Subunit IV of Cytochrome bc\(_1\) Complex from Rhodobacter sphaeroides

LOCALIZATION OF REGIONS ESSENTIAL FOR INTERACTION WITH THE THREE-SUBUNIT CORE COMPLEX*

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Recombinant subunit IV mutants which identify the regions essential for restoration of bc\(_1\) activity to the three-subunit core complex of Rhodobacter sphaeroides were generated and characterized. Four C-terminal truncated mutants: IV(1–109), IV(1–85), IV(1–76), and IV(1–40) had 100, 0, 0, and 0% of reconstitutive activity of the wild-type IV, indicating that residues 86–109 are essential. IV(1–109) is associated with the core complex in the same manner as the wild-type IV while mutants IV(1–85), IV(1–76), and IV(1–40) do not associate with the core complex, indicating that subunit IV requires its transmembrane helix region (residues 86–109) for assembly into the bc\(_1\) complex. Since GST-IV(86–109) fusion protein has little reconstitutive activity, some region(s) in residues 1–85 are required for bc\(_1\) activity restoration after subunit IV is incorporated into the complex through the transmembrane helix, presumably by interaction with cytochrome \(b\) in the core complex. The interacting regions are identified as residues 41–53 and 77–85, since mutants IV(21–109), IV(41–109), IV(54–109), and IV(77–109) had 95, 98, 53, and 53% of the reconstitutive activity of the wild-type IV. These two interacting regions are on the cytoplasmic side of the chromatophore membrane and closed to the DE loop and helix G of cytochrome \(b\), respectively.

The cytochrome bc\(_1\) complex from Rhodobacter sphaeroides, which catalyzes electron transfer from ubiquinol to cytochrome \(c_1\) in the cyclic photosynthetic electron transfer chain, has been purified and characterized (1). Electron transfer through this complex is coupled with translocating protons across the chromatophore membrane with a 2H\(^+\)/e\(^−\) stoichiometry. The purified complex contains four protein subunits: the largest three, housing two \(b\)-type cytochromes (\(b\)\(_{556}\) and \(b\)\(_{652}\)), one \(c\)-type cytochrome (\(c\)\(_1\)), and one high potential iron-sulfur cluster (2Fe-2S), respectively, are the core subunits; the smallest one (subunit IV), containing no redox prosthetic group, is a supernumerary subunit (2).

Although all cytochrome bc\(_1\) complexes contain three core subunits, they vary significantly in supernumerary subunit composition. Whereas the bc\(_1\) complex from bovine heart mitochondria has eight supernumerary subunits (3) and that from yeast has seven (4), the \(R.\) sphaeroides complex has one (5), and those from \(R.\) capsulatus \(\Delta IV\) (6), \(P.\) denitrificans (7), and \(R.\) capsulatus (8) have none. The complexes containing no supernumerary subunit are less stable and have lower activity than those with supernumerary subunit (4). The turnover rates for the cytochrome bc\(_1\) complexes purified from mitochondria of bovine heart and yeast and chromatophores of \(R.\) sphaeroides and \(R.\) capsulatus, by a single chromatographic procedure are: 1152, 219, 128, and 64 s\(^−1\) (4). It is possible that the increased enzymatic activity for the mitochondrial and \(R.\) sphaeroides complexes results from interaction of the core complex with supernumerary subunit. However, the study of supernumerary/core subunit interactions in the mitochondrial complex has been complicated by the presence of multiple supernumerary subunits. Since \(R.\) sphaeroides bc\(_1\) complex has only one supernumerary subunit, it is an ideal system for studying supernumerary/core subunit interactions.

The gene encoding subunit IV (\(fbc\)Q) was cloned and sequenced (9). Genomic mapping indicates that the \(fbc\)Q gene is at least 900 kilobase pairs away from the \(fbc\) operon, which encodes the three core subunits, in the \(R.\) sphaeroides chromosome (10). Subunit IV contains 124 amino acid residues with a molecular weight of 14,384 (9). The amino acid sequence of subunit IV bears no homology with any of the eight supernumerary subunits of the bovine heart mitochondrial bc\(_1\) complex, whose three-dimensional structure at 2.9-A resolution recently became available (11, 12). Based on the hydropathy plot of its amino acid sequence, subunit IV contains only one transmembrane helix (amino acid residues 86–109) with 86 and 15 residues at the N-terminal and C-terminal ends, respectively. The sidedness of the membrane is unknown, although there has been speculation based on preliminary results of proteolytic enzyme digestion (13).

When the \(fbc\)Q gene is deleted from the \(R.\) sphaeroides chromosome, the resulting strain (\(RS\)\(_{AI} IV\)) requires a period of adaptation (48 h) before the start of photosynthetic growth (14). The cytochrome bc\(_1\) complex in adapted chromatophores is labile to detergent treatment (75% inactivation) and shows a 4-fold increase in the \(K_m\) for Q\(_2\)H\(_2\)\(^+\) (14). Introducing wild-type \(fbc\)Q on a stable low copy number plasmid, pKH415, into \(RS\)\(_{AI} IV\) restores photosynthetic growth behavior, the apparent \(K_m\) for Q\(_2\)H\(_2\), and tolerance to detergent treatment to that of wild-type cells. Cytochrome bc\(_1\) complex purified from adapted \(RS\)\(_{AI} IV\) contains only three core subunits. It has only 25% of the activity of the four-subunit enzyme purified from complement or wild-type cells. This low activity is accompanied by an increase of the apparent \(K_m\) for Q\(_2\)H\(_2\) from 3 to 13 \(\mu\)M. We attribute the changes observed in photosynthetic growth be-

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1 The abbreviations used are: Q\(_2\)H\(_2\), 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinol; Q, ubiquinone; ISP, Rieske iron-sulfur protein; GST, glutathione S-transferase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.
Subunit IV of Cytochrome bc$_1$ Complex

havior (requires adaptation time) and in detergent liability of the cytochrome bc$_1$ complex activity (75% inactivation) to a structural role for subunit IV; the change in Q-binding parameter (K$_m$) increase) to its Q-binding function.

Although the observation of increased K$_m$ for Q$_H_2$ for the three-subunit core complex is consistent with the identification of subunit IV as one of the Q-binding proteins in this complex by photoaffinity labeling technique using 3H-azido-Q derivatives (15), its Q-binding role has always been regarded as auxiliary. The other Q-binding protein is cytochrome b. Like subunit IV in R. sphaeroides bc$_1$ complex, subunit VII is identified as the small molecular weight Q-binding protein in beef heart mitochondrial bc$_1$ complex (16). It was suggested that the presence of subunit IV or subunit VII stabilizes the Q-binding sites located in cytochrome b, in their respective complexes. However, the idea that subunit VII participates in the Q-binding site of the beef complex was abandoned after the three-dimensional structure of this complex became available in 1997 (11). Subunit VII is too far from the Q-binding sites of cytochrome b. Perhaps the observed azido-Q labeling on subunit IV is a result of this subunit being a Q sequestering protein. The Q binding activity of mitochondrial subunit VII has been established spectrophotometrically (17). The decrease in K$_m$ for Q$_H_2$ and increase in enzymatic activity for the wild-type or complement complex may result from a conformational change in the three-subunit core complex upon interaction with subunit IV. The conformation of the wild-type or complement complex is now more favorable for Q binding.

Some essential amino acid residues in subunit IV have been identified by site-directed mutagenesis followed by gene complementation (in vivo reconstitution) (18). Although this approach is very useful in structure-function studies of subunit IV, it is often complicated by mutational effects on complex assembly and by mutant protein stability (18). Recently we overexpressed subunit IV in Escherichia coli as a glutathione S-transferase (GST) fusion protein (19) and purified recombinant GST-IV fusion protein from cell extracts on glutathione-agarose gel. Purified recombinant subunit IV is obtained by thrombin digestion of recombinant fusion protein followed by gel filtration to remove uncleaved fusion protein and thrombin. Addition of recombinant IV to the three-subunit core complex, which has a fraction of the wild-type bc$_1$ complex activity and a 4-fold increase of the K$_m$ for Q$_H_2$ (14), restores enzymatic activity and Q-binding parameter to that of the wild-type complex, indicating that recombinant subunit IV is properly assembled into the bc$_1$ complex (19). Thus we can use reconstitutively active recombinant subunit IV to study its interaction in the bc$_1$ complex by site-directed mutagenesis coupled with in vitro reconstitution. Herein we report generation and characterization of subunit IV mutants which identify the regions of subunit IV required for interaction with the core complex to restore bc$_1$ activity. The topological arrangement of subunit IV in the chromatophore membrane is determined by monospecific polyclonal antibodies for various segments of subunit IV. The docking model for subunit IV in the bc$_1$ complex is presented. The effect of the transmembrane helix region of subunit IV on its assembling into the bc$_1$ complex is investigated. The effect of Rieske iron-sulfur protein on the incorporation of subunit IV to the bc$_1$ complex is also examined.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dodecyl maltoside was purchased from Anatrace. The Na-NTA resin used for purification of the His$_6$-tagged cytochrome bc$_1$ complex was purchased from Qiagen. Glutathione-agarose gel used for purification of GST-IV fusion proteins was from Sigma. pSELECT-1 vector, R408 helper phage, and BMH 71-18 mutS and JM 109 E. coli strains used in mutagenesis were from Promega. Restriction endonucleases and other DNA-modifying enzymes were purchased from Promega, Life Technologies, Inc., and New England Biolabs. Expression vector pGEX-2T and Superose 12 FPLC column were from Amersham Pharmacia Biotech. Primers and oligonucleotides were synthesized by the DNA/Protein Core Facility of Oklahoma State University. 2,3-Dimethoxy-5-methyl-6-geranyl-1,4-benzoquinol (Q$_2$H$_2$) was synthesized in our lab as described previously (20). bc$_1$ complex was prepared according to previously reported methods.

**Growth of Bacteria**—E. coli cells were grown at 37 °C on LB medium. Extra-rich medium (TYP) was used in procedures for the rescue of single-stranded DNA. For photosynthetic growth of the plasmid-bearing R. sphaeroides cells, an enriched Sistrom medium containing 5 mM glutamate and 0.2% casamino acids was used (23). Antibiotics were added to the following concentrations: ampicillin, 100–125 μg/ml; tetracycline, 10–15 μg/ml for E. coli and 1 μg/ml for R. sphaeroides; kanamycin sulfate, 30–50 μg/ml for E. coli and 20 μg/ml for R. sphaeroides.

**Generation of R. sphaeroides Strains Expressing His-tagged, Four-subunit and Three-subunit Cytochrome bc$_1$ Complexes**—The expression vector for His-tagged, wild-type, four-subunit cytochrome bc$_1$ complex (pRRD(bcFBC$_1$Q)) was constructed previously in our laboratory (23). The expression vector for the subunit IV-deficient, three-subunit core complex (pRRD(bcFBC$_1$)) was generated by digestion of pFBC$_1$Q plasmid with BamHI and HindIII to remove the pBC gene, filling in with Klenow and annealing the ends. pRRD(bcFBC$_1$Q) and pRRD(bcFBC$_1$) were mobilized into BC17 and RS419, respectively, by parental conjugation (25) to generate pRRD(bcFBC$_1$Q/BC17 and pRRD(bcFBC$_1$Q/RS419, for expression of His-tagged four-subunit and three-subunit complexes, respectively.

**Preparations and Assay of His$_6$-tagged Cytochrome bc$_1$ Complexes**—The His$_6$-tagged four-subunit wild-type complex, three-subunit core complex, and two-subunit (cytochromes b and c$_1$) complex were purified from chromatophores of photosynthetically grown pRRD(bcFBC$_1$Q/BC17, pRRD(bcFBC$_1$Q/RS419, and pRRD(bcFBC$_1$Q/ADVBC17/BC12) cells, respectively, according to the previously described method (23).

**Cytochrome bc$_1$ complex activity was assayed as previously reported (23). An appropriate amount of enzyme preparation was added to an assay mixture (1 ml) containing 50 mM Na’K’ phosphate buffer, pH 7.4, 1 mM EDTA, 100 μM cytochrome c, and 25 μM Q$_2$H$_2$. Activity was determined by measuring the reduction of cytochrome c (the increase in absorbance at 550 nm) in a Shimadzu UV-2101PC at 23 °C. Nonenzymatic oxidation of Q$_2$H$_2$ was determined under the same conditions in the absence of cytochrome c. The enzyme extinction coefficient of 18.5 was used to calculate the concentration of cytochrome c.

**Recombinant DNA Techniques**—Restriction enzyme digestion, large scale isolation and mini-preparation of plasmid DNA, agarose electrophoresis, purification of DNA fragments from gel matrices, and immunological screening of transformants for production of subunit IV mutants with antibodies against subunit IV were performed according to the protocols described by Sambrook et al. (26). Goat anti-rabbit IgG alkaline phosphatase conjugate was used as the second antibody in the screening of transformants.

**Generation of E. coli Strains Expressing GST-mutated IV Fusion Proteins**—The expression vectors for mutants IV(1–40), IV(1–76), IV(1–85), and IV(1–109) were constructed by introducing the stop codon (TGA) at positions 121, 229, 256, and 328 base pairs downstream from the start codon of the pBC gene in the pSelect/IV plasmid, respectively. They were achieved by site-directed mutagenesis using the Altered Sites System from Promega Corp. Mutations were confirmed by DNA sequencing. The mutant oligonucleotides used were: IV(1–40), CGCCTGGTGAGGAGAGGCTTACCA; IV(1–76), GCCAGGCCGAGTTGCGAGTAGAGCTGCTGCTGCAC; IV(1–85), GCACGCCGCAGCTGGCTGGCTGCTGCTGCAC; IV(1–109), GCCAGGCCGAGTTGCGAGTAGAGCTGCTGCTGCTGCTGCAC. The BamHI fragments from pSelect/IVm plasmids containing mutated subunit IV genes were ligated into the BamHI site of pGEX to generate pGEX/IV(1–40), pGEX/IV(1–76), pGEX/IV(1–85), and pGEX/IV(1–109) plasmids which were transformed into E. coli KS1000 cells.

**Expression Vectors for Mutants IV(86–109), IV(77–109), IV(54–109), IV(41–109), and IV(21–109) were generated by the PCR amplification method using the pGEX/IV(1–109) plasmid as a template, oligonucleotide GAAAGCTTCCATGATGGCCC as a reverse primer, and one of the following oligonucleotides as a forward primer: IV(86–109), CCCGCCTGAGTCCGGGCTGCTTG; IV(77–109), CCCTCGTGGATCATGCATCG; IV(54–109), CCCGCCTGAGTCCGGGCTGCTTG; IV(41–109), CCCGCCTGAGTCCGGGCTGCTTG; IV(21–109),
CCGCTGGATCCGCCGCTCGAGAAA. For construction of the IV(86–124) expression vector, the pGEX/IV plasmid was used as the template and oligonucleotides, CCGCTGGATCCGCCGCTCCGCG and GAAGCTTCAGATGCGC, were used as forward and reversed PCR primers, respectively.

PCR amplification was performed in a minicycler from M. J. Research. The thermal cycle was set-up as follows: step 1, 94 °C for 1 min; step 2, 92 °C for 40 s for denaturation; step 3, 60 °C for 40 s for annealing; and step 4, 75 °C for 90 s for extension. A total of 30 cycles were performed with a final extension step of 5 min. The annealing time for the PCR amplification of the IV(77–109) fragment was set for 70 s. PCR products were confirmed by agarose gel electrophoresis and cloned into the pCR cloning vector pCR2 from Invitrogen to generate pCR/IVm plasmids. The BamH1/HindIII fragments from pCR/IVm plasmids were ligated into pGEX-2TH vectors to generate pGEX/IVm plasmids. These pGEX/IVm plasmids were individually transformed into E. coli KS1000. Transformsants producing the GST-IVm fusion proteins were identified by immunological screening of colonies with antibodies against subunit IV and/or against GST.

Isolation of Recombinant Wild-type and Mutants Subunit IVs—Production of GST-IV or GST-IVm fusion protein by E. coli KS1000 carrying pGEX/RSIVm or pGEX/RSIVm was essentially the same as that described previously (18) with modifications. 250-ml of overnight cultures were used to inoculate 12 liters of LB broth containing 125 μg/ml ampicillin and 2% glucose. The resulting culture was incubated at 37 °C with vigorous shaking until the A600 nm reached 1.0 (approximately 2.5 h). The cultures were cooled to 27 °C, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.2 mM, and grown at 27 °C for 3 h before being harvested by centrifugation at 8,000 × g for 30 min. About 52 g cells were obtained and resuspended in 150 μl of 12 mM Na2/K2 phosphate, pH 7.3, containing 140 mM NaCl and 2.7 mM KCl (phosphate-buffered saline). Cells were broken in a French press at 1000 p.s.i. During French pressing phenylmethylsulfonyl fluoride (500 μg in dimethyl sulfoxide) was added to the cell suspension to a final concentration of 1 mM. Triton X-100 was added to the broken cell suspension to a final concentration of 1%. The suspension was stirred for 30 min at 0 °C and centrifuged at 40,000 × g for 20 min. The supernatant was mixed with 20 ml of glutathione-agarose gel equilibrated with phosphate-buffered saline. This gel mixture was shaken gently for 30 min at room temperature and packed into a column (1.6 × 20 cm). After washing five times with 500 ml of phosphate-buffered saline, the GST/subunit IV fusion protein was eluted from the column with 10 ml glutathione in 50 ml Tris-HCl, pH 8.0, and concentrated to a protein concentration of 10 mg/ml by Centriprep-10. The fusion protein was treated with thrombin (1,500 units, w/w) at room temperature for 1 h to release subunit IV from GST. The thrombin-digested sample was then treated with a small amount of glutathione beads to partially remove the released GST and then subjected to gel filtration with a Superose-12 FPLC column to completely remove GST and thrombin.

Production and Purification of Antibodies against the C-terminal Peptide and a Near N-terminal Peptide of Subunit IV—A 15-amino acid residue C-terminal peptide (NH2-SSDAGNRPSVVYPE-COOH) corresponding to residues 110–124 and a near N-terminal peptide containing 15 amino acid residues (NH2-KRRMPSREEVARQK-COOH) corresponding to residues 59–73 were synthesized, purified, and used as antigens, after conjugation with ovalbumin, to raise antibodies in rabbits (27). Boosters were given weekly for 5 weeks, and sera were collected by cardiac puncture.

Purification of antibodies and preparation of the antibody Fab' fragment-horseradish peroxidase conjugates were as previously reported (28). Horseradish peroxidase activity of the purified conjugate was assayed using TMB peroxidase substrate kit (Bio-Rad) according to the manufacturer instructions.

Other Biochemical Methods—SDS-PAGE were performed by the method of Laemmli (29) or Schagger and von Jagow (30). Western blots (2), protein (31), and cytochromes b and c1 contents (23) were determined as described previously.

RESULTS AND DISCUSSION

Requirement of Residues 86–109 for Reconstitutive Activity of Subunit IV—It was reported (19) that addition of purified recombinant subunit IV to the three-subunit complex restores the bc1 complex activity to the same level as that of wild-type or complement-four-subunit complex, indicating that recombinant subunit IV is properly assembled into the complex. To localize the regions required for reconstitutive activity, recombinant subunit IVs with progressive deletion of amino acid residues from the C terminus were generated and characterized. These are: IV(1–109), IV(1–85), IV(1–76), and IV(1–40),
FIG. 2. SDS-PAGE of proteins eluted from Ni-NTA gels. 3-μl aliquots of His-tagged three-subunit core complex, 320 μg cytochrome b, were added to 100 μl of TDE buffer (50 mM Tris-Cl, pH 8.0, containing 0.01% dodecyl maltoside and 1 mM EDTA) containing 42 μg of wild-type IV (lane 2), 39 μg of IV(1–109) (lane 3), 33 μg of IV(1–85) (lane 4), and 24 μg of IV(41–109) (lane 5), and none (lane 6). The mixtures were incubated at 4 °C for 1 h before being mixed with 0.2 ml of Ni-NTA gel. After washing the gel with 1 ml of B-100 buffer containing 0.01% dodecyl maltoside and 100 mM NaCl) twice, absorbed proteins on the gel were eluted with 50 μl of B-100 buffer containing 200 mM histidine, and subjected to SDS-PAGE. The protein standard is in lane 1.

TABLE II
Reconstitutive activities of N-terminal truncated mutants of IV(1–109)

| Recombinant IV | Fused GST | Free |
|----------------|-----------|------|
| Wild-type IV   | 100       | 100  |
| IV(1–109)      | 99        | 97   |
| IV(21–109)     | 95        | 90   |
| IV(41–109)     | 98        | 96   |
| IV(54–109)     | 53        | ND*  |
| IV(77–109)     | 53        | 50   |
| IV(86–109)     | 4         | __b |
| IV(86–124)     | 0         | __b |

* ND, not be determined due to protein aggregation.

** Unavailability of the samples.

with deletions of 15, 39, 48, and 84 amino acid residues from the C terminus of subunit IV, respectively.

These four C-terminal truncated subunit IV mutants are produced in E. coli as GST fusion proteins using constructed expression vectors, pGEX/IV(1–109), pGEX/IV(1–85), pGEX/IV(1–76), and pGEX/IV(1–40), respectively. These expression vectors were constructed by in-frame ligation of the subunit IV gene having the stop codon genetically engineered at positions 328, 256, 229, and 121 base pairs downstream from the start codon, respectively, into the GST gene in the pGEX-2TH vector.

Table I shows reconstitutive activities of the C-terminal truncated subunit IV mutants, with and without fused GST. Addition of purified recombinant IV(1–109) to the three-subunit complex restores the cytochrome bc1 complex activity to the same level as that of the recombinant wild-type IV, suggesting that 15 amino acid residues (residues 110–124) on the C terminus of subunit IV are not essential. This result is consistent with the in vivo gene complementation study which showed that deletion of residues 120–124 of subunit IV does not impair the cytochrome bc1 complex activity (17). While the IV(1–109) mutant possesses full reconstitutive activity, the IV(1–85) mutant shows no reconstitutive activity, indicating that residues 86–109 are essential. As expected, mutants IV(1–76) and IV(1–40) have no reconstitutive activity because the essential residues 86–109 are missing.

As shown in Table I, when these four C-terminal truncated subunit IVs fused with GST are added to the three-subunit core complex, the percent activity restoration is the same as when non-fusion proteins are added, indicating that a free N-terminal of subunit IV may not be essential for its interaction with the three-subunit core complex.

Subunit IV Requires Its Transmembrane Helix Region for Assembly into the Cytochrome bc1 Complex—Since residues 86–109 are essential for reconstitutive activity of subunit IV, it is important to elucidate the role this fragment plays during reconstitution. In the proposed structure of subunit IV (9), constructed based on hydrophathy plots of the deduced amino acid sequence and possible α- and β-sheets, residues 86–109 comprise the only transmembrane helix. Therefore, the role of this fragment may be to provide membrane anchoring of subunit IV in the bc1 complex. To confirm this speculation a His6-tagged core complex was generated, characterized, and used for incorporation studies of the C-terminal truncated subunit IVs, using Ni-NTA columns.

The three-subunit core complex with a 6-histidine tagged at the C terminus of cytochrome c1 is constructed by deleting the subunit IV gene (fbcQ) from pRDK(bcFBC)Q. The resulting plasmid pRDK(bcFBC)Q is immobilized into an R. sphaeroides strain lacking the subunit IV gene (RSIV). The His6-tagged core complex is purified from dodecyl maltoside-solubilized chromatophores in one step on a Ni-NTA column (23). The purity, activity, and cytochrome content of this His6-tagged
three-subunit core complex are similar to those of the untagged complex. However, the yield of purified His$_6$-tagged core complex is twice that from untagged $bc_1$ preparations and is obtained in $\frac{1}{5}$ the time. When recombinant wild-type subunit IV is added to this His$_6$-tagged core complex, the extent of activity restoration is the same as that with the untagged complex, indicating that this His$_6$-tagged core complex is suitable for incorporation studies of recombinant subunit IV mutants.

When the His$_6$-tagged core complex is mixed with mutants IV(1–109), IV(1–85), IV(1–76), and IV(1–40), respectively, for 1 h at 0 °C before being applied to Ni-NTA columns, the IV(1–109) is associated with the core complex in the same manner as the wild-type IV (see Fig. 2, lanes 2 and 3), while IV(1–85) (see Fig. 2, lane 4), IV(1–76), and IV(1–40) (data not shown) do not associate with the His$_6$-tagged core complex. This result indicates that subunit IV requires its transmembrane helix region for assembly into the $bc_1$ complex. The lack of reconstitutive activity for mutants IV(1–85), IV(1–76), and IV(1–40) (see Table I) results from an inability of these mutants to bind to the complex because the transmembrane helix region is missing.
The Rieske iron-sulfur protein (ISP) was also reported to require its transmembrane helix for assembly into the bc\(_1\) complex (32).

**The Iron-Sulfur Protein Is Not Involved in The Association of Subunit IV with the bc\(_1\) Complex**—The finding that a purified two-subunit complex (cytochromes b and c\(_1\)), lacking both subunit IV and ISP, is obtained from chromatophores of mutants with altered ISP necks, either by deletion (\(\Delta\)ADV) (23) or double cysteine substitution (33), encouraged us to investigate the effect of ISP on incorporation of subunit IV. The His\(_6\)-tagged, three-subunit and two-subunit complexes were incubated with recombinant subunit IV at dodecyl maltoside concentrations ranging from 0.01 to 1%, for 1 h at 0°C, and passed through Ni-NTA columns. The amount of subunit IV recovered in the
column eluate of the three-subunit and two-subunit complexes were the same (data not shown), indicating that incorporation of subunit IV into the bc₁ complex does not need the presence of ISP. The simultaneous loss of ISP and subunit IV when the ISP neck is altered suggests that interaction between subunit IV and cytochrome b and/or c₁ involves ISP indirectly; alteration of the ISP neck may induce some changes on b or c₁ and thus decrease their affinity to subunit IV.

Identification of Residues 77–86 and 44–54 as Essential for Reconstitutive Activity of Subunit IV—Although the transmembrane helix region (residues 86–109) is required for subunit IV to be assembled into the bc₁ complex, addition of GST-IV(86–109) to the three-subunit core complex restores little bc₁ activity (see Table II). This suggests that the reconstitutive activity of subunit IV involves interaction with the core subunits after incorporation into the complex through the transmembrane helix. Since the IV(1–109) mutant has the same reconstitutive activity as that of recombinant wild-type IV, the interacting region must be in residues 1–85, as residues 86–109 are the transmembrane helix region. Therefore, to identify the regions interacting with the core subunits, four other N-terminal truncated mutants of IV(1–109): IV(21–109), IV(41–109), IV(54–109), and IV(77–109), with progressive deletion of 20, 40, 53, and 76 amino acid residues were generated and characterized.

The purity and yield of IV(21–109), IV(41–109), and IV(77–109), isolated free (see Fig. 3B) or as the GST fusion proteins (see Fig. 3A), are comparable to those of recombinant wild-type IV or the IV(1–109) mutant. When the His-tagged core complex is mixed with these three mutants at 0 °C for 1 h and then applied to Ni-NTA columns, all of them are associated with the core complex in the same manner as the wild-type IV (data shown only for the IV(41–109) in Fig. 2, lane 5). This result is consistent with the finding that the transmembrane helix region is required for subunit IV to be assembled into the bc₁ complex. Although the GST-IV(54–109) fusion protein (Fig. 3A, lane 5) is soluble in aqueous solution and has the purity and yield similar to those of the GST-IV fusion protein, the IV(54–109), once cleaved from GST by thrombin digestion, becomes insoluble. Including 0.02% dodecyl maltoside in the fusion protein preparation during thrombin digestion did not prevent the released mutant protein from aggregation. No binding experiment was performed with the aggregated IV(54–109) mutant. The yield of recombinant GST-IV(86–109) (Fig. 3A, lane 3) and GST-IV(86–124) (Fig. 3A, lane 2) is low, about 10% of that obtained with recombinant wild-type IV, because a severe protease digestion occurred during the fusion protein preparation. When these two mutant fusion proteins are subjected to thrombin digestion, no product release or substrate disappearance is observed, as determined by high resolution SDS-PAGE. The reason for these two fusion proteins being resistant to thrombin digestion is unknown. Nevertheless, the unavailability of mutants IV(86–109) and IV(86–124) has prevented us from ob-

![Fig. 7. The docking between subunit IV and cytochrome b in R. sphaeroides bc₁ complex. Cytochrome b is indicated in blue and subunit IV is in red. The boxed areas (interaction regions) shown in the figure at the upper left are zoomed to make details visible.](http://www.jbc.org/Downloaded_from_by_guest_on_July_18,2018)
Subunit IV of Cytochrome bc$_1$ Complex

Table II compares maximum reconstitutive activities of mutants IV(86–124), IV(77–109), IV(54–109), IV(41–109), and IV(21–109), with and without fused GST. The GST-IV(86–109) fusion protein has less than 3% of reconstitutive activity of GST-IV or wild-type IV. The IV(77–109) mutant has 50% of the reconstitutive activity of the wild-type IV, indicating that residues 77–85 are involved in activation of the core complex. The increase in reconstitutive activity of the IV(77–109) mutant is not due to an increase in its hydrophobicity upon addition of residues 77–85 because the GST-IV(86–124) fusion protein, with 15 downstream amino acid residues added to the transmembrane helix, has little or no reconstitutive activity. This result further confirms that residues 110–124 are not required.

The GST-IV(54–109) fusion protein has essentially the same reconstitutive activity as that of the GST-IV(77–109) fusion protein or IV(77–109) mutant, indicating that residues 54–76 are not essential. The IV(41–109) mutant has the same reconstitutive activity as the recombinant wild-type IV (a 2-fold increase of reconstitutive activity of the GST-IV(54–109) indicating that residues 41–53 are essential. As expected, the IV(21–109) mutant, with residues 21–40 added to the IV(41–109) mutant, has the same reconstitutive activity as that of wild-type IV, indicating that residues 21–40 are not involved in interacting with the core complex. Thus, two regions of subunit IV, residues 41–53 (first domain) and 77–85 (second domain), in addition to the transmembrane helix region (residues 86–109) (third domain), are essential for optimal interaction with the core complex to restore the bc$_1$ complex activity.

Localization of Interacting Regions of Subunit IV in the Proposed Structure of R. sphaeroides Cytochrome bc$_1$ Complex—Although the structural model for subunit IV (4) was constructed on hydrophathy plots of its amino acid sequence, using the program of Kyte and Doolittle (34), and on predicted a and $\beta$ helices, the wideness of the membrane is unknown. Lack of knowledge of the topological arrangement of subunit IV in the chromatophore membrane makes it difficult to localize the putative interacting regions of subunit IV in the bc$_1$ complex without the three-dimensional structure of this bacterial complex, which is not yet become available. Therefore, the wideness of the membrane in the proposed structural model of subunit IV was determined using Fab’-horseradish peroxidase conjugates, prepared from antibodies against synthetic peptides corresponding to residues 59–73 (the near N-terminal peptide) and 110–124 (the C-terminal peptide), in sealed and broken chromatophores.

When sealed (inside-out) and broken chromatophore preparations are treated with Fab’-fragment-horseradish peroxidase conjugates prepared from anti-C-terminal peptide antibodies, peroxidase activity is observed only with the broken chromatophores (Fig. 4), indicating that the C-terminal end is exposed on the periplasmic side of the chromatophore membrane. When sealed and broken chromatophore preparations are treated with Fab’-fragment-horseradish peroxidase conjugates prepared from anti-near N-terminal peptide antibodies, peroxidase activity is observed with both sealed and broken chromatophores (Fig. 4), indicating that the C-terminal end is exposed (26).

REFERENCES

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Subunit IV of Cytochrome bc$_1$ Complex

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Subunit IV of Cytochrome bc1 Complex from Rhodobacter sphaeroides: LOCALIZATION OF REGIONS ESSENTIAL FOR INTERACTION WITH THE THREE-SUBUNIT CORE COMPLEX

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