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Formation of Engineered Intersubunit Disulfide Bond in Cytochrome bc_1 Complex Disrupts Electron Transfer Activity in the Complex

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Summary

Protein domain movement of the Rieske iron-sulfur protein has been speculated to play an essential role in the bifurcated oxidation of ubiquinol catalyzed by the cytochrome bc_1 complex. To better understand the electron transfer mechanism of the bifurcated ubiquinol oxidation at Qp site, we fixed the head domain of ISP at the cyt c_1 position by creating an intersubunit disulfide bond between two genetically engineered cysteine residues: one at position 141 of ISP and the other at position 180 of the cyt c_1 [S141C(ISP)/G180C(cyt c_1)]. The formation of a disulfide bond between ISP and cyt c_1 in this mutant complex is confirmed by SDS-PAGE and Western blot. In this mutant complex, the disulfide bond formation is concurrent with the loss of the electron transfer activity of the complex. When the disulfide bond is released by treatment with β-mercaptoethanol, the activity is restored. These results further support the hypothesis that the mobility of the head domain of ISP is functionally important in the cytochrome bc_1 complex. Formation of the disulfide bond between ISP and cyt c_1 shortens the distance between the [2Fe-2S] cluster and heme c_1, hence the rate of intersubunit electron transfer between these two redox prosthetic groups induced by pH change is increased. The intersubunit disulfide bond formation also decreases the rate of stigmatellin induced reduction of ISP in the fully oxidized complex, suggesting that an endogenous electron donor comes from the vicinity of the *b* position in the cytochrome *b*.

Keywords

bacterial cytochrome bc_1 complex; formation of a disulfide bond between ISP and cyt c_1; head domain of ISP; *Rhodobacter sphaeroides*

1. Introduction

The cytochrome bc_1 complex (also known as ubiquinol-cytochrome *c* oxidoreductase or complex III) is a multi-subunit dimeric integral membrane protein complex. It is an essential segment of cellular energy-conserving electron transport chains in animals, plants, and bacteria [1,2]. This complex catalyzes electron transfer from ubiquinol to cytochrome *c* (or cytochrome c_2 in bacteria) and concomitantly translocates protons across the membrane to generate a

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membrane potential and pH gradient for ATP synthesis. Although the cytochrome bc_1 complexes from different sources vary in their polypeptide compositions, they all contain four redox prosthetic groups: two *b*-type cytochromes (b_L and b_H), one *c*-type cytochrome (c_1), and one high potential Rieske iron-sulfur cluster [2Fe-2S]. Structural information obtained from X-ray crystallographic studies [3–5] has revealed the arrangement of the redox centers, transmembrane helices, inhibitor binding sites and the intertwined dimeric structure. The longer than expected distance between [2Fe-2S] and heme c_1 and the less defined structure of the head domain of the iron-sulfur protein suggest a domain movement of ISP during the electron transfer in bc_1 complex. The domain movement hypothesis was further supported by the observation of various positions of the [2Fe-2S] cluster in different crystal forms [4,5] or in complexes loaded with different inhibitors [5,6].

Structurally, the iron-sulfur protein (ISP) in cytochrome bc_1 complex can be divided into three domains: the soluble C-terminal extramembrane domain (head), which houses the [2Fe-2S] cluster at its tip, the membrane-spanning N-terminal domain (tail), and the flexible linking domain (neck), which links the head and tail domains [7,8]. Bending of the neck is required for movement of the ISP head domain between the two positions (heme *b* and heme c_1). This movement is essential for electron transfer [9–12].

The study of the neck region of ISP using *Rhodobacter sphaeroides* cytochrome bc_1 complex [9,12] provided the first evidence for movement of the head domain of ISP during cytochrome bc_1 complex catalysis. Since the head and tail domains are rather rigid, a flexible neck region is necessary for head domain movement. Mutants with increased neck rigidity, generated by deletion or double- or triple-proline substitution, have greatly reduced electron transfer activity with an increase in activation energy [12]. Formation of a disulfide bond between two engineered cysteines with only one amino acid residue between them in the neck region near the transmembrane helix also drastically reduces electron transfer activity [9], presumably because of increased neck rigidity. As expected, cleavage of the disulfide bond by reduction or alkylation restores activity to that of the wild type enzyme [9]. Elongation of the neck region by insertion of multiple alanine residues significantly slows down the movement of the head domain of ISP and affects the bc_1 complex activity [13]. All of these results clearly demonstrate a need for neck flexibility in catalysis.

To further establish that the movement of the head domain of ISP is essential for the bc_1 complex, the mutant expressing His_6-tagged bc_1 complex with a pair of cysteine substituted residues at the interface between cytochrome *b* (A185) and the head domain of ISP (K70) [11] was generated and characterized. The disulfide bond between the engineered cysteine pair arrests the mobility of ISP to the fixed state (*b* position) and thus decreases electron transfer activity.

While the requirement of head domain movement of ISP is well established, the bifurcated ubiquinol oxidation at Qp site is not yet fully understood. It is generally believed that ISP is reduced by ubiquinol when its head domain is located at the *b* position. In other words, if the head domain is at c_1 position the ISP will not be able to accept electron from the substrate. To test this hypothesis, we recently generated the mutant [S141C(ISP)/G180C(cyt c_1)] with head domain of ISP fixed at the c_1 position by mutating residues S141 of ISP and G180 of cyt c_1 to cysteines to form an intersubunit disulfide bond (Fig. 1). Mutants with single cysteine substitution at the indicated positions were also generated and characterized to confirm that the residues are not at a critical for the activity or other properties of the bc_1 complex. The photosynthetic growth behavior, electron transfer activity, SDS-PAGE patterns, Western blot, pH-induced reduction and oxidation of ISP and cytochrome c_1 in the partially reduced complex, redox potential titration and EPR characteristics of the [2Fe-2S] cluster in purified complexes from wild type and mutant strains were examined and compared.

To study whether the formation of an intersubunit disulfide bond between ISP and cytochrome c_1 fixes the [2Fe-2S] cluster close to heme c_1, the rate of electron transfer between these two redox prosthetic groups, induced by pH change, is tested. Stigmatellin is also used to induce the reduction of ISP, in the absence of exogenous electron donor, in the fully oxidized complex to study whether the formation of the disulfide bond between cyt c_1 and ISP could pull the [2Fe-2S] cluster away from the Qp site. The EPR signals of ISP mutant complex with or without stigmatellin are examined and compared with wild type complex. All results show that the mutant S141C(ISP)/G180C(cyt c_1) complex obtained is indeed the one whose head domain of ISP is fixed at the cyt c_1 position when the disulfide bond is formed between the two engineered cysteines.

2. Materials and methods

2.1. Materials

Cytochrome *c* (horse heart, type III) was purchased from Sigma. N-Dodecyl-β-D-maltoside (LM) and N-octyl-β-D-glucoside (OG) were from Anatrace. 2,3-Dimethoxy-5-methyl-6-(10-bromodecyl)-1,4-benzoquinol ($Q_0C_{10}BrH_2$) was prepared in our laboratory as previously reported [14]. Ni-NTA gel, Qiaprep Spin Miniprep kit and PCR purification kit were from Qiagen. All other chemicals were of the highest purity commercially available.

2.2. Growth of Bacteria

Escherichia coli cells were grown at 37 °C in LB medium. *R. sphaeroides* BC17 cells [15] were grown photosynthetically at 30 °C in an enriched Siström's medium containing 5 mM glutamate and 0.2% casamino acids. Photosynthetic growth conditions for *R. sphaeroides* were essentially as described previously [16]. The concentrations and antibiotics used were: ampicillin, 125 μg/ml; kanamycin sulfate, 30 μg/ml; tetracycline, 10 μg/ml for *E. coli*, and 1 μg/ml for *R. sphaeroides*; and trimethoprim, 100 μg/ml for *E. coli* and 30 μg/ml for *R. sphaeroides*.

2.3. Generation of R. sphaeroides Strains Expressing the His_6-tagged bc_1 Complexes with Single or Pair of Cysteine Substitutions on Cytochrome c_1 and ISP

The QuickChange™ XL site-directed mutagenesis kit from Stratagene was used for mutagenesis. The oligonucleotides used for mutagenesis were as follows: S141C (ISP), F, 5′ CCGATCGGCGGCGTGT GCGGTGACTTCGGGGGC 3′; R, 5′ GCCCCC GAAGTCACCGCACACGCCGCCGATCGG 3′; G180C (cyt c_1), F, 5′ GCCTGCTCGTGGAT CGCCATG 3′; R, 5′ CTAGGCGATCCACGAG CAGGC 3′; H111C (cyt c_1), F, 5′ GCGCGGGC GGGCTTCTGCGGCCCGATGGGCACC; R, 5′ GGTGCCCATCGGGCCGCAGAAGCCCGCCCGC GC; G112C (cyt c_1), F, 5′ CGGGCGGGCTTCCACTGCCCGATGG GCACCGGC; R, 5′ GCCGGTGCCCATCGGGCAGTGGAAGCCCGCCCG; M114C (cyt c_1), F, 5′ GGCTTCCA CGGCCCGTGCGGCACCGGCATGAGC; R, 5′ GCTCATGCCGGTGCCGCACGGGCCGTG GAAGCC.

The double-stranded plasmid pGEM-7Zf(+)-*fbcFB* was used as the template for mutagenesis. Forward and reverse primers were used for PCR amplification. The pGEM-7Zf(+)-*fbc*FB plasmid was constructed by ligating the *Eco*R I-*Xba* I fragment from pRKD418-*fbc*FBC_HQ [15] into *Eco*R I and *Xba* I sites of the pGEM-7Zf(+) plasmid. After the mutagenesis, the fragment containing the mutant from pGEM-7Zf(+)-*fbc*F_mB was inserted into pRKD418-*fbc*$FB_{Km}C_HQ$ by *Eco*R I and *Nsi* I double digestion and ligation reaction. Loss of kanamycin resistance was then used to screen for the recombinant cloning containing the mutant ISP gene (pRKD418-*fbc*F_mBC_HQ). A similar process was used to get the clones with the mutant cytochrome c_1 gene (pRKD418-*fbc*$FBC_{Hm}Q$) and double mutant pRKD418-*fbc*$F_mBC_{Hm}Q$.

The pRKD418-*fbc*$F_mBC_{Hm}Q$ plasmid in *Escherichia coli* S17-1 cells was mobilized into *R. sphaeroides* BC17 cells by the plate-mating procedure [15]. The presence of engineered mutations was confirmed by DNA sequencing before and after photosynthetic or semi-aerobic growth of the cells as previously reported [11,15]. Expression plasmid pRKD418-*fbc*$F_mBC_{Hm}Q$ was purified from an aliquot of a photosynthetic or semi-aerobic culture using the Qiagen Plasmid Mini Prep kit. Because *R. sphaeroides* cells contain more than one type of endogenous plasmids [11], the isolated plasmids lack the purity and concentration needed for direct sequencing. Therefore, the DNA segments containing the mutation sequence in ISP and cytochrome c_1 were amplified from the isolated plasmids by the polymerase chain reaction. The PCR products were purified with an extraction kit from Sigma. The DNA sequencing was performed by the Recombinant DNA/Protein Core Facility at the Oklahoma State University.

2.4. Enzyme Preparations and Assay of Cytochromes

Chromatophore membranes were prepared from frozen cell paste and cytochrome bc_1 complexes with a His_6-tag placed at the C-terminus of cytochrome c_1 were purified from chromatophores as described previously [16] and stored at −80 °C in the presence of 10% glycerol. Protein concentrations were determined by the absorbance at 280 nm, using a converting factor of 1 OD_{280} = 0.56 mg/ml. The concentrations of cytochromes *b* and c_1 were determined spectrophotometrically using published molar extinction coefficients [17–19].

2.5. Activity Assay of the Cytochrome bc_1 Complex

To assay ubiquinol-cytochrome *c* reductase activity, chromatophores or purified cytochrome bc_1 complexes were diluted with 50 mM Tris-Cl, pH 8.0, containing 200 mM NaCl and 0.01% N-dodecyl-β-D-maltoside (LM) to a final concentration of cytochrome *b* of 1 μM unless otherwise specified. Appropriate amounts of the diluted samples were added to 1 ml of assay mixture containing 100 mM Na^+/K^+ phosphate buffer, pH 7.4, 0.3 mM EDTA, 100 μM cytochrome *c*, and 25 μM $Q_0C_{10}BrH_2$. Activities were determined by measuring the reduction of cytochrome *c* (the increase of the absorbance at wavelength 550 nm) in a Shimadzu UV-2401 PC spectrophotometer at 23 °C, using a millimolar extinction coefficient of 18.5 for calculation. The non-enzymatic oxidation of $Q_0C_{10}BrH_2$, determined under the same conditions, in the absence of enzyme, was subtracted during specific activity calculations. Although the chemical properties of $Q_0C_{10}BrH_2$ are comparable with those of $Q_0C_{10}H_2$, the former is a better substrate for the cytochrome bc_1 complex [14].

2.6. Gel Electrophoresis and Western Blot Preparation

SDS-PAGE was performed according to the method of Laemmli [20] using a Bio-Rad Mini-Protean dual slab vertical cell. Samples were digested with 10 mM Tris-Cl buffer, pH 6.8, containing 1% SDS and 3% glycerol in the presence or absence of 0.4% β-mercaptoethanol (β-ME) for 10 min at room temperature before being subjected to electrophoresis. Western blotting was performed with polyclonal rabbit antibodies against cytochrome c_1 or ISP of the *R. Sphaeroides* bc_1 complex. The polypeptides separated by SDS-PAGE gel were transferred to 45 Micron nitrocellulose membrane for immunoblotting. Protein A conjugated to horseradish peroxidase (HRP) was used as the second antibody. Color development was carried out using a HRP color development solution.

2.7. Determination of the Redox Potential of Cytochromes c_1 in the Wild Type and the Mutant Cytochrome bc_1 Complexes

Redox titrations of cytochromes c_1 in the wild type and the mutant bc_1 complexes were determined essentially according to the previously published method [21,22]. 3 ml aliquots of the bc_1 complex (7 μM cytochrome c_1) in 0.1 M Na^+/K^+ phosphate buffer, pH 7.0, containing 25 μM 1,4-benzoquinone (Em, 293 mV), 25 μM 2,3,5,6-tetramethyl-p-phenylenediamine (Em,

260 mV), 25 μM 1,2-naphthoquinone (Em, 143 mV), 20 μM phenazine methosulfate (Em, 80 mV), 20 μM phenazine ethosulfate (Em, 55 mV), 25 μM 1,4-naphthoquinone (Em, 36 mV) and 25 μM duroquinone (Em, 5 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 Eh values during the redox titration, absorption spectra, 600 to 500 nm, were taken with a Shimadzu model UV-2401 PC spectrophotometer. The optical density at 550 nm, minus that at 535 nm, was used for cytochrome c_1 measurement. The midpoint potential of cytochrome c_1 was calculated by fitting the redox titration data, obtained for cytochrome c_1, using the Nernst equation for a one-electron carrier (n = 1) by Kaleidagraph [21].

2.8. Determination of pH-induced Reduction and Oxidation of ISP and Cytochrome c_1 in the Partially Reduced Wild Type and Mutant bc_1 Complexes

The wild type or mutant bc_1 complex was diluted in 3 ml of 20 mM Tris-Cl buffer, pH 8.0, containing 200 mM NaCl and 0.01% LM. The concentration of cytochrome c_1 was adjusted to about 10 μM. Different amounts of NaOH or HCl were added to give the indicated pHs. Fully oxidized or reduced cytochrome c_1 and ISP were obtained by addition of $K_3Fe(CN)_6$ or sodium ascorbate. Reduction of cytochrome c_1 was followed by measuring the increase of the α-absorption (550–535 nm) in a Shimadzu UV-2401 PC spectrophotometer. Reduction of ISP was followed by measuring the negative CD peak at 500 nm of partially reduced ISP minus fully oxidized complex in a JASCO J-715 spectropolarimeter [11,23,24]. The same samples were used for the absorption and CD measurement. Instrument settings for the spectropolarimeter were: scan speed, 100 nm/min; step resolution, 1 nm; accumulation, 10 traces for averaging; response, 1 s; bandwidth, 2.0 nm; sensitivity, 10 mdeg; and slit width, 500 μm.

2.9. Determination of the pH induced Electron Transfer Rates between the [2Fe-2S] Cluster and Heme c_1 in the Wild Type and the Mutant bc_1 Complexes

The method used is essentially the same as that previously reported [11,24]. The cytochrome bc_1 was diluted in 20 mM Tris-Cl buffer, pH 8.0, containing 200 mM NaCl and 0.01% LM to a cytochrome c_1 concentration around 10 μM. The percentage of cytochrome c_1 reduction in the sample was adjusted to around 50% at pH 8.0 by adding sodium ascorbate or ferricyanide. For the electron transfer from the [2Fe-2S] cluster to heme c_1, the pH of the sample was decreased to pH 6.9 by adding HCl. This solution was rapidly mixed with an equal volume of the same buffer but containing enough NaOH to cause the pH change from 6.9 to 8.9 in the stopped flow spectrophotometer at room temperature. Increased reduction of cytochrome c_1 was monitored spectrophotometrically. The reaction was monitored at 550nm–535nm. Complex mixed with an equal volume of the same buffer at pH 6.9 was used as a baseline.

2.10. Stigmatellin Induced Reduction Rate of ISP in the Absence of Exogenous Electron Donor in the Fully Oxidized Complex

In order to know whether position of [2Fe-2S] cluster in ISP was pulled toward the cytochrome c_1, far away from the Qp site by the formation of the disulfide bond between cyt c_1 and ISP, stigmatellin is used to induce the reduction of ISP in the absence of exogenous electron donor in the fully oxidized complex. The bc_1 complexes from wild type, S141C(ISP)/G180C(cyt c_1), and A185C(cyt *b*)/K70C(ISP) [11] were diluted to 10 μM of cytochrome c_1 in 3 ml of 20 mM Tris-Cl buffer, pH 8.0, containing 200 mM NaCl and 0.01% LM. $K_3Fe(CN)_6$ was used to fully oxidize the bc_1 complexes, and the fully oxidized state of complexes was confirmed by measuring CD from 450 nm to 550nm in a JASCO J-715. Stigmatellin induced reduction of ISP were measured after incubating the fully oxidized bc_1 complexes with 50 μM inhibitor for 5 min, 1 hr and 2hr, respectively. Fully reduced ISP spectrum was obtained after the bc_1

complex was reduced by sodium ascorbate. The reduction of ISP was calculated by the negative CD peak at 500 nm of reduced ISP minus fully oxidized spectrum.

2.11. Stigmatellin Induced Oxidation Rate of Cytochrome c_1 in the Partially Reduced Complex

It is well known that binding of stigmatellin to cytochrome bc_1 complex causes substantial elevation of the mid-point potential of ISP [25,26]. As a result, an intersubunit electron transfer between the [2Fe-2S] cluster and heme c_1 is expected to take place after stigmatellin is added into the complex. The bc_1 complex was diluted to cytochrome c_1 5 μM in the buffer containing 20 mM Tris-Cl buffer, pH 8.0, containing 200 mM NaCl and 0.01% LM. Sodium ascorbate was used to adjust the reduction of cyt c_1 to around 50%. Stigmatellin was added to the complex to a final concentration 25 μM. The redox state of cytochrome c_1 was monitored (550–535 nm) by a Shimadzu UV-2401 PC spectrophotometer at 23 °C.

2.12. EPR Experiments

EPR spectra of the ISP were recorded by a Bruker EMX Spectrometer [27]. The instrument settings were as follows: microwave frequency, 9.28 Hz; microwave power, 20 milliwatt; modulation amplitude, 20 G; modulation frequency, 100 kHz; time constants, 0.1 s; and scan rate, 20 respectively. 50 μM bc_1 complexes were incubated with 200 μM sodium ascorbate on ice for 20 min and frozen in liquid nitrogen. After EPR spectra were recorded, the complexes were thawed on ice and stigmatellin was added to 250 μM. The samples were put into liquid nitrogen again after incubation with stigmatellin on ice for 15 min. EPR spectra were record at −156°C.

3. Results and discussion

3.1. Photosynthetic Growth Behaviors of the Wild Type and Mutants

Since cytochrome bc_1 complex is absolutely required for photosynthetic growth of *R. sphaeroides*, a mutant with cysteine substitution at a critical position will not grow photosynthetically, whereas mutants with substitution at noncritical positions will grow. Thus, observing the photosynthetic growth behavior, one can determine whether the engineered cysteines are at critical positions. In mid-log phase, semiaerobically dark grown wild type and mutant cells were inoculated into enriched Siström's medium and subjected to anaerobic photosynthetic growth conditions. Mutants grew at rates comparable with that of the wild type (Table I), indicating that the engineered cysteine at these positions are noncritical to the complex. However, mutants H111C(cyt c_1), G112C(cyt c_1) and S141C(ISP)/H111C(cyt c_1), S141C(ISP)/G112C(cyt c_1) grew at a retarded rate, suggesting that substitutions of H111C(cyt c_1), G112C(cyt c_1) affect the activity of cytochrome bc_1 complex and thus decrease the growth rate of *R. sphaeroides*.

3.2. Formation of a Disulfide Bond between the ISP Head Domain and Cytochrome c_1 in the S141C(ISP)/G180C(cyt c_1) Mutant bc_1 Complex

Chromatophores freshly prepared from the S141C(ISP), G180C(cyt c_1), and S141C(ISP)/G180C(cyt c_1) mutant cells have, respectively, 100, 78, and 26% of the bc_1 activity found in the wild type chromatophores (Table 1). The purified His_6-tagged bc_1 complexes isolated from freshly prepared chromatophores have, 100, 80, and 8% of the wild type bc_1 activity respectively (Table 1), based on cytochrome *b* content. Both of the single mutants, S141C(ISP) and G180C(cyt c_1) have good activity, indicating the mutagenesis has little effect. The reason that the double mutant S141C(ISP)/G180C(cyt c_1) can grow well photosynthetically yet the activity of both the chromatophore and purified bc_1 complex are low is because few disulfide bonds are present in the intact cells. As formation of a disulfide bond is an oxidative process, no disulfide bond can be formed in anaerobic conditions, even if the two cysteines are in

favorable positions. Since photosynthetic growth is under strict anaerobic conditions, disulfide bond formation between the two engineered cysteines in mutant S141C(ISP)/G180C(cyt c_1) is not possible. Hence, the double mutant cell can grow photosynthetically. However, during preparation of chromatophores and purification of the bc_1 complex, both engineered cysteines are exposed to the air (oxygen); formation of the disulfide bond occurs, resulting in a decrease of activity.

The specific activity of aerobically purified complex from mutant S141C(ISP)/G180C(cyt c_1) is around 8% of the wild type complex. It is of interest to know whether this residual activity is from the disulfide linked complex or due to the incomplete disulfide bond formation. One way to know the nature of this residual activity is to determine the sensitivity of this activity to antimycin. Results show that 90% of activity in the mutant was blocked by antimycin, similar to the sensitivity of activity of the wild type complex (data not shown), indicating that the residual activity of mutant complex is resulted from the presence of small portion bc_1 complex without disulfide linkage between ISP and cyt c_1 subunits. The presence of a faint band of free ISP and cytochrome c_1 in the Western blot analysis with antibodies against *R. sphaeroides* cytochrome c_1 or ISP of mutant S141C(ISP)/G180C(cyt c_1) complex in the absence of β-ME supports this conclusion.

To see whether or not the loss of the bc_1 complex activity observed in the mutant S141C(ISP)/G180C(cyt c_1) results from disulfide bond formation during the purification, SDS-PAGE patterns of the purified mutant complexes, with and without β-ME treatment, were examined (Fig. 2). The purified complexes were treated with SDS in the absence or presence of β-ME (Fig. 2) at room temperature for 10 min and subjected to electrophoresis. The same process was done with bc_1 of the wild type, S141C(ISP), and G180C(cyt c_1) mutants. When the β-ME was present, all bc_1 complexes showed the same pattern in SDS-PAGE: 4 protein bands, cyt *b* (41 kDa), cyt c_1 (30.4 kDa), ISP (21 kDa), and subunit IV (14 kDa). However, in the absence of β-ME, the two single mutants had the same electrophoretic pattern as the wild type but the mutant S141C(ISP)/G180C(cyt c_1) showed two differences: (i) An adduct protein band of the cross-link protein was present and its molecular weight was around 51 kDa, the sum of cyt c_1 (30.4 kDa) and ISP (21 kDa); (ii) The density of cyt c_1 and ISP bands showed a sharp decrease, indicating that most cyt c_1 and ISP are present in the cross-link protein. The intersubunit disulfide bond linked adduct protein from the mutant S141C(ISP)/G180C(cyt c_1) was identified as a smear protein band in SDS-PAGE (Fig. 2), consistent with the characteristic of cytochrome c_1 which also shows as a smear band in SDS-PAGE in the absence of β-ME. It has been reported that cyt c_1 of *R. sphaeroides* shows as a smear band in SDS-PAGE patterns under non-reducing conditions [28]. It was recently reported that [28], under reducing conditions (in the presence of β-ME), protein with covalently bound heme (such as *c*-type cytochrome) shows as a sharp band in SDS-PAGE, whereas the protein band of *c*-type cytochromes become smears in SDS-PAGE when β-ME is absent.

Western blot analysis with antibodies against *R. sphaeroides* cytochrome c_1 or ISP was done to characterize the adduct protein band (Fig. 3). The results show that the adduct protein band can be blotted by both anti- cyt c_1 and anti-ISP antibodies, indicating the adduct protein contains both cyt c_1 and ISP. Trace amount of cytochrome c_1 and ISP was also observed in the Western blot analysis of the double mutant complex.

From gene sequence, there are nine cysteine residues in the *R. sphaeroides* cytochrome bc_1 complex: one in cytochrome *b*, four in cytochrome c_1, and four in ISP. Two cysteines (Cys129 and Cys149) in ISP serve as ligands for the [2Fe-2S] cluster [29] and two cysteines (Cys37 and Cys40) in cytochrome c_1 are covalently bonded to heme c_1 [30]. Thus, there are five cysteines in the complex: one in cytochrome *b* (Cys54), two in ISP (Cys134 and Cys151) and two in cytochrome c_1 (Cys145 and Cys169). In the reported stigmatellin loading in cytochrome

bc_1 complex structure [10], Cys134 is on loop 4–5 and Cys151 is on loop 6–7 of ISP. The distance between these two cysteines is 2.0 Å and they form an intrasubunit disulfide bond that brings the two loops together and stabilizes this region [29]. The distances between the engineered cysteine S141C (ISP) to Cys129 (ISP), Cys134 (ISP), Cys 149(ISP), and Cys 151 (ISP) are 13.3 Å, 11.2 Å, 14.4 Å, and 13.7 Å, respectively. Therefore, they are not available for intrasubunit disulfide bond formation between the engineered and endogenous cysteines in ISP. Since it has been established that the pair of cysteines, Cys145 and Cys169 in the head domain of cytochrome c_1 form a disulfide bond to maintain the structural integrity [28], their participation in forming a disulfide bond with the engineered cysteine in cytochrome c_1 (G180C) is unlikely. Also, the distances between Cys 180 (cyt c_1) and Cys145 (cyt c_1) and Cys169 (cyt c_1) are 10.0 Å and 8.6 Å, too far apart for intrasubunit disulfide bond formation, as the head domain of cytochrome c_1 is rather rigid. Although the distance between two engineered residues, S141 (ISP) and G180 (cyt c_1) is 18.6 Å when the head domain of ISP is at the *b* position (Fig. 1), this distance is expected to be much less when the head domain moves ~23 Å to c_1 position to deliver the electron [31]. Therefore, the formation of an intersubunit disulfide bond is likely.

The low bc_1 complexes activities (Table I) and the retarded rate of photosynthetic growth in mutants H111C(cyt c_1), G112C(cyt c_1) and S141C(ISP)/H111C(cytc_1), S141C(ISP)/G112C (cyt c_1), indicate that the mutation on these two amino acid residues have considerable effect on the bc_1 complexes. Double mutant S141C(ISP)/M114C (cyt c_1), however, has good bc_1 complex activity and normal growth rate, suggesting that no significant change in the mutant complexes. SDS-PAGE results (data not shown) show there is no adduct protein band in either S141C(ISP)/H111C(cyt c_1), S141C(ISP)/G112C (cyt c_1) or S141C(ISP)/M114C(cyt c_1), indicating that no disulfide bond is formed between ISP and cytochrome bc_1 in these double mutant complexes.

Based on the crystal structure of *R. sphaeroide* bc_1 complex determined in the presence of stigmatellin [10], the distances from S141C(ISP) to H111(cyt c_1), G112(cyt c_1) and M114(cyt c_1) are 20.0, 23.5 and 24.1 Å, respectively, farther than the distance between S141C(ISP) and G180C(cyt c_1) (18.6 Å). The reason of designing these 3 mutants was to test whether they are on the path of the head domain of ISP moving to cytochrome c_1 position. The results showed that none of them can form disulfide bond with S141C of ISP, suggesting they are not in the proposed path of ISP head domain movement. However, since disulfide bond can be formed between G180C(cyt c_1) and S141C(ISP), G180C is located in the proposed path of the ISP head domain movement. The fact, that S141C(ISP)/G180C(cyt c_1) can form the disulfide bond between cytochrome c_1 and ISP only in the absence and not in the presence of stigmatellin (data not show), suggests that cytochrome bc_1 complex structure obtained in the presence of stigmatellin is different from the native one and the head domain of cytochrome c_1 may undergo a certain movement in the native structure. A high resolution structure of cytochrome bc_1 complex in the absence of inhibitor would resolve these speculations.

3.3. Effect of β-ME on the Activity of Cytochrome bc_1 Complex

To confirm that formation of a disulfide bond between ISP and cytochrome c_1 in the S141C (ISP)/G180C(cyt c_1) mutant causes activity loss, the effect of β-ME on bc_1 complex activity and the degree of disulfide bond formation were examined. When purified S141C(ISP)/G180C (cyt c_1) mutant complex was incubated at 4°C with 15 mM β-ME, activity increased as incubation time was prolonged (Fig 4), the activity was restored to that of single mutant G180C (cyt c_1) after a 24 hour incubation. No adduct protein of ISP and cytochrome c_1 was detected in the β-ME treated S141C(ISP)/G180C(cyt c_1) mutant complex. When this complex was purified from freshly prepared chromatophores in the presence of 100 mM β-ME, it had the same activity as that found in the G180C(cyt c_1) and no ISP- cytochrome c_1 adduct was

detected. All these results show that the loss of enzymatic activity is caused by the formation of a disulfide bond between S141C(ISP) and G180C(cyt c_1), not the presence of cysteine. It must be emphasized that the observed activity restoration is not due to nonenzymatic reduction of cytochrome *c* by added β-ME [11] nor to long term exposure to 4°C, because the same treatment has little effect on the wild type complex.

3.4. Effect of the Mutations on Redox Potential of Cytochrome c_1 in the bc_1 Complex

The redox potential of cyt c_1 in the S141C(ISP)/G180C(cyt c_1) mutant complex is 220 mV (Fig. 5), slightly lower than 235 mV observed in the wild type bc_1 complex [28,32–34], indicating the formation of a disulfide bond does not affect the redox potential of cyt c_1 significantly, and the loss of bc_1 activity cannot be attributed to a redox potential change.

3.5. pH-dependent Oxidation of ISP and Reduction of Cytochrome c_1 in the Purified Cytochrome bc_1 Complex

It has been reported that intramolecular electron transfer between the [2Fe-2S] cluster and heme c_1 in both bovine and *R. sphaeroides* cytochrome bc_1 complexes can be induced by pH change of the enzyme solution [11,24]. This is based on the fact that the redox potential of heme c_1 is independent of pH, whereas the redox potential of the [2Fe-2S] cluster is pH-dependent (negative correlation). A similar phenomenon is observed in the S141C(ISP)/G180C(cyt c_1) mutant complex, as shown in Fig. 6. Both ISP and cytochrome c_1 are 52% reduced at pH 8.0, indicating that they have the same redox potential at the given pH. However, when the pH is increased to 9.0, cytochrome c_1 reduction increases to 78% and ISP reduction decreases to 28%. When the pH is increased, the redox potential of ISP decreases while that of cytochrome c_1 remains unchanged. As a result, electrons transfer from ISP to cytochrome c_1. Similarly, when the pH is decreased from pH 8.0 to 7.0, cytochrome c_1 reduction decreases from 52% to 30%, whereas ISP reduction increases from 52% to 82%. A linear relationship between ISP oxidation and cytochrome c_1 reduction is observed (Fig. 6). These results suggest that formation of an intersubunit disulfide bond does not alter the pH dependency of midpoint potentials of either ISP or cytochrome c_1.

3.6. Effect of Disulfide Formation, between ISP and Cytochrome c_1, on the Rate of Intramolecular Electron Transfer Between the [2Fe-2S] Cluster and Heme c_1 Induced by pH Change

The rate of intramolecular electron transfer between the [2Fe-2S] cluster and heme c_1 is correlated with the distance between them. Therefore, comparing electron transfer rates in wild type and mutant complexes is a way to prove whether or not intersubunit disulfide bond formation arrests the head domain of ISP at the c_1 position. Fig. 7 shows the time trace of alkalization-induced intramolecular electron transfer between [2Fe-2S] cluster and heme c_1 in the wild type and the mutant S141C(ISP)/G180C(cyt c_1). Reduction of heme c_1 in the wild type complex appears to be biphasic, with rate constants of 29 s^{-1} and 0.6 s^{-1} (Fig. 7A) respectively. This result is consistent with some (<15%) head domains of ISP molecule being at the *b* position while others at the c_1 position and somewhere in between. Reduction of cytochrome c_1 in the mutant S141C(ISP)/G180C(cyt c_1) complex is monophasic, with the slower rate constant of 0.7 s^{-1} (Fig. 7B). We presume that a majority of ISP molecules which are at the c_1 position are reduced within the dead-time of the apparatus. In other words, the first phase of cytochrome c_1 reduction in the mutant S141C(ISP)/G180C(cyt c_1) complex is too fast to be measured by the stopped-flow apparatus. As one can see from Fig. 7B, about 80% of inducible reduction of cytochrome c_1 is completed within the dead time of the instrument. These results proved the formation of intersubunit disulfide bond between ISP and cytochrome c_1 in the mutant S141C(ISP)/G180C(cyt c_1) complex and the head domain of ISP is held at the c_1 position by the disulfide bond.

3.7. Stigmatellin Induced Reduction Rate of ISP in the Absence of Exogenous Electron Donor in the Fully Oxidized Complex

Stigmatellin is a tightly bound Qp site inhibitor [35]. The [2Fe-2S] cluster in the mutant S141C (ISP)/G180C(cyt c_1) bc_1 complex is further from bound stigmatellin than those in the wild type and A185C(cyt *b*)/K70C(ISP). As a result, its reduction rate induced by stigmatellin is the slowest in these three bc_1 complexes (Table 2) with only a small portion of ISP, in the mutant S141C(ISP)/G180C(cyt c_1) complex, being reduced. On the contrary, the reduction rate of the [2Fe-2S] cluster in the mutant A185C(cyt *b*)/K70C(ISP) bc_1 complexes, induced by stigmatellin, is the fastest. The slow reduction rate of ISP in mutant S141C(ISP)/G180C(cyt c_1) bc_1 complex shows that formation of a disulfide bond between S141C(ISP) and G180C (cyt c_1) pulls the [2Fe-2S] cluster away from the Qp site, while the fast reduction rate of ISP in A185C(cyt *b*)/K70C(ISP) complex shows the [2Fe-2S] cluster is pulled close to the Qp site. Since there is no reducing compound present in the ferricyanide oxidized complex, the source of the electron donor is unknown; it might come from amino acid residues located near Qp site. The possibility that the reducing compound is ferricyanide has been ruled out because similar reduction is observed in a complex oxidized by cytochrome *c* oxidase and cytochrome *c*.

3.8. Stigmatellin Induced Oxidation of Cytochrome c_1 in the Partially Reduced Cytochrome bc_1 Complex

In the partially reduced wild type complex, a rapid oxidation of cytochrome c_1 is observed upon addition of stigmatellin. This oxidation is the result of an elevation of the mid-point potential of ISP by stigmatellin bound at the Qp site and the fixation of the head domain of ISP at the *b* position. In the mutant S141C(ISP)/G180C(cyt c_1) complex, the head domain of ISP is linked to the c_1 position through formation of the disulfide bond, the binding of stigmatellin is therefore no longer able to fix the head domain of ISP at the *b* position and no elevation of the mid-point potential of ISP will occur. Thus cytochrome c_1 in the mutant S141C (ISP)/G180C(cyt c_1) complex will not be oxidized by ISP in spite of the short distance between heme c_1 and the [2Fe-2S] cluster. This result confirms the observation that the elevation of ISP mid-point potential caused by stigmatellin binding is due to the fixation of ISP head domain at *b* position and not due to the binding of the inhibitor to ISP [26]. Fig. 8 compares the stigmatellin induced oxidation of cytochrome c_1 in the partially reduced bc_1 complexes of wild type and mutant S141C(ISP)/G180C(cyt c_1). Only a small portion of cytochrome c_1 in the mutant complex is oxidized upon the addition of stigmatellin and the rate of oxidation is much smaller than in the wild type. Although the rate of stigmatellin induced cytochrome c_1 oxidation in the wild type complex is much faster than that in the mutant complex, this rate is still much smaller than that induced by pH. The reason is because, in the case of pH induced oxidation, ISP potential is directly affected by pH, regardless of the location of head domain of ISP. In stigmatellin induced reduction, the potential change of iron-sulfur cluster is due to the fixation of the head domain of ISP at the *b* position. Since in the S141C(ISP)/G180C(cyt c_1) mutant complex stigmatellin can no longer fix head domain of ISP at the *b* position in the mutant complex, little potential elevation is expected. Therefore, little oxidation of cytochrome c_1 occurs, in spite of the short distance between the two redox centers.

3.9. Effect of the Mutations on EPR Characteristics of the [2Fe-2S] Cluster in the bc_1 Complex with and without stigmatellin binding

As shown in Fig. 9, the [2Fe-2S] cluster in the S141C(ISP)/G180C(cyt c_1) mutant has an EPR spectrum similar to that of the wild type complex, indicating that the microenvironments of the [2Fe-2S] cluster in the mutated bc_1 complexes are not affected by disulfide bond formation between the two engineered cysteines. Therefore, the loss of bc_1 activity is not due to the change of microenvironments of ISP but to the formation of an intersubunit disulfide. SDS-PAGE

results also show that the disulfide bond in the mutant complex remains under the conditions (in the presence of sodium ascorbate) used for the EPR measurement.

The EPR signal of ISP increases after the binding Qp site inhibitor, stigmatellin (Fig. 9). However, the amount of increase in the S141C(ISP)/G180C(cyt c_1) mutant is much less than that in the wild type complex, indicating the [2Fe-2S] cluster in the mutant is further to the *b* position in the Qp pocket so the effect of stigmatellin binding is less than that in the wild type complex. It is known that addition of stigmatellin to isolated reduced ISP has little effect on its EPR signals. Stigmatellin also has no effect on EPR signal of ISP if the cytochrome bc_1 complex is in the delipidated form. The effect is the result of the fixation of head domain of ISP at *b* position. In the isolated S141C(ISP)/G180C(cyt c_1) mutant complex, the head domain of ISP is fixed at c_1 position and it can not be moved to *b* position upon addition of the inhibitor, therefore no effect on EPR signal is observed. When the mutant complex is pretreated with β-ME to release the disulfide bond, the effect of stigmatellin on EPR signals of ISP is similar to that observed in the wild type complex (data not shown).

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The abbreviations used are

cyt
: cytochrome

LM
: N-Dodecyl-β-D-maltoside

EPR
: electron paramagnetic resonance

[2Fe-2S]
: Rieske iron-sulfur center

ISP
: Rieske iron-sulfur protein

PAGE
: polyacrylamide gel electrophoresis

$Q_0C_{10}BrH_2$
: 2,3-Dimethoxy-5-methyl-6-(10-bromodecyl)-1,4-benzoquinol

β-ME
: β-mercaptoethanol

Em
: redox midpoint potential

CD
: circular dichroism

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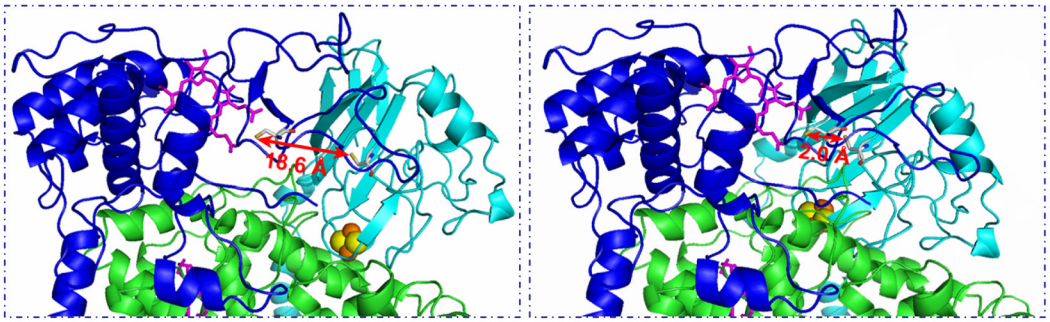

Fig. 1.

Relative distances between engineered cysteines in ISP and cytochrome c_1 in the cytochrome bc_1 complex from *R. sphaeroides* mutant S141C(ISP)/G180C(cyt c_1). Pictures show the distances between two engineered cysteines when the head domain of ISP is at the *b* position (left) and the 'c_1' position (right). Cytochrome c_1 is shown by the blue ribbon, cytochrome *b* is in green, and ISP (from the other monomer) is in cyan. The b_H and c_1 heme groups are shown as pink stick models, and the [2Fe-2S] cluster is depicted in gold and yellow spheres.

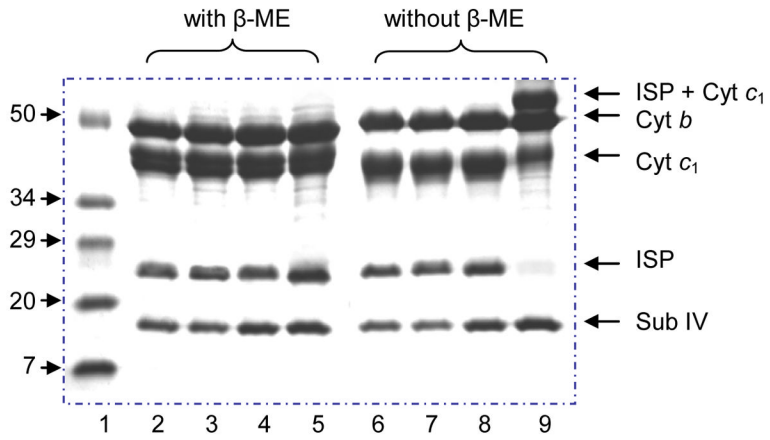

Fig. 2.

SDS-PAGE of the wild type and the cysteine mutant cytochrome bc_1 complexes. Lane 1, protein standard (Std); Lane 2 & 6, the wild type; Lane 3 & 7, S141C(ISP); Lane 4 & 8, G180C (cyt c_1); Lane 5 & 9, S141C(ISP)/G180C(cyt c_1). Adduct cross-link protein bond (ISP + Cyt c_1, lane 9) is disappeared when treated with β-ME (lane 5). Moreover, the intensity of ISP and cyt c_1 bands are obviously decreased compared to the corresponding bonds in the lane 5.

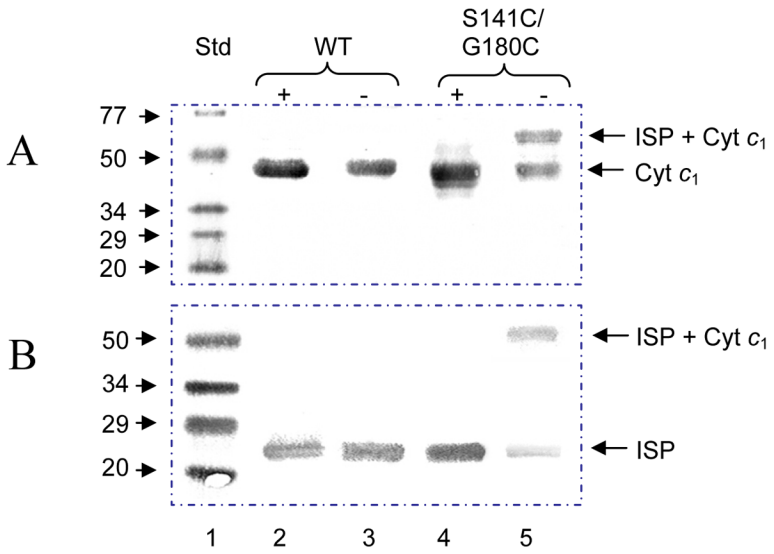

Fig. 3.

Western blot analysis of ISP or cytochrome c_1 in cytochrome bc_1 complexes from the wild type and S141C(ISP)/G180C(cyt c_1). Aliquots of the wild type and S141C(ISP)/G180C(cyt c_1) were incubated in the loading buffer containing 1% SDS with (+) or without (−) 0.4% β-ME at room temperature for 10 min. Digested samples containing 200 pmol of cytochrome *b* were subjected to electrophoresis. Samples in the gel were transferred to 45 micron nitrocellulose membrane and treated with antibody against *R. sphaeroides* cytochrome c_1 (A) or ISP (B). Protein A-horseradish peroxide conjugate was used as a second antibody. Std, protein standard. WT, the wild type.

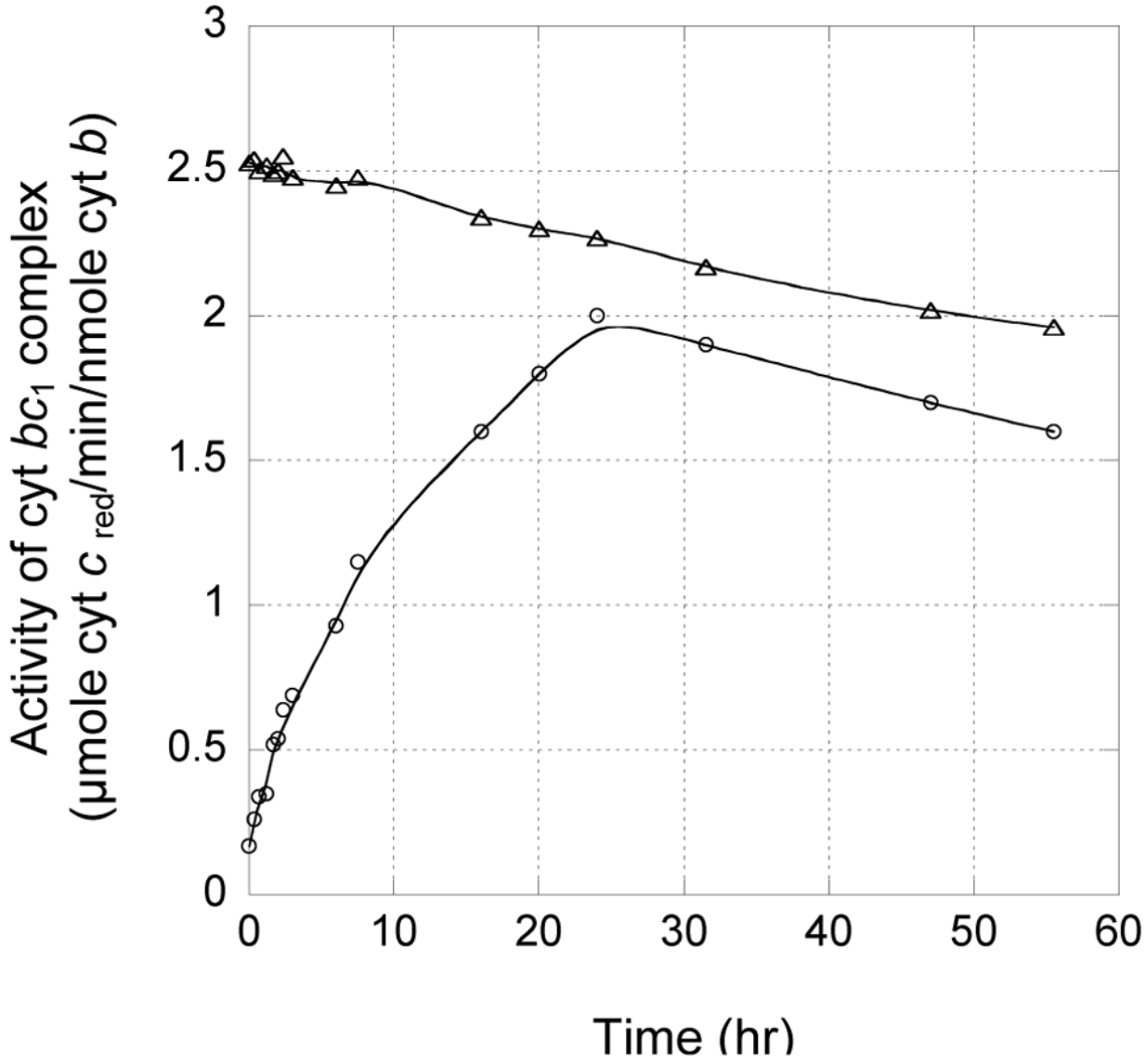

Fig. 4.

Effect of β-ME on the cytochrome bc_1 activity in purified complexes from the wild type (triangle) and the mutant S141C(ISP)/G180C(cyt c_1) (circle) at 4°C. 10 μM cytochrome bc_1 was incubated at 4°C with 15mM β-ME.

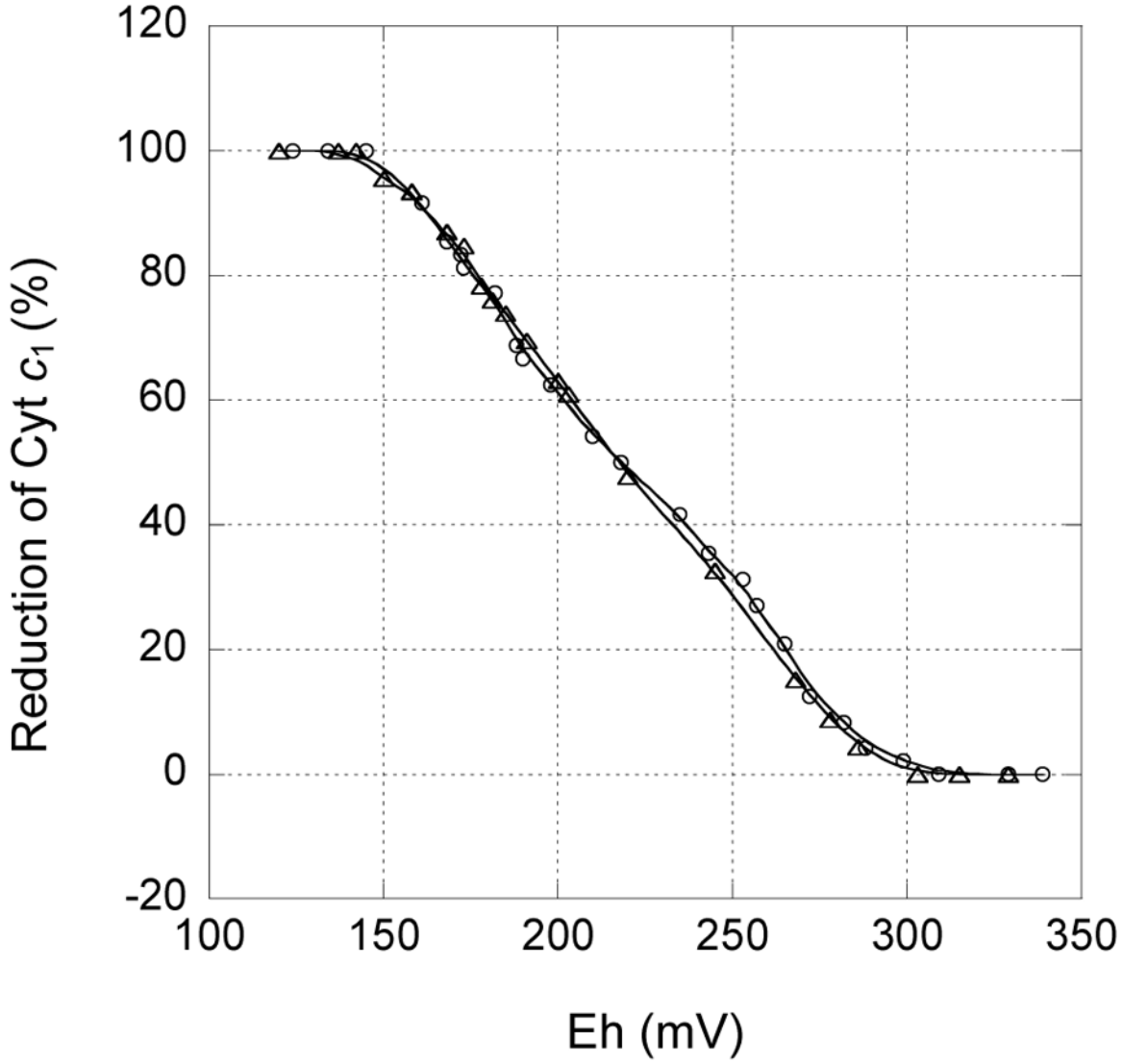

Fig. 5.

Redox potential titration of cytochrome c_1 in S141C(ISP)/G180C(cyt c_1) bc_1 complex. Oxidative and reductive titrations were performed as described in "Experimental Procedures." Triangle curve shows oxidative titration by ferricyanide and the circle shows reductive titration by dithionite. The data were fit to the Nernst equation when $n = 1$

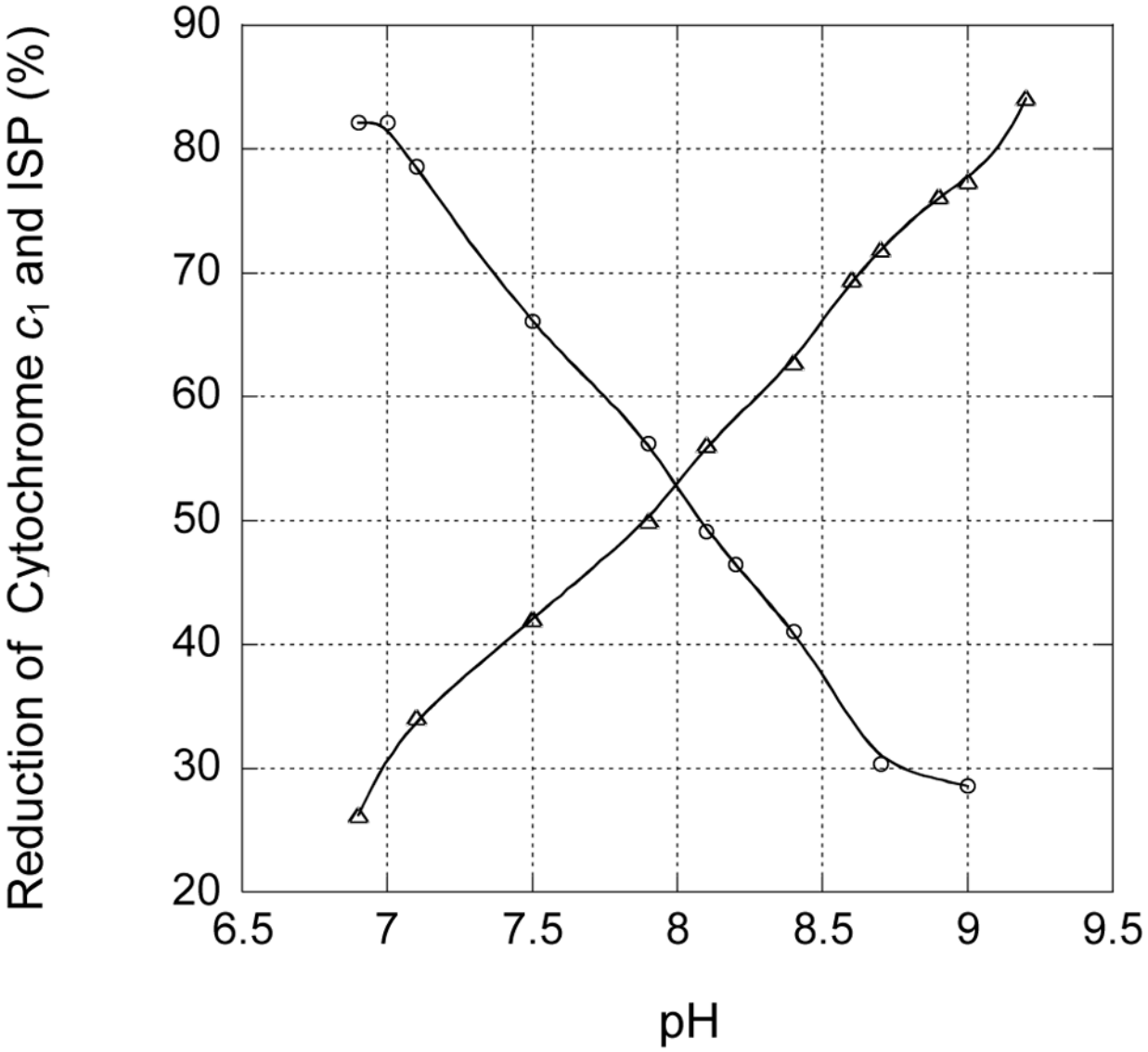

Fig. 6.

pH induced reduction and oxidation of ISP (circle) and cytochrome c_1 (triangle) in the partially reduced cytochrome bc_1 complex from S141C(ISP)/G180C(cyt c_1) mutant. The experimental conditions and instrument settings are detailed under “Experimental Procedures.”

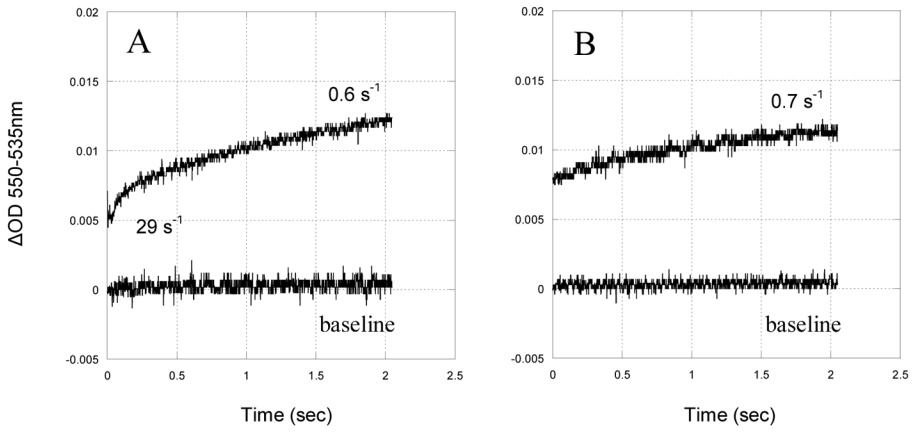

Fig. 7.

Time trace of the pH induced reduction of cytochrome c_1 in the partially reduced cytochrome bc_1 complex. 10 μM cytochrome bc_1 complex in pH 6.9 phosphate buffer was rapidly mixed with equal volume of the same buffer with enough NaOH to cause the pH change from 6.9 to 8.9 in the stopped flow spectrophotometer at room temperature. A, reduction of cytochrome c_1 in the wild type. B, reduction of cytochrome c_1 in mutant S141C(ISP)/G180C(cyt c_1).

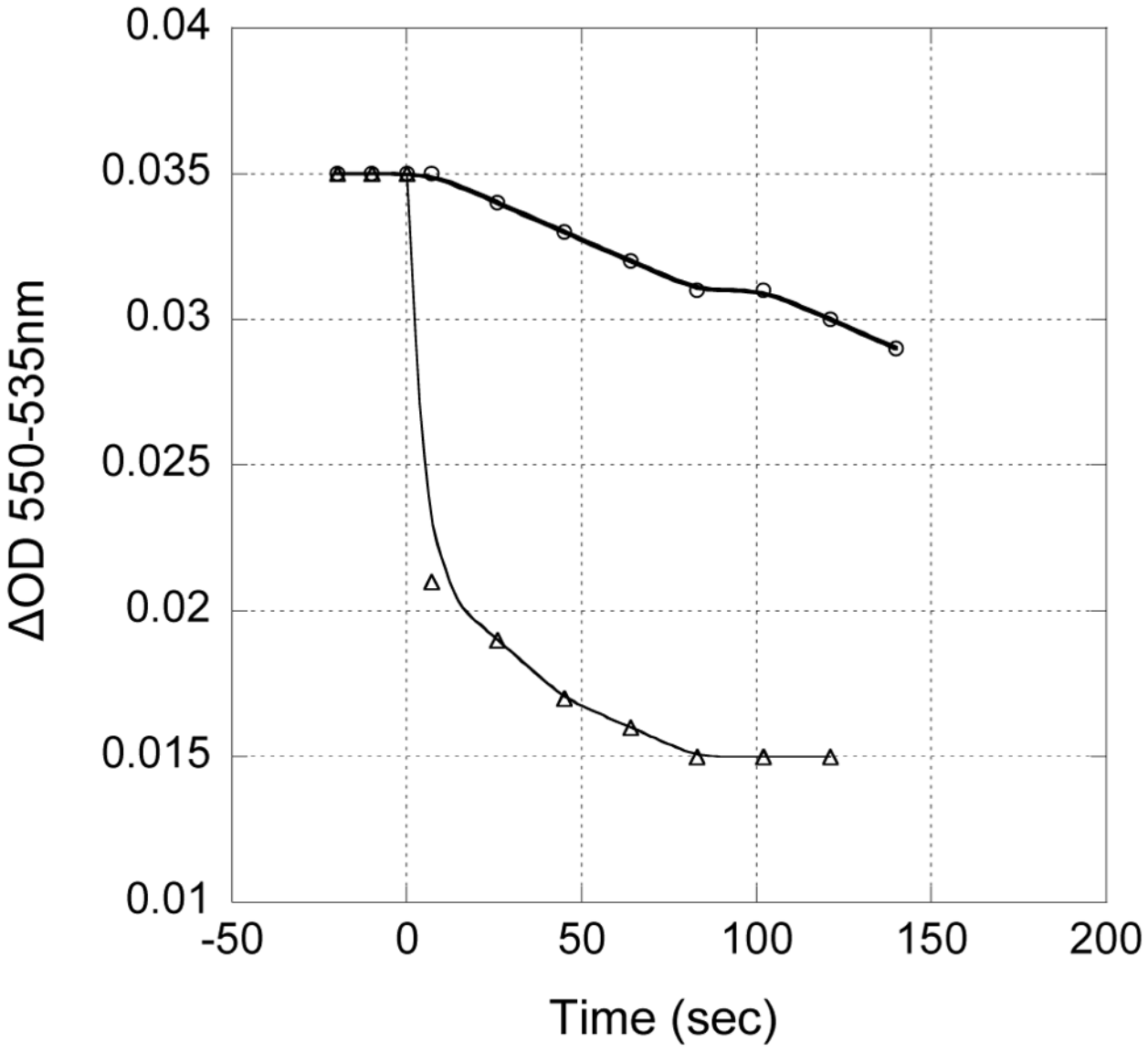

Fig. 8.

Time trace of stigmatellin induced oxidation of cytochrome c_1 in the partially reduced cytochrome bc_1 complex. 5 μM of the wild type (triangle) and the mutant S141C(ISP)/G180C (cyt c_1) (circle) cytochrome bc_1 complexes were treated with 25 μM stigmatellin. The oxidation of cytochrome c_1 is followed spectrophotometrically.

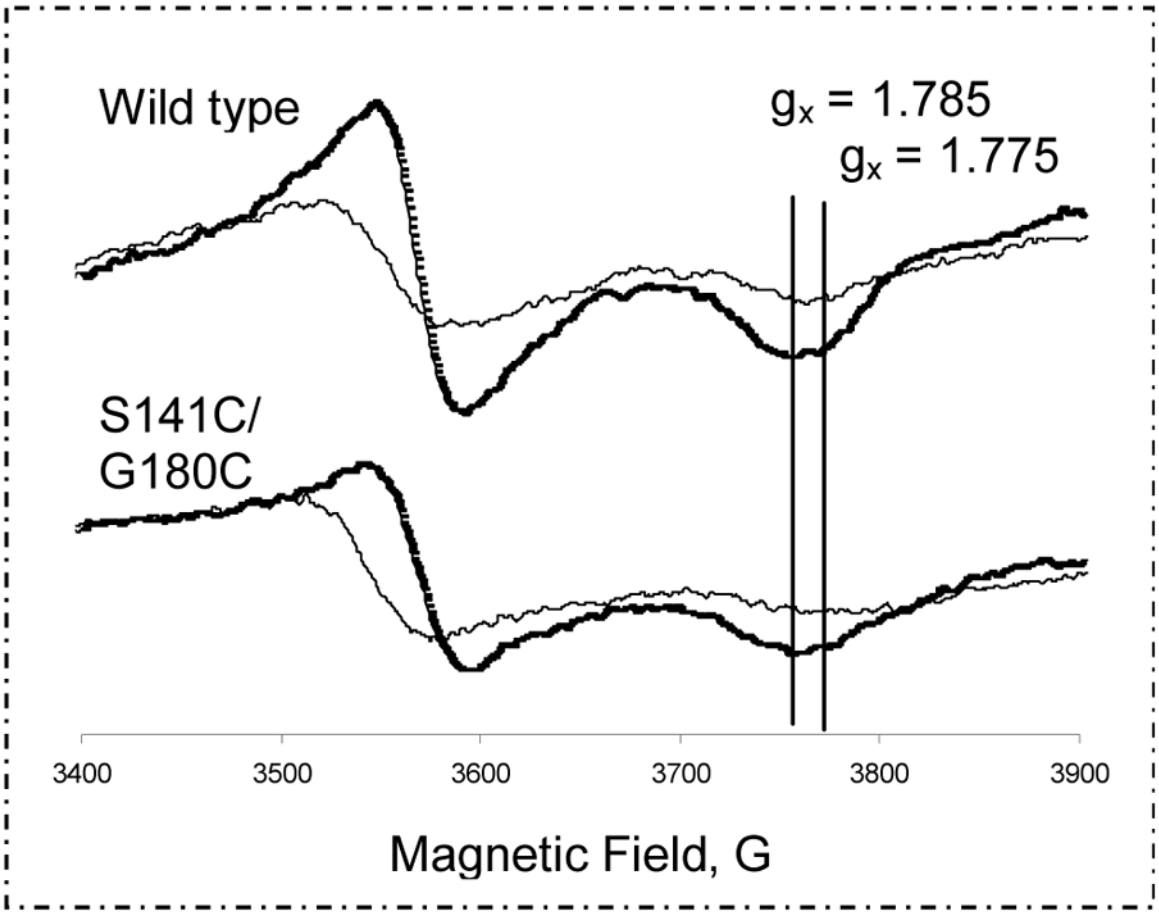

Fig. 9.
EPR spectra of the [2Fe-2S] cluster in the cytochrome bc_1 complexes of the wild type (top) and S141C(ISP)/G180C(cyt c_1) (bottom) with (thick) or without (thin) stigmatellin. The experimental conditions and instrument settings are detailed under "Experimental Procedures."

TABLE 1

Characterization of mutants. The cytochrome bc_1 complexes were in 50 mM Tris-Cl, pH 8.0, containing 200 mM NaCl, 200 mM histidine, 0.5% octyl glucoside and 10% glycerol.

Strains	Ps [a]	Activity [b]	
		Chromatoxphore	Purified complex
Wild type	+ + +	2.3 (100%)	2.5 (100%)
S141C(ISP)	+ + +	2.3 (100%)	2.5 (100%)
G180C(cyt c_1)	+ + +	1.8 (78%)	2.0 (80%)
S141C(ISP)/G180C(cyt c_1)	+ +	0.6 (26%)	0.2 (8%)*
H111C(cyt c_1)	+	0.2 (9%)	0.6 (24%)
S141C(ISP)/H111C(cyt c_1)	+	0.2 (9%)	0.6 (24%)
G112C(cyt c_1)	+	0.8 (35%)	0.9 (36%)
S141C(ISP)/G112C(cyt c_1)	+	0.8 (35%)	0.9 (36%)
M114C(cyt c_1)	+ + +	2.3 (100%)	2.5 (100%)
S141C(ISP)/M114(cyt c_1)	+ + +	2.3 (100%)	2.5 (100%)

[a] Ps=photosynthetic growth.

[b] The enzymatic activity is expressed as μmol of cytochrome *c* reduced/min/nmol cytochrome *b* at room temperature.

* If purification is carried out in the presence of β-ME, this value will be 2.0.

TABLE 2

Reduction of ISP induced by stigmatellin in the absence of exogenous electron donor. The bc_1 complexes from the wild type, S141C(ISP)/G180C(cyt c_1), and A185C(cyt *b*)/K70C(ISP) were diluted to 10 μM in 3 ml of 20 mM Tris-Cl buffer, pH 8.0, containing 200 mM NaCl and 0.01% LM. After incubated with 50 μM stigmatellin for 5 min, 1 hr, and 2 hr, the bc_1 complexes was subjected to the CD to measure the reduction of ISP.

	Reduction percentage of ISP		
	5 min	**1 hr**	**2 hr**
Wild type	54%	81%	100%
S141C(ISP)/G180C(cyt c_1)	43%	59%	76%
K70C(ISP)/A185C(cyt *b*)	91%	98%	100%