to that of the column effluent (unabsorbed plus wash). 2-µl aliquots were withdrawn from column elute (A) and column effluent (B) fractions and subjected to SDS-PAGE. Samples in the gel were transferred to a 22-µm nitrocellulose membrane and treated with antibodies against R. sphaeroides ISP and subunit IV. ProteinA-horseradish peroxide conjugate was used as a second antibody. Lane 1 shows low range prestained standards.
These results are consistent with the presence of increasing amounts of ISP and subunit IV in the Ni-NTA column effluents of cyt$b\Delta$(433–445), cyt$b\Delta$(427–445), cyt$b\Delta$(425–445), and cyt$b\Delta$(421–445) (see Fig. 20B). It should be noted that subunit IV of the $bc_1$ complex produced by $R. sphaeroides$ BC-17 cells carrying the pRKD418/fbcFBCQ plasmid includes chromosomal and plasmid copies, whereas cytochrome $b$, $c_1$, and ISP have only the plasmid copy. These results indicate that the binding affinities of ISP and subunit IV to the complex are affected by the first 12 residues (residues 421–432) at the N-terminal end of the C-terminal extra fragment of cytochrome $b$; binding affinity decreases as the fragment size decreases.

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

The Abnormality of Electrophoretic Mobilities of C-terminal Truncated Cytochrome $b$ Mutants
Fig. 21. Comparison of electrophoretic behavior of cytochrome b in purified complement and mutant cytochrome bc₁ complexes under various conditions. Purified complement and mutant cytochrome bc₁ complexes (50 pmol of cytochrome c₁) were subjected to SDS-PAGE with a separation gel containing T=12.5% and C=3% (panel A); and T=16.5% and C=4% (panel B). Lane 1, pre-stained molecular weight standards; lanes 2-6, cytochrome bc₁ complexes from wild-type, cyt bΔ(433–445), cyt bΔ(427–445), cyt bΔ(425–445), and cyt bΔ(421–445), respectively. T stands for the total concentration of acrylamide and bisacrylamide, C stands for cross-linker concentration.
Fig. 21. shows electrophoretic mobility of wild-type and C-terminal-truncated cytochrome b in separation gels containing two concentrations of acrylamide (T) and bisacrylamide (C). In a separating gel having T =12.5% and C = 3%, a system used routinely for SDS-PAGE analysis of the R. sphaeroides bc₁ complex, wild-type and mutant bc₁ complexes of cytochrome b of cyt bΔ(433–445), cyt bΔ(427–445), cyt bΔ(425–445), and cyt bΔ(421–445) have $R_f$ values of 16.2, 17.5, 18.5, 19, and 19 mm, respectively (see Fig. 21A). As expected, the $R_f$ values of cytochrome c₁, ISP, and subunit IV in mutant bc₁ complexes are the same as those in the wild-type complex, because shortening the C-terminal extra fragment of cytochrome b should not affect the molecular masses of other subunits in the complex. The $R_f$ values of cytochrome b obtained in these mutant complexes are larger than the calculated values, as the molecular mass of cytochrome b is decreased by 1200, 1947, 2206, 2588, respectively, from that of wild-type cytochrome b. It should be noted that the larger than calculated $R_f$ values observed are not because of reduction of the molecular mass of mutant b proteins by proteolytic enzyme digestion, because the molecular mass of cytochrome b in the cyt bΔ(421–445) mutant complex, determined by MALDI-TOF mass analysis, is 47,264, which corresponds to the calculated value.

To further confirm that C-terminal-truncated cytochrome b have abnormal electrophoretic mobility in SDS-PAGE, purified wild-type and mutant cytochrome bc₁ complexes were subjected to SDS-PAGE using a separating gel having T =16% and C = 4% (see Fig. 21B). The electrophoretic mobility of wild-type R. sphaeroides cytochrome b, relative to cytochrome c₁, is decreased when the concentrations of T and C in a separation gel is increased. This increased distance between wild type cytochrome b
and $c_1$ enables us to obtain the $R_f$ values for the cytochrome $b$ mutants, relative to cytochrome $c_1$. The values are: 0.70, 0.76, 0.81, 0.84, and 0.86, respectively. These values are larger than those calculated. Thus, cytochrome $b$ with decreasing lengths of the C-terminal extra fragment exhibits abnormal electrophoretic mobility in SDS-PAGE. Perhaps removal of the C-terminal extra fragment in cytochrome $b$ makes cytochrome $b$ protein assume a molecular shape more globular than the wild-type protein, in the presence of SDS, which moves faster in SDS-PAGE than expected.

Effect of Mutations on the Rieske Iron-Sulfur Cluster

Western blotting and SDS-PAGE analysis indicate that the amount of ISP in the purified $bc_1$ complex decreases as the C-terminal extra fragment of cytochrome $b$ decreases; no ISP is detected in the complex with cytochrome $b$ lacking this extra fragment. To see whether or not the mutations on the C-terminal extra fragment of cytochrome $b$ also affect the microenvironments of the iron sulfur cluster, EPR spectra of the $[2\text{Fe}-2\text{S}]$ cluster in complement and mutant $bc_1$ complexes were determined and compared (see Fig.22).

When complement and mutant complexes of cyt$b\Delta(433–445)$, cyt$b\Delta(427–445)$, cyt$b\Delta(425–445)$, and cyt$b\Delta(421–445)$, at a cytochrome $b$ concentration of 230 µM, were reduced by a small excess of ascorbate, the EPR signal of the Rieske iron sulfur cluster in the complement complex is essentially the same as that previously reported for the wild-type $R. \text{sphaeroides bc}_1$ complex (8,9), with resonance at $g_x=1.80$, $g_y=1.90$, and $g_z = 2.02$.  


Fig. 22. EPR spectra of the [2Fe-2S] cluster of the Rieske iron-sulfur protein in purified $bc_1$ complexes from the complement and mutants cytb$\Delta$(433–445), cytb$\Delta$(427–445), cytb$\Delta$(425–445), and cytb$\Delta$(421–445). Purified complement and mutant $bc_1$ complexes (230 µM cytochrome $b$) were treated with a small excess of ascorbate solution to fully reduce cytochrome $c_1$ and frozen in liquid nitrogen. Panel A, spectra obtained from samples scanning once from 3200 to 4000 G magnetic field; panel B, enlarged spectra at the $g_z$ region. The $g_y$ signal amplitude in mutant complexes have been adjusted to about the same as that in the complement complex by scanning the cytb$\Delta$(433–445), cytb$\Delta$(427–445), and cytb$\Delta$(425–445) complexes, through the magnetic field, once, twice, and five times, respectively. EPR spectra were recorded at 7 K with the following instrument settings: microwave frequency, 9.3 GHz; microwave power, 2.2 milliwatts; modulation amplitudes, 6.3 G; modulation frequency, 100 kHz; time constant, 665.4 ms; sweep time: 167.8 s; conversion time, 163.8 ms.
(see Fig. 22A). The signatures of \( g_y \) and \( g_z \) of [2Fe-2S] cluster in the cyt\( h\Delta(433–445) \), cyt\( h\Delta(427–445) \), and cyt\( h\Delta(425–445) \) mutant complexes are the same as those detected in the complement complex, but with signal amplitudes decreasing as the C-terminal extra fragment of cytochrome \( b \) shortens (see Fig. 22A). No EPR spectrum of the [2Fe-2S] cluster is detected in the cyt\( h\Delta(421–445) \) mutant complex. These results are consistent with Western blot results showing that the amount of ISP in the \( bc_1 \) complex decreases as the C-terminal extra fragment of cytochrome \( b \) decreases and no ISP is detected in the complex that has cytochrome \( b \) lacking the entire C-terminal extra fragment.

In contrast to \( g_y \) and \( g_z \), the \( g_x \) signal of [2Fe-2S] in mutant complexes of cyt\( h\Delta(433–445) \), cyt\( h\Delta(427–445) \), and cyt\( h\Delta(425–445) \) mutant complexes change progressively from a relatively sharp peak with \( g=1.80 \) to a broadened peak with \( g=1.76 \) (see Fig. 22B). Whereas the \( g_x \) signal from the cyt\( h\Delta(433–445) \) mutant complex is quite sharp with \( g=1.80 \) peak, that from the cyt\( h\Delta(425–445) \) mutant complex is broader with \( g=1.76 \) peak. These results indicate that the alteration of the microenvironments of the [2Fe-2S] cluster increases as the C-terminal extra fragment of cytochrome \( b \) decreases. This finding is somewhat surprising, because in the model structure of \( R. \ sphaeroides \) cytochrome \( bc_1 \) complex (5), constructed by using the coordinates of subunits from beef heart mitochondrial \( bc_1 \) complex, the Rieske iron-sulfur cluster is located at the head domain of ISP on the periplasmic side of the chromatophore membrane (positive side), whereas the C-terminal extra fragment of cytochrome \( b \) is located at the cytoplasmic side of this chromatophore membrane. Thus, the effect of shortening the C-terminal extra fragment of cytochrome \( b \) on the EPR signature of the Rieske [2F-2S] center would appear to be long range.
The line shape of the $g_x$ signature of [2Fe-2S] clusters is thought to be sensitive to the redox state of ubiquinone present in the Qo center (8-13). The $g_x$ of $bc_1$ from wild-type $R.\ sphaeroides$ is at $g=1.80$ when oxidized ubiquinone is present but shifts to 1.76 and becomes much broader when ubiquinol is present. In a study of the effect of extraction of ubiquinone, from chromatophore membranes, on the iron-sulfur cluster, Ding et al. (13) found that the $g_x$ signal became very broad and was located at ~1.765 upon deletion of ubiquinone from the $R.\ capsulatus$ chromatophore membrane. Although the broadened, $g_x=1.76$ resonance observed in the cyt$\text{b}\Delta(427–445)$ and cyt$\text{b}\Delta(425–445)$ mutant complexes resembles the “reduced state” or the “depleted state” spectrum, it is not because of changes the redox state of Q or a decrease of Q in the mutant complex, because no EPR spectrum of [2Fe-2S cluster] is detected in these two mutant complexes without treatment with ascorbate and the amount of Q in these two mutant complexes is the same as that in the complement complex. It should also be noted that upon complete reduction by addition of dithionite, the broadened, $g_x=1.76$ signal observed in the mutant complexes remains unchanged, whereas the $g_x=1.80$ signals becomes broadened and shifts to a $g_x=1.76$ signal, similar to the changes observed in the complement complex (8,9).

The broadened, $g_x=1.76$ EPR signal observed in the cyt$\text{b}\Delta(427–445)$ and cyt$\text{b}\Delta(425–445)$ mutant complexes is similar to the $g_x$ signal observed for the substitution of Leu for Phe-144 (F144L) in the cytochrome $b$ from $R.\ capsulatus$ (12) and of serine for Thr-160 in cytochrome $b$ from $R.\ sphaeroides$ (14). The F144L $bc_1$ complex in $R.\ capsulatus$ and the T160S mutant complex in $R.\ sphaeroides$ chromatophores were reported to have a decreased turnover rate with a broadened, redox state insensitive
The *gs* value at 1.765. It was suggested that these properties of the F144L and T160S complexes resulted from a reduced affinity for quinone and quinol at the Qo site of the mutated complex. One possibility, which could account for the decreased turnover rate of the *cytbΔ*(427–445) and *cytbΔ*(425–445) mutant complexes and their reduced state or the high field shift of the *gs* EPR signal, is that shortening the C-terminal extra fragment to less than 6 residues induces conformational changes at the Qo site, which raise the effective redox potential of bound ubiquinol beyond the optimal range for transfer to the [2Fe-2S] cluster.

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

Fig. 23 shows redox titration curves of cytochrome *b* in the complement and C-terminal truncated cytochrome *b* mutant complexes. Two redox components (*b*<sub>L</sub> and *b*<sub>H</sub>) are resolved from each of the titration curves and their redox potentials were calculated (see Fig. 23 and Table 4). The redox potentials of *b*<sub>L</sub> and *b*<sub>H</sub> in the complement complex are -87 and 41 mV, respectively, similar to previously reported values (9). The redox potentials of *b*<sub>L</sub> and *b*<sub>H</sub> in the *cytbΔ*(433–445) mutant complex are -73 and 49 mV, respectively, indicating that deleting 13 residues has little effect on redox potentials of cytochrome *b*. In contrast, the redox potential of *b*<sub>L</sub> in mutant complexes of *cytbΔ*(427–445), *cytbΔ*(425–445), and *cytbΔ*(421–445) decreases by 28, 58, and 73 mV, respectively, and that of *b*<sub>H</sub> decreases by 16, 82, and 111 mV, respectively, compared with counterparts in the complement complex. Thus residues 421–432 in the C-terminal extra fragment are
essential for maintaining redox potentials of \( b \) cytochromes. The effect is larger in \( b_H \) than in \( b_L \). It should be noted that the amplitudes of the reductive and oxidative titrations, shown in Figs. 23 and 25, match so well because no protein denaturation occurs during the titrations, as a freshly thawed sample from the same batch of a given \( bc_1 \) complex was used for each trial of reductive and oxidative titrations.

Fig. 24 shows EPR spectra of the \( b \) cytochromes from the complement and mutant complexes, taken after the samples were reduced with sodium ascorbate to eliminate the overlapping signal from cytochrome \( c_1 \). The complement complex has features at \( g=3.53 \) and \( g=3.77 \) previously assigned to cytochrome \( b_H \) and \( b_L \), respectively, in the wild-type \( bc_1 \) complex (8). The \( g=4.3 \) signal is thought to be nonspecific bound iron (III). Similar EPR spectra were reported for the mitochondrial \( bc_1 \) complex (15-18). The cytochrome \( b_H \) and \( b_L \) signals in the cyt\( b\Delta(433–445) \) mutant complex are the same as those in the complement complex, indicating that deletion of 13 residues does not perturb the heme environments of the \( b \) cytochromes in the complex. A small effect on cytochrome \( b_L \) EPR signal is observed in the cyt\( b\Delta(427–445) \) mutant complex. The positions of cytochrome \( b_H \) and \( b_L \) features in the cyt\( b\Delta(425–445) \) and cyt\( b\Delta(421–445) \) mutant complexes are shifted to 3.57 and 3.72, respectively, and the cytochrome \( b_H \) signals are broadened significantly. These results indicate that the first 6 residues (residues 421–426) at the N-terminal end of this C-terminal extra fragment are essential for maintaining heme environments of cytochrome \( b \), especially for \( b_L \).
Fig. 23. Potentiometric titration of cytochrome $b$ in purified complement and mutant $bc_1$ complexes. The filled circles are reductive titration data and open circles are oxidative titration data. For each $bc_1$ complex two trials of oxidative and two trials of reductive titrations were performed. For each trail of reductive and oxidative titrations, a freshly thawed sample from the same batch of a given $bc_1$ complex was used. The data points in oxidative and reductive titration curves are average of these two trials. The solid lines represent the calculated $n=1$ redox titrations with an equal contribution from $b_L$ and $b_H$ having the indicated midpoint redox potentials.
Table 4. The redox potentials (mV) of cytochromes $b_L$, $b_H$, and $c_1$ in wild type and mutant $bc_1$ complexes.

<table>
<thead>
<tr>
<th>Preparations</th>
<th>Midpoint potentials (mV)$^a$</th>
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</thead>
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<tr>
<td></td>
<td>$cyt b_H$</td>
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<tr>
<td>complement</td>
<td>41±8</td>
</tr>
<tr>
<td>$cyt b\Delta(433-445)$</td>
<td>49±5</td>
</tr>
<tr>
<td>$cyt b\Delta(427-445)$</td>
<td>25±10</td>
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<tr>
<td>$cyt b\Delta(425-445)$</td>
<td>-41±6</td>
</tr>
<tr>
<td>$cyt b\Delta(421-445)$</td>
<td>-70±5</td>
</tr>
</tbody>
</table>

$^a$Data on standard deviations of the mid-point potentials were obtained from four trials of titrations.
Fig. 24. EPR spectra of cytochromes $b_H$ and $b_L$ in purified complement and mutant cytochrome $bc_1$ complexes. Sample preparations and instrument settings were the same as those described in the legend to Fig. 22, except that the microwave power used was 108.1 milliwatts and modulation amplitude was 20 G.
Effect of the C-terminal Extra Fragment of Cytochrome $b$ on Cytochrome $c_1$

Fig. 25 shows redox titration curves of cytochrome $c_1$ in complement and mutant complexes. The redox potentials of cytochrome $c_1$ in these $bc_1$ complexes, calculated to be 237, 227, 165, 170, and 187 mV, respectively (see Fig. 25 and Table 4), indicate that the redox potential of cytochrome $c_1$ is affected by deleting more than 13 residues from the C terminus of cytochrome $b$. In the proposed structural model of the $R. sphaeroides$ cytochrome $bc_1$ complex, the C-terminal extra fragment of cytochrome $b$ is located on the cytoplasmic side of the chromatophore membrane where N-terminal portions of cytochrome $c_1$, ISP, and cytochrome $b$ also reside. Heme $c_1$ is located on the periplasmic side of the chromatophore membrane. Perhaps interactions between the first 12 residues from the N-terminal end of the C-terminal extra fragment of cytochrome $b$ and residues of cytochrome $b$, cytochrome $c_1$, or ISP on the cytoplasmic side stabilize the structure of cytochrome $c_1$ and maintain its redox potential. Thus, the effect on the redox potential of cytochrome $c_1$ by the C-terminal extra fragment of cytochrome $b$ is a long range effect. It has been previously reported that redox midpoint potentials of cytochrome $c_1$ are affected by substitutions in cytochrome $b$ (17). Moreover, the redox potential of heme $c_1$, heme $b_L$, and heme $b_H$ of the deletion mutants cytbΔ-(427-445), cytbΔ-(425-445), and cytbΔ-(421-445) are lower than those of the w.t. It is well known that redox potentials are pH dependent (19). For example, the redox potential of ISP is pH dependent with a slope of $ca.$ -60 mV per pH unit (20). It is possible that deletion of the first 12 residues from the
N-terminal end of the C-terminal extra fragment of cytochrome b would change the local pH of heme $c_1$, heme $b_L$ and heme $b_H$. Heme is iron-protoporphyrin IX with two propionate side chains attached. The higher redox potentials of the w.t. in comparison with those of the mutants indicate that the one or both propionate might be protonated, due to the decrease of the local pH, which in turn, increases the electron affinity of the hemes.

It should be noted again that the C-terminal extra fragment of cytochrome b is located on the cytoplasmic side of the chromatophore membrane, yet its deletion affects redox components, such as heme $b_H$, iron-sulfur cluster, and heme $c_1$, located on the periplasmic (opposite) side of the chromatophore membrane. Therefore, these effects cannot be explained by direct interaction between the extra fragment of cytochrome b and the redox components or their ligands or their vicinity peptides. These long range effects are probably because of globular changes in the deleted mutant complex. That deleted cytochrome b proteins have electrophoretic mobilities greater than those of comparable molecular mass, in SDS-PAGE, suggests that the deleted cytochrome b proteins are more globular than the unaltered protein.

Very recently, our collaborator Di, X. got a new *R. sphaeroides* bc$_1$ crystal structure at 2.9 Å (unpublished data). In this structure the assembly of the three core subunits, cytochrome b, cytochrome $c_1$ and the iron-sulfur-protein (Fig. 26) are very similar to those in mitochondrial bc$_1$ (21). The iron-sulfur protein in *R. s. bc$_1$* crosses over as in mitochondrial bc$_1$, connecting one molecule of cytochrome b to the adjacent one. Since *R. s. bc$_1$* is structurally unsupported by supernumerary subunits (with the exception of subunit IV which appears to be too loosely associated with the core subunits
to crystallize with them), it must depend entirely on insertions and extensions both at the amino- and carboxyl termini of cytochrome $b$ in order to maintain a global structure. This 3D-structure reveals that the last 15 residues are flexible and unlikely to be involved in maintaining the stability of the complex, which agrees well with our result that the fragment (residues from 432 to 445) is not essential for the function or assembly of the complex. The removal of i-helix (425-433) is expected to destabilize de-helix to which it is bound via several hydrogen bonds. The i-helix, the de-helix and the C-terminus of cyt$c_1$ are located very close to each other, suggesting mutual structural dependence. Consequently, removal of the i-helix explains the observed reduction of cyt $c_1$ binding and in turn the loss of the ISP, which also agree well with our result that the first 12 residues (residues 421-432) affect ISP and cyt$b$ binding affinity for cyt$c_1$. As a whole, the new structural data about the C-terminal extra-fragment of cyt$b$ are consistent with our biophysical and biochemical experimental conclusion that this extra-fragment is very important for maintaining the structure stability of cyt$b$. 
Fig. 25. Potentiometric titration of cytochrome $c_1$ in purified complement and mutant $bc_1$ complexes. The filled circles are reductive titration data and open circles are oxidative titration data. The solid lines represent the calculated $n=1$ redox titrations with the indicated midpoint redox potentials.
Fig. 26. The new crystal structure of *R. sphaeroides* bc$_1$ at 2.9 Å (unpublished data). Cytochrome $b$ is light green, cytochrome $c_1$ is blue, ISP is yellow, extra fragments are red.
References


Chapter IV

Differential Scanning Calorimetry Study of Extra Fragment of Iron-sulfur Protein (Residues 96-107) of Cytochrome \( bc_1 \) Complex from *Rhodobacter sphaeroides*

Abstract

To confirm that the extra fragment of ISP of *R. sphaeroides* cytochrome \( bc_1 \) complex is required for protein stability, we studied the thermotropic properties of cytochrome \( bc_1 \) complexes from wild type and the mutant of ISP(96-99)A in the extra fragment of ISP by differential scanning calorimetry (DSC). When purified cytochrome \( bc_1 \) complexes from wild type and mutant were subjected to DSC analysis, they went endothermic thermodenaturation with transition temperatures (\( T_m \)) at 46.3 and 40.7°C, respectively. The change of enthalpy (\( \Delta H \)) (46.3 Kcal/mol) of wild type protein was much higher than that (27.5 Kcal/mol) of mutant \( bc_1 \) complex. These results further establish that the loss (or decrease) of \( bc_1 \) activity in extra fragment mutants results from a lack (or decrease) of ISP in the membrane due to ISP protein instability.
Introduction

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 chain of the mitochondria and many respiratory and photosynthetic bacteria (1). This complex catalyzes electron transfer from ubiquinol to cytochrome $c$ ($c_2$ in bacteria) and concomitantly translocates protons across the membrane to generate a membrane potential ($\Delta\Psi$) and pH gradient ($\Delta$pH) subsequently used by the ATP synthase to produce ATP. All the cytochrome $bc_1$ complexes contain three core subunits, cytochrome $b$, cytochrome $c_1$, and Rieske iron sulfur protein (ISP), which house two $b$-type hemes ($b_L$ and $b_H$), one $c$-type heme (heme $c_1$), and a high potential [2Fe-2S] cluster, respectively. In addition to these three core subunits, the cytochrome $bc_1$ complex also contains varying numbers (one to eight) of non-redox containing subunits, known as supernumerary subunits (2,3).

Although the function of supernumerary subunits in the cytochrome $bc_1$ complex is not yet fully understood, it has been suggested that the structure of a core subunit in the mitochondrial complex may be stabilized through interactions between a core subunit and its neighboring supernumerary subunits (4), thus keeping its redox groups in defined positions. Since the bacterial complexes contain none (or one) supernumerary subunit, maintaining the structural stability of a core subunit(s) in the bacterial complex may result from interactions between a part of a core subunit and another part of the same subunit or another core subunit. This speculation finds some support from the fact that
core subunits in bacterial complexes are generally bigger than their counterparts in the mitochondrial complex.

Sequence alignment of bacterial ISP, cytochrome $b$, and cytochrome $c_1$ with their counterparts in the mitochondrial complexes reveals four extra fragments in bacterial cytochrome $b$ and one each in bacterial ISP and cytochrome $c_1$ (5). These extra fragments are modeled into the structure of *Rhodobacter sphaeroides* $bc_1$ complex by using coordinates from the corresponding supernumerary subunits in the mitochondrial enzyme (5). These findings encouraged us to suggest that these extra fragments may possess mitochondrial supernumerary subunit function in stabilizing the structure of the core subunits in the bacterial complex. This suggestion is further supported by the recent finding that the extra fragments of *R. sphaeroides* ISP (residues 96-107) and cytochrome $b$ (residues 421-445) are found to be essential for maintaining structural integrity of the $bc_1$ complex (6,7) (see Fig. 16). Additionally, Xiao et al (8) reported that the presence of more supernumerary subunits in the mitochondrial cytochrome $bc_1$ complex apparently contributes to the thermal stability of the complex based on comparison of the thermotropic properties of mitochondrial $bc_1$ and bacterial $bc_1$ through the differential scanning calorimetry (DSC) study.

DSC is a thermal analysis technique which has been used to measure the energy absorbed or emitted by a sample during its thermodenaturation. When thermal transition occurs in the sample, DSC provides a direct calorimetric measurement of the transition energy at the temperature of the transition. Such measurements provide qualitative and quantitative information about physical and chemical changes that involve endothermic and exothermic processes, or changes in heat capacity. DSC can be used for a variety of
applications, such as studying stability and domain structure of proteins and nucleic acids; detecting impurity or minor components in phospholipid sample; studying protein:protein, protein:ligand or protein:lipid interaction in membrane or in the detergent dispersed form. The thermodenaturation temperature ($T_m$) and the change of enthalpy ($\Delta H$) are the two parameters used to assess the thermotropic properties of the samples.

To further establish that the extra fragment of ISP of *R. sphaeroides* cytochrome $bc_1$ complex is required for protein stability, we studied the thermotropic properties of cytochrome $bc_1$ complexes from wild type and mutants in the extra fragment of ISP. Decrease in $T_m$ and $\Delta H$ in mutant is expected. Herein, we report the procedure and results of DSC study of mutant of ISP(96-99)A in the extra fragment of ISP.

**Results and Discussion**

Choice of the mutant of ISP(96-99)A among the extra fragment ISP mutants

To study the role of the extra fragment of ISP in the *R. sphaeroides* $bc_1$ complex, several mutants expressing His-tagged cytochrome $bc_1$ complexes with deletion or single- or multiple-alanine substitution at various positions of this fragment (residues 96-107) were generated. They are: full deletion mutant ISP$\Delta$(96-107), alanine substitution mutants ISP(96-107)A, ISP(96-99)A, ISP(100-103)A, ISP(104-107)A, ISP(D104A), ISP(G106A), ISP(D104A/G106A), ISP(G106L), ISP(N100A), ISP(I103A), ISP(T96A),
Because the \( bc_1 \) complex is absolutely required for the photosynthetic growth of \( R.\ sphaeroides \), whether this ISP extra fragment is crucial to the complex can be determined by assaying photosynthetic growth. Both full deletion mutant [ISP\( \Delta \)(96-107)] and full alanine substitution [ISP(96-107)A] cell can not grow photosynthetically, indicating that inability to grow photosynthetically of full deletion mutant cell is due to the essentiality of the extra fragment for the \( bc_1 \) complex, not due to improper assembly or folding because of the large deletion, and this region maybe required for \( bc_1 \) complex activity.

The amino acid residues, rather than the length of the extra fragment, are critical, because the alanine substituted fragment should have the same length as the wild type fragment.

To locate the critical regions of the extra fragment, three alanine substitution mutants, ISP(96-99)A, ISP(100-103)A, ISP(104-107)A, were generated. The first four residues (residues 96-99) are not critical as the ISP(96-99)A mutant grows photosynthetically at a rate comparable to that of wild type cells. Residues 100-103 may possess the supernumerary subunit’s function, since the ISP(100-103)A mutant has a growth behavior similar to that of the subunit IV lacking \( R.\ sphaeroides \) (RSAIV) cells (9), the last four residues (residues 104-107) are critical because the ISP(104-107)A mutant does not grow photosynthetically. The essentiality of the ISP extra fragment to the \( bc_1 \) complex is in the order residues 104-107>residues100-103> residues 96-99, since the ISP(96-99)A, ISP(100-103)A, and ISP(104-107)A mutant membranes have 48%, 9%, and 0% of the ubiquinol-cytochrome \( c \) reductase activity found in the complement chromatophores. The role played by the extra fragment of ISP is not individual amino acid specific but is a combination effect of several residues because all the single-
substitution mutant chromatophores, T96A, N97A, R99A, N100A, N10A, I103A, D104A, G106A and G106L, have ubiquinol-cytochrome c reductase activity similar to that found in complement chromatophores.

Absorption spectral analysis revealed that mutant membranes of ISPΔ(96-107), ISP(96-107)A, ISP(104-107)A, ISP(100-103)A, and ISP(96-99)A have cytochrome b and $c_1+c_2$ contents and spectral characteristics similar to those of complement chromatophores, indicating that the mutation in the extra fragment of ISP does not affect the assembly of cytochrome $b$ and $c_1/c_2$ into the membrane. Western blotting analysis using antibodies against subunit IV shows that the mutations do not affect assembly of subunit IV into the membrane, as all of the mutant membranes contain the same amount of subunit IV as the complement membrane. When the cytochrome $bc_1$ complexes were purified from mutant membranes by dodecyl maltoside solubilization and Ni-NTA column chromatography, all but the ISP(96-99)A complex contained two subunits corresponding to cytochrome $b$ and $c_1$. Purified ISP(96-99)A complex contains four protein subunits with ISP and subunit IV in decreased amounts compared to the purified wild-type complex. The ratio of $b/c_1$ in all purified mutant complexes is similar to that in the wild-type complex, which indicates that mutation does not affect the binding affinity of cytochrome $b$ to cytochrome $c_1$, but greatly decreased the binding affinity of subunit IV to cytochrome $c_1$ or to cytochrome $b$. The simultaneous loss of ISP and subunit IV when the ISP extra fragment is altered may result from induced changes on subunit IV which decrease its affinity for cytochrome $b$ or $c_1$ in the purified complex. The two-subunit $bc_1$ complexes purified from ISPΔ(96-107), ISP(96-107)A, ISP(100-103)A, and ISP(104-107)A are functionally active, because the activity of cytochrome $bc_1$
complex is almost restored after the incubation of these two subunit $bc_1$ complexes with subunit IV and purified ISP.

However, Western blot analysis with antibody against $R. sphaeroides$ ISP revealed that mutant membranes of ISPΔ(96-107), ISP(96-107)A, ISP(104-107)A contain no detectable ISP and that the ISP(100-103)A, and ISP(96-99)A mutant membranes have 5% and 50%, respectively, of the amount of ISP found in the complement membrane, which indicate that the loss (or decrease) of cytochrome $bc_1$ complex activity in the ISP extra fragment mutant membranes is due to a lack (or decrease) of ISP. Although ISP(100-103)A, and ISP(96-99)A mutation cause a decrease in the amount of ISP in the membrane, they do not affect the microenvironment of the iron-sulfur cluster because the [2Fe-2S] clusters in these two mutant membranes had EPR spectral features identical to those observed in complement chromatophores. No [2Fe-2S] cluster is detected in mutant membranes of ISPΔ(96-107), ISP(96-107)A, ISP(104-107)A, since they contain no ISP.

The observation of a lack of (or decrease in) ISP in mutant membranes of ISPΔ(96-107), ISP(96-107)A, ISP(104-107)A, ISP(100-103)A, and ISP(96-99)A is due to instability of the mutant protein, not due to the instability of the mutant mRNA because all mutant cells were found to have the same amounts of ISP mRNA as complement cells. The amounts of ISP in the cell lysate, 200,000g supernatant and precipitate (membrane) fractions, from mutant cells during membrane preparation were measure and compared with those obtained from complement cells in order to further confirm that a lack (or decrease) of ISP in mutant membranes results from mutant ISP protein instability. Freshly prepared mutant cell lysates of ISPΔ(96-107), ISP(96-107)A, ISP(96-99)A, ISP(100-103)A, and ISP(104-107)A have, respectively, 10%, 10%, 50%, 30%, and 10% of the ISP
content of complement cell lysates as determined by Western blot. No ISP was found in any of the supernatant fractions after centrifugation of these cell lysates. Less than 60% and 10% of the ISPs in the ISP(96-99)A and ISP(100-103)A mutant cell lysates are recovered in the membrane fraction, respectively. No cell lysate ISP from the ISPΔ(96-107), ISP(96-107)A, ISP(104-107)A mutants is recovered in their respective membrane fractions. The low (or lack of) recovery of ISP in the membrane fractions of these mutants results from degradation of mutant ISP during centrifugation. Time course analysis revealed a progressive decrease in ISP in these mutant cell lysates, but not in the complement cell lysate.

All of these observations suggested to us that a decrease in ISP in the mutant membrane is due to the instability of assembled mutant ISP. The confirmation of this idea may be obtained from the comparison of thermotrophic properties of purified cytochrome $bc_1$ complexes from wild type and mutants. Among the mutants of the extra fragment of ISP, only mutant ISP(96-99)A has decreased $bc_1$ activity although it still has four subunits, ISP, cyt$b$, cyt$c_1$, and subunit IV, which is the same as wild type. To unambiguously establish the idea mentioned, the protein from mutant ISP(96-99)A might be the best candidate to be chosen for the further study.

Differential Scanning Calorimetry (DSC) study of ISP(96-99)A

DSC has been widely used to study protein stability. The thermodenaturation temperature ($T_m$) and the change of enthalpy ($\Delta H$) are the two parameters used to assess this thermostability. Since the $bc_1$ complex purification involves detergents, the amount
and type of detergents used greatly affect the protein stability, for systematic comparison of protein stability, all bc1 preparations used in these DSC studies were in 0.5% octylglucoside. When purified wild type cytochrome bc1 complex underwent thermodenaturation, it showed endothermic thermodenaturation with a $T_m$ at 46.3°C and $\Delta H$ of 46.3 kcal/mol. Under identical conditions the mutant ISP(96-99)A has a $T_m$ of 40.7 °C and $\Delta H$ of 27.5 kcal/mol (see Fig. 27.). The decrease in $T_m$ and $\Delta H$ of the mutant complex indicates that it is less stable than the wild-type complex.

From all the results mentioned above, the loss (or decrease) of bc1 activity in these mutant membranes results from a lack (or decrease) of ISP in the membrane due to ISP protein instability and not from mutations affecting the assembly of cytochromes b and c1 into the membrane, the binding affinity of cytochrome b to cytochrome c1, or the ability of these two cytochromes to interact with ISP or subunit IV.
Fig. 27. DSC thermograms of ISP(96-99)A and wild-type $bc_1$ complexes. The red line is the thermogram of wild-type cytochrome $bc_1$ complex. The blue line is the thermogram of ISP(96-99)A cytochrome $bc_1$ complex.
References


Chapter V

5-Br-Q_0C_{10} Binding Site in *Rhodobacter sphaeroides* bc_1 complex

(On going project)

Abstract

The preliminary structural analysis of the low resolution of co-crystal of bovine bc_1 complex with 5-Br-Q_0C_{10} suggests that the binding site of 5-Br-Q_0C_{10} is different from Qi site and Qo site. Additionally, the crystal structure indicates that there is a channel connected to the cavity for the binding of 5-Br-Q_0C_{10}. To elucidate this novel Q binding site, two sets of *R. sphaeroides* cytochrome b mutants expressing His_6-tagged bc_1 complexes were generated and characterized. One set of mutants, Y399F/W400F, Y399A/W400A, M343F/L370F, are constructed to interact with 5-Br-Q_0C_{10}. Another set of mutants, I106C/V375C, Y109C/F374C, L110C/A371C, X(106,109,374,375)C, are designed to block the entrance of the putative channel. All these mutants grew photosynthetically at a rate comparable to that of wild-type cells. The bc_1 complexes prepared from these mutants have the similar activity as that of the wild type complex. The Kms for Q_0C_{10}BrH_2 determined with mutant chromatophores of cyt_b-(M343F), cyt_b-(L370F), and cyt_b-(M343F/L370F), or with mutant complexes of cyt_b-(Y399F/W400F),
cytb-(Y399A/W400A), and cytb-(M343F), are comparable with that of the wild type chromatophore or complex, respectively. Also, the 5-Br-Q_0C_{10} titration curves of all mutants showed the same pattern as that of the wild type, which is that low concentration of 5-Br-Q_0C_{10} can activate bc_1 complex, high concentration of 5-Br-Q_0C_{10} inhibits the bc_1 complex.

Introduction

The mitochondrial respiratory chain, which provides more than 90% of the energy needed for aerobic cells by oxidative phosphorylation process, a universal process that converts most of the energy provided by foodstuffs into the general energy source ATP, contains four electron transfer complexes and an ATP synthase complex (1-3). The cytochrome bc_1 complex plays a crucial role in oxidative phosphorylation. Within this process, the cytochrome bc_1 complex connects hydrophobic ubiquinol and water-soluble cytochrome c, transferring electrons between these two freely diffusible intermediates and thereby linking the exergonic reaction to generating a proton gradient and membrane potential for ATP synthesis by ATP synthase (4,5).

Although the study of cytochrome bc_1 has been intensive since its discovery in the early 1960s, our understanding of the bc_1 complex has been greatly enhanced by the availability of crystallographic structures (6-10). Unfortunately, none of the reported structures for cytochrome bc_1 complex shows direct ubiquinone or ubiquinol binding. The reported structures for cytochrome bc_1 complex only shows the binding of Qo site
and Qi site inhibitors (11). Because most of the Qo site inhibitors are reported to be noncompetitive inhibitors (12,13), their binding sites cannot be assumed to be the ubiquinol binding site. Although the essential role of ubiquinone in mitochondrial electron transfer is well established (14-16), the interaction between Q and protein are not yet fully understood. Establishing the nature of ubiquinol binding site is very important in the mechanistic study of this complex.

To study the ubiquinol binding site, the most direct approach is to co-crystallize the bc$_1$ complex with ubiquinol or ubiquinol-like compound. However, typical difficulty in co-crystallize with ubiquinol is that ubiquinol can not be seen in the crystal. To overcome this, one of the feasible methods is synthesizing Q-derivatives to enhance the diffraction signal. Recently, we synthesized 5-Br-Q$_0$C$_{10}$, whose structure is showed in Fig. 28. Due to the special property of the bromine in this Q derivative, we can locate bromine first, and then this Q-like compound can be located. More recently, we co-crystallized bovine mitochondrial bc$_1$ with 5-Br-Q$_0$C$_{10}$ at low resolution. Surprisingly, the preliminary structural data shows that the binding site of 5-Br-Q$_0$C$_{10}$ is different from putative Qo and Qi site, it also show there is a channel connected to the cavity for the binding of 5-Br-Q$_0$C$_{10}$ (see Fig.29.). There was electron density among amino acid residues L357 and Y358 of helix H (Y399 and W400 in R. sphaeroides), residue L328 of helix G (L370 in R. sphaeroides), and residue L301 of helix F (M343 in R. sphaeroides) surrounding the putative cavity for the 5-Br-Q$_0$C$_{10}$ binding. Residues I92, Y95, M96, V329, L332, L333 (I106, Y109, L110, A371, F374, and V375, respectively, in R. sphaeroides) are probably involved in the putative channel connected to the cavity of the binding of 5-Br-Q$_0$C$_{10}$.
Fig. 28. Structure of ubiquinol and 5-BrQ\textsubscript{6}C\textsubscript{10}
Fig. 29. The relative binding location of 5-Br-Q$_0$C$_{10}$ to one of Qo site inhibitor (stigmatellin), Qi site inhibitor (antimycin) and redox centers in the cytochrome $bc_1$ complex. Stigmatellin in green, antimycin in red, and heme $b_L$ and $b_H$ in yellow. The residues (Y399, W400) of helix H, residue (L370) of helix G, and residue (M343) of helix F surrounding the cavity for the 5-Br-Q$_0$C$_{10}$ binding site.
An effective way to test this hypothesis is to systematically mutate the residues involved in the binding of 5-Br-Q_0C_{10} and to follow the biochemical biophysical characterization of the resulted mutant proteins. Herein, we report procedures for generating *R. sphaeroides* mutants with mutations involved in this putative Q binding site in the *bc_1* complex. The photosynthetic growth behavior, cytochrome *bc_1* complex activity, and Km titrations in *bc_1* complexes from wild type and mutant strains were examined and compared, as were the titrations of 5-Br-Q_0C_{10}.

**Experimental procedures**

*Materials*—Cytochrome c (horse heart, type III) was from Sigma. N-Dodecyl-β-D-maltoside and N-dodecyl-β-D-glucoside were from Anatrace. Ni-NTA gel and Qiaprep Spin Miniprep kit were from Qiagen. 2,3-Dimethoxy-5-methyl-6-(10-brimodecyl)-1,4-benzoquinol (Q_0C_{10}BrH_2) was prepared in our laboratory as previously reported (17). All other chemicals were of the highest purity commercially available.

*Growth of Bacteria*—*Escherichia coli* cells were grown at 37°C in LB medium. Photosynthetic growth conditions for *Rhodobacter sphaeroides* were essentially as described previously (18). The concentration and antibiotics used were: ampicillin, 125 μg/ml; kanamycin sulfate, 30 μg/ml; tetracycline, 10 μg/ml for *E. coli*, 1 μg/ml for *R.*
sphaeroides; and trimethoprim, 100 µg/ml for E. coli, 30 µg/ml for R. sphaeroides.

Generation of R. sphaeroides Strains Expressing the His₆-tagged bc₁ Complexes With Mutations at the Binding Site of 5-Br-Q₀C₁₀ and the Entrance of That Site—Mutations were constructed by using the QuikChange™ XL site-directed mutagenesis kit from Stratagene. A double-stranded plasmid pGEM7Zf(+)−fbcFB was used as a template and forward and reverse primers were used for PCR amplification. The pGEM7Zf(+)−fbcFB plasmid was constructed by ligating the EcoRI-XbaI fragment from pRKDfbcFBC₆H₉Q (19) into EcoRI and XbaI sites of the pGEM7Zf(+) plasmid. The primers used are given in Table 5.

There are two kinds of fragments: one is a 962-base pair BstEI-XbaI fragment, the other is a 3379-base pair EcoRI-XbaI fragment depend on the location of mutation site(s) on cytochrome b after mutagenesis. These two fragments were ligated into BstEI-XbaI or EcoRI-XbaI sites of pRKD418fbcFBₖmBP₆H₉Q plasmid (19). Loss of kanamycin resistance was then used to screen for recombinant plasmids containing the mutant cytochrome b gene.

The pRKD418fbcFBₖmBP₆H₉Q plasmid in E.coli S17-1 cells was mobilized into R. sphaeroides BC-17 cells by a plate-mating procedure (19). The presence of engineered mutations was confirmed by DNA sequencing of the 962-base pair BstEI-XbaI or 3379-base pair EcoRI-XbaI fragment after photosynthetic growth as previously reported (19). DNA sequencing was performed by the Recombinant DNA/Protein Core Facility at Oklahoma State University. The primers were synthesized by Invitrogen™.
Table 5. Oligonucleotide used for site-directed mutagenesis for study the
5-Br-Q<sub>0</sub>C<sub>10</sub> binding site.

<table>
<thead>
<tr>
<th></th>
<th>Oligonucleotide used for site-directed mutagenesis for study the 5-Br-Q&lt;sub&gt;0&lt;/sub&gt;C&lt;sub&gt;10&lt;/sub&gt; binding site.</th>
</tr>
</thead>
<tbody>
<tr>
<td>I106C(F)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>GCCTCGCTGTTTTTCTGCACCGCGGTATCATCTCTG</td>
</tr>
<tr>
<td>I106C(R)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CAGATAGACCCGCGAGAAAGAACAGCGAGG</td>
</tr>
<tr>
<td>I109C(F)</td>
<td>CTTCATCGCGGTCTGCTGCACATCTTCC</td>
</tr>
<tr>
<td>I109C(R)</td>
<td>GGAAGATGTCAGCGAGACACCGAGCATG</td>
</tr>
<tr>
<td>L110C(F)</td>
<td>GTCCTTTCATCGCGGTCATATCCACATCTTCCGCAGG</td>
</tr>
<tr>
<td>L110C(R)</td>
<td>GCCCGCAGAGATGTGGAATAGACGCAGATGAAAGAC</td>
</tr>
<tr>
<td>A371C(F)</td>
<td>CTACTTTGTGCTGCATCGTCATCTGACCTGG</td>
</tr>
<tr>
<td>A371C(R)</td>
<td>CAGGATAGCGTAGACCAAGAGAGGAGG</td>
</tr>
<tr>
<td>L110C(R)</td>
<td>CAGGATAGCGTAGACCAAGAGAGGAGG</td>
</tr>
<tr>
<td>Y399A(F)</td>
<td>CATTCTCGGTGTTCGAGGCTGAGG</td>
</tr>
<tr>
<td>Y399A(R)</td>
<td>GGAAGATGCGAGGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>W400A(F)</td>
<td>CTACGCGAGGCACCGGCGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>W400A(R)</td>
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</tr>
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<td>M343F(F)</td>
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<td>M343F(R)</td>
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<td>L370F(R)</td>
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<td>X(106,109)C(F)</td>
<td>CTCGCGGCCTTTCTGACCGGCGGCGGCTGCTGACGATCTG</td>
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<td>X(106,109)C(R)</td>
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<tr>
<td>X(374,375)C(F)</td>
<td>CTGCTCGCGGCGGACTGCTGACGATCTGAGG</td>
</tr>
<tr>
<td>X(374,375)C(R)</td>
<td>CCCAGGATCAGGATGACGAGCGGCAGCAG</td>
</tr>
<tr>
<td>X(399,400)A(F)</td>
<td>CTACGCGATCGCGGCGGCGGCGGCGG</td>
</tr>
<tr>
<td>X(399,400)A(R)</td>
<td>GGAAGATGCGAGGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>X(399,400)F(F)</td>
<td>CTACGCGATCGCGGCGGCGGCGGCGG</td>
</tr>
<tr>
<td>X(399,400)F(R)</td>
<td>GGAAGATGCGAGGAGGAGGAGGAGG</td>
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</table>

<sup>a</sup> F and R in the parentheses denote forward and reverse primers, respectively.
Enzyme Preparation and Activity Assay—Chromatophores were prepared from frozen cell paste, and cytochrome bc\textsubscript{1} complexes with a His\textsubscript{6} tag placed at the C terminus of cytochrome c\textsubscript{1} were purified from chromatophores as previously reported (20). To assay the cytochrome bc\textsubscript{1} complex activity, chromatophores or purified cytochrome bc\textsubscript{1} complexes were diluted with 50 mM Tris-Cl, pH 8.0, containing 200 mM NaCl and 0.01% N-dodecyl-\(\beta\)-D-maltoside (DM) to a final concentration for cytochrome b of 3 \(\mu\)M. Appropriate amounts of the diluted samples were added to 1 ml of assay mixture containing 100mM Na\textsuperscript{+}/K\textsuperscript{+} phosphate buffer, pH 7.4, 1 mM EDTA, 100 \(\mu\)M cytochrome c, and 25 \(\mu\)M \(Q_0C_{10}BrH_2\). 30 \(\mu\)M potassium cyanide was added to the assay mixture when bc\textsubscript{1} activity in chromatophores was determined. For determination of apparent Km for \(Q_0C_{10}BrH_2\), various concentrations of \(Q_6C_{10}BrH_2\) were used. Activity was determined by measuring the reduction of cytochrome c (the increase of absorbance at 550 nm) in a Shimadzu UV 2101 PC spectrophotometer at 23\textdegree C, using a millimolar extinction coefficient of 18.5 for calculation. The nonenzymatic oxidation of \(Q_0C_{10}BrH_2\), determined under the same conditions, in the absence of enzyme, was subtracted. Although the chemical properties of \(Q_0C_{10}BrH_2\) are comparable with those of \(Q_6C_{10}H_2\), it is a better substrate for the cytochrome bc\textsubscript{1} complex (17). The unit of specific activity is \(\mu\)mol of cytochrome c reduced/min/nmol of cytochrome b.

Titration of the Cytochrome bc\textsubscript{1} Complex Activity in Complement and Mutants

Chromatophores or Proteins with Various Concentrations of \(Q_0C_{10}BrH_2\) or 5-Br \(Q_0C_{10}\). Chromatophores or purified cytochrome bc\textsubscript{1} complexes were diluted with 50 mM Tris-Cl, pH 8.0, containing 200 mM NaCl and 0.01% N-dodecyl-\(\beta\)-D-maltoside to a final
concentration for cytochrome $b$ of 3µM, 3 µl of the diluted sample were added to 1 ml of
assay mixture containing 100 mM Na$^+$/K$^+$ phosphate buffer, pH 7.4, 1 mM EDTA, 50 µM
cytochrome $c$, and indicated concentrations of $Q_0C_{10}BrH_2$ or 5-Br-$Q_0C_{10}$.

*Other Biochemical and Biophysical Techniques*—The contents of cytochrome $b$ (21) and
cytochrome $c_1$ (22) were determined according to published methods.

**Results and Discussion**

*Choice of Residues and Strategy for the Study*—The low resolution co-crystal structure of
bovine mitochondrial $bc_1$ complex with 5-Br-$Q_0C_{10}$ showed that amino acid residues
L357, Y358 of helix H, residue L328 of helix G, and residue L301 of helix F are
surrounding the putative cavity for the 5-Br-$Q_0C_{10}$ binding, and residues I92, Y95, M96,
V329, L332, L333 are probably involved in the putative channel connected to the cavity
of the binding of 5-Br-$Q_0C_{10}$. According to the sequence alignment, we picked up the
correspondent residues of *R. sphaeroides* $bc_1$ complex to study the 5-Br-$Q_0C_{10}$ binding
site, including (Y399, W400) of helix H, residue (L370) of helix G, and residue (M343)
of helix F, and the mutations blocking the entrance of the channel, including I106, Y109,
L110, A371, F374, and V375.

There are mainly two ways of quinone interacting with amino acid residues, one
is form hydrogen bond between the hydroxyl group of the quinol and that of amino acid
residue, the other is the interaction between the aromatic ring of quinone and aromatic ring of amino acid residues, such as W and Y. Therefore, we generated two double mutants, one is X(399,400)F to get rid of hydrogen bonding, the other is X(399,400)A to get rid of aromatic-aromatic interaction. On the other hand, the crystal structure shows that the cavity of the 5-Br-Q\textsubscript{0}C\textsubscript{10} binding is very compact, so we mutated the relative small residues, such as L370 and M343, in this cavity to the bulky residues, such as F, in order to squeeze out 5-Br-Q\textsubscript{0}C\textsubscript{10}, we generated three mutations, L370F, M343F, and X(343,370)F.

In order to blocking the entrance of the channel, we generated the mutants with double or tetra cysteine substitution, such as I106C/V375C, Y109C/F374C, L110C/A371C, X(106,109,374,375)C.

**Titration of the Cytochrome bc\textsubscript{1} Complex Activity in Complement and Mutants**

**Chromatophores or Proteins with Various Concentrations of Q\textsubscript{0}C\textsubscript{10}BrH\textsubscript{2}**—Since we hope we can extrapolate the quinol binding site by using Q-derivative compound, if the hypothesis is correct, the Km for the mutants should be different from that of the w..t..

Therefore, we titrated the cytochrome bc\textsubscript{1} complex activity with various concentrations of Q\textsubscript{0}C\textsubscript{10}BrH\textsubscript{2}. We did following titration: X(399,400)F (Fig.30.), M343F (Fig.31.), L370F (Fig.33), X(343,370)F (Fig.34). To rule out the affect of detergent on Km, we also did titration of M343F chromatophore (Fig.32.). Because the Km of M343F chromatophore is similar to that of purified protein of M343F, the detergent affect can be omitted. As Table 6 shows, the Kms of the mutants is similar to that of the wild type.

**Titration of the Cytochrome bc\textsubscript{1} Complex Activity in Complement and Mutants**
Chromatophores or Proteins with Various Concentrations of 5-BrQ<sub>0</sub>C<sub>10</sub>—We also did 5-BrQ<sub>0</sub>C<sub>10</sub>. We titrated X(343,370)F (Fig.35), X(399,400)A (Fig.36). For X(399,400)A, although the pattern of titration curve is similar to that of w.t. (Fig.36), the value of every points is higher than that of w.t., so we incubated the X(399,400)A with endogenous Q<sub>0</sub>C<sub>10</sub>, then titrated with 5-BrQ<sub>0</sub>C<sub>10</sub> to see any difference (Fig.36). The possible explanation of the high value of every points is that X(399,400)A makes bc<sub>1</sub> more accessible to 5-BrQ<sub>0</sub>C<sub>10</sub>. We also did regular Km titration by using the samples at the same day, the Km and Vmax of X(399,400)A are similar to those of w.t.. We also did X(399,400)F titration, the sample was incubated with or without endogenous Q<sub>0</sub>C<sub>10</sub> (Fig.37).

We also titrated the mutants with double or tetra cysteine substitution, such as I106C/V375C (Fig.38), Y109C/F374C (Fig.39), L110C/A371C (Fig.40), X(106,109,374,375)C (Fig.41) with 5-BrQ<sub>0</sub>C<sub>10</sub>.

All of the 5-BrQ<sub>0</sub>C<sub>10</sub> titration curve of mutants show the same pattern as that of w.t., which is that low concentration of 5-BrQ<sub>0</sub>C<sub>10</sub> can active bc<sub>1</sub> complex, high concentration of 5-BrQ<sub>0</sub>C<sub>10</sub> inhibits the bc<sub>1</sub> complex. One explanation for this phenomenon is that the structural data of 5-BrQ<sub>0</sub>C<sub>10</sub> site is not accurate at all.
Table 7. Km of the different mutants.

<table>
<thead>
<tr>
<th>X(399,400)F(P) (a)</th>
<th>M343F(C) (b)</th>
<th>L370F(C)</th>
<th>X(343,370)F(C)</th>
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</thead>
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<tr>
<td>Km ((\mu)M)</td>
<td>1.69</td>
<td>1.27</td>
<td>1.67</td>
</tr>
</tbody>
</table>

\(a\): purified protein

\(b\): chromatophore
Fig. 30. Km titration of X(399,400)F (purified protein).
Fig. 31. Km titration of M343F (purified protein).
Fig. 32. Km titration of M343F (chromatophore)
Fig. 33. Km titration of L370F (chromatophore).
Fig. 34. Km titration of X(343,370)F (chromatophore).
Fig. 35. 5-Br-Q$_0$C$_{10}$ titration of X(343,370)F (protein) compared with w.t. (narrow range)
Fig. 36. 5-Br-Q$_{0}$C$_{10}$ titration of X(399,400)A compared with w.t. (the purified protein incubated w/o endogenous Q$_{0}$C$_{10}$)
Fig. 37.5-Br-Q0C10 titration of X(399,400)F (purified protein incubated w/o Q0C10)
Fig. 3.8.5-Br-Q_0C_{10} titration of X(106,375)C (purified protein incubated w/o Q_0C_{10}).
Fig. 39. 5-Br-Q₀C₁₀ titration of X(109,374)C compared with w.t.
Fig. 40. 5-Br-Q0C10 titration of X(110,371)C compared with w.t.
Fig. 41. 5-Br-Q\textsubscript{10} titration of X(106,109,347,375)C compared with w.t.
References

Chapter VI

Saturation Transfer Electron Paramagnetic Resonance and Differential Scanning Calorimetry Studies of the Interaction between Cytochrome $ca_{a3}$ and $F_{1}F_{0}$-ATP Synthase from Alkaliphilic Bacillus pseudofirmus OF$_{4}$

Abstract

The interaction between cytochrome $ca_{a3}$ and $F_{1}F_{0}$-ATP synthase from the alkaliphilic Bacillus pseudofirmus OF$_{4}$ was studied by differential scanning calorimetry (DSC) and by saturation transfer electron paramagnetic resonance (STEPR). When these two purified complexes are embedded in phospholipid vesicles individually [(cca$_{3}$) × PL, (F$_{1}$F$_{0}$) × PL] or in combination [(cca$_{3}$+F$_{1}$F$_{0}$) × PL] and subjected to DSC analysis, they undergo exothermic thermodenaturation with transition temperatures at 69, 57, and 46/75 °C, respectively. The $\Delta$H (-8.8 Kcal/mmol) of protein-phospholipid vesicles containing both cytochrome $ca_{a3}$ and F$_{1}$F$_{0}$ is smaller than that (-12.4 Kcal/mmol) of a mixture of protein-phospholipid vesicles formed from each individual electron transfer complex [(cca$_{3}$ × PL) + (F$_{1}$F$_{0}$ × PL)]. These results suggest that a specific interaction between cytochrome $ca_{a3}$ and F$_{1}$F$_{0}$ exists in the membrane. Further evidence for the interaction
between these two complexes is provided by STEPR studies in which the rotational correlation time of spin-labeled caa3 (65 µs) increases significantly when the complex is mixed with F1F0 prior to being embedded in phospholipids vesicles (270 µs). From these results, it is concluded that at least a part of cca3 and a part of F1F0 form a supermacromolecular complex in this bacterial membrane.

Introduction

The facultative alkaliphile, *Bacillus pseudofirmus* OF4, grows well over a pH range extending from 7.5 to above 10.5 (1). The organism possesses a branched respiratory chain with at least two terminal oxidases, cytochrome bd and cytochrome caa3 (2). Increased amounts of cytochrome caa3 are associated with two distinct growth conditions where the bulk electrochemical proton gradient (∆p) is low: pH 10.5 (3) or pH 7.5 in the presence of protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) (4). Additionally, a CCCP-resistant mutant strain was found to contain constitutively elevated levels of cytochrome caa3 when grown at pH 7.5 (4).

Like mitochondria of eukaryotic organisms and most bacteria, alkaliphilic *Bacillus pseudofirmus* OF4 synthesizes ATP using a proton-coupled F1F0-ATP synthase via oxidative phosphorylation (5). The widely confirmed chemiosmotic mechanism for ATP synthesis and other membrane-associated bioenergetic work requires establishment of ∆p during respiration and photosynthesis. Coupling is achieved by the proton-translocating nature of the reversible F1F0-ATP synthase (6). Two properties of proton-coupled oxidative phosphorylation by alkaliphilic *Bacillus* species suggest that above pH
9.5, ATP synthesis utilizes a mechanism that depends upon the bulk transmembrane electrical potential, the $\Delta\Psi$, but also depends upon a sequestered transfer of protons from the respiratory chain to the ATP synthase. Firstly, the protonmotive driving force is very low at pH 10.5 and above, because the needs of pH homeostasis produce a large $\Delta pH$, acid in. Nonetheless, the ATP synthesized, as reflected in the observed phosphorylation potential, is even greater than at pH 7.5, where the $\Delta p$ is three times higher. Secondly, artificially imposed diffusion potentials fail to energize ATP synthesis at pH values above 9.5 (7).

We have hypothesized that robust alkaliphile oxidative phosphorylation at very alkaline pH values involves the required respiratory chain proton pumping to the bulk phase to establish a $\Delta p$ and also involves use of one or more specific respiratory chain complexes to transfer protons to $F_0$ sector of the $F_1F_0$ ATP-synthase during dynamic protein-protein interactions (7). In this way, the proton-translocating $F_1F_0$-ATPase accesses protons that have not completely equilibrated with the alkaline medium, accounting for greater synthesis than anticipated from the measured bulk $\Delta p$ and for the inefficacy of an imposed bulk potential. The $caat$ terminal oxidase is the best candidate for that interacting partner both because of its alkali-dependent expression and because of the observation that small mutational decreases in the level of this oxidase lead to a non-alkaliphilic phenotype on non-fermentative substrates (3,8). An alternative hypothesis that would not involve protein-protein interactions between a respiratory chain complex and the ATP synthase is that alkaliphile oxidative phosphorylation at high pH utilizes protons that are sequestered near the membrane surface in some trapped, delocalized manner (9), but such hypotheses have not been experimentally supported and do not
account from the requirement of specific features of the alkaliphile F$_1$F$_0$-ATPase for ATP synthesis at high pH (10). Using methods of differential scanning calorimetry (DSC) and saturation transfer electron paramagnetic resonance (STEPR), we had earlier been able to detect protein-protein interactions between the bovine heart mitochondrial cytochrome $c$ oxidase and F$_1$F$_0$-ATP synthase in the native membrane state (11). Recently, we used these methods to study the interaction between cytochrome $ca_{a3}$ and ATP synthase from alkalphilic Bacillus pseudofirmus OF$_4$ in an attempt to test the hypothesis that protein-protein interactions are involved in ATP synthesis. If the assays supported such interactions, they could then be used to assess the role of specific features of the ATP synthase and oxidase. The DSC study is based on the assumption that if two lipoprotein complexes exist separately in a phospholipid vesicle, no difference in thermotropic properties will be observed between protein-phospholipid vesicles formed from a mixture of two complexes and a mixture of protein-phospholipid vesicles formed individually from each complex. Differences in the thermodenaturation temperatures and enthalpy changes would suggest formation of a physical complex between cytochrome $ca_{a3}$ and ATP synthase. In the STEPR study, the formation of a physical complex between cytochrome $ca_{a3}$ and ATP synthase will be indicated by an increase of rotational correlation time of spin-labeled cytochrome $ca_{a3}$. Herein, we report experimental details and results of DSC and STEPR studies with cytochrome $ca_{a3}$ and ATP synthase embedded in phospholipid vesicles. The results of DSC and STEPR indicate that cytochrome $ca_{a3}$ does indeed interact directly with the ATP synthase in phospholipid vesicles.
Material and methods

*Materials.* 4-Maleimide-2,2,6,6-tetramethyl-1-piperidinyl-N-oxyl (MSL), Cytochrome c (horse heart, type III) and sodium cholate were from Sigma. N-Dodecyl-β-D-maltoside (DM) and N-octyl-β-D-glucoside were from Anatrace. Asolecion was obtained from Associated Concentrates, Inc., and purified according to the procedure reported by Kagama *et al* (12). Centriprep-30 and Centricon-30 were bought from Amicon. Other chemicals were of the highest purity commercially available.

*Enzyme Preparations and Assays.* Highly purified cytochrome *caa*$_3$ and *F*$_1$*F*$_0$-ATP synthase from alkaliphilic *Bacillus pseudofirmus* OF$_4$ were prepared and assayed essentially as reported previously (3,5).

*Preparation of Maleimide Spin-Labeled (MSL) Cytochrome *caa*$_3$.* Alkaliphile, *Bacillus pseudofirmus* OF$_4$ cytochrome *caa*$_3$, 20 mg/ml in 20 mM Tris-Cl, pH 8.0, containing 0.35 M NaCl, 1 mM EDTA, 0.1 mM PMSF, 0.05 % dodecyl maltoside, and 10 % glycerol was incubated with a 5 molar excess of 4-maleimide-2,2,6,6-tetramethyl-1-piperidinyl-N-oxyl (MSL) for 1 hour at room temperature. The stock solution of MSL (10 mM) was made in 10 mM Tris-Cl/sucrose buffer, pH 8.0, containing 20% methanol. After incubation, the unreacted MSL was removed by passage through a D-Salt$^\text{TM}$ Excellulose Desalting Column from Pierce, equilibrated with 10 mM Tris-Cl buffer, containing 0.05 % dodecyl maltoside. Fractions containing MSL-cytochrome *caa*$_3$ were pooled and concentrated by Centriprep-30 and Centricon-30 to a protein concentration of approximately 20 mg/ml. MSL-cytochrome *caa*$_3$ obtained by this method contains no free spin-label. The absence of free spin-label in the preparation was confirmed by the conventional EPR spectra.
Preparation of Cytochrome \( \text{ca}_{3} \) and \( F_{1}F_{0} \) Complex-Phospholipid Vesicles. The protein-phospholipid vesicles were prepared by the cholate-dialysis method reported by Racker (13). Cytochrome \( \text{ca}_{3} \) complex, with or without MSL labeling, alone or in combination with \( F_{1}F_{0} \), at a protein concentration of approximately 30 mg/ml, was mixed with an asolectin micellar solution (20 mg/ml in 50 mM phosphate buffer, pH 7.4) and a sodium cholate solution [20% (w/v) in water]. The final solution contained 7 mg/ml protein, 10 mg/ml sodium cholate, and 10.5 mg/ml asolectin. After incubation at 4°C for 30 min, the solution was dialyzed overnight against 500 volumes of 50 mM phosphate buffer, pH 7.4, with four changes of buffer to form vesicles. The protein-phospholipid vesicles formed were collected as precipitated by centrifugation at 80,000 g for 1 h and were resuspended in 50 mM phosphate buffer, pH 7.4, to a protein concentration of suitable number. The suspensions were used for the DSC and STEPR experiments.

Differential Scanning Calorimetry. All calorimetric measurements were performed with a CSC 6100 NanoII DSC from Calorimetry Science Corp. The reference and sample solutions were carefully degassed under vacuum for 15 min prior to use. A 0.50-ml sample in 50 mM \( K^{+}/Na^{+} \) phosphate buffer, pH 7.4, was placed in the sample capillary cell, and the same amount of buffer was placed in the reference capillary cell. All DSC scans reported in this study were run at a rate of 2°C/min. After the first scan, the samples were cooled to the original temperature and rescanned. Since after the first scan the protein was completely and irreversibly denatured, no thermotransition peaks were observed in the second scan. Thus the second scan could be used as a baseline. All thermodynamic analyses were carried out according to the program known as \( \text{CpCal} \) from the Nano DSC program group.
**EPR Measurements.** All EPR measurements were made with a Bruker EMX EPR spectrometer, using an aqueous quartz flat cell. The temperature of the microwave cavity was controlled by circulation of cooled nitrogen gas from a modified variable temperature housing assembly equipped with an electric temperature sensor. Conventional EPR spectra were recorded with instrument settings as follows: field modulation frequency, 100 kHz; modulation amplitude, 8 G; microwave frequency, 9.757 GHz; microwave power, 10.78 mW; time constant, 1310.72 ms. Saturation transfer EPR spectra were recorded using the same instrument settings as those described by Thomas *et al.* (14) and Poore *et al.* (15). A field modulation of 8 G and microwave frequency of 9.757 GHz were employed with phase-sensitive detection at 100 Hz (second harmonic) 90\(^0\) out of phase. Incident microwave power was 107.80 mW. The phase was adjusted to minimize the second harmonic signal. Approximate rotational correlation time (\(\tau_2\)) was obtained from the ratio of the two field lines (L”/L). The calibration curve of Thomas *et al.* (14) derived from isotropic tumbling of MSL-labeled hemoglobin was used in the calculation.

**Other Analytical Methods.** Protein concentration was determined by the biuret method (16), using bovine serum albumin as the standard (assuming 1 mg/ml has an \(A_{279}\) of 0.667). Absorption spectra were measured in a Shimadzu UV-2401 PC spectrophotometer.

**Results and discussion**

**Thermotropic Properties of Cytochrome caa\(_3\) and F\(_1\)F\(_0\)-synthase Embedded in Phospholipid Vesicles.** To unambiguously study the interaction between cytochrome caa\(_3\)
and $F_1F_0$-synthase from alkaliphilic *Bacillus pseudofirmus* OF$_4$, DSC studies were carried out with both complexes embedded in phospholipid (asolectin) vesicles, because these enzymes in protein-phospholipid vesicles should have an environment similar to that in membrane. The isolated complexes, singly or in combination, were embedded in phospholipids vesicles by the cholate dialysis method (13). A constant phospholipid to protein ratio of 1.5 was used. The ratio between $F_1F_0$-synthase and cytochrome $caaa_3$ varies from 0 to 1.5. If the two lipoprotein complexes have no specific interaction, then no difference in DSC characteristics should be observed between phospholipids vesicles embedded with a mixture of two complexes and a mixture of phospholipid vesicles embedded with one or the other complex. In other words, differences in the thermodenaturation temperatures ($T_m$) and enthalpy changes ($\Delta H$) would suggest formation of a physical complex between these two lipoproteins.

Fig. 42 show the differential scanning calorimetric curves of alkaliphilic *Bacillus pseudofirmus* OF$_4$ $F_1F_0$-synthase and cytochrome $caaa_3$ embedded in phospholipids singly or in combination. When $F_1F_0$-synthase was incorporated into phospholipid vesicles and subjected to DSC analysis, an exothermic peak at 57.2 °C with the enthalpy change of -7.3 Kcal/mmol of protein was observed (see Fig. 42.A). Purified cytochrome $caaa_3$ also shows a single transition with $\Delta H = -17.8$ Kcal/mmol of protein and $T_m = 68.5$ °C when embedded into phospholipids vesicles as shown in Fig.42.B. As expected, when the mixture of protein-phospholipid vesicles formed individually from $F_1F_0$-synthase and cytochrome $caaa_3$ was subjected to DSC analysis under the identical condition, two exothermic transient peaks at 56.8 and 68.9 °C with the $\Delta H$ of -12.4 Kcal/mmol of protein were obtained (see Fig. 43D). These data are sum of the thermotropic properties
of $F_1F_0$-synthase-phospholipid vesicles and cytochrome $caa_3$-phospholipid vesicles. However, the protein-phospholipid vesicles formed from a mixture of $F_1F_0$-synthase and cytochrome $caa_3$ have $Tm1 = 45.5^\circ C$ and $Tm2 = 74.6^\circ C$ with $\Delta H = -8.8$ Kcal/mmol of protein (see Fig. 42C). These data are significantly different from those observed with a mixture of phospholipid vesicles embedded individually with $F_1F_0$-synthase or cytochrome $caa_3$, suggesting that there is some interaction between these two complexes.

Fig. 43 compares the thermodenaturation enthalpy changes of phospholipid vesicles formed with mixtures of cytochrome $caa_3$ and $F_1F_0$-synthase at various molar ratios and of mixtures of phospholipid vesicles of individual complexes. The value of the difference in $\Delta H$ increases as $F_1F_0$-synthase is increased. The maximum difference is obtained when approximately one mol of $F_1F_0$-synthase per mol cytochrome $caa_3$ is used. This suggests that the interaction between cytochrome $caa_3$ and $F_1F_0$-synthase is specific. The accuracy of assessment of the stoichiometry between the two complexes may have been compromised by uncertainty concerning the intactness of each complex.

As discussed earlier (11,17-19), the energy for the exothermic transition of an electron transfer complex embedded in phospholipid vesicles is derived from the collapse, upon thermodenaturation, of a strained interaction between unsaturated fatty acyl groups of phospholipids and a protein surface on the electron transfer or other lipoprotein complex which was exposed, by removal of an interacting protein from a complex or a complex from a supermacromolecular complex, during the isolation. Such an interaction occurs only when a vesicle is formed. Little exothermic transition was observed in mitochondrial or submitochondrial preparations because there is no such exposed area in the native complex or supercomplex to interact with phospholipids under
Fig. 42. DSC curves of alkaliphile *B. firmus* OF$_4$ F$_1$F$_0$ and cytochrome *caa*$_3$ embedded in phospholipids singly or in combination. The molar ratio of F$_1$F$_0$ and *caa*$_3$ is 1 and the weight ratio of phospholipids to protein is 1.5 in all cases. These vesicles are prepared by the cholate dialysis method. Spectrum A shows the exothermic thermodenaturation of 0.5 mg F$_1$F$_0$ embedded in phospholipid vesicles. Spectrum B is the DSC thermogram of 0.105 mg/ml *caa*$_3$ embedded in phospholipid vesicles. Spectrum C is the DSC profile of phospholipid vesicle embedded with a mixture of 0.5 mg F$_1$F$_0$ and 0.105 mg *caa*$_3$. Spectrum D is a mixture of phospholipid vesicles, which include 0.5 mg F$_1$F$_0$ embedded in phospholipid vesicles and 0.105 mg *caa*$_3$ embedded in phospholipid vesicles.
Fig. 43. Comparison of thermodenaturation enthalpy changes of phospholipids vesicles formed with mixtures of cytochrome $\text{caa}_3$ and $F_1F_0$-synthase from alkaliophile $B. \text{firmus OF}_4$ at various molar ratios and of mixtures of phospholipids vesicles of individual complexes. The molecular masses used in calculation of molar ratios were 517,000 and 105,500 daltons for $F_1F_0$-synthase and cytochrome $\text{caa}_3$, respectively. The ratio of phospholipids to protein was 1.5 by weight in all cases.
strained conditions. When two interacting complexes are mixed together before being embedded in phospholipids vesicles, the exposed area on the protein surface is greatly diminished through the protein-protein interaction. Therefore, less strained interaction occurs upon vesicle formation, and less enthalpy change of exothermic denaturation is observed. It has been reported that thermodenaturation of the mitochondrial membrane under aerobic condition is accompanied by a heat release, which was attributed to the autooxidation of iron-sulfur protein (20). This explanation is not applicable to the present study because there are no iron-sulfur proteins in either cytochrome $ca_{a3}$ or $F_{1}F_{0}$-synthase.

**STEPR Studies of Spin-labeled Cytochrome $ca_{a3}$ Embedded in Phospholipid Vesicles in the Absence and Presence of $F_{1}F_{0}$-synthase.** To confirm the existence of a specific interaction between cytochrome $ca_{a3}$ and $F_{1}F_{0}$-synthase, cytochrome $ca_{a3}$ was labeled with 4-maleimide-2,2,6,6-tetramethyl-1-piperidinyl-N-oxyl (MSL) as described under Materials and Methods. This MSL-cytochrome $ca_{a3}$, which is enzymatically active, was embedded in phospholipids vesicles alone or together with $F_{1}F_{0}$-synthase. The electron paramagnetic resonance (EPR) measurements of these electron transfer complex-phospholipid vesicles show typical spin-immobilized spectra (see spectra A and B of Fig.44.). The spectra are identical regardless of whether the protein-phospholipid vesicles contained only cytochrome $ca_{a3}$ or cytochrome $ca_{a3}$ and $F_{1}F_{0}$-synthase complexes (Fig. 44.A & B). This suggests that the difference in mobility of the spin-label on cytochrome $ca_{a3}$, in the absence and presence of $F_{1}F_{0}$-synthase, is too small to be measured by conventional EPR (14). Therefore, the protein rotational diffusion of the spin-labeled complex was measured by saturation transfer electron paramagnetic
resonance (STEPR) because ST-EPR is an excellent method for study of the motional anisotropy and dynamics of spin-labeled proteins embedded in phospholipid bilayers (21-22). The spin correlation time, $\tau_2$, which represents the speed of motion of spin-labeled proteins, can be calculated from the ratio of the low field signals ($L''/L$) in the ST-EPR spectrum (e.g., see spectra C and D of Fig. 44) (21). The longer the rotational correlation time, the slower the protein moves (22). It was reported that the rotational correlation time of spin-labeled protein is increased when two proteins form a supermacromolecular complex (11,18). Table 5 shows the effect of additions on the rotational correlation time of spin-labeled cytochrome $ca_{a3}$. When MSL-cytochrome $ca_{a3}$ mixed with $F_1F_0$-ATP synthase before being embedded in phospholipid vesicles, a significant increase in $\tau_2$ (270 $\mu$s) was observed in comparison with that of MSL-cytochrome $ca_{a3}$ embedded in phospholipid vesicle alone (65 $\mu$s), indicating that MSL-cytochrome $ca_{a3}$ does interact with $F_1F_0$-ATP synthase to form supermacromolecular complex. However, the $\tau_2$ of spin-labeled cytochrome $ca_{a3}$ was not affected by the addition of cytochrome $bc_1$ complex or ATP-synthase from bovine heart prior to the formation of vesicles; and the mixture of spin-labeled cytochrome $ca_{a3}$ complex and $F_1F_0$-synthase phospholipid vesicles showed the same $\tau_2$ as that of cytochrome $ca_{a3}$ phospholipid vesicles alone (see Table 7), because the MSL-cytochrome $ca_{a3}$ can’t form complex with neither mitochondrial $F_1F_0$-synthase nor mitochondrial $bc_1$ complex.

To ensure that the observed $\tau_2$ increase upon mixing $F_1F_0$-synthase with spin-labeled cytochrome $ca_{a3}$ is indeed due to the specific interaction between these two complexes and the formation of a binary complex, and not due to the change of protein concentration or self-aggregation upon addition of $F_1F_0$-synthase, a titration of spin-
Table 7. Effect of additions on the rotational correlation time ($\tau_2$) of spin-labeled cytochrome $caa_3$\(^1\)

<table>
<thead>
<tr>
<th>Preparations</th>
<th>L''/L</th>
<th>$\tau_2$ (µs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(MSL-$caa_3 \times PL$)</td>
<td>0.70</td>
<td>65</td>
</tr>
<tr>
<td>[(MSL-$caa_3 + bF_1F_0^2) \times PL]</td>
<td>1.06</td>
<td>270</td>
</tr>
<tr>
<td>[(MSL-$caa_3 \times PL) + (bF_1F_0 \times PL)]</td>
<td>0.71</td>
<td>70</td>
</tr>
<tr>
<td>[(MSL-$caa_3 + mF_1F_0^3) \times PL]</td>
<td>0.71</td>
<td>70</td>
</tr>
<tr>
<td>[(MSL-$caa_3 + mbc_1^4) \times PL]</td>
<td>0.70</td>
<td>65</td>
</tr>
</tbody>
</table>

\(^1\)The molar ratio of cytochrome $caa_3$ to other proteins used was 1:1.

\(^2\)The bF\(_1\)F\(_0\) was F\(_1\)F\(_0\)-synthase from bacterial alkaliphile *B. firmus* OF\(_4\).

\(^3\)The mF\(_1\)F\(_0\) was F\(_1\)F\(_0\)-synthase from bovine mitochondria.

\(^4\)The mbc\(_1\) was cytochrome *bc\(_1\)* complex from bovine mitochondria.
labeled cytochrome *caa*3 with F1F0-synthase was carried out. If a specific interaction between these two complexes exists, it is expected that a maximum $\tau_2$ will be obtained when the two components complex reaches to a specific stoichiometry. As shown in Figure 45, the break point in $\tau_2$ was obtained when the ratio of F1F0-synthase to cytochrome *caa*3 approached one, which is in consistent with the number obtained from the DSC data.

A similar effect of succinate-Q reductase on $\tau_2$ of spin-labeled ubiquinol-cytochrome *c* reductase (18), and of F1F0-synthase on $\tau_2$ of spin-labeled cytochrome *c* oxidase from bovine mitochondria (11), has been reported from this group. It is conceivable that at least part of the observed effect resulted from a change in the fluidity of the membrane by inclusion of protein complexes other than the spin-labeled complex. Also, it should be mentioned that the rotational correlation time obtained from STEPR is only an approximate value; it is based on the calibration curve derived from the isotropic motion of spin-label. The values obtained, however, agree with those obtained by other methods, such as flash photolysis (23). Although in this study our main concern is with the relative $\tau_2$ of spin-labeled cytochrome *caa*3 in the absence and presence of the F1F0-synthase from alkaliphilic *Bacillus pseudofirmus* OF4, the $\tau_2$ values obtained are in agreement with the DSC data.

From the results of DSC and STEPR experiments, we conclude that cytochrome *caa*3 and F1F0-synthase from alkaliphilic *Bacillus pseudofirmus* OF4 may exist as a supermacromolecule complex in the membrane. This conclusion differs significantly from the free diffusible model of electron transfer complexes derived from results of membrane fusing (24) and fluorescence recovery, after photobleaching, measurements
However, it has been clearly established that some mitochondrial electron-transfer complexes specifically interact to form supermolecular structures called supercomplexes from the work on the yeast *Saccharomyces cerevisiae* (26,27), beef (11,18,27,28), and plants (29-32). Similar supermolecular structures were also described for the respiratory chains of bacteria (33-37). The roles that have been attributed to respiratory supercomplexes are substrate-channeling, catalytic enhancement, sequestration of reactive intermediates (27), stabilization of protein complexes (38), increasing the capacity of the inner mitochondrial membrane for protein insertion (26), and generating mitochondrial cristae morphology (39). Furthermore, the dynamic formation of such supercomplexes is speculated to serve some regulatory function in the energy generation in mitochondria from plants (29) and beef (11,29). Similarly, the formation of a supermacromolecule complex between cytochrome *caa*3 and F1F0-synthase from alkalophilic *Bacillus pseudofirmus* OF4 may help control energy generation in this alkalophilic bacterium. The formation of supermacromolecule complexes from some electron transfer and F1F0-synthase complexes indicates that some of these complexes do not follow the random diffusion model, even though they are capable of doing so.
Fig. 44. EPR spectra of spin-labeled alkaliphile *B. firmus* OF₄ cytochrome *caa₃* in the absence and presence of OF₄ F₁F₀-synthase complex. Spectra A and B are conventional EPR spectra of spin-labeled OF₄ cytochrome *caa₃* embedded in phospholipid vesicles in the absence or presence of OF₄ F₁F₀-synthase complex. Spectra C and D are the saturation transfer EPR spectra of the same samples. The protein concentrations were 6 and 36 mg/ml for *caa₃* and (*caa₃* + F₁F₀) vesicles, respectively.
Fig. 45. Effect of $F_1F_0$-synthase on STEPR of spin-labeled cytochrome $ca_{a3}$. Increasing amounts of $F_1F_0$-synthase were added to a constant amount of spin-labeled cytochrome $ca_{a3}$. The solutions were incubated for 60 min at 4 °C before embedded in phospholipids vesicles. 1.5 mg of phospholipid/mg of protein was used. $L''/L$ was calculated from the saturation transfer EPR spectra of each sample. Instrument settings are given under Materials and Methods.
References

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Vita

Xiaoying Liu

Candidate for the Degree of

Doctor of Philosophy

Thesis: STUDIES OF EXTRA FRAGMENTS OF CYTOCHROME \( bc_1 \) COMPLEX FROM 

\textit{RHODOBACTER SPHAEROIDES} AND THE INTERACTION BETWEEN CYTOCHROME 

\( \text{CAA}_3 \) AND \( F_1F_0 \)-ATP SYNTHASE FROM ALKALIPHILIC \textit{BACILLUS} 

\textit{PSEUDOFIRMUS} OF \( F_1 \)

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Scope and Method of Study: Although bacterial enzymes have simpler subunit composition than their mammalian counterparts, the sizes of core subunits (cytochrome b, ISP and cytochrome c₁) are generally larger. Are these extra fragments required for bacterial bc₁ complex? To answer this question, we studied two of these extra fragments through the molecular genetic manipulation of Rhodobacter sphaeroides (R.S.) bc₁ complex at various positions of residues 421-445 in cytochrome b and residues 96-107 in ISP. bc₁ activity assay, SDS-PAGE, western-blotting, DSC, and EPR, were used to characterize the mutants. To elucidate the quinone binding site of the bc₁ complex, we generated and characterized some mutants in R.S. bc₁ complex based on the structural analysis of the low resolution of co-crystal of bovine bc₁ complex with 5-Br-Q₀C₁₀. There is a hypothesis alkaliphile oxidative phosphorylation at very alkaline pH values involves the required respiratory chain proton pumping to the bulk phase to establish a membrane potential and also involves the use of one or more specific respiratory chain complexes to transfer protons to F₀ sector of the F₁F₀ ATP-synthase during dynamic protein-protein interaction. To test this hypothesis, the interaction between cytochrome caa₃ and F₁F₀-ATP synthase from alkaliphilic Bacillus pseudofirmus OF₄ was studied by using DSC and saturation transfer EPR.

Findings and Conclusions: Our data demonstrated that both extra fragments are required for bacterial bc₁ complex. The first 12 residues (residues 421-432) of the C-terminal extra fragment of cytochrome b in the cytochrome bc₁ complex from Rhodobacter sphaeroides are essential for maintaining structural integrity of the bc₁ complex. The ISP extra fragment (residues 96-107) is found to be required for the structural stability of ISP in this bacterial bc₁ complex. Both of these findings suggest that these two extra fragments possess the supernumerary subunit function in stabilizing the structure of the bacterial complex. Quinone binding site is an ongoing project, we haven’t got very good results yet. From the results of DSC and ST-EPR experiments, we concluded cytochrome caa₃ and F₁F₀-synthase from alkaliphilic Bacillus pseudofirmus OF₄ exist as a supermacromolecular complex in the membrane.