The Extra Fragment of the Iron−Sulfur Protein (Residues 96-107) of *Rhodobacter sphaeroides* Cytochrome bc_1 Complex Is Required for Protein Stability†

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ABSTRACT: Sequence alignment of the Rieske iron−sulfur protein (ISP) of cytochrome bc_1 complex from various sources reveals that bacterial ISPs contain an extra fragment. To study the role of this fragment in bacterial cytochrome bc_1 complex, *Rhodobacter sphaeroides* 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 and characterized. The ISPΔ(96−107), ISP(96−107)A, and ISP(104−107)A mutant cells, in which residues 96−107 of ISP are deleted, and residues 96−107 and 104−107 are substituted with alanine, respectively, do not grow photosynthetically and show no bc_1 complex activity in intracytoplasmic membranes prepared from these mutant cells. The ISP(96−99)A, in which residues 96−99 are substituted with alanine, grows photosynthetically at a rate comparable to that of the complement cells, whereas ISP(100−103)A, in which residues 100−103 are substituted with alanine, has a longer lag period prior to photosynthetic growth. Chromatophores prepared from these two mutant cells have 48% and 9% of the bc_1 activity found in the complement chromatophores. The loss (or decrease) of bc_1 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 c_1 into the membrane, the binding affinity of cytochrome *b* to cytochrome c_1, or the ability of these two cytochromes to interact with ISP or subunit IV. The order of essentiality of residues in this fragment is residues 104−107 > residues 100−103 > residues 96−99.

Rhodobacter sphaeroides cytochrome bc_1 complex catalyzes electron transfer from ubiquinol to cytochrome c_2 with concomitant translocation of protons across the membrane to generate a proton gradient and membrane potential for ATP synthesis (*1*). Purified complex contains four protein subunits: the largest three, housing two *b*-type cytochromes (b_{566} and b_{562}), one *c*-type cytochrome (c_1), and one high-potential Rieske iron−sulfur cluster ([2Fe−2S]),[1] respectively, are the core subunits; the smallest one (subunit IV), containing no redox prosthetic group, is a supernumerary subunit (*2*). The genes for these four subunits were cloned and sequenced (*3*, *4*). The protocol for generation of *R. sphaeroides* expressing His_6-tagged, wild-type and mutant cytochrome bc_1 complexes, has been well developed (*5*, *6*). Since this four-subunit bacterial complex is functionally analogous to the mitochondrial enzyme, has simpler subunit composition, and is readily manipulated genetically, it has been used as a model for structural and functional studies of mitochondrial cytochrome bc_1 complexes.

Recently, the 3-D structures of mitochondrial bc_1 complexes from beef (*7*, *8*), chicken (*9*), and yeast (*10*), which contain seven to eight supernumerary subunits in addition to the three core subunits, have been obtained. This structural information suggests an unexpected feature for this complex, e.g., the movement of the head domain of Rieske iron−sulfur protein (ISP) during bc_1 catalysis (*7*−*9*, *11*, *12*). The structure of ISP can be divided into three domains: the membrane-spanning N-terminal domain (tail), the soluble C-terminal extramembrane domain (head), and the flexible linking domain (neck). The [2Fe−2S] cluster, located at the tip of the head, accepts the first electron from ubiquinol and transfers it to heme c_1 in the proposed Q-cycle mechanism (*13*).

Biochemical evidence for this movement hypothesis was first provided by molecular genetic studies of the ISP neck in the *R. sphaeroides* complex (*5*, *14*). Because the three-dimensional structures of the head and tail domains are rigid and are the same in the fixed and released states, a bending of the neck is required for movement of the head domain. For the neck region to bend, some flexibility is imperative (*5*, *14*). Mutants with increased neck rigidity, generated by deletion or double- or triple-proline substitution, have greatly reduced electron-transfer activity with increased activation energy (*5*). Formation of a disulfide bond between two engineered cysteines, having only one amino acid residue between them, in the neck region near the transmembrane helix, drastically reduces electron-transfer activity (*14*),

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[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; cyt, cytochrome; ISP, Rieske iron−sulfur protein; $Q_0C_{10}H_2$, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinol; [2Fe−2S], Rieske iron−sulfur center; EPR, electron paramagnetic resonance; HRP, horseradish peroxides; Ni-NTA, nickel nitrilotriacetic acid; β-ME, β-mercaptoethanol.

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presumably because of increased neck rigidity. Cleavage of the disulfide bond by reduction or alkylation restores activity to that of the wild-type enzyme. These results clearly demonstrate a need for neck flexibility during catalysis. Molecular genetic studies of the ISP neck of the yeast (*15*−*17*) and *Rhodobacter capsulatus* (*18*, *19*) bc_1 complexes support this conclusion.

The movement hypothesis is further supported by the loss of bc_1 activity in *R. sphaeroides* bc_1 complexes having the ISP head locked in the fixed position by formation of an intersubunit disulfide bond from a pair of engineered cysteines, one each at the head domain of ISP and cytochrome *b* (*20*). The rates of intra electron transfer between heme c_1 and [2Fe−2S], induced by a pH change (*20*) or by a photoinduced ruthenium compound (*21*, *22*), in this intersubunit disulfide bond forming mutant complex are much lower than that in the wild-type or in their respective single cysteine mutant complexes, indicating that formation of an intersubunit disulfide bond between cytochrome *b* and ISP arrests the head domain of ISP in the "fixed state" position, too far away for electron transfer to heme c_1 (*20*, *22*).

Since the redox potentials and EPR characteristics of [2Fe−2S] in *R. sphaeroides* bc_1 complex are similar to those of the mitochondrial complex, the microenvironments of the [2Fe−2S] cluster in these two complexes must be quite similar. However, due to the presence of different numbers of supernumerary subunits in these two complexes, zero to one in the bacterial complexes and seven to eight in the mitochondrial complexes, maintaining the structural stability of ISP in these two complexes may be different. It has been reported (*23*) that subunit IX of the yeast mitochondrial cytochrome bc_1 complex is required for insertion of an EPR-detectable iron−sulfur cluster into the Rieske iron−sulfur protein and that the iron−sulfur protein is lost from the bc_1 complex during purification if subunit IX is deleted. Thus, the structure of ISP in the mitochondrial complex may be stabilized through interactions between ISP and its neighboring supernumerary subunits. Since bacterial complexes lack a supernumerary subunit in the vicinity of ISP, perhaps interactions between an ISP fragment and another part of ISP or between cytochrome *b* or cytochrome c_1 may contribute to the stability of ISP.

Sequence alignment of ISP in *R. sphaeroides* bc_1 complex with its counterpart in the beef heart mitochondrial complex reveals an extra fragment in the bacterial protein, corresponding to residues 96−107 (*2*) (see Figure 1). This extra fragment is also present in ISPs from other bacterial bc_1 complexes, such as *R. capsulatus* and *Paracoccus denitrificans*. ISPs from other mitochondrial complexes, such as from humans and yeast, lack this extra fragment. Perhaps this extra fragment is required for bacterial ISP's structural stability in the bc_1 complex, a function similar to that suggested for supernumerary subunit IX in the yeast mitochondrial complex.

This extra fragment, located at the near middle portion of the ISP, shows an α-helical structure in a homology-based 3-D structure model of *R. sphaeroides* bc_1 complex (see Figure 2) constructed using coordinates of subunits from the bovine complex (*2*). The ISP extra fragment is modeled into the bacterial complex using coordinates from residues 34−52 of the bovine subunit IX. The insight II homology

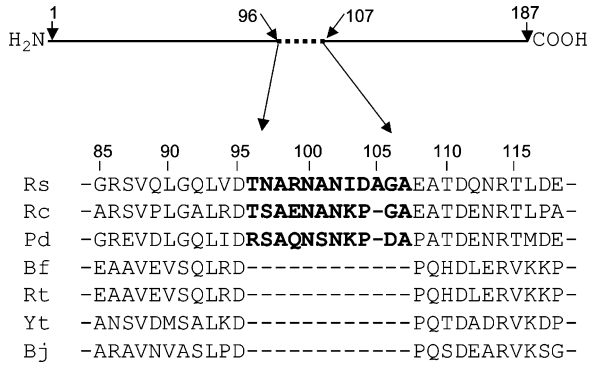

FIGURE 1: Partial sequence comparison in the extra fragment of various ISPs: Rs, *R. sphaeroides*; Rc, *R. capsulatus*; Pd, *P. denitrificans*; Bf, beef; Rt, rat; Yt, yeast; Bj, *Bradyrhizobium japonicum.*

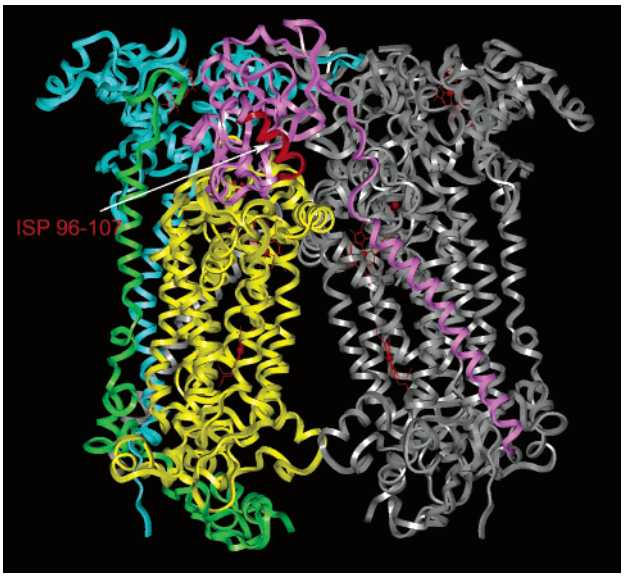

FIGURE 2: Location of the extra fragment of ISP in the proposed structural model of *R. sphaeroides* cytochrome bc_1 complex. In the left monomer, the cytochrome *b* is yellow, cytochrome c_1 blue, subunit IV green, and ISP purple. The extra fragment (residues 96−107) is colored in red. The other subunits are in gray for clarity.

modeling modular (*24*) using the GORII method (*25*) also predicts the presence of an α-helical structure for this extra fragment. In this structural model of *R. sphaeroides* bc_1 complex, the extra fragment is 20−24 Å (Thr 96---Fe II, 20 Å; ASN 102---Fe II, 21 Å; ALA 107---Fe II, 24 Å) away from the [2Fe−2S] cluster of ISP. Thus, direct participation of this fragment in electron-transfer activity of ISP is unlikely. On the other hand, the feasibility of using coordinates of supernumerary subunit IX of mitochondria to model this extra fragment into the structure of bacterial bc_1 complex (*2*) supports the idea that the extra fragment of ISP functions as a supernumerary subunit.

To investigate the role of this extra fragment of ISP in the bc_1 complex, we generated *R. sphaeroides* mutants expressing His_6-tagged bc_1 complexes with deletion or substitution at various positions in this fragment. The photosynthetic behavior of the mutants was examined and compared with that of the complement strain to see whether this extra fragment of ISP is an essential component of the bc_1 complex and to identify the critical amino acid residue(s). The bc_1 activity, the amount of ISP protein, and the EPR

characteristics of ISP, in the membrane and the purified state, of the complement and mutants were examined and compared.

EXPERIMENTAL PROCEDURES

Materials. Cytochrome *c* (horse heart, type III) was purchased from Sigma Chemical Co. *n*-Dodecyl β-D-maltoside and *n*-octyl β-D-glucoside were from Anatrace. Nickel nitrilotriacetic acid (Ni-NTA) gel and a Qiaprep spin Miniprep kit were from Qiagen. 2,3-Dimethoxy-5-methyl-6-decyl-1,4-benzoquinol ($Q_0C_{10}H_2$) was prepared in our laboratory (*26*), and purified ISP was obtained from *R. sphaeroides* bc_1 complex as previously described (*27*). 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 and semiaerobic growth conditions for *R. sphaeroides* were essentially as described previously (*28*). The concentrations of antibiotics used were as follows: ampicillin, 125 μg/mL; kanamycin sulfate, 30 μg/mL; tetracycline, 10 μg/mL for *E. coli* and 1 μg/mL for *R. sphaeroides*; trimethoprim, 100 μg/mL for *E. coli* and 30 μg/mL for *R. sphaeroides*.

Generation of R. sphaeroides Strains Expressing the His_6-Tagged bc_1 Complexes with Mutations at the ISP Extra Fragment (Residues 96−107). Mutations were constructed by site-directed mutagenesis using the Altered Sites II mutagenesis system from Promega or the QuickChange site-directed mutagenesis kit from Stratagene. The ISP(96−107)A mutant and all single-substitution mutants were constructed by the Altered Sites II mutagenesis method using single-stranded pSELNB3503 (*5*) as template and one of the following mutant oligonucleotide as the annealing primer: [ISP(96−107)A] CAGCTCGGCCAGCTGGTCGAC*GCCG-CCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCC*GAG-GCGACGGACCAGAACCGC; [ISP(T96A)] CAGCTGGT-CGAC*GCC*AATGCCCGCAACGCG; [ISP(N97A)] CAGCTG-GTCGACACC*GCC*GCCCGCAACGCGAACA; [ISP(R99A)] TGGTCGACACCAATGCC*GCC*AACGCGAACATCGA-CGCC; [ISP(N100A)] GTCGACACCAATGCCCGC*GCC*-GCGAACATCGA; [ISP(N102A)] ACCAATGCCCGCAA-CGCG*GCC*ATCGACGCCGGCGCCG; [ISP(I103A)] CG-CAACGCGAAC*GCC*GACGCCGGCGCCGAGGCGA-CGG; [ISP(D104A)] GCAACGCGAACATC*GCC*GCCG-GCGCCGAGGCG; [ISP(G106A)] AACGCGAACATCGACGCC-*GCC*GCCGAGGCGACGGACCA; [ISP(G106L)] AACGC-GAACATCGACGCC*CTC*GCCGAGGCGACGGACCA.

ISPΔ(96−107), ISP(96−99)A, ISP(100−103)A, and ISP-(104−107)A were constructed by the QuickChange site-directed mutagenesis method, using a supercoiled double-stranded pGEM7Zf(+)−*fbcFB* as template and a forward and a reverse primer for PCR amplification. The pGEM7Zf-(+)−*fbcFB* plasmid was constructed by ligating the *Eco*RI−*Xba*I fragment from pSELNB3503 into *Eco*RI and *Xba*I sites of the pGEM7Zf(+) plasmid. The primers used were as follows: [ISP(96−107)] (forward primer) CAGCTCGGC-CAGCTGGTCGACGAGGCGACGGACCAGAACC-GC; (reverse primer) GCGGTTCTGGTCCGTCGCCTCG-TC-GACCAGCTGGCCGAGCTG; [ISP(96−99)A] (forward primer) CGGCCAGCTGGTCGAC*GCCGCCGCCGCC*AAC-GCGAAC; (reverse primer) GTTCGCGTT*GGCGGCGGC-GGC*GTCGACCAGCTGGCCG; [ISP(100−103)A] (forward primer) CAATGCCCGC*GCCGCCGCCGCC*GACGCCG-GCGCCGAGGCGACG; (reverse primer) CGTCGCCTCG-GCGCCGGCGTC*GGCGGCGGCGGC*GCGGGCAT-TG; [ISP(104−107)A] (forward primer) GCAACGCGAACATC-*GCCGCCGCCGCC*GAGGCGACGGAC; (reverse primer) GTCCGTCGCCTC*GGCGGCGGCGGC*GATGTTCGCGT-TGC.

A plate-mating procedure (*28*) was used to mobilize the pRKD*fbc*F_mBC_HQ plasmid in *E. coli* S17-1 cells into *R. sphaeroides* BC17 cells. The presence of engineered mutations was confirmed twice by DNA sequencing before and after photosynthetic or semiaerobic growth of the cells as previously reported (*28*). DNA sequencing and oligonucleotide syntheses were performed by the Recombinant DNA/Protein Core Facility at Oklahoma State University.

Enzyme Preparations and Activity Assay. Chromatophores were prepared and His_6-tagged cytochrome bc_1 complexes were purified from chromatophores or ICM as previously reported (*5*). To assay the cytochrome bc_1 complex activity, membrane or purified cytochrome bc_1 complexes were diluted with 50 mM Tris−Cl, pH 8.0, 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, 300 μM EDTA, 100 μM cytochrome *c*, and 25 μM $Q_0C_{10}H_2$. Activities were determined by measuring the reduction of cytochrome *c* (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 for calculation. The nonenzymatic oxidation of $Q_0C_{10}H_2$, determined under the same conditions, in the absence of enzyme, was subtracted.

Other Biochemical and Biophysical Techniques. Protein concentration was measured by the method of Bradford (*29*). Cytochrome *b* (*30*) and cytochrome c_1 (*31*) were determined according to published methods. SDS−PAGE was performed according to Laemmli (*32*) using a Bio-Rad Mini-protean dual-slab vertical cell. Western blotting used rabbit polyclonal antibodies raised against ISP of *R. sphaeroides*. The polypeptides separated in the SDS−PAGE gel were transferred to a 0.22 μM nitrocellulose membrane for immunoblotting. Protein A conjugated to horseradish peroxidase was used as the second antibody. Total RNA from wild-type or mutant *R. sphaeroides* was prepared from cells harvested at mid-log phase using the RNeasy Mini kit from Qiagen. The DNA probe containing the ISP gene was prepared by polymerase chain reaction using 5′-ACGCCACGGCCGGAGCCGG-GGCGGTG-3′ and 5′-CTCCGCTGCCTGGTCTTGGC-3′ as forward and reverse primers, respectively. Northern blot was performed using the North2South direct HRP labeling and detection kit from Pierce. EPR spectra were recorded with a Bruker ER 200D equipped with a liquid N_2 Dewar at 77 K. Instrument settings are detailed in the captions of the relevant figures. Differential scanning calorimetric measurements were performed with a CSC 6100 NanoII DSC instrument. All reference and sample solutions were degassed prior to use. A 0.33 mL sample of bc_1 solution, 2 mg/mL, in 50 mM K^+/Na^+ phosphate buffer, pH 7.4, containing 100 mM KCl and 0.5% octyl glucoside was placed in the sample capillary cell, and the same amount of buffer was placed in the reference capillary cell. The scanning rate was 1 °C/min for

both heating and cooling. All thermodynamic analyses were according to the program known as CpCalc from the Nano DSC program group.

RESULTS AND DISCUSSION

Essentiality of the ISP Extra Fragment for the Cytochrome bc_1 Complex. The *R. sphaeroides* ISP extra fragment that corresponds to residues 96−107 with a sequence of TNARNANIDAGA is located at the near middle portion of the ISP sequence (see Figures 1 and 2). To probe the role of this fragment, *R. sphaeroides* mutants expressing His_6-tagged bc_1 complexes with deletion or substitution at various positions of the extra fragment were generated and characterized. Because the bc_1 complex is absolutely required for the photosynthetic growth of *R. sphaeroides*, whether this ISP extra fragment is critical to the complex can be determined by assaying photosynthetic growth.

When this extra fragment is deleted from the ISP sequence, the resulting cells [ISPΔ(96−107)] are unable to support photosynthetic growth, indicating that this region is required for bc_1 complex activity. To further confirm that the inability of the ISPΔ(96−107) mutant cells to grow photosynthetically results from the essentiality of the extra fragment for the bc_1 complex, and not from improper protein assembly or folding due to the large deletion, a mutant with an extra fragment substituted with alanine [ISP(96−107)A] was generated and shown to be unable to grow photosynthetically. This result supports the essentiality of this ISP extra fragment in the bacterial bc_1 complex, and suggests that the amino acid residues, rather than the length or α-helical structure of the extra fragment, are critical, since the alanine-substituted fragment should have the same length as the wild-type fragment and the same α-helical structure.

To identify critical amino acid residues in the ISP extra fragment, we first located the critical regions. Residues in three portions of the extra fragment were replaced with alanine to generate three mutants, ISP(96−99)A, ISP(100−103)A, and ISP(104−107)A, which were subjected to photosynthetic growth conditions. The ISP(96−99)A mutant grows photosynthetically at a rate comparable to that of wild-type cells, the ISP(100−103)A mutant, after a long lag time (when the wild-type cells have grown to stationary phase under the same conditions), starts to grow at a rate comparable to that of the wild-type cells, and the ISP(104−107)A mutant does not grow photosynthetically (see Figure 3). These results indicate that the first four residues (residues 96−99) in the ISP extra fragment are not critical, the second four residues (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* (RSΔIV) cells (*33*), and the last four residues (104−107) are critical. It should be noted that photosynthetic growth of the ISP(100−103)A mutant cells after a long lag period is not due to an additional mutation occurring somewhere in the bc_1 genes because sequencing of the cytochrome bc_1 genes carried by the plasmid $pRKDfbcF_mBC_{His6}Q$ extracted from these mutant cells harvested after photosynthetic growth revealed no mutation besides the alanine substitutions at residues 100−103 of ISP. However, we cannot rule out a remote possibility that photosynthetic growth of the ISP(100−103)A mutant cells results from an additional mutation occurring somewhere on the *R. sphaeroides* chromosomes.

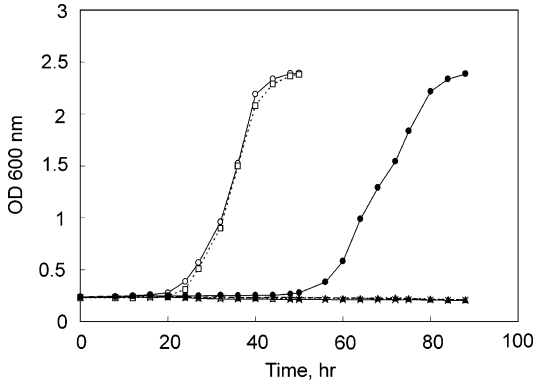

FIGURE 3: Photosynthetic growth of various *R. sphaeroides* strains. A 60 mL sample of mid-log phase aerobically grown complement (○), ISPΔ(96−106) (×), ISP(96−106)A (△), ISP(96−99)A (□), ISP(100−103)A (●), or ISP(104−107)A (▲), with the same cell density, were inoculated into 1 L of Sistrom medium containing 25 μg/mL kanamycin and 30 μg/mL thrimethoprim and subjected to photosynthetic growth conditions as described in the Experimental Procedures. Growth was monitored at OD = 600 nm.

Since residues 104−107 of ISP have an amino acid sequence of −D−A−G−A−, the inability of ISP(104−107)A mutant to grow photosynthetically must result from alanine replacement at D104 and/or G106. Hence, two single-alanine mutants (D104A and G106A) and a double-alanine mutant (D104A/G106A) were generated and subjected to anaerobic photosynthetic growth conditions. Both single-alanine mutants grow at a rate comparable to that of the wild-type cell and the double-alanine mutant cannot grow photosynthetically. This indicates that the inability of the ISP-(104−107)A mutant to grow photosynthetically results from the combined effect of alanine substitutions at both D104 and G106.

Table 1 shows ubiquinol-cytochrome *c* reductase activities in membranes prepared from complement and mutant cells. Intracytoplasmic membranes (ICMs) from mutants ISPΔ-(96−107) and ISP(96−107)A have no ubiquinol-cytochrome *c* reductase activity. This is as expected, since bc_1 complex is required for photosynthetic growth and neither mutant grows photosynthetically. The ISP(96−99)A, ISP(100−103)A, and ISP(104−107)A mutant membranes have, respectively, 48%, 9%, and 0% of the ubiquinol-cytochrome *c* reductase activity found in the complement chromatophores, suggesting the essentiality of the ISP extra fragment to the bc_1 complex is in the order residues 104−107 > residues 100−103> residues 96−99. It should be noted that all the single-substitution mutant chromatophores, ISPs-(T96A), (N97A), (R99A), (N100A), (N102A), ISP(I103A), (D104A), (G106A), and (G106L), have ubiquinol-cytochrome *c* reductase activity similar to that found in complement chromatophores. This indicates that the role played by the extra fragment of ISP is not individual amino acid specific but is a combination effect of several residues.

Requirement of the ISP Extra Fragment for ISP Protein Stability. To confirm that the loss (or decrease) of cytochrome bc_1 activity in the ISP extra fragment mutant membranes results from a lack (or decrease) of ISP, but not cytochrome *b* or c_1, the amounts and spectral characteristics of cytochromes *b* and c_1 and ISP in mutant and complement

Table 1: Characterization of the ISP Extra Fragment Mutants

		cytochrome bc_1 complex			
		membrane	purified complex		
strain	Ps[a]	specific activity[e]	specific activity	subunit composition	reconstituted activity[g]
complement	+++[b]	2.3	2.5	FBCQ[f]	
ISPΔ(96−107)	−[c]	0	0	BC	1.1
ISP(96−107)A	−	0	0	BC	1.1
ISP(96−99)A	+++	1.1	0.9	FBCQ	
ISP(100-103)A	++[d]	0.2	0	BC	1.2
ISP(104−107)A	−	0	0	BC	1.3
ISP(D104A)	+++	2.2	2.4	FBCQ	
ISP(G106A)	+++	2.2	2.4	FBCQ	
ISP(D104A/G106A)	−	0	0	BC	1.3
ISP(G106L)	+++	2.2	2.4	FBCQ	
ISP(N100A)	+++	2.2	2.4	FBCQ	
ISP(I103A)	+++	2.3	2.3	FBCQ	
ISP(T96A)	+++	2.2	2.4	FBCQ	
ISP(N97A)	+++	2.2	2.5	FBCQ	
ISP(R99A)	+++	2.3	2.5	FBCQ	

[a] Ps = photosynthetic growth. [b] +++ indicates the growth rate was essentially the same as that of the complement cells. [c] − indicates no photosynthetic growth within 4 days. [d] ++ indicates the cells can grow photosynthetically but the growth rate was slower than that of the complement cells. [e] Enzymatic activity is expressed as μmol of cytochrome *c* reduced/min/nmol of cytochrome *b*. [f] FBCQ indicates gene products of the *fbc*F (ISP) (F), *fbc*B (cytochrome *b*) (B), *fbc*C (cytochrome c_1) (F), and *fbc*Q (subunit IV) (Q), respectively. [g] Activity was assayed after the mutant complex was mixed with recombinant subunit IV and purified ISP.

membranes were compared. 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 (data not shown), 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 blot analysis with antibodies against *R. sphaeroides* ISP revealed that mutant membranes of ISPΔ(96−107), ISP(96−107)A, and ISP(104−107)A contain no detectable ISP and that the ISP(96−99)A and ISP(100−103)A mutant membranes have 50% and 5%, respectively, of the amount of ISP found in the complement membrane (see Figure 4). These results 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. It has been reported that mutations of conserved residues (liganding or nonliganding of the [2Fe−2S] cluster) at the two highly conserved hexapeptide motifs in the carboxyl-terminal part of *R. capsulatus* ISP cause the Rieske protein not be assembled properly into the cytochrome bc_1 complex (*34*, *35*).

As expected, no [2Fe−2S] cluster is detected in mutant membranes of ISPΔ(96−107), ISP(96−107)A, and ISP(104−107)A, since they contain no ISP. Although the ISP(96−99)A and ISP(100−103)A mutant membranes had decreased amounts of ISP, the [2Fe−2S] clusters in these two mutant membranes had EPR spectral features identical to those observed in complement chromatophores, with resonance at $g_x = 1.80$ and $g_y = 1.90$ (see Figure 5). These results indicate that, despite causing a decrease in the amount of ISP in the membrane, these mutations do not affect the microenvironment of the iron−sulfur cluster.

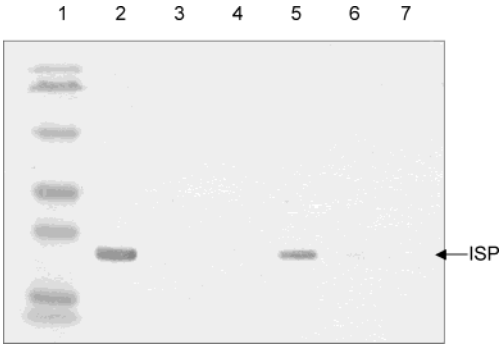

FIGURE 4: Western blot analysis of ISP in complement and mutant membranes. Membrane samples containing 35 pmol of cytochrome *b* were loaded into each well and subjected to SDS−PAGE. The proteins in the gel were transferred electrophoretically to a 0.22 μM nitrocellulose membrane and treated with antibodies against *R. sphaeroides* ISP. Protein A horseradish peroxidase was used as the second antibody. Key: lane 1, prestained molecular mass standards; lanes 2−7, membranes from the complement, ISPΔ(96−107), ISP(96−107)A, ISP(96−99)A, ISP(100−103)A, and ISP(104−107)A, respectively.

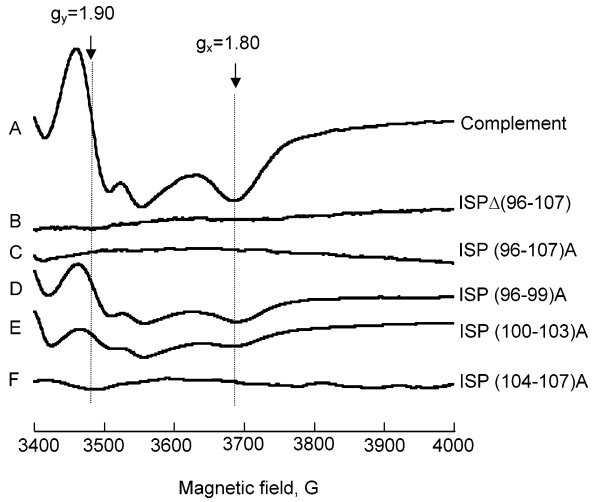

FIGURE 5: EPR spectra of the [2Fe−2S] cluster of the Rieske iron−sulfur protein in membranes from the complement and mutants ISPΔ(96−107), ISP(96−107)A, ISP(96−99)A, ISP(100−103)A, and ISP(104−107)A. Membrane pastes were partially reduced by addition of 5 mM sodium ascorbate on ice for 30 min and frozen in liquid nitrogen. EPR spectra were recorded at 77 K with the following instrument settings: microwave frequency, 9.28 Hz; microwave power, 20 mW; modulation amplitude, 20 G; modulation frequency, 100 kHz; time constants, 0.1 s; scan rate, 20 G/s.

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 can result from instability of the mutant protein or the mutant mRNA. To test these two possibilities, the amounts of ISP mRNA from complement and mutant cells were compared by Northern analysis using an HRP-labeled DNA probe containing the ISP gene. All mutant cells were found to have the same amount of ISP mRNA as the complement cells (data not shown), indicating that mutation of the ISP extra fragment did not affect the stability of ISP mRNA.

To confirm that a lack (or decrease) of ISP in mutant membranes results from mutant ISP protein instability, the amounts of ISP in the cell lysate, 200000*g* supernatant and precipitate (membrane) fractions, from mutant cells during membrane preparation were measured and compared with

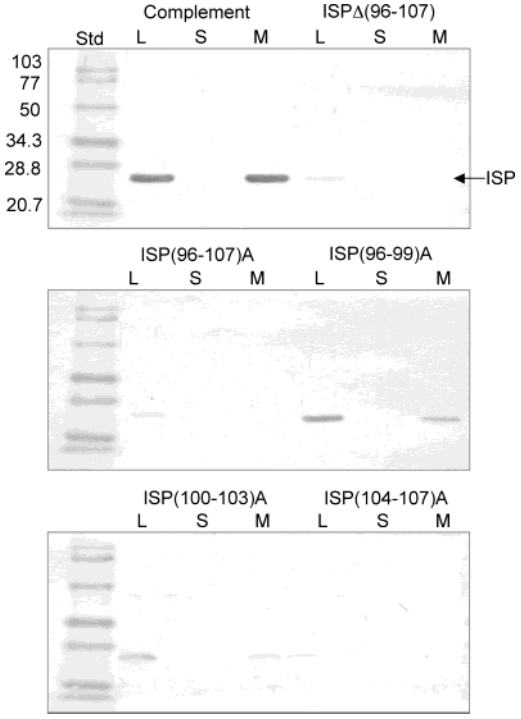

FIGURE 6: Western blot analysis of ISP in cell lysates, 200000*g* supernatant and 200000*g* precipitate fractions, from complement and mutant cells. Samples of 30 g wet weight of the complement, ISPΔ(96−107), ISP(96−107)A, ISP (96−99)A, ISP(100−103)A, and ISP(104−107)A were suspended with 50 mM Tris−Cl, pH 8.0, containing 200 mM NaCl to a final volume of 90 mL, subjected to sonification to break the cells, and centrifuged at 12000*g* for 15 min to remove unbroken cells. The cell lysates were then centrifuged at 200000*g* for 2.5 h to separate the soluble fractions from the membrane fractions. The membrane fractions were suspended with 50 mM Tris−Cl, pH 8.0, containing 1 mM EDTA to a final volume of 90 mL. Aliquots of 40 μL were withdrawn from the cell lysate (L), 200000*g* supernatant (S) and membrane (M) suspension fractions, mixed with 10 μL of 5× digestion buffer, and incubated 2 h at 37 °C. Aliquots of 5 μL of digested samples were applied to each well of the SDS−PAGE gel. Western blotting was performed as described for Figure 4.

those obtained from complement cells (see Figure 6). 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 blots. When these cell lysates are centrifuged at 200000*g* for 2.5 h to separate the supernatant from the membrane fraction, no ISP is found in any of the supernatant fractions. While nearly all of the ISP in the complement cell lysates is recovered in the membrane fraction, 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, and 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 (data not shown). The amounts of ISPs remaining in the ISP(96−99)A and ISP-(100−103)A mutant cell lysates 3 h after sonication at 0 °C are about 40% and 10%, respectively. These values correlate well with the amounts of mutant ISPs recovered in the membrane fractions of these mutants after centrifugation of cell lysates.

Although these results clearly demonstrate that the mutations in the extra fragment of ISP cause ISP protein instability, they do not prove that a lack of (or decrease in) ISP in mutant membranes results from degradation of the assembled mutant ISP or from decreasing the ability of mutant ISP to be incorporated into the bc_1 complex and accelerating the protease digestion of unassembled mutant ISP. Apparently, the degree of protein instability increases with mutation of residues toward the end (residues 104−107) of the ISP extra fragment.

When the thermotrophic properties of complement and mutant ISP(96−99)A complexes were measured by differential scanning calorimetry, the mutant complex exhibited a thermodenaturation temperature (T_m) of 40.7 °C with a heat capacity change (ΔH) of 27.5 kcal/mol whereas the complement complex showed a T_m of 46.3 °C with a ΔH of 46.3 5 kcal/mol. The lower T_m and ΔH of the mutant complex indicate that it is less stable than the wild-type complex. This result supports the idea that a decrease in ISP in the mutant membrane is due to the instability of assembled mutant ISP.

Mutation on the Extra Fragment of ISP Does Not Affect the Assembly of Subunit IV. As shown in the preceding section, mutation of the extra fragment of ISP does not affect assembly of cytochromes *b* and c_1 into the membrane. Since the *R. sphaeroides* bc_1 complex contains a supernumerary subunit, subunit IV, in addition to cytochrome *b*, cytochrome c_1, and ISP, we tested the effect of the extra fragment of ISP on assembly of subunit IV into the membrane. Western analysis using antibodies against subunit IV showed that all of the mutant membranes contain the same amount of subunit IV as the complement membrane (see Figure 7A), indicating that mutation does not affect assembly of subunit IV into the 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 cytochromes *b* and c_1 (see Figure 7B). 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, indicating that mutation did 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*. Since it has been reported that incorporation of recombinant subunit IV into the bc_1 complex does not require the presence of ISP (*36*), 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.

To determine whether 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, the effect of the addition of ISP was examined. The two-subunit mutant complexes were incubated with purified recombinant subunit IV for 30 min at 0 °C prior to the addition of purified, wild-type ISP. The activity of cytochrome bc_1 complex is restored

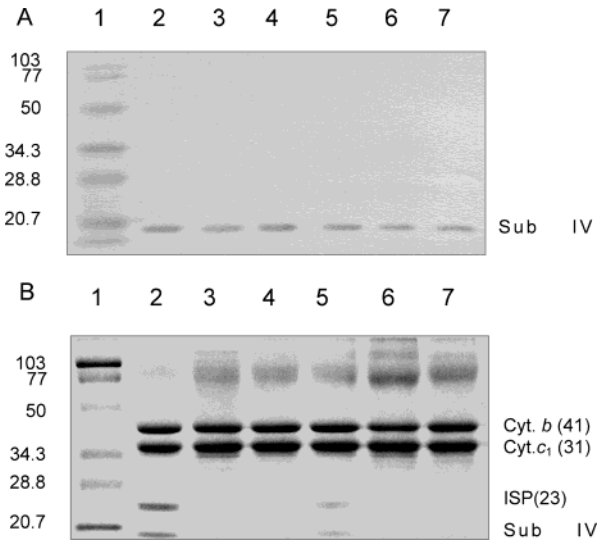

FIGURE 7: Western blot analysis of subunit IV in complement and mutant membranes (A) and SDS−PAGE analysis of the purified cytochrome bc_1 complexes (B). (A) Complement and mutant membranes containing 35 pmol of cytochrome *b* were subjected to SDS−PAGE. The proteins in the gel were transferred electrophoretically to a 0.22 μM nitrocellulose membrane and treated with antibodies against *R. sphaeroides* subunit IV. Protein A horseradish peroxidase was used as the second antibody. (B) Purified complement and mutant cytochrome bc_1 complexes containing 150 pmol of cytochrome *b* were incubated with 1% SDS and 0.4% β-ME at 37 °C for 2 h and subjected to electrophoresis. Key: lane 1, prestained molecular mass standards protein standard (*Std*) containing phosphorylase B (103 kDa), bovine serum albumin (77 kDa), ovalbumin (50 kDa), carbonic anhydrase (34.3 kDa), soybean trypsin inhibitor (28.8 kDa), and lysozyme (20.7 kDa).; lanes 2−7, proteins or membranes from the complement, ISPΔ(96−107), ISP-(96−107)A, ISP(96−99)A, ISP(100−103)A, and ISP(104−107)A, respectively.

after the addition of ISP (see Table 1), indicating that cytochromes *b* and c_1 subunits are indeed functionally active.

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