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Essentiality of the Molecular Weight 15 000 Protein (Subunit IV) in the Cytochrome b–c_1 Complex of *Rhodobacter sphaeroides*†

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ABSTRACT: The cytochrome b–c_1 complex from *Rhodobacter sphaeroides* was resolved into four protein subunits by a phenyl-Sepharose CL-4B column eluted with different detergents. Individual subunits were purified to homogeneity. Antibodies against subunit IV (M_r = 15 000) were raised and purified. These antibodies had a high titer with isolated subunit IV and with the b–c_1 complex from *R. sphaeroides*. They inhibited 95% of the ubiquinol–cytochrome c reductase activity of the cytochrome b–c_1 complex, indicating that subunit IV is essential for the catalytic function of this complex. When detergent-solubilized chromatopores were passed through an anti-subunit IV coupled Affi-Gel 10 column, no ubiquinol–cytochrome c reductase activity was detected in the effluent, and four proteins, corresponding to the four subunits in the isolated complex, were adsorbed to the column. This indicated that subunit IV in an integral part of the cytochrome b–c_1 complex. No change in the apparent K_ms for Q_2H_2 and for cytochrome c was observed with anti-subunit IV treated complex. Antibodies against subunit IV had little effect on the stability of the ubisemiquinone radical in this complex, suggesting that they do not bind to the subunit near its ubiquinone-binding site.

The cytochrome b–c_1 complex of the photosynthetic bacterium *Rhodobacter sphaeroides*, which catalyzes electron transfer from ubiquinol to cytochrome c_2 has been purified and characterized in several laboratories (Gabellini et al., 1982; Takamiya et al., 1982; Yu et al., 1984; Ljungdahl et al., 1986; Andrews et al., 1990; McCurley et al., 1990; Purvis et al., 1990). In spite of the fact that different bacterial strains and different isolation procedures were used, all the cytochrome b–c_1 complex preparations from *R. sphaeroides* contain four protein subunits with apparent molecular weights of 43 000, 30 000, 21 000, and 15 000. The three larger molecular weight proteins are identified as cytochromes b and c_1 and iron–sulfur protein, respectively. The M_r = 15K subunit has recently been identified as one of the ubiquinone (Q)[1]-binding sites in the

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[1] Abbreviations: DTE, dithioerythritol; PBS, phosphate-buffered saline; Q, ubiquinone; Q_2H_2, 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinol; SDS, sodium dodecyl sulfate; TMD, 50 mM Tris-HCl, pH 8.0, containing 1 mM $MgSO_4$ and 0.01% dodecyl maltoside.

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complex by photoaffinity labeling techniques using an azido-Q derivative (Yu & Yu, 1987). Another Q-binding site is located in cytochrome b. The amino acid sequences for cytochromes b and c_1 and iron–sulfur protein have been obtained through nucleotide sequencing (Andrews et al., 1990), and the partial amino acid sequence for subunit IV has also been reported (Purvis et al., 1990).

Recently, an active three-subunit cytochrome b–c_1 complex was isolated from *Rhodospirillum rubrum* (Kriauciunas et al., 1989). The three subunits correspond to the three larger molecular weight proteins in the cytochrome b–c_1 complex of *R. sphaeroides*. The absence of a fourth subunit in *Rs. rubrum*'s complex questions the intrinsic nature of subunit IV in *R. sphaeroides*. Clarification of the functional role of subunit IV is needed if we are to understand the transfer mechanisms in this region of the electron-transfer chain.

There are two ways to investigate the function of a protein subunit in an enzyme complex: resolution and reconstitution, or immunoinhibition. Both approaches require the purified protein subunit. We have recently developed a simple method to obtain pure subunit IV in large quantities. However, the unavailability of a subunit IV deficient (or depleted) cytochrome b–c_1 complex at the present time has prevented us from using the reconstitution approach. On the other hand, antibodies against subunit IV has been raised in rabbits, enabling us to take an immunological approach. Herein we report experiments which establish the essentiality of subunit IV in the cytochrome b–c_1 complex. The effects of antibodies against subunit IV on its kinetic parameters and on the Q radical in the cytochrome b–c_1 complex are also described.

Materials and Methods

Materials

Affi-Gel 10 and DEAE-Bio-Gel A were obtained from Bio-Rad. Cytochrome c, DEAE-Sepharose CL-6B, and phenyl-Sepharose CL-4B were from Sigma. Calcium phosphate was prepared according to Jenner (1973).

Methods

SDS–PAGE was done according to Laemmli (1970), and 14% acrylamide was used in the separating gel. Published methodology was used to measure protein (Lowry et al., 1951) and cytochromes (McCurley et al., 1990). Ubiquinol–cytochrome c reductase activity of the cytochrome b–c_1 complex was determined as previously reported (McCurley et al., 1990). An appropriate amount of enzyme, dissolved in 50 mM Tris-HCl buffer, pH 8.0, containing 0.01% dodecyl maltoside and 100 mM NaCl, was added to a 1-mL reaction mixture containing 50 mM sodium/potassium phosphate buffer, pH 7.0, 50 μM cytochrome c, 1 mM EDTA, and 25 μM Q_2H_2. The reduction of cytochrome c was monitored at 550 nm in a Cary spectrophotometer, Model 219, at 23 °C. The nonenzymatic reduction of cytochrome c by Q_2H_2, determined under the same conditions in the absence of enzyme, was used to correct the activity. The EPR measurements were made with a Bruker ER-200D spectrometer at 77 K (McCurley et al., 1990).

Isolation of Cytochrome b-c_1 Complex from Rhodobacter sphaeroides Wild Type. The cytochrome b–c_1 complex was isolated from chromatophores of *R. sphaeroides* wild type essentially according to as reported (McCurley et al., 1990); 144 g of frozen cell paste was thawed and mixed with 432 mL of 20 mM Tris–succinate, pH 8.0, containing 1 mM EDTA. Cells were broken with a French pressure cell press, at a pressure of 1000 psi and a flow rate of 20 mL/min. DNase (2.88 mg) and phenylmethanesulfonyl fluoride (PMSF) (360 mg in 14 mL of ethanol) were added to the broken cell suspension before it was centrifuged at 17000g for 15 min. The precipitates were discarded, and the supernatant was centrifuged at 200000g for 90 min. The precipitates thus obtained were suspended with 50 mM Tris-HCl, pH 8.0, containing 1 mM $MgSO_4$ and 1 mM PMSF (TMP buffer) to a final volume of 400 mL and centrifuged at 200000g for 90 min. The precipitate (chromatophore) obtained was homogenized with TMP buffer containing 10% glycerol and adjusted to a protein concentration of 10 mg/mL with the same buffer. The chromatophores thus obtained either were directly used for the isolation of cytochrome b–c_1 complex or were frozen at −80 °C for future use. The prepared chromatophore is stable at −80 °C for several months.

The cytochrome b–c_1 complex was solubilized from chromatophore by Triton X-100 and purified by DEAE-Bio-Gel A and DEAE-Sepharose 6B column chromatography in the presence of 0.01% dodecyl maltoside. Triton X-100 (70 μL of 15% Triton X-100/mL of suspension) was added to the chromatophore suspension, and the mixture was stirred for 30 min at 0 °C before being centrifuged at 27000g for 30 min. The hard precipitates at the bottom of the centrifuge tubes were discarded, and the loose pellets and supernatant were collected and mixed with NaCl (25 μL of 4 M NaCl/mL of solution). After being stirred for 1 h at 0 °C, the mixture was centrifuged at 200000g for 90 min. The supernatant obtained was applied to a DEAE-Bio-Gel A column equilibrated with, in sequence, 100 mL of 4 M NaCl, 100 mL of 1 M Tris-HCl, pH 8.0, and 700 mL of 50 mM Tris-HCl, pH 8.0, containing 1 mM $MgSO_4$, 0.01% dodecyl maltoside (TMD buffer), and 100 mM NaCl. After application, the column was washed with, in sequence, 1000 mL of TMD buffer containing 100 mM NaCl, 200 mL of TMD buffer containing 150 mM NaCl, and 500 mL of TMD buffer containing 200 mM NaCl. Crude cytochrome b–c_1 complex was then eluted from the column with TMD buffer containing 300 mM NaCl. The collected b–c_1 complex was diluted with an equal volume of TMD buffer containing 100 mM NaCl and then applied to a DEAE-Sepharose 6B column equilibrated with the same buffer. The column was washed with 100 mL each of TMD buffer containing 200 mM NaCl and 300 mM NaCl. Pure cytochrome b–c_1 complex was then eluted from the column with TMD buffer containing 400 mM NaCl and concentrated by an Amicon membrane concentrator to about 10 mg/mL. This concentrated solution was mixed with glycerol to a final concentration of 10% and stored at −80 °C until use. About 30 mg of purified cytochrome b–c_1 complex was routinely obtained from 144 g of frozen cell paste.

Resolution of the Cytochrome b–c_1 Complex and Purification of Subunits. All the procedures were carried out at between 0 and 4 °C, unless otherwise stated. Twelve milligrams of purified cytochrome b–c_1 complex in 50 mM Tris-HCl, pH 8.0, containing 400 mM NaCl, 1 mM EDTA, 1 mM $MgSO_4$, 0.01% dodecyl maltoside, and 10% glycerol was concentrated by 80% ammonium sulfate precipitation. The precipitate was collected by centrifugation at 28000g for 10 min and redissolved in 4 mL of 25 mM Tris-HCl, pH 8.0, containing 1 mM dithioerythritol (DTE), 1 mM EDTA (buffer A), and 0.25% sodium cholate. The mixture was then incubated at 0 °C for 1 h before being applied to a phenyl-Sepharose CL-4B column (1.6 × 4.5 cm), equilibrated with buffer A containing 0.25% sodium cholate. Brownish pink colored protein (iron–sulfur protein) was recovered in the effluent while cytochromes b and c_1 and subunit IV were

absorbed to the column. When this column was washed with 30 mL of buffer A containing 2% sodium cholate, cytochrome c_1 was eluted as a pink-colored protein. Subsequently, the column was washed with 5 mL of buffer A containing 1.5 M guanidine hydrochloride and 2% sodium cholate followed with 30 mL of buffer A containing 2% sodium cholate. A light greenish colored pigment fraction was obtained. Then the remaining cytochrome *b* and subunit IV on the column were eluted at room temperature with 120 mL of 50 mM Tris-HCl, pH 8.0, containing 2 M urea and 0.1% sodium dodecyl sulfate (mainly subunit IV), and with 20 mL of 50 mM Tris-HCl, pH 8.0, containing 0.2% sodium dodecyl sulfate and 2 M urea (mainly cytochrome *b*).

The iron–sulfur protein fraction from the phenyl-Sepharose CL-4B column was concentrated by ultrafiltration using an Ultrafilter PM-10 and chromatography on a Sephacryl S-200 superfine column equilibrated with 0.1 M Tris-HCl, pH 7.5, containing 0.5% sodium cholate, 0.1 M NaCl, 1 mM DTE, and 20% glycerol. Purified iron–sulfur protein was eluted in a peak at the exclusion volume of the column.

The cytochrome c_1 fraction from the phenyl-Sepharose CL-4B column was applied to a calcium phosphate/cellulose (3:1) column, equilibrated with 50 mM sodium/potassium phosphate buffer, pH 7.4, containing 0.5% sodium cholate. After the column was extensively washed with the same buffer, cytochrome c_1 was eluted with a linear gradient formed from 50 and 300 mM potassium phosphate buffer, pH 8.0, containing 0.5% sodium cholate. The cytochrome c_1 fractions were combined and concentrated by ultrafiltration.

Cytochrome *b* and the M_r 15000 fractions from the phenyl-Sepharose CL-4B column were concentrated by Aquacide II and applied to a Sephacryl S-200 HR column (1.2 × 37 cm) equilibrated with 50 mM Tris-HCl, pH 8.0, containing 0.1% SDS and 2 M urea. The fractions containing pure subunit IV and cytochrome *b* were combined and concentrated by membrane filtration using Centricon-10 and Centricon-30, respectively.

Production and Purification of Antibody against Subunit IV. Purified subunit IV (1 mg) was precipitated by 50% acetone at 0 °C, resuspended in 0.5 mL of 50 mM sodium phosphate, pH 7.4, containing 0.15 M NaCl (PBS), emulsified with an equal volume of Freund's complete adjuvant, and injected into a rabbit subcutaneously. Boosters, 0.5 mg of subunit IV, were given weekly for 1 month, and blood samples were collected by cardiac puncture.

Purification of antibodies was carried out at 0–4 °C. Sera were brought to 50% ammonium sulfate saturation with a neutralized saturated ammonium sulfate solution and centrifuged at 28000*g* for 30 min. The precipitate was dissolved in 20 mM Tris-HCl buffer, pH 8.0, containing 50 mM NaCl and dialyzed against the same buffer. The dialyzed solution was applied to a DEAE-Affi-Gel blue column (5 times the serum volume) equilibrated with 20 mM Tris-HCl buffer, pH 8.0, containing 50 mM NaCl. The effluent containing IgG was collected and concentrated by ultrafiltration with an Amicon PM-30 membrane. IgG was further purified on a Sephacryl S-300 SF column equilibrated with PBS. The purity of IgG was confirmed by SDS–PAGE. Control antibodies were prepared in the same way using serum taken from the same rabbit prior to immunization.

Coupling of Antibodies against Subunit IV to Affi-Gel 10 Support. Affi-Gel 10 support is a *N*-hydroxysuccinimide ester of a derivatized agarose gel bead. One millilitr of Affi-Gel 10 slurry was transferred to a small glass filter funnel, the supernatant solvent was drained, and the gel was washed with

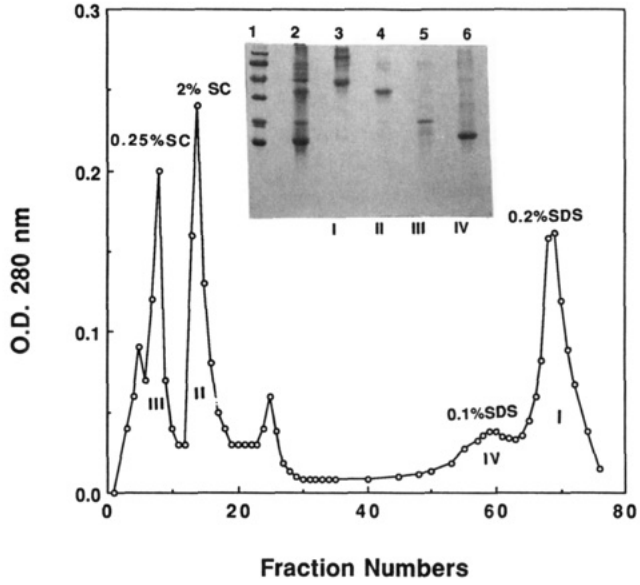

FIGURE 1: Resolution of the cytochrome b–c_1 complex by a phenyl-Sepharose CL-4B column with different detergents. The conditions were as described under Materials and Methods. Fractions eluted with different detergents were analyzed by SDS–PAGE. Lane 1, molecular weight standards (phosphorylase *b*, 97400; bovine serum albumin, 66200; ovalbumin, 42700; carbonic anhydrase, 31000; soybean trypsin inhibitor, 21500; and lysozyme, 14400); lane 2, cytochrome b–c_1 complex; lanes 3–6 are fractions eluted with 0.2% SDS (subunit I), 2% sodium cholate (subunit II), 0.25% sodium cholate (subunit III), and 0.1% SDS (subunit IV), respectively.

20 mL of cold distilled water. Antibodies against subunit IV, 8 mg in 3 mL of 0.1 M sodium bicarbonate buffer, pH 8.5, were added to the gel and gently agitated at 4 °C overnight. Excess ligand was washed off with 0.1 M sodium bicarbonate buffer, pH 8.5, and any remaining active esters were blocked with Tris-HCl buffer, pH 8.0, for 1 h at room temperature. The gel was then transferred to a column and washed with three cycles of alternating pH. Each cycle consisted of a wash with 0.1 M Tris-HCl buffer, pH 8.0, containing 0.5 NaCl, followed by a wash with 0.1 M acetate buffer, pH 4.0, containing 0.5 M NaCl. The gel was stored in 0.02% sodium azide in a cold room until use.

Results and Discussion

Isolation and Purity of Subunits of the Cytochrome b–c_1 Complex of R. sphaeroides. Purified cytochrome b–c_1 complex of *R. sphaeroides* contains four subunits. Resolution of these subunits was achieved by phenyl-Sepharose CL-4B column chromatography. Figure 1 shows the elution profile. The insert shows the SDS–PAGE patterns of the four fractions of the column resolved by the phenyl-Sepharose CL-4B column. Iron–sulfur protein (subunit III) was eluted with 0.25% sodium cholate and cytochrome c_1 (subunit II) by 2% sodium cholate. Subunit IV was eluted with 0.1% sodium dodecyl sulfate and cytochrome *b* (subunit I) by 0.2% sodium dodecyl sulfate. These results suggest that the list of these four subunits, in order of decreasing hydrophobicity, is cytochrome *b*, subunit IV (M_r = 15000 protein), cytochrome c_1, and the iron–sulfur protein. A close relationship between subunit IV and cytochrome *b* in the complex is confirmed by the fact that they are both present in the same fraction when the complex is treated with high concentrations of detergents such as sodium cholate or Triton-X-100, to split cytochrome *b* from cytochrome c_1. The finding that the iron–sulfur protein is the least hydrophobic of these subunits is consistent with the reported observation that the iron–sulfur protein is removed from

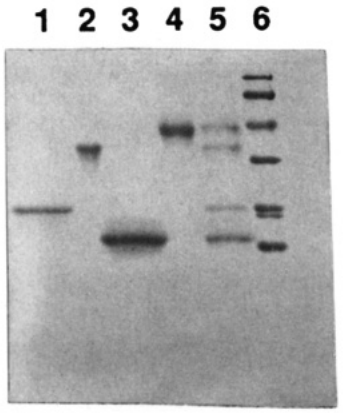

FIGURE 2: SDS–PAGE of purified subunits of the cytochrome b–c_1 complex. Lane 1, iron–sulfur protein (subunit III); lane 2, cytochrome c_1 (subunit II); lane 3, M_r 15 000 protein (subunit IV); lane 4, cytochrome b (subunit I); lane 5, cytochrome b–c_1 complex; lane 6, molecular weight standards.

the rest of the subunits during gel permeation chromatography of cytochrome b–c_1 complex in the presence of 200 mM NaCl (Purvis et al., 1990).

The individual subunits obtained from the phenyl-Sepharose CL-4B column were purified to homogeneity by Sephacryl-S200 column chromatography using 0.5% sodium cholate (for the iron–sulfur protein) and 0.1% SDS and 2 M urea (for subunits I and IV), or by calcium phosphate/cellulose column chromatography (for cytochrome c_1). The purity of these subunits is evidenced by a single protein band on SDS–PAGE analysis (Figure 2). About 6.1, 7.7, 6.1, and 2.0 mg of pure subunits I, II, III, and IV, respectively, was recovered in purified form from 100 mg of purified complex. These correspond to yields of 15.9, 27.9, 26.5, and 14.9%, respectively, based on molecular weights of 43 000, 30 000, 21 000, and 15 000.

Purified cytochrome c_1 and iron–sulfur protein have spectral properties (absorption and EPR) identical with those of the respective proteins present in the complex. The electron-transfer activity of cytochrome c_1 (to horse cytochrome c or bacterial cytochrome c_2) is the same as that of cytochrome c_1 prepared from a strain of R-26 (Yu et al., 1986). These results indicate that the purified cytochrome c_1 and iron–sulfur protein are functionally and structurally intact. Although purified cytochrome b (subunit I) had absorption spectra similar to those of cytochrome b in the cytochrome b–c_1 complex, it contained a less than stoichiometric amount of heme to protein and showed no spectral multiplicity, indicating that it may not be functionally active. Purified subunit IV is a colorless protein, since associated ubiquinone and phospholipid are removed during the extensive detergent washing steps. The described purification procedure simultaneously provides sufficient quantities of all four subunits for chemical, structural, and immunological studies.

While the participation of cytochrome b, cytochrome c_1, and iron–sulfur protein in the redox cycle is very apparent, the involvement of subunit IV in the catalytic cycle of this complex has not yet been established. Although all available cytochrome b–c_1 complex preparations (Gabellini et al., 1982; Takamiya et al., 1982; Yu et al., 1984; Ljungdahl et al., 1986; Andrews et al., 1990; McCurley et al., 1990; Purvis et al., 1990) from. *R. sphaeroides*, prepared by different methods, contain this subunit, its absence from the functionally active, cytochrome b–c_1 complex of *Rs. rubrum* raises questions about the intrinsic nature of this subunit in the cytochrome b–c_1 complexes of photosynthetic bacteria. With purified subunit IV in hand, we could determine whether or not this protein

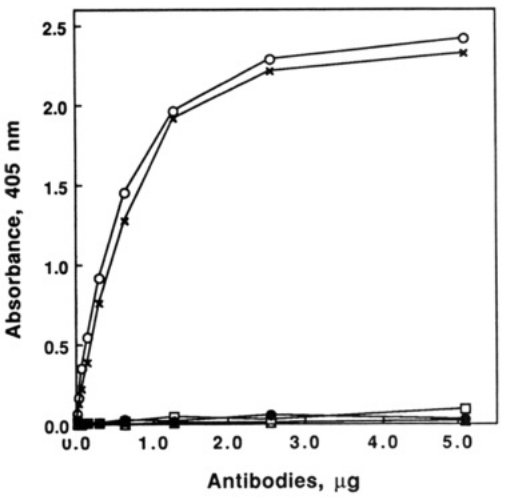

FIGURE 3: Immunological specificity of antibodies against subunit IV, analyzed by ELISA. The antigens, 3 μg of subunit IV (○), 10 μg of bacterial cytochrome b–c_1 complex (×), 10 μg of mitochondrial cytochrome b–c_1 complex (△), 10 μg of chloroplast cytochrome b_6–f complex (□), or 2 μg of mitochondrial QPc–9.5 kDa (●), were coated on the ELISA plate and then reacted with various amounts of purified antisubunit IV. Meanwhile, the preimmune IgG (▲) was reacted with 2 μg of subunit IV. A goat anti-rabbit IgG–alkaline phosphatase conjugate was then added. The binding was quantified by measuring the color intensity of p-nitrophenol, at 405 nm, resulting from hydrolysis of p-nitrophenyl phosphate.

is an integral part of the complex. Restoration of the functional activity of reconstitutively active, subunit IV depleted cytochrome b–c_1 complex by addition of subunit IV would prove the essentiality of the M_r = 15 000 protein. Unfortunately, the lack of reconstitutively active, subunit IV depleted cytochrome b–c_1 complex has prevented us from using this approach. An alternative approach uses immunoinhibition.

Immunological Specificity of Antibodies against Subunit IV. Antibodies against subunit IV were raised in rabbits and purified by ammonium sulfate fractionation, DEAE-Affi-Gel blue, and molecular sieving column chromatographies. Figure 3 shows the ELISA of the antigen–antibody interaction. Fixed concentrations of antigens, beef heart mitochondrial ubiquinol–cytochrome c reductase, mitochondrial QPc–9.5 kDa (Usui et al., 1990), chloroplast cytochrome b_6–f complex, *R. sphaeroides* cytochrome b–c_1 complex, and subunit IV were titrated with varying amounts of antibodies specific for subunit IV and preimmune IgG preparations. Antibodies against subunit IV reacted only with *R. sphaeroides* cytochrome b–c_1 complex and isolated subunit IV. No binding was observed with mitochondrial ubiquinol–cytochrome c reductase, cytochrome b_6–f complex, or mitochondrial QPc–9.5 kDa.

The immunological specificity of subunit IV antibodies was further characterized by a Western blot using beef heart mitochondrial ubiquinol–cytochrome c reductase, spinach chloroplast cytochrome b_6–f complex, *R. sphaeroides* cytochrome b–c_1 complex, and subunit IV as antigens (Figure 4). Antibodies against subunit IV bound specifically to isolated subunit IV and to *R. sphaeroides* cytochrome b–c_1 complex, confirming the results observed with ELISA. These results indicate that subunit IV of the *R. sphaeroides* cytochrome b–c_1 complex is immunologically different from the small molecular weight subunits in the cytochrome b–c_1 complexes of beef heart mitochondria and spinach chloroplasts.

Immunoinhibition of the Cytochrome b–c_1 Complex by Antibodies against Subunit IV. Figure 5 shows the effect of subunit IV antibodies on the activity of the cytochrome b–c_1 complex. When the complex, 0.3 mg/mL, in 50 mM Tris-HCl buffer, containing 0.01% dodecyl maltoside and 100 mM

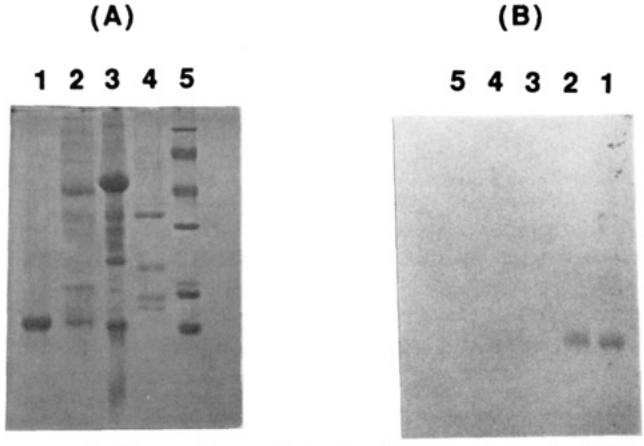

FIGURE 4: Western blot analysis of antibodies against subunit IV. (A) SDS–PAGE of subunit IV (lane 1), *R. sphaeroides* cytochrome b–c_1 complex (lane 2), mitochondrial cytochrome b–c_1 complex (lane 3), spinach cytochrome b_6–f complex (lane 4), and molecular weight standards (lane 5). SDS–PAGE was carried out according to the method of Laemmli using a 14% gel. (B) Protein bands on the gel of (A) were electrophoretically transferred to a nitrocellulose membrane without staining, and the membrane was then reacted with antisubunit IV. After the unreacted antibodies were washed off, the membrane was treated with protein A–horseradish peroxidase conjugate. Detection of peroxidase activity was with a horseradish peroxidase color development kit (Bio-Rad).

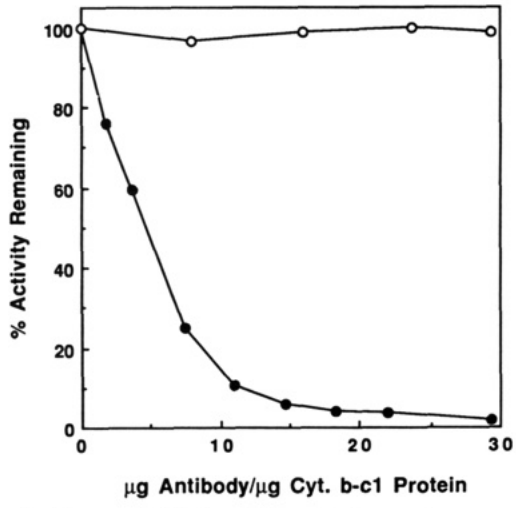

FIGURE 5: Immunoinhibition of the cytochrome b–c_1 complex by antibodies against subunit IV. 30-μL aliquots of the cytochrome b–c_1 complex, 0.45 mg/mL, in 50 mM Tris-Cl buffer, pH 8.0, containing 0.01% dodecyl maltoside, 1 mM EDTA, and 100 mM NaCl were added to 20-μL aliquots of PBS buffer containing the indicated concentration of antibodies against subunit IV (●) and preimmune IgG (○). The ubiquinol–cytochrome c reductase activity was assayed after samples were incubated overnight at 4 °C.

NaCl, was incubated overnight at 4 °C with increasing amounts of antibody, ubiquinol–cytochrome c reductase activity decreased as the amount of antibody increased. A maximal inhibition of 95% was reached when 20 μg of antibody was used per microgram of cytochrome b–c_1 protein. Under identical conditions, no decrease in activity was observed with untreated complex or with complex treated with preimmune IgG. These results strongly suggest that subunit IV is essential for the catalytic function of this complex.

Isolation of the Cytochrome b–c_1 Complex with Antibodies against Subunit IV. To further confirm that subunit IV is an intrinsic component of the cytochrome b–c_1 complex, an antisubunit IV affinity column was prepared and used to isolate proteins from chromatophores. This affinity column was prepared by coupling the amino ligands of antibodies to an

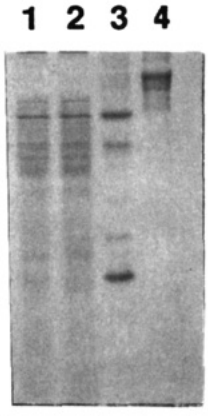

FIGURE 6: SDS–PAGE of proteins eluted from antibody affinity columns. Five milliliters chromatophore, 10 mg/mL, in TMP buffer was solubilized with dodecyl maltoside (0.7 mg/mg of protein) and 100 mM NaCl; 0.7-mL aliquots of solubilized solution were applied to columns (0.7 mL of column bed volume) equilibrated with 50 mM Tris-HCl, pH 8.0, containing 1 mM $MgSO_4$ and 0.01% dodecyl maltoside. Columns were packed with Affi-Gel 10 coupled to antibodies against subunit IV (lanes 2 and 3) or preimmune IgG (lanes 1 and 4). After the columns were washed with 5 mL of the equilibrating buffer (lanes 1 and 2), proteins absorbed to the column were eluted with 0.1 M Tris–glycine buffer, pH 2.5, containing 0.01% dodecyl maltoside (lanes 3 and 4). The eluted samples were dialyzed against water before being subjected to SDS–PAGE.

Affi-Gel 10–agarose support. When detergent-solubilized chromatophore was passed through this antibody affinity column, equilibrated with 50 mM Tris-HCl buffer, pH 8.0, containing 0.01% dodecyl maltoside and 100 mM NaCl, ubiquinol–cytochrome c reductase activity was completely removed. Under identical conditions, when solubilized chromatophore was passed through a preimmune IgG affinity column, less than 10% of the activity was lost. When the proteins absorbed to the subunit IV affinity column were eluted with 0.1 M Tris–glycine, pH 2.5, containing 0.01% dodecyl maltoside and subjected to SDS–PAGE, cytochrome b, cytochrome c_1, iron–sulfur protein, and subunit IV were detected (see Figure 6). None of these proteins were found in the eluates of the preimmune IgG affinity column. These results clearly indicate that subunit IV not only is essential for the catalytic activity of the cytochrome b–c_1 complex of *R. sphaeroides* but also is an integral part of this complex.

Although the essentiality of subunit IV in the *R. sphaeroides* cytochrome b–c_1 complex, and of a small subunit (M_r 9500) in the mitochondrial cytochrome b–c_1 complex, is certain, the need of a small protein in other bacterial cytochrome b–c_1 complexes is uncertain. Cytochrome b–c_1 complexes from *Rs. rubrum* (Kriauciunas et al., 1989) and from *Paracoccus denitrificans* have only three subunits yet show good ubiquinol oxidation activity and generate a proton gradient (Yang & Trunpower, 1986). The three-subunit cytochrome b–c_1 complexes seem to be present in organisms which have high ubiquinone content in their membranes (Carr & Exell, 1965). Perhaps the quinone reduction site (Q_i) of cytochrome b–c_1 complexes present in membranes having a high quinone content differs from that in complexes of low quinone content membranes. A careful comparison of the K_ms for quinone in these different complexes would be informative. These will be determined when all the complexes become available to us.

Effect of Antibodies against Subunit IV on Kinetic Parameters in the Cytochrome b–c_1 Complex. The nature of the immunoinhibition of ubiquinol–cytochrome c reductase activity by antibodies against subunit IV was studied by examining some kinetic parameters of the treated complex. After treatment with antibody at a level which gives 50% inhibition,

the apparent K_ms for Q_2H_2 and for cytochrome c, calculated from Lineweaver–Burk plots, were the same as those for the untreated complex, with or without incubation with preimmune IgG. A 50% decrease in V_{max} was observed with the antibody-treated sample. These results differ from those observed with the mitochondrial cytochrome b–c_1 complex treated with antibody against the small molecular weight Q-binding protein (QPc–9.5 kDa). In this case, antibody binding increases the K_m for Q_2H_2 without affecting V_{max} (Usui et al., 1990). The observation that antibody binding does not change the K_m for Q_2H_2 of the *R. sphaeroides* cytochrome b–c_1 complex suggests that antibody binding has little effect on the substrate-binding site.

Effect of Antibodies against Subunit IV on Ubisemiquinone Radical in the Cytochrome b–c_1 Complex. Since subunit IV has been identified as one of the Q-binding proteins in this complex, it is of interest to see whether or not binding of antibody affects the formation of ubisemiquinone radical. When cytochrome b–c_1 complex was treated with a catalytic amount of beef heart mitochondrial succinate–cytochrome c reductase, in the presence of a fumarate/succinate mixture (10:1), cytochrome c_1 and iron–sulfur protein of the complex were reduced, and formation of ubisemiquinone was detected by EPR at liquid nitrogen temperature. The ubisemiquinone radical of the complex treated with antibodies against subunit IV had EPR signal characteristics identical with those of the untreated complex (data not shown). The signal had a g value of 2.005 and a band width of 8 G (McCurley et al., 1990). A power saturation study (data not shown) indicated that the ubisemiquinone radical in the complex treated with antibody relaxed as in the untreated complex. Also, the intensity of the EPR signal from the ubisemiquinone radical or the treated complex was very similar to those from untreated complex and from the complex treated with preimmune IgG. This suggests that the stability of the ubiquinone radical is not altered by antibody treatment. Since the ubisemiquinone radical is believed to be generated at the Q_i site (quinone reduction site) of the complex, this site is apparently not involved in the antibody–antigen interaction. This differs from the observation with the mitochondrial QPc–9.5-kDa protein. In this case, the antibody–antigen interaction inhibits antimycin-sensitive PQ reduction at the Q_i site of beef heart mitochondrial b–c_1 complex (Usui et al., 1990).

That antibody inhibits ubiquinol–cytochrome c reductase activity without affecting the K_m for Q_2H_2, or the formation of ubisemiquinone radical can be explained in at least two ways: (1) Subunit IV is part of the Q-binding site or domain of the complex, but since the antigenicity of the Q-binding site or domain is very low, antibodies against the epitopes of the quinone-binding domain are not present in purified IgG. The immunoinhibition observed is due to the binding of antibody to epitopes other than those at the quinol oxidation (Q_o) or reduction (Q_i) sites. (2) Subunit IV is essential structurally and required for assembly of the complex. Immunoinhibition results from indirect conformational change induced by interaction between subunit IV and its antibodies, not from direct interference of electron transfer at the redox centers.

The lack of a fourth subunit in isolated cytochrome b–c_1 complexes from other species, such as *P. dentrificans* or *Rs. rubrum*, does not rule out the possibility that such a protein is present in these organisms but is lost during the isolation. Conclusive evidence for or against the existence of such a small molecular weight subunit in these species can be obtained from the analysis of genomic libraries of these bacteria, using the gene for *R. sphaeroides* subunit IV as a probe. This gene has recently been cloned and sequenced (Usui et al., 1991).

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