Evidence for the Head Domain Movement of the Rieske Iron-Sulfur Protein in Electron Transfer Reaction of the Cytochrome bc₁ Complex

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The three-dimensional structure of the mitochondrial cytochrome bc₁ complex suggests that movement of the extramembrane domain (head) of the Rieske iron-sulfur protein (ISP) may play an important role in electron transfer. Such movement requires flexibility in the neck region of ISP, since the head and transmembrane domains of the protein are rather rigid. To test this hypothesis, Rhodobacter sphaeroides mutants expressing His-tagged cytochrome bc₁ complexes with cysteine substitution at various positions in the ISP neck (residues 39–48) were generated and characterized. The mutants with a single cysteine substitution at Ala₄₂ or Val₄₄ and a double cysteine substitution at Val₄₄ and Ala₄₆ (VQA-CQC) or at Ala₄₂ and Ala₄₆ (ADVQA-CDVQC) have photosynthetic growth rates comparable with that of complement cells. Chromatophore membrane and intracytoplasmic membrane (ICM) prepared from these mutants have cytochrome bc₁ complex activity similar to that in the complement membranes, indicating that flexibility of the neck region of ISP was not affected by these cysteine substitutions. Mutants with a double cysteine substitution at Ala₄₂ and Val₄₄ (ADV-CDC) or at Pro₄₀ and Ala₄₂ (PSA-CSC) have a retarded (50%) or no photosynthetic growth rate, respectively. The ADV-CDC or PSA-CSC mutant ICM contains 20 or 0% of the cytochrome bc₁ complex activity found in the complement ICM. However, activity can be restored by the treatment with β-mercaptoethanol (β-ME). The restored activity is diminished upon removal of β-ME but is retained if the β-ME-treated membrane is treated with the sulfhydryl reagent N-ethylmaleimide or p-chloromercuribenzoic acid. These results indicate that the loss of bc₁ complex activity in the ADV-CDC or PSA-CSC mutant membranes is due to disulfide bond formation, which increases the rigidity of ISP neck and, in turn, decreases the mobility of the head domain. Using the conditions developed for the isolation of His-tagged complement cytochrome bc₁ complex, a two-subunit complex (cytochromes b and c₁) is obtained from all of the double cysteine-substituted mutants. This suggests that introduction of two cysteines in the neck region of ISP weakens the interactions between cytochromes b, ISP, and subunit IV.

The cytochrome bc₁ complex (ubiquinol-cytochrome c reductase) is an essential segment of the energy-conserving electron transfer chains of mitochondria and many respiratory and photosynthetic bacteria (1). This complex catalyzes electron transfer from ubiquinol to cytochrome c and concomitantly translocates protons across the membrane to generate a membrane potential and pH gradient for ATP synthesis. Although the cytochrome bc₁ complexes from different sources vary in their polypeptide compositions, they all contain four redox prosthetic groups: two b-type cytochromes (b₅₆₆ or b₁, and b₅₆₂ or b₄), one c-type cytochrome (cytochrome c₁), and one high potential Rieske iron-sulfur cluster [2Fe-2S]. The proton-motive Q cycle model (2) has been favored for electron transfer and proton translocation in the complex. The key feature of this model is the presence of two separate ubiquinone- or ubiquinol-binding sites: a ubiquinol oxidation site near the P side of the inner mitochondrial membrane and a ubiquinone reduction site near the N side of the membrane.

Recently, the cytochrome bc₁ complex from beef heart mitochondria was crystallized and its three-dimensional structure solved at 2.9-Å resolution (3, 4). The structural information obtained not only answered a number of questions concerning the arrangement of the redox centers, transmembrane helices, and inhibitor binding sites but also suggested movement of an extramembrane domain within the iron-sulfur protein (ISP) during electron transfer (4). This suggestion arose from observation of an uneven electron density in the I₄₁₂₂ crystal data of native bovine cytochrome bc₁ complex. A particularly low electron density area is observed in the intermembrane space portion of the complex, where the extramembrane domains of ISP and cytochrome c₁ reside (4). This movement hypothesis was further supported by the finding that the position of the iron-sulfur cluster in the complex is affected by ubiquinol oxidation site inhibitor binding (5, 6, 8) and by the crystal form (7, 9).

The anomalous light scattering signal of the [2Fe-2S] cluster is enhanced in co-crystals with stigmatellin or UHDBT but is diminished in the co-crystal with (E)-methyl-3-methoxy-2-(4′-trans-stilbene) acrylate (5). Thus, binding of stigmatellin or UHDBT arrests the movement of the extramembrane domain of ISP, fixing the iron-sulfur cluster 27 Å from heme b₁ and 31 Å from heme c₁ (referred to as the “fixed state” of ISP), the same position it occupies in the I₄₁₂₂ crystal of native bovine cytochrome bc₁ complex. The position of the iron-sulfur cluster changes from the fixed state to somewhere closer to heme c₁ (referred to as the “released state” of ISP) upon (E)-methyl-3-

† The abbreviations used are: [2Fe-2S] cluster, iron sulfur cluster of Rieske iron-sulfur protein; b₁, low potential heme b; b₅₆₂, high potential heme b; DM, dodecylmaltoside; NTA, nitrilotriacetic acid; ICM, intracytoplasmic membrane(s); PAGE, polyacrylamide gel electrophoresis; ISP, iron-sulfur protein; β-ME, β-mercaptoethanol; NEM, N-ethylmaleimide; PCMB, p-chloromercuribenzoic acid.
methoxy-2-(4′-trans-stilbenyl) acrylate binding. The recent report of Iwata et al. (9), showing the iron-sulfur cluster at two different positions in two crystal forms, further supports the presence of a variable position in the "released state" of the iron-sulfur cluster.

Movement of the head domain of ISP during electron transfer in cytochrome bc₁ complex can be explained as follows. The [2Fe-2S] cluster is reduced by the first electron of ubiquinol at a position 27 Å from heme b₄ and 31 Å from heme c₁ (ISP in "fixed state"). Since a reduced [2Fe-2S] cluster cannot donate an electron to cytochrome c₁ before the second electron of ubiquinol is transferred to heme b₄, it was postulated that either the change of the ubiquinone binding position during reduction of b₄ or the electron transfer from b₄ to b₅ causes a conformational change in cytochrome b, which forces or allows reduced [2Fe-2S] to move close enough to heme c₁ (ISP in "released state") for electron transfer (5, 7). This model would also explain why ebisemiquinone, a more powerful reductant than ubiquinol, reduces b₄, but not the [2Fe-2S] cluster, during ubiquinol oxidation.

ISP has three domains: the membrane-spanning N-terminal domain consisting of residues 1–62 (tail), the soluble C-terminal extramembrane domain consisting of residues 73–196 (head), and the flexible linking domain comprising residues 63–72 (neck). ISP is associated with the complex primarily via the membrane-spanning N-terminal domain (4, 7, 9). The [2Fe-2S] cluster is located at the tip of the head domain (12, 13). Since the three-dimensional structures of the head and tail domains are the same in the fixed and released states, movement of the head domain of ISP in the bc₁ complex requires flexibility of the neck region.

If movement of the head domain of ISP is required for bc₁ catalysis and the neck region of ISP confers the required mobility, decreasing the flexibility of the neck region of ISP should affect bc₁ complex activity. This hypothesis can be tested by site-directed mutagenesis followed by biochemical and biophysical characterization of mutant expressing cytochrome bc₁ complexes with altered ISP necks. However, site-directed mutagenesis in bovine heart mitochondria is not practical. R. sphaeroides is an ideal system to study the neck region of ISP by molecular genetics approach. The four-subunit complex is an ideal system to study the neck region of ISP in the bc₁ complex with altered ISP necks by introducing single cysteines or a pair of cysteines at different positions. The photosynthetic growth behavior, the cytochrome bc₁ complex activity, and the EPR characteristics of the Rieske [2Fe-2S] cluster in membranes and the purified state from complement and mutant strains are examined and compared. The effect of sulphydryl reagents on cytochrome bc₁ complexes from complement and mutant membranes is also examined.

EXPERIMENTAL PROCEDURES

Materials—2-Mercaptoethanol (β-ME), N-ethylmaleimide (NEM), and p-chloromercuribenzoic acid (PCMB) are from Sigma. All other chemicals are of the highest purity commercially available.

Generation of R. sphaeroides Strains Expressing the bc₁ Complexes with Altered ISP—Mutations were constructed by site-directed mutagenesis using the Altered Sites system from Promega. The oligonucleotide primers used for mutagenesis were as follows: ADV(42–44)-C-DCA, ACATGAACTCCTGACGTGCGAC-CTCGCCACCTCCTCGTGC; A42C, CAATAAGTATTCCGACGTGAC-GTGCAACCTCCTCGCCTACT; VQA(44–46)-CQC, ACCAGGAT-AATCTGCGCAGTGGCAGCTGTGCTGCTCCCATCCTTCGTCGTG- GTCGTA; PSA(40–42)-CSC, TGCCGCGTACGAACAAATGATGTG-GTCGTTGACCGTGCCCCGTCCCTGCATCATCCT; ADVQA(42–46)-C-DVQGC, TCAGTGGACGTCCAGTGCCGCTTGGCCTCCCTGATCCATCCATCTT.

The method for construction of ISP mutants is essentially the same as previously reported by Tien et al. (14). The ADVQA(42–46)-CDVQGC-rich medium was used in cloning the oligonucleotide primers with a single-stranded pSLEN53503 carrying a A42C mutation in ISP. The presence of engineered mutations were confirmed by DNA sequencing before and after photosynthetic or semiaerobic growth of the cells. Expression plasmid pRKGhfbF_Bc₁Q was purified from a plasmid of a photosynthetic or semiaerobic culture using the Qiagen plasmid Mini Prep kit. Since R. sphaeroides cells contain four types of endogenous plasmids, isolated plasmids are not pure and concentrated enough for direct sequencing. Thus, the 1.2-kilobase pair DNA segment containing the mutation sequence was amplified from the isolated plasmids by polymerase chain reaction and purified by 1% agarose gel electrophoresis. The 1.2-kilobase pair polymerase chain reaction product was recovered from the gel by a gel extraction kit from Qiagen.

Growth of Bacteria—Escherichia coli was grown at 37 °C in an enriched medium (TPV) in order to shorten growth time and increase plasmid yield (15). For photosynthetic growth of the plasmid-bearing R. sphaeroides BC17 cells, an enriched Sistrom’s medium containing 5 mM glutamate and 0.2% casamino acids was used. The pH of the medium was adjusted to 7.1 with a mixture of 6 N NaOH and 2 N KOH to increase the sodium and potassium ion content of the medium to a more physiological level (16). Photosynthetic growth condition of R. sphaeroides was essentially as described previously (14); cells harboring mutated bcf genes on the pRKGhfbF_Bc₁Q plasmid were grown photosynthetically for one or two serial passages to minimize any pressure for reversion. For semiaerobic growth of R. sphaeroides, an enriched Sistrom’s medium supplemented with 20 amino acids and extra rich vitamins was used. These semiaerobic cultures were grown in 0.5 liters of enriched medium in 2-liter Bellco flasks with vigorous shaking (220 rpm) for 26 h. The inoculation volumes used for both photosynthetic and semiaerobic cultures were at least 5% of the total volume. Antibiotics were added to the following concentrations: ampicillin (125 mg/liter), tetracycline (10 mg/liter for E. coli and 1 mg/liter for R. sphaeroides), kanamycin sulfate (30 mg/liter for E. coli and 20 mg/liter for R. sphaeroides), trimethoprim (57.5 mg/liter for E. coli).
chromophores by the method of Tian et al. (14). His6 tag is located at the C terminus of the cytochrome c1 subunit.

Ubiqunol-cytochrome c reductase activity was measured at 23 °C in a 1-mL assay mixture containing 100 mM sodium/potassium phosphate buffer, pH 7.4, 0.3 mM EDTA, 100 μM cytochrome c, 25 μM 2,3-dimethoxy-5-methyl-6(10-bromodecyl)-1,4-benzoquinol, and an appropriate amount of membrane or purified cytochrome bc1 complex. Chromatophores or ICM were diluted with 50 mM Tris-Cl, pH 8.0, containing 20% glycerol and 1 mM MgSO4 to a final concentration of cytochrome c1 of 5 μM. No detergent was added to the diluted mixture in order to preserve the bc1 activity. 5 μl of diluted membrane was added to the assay mixture. Activity was determined by measuring the reduction of cytochrome c (the absorbance increase at 550 nm), using a millimolar extinction coefficient of 18.5 cm−1 mM−1. Nonenzymatic oxidation of 2,3-dimethoxy-5-methyl-6(10-bromodecyl)-1,4-benzoquinol, determined under the same conditions in the absence of enzyme, was subtracted.

Freshly prepared NEM (100 mM in H2O) was added to the chromatophore or ICM and PCMB was removed by repeated washing and centrifuging, and excess NEM treatment of membranes with NEM or PCMB—such as described above—was used as the second antibody.

EPR spectra were recorded with a Bruker ER 200D apparatus equipped with a liquid N2 Dewar at 77 K. Instrument settings are detailed in the figure legends.

**RESULTS AND DISCUSSION**

Characterization of the Mutants Carrying Cysteine Substitutions in the Neck Region of the Iron-Sulfur Protein—Six *R. sphaeroides* mutants expressing His6-tagged cytochrome bc1 complexes with single or double cysteine substitutions at various positions in the ISP neck region were generated to test the hypothesis that neck flexibility allows ISP head domain movement required for bc1 catalysis. The flexibility of the neck should decrease when a disulfide bond is formed between a pair of substituted cysteines.

Of the six mutants, two, A42C and V44C, are single substitutions, in which Ala-42 or Val-44 is replaced with cysteine. The other four are double cysteine substitutions, PSA-CSC, ADV-CDC, VQA-CQC, and ADVQA-CDVQC, in which Pro40 and Ala42, Ala42 and Val14, Val14 and Ala46, or Ala32 and Ala46 are replaced with cysteines. A plate mating technique was used (22) to transfer the pRkD/pbFbc1BC17Q plasmid from *E. coli* S17 to *R. sphaeroides* BC17. The mating took place in less than 16 h on the LB/SIS plates. *R. sphaeroides* BC17 cells harboring pRkD/pbFbc1BC17Q plasmid were selected by spreading the conjugated cell mixture on enriched Sistrom’s plate containing tetracycline and kanamycin sulfate. It took 4 days for the A42C, V44C, ADVQA-CDVQC, and VQA-CQC mutant colonies to show up on the plate, the same time period as that required for complement colonies. However, it took about 7 days for the ADV-CDC and PSA-CSC mutant colonies to appear. This slower growth rate on the plates is an indication of zero or reduced bc1 activity in the virtual absence of environmental selection pressure. A similar phenomenon was observed with several cytochrome b mutants that had no bc1 activity.2

When mid-log phase, aerobically grown complement and mutant cells were inoculated into enriched Sistrom medium and subjected to anaerobic photosynthetic growth conditions, the A42C, V44C, VQA-CQC, and ADVQA-CDVQC mutants grow at a rate comparable with that of complement cells, the ADV-CDC mutant has a retarded (50%) growth rate, and PSA-CSC does not grow photosynthetically (Table I). Chromatophores from the A42C, V44C, and ADVQA-CDVQC mutant cells have cytochrome bc1 complex activity comparable with that of the complement chromatophores. The VQA-CQC and ADV-CDC mutant chromatophores have, respectively, 77 and 68% of the bc1 activity.

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**Table I** Characterization of ISP neck cysteine mutants

| Strains | Position of cysteine substitution(s) | Photosynthetic growth | Enzymatic activity* | Subunit composition |
|--------|-------------------------------------|-----------------------|---------------------|-------------------|
|        |                                     |                       | Chromophore | ICM | Purified complex | Chromophore or ICM | Purified complex |
| Complement | None | ++| 2.2 | 2.1 | 2.5 | FBCQ' | FBCQ |
| A42C  | Ala42   | ++| 1.8 | − | 2.8 | FBCQ | FBCQ |
| V44C  | Val14 | ++| 1.9 | − | 2.6 | FBCQ | FBCQ |
| ADVQA-CDVQC | Ala42, Ala46 | ++ | 1.9 | 2.0 | 0 | FBCQ | BC |
| VQA-CQC  | Ala44, Ala46 | ++ | 1.7 | 1.8 | 0 | FBCQ | BC |
| PSA-CSC  | Pro40, Ala42 | − | 1.0 | 0 | 0 | FBCQ | BC |
| ADV-CDC  | Ala42, Ala46 | + | 1.5 | 0.3 | 0 | FBCQ | BC |

*The enzymatic activity is expressed as μmol of cytochrome c reduced/min/mg of cytochrome b.
*FBCQ indicates gene products of the fbcF (ISP) (F), fbcB (cytochrome b1) (B), fbcC (cytochrome c1) (C), and fbcQ (subunit IV) (Q), respectively.

†, −, no photosynthetic growth within 4 days.

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2 H. Tian, S. White, L. Yu, and C-A. Yu, unpublished data.
complex activity found in complement chromatophores. ICM from the PSA-CSC mutant have no ubiquinol-cytochrome c reductase activity. This was expected, since bc1 complex is required for photosynthetic growth and this mutant does not grow photosynthetically.

To determine whether the loss (or decrease) of the cytochrome bc1 complex activity in the mutant membranes results from a lack of or improper assembly of ISP protein in the membrane, the amount of ISP and its EPR characteristics in mutant and complement membranes were compared. Western blot analysis with antibodies against R. sphaeroides cytochrome bc1, cytochrome c1, ISP, and subunit IV revealed that the amount of these four subunits in the six mutant membranes is the same as that in the complement membrane (Fig. 1, lanes 2–8). Absorption spectral analysis shows that the content of cytochrome b and c1/c2 in all of these mutant membranes is the same as that in complement membrane. These results indicate that the mutations did not affect the assembly of ISP protein into the membrane. The [2Fe-2S] cluster in all of these mutant membranes has an EPR spectrum identical to that observed in complement chromatophores, with resonance at $g_x = 1.80$ and $g_y = 1.90$ (Fig. 2). Thus the mutations did not change the microenvironment of the iron-sulfur cluster.

**Effect of Mutation on the Disulfide Bond Formation in the Neck Region of the Iron-Sulfur Protein—**The bc1 complex activity decreased by 23, 32, 14, and 100% in the VQA-CQC, ADV-CDC, ADVQA-CDVQC, and PSA-CSC mutant membranes, respectively, with no change in the amount of ISP incorporated into the membrane and the EPR characteristics of the [2Fe-2S] cluster. Did this activity loss result from increased neck region rigidity due to the formation of a disulfide bond? We addressed this question by comparing cytochrome bc1 complex activity in mutant and complement membranes with and without β-ME treatment. Also, we used molecular modeling to examine the feasibility of disulfide bond formation in the PSA-CSC mutant.

When ICM from the PSA-CSC mutant, which has no bc1 activity, was treated with β-ME, about 60% of the activity found in complement ICM was restored (Table II). It should be emphasized that the observed activity restoration is not due to nonenzymatic reduction of cytochrome c by β-ME, because less than 0.05 μM β-ME is present in the assay mixture, and the restored activity is sensitive to antimycin. This restored cytochrome bc1 complex activity is diminished when β-ME is removed by repeated centrifugation and suspension. However, the restored bc1 activity is retained if the β-ME-treated membrane is reacted with sulfhydryl modifying reagents, such as NEM or PCMB, at 5-fold molar excess to β-ME, followed by repeated centrifugation and suspension (Table II). Activity restoration is not observed when the PSA-CSC mutant membrane is treated with NEM or PCMB without prior reduction with
β-ME. These results indicate that the complete lack of the bc1 activity in the PSA-CSC mutant membrane results from decreased mobility of the ISP head domain, due to a neck region made rigid by the formation of a disulfide bond between Cys40 and Cys42. The addition of β-ME reduces this disulfide bond, restoring flexibility to the neck region. Modification of β-ME released SH– groups with NEM or PCMB has no effect on the flexibility of the neck region but blocks reformation of the disulfide bond, thus guaranteeing retention of the restored activity.

Formation of a disulfide bond between Cys40 and Cys42 in the ISP neck of the PSA-CSC mutant is also supported by molecular modeling using the peptide sequence of NCSCQVQLA, corresponding to the neck sequence of the PSA-CSC mutant. In the bovine bc1 structure (4), the distance between C-β atoms of Ala66 and Ala70 (corresponding to residues 40 and 42 of R. sphaeroides ISP) is 5.2 Å, and the side chains of the two residues point in the same direction relative to the backbone of this stretch. Molecular modeling, based upon this structural information, indicates that a disulfide bond can be formed. This would result in an 11-membered ring structure. A distance of 5.2 Å for disulfide bond formation is slightly longer than the 4.1 Å observed for the two C-β atoms of the disulfide bond, between Cys160 and Cys144 of ISP, in bovine bc1 crystals (4).

The possibility that the lack of bc1 activity in the PSA-CSC mutant ICM is due to the formation of a intermonomer disulfide bond between one of the two cysteines at positions 40 or 42 and a cysteine in the other monomer is ruled out because bc1 complex activity in the A42C mutant chromatophore or ICM is the same as that in complement membranes and is not activated by the addition of β-ME. Furthermore, the distance between the two cysteines at position 40 and 40’ in ISP from different monomers appears to be over 33 Å, as estimated from the distance between Ala42 and Cys44 of ISP, in bovine bc1 crystals (4).

The distances between C-β atoms of Val68 and Ala70 of ISP (Val44 and Ala46 in R. sphaeroides) and between C-β atoms of Ala66 and Ala70 (Ala42 and Ala46 in R. sphaeroides) are 7.35 and 12 Å, respectively. These exceed the distance that permits disulfide bond formation. Since no disulfide bond is formed, the flexibility of the ISP neck should not change in these mutant chromatophores. Thus, the decreased bc1 activity in these two mutant chromatophores cannot be attributed to decreased mobility of the head domain of ISP.

The finding that the ADV-CDC mutant ICM has only 20% as much bc1 complex activity as does its chromatophore membrane is rather surprising (Table I), because bc1 complex activities in chromatophore and ICM are comparable in all of the other cysteine-substituted mutants and in complement cells (Table I). The addition of β-ME to ADV-CDC mutant membrane has no effect on bc1 complex activity in chromatophores, while increasing the activity in ICM to the level observed in untreated chromatophores (Table II). The restored activity in the mutant ICM is diminished by removal of β-ME. The addition of 5-fold molar excess NEM or PCMB to the β-ME-treated mutant ICM preserves the restored activity in the membrane. These results indicate that the loss of bc1 activity in the ADV-CDC mutant ICM, compared with that in its chromatophore, results from the decreased mobility of the head domain of ISP, due to formation of a disulfide bond between Cys42 and Cys44 in the neck region of ISP. However, a similar explanation does not account for the lower (32% less than complement) bc1 complex activity in ADV-CDC mutant chromatophore or in β-ME-treated ICM, because neither contain disulfide bonds. Apparently, some other effect of cysteine substitution decreased the bc1 activity.

It should be noted that the loss of bc1 complex activity in the ADV-CDC mutant ICM is not due to the formation of an intermonomer disulfide bond, because the distance between potential cysteine pairs in the two monomers is over 30 Å. Recall that the bc1 activity in the single cysteine substitution mutants A42C and V44C was not affected by the addition of β-ME. In the bovine bc1 complex structure, the distance between C-β atoms of Ala66 and Val68 (corresponding to residues Ala42 and Val44 in R. sphaeroides) is 7 Å, and the orientations of the side chains are opposite each other. However, since the neck region is flexible, a different crystal form might result in a different conformation. This comparison is at the “noise” level of homologous sequence similarities, e.g. bovine versus R. sphaeroides. Therefore, formation of a disulfide bond between Cys42 and Cys44 in ADV-CDC mutant ICM is not impossible.

Since disulfide bonds occur in ICM, but not in the chromatophore, of the ADV-CDC mutant, probably disulfide bond formation in this mutant ISP neck region depends on the oxygen supply in the cell culture and is related to the functionality of the bc1 complex in the membranes. When these mutant cells are grown in the dark with an ample oxygen supply, no bc1 activity is detected in the cytoplasmic membrane due to the formation of a disulfide bond in the ISP neck region. The

| Strains       | Chromatophore | ICM        |
|---------------|---------------|------------|
|               | No treatment  | β-ME       | NEM/PCMBa | No treatment | β-ME | NEM/PCMBa |
| Complement    | 2.2           | 2.2        | 2.2       | 2.1          | 2.0 | 2.0       |
| A42C          | 1.8           | 1.8        | 1.8       | 1.8          | 1.8 | 1.8       |
| V44C          | 1.9           | 1.9        | 1.9       | 1.9          | 1.9 | 1.9       |
| ADVQA-CDVQC   | 1.9           | 1.9        | 1.9       | 2.0          | 2.0 | 2.0       |
| VQA-CQC       | 1.7           | 1.7        | 1.7       | 1.8          | 1.8 | 1.8       |
| PSA-CSC       | —             | —          | 0.3       | 1.4          | 1.4 | 1.4       |
| ADV-CDC       | 1.5           | 1.5        | 1.5       | 1.4          | 1.4 | 1.4       |

a NEM or PCMB was added at 5-fold molar excess to the β-ME-pretreated membrane.

b Sample was not available.
addition of β-ME to the cytoplasmic membrane can restore bc1 activity to the level observed in the chromatophore membrane. This is consistent with the fact that bc1 complex is not required for dark aerobic growth of this organism because the cells can utilize quinol oxidases. When the O2 supply is limited, the cells for dark aerobic growth of this organism because the cells can adjust their protein expression to prepare for photosynthetic growth, as indicated by the appearance of chlorophyll. The mutation that prevents disulfide bond formation between Cys42 and Cys44. When oxygen is completely depleted from the culture, the cells undergo photosynthetic growth and require active bc1 complex. Apparently, no disulfide bonds are formed in the mutant chromatophores, and bc1 activity increases greatly (1.5 μmol of cytochrome c reduced per min per nmol of b; Table I).

**Fig. 3.** The effect of dodecylmaltoside concentration on the activity and the solubilization efficiency of bc1 complex from membranes of the double cysteine-substituted mutants and the complement cells. The bc1 complex activities in the extraction mixture (A) as well as in the supernatant fractions (B) obtained from membranes treated with various concentrations of dodecylmaltoside are expressed as the percentage of that of the untreated sample. Extraction efficiency (C) was calculated as the ratio of the cytochrome b present in the detergent-solubilized supernatant fraction to the total amount of cytochrome b in the extraction mixture. The β-ME-restored bc1 activity in the PSA-CSC mutant ICM was used as 100% activity for this mutant membrane. Membranes from the complement (●), VQA-CQC (+), ADV-CDC (□), ADVQA-CDVQC (○), and PSA-CSC (○) cells were adjusted to 25 μM cytochrome b with 50 mM Tris-Cl, pH 8.0, containing 20% glycerol. Dodecylmaltoside was added to give the final concentrations as indicated.

**Fig. 4.** SDS-PAGE of purified His6-tagged bc1 complexes. Aliquots of cytochrome bc1 complexes (75 pmol of cytochrome b), recovered from the Ni2+-NTA column of DM-solubilized mutant and complement membrane fractions were loaded into each well of a SDS-PAGE gel. Lane 1, prestained molecular weight standards; lane 2, cytochrome bc1 complex from the wild-type complement strain; lanes 3–5, the bc1 complexes from mutants A42C, V44C, VQA-CQC, ADV-CDC, ADVQA-CDVQC, and PSA-CSC, respectively.

**Effect of Mutation on Binding Affinity of the Iron-Sulfur Protein in the bc1 Complex—**Although chromatophores from mutants with a double cysteine mutation in the ISP neck region (VQA-CQC, ADV-CDC, and ADVQA-CDVQC) have ISP amounts, EPR properties, and ISP neck flexibility similar to those in complement chromatophores, the bc1 activities are 23, 32, and 14% less, respectively. Is this activity loss due to a decreased binding affinity of ISP to the whole complex? Assuming that ISP binding is sensitive to detergent treatment, any difference in subunit composition of purified His-tagged bc1 complexes from mutant and complement strains will indicate detergent lability and binding affinity differences between normal and mutant ISPs. When chromatophores from these three mutant cells, VQA-CQC, ADV-CDC, and ADVQA-CDVQC, at a cytochrome b concentration of 25 μM were mixed with various amounts of dodecymaltoside (up to 0.44 mg/nmol of cytochrome b), the cytochrome bc1 complex activities decreased as the concentration of dodecylmaltoside (DM) increased (Fig. 3A). More than 95% of the bc1 complex activity in all of these mutant chromatophores was inactivated by 0.44 mg of DM/nmol of cytochrome b. Under identical conditions, complement chromatophores did not lose activity (Fig. 3A), indicating that the mutant cytochrome bc1 complexes (two-cysteine substitution) are more labile to detergent.

Since DM solubilizes the bc1 complex from *R. sphaeroides* chromatophores, it is important to establish that DM denaturation of the bc1 complex in mutant chromatophores is due to ISP dissociation from the complex and not due to a decreased affinity for membrane. This is done by centrifuging DM-treated chromatophores from mutants and complement cells at 100,000 × g for 30 min to separate the solubilized from the unsolubilized fractions and measuring cytochrome content, ISP amount, and bc1 activity in both fractions. Since the amount of cytochrome bc1 complex protein (cytochromes b and c1, ISP, and subunit IV) in the supernatant fractions of the three mutant chromatophores is comparable with that in the supernatant fraction from complement chromatophores at a given concentration of detergent, it is apparent that DM solubilization of the cytochrome bc1 complex is not affected by mutation (data not shown). However, the specific activity of cytochrome bc1 complex, based on b content in the supernatant fractions from mutant chromatophores, decreased as the detergent concentration increased (Fig. 3B). Under identical conditions, the bc1 activity in supernatant fractions from complement chromatophores does not drastically decrease. The extraction efficiency, based on cytochrome b content, is the same for all mutants and complement membranes (Fig. 3C).
To confirm that the loss of bc1 activity in the DM-solubilized fractions resulted from dissociation of ISP from the complex, the supernatant fractions, extracted by 0.5 or 1% of DM, were passed through a Ni2+-NTA column. The column eluents and eluates were examined for bc1 complex subunit composition. Two subunits, corresponding to cytochromes b and c1, were found in the Ni2+-NTA column eluates (Fig. 4, lanes 5–7). ISP and subunit IV were detected in the column eluents by Western blot analysis (Fig. 1, lanes 9–11). On the other hand, four subunits (cytochrome b, cytochrome c1, ISP, and subunit IV) in unit stoichiometry are in the Ni2+-NTA column eluates from detergent-solubilized complement chromatophores (Fig. 4, lane 2). These results indicate that a double cysteine substitution in the ISP neck region decreases the binding affinity of ISP for the bc1 complex. Therefore, detergents dissociate ISP from the complex.

Since no ISP is found in the Ni2+-NTA column eluates from the DM (0.5%)-solubilized mutant chromatophore fractions having 40% of the untreated cytochrome bc1 complex activity, the activity detected in this fraction reflects the ability of dissociated ISP to reconstitute into a functionally active bc1 complex after dilution of detergent in the assay mixture. Perhaps the activity decrease in untreated mutant chromatophores indicates the extent of ISP dissociation from the complex. However, the 14% decrease in bc1 complex activity in the A42C or V44C mutant chromatophores is probably due to a deviation in the activity assay and not to a mutational effect, since their activities are not labile to detergents. Indeed, all four subunits of the bc1 complex are absorbed on the Ni2+-NTA column after the detergent solubilization (Fig. 4, lanes 2 and 3). The purified complexes from these two mutants have specific activities comparable with that of the complement (Table I).

Although the restored bc1 complex activity in the PSA-CSC mutant ICM treated with β-ME is more labile to DM treatment than are the bc1 activities in VQA-CQC, ADV-CDC, and AD-VQA-CDVQC mutant chromatophores (Fig. 3), the effectiveness of dodecylmaltoside in solubilizing bc1 complex from PSA-CSC mutant ICM and the bc1 complex subunit composition of the Ni2+-NTA column eluents and eluates (Fig. 1, lane 11, and Fig. 4, lane 8) are the same as that for the three double cysteine-substituted mutant chromatophores. Probably the binding affinity of ISP for the bc1 complex in PSA-CSC is weaker than in the other double cysteine mutant chromatophores. Thus, ISP is more easily dissociated from the complex by detergent. Since only 60% of the bc1 activity found in complement chromatophore is restored to the PSA-CSC mutant ICM upon the addition of β-ME, it is likely that 40% of the ISP in this mutant ICM is dissociated from the bc1 complex.

The activity of detergent-dissociated ISP to reconstitute into functionally active bc1 complex depends on the detergent concentration in the reconstituting mixture (or in the assay mixture) and the status of the dissociated ISP. It has been reported (23) that including reducing reagents, such as dithiothreitol or β-ME, during isolation of ISP from the complex protects free ISP from oxidation by oxygen and that the addition of phospholipid to the detergent-containing solution reduces effective detergent concentration. Therefore, we examined the effect of β-ME and phospholipid on the reassociation of ISP into functionally active bc1 complex in the DM-solubilized chromatophore fraction from the ADV-CDC mutant cells. As described above, β-ME has no effect on the bc1 activity in this mutant chromatophore or on the solubilization efficiency of DM. β-ME preserves the activity of mutant ISP after its dissociation from bc1 complex. In the presence of β-ME, when the mutant chromatophore is treated with 1.2% dodecylmaltoside, the cytochrome bc1 complex activity in the supernatant fraction is 10-fold higher than that in the supernatant fraction obtained from mutant chromatophore without pretreatment with β-ME.

The activity of the supernatant with β-ME is about 50% of that in the chromatophore before detergent solubilization. When the detergent-solubilized fraction obtained from the β-ME-treated mutant chromatophore is incubated with phospholipid (10 mg/ml), the cytochrome bc1 complex activity increases to 95% of that in the untreated chromatophore. Without prior addition of β-ME, the supernatant has minimal activity (2%), which only increases slightly after the addition of phospholipid. These results indicate that the addition of β-ME to the ADV-CDC chromatophore prior to detergent solubilization prevents denaturation of dissociated ISP and subunit IV and thus enables them to reconstitute with cytochromes b and c1 to form functionally active bc1 complex after excess detergent is removed by phospholipid and dilution with the assay mixture.

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