Role of Subunit IV in the Cytochrome b–c_1 Complex from *Rhodobacter sphaeroides*†

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ABSTRACT: *Rhodobacter sphaeroides* mutants lacking subunit IV (M_r = 14 384) of the cytochrome b–c_1 complex (representative mutant strain, RSΔIV-2) have been constructed by site-specific recombination between the wild-type genomic subunit IV structural gene (*fbc*Q) and a suicide plasmid containing a defective *fbc*Q sequence. RSΔIV-2 gives rise to a photosynthetically competent phenotype after a period of adaptation. The chemical compositions, spectral properties, and cytochrome b–c_1 complex activities in subunit IV-deficient chromatophores from adapted RSΔIV-2 are similar to those in wild-type chromatophores. However, the apparent K_m for Q_2H_2 for the b–c_1 complex in subunit IV-deficient chromatophores from adapted RSΔIV-2 cells is about four times higher than that in chromatophores from wild-type cells. The cytochrome b–c_1 complex activity in subunit IV-deficient chromatophores of adapted RSΔIV-2 cells is more labile to detergent treatment than that from wild-type cells. The specific activities of dodecylmaltoside-solubilized fractions of RSΔIV-2, based on cytochrome b, are only one-fourth that of the untreated chromatophores. Introducing a wild-type *fbc*Q operon on a stable low copy number plasmid, pRK415, into RSΔIV-2 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 b–c_1 complex purified from adapted RSΔIV-2 contains only three subunits. It has only 25% of the activity of the four-subunit enzyme. This low activity is accompanied by an increase of the apparent K_m for Q_2H_2 from 3 to 13 μM, suggesting that subunit IV may be involved in quinone binding in addition to its structural role.

The cytochrome b–c_1 complex, which catalyzes electron transfer from ubiquinol to cytochrome c_2, has been purified from the photosynthetic bacterium *Rhodobacter sphaeroides* and characterized in several laboratories (Gabellini *et al.*, 1982; Yu *et al.*, 1984; Ljungdahl *et al.*, 1987; McCurley *et al.*, 1990; Andrew *et al.*, 1990; Purvis *et al.*, 1990). 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, respectively. The smallest polypeptide (subuint IV) has been identified as one of the ubiquinone (Q)[1]-binding proteins in the complex by photoaffinity labeling techniques using azido-Q derivatives (Yu & Yu, 1987). The other Q-binding site(s) is(are) on the cytochrome b of the complex.

Since the cytochrome b–c_1 complexes isolated from *Rhodospirillum rubrum* (Kriauciunas *et al.*, 1989), *Rhodobacter capsulatus* (Robertson *et al.*, 1993), and *Paracoccus denitrificans* (Yang & Trumpower, 1988) contain only the three protein subunits that correspond to the three largest subunits of the *R. sphaeroides* complex, the essential role of subunit IV has been questioned. Recently, immunochemical studies established the essentiality of subunit IV (Yu & Yu, 1991) in the *R. sphaeroides* system. Antibodies against subunit IV react with the cytochrome b–c_1 complex and inhibit 90% of ubiquinol–cytochrome c reductase activity, indicating that this subunit is involved in the catalytic function of the complex. Furthermore, when detergent-solubilized chromatophores are passed through an anti-subunit IV antibody affinity column, no ubiquinol–cytochrome c reductase activity is found in the column effluent. Four polypeptides (corresponding to cytochromes b and c_1, iron–sulfur protein, and subunit IV) are adsorbed to the column and subsequently eluted, indicating that subunit IV is an integral part of the b–c_1 complex (Yu & Yu, 1991).

The gene for *R. sphaeroides* subunit IV (*fbc*Q) was recently cloned and sequenced (Usui & Yu, 1991). The *fbc*Q cistron is 372 base pairs long and encodes 125 amino acid residues. Genomic mapping indicates that the *fbc*Q gene is at least 900 kb away from the *fbc* operon.[2] A hydropathy plot of the predicted amino acid sequence reveals only one transmembrane helix, located near the C-terminal end. The Q-binding domain has been identified through isolation and sequencing of a [^{3}H]-azido-Q binding peptide from a preparation of the [^{3}H]azido-Q photoaffinity labeled cytochrome b–c_1 complex. This domain is near the surface of the membrane (Usui & Yu, 1991).

Although results from biochemical, immunological, and photoaffinity labeling studies have established the essentiality and the possible function of subunit IV in the isolated cytochrome b–c_1 complex of *R. sphaeroides*, there is no genetic evidence for an obligate role of this subunit. Therefore, we have used molecular genetics to study the essentiality of subunit IV. A subunit IV-deficient mutant has been generated by the disruption of or by the deletion of the *fbc*Q gene. Herein, we report the details of the construction of subunit IV-deficient mutants of *R. sphaeroides* and the characterization of mutant (three-subunit) and wild type (four-subunit) cytochrome b–c_1 complexes.

EXPERIMENTAL PROCEDURES

Materials. All restriction endonucleases were obtained from either Promega or Bethesda Research Laboratories. pUC4K

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[1] Abbreviations: DMG, decanoyl-*N*-methylglucamide; ICM, intracytoplasmic membrane; Q, ubiquinone; Q_2H_2, 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone; ISP, Rieske iron–sulfur protein.

[2] S. Kaplan, personal communications.

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plasmid was from Pharmacia. pGEM-3ZF (+) plasmid, *Sac*I-phosphorylated linker, 5′-d(pCGAGCTCG)-3′, Klenow DNA polymerase, T4 DNA polymerase, and calf intestine phosphatase were from Promega. pSup202, pRK415, and *Escherichia coli* T4 bacteriophage were gifts from Dr. R. Gennis, University of Illinois. Biotin-21-dUTP was from Clontech. Goat anti-rabbit IgG alkaline phosphatase conjugate was from Bio-Rad. LB agar and LB broth base were from Gibco. Nylon transfer membrane for Southern hybridization and pure nitrocellulose membrane for Western blots were from Schleicher & Schuell. 2,3-Dimethoxyl-5-methyl-6-geranyl-1,4-benzoquinone (Q_2H_2) was synthesized in our laboratory (Yu & Yu, 1982). Horse cytochrome *c*, type III, was from Sigma. Dodecyl-β-maltoside was from Calbiochem. Lumi-Phos 530 was from Boehringer Mannheim. All other chemicals were of the highest purity commercially available.

Growth of Bacteria. A culture of *R. sphaeroides* wild-type strain, NCIB8253, was a gift from Dr. R. A. Niederman, Rutgers University. Cells were grown at 30 °C chemoheterotrophically in Sistrom's medium A (Leuking *et al.*, 1978) with vigorous gyratory shaking. Liquid photoheterotrophic cultures were maintained in completely filled culture vessels at 1800 Lux light intensity and 30 °C. Dark anaerobic growth was accomplished in Sistrom's minimal medium lacking succinate by supplementating with 20 mM glucose and 80 mM dimethyl sulfoxide as a final electron acceptor. Cell growth was monitored by measuring the increase of optical density at 660 nm. Where appropriate, 2 μg/mL tetracycline or 50 μg/mL kanamycin was used for the growth of the mutants of *R. sphaeroides*.

E. coli S17-1 used as donor for conjugations and *E. coli* strains harboring pGEM-3 ZF (+), pUC8, pSup202, or pRK415 and their derivatives were grown in LB medium at 37 °C in the presence of ampicillin (50 μg/mL), kanamycin (50 μg/mL), or tetracycline [(20 μg/mL) for pSup and (10 μg/mL) for pRK 415 vectors] where appropriate. *E. coli* S17-1 was a gift from Dr. R. Gennis, University of Illinois.

Conjugation Mating and Recombinant DNA Techniques. Plasmid DNA was mobilized into *R. sphaeroides* using *E. coli* S17-1 as a donor. Conjugation between *E. coli* and *R. sphaeroides* was performed essentially as described by Donohue *et al.*, (1988).

Restriction enzyme digestion, large-scale isolation, and mini-preparation of plasmid DNA (Sambrook *et al.*, 1989) and isolation and purification of *R. sphaeroides* DNA from chemoheterotrophic cells (Usui & Yu, 1991) were performed according to these reported methods. Electrophoretic analysis and purification of DNA molecules from gel matrices have been described (Usui & Yu, 1991). Southern hybridization with the biotinylated DNA probes was performed according to Leary *et al.* (1983). Biotinylated DNA probes were prepared by the nick translation or mixed primer extension technique with Biotin-21-dUTP from Clontech. After hybridizing the biotinylated DNA probe to specific sequences bound on nylon filter, Lumi-Phos 530 was used as a chemiluminescent substrate for streptavidin–alkaline phosphatase for the detection of hybridized DNA.

Enzyme Preparations and Assays. Chromatophores of photosynthetically grown and cytoplasmic membranes of dark aerobically grown *R. sphaeroides* were prepared from wild-type, complement, and subunit IV deletion strains according to our previously reported method (Yu & Yu, 1991). The b-c_1 complexes were prepared from chromatophores by the method of Ljungdahl *et al.* (1987) with modifications (Yu & Yu, 1991). The cytochrome b-c_1 complex activity in membrane preparations or isolated complexes was assayed as previously reported (McCurley *et al.*, 1990). An appropriate amount of enzyme preparation was added to an assay mixture (1 mL) containing 50 mM sodium/potassium phosphate buffer, pH 7.0, 1 mM EDTA, 100 μM cytochrome *c*, and 25 μM Q_2H_2. For the determination of K_m, various concentrations of Q_2H_2 were used. The cytochrome b–c_1 complex activity was determined by measuring the reduction of cytochrome *c* (the increase in absorbance at 550 nm) in a Cary spectrophotometer, Model 219, or a Shimadzu UV-210IPC spectrophotometer at 22 °C. A millimolar extinction coefficient of 18.5 was used to calculate activity. The nonenzymatic oxidation of Q_2H_2 was determined under the same conditions in the absence of enzyme.

Absorption spectra were recorded at room temperature in a Shimadzu UV-210IPC spectrophotometer. Samples were fully oxidized by the addition of aliquots of 10 mM potassium ferricyanide. The *c*-type cytochromes were reduced by adding a few crystals of sodium ascorbate, and the *b*-type cytochromes were reduced by adding a few crystals of sodium dithionite.

Analytical SDS–PAGE was performed on a 15% polyacrylamide gel in a Bio-Rad Mini-Protean dual slab cell using the Laemmli system (Laemmli, 1970). Pigments present in the membrane preparation (cytoplasmic or chromatophore), which interfere with the separation of subunit IV on SDS–PAGE gel, were removed by an actone:methanol (7:2) extraction at 0 °C for 20 min. The extracted membrane precipitate was dissolved in 1% SDS after the residual solvent in the precipitate was removed by flushing with a stream of argon gas. The pigment-less membrane solution (5 mg/mL) was digested with 2% SDS and 1% β-mercaptoethanol for 2 h at 37 °C before being applied to SDS–PAGE gel. Western blotting was carried out according to the method (Towbin *et al.*, 1979) using rabbit antibody against purified subunit IV (Yu & Yu, 1991). The polypeptides separated on SDS–PAGE gel were transferred to 0.4-μm nitrocellulose for immunoblotting. Goat anti-rabbit IgG conjugated to alkaline phosphatase was used as the second antibody.

Protein (Lowry *et al.*, 1951), cytochromes *b* (Berden & Slater, 1970) and c_1 (Yu *et al.*, 1986), phospholipids (Ames & Dubin, 1960), ubiquinone (Redfearn, 1967), iron–sulfur cluster (McCurley *et al.*, 1990), chlorophyll (Cohen-Bazire *et al.*, 1957), and carotenoid (Cohen-Bazire *et al.*, 1957)) contents were determined according to reported methods. Ubiquinone in chromatophore preparations was isolated by methanol/hexane extraction (Redfearn, 1967) followed by TLC separation on a silica gel plate developed with an ether: hexane (3:1) mixture. The band with an R_f value of 0.39 was eluted with ether, concentrated, and assayed.

RESULTS AND DISCUSSION

Construction of Subunit IV-Deficient Mutants. R. sphaeroides lacking subunit IV was constructed by site-specific recombination between the wild-type genomic *fbc*Q and a suicide plasmid containing a defective *fbc*Q sequence. This procedure is adapted from the one used by Donohue *et al.*, (1988) for the construction of cytochrome c_2-deficient mutants of *R. sphaeroides*.

Figure 1 summarizes the strategy used for the generation of defective *fbc*Q genes. The *fbc*Q sequence was destroyed either by the deletion of the *fbc*Q gene accompanied by insertion of a Kn^R gene or by the insertion of a Kn^R gene into the *fbc*Q gene. The *fbc*Q gene is contained in a 1.6-kb *Bam*HI restriction endonuclease fragment within the 4.7-kb *Bam*HI–*Xho*I fragment. A 288-bp *Sac*I and *Sph*I fragment of the

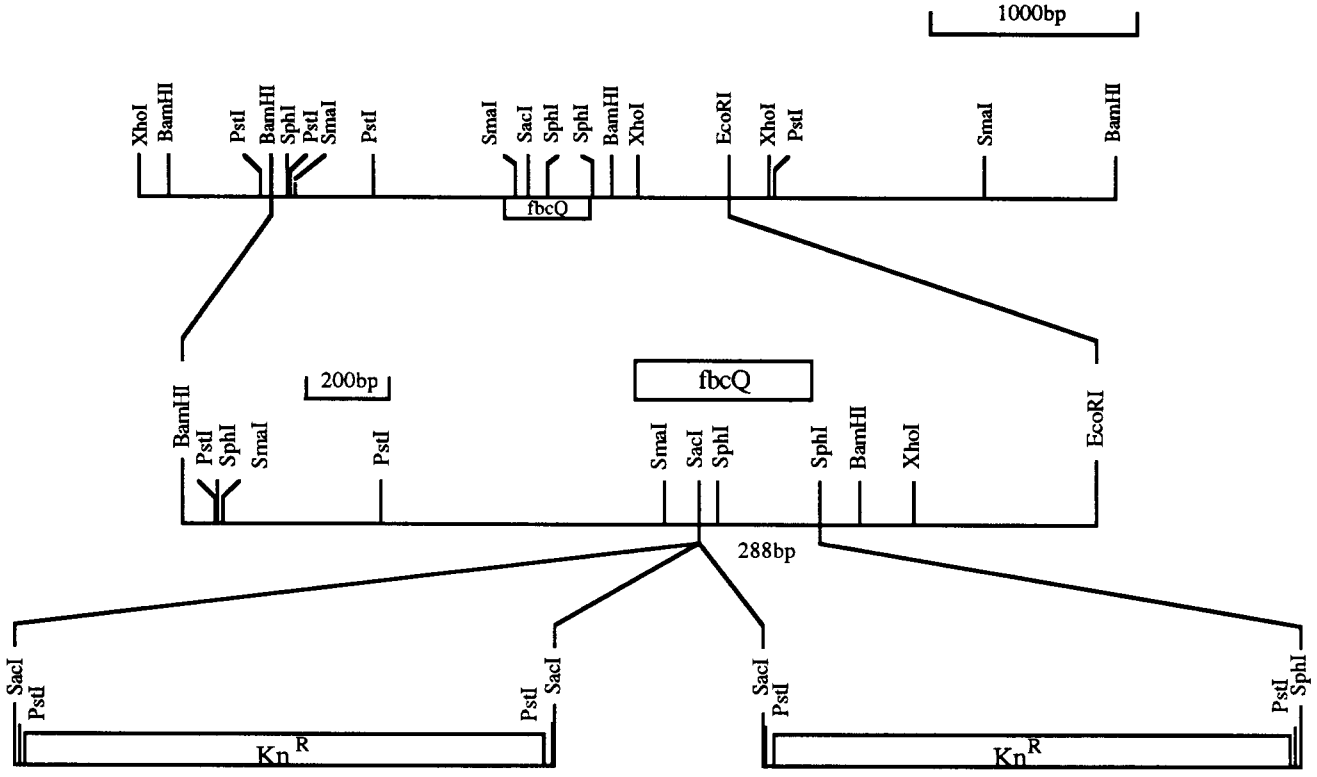

FIGURE 1: Strategy for mutagenesis of *R. sphaeroides fbc*Q gene. Defective *fbc*Q genes were constructed by deletion–insertion mutations (right) and by insertion mutations (left). Detailed procedures are described in the text.

*fbc*Q, encoding 81 amino acid residues from the C-terminal end of subunit IV, was deleted from the pRSQ-B11 plasmid and replaced with a 1.3-kb *Sac*I–*Sph*I fragment from pGEM-Kn1 plasmid containing a Kn^R gene to generate pRS-BS1:Kn1(+) or pRS-BS1:Kn1(–) plasmids. The direction of the Kn^R gene transcription is the same (+) or opposite (–) that of *fbc*Q gene transcription. The pRSQ-B11 plasmid was generated by subcloning the 1.6-kb *Bam*HI fragment into the *Bam*HI site of pGEM-3ZF(+)Δ*Sph*I plasmid. A pGEM-Kn1 plasmid was constructed by ligating the *Pst*I-cleaved Kn^R fragment from pUC4K into the *Pst*I site of the pGEM-3ZF(+)Δ(*Kpn*I–*Sal*I) plasmid. Since the 1.3-kb *Sac*I fragment, upstream from the *fbc*Q gene, was removed from pRSQ-B11 during the construction of the pRS-BS1:Kn1 plasmid, a 1.3-kb *Sac*I fragment from the pRSQ-B1 plasmid was ligated back into the *Sac*I site of pRS-BS1:Kn1(+) or (–) to generate the plasmids pRSQ-Kn1(+) or (–) plasmid.

The 2.7-kb *Bam*HI fragment from pRSQ-Kn1(+) or (–) was ligated into the *Bam*HI site of the pUC8N-BE plasmid to generate pUC8N-BBE Kn(+) or (–). The pUC8N-BE plasmid was constructed by ligating the 0.55-kb *Bam*HI–*Eco*RI fragment, downstream from the *fbc*Q gene, to the *Bam*HI and *Eco*RI sites of the pUC8N plasmid in which the *Sal*I site had been converted to the *Nco*I site using an *Nco*I linker (GCCATGGC).

The 3.2-kb *Nco*I–*Eco*RI fragment from pUC8N-BBE:Kn1-(+) or (–) was cloned into the *Nco*I and *Eco*RI sites of the chloramphenicol resistance gene of a suicide plasmid, pSup202, to generate pSupRSQ-Kn1(+) or (–). This construction leaves approximately 1160 and 690 bp of *R. sphaeroides* DNA upstream and downstream of the Kn^R cartridge, respectively.

A defective *fbc*Q gene was also constructed by inserting a 1.3-kb Kn^R cartridge as a *Sac*I fragment from the pGEM-Kn2 plasmid into the *Sac*I site of the pUC8N-BBE plasmid (indicated at the left middle of Figure 1). The pGEM-Kn2 plasmid was generated by converting the *Sph*I site in pGEM-Kn1 to a *Sac*I site. The pUC8N-BBE plasmid was generated by ligating a 1.6-kb *Bam*HI fragment containing the *fbc*Q gene into the *Bam*HI site of pUC8N-BE. This insertion results in the plasmid pUC8N-BBEKn2(+) or (–), such that the direction of the Kn^R gene transcription is the same (+) or opposite (–) that of the *fbc*Q gene transcription.

A 3.5-kb *Nco*I–*Eco*RI restriction fragment from pUC8N-BBEKn2(+) or (–) was then subcloned into the *Nco*I and *Eco*RI sites of pSup202 to generate the plasmid pSupRSQ-Kn2(+) or (–) with 1160 and 980 bp of *R. sphaeroides* DNA upstream and downstream of the Kn^R cartridge, respectively.

pSupRSQ-Kn1(+) or (–) and pSupRSQ-Kn2(+) or (–) were transformed into *E. coli* S17-1 cells. *R. sphaeroides* subunit IV-deficient mutants were constructed by conjugation with *E. coli* S17-1 [pSupRSQ-Kn] donors and by selection for Kn^R *R. sphaeroides* exconjugants under chemoheterotrophic plating conditions. Since pSup202 and its derivatives cannot replicate in *R. sphaeroides*, the only way to maintain the kanamycin resistance phenotype in the cell is by homologous recombination between genomic *fbc*Q and the inactivated *fbc*Q in the pSup202 plasmid. *R. sphaeroides* Kn^R strains arising from double-crossover events should contain an inactive *fbc*Q in place of the wild-type genomic copy and be sensitive to tetracycline (encoded by pSup202). Strains containing the suicide plasmid incorporated into the genome (single crossover) are both kanamycin (Kn^R) and tetracycline (Tc^R) resistant. The other selectable markers on pSup202 are ineffective for screening *R. sphaeroides* since this bacterium is naturally ampicillin resistant and does not express the pSup chloramphenicol resistance gene *in vivo*. (Donohue *et al.*, 1988).

Nine out of 127, 9 out of 134, 13 out of 138, and 12 out of 113 Kn^R *R. sphaeroides* exconjugants selected from the conjugation experiments using *E. coli* S17-1 bearing pSupRSO-Kn1(+), pSupRSO-Kn1(–), pSupRSO-Kn2(+), or pSupRSO-Kn2(–), respectively, as a donor were sensitive to

tetracycline. This indicates that about 7–11% of Kn^R exconjugants arose from a double-crossover event, a frequency similar to that reported by Donohue *et al.*, (1988) and Yun *et al.*, (1990) in the construction of *R. sphaeroides* cytochrome c_2-deficient and cytochrome b-c_1 complex-deficient mutants, respectively. Four Kn^RT^S exconjugants, one from each of the four conjugation experiments, were isolated, purified, and named RSΔIV-1, RSΔIV-2, RSΔIV-3, and RSΔIV-4, respectively. RSΔIV-2 was used for characterization throughout this investigation.

Southern Analysis of Subunit IV-Deficient Mutants. The exchange of genomic *fbc*Q gene for the defective *fbc*Q gene in Kn^RTc^S exconjugants was confirmed by Southern hybridization using biotinylated DNA probes (data not shown). Chromosomal DNAs isolated from wild-type and RSΔIV-2 were digested with *Bam*HI, *Bam*HI–*Hin*dIII, *Bam*HI–*Xho*I, or *Xho*I and probed with a biotinylated 1.6-kb *Bam*HI fragment carrying the *fbc*Q gene, a 232-bp *Sph*I fragment in the *fbc*Q gene; and a 1.3-kb *Pst*I fragment containing a Kn^R cartridge from pUC4. One *Bam*HI fragment (2.6-kb bp), two *Bam*HI–*Hin*dIII fragments (1890 and 790 bp), two *Bam*HI–*Xho*I fragments (2444 and 238 bp), and two *Xho*I fragments (3054 and 365 bp) in RSΔIV-2 DNA hybridized to both the *Bam*HI and the Kn^R DNA probes. No restriction endonuclease fragments derived from genomic RSΔIV-2 DNA hybridized to the 232-bp *fbc*Q-specific *Sph*I restriction endonuclease fragment probe. On the other hand, one *Bam*HI fragment (1600 bp), one *Bam*HI–*Hin*dIII fragment (1600 bp), one *Bam*HI–*Xho*I fragment (1600 bp), and one *Xho*I fragment (2337 bp) in wild-type DNA hybridized to both the 1.6-kb *Bam*HI and the 232-bp *Sph*I DNA probes. No restriction endonuclease fragments derived from genomic wild-type DNA hybridized to the Kn^R gene probe. These results demonstrate that the 2.6-kb *Bam*HI restriction endonuclease fragment in RSΔIV-2 DNA is homologous to the 1.6-kb *fbc*Q containing *Bam*HI restriction endonuclease fragment contained in wild-type DNA. RSΔIV-2 DNA lacked detectable homology with the 232-bp *fbc*Q-specific *Sph*I restriction endonuclease fragment that was replaced by the Kn^R gene. The interrupted *fbc*Q and Kn^R genes in the 2.6-kb *Bam*HI fragment from RSΔIV-2 genomic DNA are collinear. In addition, the direction of transcription of the Kn^R gene in the RSΔIV-2 genome was opposite that of the *fbc*Q gene.

Western Analysis and Growth Characteristics of Subunit IV-Deficient Strains of R. sphaeroides. When single colonies were picked from the subunit IV-deficient (RSΔIV-2) and wild-type *R. sphaeroides* culture plates, inoculated into 2-mL Sistrom A minimal medium, and shaken vigorously in the dark at 30 °C, both innoculums showed the same growth rate. Since the respiratory chain of *R. sphaeroides* is branched (Crofts & Wang, 1990) and there are several quinol oxidases, we initially attributed this result to respiration through cytochrome b–c_1 complex-independent quinol oxidase in RSΔIV-2 cells and through the cytochrome b–c_1-dependent and/or -independent quinol oxidase (Zannoni *et al.*, 1976) in wild-type cells. This explanation was based on the assumption that the *same* cytochrome b–c_1 complex (four subunit) is functioning in both the cyclic photosynthetic electron transfer chain and the respiratory chain. However, when membrane preparations from these two aerobically grown cell lines were subjected to Western blot analysis using antibodies against subunit IV, no subunit IV protein was detected (Figure 2, lanes 2 and 4). The absence of subunit IV protein in the aerobically grown wild-type cells indicates that subunit IV is not expressed and, thus, is not involved in the aerobic growth of *R. sphaeroides*. Therefore, deletion of the subunit IV gene

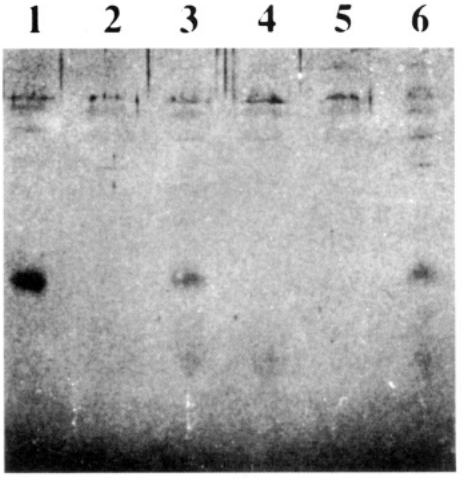

FIGURE 2: Western analysis of subunit IV in the membrane fraction of various preparations. Membranes were prepared from wild-type cells grown under dark aerobic (lane 2) and anaerobic photosynthetic conditions (lane 3); the subunit IV-deficient mutant strain (RSΔIV-2) under aerobic (lane 4) or photosynthetic conditions (lane 5); and RSΔIV-2 complemented with intact *fbc*Q in a 1.6-kb *Bam*HI *R. sphaeroides* DNA fragment on pRK415 plasmid under photosynthetic conditions (lane 6). Membrane proteins (200 μg each) and the purified *R. sphaeroides* cytochrome b–c_1 complex from the photosynthetically grown wild-type strain (2 μg, lane 1) were analyzed by SDS–PAGE using 14% gel. Proteins were blotted onto a nitrocellulose membrane electrophoretically and reacted with anti-subunit IV antibodies. Goat anti-rabbit IgG alkaline phosphatase conjugate was used as the second antibody.

from *R. sphaeroides* should not affect the aerobic growth of the cells regardless of the respiratory chains the cell utilizes. If the cytochrome b–c_1 complex-dependent oxidase system is involved in the aerobic growth of *R. sphaeroides*, then the cytochrome b–c_1 complex in the respiratory chain may differ from that in the photosynthetic cyclic electron transfer chain.

Subunit IV protein was detected in the chromatophore preparation obtained from light anaerobically grown wild-type cells (Figure 2, lane 3). To determine whether the expression of subunit IV protein is induced by deoxygenation or light, Western blots were performed with membranes obtained from cells grown under different conditions. Subunit IV protein was present in intracytoplasmic membrane (ICM) (Kiley & Kaplan, 1988) obtained from wild-type cells grown in the dark with reduced O_2 tension and in membrane preparations obtained from cells grown in the dark under anaerobic conditions using dimethyl sulfoxide as the electron acceptor (data not shown). These results indicate that subunit IV protein in *R. sphaeroides* is expressed when oxygen is depleted from the medium. The amount of subunit IV protein in these membrane preparations correlates well with the amounts of chlorophylls and carotenoids in the preparation, indicating the involvement of subunit IV in the photosynthetic growth of *R. sphaeroides*.

When mid-log phase, aerobically grown wild-type and subunit IV-deficient (RSΔIV-2) cells were inoculated into Sistrom A minimal medium at the same cell density and subjected to anaerobic photosynthetic growth conditions (Figure 3), the wild-type cells began exponential growth after a lag phase of 22 h, whereas RSΔIV-2 cells began growth after a lag of 52 h. To confirm that the delay of photosynthetic growth of the subunit IV-deficient strain is due to disruption of the subunit IV gene, the intact *fbc*Q gene contained in a 1.6-kb *Bam*HI *R. sphaeroides* DNA fragent on a broad host range, low copy plasmid pRK415 was mobilized into the cell by parental conjugation. The resulting strain (complement) grew photosynthetically at a rate similar to that of a wild-type

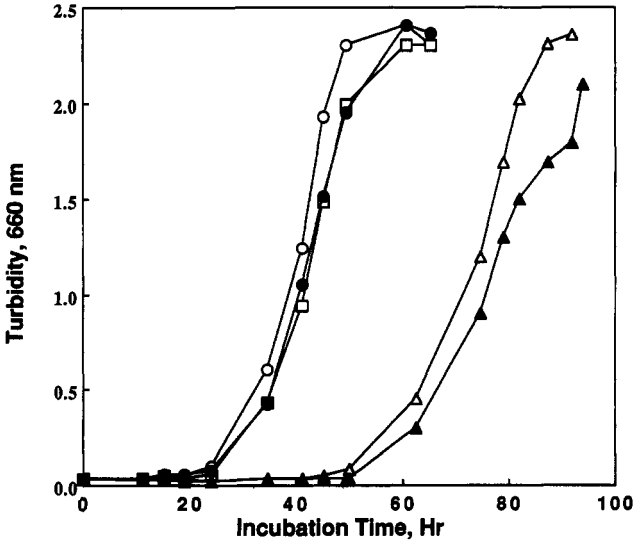

FIGURE 3: Photosynthetic growth in Sistrom's minimal medium A of various *R. sphaeroides* strains. Aliquots of mid-log phase aerobically grown wild-type parent (○), wild-type + pRK415 (●), subunit IV-deficient (RSΔIV-2) (△), and RSΔIV-2 complemented with *fbc*Q on pRK415 plasmid (□) and with pRK415 plasmid only (▲) were inoculated into 8-mL Sistrom A minimal medium containing 25 μg/mL kanamycin (except for wild-type strain and wild-type + pRK415 plasmid) and subjected to photosynthetic growth conditions as described in Experimental Procedures. Three tubes were used for each bacterial strain. Growth was monitored at OD_{660nm}.

strain harboring the pRK415 plasmid. It should be noted that *R. sphaeroides* cells harboring pRK415 plasmid have growth rates slightly lower than those without plasmid. Western blots confirmed the presence of subunit IV protein in chromatophores prepared from the complement strain (Figure 2, lane 6).

It is rather surprising that after a long lag time (when the wild-type cells have grown to stationary phase under the same conditions) the RSΔIV-2 cells start to grow photosynthetically (Figure 3) at a rate comparable to that of the wild-type cells. Western blot analysis revealed that subunit IV protein was not present in the chromatophore preparation obtained from such photosynthetically grown RSΔIV-2 cells (Figure 2, lane 5), suggesting that a subunit IV-deficient cytochrome b–c_1 complex can function in the photosynthetic cyclic electron transfer chain of *R. sphaeroides*. At present, we do not know how the photosynthetically incompetent subunit IV-deficient strain develops into a photosynthetically competent phenotype, but we do know that this event is a consequence of adaptation rather than mutation elsewhere in the genome: petri plates seeded with steady-state chemoheterotrophically grown RSΔIV-2 cells were incubated under photoheterotrophic conditions, and all of the cells acquired photosynthetic growth capability after a long incubation time. Adaptation was further confirmed by regrowing the adapted cells under aerobic conditions and observing a lag in the growth curve when these cells were again grown under photosynthetic conditions. There are several possible explanations for the observed conversion of the subunit IV-deficient strain from photosynthetically incompetent to photosynthetically competent: (i) increased synthesis of one or more of the functionally cryptic *R. sphaeroides* periplasmic redox carriers (Myer *et al.*, 1985); (ii) change of membrane structure to allow electrons to be directly transferred from the reaction center to the three-subunit b–c_1 complex; or (iii) synthesis of a protein which can functionally replace subunit IV in the cytochrome b–c_1 complex (isosubunit IV), analogous to the isocytochrome c_2 recently found in *R. sphaeroides* (Rott et al., 1993) and *R. capsulatus* (Jenney et al., 1993). These possibilities are currently under investigation in our laboratory.

Table 1: Comparison of Chemical Compositions and Cytochrome b–c_1 Complex Activity in Chromatophore Preparations from Photosynthetic Grown Wild-Type and Adapted Subunit IV-Deficient *R. sphaeroides* Strain (RSΔIV-2)[a]

components	wild type	RSΔIV-2
cytochrome b	0.89[b]	0.87[b]
cytochromes (c_1+c_2)	0.38[b]	0.42[b]
ubiquinone	2.5[b]	2.4[b]
chlorophylls	146[c]	131[c]
carotenoids		
yellow	21.8[c]	20.9[c]
red	4.5[c]	2.7[c]
enzymatic activity with 25 μM Q_2H_2	1.24[d]	1.22[d]
K_m for Q_2H_2	2.5[d]	10.8[d]

[a] Data are the average values of three batches of chromatophore preparations. The deviation among the different batches was less than 5%. [b] In nmol/mg of protein. [c] In μg/mg of protein. [d] In μmol of cytochrome c reduced min^{-1} (nmol of cytochrome b)$^{-1}$ at 22 °C.

Chemical Compositions, Spectral Characteristics, and Enzymatic Properties of Cytochrome b–c_1 Complex in Chromatophore Membranes from Wild-Type and Adapted RSΔIV-2 Cells. As described in the previous section, the subunit IV-deficient strain becomes photosynthetically competent after an adaptation period. Therefore, systematic comparison of the essential components and spectral and enzymatic properties of the cytochrome b–c_1 complex in the adapted RSΔIV-2 chromatophore with those in the wild-type chromatophore should yield information about the role of subunit IV. As indicated in Table 1, no significant difference in essential components, such as cytochromes b and c_1, chlorophylls, carotenoids, and ubiquinone, is observed. Spectral characteristics of cytochromes b and c_1 in these two chromatophore preparations are also similar (data not shown). The cytochrome b–c_1 complex activities of these two preparations, using 25 μM of Q_2H_2 as substrate, are comparable: 1.2 μmol of cytochrome c reduced min^{-1} (nmol of cytochrome b)$^{-1}$ at room temperature. However, the Q_2H_2-dependent activity titration curve for the cytochrome b–c_1 complex in the adapted RSΔIV differs from that in the wild-type chromatophore (see Figure 4). The apparent K_m for Q_2H_2 for the cytochrome b–c_1 complex in adapted RSΔIV-2 and wild-type chromatophores, as determined by Lineweaver–Burk plots, are 10.8 and 2.5 μM, respectively, suggesting that in the absence of subunit IV the binding affinity for Q decreases. It should be noted that, although the change in apparent K_m for Q is not great, it was consistently observed in each group of experiments. This is in line with our previous results identifying subunit IV as one of the Q-binding proteins in the photosynthetic b–c_1 complex of *R. sphaeroides*. Furthermore, the difference in enzymatic parameters seems to favor a membrane structural modification, over production of an isosubunit IV, during adaptation in the subunit IV-deficient mutant.

Effect of Detergents on Cytochrome b–c_1 Complex in Chromatophore Preparations from Wild-Type, Complement, and Adapted RSΔIV-2. In order to facilitate the functional study of subunit IV, it is necessary to isolate the cytochrome b–c_1 complex from chromatophore preparations. Dodecylmaltoside, the most effective detergent for solubilizing the cytochrome b–c_1 complex from chromatophores of wild-type *R. sphaeroides*, was used to solubilize the b–c_1 complexes from chromatophores of adapted subunit IV-deficient (RSΔIV-2) and complement strains. When chromatophores from adapted RSΔIV-2 cells, at a protein concentration of 10 mg/

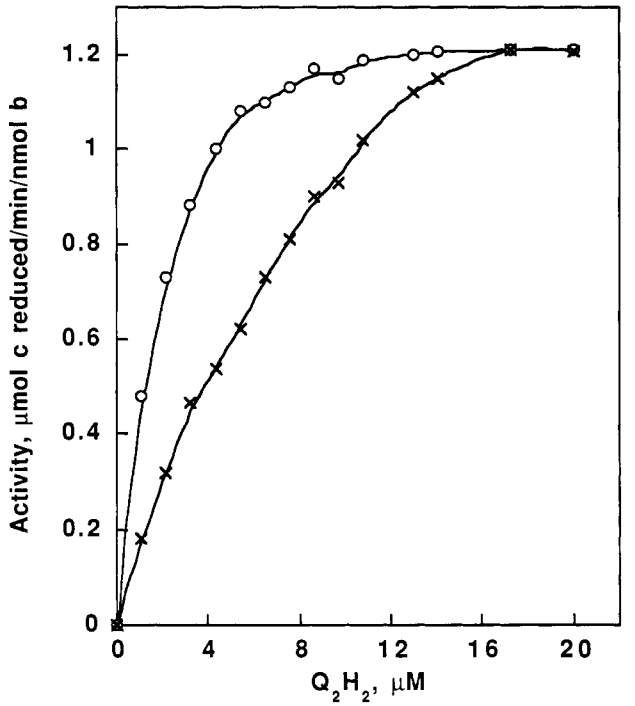

FIGURE 4: Titration of the cytochrome b–c_1 complex activity in chromatophores of wild-type and adapted RSΔIV with various concentrations of Q_2H_2. Aliquots of wild-type chromatophore (O) and adapted RSΔIV chromatophore (X), 0.8 nmol of cytochrome b/mL in 50 mM Tris-HCl, pH 8.0, containing 1 mM $MgSO_4$ and 100 mM NaCl were added to a 1-mL assay mixture containing indicated concentrations of Q_2H_2. Each data point was an average value of duplicate assays of three different batches of chromatophore preparations.

mL, are mixed with various amounts of dodecylmaltoside (up to 0.8 mg/mg of protein), the cytochrome b–c_1 complex activity decreases as the amount of dodecylmaltoside added increases (Figure 5B, open circles). Under the same conditions, no change in b–c_1 complex activity is observed for dodecylmaltoside-treated chromatophores from wild-type and complement cells (Figure5A and C, open circles), indicating that, in the absence of subunit IV the cytochrome b–c_1 complex becomes labile to detergent denaturation.

To determine whether or not the loss of the cytochrome b–c_1 complex activity in the dodecylmaltoside-treated chromatophores of adapted RSΔIV-2 cells results from detachment of the complex from the membrane, the dodecylmaltoside-treated chromatophores from wild-type, adapted RSΔIV-2, and complement cells were centrifuged at 100000g for 90 min to separate the solubilized fraction from the unsolubilized, and the cytochrome content and the b–c_1 complex activity were assayed in both fractions. The effectiveness of dodecylmaltoside in solubilizing cytochrome b–c_1 complex is the same in subunit IV-deficient chromatophores of adapted RSΔIV-2, wild-type, and complement cells. The cytochrome b content in the supernatant fractions increases as the amount of dodecylmaltoside increases (Figure 5, solid triangles). About 95% of the cytochrome b was solubilized from all three preparations when 0.8 mg of dodecylmaltoside was used per milligram of protein. The cytochrome b–c_1 complex activities, based on cytochrome b content, in supernatant (open triangle/solid triangle) and residual membrane (precipitate) (open square/solid square) fractions obtained from wild type (Figure 5A) and complement (Figure 5C) chromatophores, treated with various concentrations of dodecylmaltoside, were the same as for untreated chromatophores. However, the supernatant fractions obtained from chromatophores of adapted RSΔIV-2

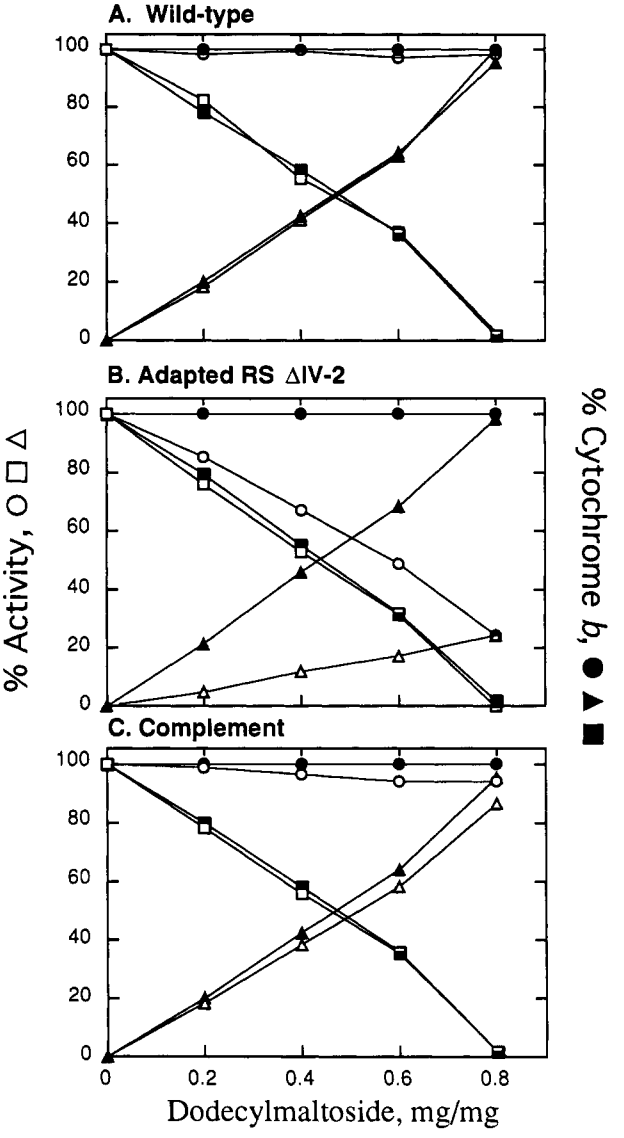

FIGURE 5: Effect of dodecylmaltoside concentration on the solubilization of the cytochrome b–c_1 complex from chromatophore preparations. 1-mL aliquots (10 mg/mL) of chromatophore preparations from wild-type (A), RSΔIV-2 (B), and RSΔIV-2 complemented with *fbc*Q in pRK415 (C) were incubated with indicated amounts of dodecylmaltoside. After incubation at 0 °C for 30 min, the mixtures were centrifuged at 100000g for 30 min. The precipitates were suspended in 3 mL of 50 mM Tris-HCl buffer, pH 8.0, containing 1 mM $MgSO_4$ and 1 mM EDTA. The cytochrome b content (●, ▲, ■) and cytochrome b–c_1 complex activity (O, △, □) in dodecylmaltoside-treated (O, ●), supernatant (△, ▲), and precipitate (□, ■) fractions were determined. The 100% cytochrome b content (nmol/mg and enzymatic activity (μmol of cytochrome c reduced min^{-1} nmol of cytochrome b) used were 8.9 and 1.23 for wild-type, 9.1 and 1.2 for RSΔIV-2, and 9.2 and 1.21 for complement strains.

treated with various amounts of dodecylmaltoside (Figure 5B) all have only 25% of the cytochrome b–c_1 complex activity found in residual (precipitate) fractions or in untreated chromatophores, indicating that in the absence of subunit IV the cytochrome b–c_1 complex becomes labile toward detergent treatment. This could be due to modification of the lipid environment of the complex or loss or change of a specific interaction with some other, yet to be identified, protein components when the reductase complex is detached from the membrane.

The lability toward dodecylmaltoside of the b–c_1 complex lacking subunit IV is also observed with other detergents. When cholate, deoxycholate, lubriol, decanoyl-*N*-methylglu-

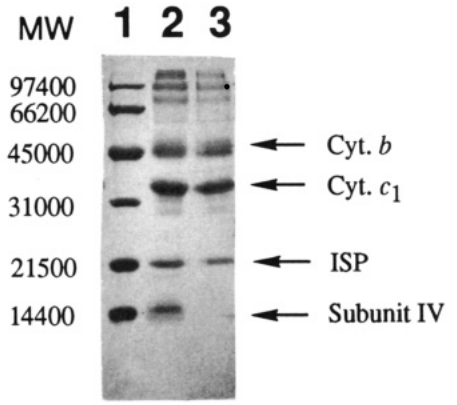

FIGURE 6: SDS–PAGE of purified cytochrome b–c_1 complexes from wild-type and subunit IV-deficient *R. sphaeroides* strains. Lane 1, the molecular weight reference proteins used were phosphorylase b (97 400), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), trypsin inhibitor (21 500) and lysozyme (14 400); lane 2, the purified cytochrome b–c_1 complex (15 μg) from wild-type cells; lane 3, the purified cytochrome b–c_1 complex (13 μg) from adapted RSΔIV-2 cells. Samples were treated with 1% SDS and 1% β-mercaptoethanol and incubated at 37 °C for 2 h prior to SDS–PAGE.

camide–sodium cholate, Tween-80, and Trition X-100 were mixed with chromatophore preparations of wild-type, adapted RSΔIV-2, and complement cells, only Triton X-100 effectively solubilized the b–c_1 complex from the chromatophore preparations. About 70% of the cytochrome b present in chromatophore preparations from wild-type, complement, and adapted RSΔIV-2 cells was solubilized by Triton X-100 at 0.6 mg/mg of protein. Whereas no cytochrome b–c_1 complex activity was detected in the Triton-X100-solubilized chromatophore of adapted RSΔIV-2, the specific activity in Triton X-100-solubilized fractions of wild-type and complement strains is about 90% of that of the untreated chromatophore. The sensitivity of subunit IV-deficient cytochrome b–c_1 complex to detergent treatment will be a useful way of assaying subunit IV mutants from future genetic manipulations.

Comparison of Purified Cytochrome b–c_1 Complexes Obtained from Chromatophores of Wild-Type and Adapted Subunit IV-Deficient Strains. Since the cytochrome b–c_1 complex solubilized from chromatophores of adapated RSΔIV-2 cells by dodecylmaltoside retained partial (25%) activity and can be purified, this complex and the complexes obtained from wild-type or complement cells purified by the same procedure were compared. Purification from dodecylmaltoside-solubilized chromatophores involved DEAE-BioGel A and DEAE-Sepharose 4B column chromatography. The percent recoveries of cytochromes b and c_1 in the final purification step were the same for all complexes. The specific cytochrome b–c_1 complex activities measured with 25 μM Q_2H_2 as substrate at 22 °C were about 1.2 and 0.3 μmol of cytochrome c reduced min^{-1} (nmol of cytochrome b)$^{-1}$ for wild-type and adapted RSΔIV-2 cytochrome b–c_1 complexes, respectively. These specific activities are identical to those activities in their respective dodecylmaltoside-solubilized chromatophore fractions, indicating that the cytochrome b–c_1 complex activity remains unchanged throughout the entire purification procedure after solubilization from the membrane by dodecylmaltoside.

When these purified complexes were subjected to SDS–PAGE (Figure 6), four protein subunits with molecular masses of 43 (cytochrome b), 31 (cytochrome c_1), 23 (iron–sulfur protein) and 15 (subunit IV) kDa were detected in the b–c_1 complex from wild-type cells, and only three proteins (corresponding to cytochrome b, cytochrome c_1, and iron–sulfur protein of wild-type b–c_1 complex) were detected in the complex isolated from adapted RSΔIV-2 cells. Neither subunit IV nor a substituting proetin was detected in the latter, indicating that it is the deletion of subunit IV that forces the b–c_1 complex to undergo certain adaptations to replace the role normally played by subunit IV. However, this does not rule out the possibility of that a new protein (isosubunit IV) may be synthesized to replace subunit IV, since such a protein may be tightly bound to the membrane or to other electron transfer complexes such as the reaction center and, therefore, incapable of being solubilized by dodecylmaltoside. Alternatively, this isosubunit IV protein may be only loosely bound to the cytochrome b–c_1 complex and consequently lost during the purification procedure.

Table 2: Comparison of Chemical Compositions and Enzymatic Activity of Purified Cytochrome b–c_1 Complexes from Chromatophores of Wild-Type and Adapted RSΔIV-2[a]

components	purified cytochrome b–c_1 complex concns	
	wild type	RSΔIV-2
cytochrome b	15.9[b]	16.1[b]
cytochrome c_1	8.8[b]	10.0[b]
phospholipids (phosphorus)	86.4[b]	82.9[b]
ubiquinone-10	10.8[b]	8.5[b]
enzymatic activity with 25 μM Q_2H_2	1.23[c]	0.34[c]
K_m for Q_2H_2	3[c]	13[c]

[a] Data are average values of three batches of cytochrome b-c_1 complex preparations. The deviation among the different batches was less than 5%. [b] In nmol/mg. [c] In μmol of cytochrome c reduced min^{-1} (nmol of cytochrome b)$^{-1}$ at 22 °C.

The contents of cytochromes b and c_1 and phospholipid were about the same in the two complexes (Table 2) from wild-type and adapted RSΔIV-2 cells. The Q content in the three-subunit cytochrome b–c_1 complex is lower (15%) than in the four-subunit complex. Figure 7 shows the Q_2H_2-dependent ubiquinol–cytochrome c reductase activities in purified three-subunit and four-subunit cytochrome b–c_1 complexes. The apparent K_m values for Q_2H_2 for the cytochrome b–c_1 complex activity in four-subunit and three-subunit complexes, as determined by Lineweaver–Burk plots, are 3 and 13 μM, respectively, indicating that the binding affinity of the b–c_1 complex for Q decreases in the absence of subunit IV. This decrease is also reflected by a lower Q content in the three-subunit complex. These results are compatible with the Q-binding role of subunit IV observed in photoaffinity studies. Since the three-subunit b–c_1 complex is still partially functional, the role of this subunit in Q-binding may be only auxiliary.

Since the spectral characteristics of cytochromes b and c_1 in the four-subunit complex are the same as those in the three-subunit complex, subunit IV has no effect on the structural integrity of the heme environments of cytochromes b and c_1. The ratio of cytochrome b to cytochrome c_1 in the four-subunit complex was slightly higher than that in the three-subunit complex. The presence of subunit IV may prevent the loss of cytochrome b heme during purification since cytochrome b protein is known to associate with subunit IV (Purvis *et al.*, 1990; Yu & Yu, 1991). The effect of subunit IV on the structural integrity of the iron–sulfur cluster is currently under investigation.

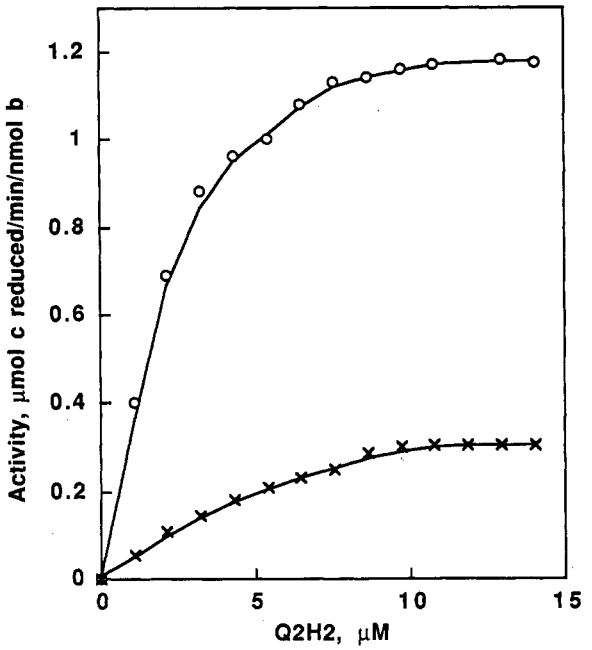

FIGURE 7: Titration of the cytochrome b–c_1 complex activity in four-subunit and three-subunit cytochrome b–c_1 complex preparations with various concentrations of Q_2H_2. Aliquots of purified wild-type cytochrome b–c_1 complex (O), 0.8 nmol cytochrome b/mL, and of adapted RSΔIV cytochrome b–c_1 complex (X), 4 nmol cytochrome b/mL, in 50 mM Tris-HCl, pH 8.0, containing 300 mM NaCl and 0.01% dodecylmaltoside were added to a 1-mL assay mixture containing indicated concentrations of Q_2H_2. Each data point was an average value of duplicate assays of three batches of cytochrome b–c_1 complex preparations.

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