Subunit IV of Rhodobacter sphaeroides cytochrome b-c1 complex was over-expressed in Escherichia coli JM109 cells as a glutathione S-transferase fusion protein (GST-RSIV) using the expression vector, pGEX/RSIV. Maximum yield of soluble active recombinant fusion protein was obtained from cells harvested 3 h after induction of growth at 37 °C in LB medium. Subunit IV was released from the fusion protein by proteolytic cleavage with thrombin. When subjected to SDS-polyacrylamide gel electrophoresis, isolated recombinant subunit IV showed one protein band corresponding to subunit IV of R. sphaeroides cytochrome b-c1 complex. Although the isolated recombinant subunit IV is soluble in aqueous solution, it is in a highly aggregated form, with an apparent molecular mass of over 3000 kDa. The addition of detergent deaggregates the isolated protein, suggesting that the recombinant protein exists as a hydrophobic aggregation in aqueous solution. When the three-subunit core cytochrome b-c1 complex, purified from RSΔIV-adapted chromatophores containing a fraction of the wild-type cytochrome b-c1 complex activity, was reacted with varying amounts of recombinant subunit IV, the activity increased as the subunit IV concentration increased. Maximum activity restoration was reached when 1 mol of subunit IV/mol of three-subunit core complex was used. The reconstituted cytochrome b-c1 complex is similar to the wild-type complex in molecular size, apparent K_m for Q_2H_2, and inhibitor sensitivity, indicating that recombinant subunit IV is properly assembled into the active cytochrome b-c1 complex. A tryptophan residue in subunit IV was found to be involved in the interaction with the three-subunit core complex.

The Rhodobacter sphaeroides cytochrome b-c1 complex, which catalyzes the electron transfer from ubiquinol to cytochrome c_2 (1), has been purified and characterized in several laboratories (2–6). The purified complex contains four protein subunits with molecular masses of 43, 31, 23, and 15 kDa. The three largest subunits house cytochrome b, cytochrome c_1, and a high potential [2Fe-2S] Rieske iron-sulfur cluster. The smallest protein subunit (subunit IV) has been proven to be an integral part of the complex by immunochemical studies (7). Subunit IV and cytochrome b have been identified as ubiquinone (Q)-binding proteins in the complex by photoaffinity labeling techniques using azido-Q derivatives (8). However, subunit IV is not present in other comparable bacterial cytochrome b-c1 complexes, such as Rhodospirillum rubrum (9), Rhodobacter capsulatus (10), and Paracoccus denitrificans (11).

The involvement of subunit IV in Q-binding and structural integrity of the R. sphaeroides cytochrome b-c1 complex has been further established by molecular genetics studies (12, 13). The gene for subunit IV (fbcQ) has been cloned and sequenced (12). The fbcQ cistron is 372 base pairs long, encodes 124 amino acid residues, and is contained in a 4.7-kilobase pair BamHI R. sphaeroides DNA fragment. When fbcQ is deleted from the R. sphaeroides chromosome, the resulting strain (RSΔIV) requires a period of adaptation before the start of photosynthetic growth (13). The cytochrome b-c1 complex in adapted chromatophores is labile to detergent treatment (75% inactivation) and shows a 4-fold increase in the K_m for Q_2H_2 (13). The first two changes (adaptation time and detergent lability) indicate a structural role of subunit IV; the third change (K_m increase) indicates its Q-binding function. Introducing wild-type fbcQ on a stable low copy number plasmid, pRK415, into RSΔIV restores photosynthetic growth behavior, the apparent K_m for Q_2H_2, and tolerance to detergent treatment to the level of wild-type cells.

The Q-binding domain in subunit IV is located at residues 77–124, as determined by isolation and sequencing of an [3H]azido-Q-labeled, V8-digested peptide (12). The most likely Q-binding region is at residues 77–86, which lies on the cytoplasmic side of the chromatophore membrane. By using site-directed mutagenesis techniques coupled with in vivo complementation, tryptophan-79 has been identified to be responsible for Q-binding, and amino acid residues 6–11 are responsible for the structural role of subunit IV (14).

Although mutagenesis coupled with in vivo complementation has generated useful information in structure-function studies of subunit IV, this approach is often complicated by mutational effects on the complex assembly and stability of the mutated protein. An approach using expressed mutated recombinant protein to reconstitute, in vitro, a subunit IV-lacking complex (three-subunit core complex) alleviates the problem of assembly and stability of mutated protein and thus complements the in vivo complementation approach. In order to employ this in vitro reconstitution approach, a reconstitutively active, three-subunit core cytochrome b-c1 complex and an over-expressed, functionally active subunit IV are needed.

The three-subunit core complex is available in our laboratory. This complex is prepared from adapted chromatophores of RSΔIV by a method involving dodecylmaltoside solubilization.
and DEAE-Biogel A and DEAE-Sepharse 6 B column chromatography (13). Recently, we have over-expressed subunit IV in Escherichia coli as a glutathione S-transferase (GST) fusion protein by using the pGEX expression vector system (15). The pGEX expression system allows one-step affinity purification of the recombinant fusion protein with glutathione-agarose gel (16). The recombinant protein is then released from the fusion protein by thrombin cleavage (17). Herein we report construction of a subunit IV expression vector, pGEX/RSIV, conditions for high expression of an active soluble form of the GST-RSIV fusion protein in E. coli JM109, and isolation and characterization of pure recombinant subunit IV. Reconstitution of the R. sphaeroides cytochrome b-c1 complex from the three-subunit core complex and recombinant subunit IV is described, and properties of the reconstituted complex are examined. The amino acid residues essential for reconstitutive activity of subunit IV are also identified.

**EXPERIMENTAL PROCEDURES**

**Materials**—All restriction endonucleases were obtained from either Promega or Life Technologies, Inc. Altered Sites in vitro Mutagenesis System was purchased from Promega. Expression vector pGEX-2T, fast protein liquid chromatography columns of Superox 6 and Superox 12 were prepared by Pharmacia Biotech Inc. Primers and oligonucleotides were synthesized by the DNA/Protein Core Facility of Oklahoma State University. T4 DNA polymerase and T4 DNA ligase were from Promega. Shrimp alkaline phosphatase was from Amersham Corp. Goat anti-rabbit IgG alkaline phosphatase conjugate and protein A- horseradish peroxidase conjugate were from Bio-Rad. Purenitrocellulose membrane for western blots was from Schleicher & Schuell. Glutathione (reduced form), glutathione-agarose gel, phenylmethylsulfonyl fluoride, leupeptin, isopropyl-β-D-thiogalactoside (IPTG), horse cytochrome c, type III, and thrombin were from Sigma. 2,3-Dimethoxy-5-methyl-6-geranyl-1,4-benzoquinol (Q2H2) was synthesized in our laboratory as described previously (7). Antibodies against subunit IV were raised in rabbits previously (18). Antibodies against subunit IV were performed according to the protocols described by Thorne et al. (23), and protein (24) and cytochromes c and b6 contents (13) were determined according to methods previously described.

**Growth of Bacteria**—R. sphaeroides wild-type (NCIB8253), R. sphaeroides cytochrome b-c1 complex (R. sphaeroides), wild-type (NCIB8253), and complement strains were grown at 30 °C in Sistrom's medium (3). T4 DNA polymerase and T4 DNA ligase were from Promega. Shrimp alkaline phosphatase was from Amersham Corp. Goat anti-rabbit IgG alkaline phosphatase conjugate and protein A-horseradish peroxidase conjugate were from Bio-Rad. Pure nitrocellulose membrane for western blots was from Schleicher & Schuell. Glutathione (reduced form), glutathione-agarose gel, phenylmethylsulfonyl fluoride, leupeptin, isopropyl-β-D-thiogalactoside (IPTG), horse cytochrome c, type III, and thrombin were from Sigma. 2,3-Dimethoxy-5-methyl-6-geranyl-1,4-benzoquinol (Q2H2) was synthesized in our laboratory as described previously (7). Antibodies against subunit IV were raised in rabbits previously (18). Antibodies against subunit IV were performed according to the protocols described by Thorne et al. (23), and protein (24) and cytochromes c and b6 contents (13) were determined according to methods previously described.

**Results and Discussion**

Construction of the Expression Vector for Subunit IV (pGEX/RSIV)—It has been reported (16) that in E. coli, recombinant polypeptides produced as fusion proteins as glutathione S-transferase (GST) using the pGEX vector system can be purified to homogeneity by a one-step affinity chromatography with glutathione-agarose gel followed by thrombin cleavage. The very high yields reported in studies with pGEX combined with the simple purification format prompted us to use this system to express recombinant cytochrome b-c1 complex from R. sphaeroides. To this end, we took advantage of the pGEX system to express recombinant subunit IV. The final strain used for the experiments was JM109/pGEX/RSIV. JM109/pGEX/RSIV was transformed into E. coli. Fig. 1 summarizes the protocol used for construction of the subunit IV expression vector, pGEX/RSIV. Because the BamH1 site located right next to the thrombin cleavage site is a unique site in the pGEX-2T vector and is missing in the subunit IV structural gene (fbQ), an in-frame fusion of the subunit IV gene with the GST gene in the pGEX-2T plasmid was achieved by generating a BamH1 fragment encoding subunit IV and subsequently ligating it into the BamH1 site of the pGEX-2T plasmid. To obtain the BamH1 fragment encoding subunit IV, a BamH1 recognition sequence (GGATCC) immediately upstream from the start codon (ATG) of subunit IV was created by site-directed mutagenesis. A 1.6-base pair BamH1 fragment cloned into the pSelect plasmid generated pSelect/RSVIAG was used as the template for mutagenesis. A mutant oligonucleotide, CTGGAGACGCCGGATCCATGTTCTCATT, and an ampicillin repair oligonucleotide were incubated in the mutagenesis system. This mutagenesis procedure produced greater than 70% mutants. The resulting plasmid, pSelect/RSVIAG, was digested with BamH1 to produce a 505-base pair fragment containing fbQ. This BamH1 fragment was ligated into pGEX-2T to generate pGEX/RSIV. pGEX/RSIV was transformed into E. coli JM109. Transformants producing the GST-RSIV fusion protein were identified by immunological screening of colonies with antibodies against subunit IV.

Expression and Purification of Recombinant Subunit IV—The production of active soluble GST-RSIV recombinant fusion protein in E. coli transformed with pGEX/RSIV plasmid was found to be IPTG induction- and growth-time dependent. Fig. 2

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shows the yields of glutathione-agarose gel-purified GST-RSIV fusion protein from cells harvested at various induction growth times. The yield of fusion protein was estimated by the color intensity of the 41-kDa protein band (GST-RSIV fusion protein), which reacted with antibodies against subunit IV and antibodies against GST. The yield increased as the induction growth time was increased to a maximum yield that was obtained when cells were harvested 3 h after growth induction. When cells were grown for 5 h, there was a 20% decrease in yield, suggesting that the recombinant protein is unstable and susceptible to protease digestion.

The isolation of GST-RSIV fusion protein by affinity glutathione-agarose gel is as described under “Experimental Procedures.” 30 μl of glutathione-agarose eluates were applied to SDS-PAGE. B, the proteins on the gel of A were electrophoretically transferred to a nitrocellulose membrane without staining and then reacted with anti-subunit IV antibodies. Protein A-horseradish peroxidase conjugate was used as a second antibody.

The susceptibility of recombinant subunit IV to the protease digestion is further evident from the presence of three smaller molecular mass protein bands, with apparent molecular sizes of 31, 28, and 26 kDa, following SDS-PAGE of glutathione-agarose eluates (see Fig. 2A). The 31- and 28-kDa protein bands, which reacted with antibodies against subunit IV and antibodies against GST and disappeared after thrombin digestion, may derive from partial C-terminal digestion of recombinant fusion protein. The 26-kDa protein band, which reacted only with antibodies against GST, may result from the complete digestion of subunit IV from the fusion protein.

The addition of protease inhibitors, such as phenylmethylsulfonyl fluoride and leupeptin, during cell extract preparation did not prevent degradation of the recombinant subunit IV. Expression of the GST-RSIV fusion protein in E. coli KS1000, which is deficient in the Tsp protease, a periplasmic protease, also did not prevent the degradation of recombinant subunit IV. The Tsp protease (26) was reported to degrade cytoplasmically expressed proteins in crude cell extracts, and presumably it can degrade proteins expressed in the periplasm as well.

It should be noted that our routine cell extract procedure included treatment with 1% Triton X-100. If this treatment was omitted, the yield of soluble recombinant GST-RSIV fusion protein decreased 55%. This suggests that some GST-RSIV fusion protein is either in a membrane fraction or an inclusion body aggregate that can be solubilized by Triton X-100 while maintaining the GST-active site recognizable by glutathione-agarose gel. Recombinant GST-RSIV fusion protein obtained from cell extracts prepared with Triton X-100 has the same molecular size and reconstitutive activity as protein obtained without Triton X-100 treatment.

Recombinant subunit IV was released from the fusion pro-
protein by thrombin digestion. When the fusion protein was incubated with thrombin at a weight ratio of 1:500 at room temperature, about 80% of the subunit IV was recovered within 1 h. Although prolonged incubation can complete cleavage, it is often accompanied by irreversible denaturation of protein. Therefore, a 1-h digestion time was used. The released GST and uncleaved GST-RSIV fusion protein in the treated sample were removed by glutathione-agarose beads, and thrombin was removed by gel filtration.

Properties of Recombinant Subunit IV—Isolated recombinant subunit IV is soluble in aqueous solution but exists in a highly aggregated form. The apparent molecular mass of the isolated recombinant protein is over 1000 kDa, as determined by fast protein liquid chromatography gel filtration with Sepharose-12 in 50 mM Tris-Cl buffer, pH 8.0. Aggregation is apparently due to the hydrophobic transmembrane segment of the peptide chain. The hydropathy plot of subunit IV suggests the presence of a definitive transmembrane helix (12) near the C-terminal end of the protein. Aggregated subunit IV was deaggregated to decamer (145,000), pentamer (75,000), and the apparent molecular mass of the highly aggregated form. The apparent molecular mass of the native subunit IV is soluble in aqueous solution but exists in a nonaggregated form. The protective effect of subunit IV on the cytochrome b-c1 complexes in wild-type, complement, and RSIV chromatophores toward detergent treatment

Properties of Recombinant Subunit IV—Isolated recombinant subunit IV is soluble in aqueous solution but exists in a highly aggregated form. The apparent molecular mass of the isolated recombinant protein is over 1000 kDa, as determined by fast protein liquid chromatography gel filtration with Sepharose-12 in 50 mM Tris-Cl buffer, pH 8.0. Aggregation is apparently due to the hydrophobic transmembrane segment of the peptide chain. The hydropathy plot of subunit IV suggests the presence of a definitive transmembrane helix (12) near the C-terminal end of the protein. Aggregated subunit IV was deaggregated to decamer (145,000), pentamer (75,000), and trimer (45,000) states in the presence of 0.01, 0.1, and 0.2% dodecylmaltoside, respectively, in 50 mM Tris-Cl buffer, pH 8.0, containing 300 mM NaCl. The soluble hydrophobic aggregation of isolated subunit IV resembles isolated recombinant mitochondrial QPC-9.5 kDa (27) in which the protein exhibits as a subunit of isolated subunit IV resembles isolated recombinant mitochondrial QPC-9.5 kDa (27) in which the protein exhibits as a subunit of isolated subunit IV resembles isolated recombinant mitochondrial QPC-9.5 kDa (27) in which the protein exhibits as a subunit.

Following SDS-PAGE, isolated subunit IV showed a single protein band that corresponds to subunit IV (M, = 14,384) of R. sphaeroides. The partial N-terminal amino acid sequence of recombinant subunit IV was determined to be GSMSFSL, indicating that two additional amino acid residues, glycine and serine, are present at the N terminus of subunit IV. These two residues result from the recombinant manipulation. The true molecular mass of recombinant subunit IV should be 201 daltons more than that of native subunit IV.

Functional Activity of Recombinant Subunit IV—Previous studies established that subunit IV is an essential subunit of R. sphaeroides cytochrome b-c1 complex. It is required for catalytic activity as well as structural integrity of the complex. Cytochrome b-c1 complex activity in subunit IV-deficient chromatophores of adapted RS IV cells is more labile to detergent treatment than that from wild-type cells, indicating that subunit IV is essential for the structural integrity of the complex. The three-subunit core complex, prepared from adapted chromatophores of RS IV cells, has only 25% of the activity of the four-subunit enzyme, indicating that subunit IV is essential for catalytic activity of the complex. Thus, the functional activity of recombinant subunit IV can be assessed by its ability to increase the tolerance of the cytochrome b-c1 complex in RS IV chromatophores to detergent treatment and its ability to restore cytochrome b-c1 complex activity to the three-subunit core complex.

Table I shows the protective effect of subunit IV on the RS IV cytochrome b-c1 complex toward detergent treatment.

Table I: The protective effect of subunit IV on the cytochrome b-c1 complexes in wild-type, complement, and RS IV chromatophores toward detergent treatment

| Preparations | Activity | Chromatophores\(a\) | DM-solubilized fraction\(b\) |
|--------------|----------|----------------------|------------------------------|
| Wild type    | 1.2      | 1.2                  |                              |
| Wild type + RS IV\(c\) | 1.21     | 1.2                  |                              |
| Complement   | 1.23     | 0.96                 |                              |
| Complement + RS IV\(c\) | 1.23     | 0.96                 |                              |
| RS IV        | 1.23     | 0.32                 |                              |
| RS IV + RS IV\(c\) | 1.23     | 0.82                 |                              |

\(a\) Data are the average values of three batches of chromatophore preparations. The deviation among the different batches was less than 5%.

\(b\) 1 ml aliquots (10 mg/ml) of chromatophore preparations were incubated with 6.7 mg dodecylmaltoside (DM) and 100 mM NaCl at 0 °C for 60 min. The mixtures were centrifuged at 100,000 × g for 90 min, and the supernatants were used for activity assay.

\(c\) Chromatophore preparations of the wild-type, complement, and RS IV were incubated with various amounts of subunit IV at 0 °C for 1 h before being subjected to dodecylmaltoside and NaCl treatment. The activities shown were the maximal activity obtained.

When RS IV chromatophores were added to varying amounts of recombinant subunit IV before being subjected to dodecylmaltoside treatment, the cytochrome b-c1 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 RS IV b-c1 complex were present in a 1:1 molar ratio. The addition of subunit IV to the wild-type or complement chromatophores had no effect on cytochrome b-c1 complex activity upon detergent solubilization. The further addition of subunit IV to the subunit IV-treated, detergent-solubilized chromatophores fraction did not further increase cytochrome b-c1 complex activity. The incomplete restoration of detergent tolerance to the cytochrome b-c1 complex in RS IV chromatophores by recombinant subunit IV may result from a decrease in binding affinity of the three-subunit core complex to recombinant subunit IV in the presence of high concentrations of dodecylmaltoside, as used in solubilization.
Subunit IV of R. sphaeroides Cytochrome b-c\textsubscript{1} Complex

FIG. 4 shows the restoration of the cytochrome b-c\textsubscript{1} complex activity from the purified three-subunit core complex by recombinant subunit IV. When the core complex was incubated with varying concentrations of subunit IV, activity increased as the concentration of subunit IV increased. Maximum restoration was reached when 1 mol of subunit IV/mole of three-subunit core complex was used. The activity was restored to the same level as that of the wild-type complex, indicating that recombinant subunit IV is fully active. Because recombinant subunit IV can fully restore cytochrome b-c\textsubscript{1} complex activity to the three-subunit core complex, the structural requirement for the amino acid residues near the N terminus of subunit IV are not stringent, as recombinant subunit IV has two amino acid residues, serine and glycine, added to the N terminus. This is in line with the gene deletion study showing that the first five amino acid residues from the N terminus are not essential for subunit IV (14). Restoration of the cytochrome b-c\textsubscript{1} complex activity to the three-subunit complex by subunit IV was found to be incubation time-dependent (see Fig. 5). Maximum activity restoration was observed after 1 h of incubation at 0°C. The incubation time dependence of reconstitution may result from deaggregation of recombinant subunit IV or conformational change of the reconstituted complex.

The addition of recombinant subunit IV to the three-subunit core complex not only restored the enzymatic activity but also the Q-binding environment. Fig. 6 shows the Q\textsubscript{2}H\textsubscript{2}-dependent activity titration curves for the wild-type, reconstituted, and RS\textsubscript{3}IV complexes in 50 mM Tris-HCl, pH 8.0, containing 0.01% dodecylmaltoside and 300 mM NaCl. Ubiquinol-cytochrome c reductase activity was assayed after incubation at 0°C for 1 h.

| Reagents used                                      | Reconstitutive activity % |
|---------------------------------------------------|----------------------------|
| None                                              | 100                        |
| Diethylpyrophosphate                              | 89                         |
| N-p-Tosyl-L-lysine chloromethyl ketone            | 95                         |
| L-1-p-Tosylamino-2-phenylchloromethyl ketone      | 96                         |
| Dioctylchexyl carbodiimide                        | 98                         |
| 1-Ethyl-(3-dimethylaminopropyl) carbodiimide      | 100                        |
| Phenylglyoxal                                     | 99                         |
| Dyclohexanedione                                  | 100                        |
| Chloramine T                                      | 100                        |
| N-Chlorosuccimide                                 | 3                          |
| N-Bromosuccimide                                  | 0                          |
| Maleic anhydride                                  | 99                         |
| Succinic anhydride                                | 100                        |

TABLE II

Effect of various amino acid modifying reagents on reconstitutive activity of recombinant subunit IV

100-μl aliquots of recombinant subunit IV, 0.73 mg/ml, in 50 mM Tris-Cl buffer, pH 8.0, containing 0.01% dodecylmaltoside and 300 mM NaCl, were incubated with 1 mM of the indicated compounds at 25°C for 10 min and dialyzed against the same buffer overnight at 4°C. 10-μl aliquots were withdrawn from each dialyzed sample, added 20 μl of three-subunit core complexes, 1 mg/ml, in the same buffer, incubated for 1 h at 0°C, and assayed for ubiquinone-cytochrome c reductase activity. The activity restored by subunit IV in which no modifying compound was added is used as 100% reconstitutive activity.

| Reagents used                                      | Reconstitutive activity % |
|---------------------------------------------------|----------------------------|
| None                                              | 100                        |
| Diethylpyrophosphate                              | 89                         |
| N-p-Tosyl-L-lysine chloromethyl ketone            | 95                         |
| L-1-p-Tosylamino-2-phenylchloromethyl ketone      | 96                         |
| Dioctylchexyl carbodiimide                        | 98                         |
| 1-Ethyl-(3-dimethylaminopropyl) carbodiimide      | 100                        |
| Phenylglyoxal                                     | 99                         |
| Dyclohexanedione                                  | 100                        |
| Chloramine T                                      | 100                        |
| N-Chlorosuccimide                                 | 3                          |
| N-Bromosuccimide                                  | 0                          |
| Maleic anhydride                                  | 99                         |
| Succinic anhydride                                | 100                        |
of wild-type, reconstituted, and three-subunit core cytochrome b-c1 complexes were 2.2, 2.5, and 10.8, respectively. The restoration of the K_m in the reconstituted complex further confirms the involvement of subunit IV in Q binding of this complex.

The ubiquinol-cytochrome c reductase activity in the reconstituted four-subunit complex is fully sensitive to antimycin treatment. A 50% inhibition was found with 1 mol of antimycin/mol cytochrome c, a level identical to that observed for the wild-type cytochrome b-c1 complex.

The reconstituted cytochrome b-c1 complex has the same molecular size as the wild-type complex as revealed by the same electrophoretic mobility of these two complexes in a non-denaturing blue gel electrophoresis (data not shown). The apparent molecular mass of these two complexes was estimated to be around 240 kDa, indicating that they exist in dimer form. The isolated three-subunit core complex also occurs in dimer form with a slightly higher electrophoretic mobility than the wild-type complex in non-denaturing blue gel. Because the isolated recombinant subunit IV in aqueous solution has an apparent molecular mass of over one million, whereas the reconstituted cytochrome b-c1 complex has only 240 kDa, deaggregation of subunit IV must occur during the reconstitution process. This correlates with the observation that incubation time is required for maximum reconstitution.

Involvement of Tryptophan Residues in Subunit IV for Its Reconstitutive Activity—Table I shows the effects of some commonly used protein modifying reagents on the reconstitutive activity of recombinant subunit IV. Among the modifiers tested, only N-chlorosuccimide or N-bromosuccimide, a tryptophan-modifying reagent, inhibited the reconstitutive activity of subunit IV, indicating that tryptophan(s) is essential for subunit IV in its interaction with the three-subunit core complex or for the catalytic activity of the complex. The latter possibility was ruled out, as the intact four-subunit complex is not sensitive to N-bromosuccimide treatment (up to 10 mol/mol protein).

Because there are five tryptophans in subunit IV, the number of tryptophans involved was unclear. To address this question, the correlation between loss of reconstitutive activity of recombinant subunit IV and tryptophan residues in subunit IV reacting with N-bromosuccimide was established. When recombinant subunit IV was incubated with various concentrations of N-bromosuccimide at room temperature for 10 min, the reaction of N-bromosuccimide with tryptophan residues was directly proportional to the loss of reconstitutive activity of subunit IV, up to a 6 mol excess of N-bromosuccimide (Fig. 7). About 70% of the reconstitutive activity of subunit IV was abolished when one tryptophan residue was modified. The direct correlation between activity loss and the tryptophan modification suggests that the first tryptophan residue modified in subunit IV is required for interaction with the three-subunit core complex. This tryptophan residue is more reactive toward N-bromosuccimide than other tryptophan residues in subunit IV. N-Bromosuccimide reacted with maximum of three tryptophans in subunit IV even though there are five tryptophans present. Because isolated recombinant subunit IV is in decamer form under the modification conditions, the N-bromosuccimide inactive tryptophans must be buried inside the aggregate. When wild-type four-subunit and three-subunit core complexes were incubated with a 10 mol excess of N-bromosuccimide at room temperature for 10 min, no loss of activity was observed. This indicates that the tryptophan residue in subunit IV responsible for interaction with other core subunits is shielded by their interacting subunit in the cytochrome b-c1 complex. Identification of the subunit IV tryptophan residue responsible for this interaction is currently in progress in our laboratory.

REFERENCES
1. Trumpower, B. L., and Gennis, R. B. (1994) Annu. Rev. Biochem. 63, 675–716
2. Yu, L., Mei, Q.-C., and Yu, C. A. (1984) J. Biol. Chem. 259, 5752–5760
3. Ljungdahl, P. O., Pennoyer, J. D., Robertson, D. E., and Trumpower, B. (1987) Biochim. Biophys. Acta 913, 227–241
4. Purvis, D. J., Theiler, R., and Niederman, R. A. (1990) J. Biol. Chem. 265, 1208–1215
5. Anderson, K., Crofts, A. R., and Gennis, B. (1990) Biochemistry 29, 2645–2651
6. Gennis, R. B., Barquera, B., Hacker, B., Van Doren, R. S., Armaud, S., Crofts, A. R., Davidson, E., Gray, K. A., and Daldal, F. (1993) J. Bioenerg. Biomembr. 25, 195–209
7. Yu, L., and Yu, C. A. (1991) Biochemistry 30, 4934–4939
8. Yu, L., and Yu, C. A. (1987) Biochemistry 26, 3658–3664
9. Kiriacuduna, A., Yu, L., Yu, C. A., Max Wynn, R., and Knaff, D. (1989) Biochim. Biophys. Acta 976, 70–76
10. Robertson, D. E., Ding, H., Chelminski, P. R., Slaughter, C., Hsu, J., Moomaw, C., Tokito, M., Daldal, F., and Dutton, P. L. (1993) Biochemistry 32, 1310–1317
11. Yang, X., and Trumpower, B. L. (1988) J. Biol. Chem. 263, 12282–12289
12. Usui, S., and Yu, L. (1993) J. Biol. Chem. 268, 15644–15649
13. Chen, Y.-R., Usui, S., Yu, C. A., and Yu, L. (1994) Biochemistry 33, 10207–10214
14. Chen, Y.-R., Shenoy, S. K., Yu, C. A., and Yu, L. (1995) J. Biol. Chem. 270, 11496–11501
15. Smith, D. B., and Johnson, K. S. (1988) Gene (Amst.) 67, 31–40
16. Johnson, K. S., Harrison, G. B. L., Lightowlers, M. W., O’Hoy, K. L., Cougle, W. G., Dempster, R. P., Lawrence, S. B., Vinton, J. G., Heath, D. D., and Rickard, M. D. (1989) Nature 338, 585–587
17. Abath, F. G. C., and Simpson, A. J. G. (1990) Peptide Res. 3, 167–168
18. Yu, C. A., and Yu, L. (1982) Biochemistry 21, 4096–4101
19. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
20. Altered Sites in vitro Mutagenesis System, Technical Manual, Promega Corporation
21. Laemmli, U. K. (1970) Nature 227, 680–685
22. Schagger, H., and Von Jagow, G. (1991) Anal. Biochem. 199, 223–231
23. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
24. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
25. Spande, T. F., and Witkop, B. (1967) Methods Enzymol. 11, 498–532
26. Siber, K. R., Keller, K. C., and Sauer, R. T. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 295–299
27. Yu, L., Ding, K.-P., and Yu, C. A. (1995) J. Biol. Chem. 270, 25634–25638
Functional Expression of Subunit IV of *Rhodobacter sphaeroides* Cytochrome b-c Complex and Reconstitution of Recombinant Protein with Three-subunit Core Complex
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