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The Role of the Supernumerary Subunit of *Rhodobacter sphaeroides* Cytochrome bc_1 Complex[1]

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The smallest molecular weight subunit (subunit IV), which contains no redox prosthetic group, is the only supernumerary subunit in the four-subunit *Rhodobacter sphaeroides* bc_1 complex. This subunit is involved in Q binding and the structural integrity of the complex. When the cytochrome bc_1 complex is photoaffinity labeled with [^{3}H]azido-Q derivative, radioactivity is found in subunits IV and I (cytochrome *b*), indicating that these two subunits are responsible for Q binding in the complex. When the subunit IV gene (*fbc*Q) is deleted from the *R. sphaeroides* chromosome, the resulting strain (RSΔIV) requires a period of adaptation before the start of photosynthetic growth. The cytochrome bc_1 complex in adapted RSΔIV chromatophores is labile to detergent treatment (60–75% inactivation), and shows a four-fold increase in the K_m for Q_2H_2. The first two changes indicate a structural role of subunit IV; the third change supports its Q-binding function. Tryptophan-79 is important for structural and Q-binding functions of subunit IV. Subunit IV is overexpressed in *Escherichia coli* as a GST fusion protein using the constructed expression vector, pGEX/IV. Purified recombinant subunit IV is functionally active as it can restore the bc_1 complex activity from the three-subunit core complex to the same level as that of wild-type or complement complex. Three regions in the subunit IV sequence, residues 86–109, 77–85, and 41–55, are essential for interaction with the core complex because deleting one of these regions yields a subunit completely or partially unable to restore cytochrome bc_1 from the core complex.

KEY WORDS: Nonredox subunit; the supernumerary subunit; subunit interaction; activity restoration.

INTRODUCTION

The protein subunits in the cytochrome bc_1 complex can be divided into two groups: those that contain a redox prosthetic group, called the core subunits, and those which do not possess a redox prosthetic group, called the supernumerary subunits (Yang and Trumpower, 1988). All the cytochrome bc_1 complexes contain three core subunits: cytochrome *b* (43 kDa), cytochrome c_1(31 kDa), and Rieske iron–sulfur protein (23 kDa), which house two *b*-type cytochromes (b_{562} and b_{565}), one *c*-type cytochrome (c_1), and one high potential Rieske iron-sulfur cluster [2Fe–2S], respectively. However, they vary significantly in supernumerary subunit composition (Yu and Yu, 1993; Trumpower and Gennis, 1994). Whereas the bc_1 complex from bovine heart mitochondria has eight supernumerary subunits and that from yeast has seven, the *R. sphaeroides* complex has one, and those from *Rhodopseudomonas rubrum*, *Paracoccus denitrificans*, and *Rhodobacter capsulatus* have none.

Biochemical and biophysical studies of the three core subunits of the bc_1 complex have been extensive and a wealth of information has been obtained. However, the functions of the supernumerary subunits are

[1] Abbreviations used: Q, ubiquinone, GST, glutathione-*S*-transferase; Q_2H_2, 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone.

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largely unknown. The delay in functional assessment of supernumerary subunits is mainly due to the unavailability of a reconstitutively active cytochrome bc_1 complex depleted of a given supernumerary subunit. By using the gene deletion and complementation approach, the functions of supernumerary subunits in the yeast bc_1 complex have been suggested. Subunit I (Tzagoloff *et al.*, 1986) and subunit II (Oudshoorn *et al.*, 1987) are believed to be essential for maintaining proper conformation of apocytochrome *b* for the addition of heme; subunit VI (Schoppink *et al.*, 1989; Schmitt and Trumpower, 1990) is involved in manipulating dimer/monomer transition; subunits VII and VIII (Maarse *et al.*, 1988) are essential for assembly of the bc_1 complex; and subunit IX (Phillips *et al.*, 1990) interacts with the iron–sulfur protein and cytochromes *b* and c_1. Recently, subunits I and II in plant and beef heart mitochondrial bc_1 complexes, were reported to associate with the mitochondrial-processing peptidase activity (Braun and Schmitz, 1995; Deng *et al.*, 1998).

The presence of multiple supernumerary subunits in the mitochondrial bc_1 complexes has made structure–function studies difficult. Since *R. sphaeroides* bc_1 complex has only one supernumerary subunit, it is an ideal system for studying supernumerary subunit function. In this article, we review structure–function relationships of subunit IV in the *R. sphaeroides* cytochrome bc_1 complex.

ESTABLISHING THE SUBUNIT IV AS A SUPERNUMERARY SUBUNIT IN *Rhodobacter sphaeroides* CYTOCHROME bc_1 COMPLEX

The cytochrome bc_1 complex, purified from chromatophores of *R. sphaeroides* in several laboratories using different purification procedures, contains four subunits with apparent molecular weights of 43, 31, 24, and 15 kDa (Yu *et al.*, 1984; Ljungdahl *et al.*, 1987; Purvis *et al.*, 1990; Andrew *et al.*, 1990.) The larger three are the core subunits housing two *b*-type cytochrome (b_{566} and b_{562}), cytochrome c_1 and the iron–sulfur cluster, respectively. The smallest subunit (subunit IV), which contains no redox prosthetic group and is absent from some comparable bacterial complexes (Yang and Trumpower, 1988; Kirauciunas *et al.*, 1989; Davidson *et al.*, 1993), was shown to be an integral part of the *R. sphaeroides* bc_1 complex as follows (Yu and Yu, 1991). When detergent-solubilized chromatophore was passed through an antisubunit antibody-affinity column, equilibrated with 50 m*M* Tris-Cl buffer, pH 8.0, containing 0.01% dodecylmaltoside and 100 m*M* NaCl, ubiquinol:cytochrome *c* reductase activity was completely removed. Under identical conditions, when solubilized chromatophore was passed through a preimmune IgG affinity column, less than 10% of the activity was lost. When the proteins absorbed to the subunit IV affinity column were eluted with 0.1 *M* Tris–glycine, pH 2.5, containing 0.01% dodecylmaltoside and subjected to SDS-PAGE, cytochrome *b*, cytochrome c_1, iron–sulfur protein, and subunit IV were detected. None of these proteins were found in the eluates of the preimmune IgG affinity column. These results clearly indicate that subunit IV is an integral part of *R. sphaeroides* cytochrome bc_1 complex.

THE STRUCTURE MODEL OF SUBUNIT IV IN THE CHROMATOPHORE MEMBRANE

The *R. sphaeroides* gene encoding for subunit IV (*fbc*Q) has been cloned and sequenced (Usui and Yu, 1991). The *fbc*Q cistron is 372 base pairs long and encodes 124 amino acid residues. The molecular mass of subunit IV, deduced from the nucleotide sequence, is 14,384 da. Figure 1 shows the proposed structure of subunit IV in chromatophore membranes. This structural model is based on the hydropathy plots of the amino acid sequence of subunit IV, predicted tendencies to form α-helices and β-sheets, and the binding of Fab′ fragment–horseradish peroxidase conjugates, prepared from antibodies against synthetic peptides corresponding to residues 59–73 and 110–124, in sealed and broken chromatophores. In this model, subunit IV has one transmembrane helix, residues 86–109, and two major β-pleated sheet structures: one near the N terminus and the other just before the transmembrane helix. Residues 1–85 are on the cytoplasmic side and residues 110–124 are on the periplasmic side of the chromatophore membrane.

The sideness of the membrane in this model was determined immunologically with Fab′ horseradish peroxidase conjugates prepared from anti-N-terminal peptide (residues 59–73) and anti-C-terminal peptide (residues 110–124) antibodies, in sealed and broken chromatophores. When sealed (inside-out) and broken chromatophore preparations (Hunter *et al.*, 1988) are treated with Fab′ fragment-horseradish peroxidase conjugates prepared from anti-C-terminal peptide antibodies, peroxidase activity is observed only on the broken chromatophores, indicating that the C-terminal end is exposed on the periplasmic side of the chromato-

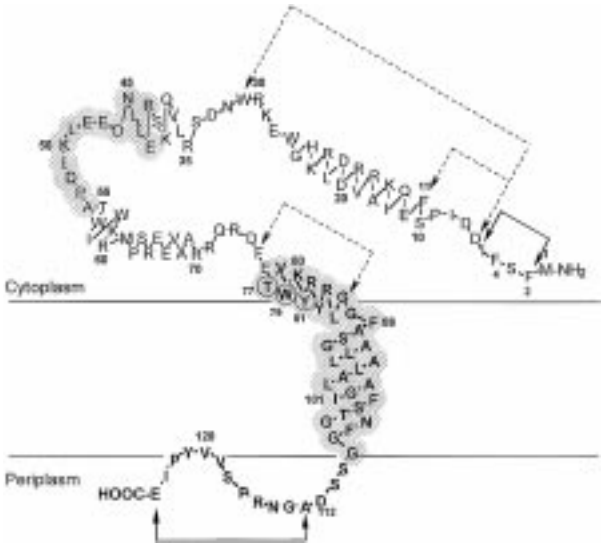

Fig. 1. Proposed subunit IV structure in the membrane. The Q-binding peptide identified by photoaffinity labeling studies is indicated by *heavy connecting bars* between the amino acid residues. Amino acid residues indicated with a *solid line* are those that can be deleted without affecting the function of the subunit, and amino acid residues indicated with a *dotted line* are those whose deletion cannot be tolerated. Amino acid residues essential for interaction with the core complex are indicated by *shaded areas*. Amino acid residues enclosed with *squares* are those whose replacement with other amino acid residues results in loss of function, whereas those enclosed with *circles* are residues that are replaceable without lose of function.

phore membrane. When sealed and broken chromatophore preparations are treated with Fab′ fragment-horseradish peroxidase conjugates prepared from anti-N-terminal peptide antibodies, peroxidase activity is observed on both sealed and broken chromatophores, indicating that the N-terminal end is exposed on the cytoplasmic side of the chromatophore membrane.

Although the three-dimensional (3-D) structure of *R. sphaeroides* bc_1 complex has not yet become available, the sequence homology between bacterial and mitochondrial core subunits enables us to obtain hypothetical structures for the three core subunits in the bacterial complex by homologous modeling using the coordinates from mitochondrial enzyme (Xia *et al.*, 1997). Subunit IV bears no sequence homology with the supernumerary subunits in mitochondrial enzyme, but is functionally homologous to subunit VII (Yu *et al.*, 1985; Yu and Yu, 1987). This subunit is incorporated into the complex by homologous modeling using the coordinates of mitochondrial subunit VII. Figure 2 shows the proposed ribbon diagrams for the *R. sphaeroides* cytochrome bc_1 complex in dimeric form.

THE INVOLVEMENT OF SUBUNIT IV IN QUINONE BINDING AND STRUCTURAL INTEGRITY OF THE CYTOCHROME bc_1 COMPLEX

When the *fbc*Q is deleted from the *R. sphaeroides* chromosome by site-specific recombination between the wild-type genomic *fbc*Q and a suicide plasmid (pSup202) containing a defective *fbc*Q sequence, the resulting strain (RSΔIV) requires a period of adaptation time (48 h) to start photosynthetic growth (Chen *et al.*, 1994). Although the chemical compositions, spectral properties, and the cytochrome bc_1 complex activities in chromatophores from adapted RSΔIV and wild-type cells are similar, the apparent K_m for Q_2H_2 for the bc_1 complex in RSΔIV chromatophores is about four times higher than that in wild-type chromatophores (Chen *et al.*, 1994). The cytochrome bc_1 complex activity in RSΔIV chromatophores is more labile to detergent treatment than that in the wild-type chromatophores (Chen *et al.*, 1994). The specific activities of dodecylmaltoside-solubilized fractions from RSΔIV chromatophores, based on cytochrome *b*, are only one-fourth that of the untreated chromatophores, while specific activities of dodecylmaltoside-solubilized fractions from the wild-type chromatophore, based on cytochrome *b*, are the same as that of the untreated chromatophores. Introducing a wild-type *fbc*Q on a stable low-copy number plasmid, pRK415, into RSΔIV restores photosynthetic growth behavior, the apparent K_m value for Q_2H_2, and tolerance to detergent treatment, to that of wild-type cells. Cytochrome bc_1 complex purified from adapted RSΔIV contains only three 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_2H_2 from 3 to 13 μM. We attribute the changes observed in photosynthetic growth behavior (requires adaptation time) and in detergent lability of the cytochrome bc_1 complex activity (75% inactivation) to a structural role for subunit IV and the change in Q-binding parameter (K_m increase) to its Q-binding function.

The Q-binding function of subunit IV is further supported by photoafffinity labeling using azido-Q derivatives (Yu and Yu, 1987). If purified *R. sphaeroides* cytochrome bc_1 complex is treated with a 50-fold molar excess of 3-azido-2-methyl-5-methoxy-6-(3,7-dimethyl[^{3}H]-octyl)1,4-benzoquinone {[^{3}H]azido-Q}derivative in the dark, no loss of activity is observed. When this azido-Q-treated sample is illuminated with

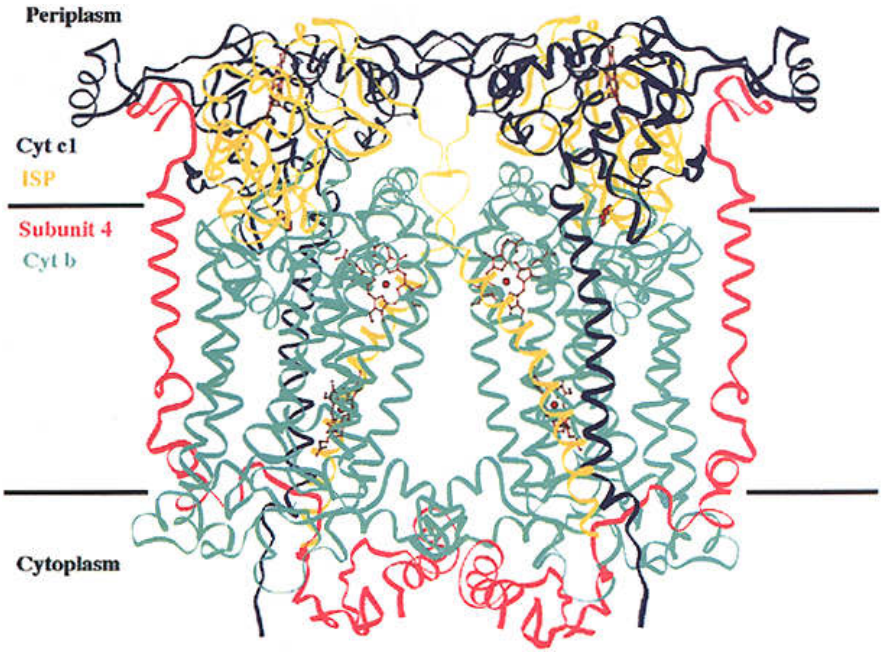

Fig. 2. Proposed ribbon diagrams for *Rhodobacter sphaeroides* bc_1 complex in dimeric form.

long wavelength UV light at 08C for 5 min, about 50% of the ubiquinol:cytochrome *c* reductase activity is lost. Since ubiquinol: cytochrome *c* reductase activity is assayed in the presence of excess Q_2H_2 (30 m*M*), the extent of inactivation of this reductase in the azido-Q-treated bc_1 complex, after illumination, is a measure of the Q-binding sites covalently linked to azido-Q. When illuminated [^{3}H]azido-Q-treated cytochrome bc_1 complex is subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), after removal of nonprotein-linked azido-Q by organic solvent extraction, followed by analysis of the radioactivity distribution among the complex subunits, subunit I (cytochrome *b*) and subunit IV are heavily labeled. The amount of azido-Q labeling in these two subunits is proportional to the extent of complex inactivation during illumination, indicating that subunit IV, along with cytochrome *b* protein, provide Q-binding site(s) in this complex (Yu and Yu, 1987).

IDENTIFICATION OF QUINONE-BINDING DOMAIN IN SUBUNIT IV

Detergent free, pure [^{3}H]azido-Q-labeled-subunit IV is obtained from the illuminated, [^{3}H]azido-Q treated bc_1 complex by a procedure involving preparative SDS-PAGE of the complex, slicing the band containing subunit IV from the gel, and electrophoretic elution of the protein from the gel slice. The SDS present in the preparation is removed by precipitation with cold (220 8C) acetone to 50%. When the V8 digested, [^{3}H]azido-Q labeled subunit IV is separated by HPLC on a SynchropakRP-8 column, most of the radioactivity is found in a peptide (fraction 56) having a retention time of 56.10 min (Usui and Yu, 1991). The partial N-terminal amino acid sequence of fraction 56 is (Thr-Val-Trp-Lys)-Tyr-Arg-Tyr-Arg-leu-Gly-Phe-leu-Ala-, with the first four amino acids in the sequencing chromatogram unidentified. This abnormality might be caused by covalent linkages between activated nitrene and these four amino acid residues. The sequence determined matches amino acid residues 81–90 of subunit IV. According to the model proposed in Fig. 1, the region (Thr-Val-Trp-Lys), thought to be covalently linked to azido-Q in the isolated Q-binding peptide, is located near the surface of the cytoplasmic side of the chromatophore membrane. Since the isolated Q-binding peptide is quite large, binding of the benzoquinone ring to amino acid residues other than those proposed is a possibility. More detailed fragmentation of the peptide is needed to establish the precise Q-binding site.

IDENTIFICATION OF AMINO ACID RESIDUES INVOLVED IN QUINONE BINDING AND STRUCTURAL FUNCTIONS OF SUBUNIT IV

Rhodobacter sphaeroides strains expressing cytochrome bc_1 complex with substitution mutations at the putative Q-binding domain of subunit IV were generated to identify the amino acid residues involved in Q binding (Chen *et al.*, 1995). Thr77, Tyr81, and Trp79 were selected for mutagenesis because the hydroxyl groups of Thr77 and Tyr81 can form hydrogen bonds with the carbonyl oxygens of quinone and Try79 may provide the needed aromatic interaction with the benzoquinone ring, similar to those found in the photosynthetic bacterial reaction center (Deisenhofer and Michel, 1989). Replacing Thr77 with valine (T77V) or replacing Tyr81 with phenylalanine (Y81F) or valine (Y81V) results in cells having photosynthetic growth behavior, tolerance to detergent, and Q-binding kinetics (K_m for Q_2H_2) similar to those of the wild-type or complement strain, indicating that Thr77 and Tyr81 are not involved in Q binding and structural integrity of subunit IV. However, when Trp79 was replaced with leucine (W79L) or phenylalanine (W79F), the resulting mutant strains showed photosynthetic growth, tolerance to detergent, and Q-binding kinetics similar to those of RSΔIV strain, indicating that this amino acid residue is essential for the structural and Q-binding functions of subunit IV (Chen *et al.*, 1995).

Rhodobacter sphaeroides strains expressing the cytochrome bc_1 complex with progressive deletion of amino acid residues from the N- or C-terminus of subunit IV were generated to identify the regions required for structural function of subunit IV (Chen *et al.*, 1995). Deleting residues 2–5 from the N-terminal end or residues 113–124 from the C-terminal end of subunit IV results in cells having photosynthetic growth behavior, tolerance to detergent treatment, and Q_2H_2 binding kinetics similar to those of wild-type and complement strains, indicating that these two segments are not essential. However, when the deletion was extended further, the resulting mutant strain contains no subunit IV protein. The failure to detect subunit IV protein is due to mutant protein instability because mutant mRNA was detected in these deletion strains. To overcome the complications arising from mutational effects of larger fragment deletion on improper complex assembly or instability of mutated protein, an approach using expressed mutated recombinant protein to reconstitute, *in vitro*, a subunit IV-lacking complex (the three-subunit core complex) is employed. This *in vitro* reconstitution approach requires the availability of a reconstitutively active, three-subunit core bc_1 complex and an overexpressed, functionally active subunit IV.

CHARACTERIZATION OF RECOMBINANT SUBUNIT IV

It has been reported (Johnson *et al.*, 1989) that in *E. coli*, recombinant polypeptides produced as glutathione-*S*-transferase (GST) fusion proteins using the pGEX vector system can be purified to homogeneity by a one-step affinity-column chromatography with glutathione agarose gel followed by thrombin cleavage. The very high yields reported in studies with pGEX combined with the simple purification format encouraged us to use the pGEX system to express *R. sphaeroides* subunit IV. The subunit IV expression vector (Chen *et al.*, 1996), pGEX/IV, is constructed by in-frame fusion of subunit IV gene with the GST gene on the pGEX-2T plasmid from Invitrogen. The production of active soluble GST–RSIV recombinant fusion protein in *E. coli* transformed with pGEX/RSIV plasmid depends on IPTG concentration, induction growth time, medium, and temperature (Chen *et al.*, 1996). About 40% of the expressed GST-IV fusion protein in *E. coli* is in a soluble form when IPTG (0.5 m*M*) induction growth is in a LB medium supplemented with 2% glucose at 25–27°C for 3.5 h. Pure recombinant IV, which shows a single band in SDS-PAGE, corresponding to subunit IV in the complex, is obtained from the fusion protein by thrombin digestion followed by fast-protein liquid chromatography gel filtration with Sepharose-12 to remove uncleaved GST-IV and thrombin.

Isolated recombinant subunit IV is soluble in aqueous solution but exists in a highly aggregated form, with an apparent molecular mass of 1000 kDa, as estimated by molecular-sieving column chromatography. Aggregated subunit IV was deaggregated to decamer (145,000), pentamer, and trimer states in the presence of 0.01, 0.1, and 0.2%, dodecylmaltoside, respectively. Purified recombinant subunit IV is functionally active since it increases the tolerance of cytochrome bc_1 complex in RSΔIV chromatophores to detergent treatment and restores cytochrome bc_1

complex activity to the three-subunit core complex (Chen *et al.*, 1996). When RSΔIV chromatophores were added to varying amounts of recombinant subunit IV, before being subjected to dodecylmaltoside treatment, the cytochrome bc_1 complex activity in the detergent-solubilized chromatophore fraction increased with the amount of recombinant subunit IV added. Maximum restoration (68%) was reached when recombinant subunit IV and the RSΔIV bc_1 complex were present in a 1:1 molar ratio. When varying amounts of purified subunit IV in 50 m*M* TrisCl, pH 8.0, containing 0.01% dodecylmaltoside, were added to a constant amount of three-subunit core complex, cytochrome bc_1 complex activity increased as the amount of subunit IV increased. Maximum restoration was reached at 1 mol of subunit IV per mol of three-subunit core complex.

IDENTIFICATION OF REGIONS OF SUBUNIT IV INVOLVED IN INTERACTION WITH THE THREE-SUBUNIT CORE COMPLEX

Since addition of recombinant subunit IV to the three-subunit core complex restores the bc_1 complex activity to the level of wild-type or complement four-subunit complex, recombinant subunit IV is properly assembled into the bc_1 complex resulting from interaction with the core complex. In order to understand the mode of interaction between subunit IV and the core complex, it is necessary to locate the region of subunit IV involved in this interaction. Four recombinant C-terminal truncate subunit IV mutants: IV(1–109,), IV(1–85), IV(1–76), and IV(1–40), deleting 15, 39, 48, and 84 amino acid residues, respectively, from the C-terminus, were generated for initial investigations. The region of subunit IV involved in interaction with the core complex is indicated by the loss (or decrease) in ability to restore the bc_1 activity to the core complex (reconstitutive activity) upon its removal from the subunit IV sequence. The IV(1–109) mutant restores the bc_1 complex activity to the same level as does wild-type IV, indicating that residues 110–124 are not essential. However, mutants IV(1–85), (1–76), and IV(1–40) are unable to restore cytochrome bc_1 complex activity to the core complex, indicating that residues 86–109 of subunit IV are essential for interaction.

Since residues 86–109 are the putative transmembrane helix, apparently reconstitutive activity of subunit IV, requires a membrane anchoring segment in the subunit. This is evident from incorporation data which show that the wild-type and the IV(1–109) mutant is associated with the His-tagged core complex on a Ni-TNA column at 1:1 molar ratio, whereas the IV(1–85) mutant is unable to associate with the core complex. This result suggests that subunit IV is assembled into the bc_1 complex by the transmembrane helix. The Reiske iron–sulfur protein has also been reported to require a transmembrane helix for assembly into the bc_1 complex (Gonzalez-Halphen *et al.*, 1991).

Although residues 86–109 are essential for subunit IV to be associated with the core complex, this fragment alone shows little reconstitutive activity. This together with the fact that the IV(1–109) has full reconstitutive activity indicates that regions within residues 1–85 are also essential for reconstitutive activity of subunit IV. Recombinant subunit IV mutants: IV(77–109), IV(54–109), IV(41–109), IV(21–109), progressive elongation of 9, 32, 45, and 65 amino acid residues, respectively, from the transmembrane helix toward the N-terminus, were generated for identifying the other essential regions of subunit IV involved in its interaction with the core complex. Addition of residues 77–85 to the transmembrane helix, the resulting mutant IV [IV(77–109)] has 50% of the reconstitutive activity of the wild-type IV, indicating that residues 77–85 are essential. Further addition of residues 55–76 to the IV(77–109) mutant did not further increase the reconstitutive activity, indicating that residues 54–76 are not essential. Addition of residues 41–53 to the IV(54–109) mutant increase the reconstitutive activity to the same level as that of the recombinant wild-type IV, indicating that residues 41–53 are essential. As expected, addition of residues 21–40 to the IV(41–109) mutant gives a mutant [IV(21–109)] having the same activity as the IV(41–109) mutant or the wild-type recombinant IV, indicating that residues 1–40 are not essential.

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