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The Small Molecular Mass Ubiquinone-Binding Protein (QPc-9.5 kDa) in Mitochondrial Ubiquinol-Cytochrome *c* Reductase: Isolation, Ubiquinone-Binding Domain, and Immunoinhibition†

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ABSTRACT: The small molecular mass ubiquinone-binding protein (QPc-9.5 kDa) was purified to homogeneity from 3-azido-2-methyl-5-methoxy-6-(3,7-dimethyl[^{3}H]octyl)-1,4-benzoquinol-labeled bovine heart mitochondrial ubiquinol-cytochrome *c* reductase. The N-terminal amino acid sequence of the isolated protein is Gly-Arg-Gln-Phe-Gly-His-Leu-Thr-Arg-Val-Arg-His-, which is identical with that of a M_r = 9500 protein in the reductase [Borchart et al. (1986) *FEBS Lett. 200*, 81-86]. A ubiquinone-binding peptide was prepared from [^{3}H]azidoubiquinol-labeled QPc-9.5 kDa protein by trypsin digestion followed by HPLC separation. The partial N-terminal amino acid sequence of this peptide, Val-Ala-Pro-Pro-Phe-Val-Ala-Phe-Tyr-Leu-, corresponds to amino acid residues 48-57 in the reported M_r = 9500 protein. According to the proposed structural model for the M_r = 9500 protein, the azido-Q-labeled peptide is located in the membrane on the matrix side. These results confirm our previous assessment that the M_r = 13 400 subunit is not the small molecular weight Q-binding protein. Purified antibodies against QPc-9.5 kDa have a high titer with isolated QPc-9.5 kDa protein and complexes that contain it. Although antibodies against QPc-9.5 kDa do not inhibit intact succinate- and ubiquinol-cytochrome *c* reductases, a decrease of 85% and 20% in restoration of succinate- and ubiquinol-cytochrome *c* reductases, respectively, is observed when delipidated succinate- or ubiquinol-cytochrome reductases are incubated with antibodies prior to reconstitution with ubiquinone and phospholipid, indicating that epitopes at the catalytic site of QPc-9.5 kDa are buried in the phospholipid environment. Antibodies against QPc-9.5 kDa cause an increase of the apparent K_m for ubiquinol 2 in ubiquinol-cytochrome *c* reductase, suggesting that the low level of inhibition of the reductase by these antibodies may be due to the use of excess ubiquinol 2 in the assay mixture. Since antibodies against QPc-9.5 kDa inhibit 75% of the antimycin-sensitive plastoquinone reduction activity in the reconstituted succinate-cytochrome *c* reductase, QPc-9.5 kDa may be involved in the Q_i site. The topological arrangement of QPc-9.5 kDa in the mitochondrial membrane was examined immunologically with an anti-QPc-9.5 kDa Fab' fragment-horseradish peroxidase conjugate. When intact mitochondria (mitoplasts) or electron-transport particles (ETP) are exposed to this conjugate, peroxidase activity is found in both preparations, with ETP having the higher activity. This suggests that QPc-9.5 kDa is transmembranous, possibly with more mass on the matrix side of the membrane.

Bovine heart mitochondrial ubiquinol-cytochrome *c* reductase, known as complex III, catalyzes electron transfer from ubiquinol to cytochrome *c*. It contains five redox-active centers: two *b*-type cytochromes (cytochrome *b*-565 and cytochrome *b*-562), one *c*-type cytochrome (cytochrome c_1), one high-potential iron-sulfur center (2Fe-2S Rieske's center), and one ubiquinone (Q).[1] Purified reductase shows seven protein bands in SDS-PAGE using the Weber-Osborn gel system. The two smaller molecular weight protein bands are resolved into six bands when subjected to a high-resolution gel system, thus giving a total of 11 protein subunits for ubiquinol-cytochrome *c* reductase (Schägger et al., 1986; von Jagow et al., 1986; Gonzalez-Halphen et al., 1988). While cytochrome *b*, cytochrome c_1, and the iron-sulfur protein, identified as subunits III-V, respectively, in both gel systems, have been purified to homogeneity and their amino acid sequences reported (Wakabayashi et al., 1980; Anderson et al., 1982; Shimomura et al., 1984; Schägger et al., 1987), information on the protein structure of the Q-associated proteins is rather limited.

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[1] Abbreviations: [^{3}H]azido-QH_2, 3-azido-2-methyl-5-methoxy-6-(3,7-dimethyl[^{3}H]octyl)-1,4-benzoquinol; ELISA, enzyme-linked immunosorbent assay; ETP, electron-transport particles; HRP, horseradish peroxidase; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PITC, phenyl isothiocyanate; PTC, phenylthiocarbamoyl; PTH, phenylthiohydantoin; PBS, phosphate-buffered saline; Q, ubiquinone; Q_2, 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone; QPc-9.5 kDa, the small molecular mass ubiquinone-binding protein in ubiquinol-cytochrome *c* reductase; QPs, a protein complex that converts succinate dehydrogenase into succinate-ubiquinone reductase; [^{3}H]Q_0C_{10}NAPA, 2,3-dimethoxy-5-methyl-6-[10-[3-(4-azido-2-nitroanilino)[^{3}H]propionoxy]decyl]-1,4-benzoquinone; SDS, sodium dodecyl sulfate; TUT, 50 mM Tris-acetate buffer, pH 7.8, containing 2 M urea and 1.5% Triton X-100.

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The existence of a specific Q-binding protein (Yu & Yu, 1981) in mitochondrial ubiquinol-cytochrome *c* reductase was demonstrated by the following observations: (i) a unique stoichiometry between Q and cytochrome c_1 exists in highly active preparations; (ii) the removal of Q does not occur simultaneously with the removal of phospholipids from the reductase during the delipidation process (Yu et al., 1978); and (iii) stable ubisemiquinone radicals are detected in isolated reductases. These radicals are sensitive to antimycin treatment and proteolytic digestion. Q-binding proteins were identified in ubiquinol-cytochrome *c* reductase by photoaffinity labeling using functionally active arylazido-Q (Yu & Yu, 1982a) and azido-Q (Yu et al., 1985) derivatives. When ubiquinol-cytochrome *c* reductase is illuminated with 3-azido-2-methyl-5-methoxy-6-(3,7-dimethyl[^{3}H]octyl)-1,4-benzoquinone ([^{3}H]-azido-Q) or 2,3-dimethoxy-5-methyl-6-[10-[3-(4-azido-2-nitroanilino)[^{3}H]propionoxy]decyl]-1,4-benzoquinone ([^{3}H]Q_0C_{10}NAPA), two proteins, identified as subunits III and VI (Yu & Yu, 1982a; Yu et al., 1985) in the Weber–Osborn gel system and as subunits III and VII (Yu & Yu, 1982b) in the Swank–Munkres gel system, are heavily labeled. The larger molecular weight Q-binding protein (subunit III) is cytochrome *b* and called "QPc-cytochrome *b*". The apparent molecular mass of cytochrome *b* determined by SDS–PAGE is 30 000–37 000 daltons, depending on the conditions used. The true molecular weight of cytochrome *b* calculated from the amino acid sequence, deduced from the gene sequence, is 42 540 (Anderson et al., 1982). The apparent molecular mass of the smaller Q-binding protein was estimated to be 17 000 daltons by SDS–PAGE using the Weber–Osborn gel system (Yu et al., 1985). By determining the N-terminal amino acid of isolated azido-Q-labeled subunits, we find that the smaller Q-binding protein corresponds to subunit VII in Schägger's gel system. The amino acid sequences of smaller molecular weight subunits, VI–XI according to the gel electrophoresis system reported by Schägger et al. (1986), have recently become available. The molecular mass of subunit VII was reported to be 95071 daltons (Borchart et al., 1986) on the basis of its amino acid sequence. We call this small molecular weight Q-binding protein QPc-9.5 kDa.

The identification of two Q-binding proteins in ubiquinol-cytochrome *c* reductase is consistent with the two Q-binding sites (Qi and Q_o) proposed in the Q-cycle mechanism (Mitchell, 1976; Berry & Trumpower, 1985). By use of the same photoaffinity labeling techniques, the Q-binding protein(s) in the cytochrome b–c_1 complex of bakers' yeast (Yu et al., 1986) and *Rhodobacter sphaeroides* R-26 (Yu & Yu, 1987) and in the chloroplast cytochrome b_6–f complex (Doyle et al., 1989) was (were) identified. In the yeast mitochondrial and photosynthetic bacterial cytochrome b–c_1 complexes, two proteins (cytochrome *b* and a small molecular weight protein) bind Q. However, there is only one plastoquinone-binding protein in the chloroplast cytochrome b_6–f complex (Doyle et al., 1989).

Recently, a protein with an apparent molecular mass of 15 kDa (subunit VII in the Weber–Osborn gel system) was isolated and identified as Q binding on the basis of the fact that it can restore enzymatic activity to α-chymotrypsin-treated ubiquinol-cytochrome *c* reductase (Wang & King, 1982). Judging from the amino acid sequence reported for this protein (Wakabayashi et al., 1985), it appears to correspond to subunit VI in the gel system of Schägger et al. (1986). The N-terminal sequence analysis of the protein directly eluted from band VI of the gel (Gonzalez-Halphen et al., 1988) showed a sequence identical with that of the M_r = 15K protein (Wakabayashi et al., 1985). Since this protein is not labeled with azido-Q derivatives by the aforementioned photoaffinity-labeling technique, the role of this protein in Q binding remains obscure.

Knowledge of the molecular structure of the Q-binding site(s) (domain) is imperative if we are to better understand the Q-mediated electron-transfer mechanism in the mitochondrial electron-transfer chain. By taking advantage of being able to isolate [^{3}H]azido-Q labeled QPc-9.5 kDa and labeled peptide from photoaffinity-labeled ubiquinol-cytochrome *c* reductase and of the amino acid sequence data for this protein (Borchart et al., 1986), one can study the Q-binding domain in QPc-9.5 kDa. Using antibodies against QPc-9.5 kDa, we have investigated the topological arrangement and the location of the catalytic site of QPc-9.5 kDa. Herein we report the isolation procedure for, the location of the Q-binding domain in, and the functional role and topological arrangement of QPc-9.5 kDa.

Experimental Procedures

Materials. Coenzyme Q_{10}, horse heart cytochrome *c* (type III), bovine serum albumin, Triton X-100, and sodium cholate were obtained from Sigma. Goat anti-rabbit IgG–alkaline phosphatase conjugate, protein A–horseradish peroxidase conjugate, avidine–horseradish peroxidase conjugate, biotinylated SDS–PAGE standards, SDS–PAGE molecular weight standards, alkaline phosphatase substrate kit, and DEAE Affi-Gel blue were purchased from Bio-Rad. Sephacryl S-300 SF and Sephacryl S-200 HR were obtained from Pharmacia. Calcium phosphate was prepared according to Jenner (1973) and mixed at a 3:1 ratio with cellulose powder prior to use in column chromatography. Microtiter plates (Nunc immunoplate II) for ELISA were obtained from Gibco. Asolectin was a product of Associated Concentrates. Other chemicals were of the highest purity commercially available.

The ubiquinone derivatives 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone (Q_2), 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinol (Q_2H_2), and 3-azido-2-methyl-5-methoxy-6-(3,7-dimethyl[^{3}H]octyl)-1,4-benzoquinone ([^{3}H]azido-Q) were synthesized in our laboratory (Yu et al., 1985). 3-Azido-2-methyl-5-methoxy-6-(3,7-dimethyl[^{3}H]octyl)-1,4-benzoquinol ([^{3}H]azido-QH_2) was prepared by enzymatic reduction of [^{3}H]azido-Q with a catalytic amount of succinate-Q reductase in the presence of succinate as previously reported (Yu et al., 1985). The specific radioactivity of synthesized [^{3}H]azido-Q was 6 Ci/mol.

Enzyme Preparations and Assays. Intact mitochondria (Harmon & Crane, 1976), mitoplasts (Greenawalt, 1974), electron-transport particles (ETP) (Harmon, 1982), succinate-cytochrome *c* reductase (Yu & Yu, 1982), succinate-Q reductases (Yu & Yu, 1982), ubiquinol-cytochrome *c* reductase (Yu & Yu, 1980), the Q- and phospholipid-depleted succinate- and ubiquinol-cytochrome *c* reductases (Yu & Yu, 1980), QPs (Yu et al., 1987), and the M_r = 13 400 protein (subunit VI) from ubiquinol-cytochrome *c* reductase (Yu et al., 1989) were prepared and assayed by reported methods.

Absorption spectra and activity assays were performed at room temperature in a Cary spectrophotometer (Model 219). Protein (Lowry et al., 1951) and cytochromes *b* and c_1 (Berden & Slater, 1970) were determined by reported methods. Analytical SDS–PAGE was carried out according to Schägger et al. (1986) in a Bio-Rad Mini-Protein dual-slab cell; 8 M urea was used in the separating gel instead of 13% glycerol, and electrophoresis was run at 15 V for 2 h and then at 35 V for another 17 h. Western blot (Towbin et al., 1979) and ELISA (Voller et al., 1980) were carried out according to published procedures.

Isolation of Small Molecular Mass Q-Binding Protein (QPc-9.5 kDa). All purification steps were carried out at 0–4 °C unless otherwise specified. QPc-9.5 kDa and [^{3}H]azido-QH_2-labeled QPc-9.5 kDa were isolated from delipidated ubiquinol-cytochrome *c* reductase and [^{3}H]azido-QH_2-labeled reductase, respectively, according to the same purification procedure. The [^{3}H]azido-QH_2-labeled reductase was prepared by incubating a Q- and phospholipid-depleted ubiquinol-cytochrome *c* reductase, 6 mg/mL, in 50 mM Tris–acetate, pH 7.8, containing 0.5% sodium cholate, with [^{3}H]-azido-QH_2 at a 2:1 molar ratio to cytochrome *b* at 0 °C for 5 min in the dark and then illuminating at −20 °C for 7 min as previously described (Yu et al., 1985). The illuminated reductase had less than 5% of the activity of the unilluminated one when assayed after reconstitution with Q_2 and asolectin. The illuminated, [^{3}H]azido-QH_2-treated ubiquinol-cytochrome *c* reductase (130 mg) was mixed with 0.65 mL of micelle asolectin (10 mg/mL in H_2O) and precipitated by 50% ammonium sulfate saturation to remove most non-protein-bound azido-QH_2. The precipitate was collected by centrifugation at 28000*g* for 10 min and dissolved in 50 mM Tris–acetate, pH 7.8, containing 2 M urea and 1.5% Triton X-100 (TUT), adjusted to a protein concentration of 7 mg/mL, and then frozen at −80 °C for 1 h. The thawed solution was centrifuged at 28000*g* for 10 min, and the supernatant was applied to a calcium phosphate–cellulose column (3.2 × 4.7 cm) equilibrated with TUT. The effluent was collected, diluted with an equal volume of 2 mM dithiothreitol, adjusted to pH 6.0 with 2 N acetic acid, and then applied to a second calcium phosphate–cellulose column (2.3 × 4.3 cm) equilibrated with 50 mM Tris-HCl, pH 6.0, containing 0.2% Triton X-100. The column was subsequently washed, in sequence, with 40 mL of 50 mM Tris-HCl, pH 6.0, containing 0.2% Triton X-100, 40 mL of 35 mM potassium phosphate, pH 7.0, containing 1% Triton X-100, 2 M urea, 0.4 M NaCl, and 2 mM dithiothreitol, and 20 mL of 0.1 M potassium phosphate, pH 7.0, containing 0.2% Triton X-100. QPc-9.5 kDa was eluted with 0.3 M potassium phosphate, pH 8.0, containing 1% Triton X-100 and 2 M guanidine hydrochloride. The fractions containing QPc-9.5 kDa were combined and dialyzed against 50 mM Tris–acetate, pH 7.8, containing 0.25 M sucrose, overnight. The dialyzed sample was mixed with an equal volume of cold acetone (−20 °C), and the precipitate formed was collected by centrifugation. This acetone precipitation concentrates the protein and removes the bound Triton X-100. The precipitate was washed with cold acetone once, dried under a stream of nitrogen, and then dissolved in 125 mM Tris-HCl, pH 7.0, containing 4% SDS, 10% 2-mercaptoethanol, and 20% glycerol. The solution was applied to a Sephacryl S-200 HR column (1.2 × 37 cm) equilibrated with 20 mM Tris-HCl, pH 8.0, containing 1% SDS at room temperature. The fractions containing QPc-9.5 kDa were combined and treated with an equal volume of cold acetone at −20 °C. After the precipitate was collected and washed once with cold acetone, the protein was further purified by repeating the Sephacryl S-200 HR column chromatography and acetone precipitation described above.

Isolation of Ubiquinone-Binding Peptide. The isolated, [^{3}H]azido-QH_2-labeled QPc-9.5 kDa protein, 1 mg/mL, in 0.1 M ammonium bicarbonate, pH 8.5, containing 0.1% SDS and 2 M urea was digested with trypsin (type XIII, TPCK treated, Sigma) at 37 °C for 16 h. A trypsin to QPc-9.5 kDa ratio of 1:50 (w/w) was used. Fifty-microliter aliquots of the trypsin-digested QPc-9.5 kDa was separated by high-performance liquid chromatography (HPLC) on a Synchropak RP-8 column (0.46 × 25 cm) using gradients of acetonitrile in 0.1% trifluoroacetic acid with a flow rate of 1 mL/min. A 1-mL fraction was collected. The absorbance at 214 nm and radioactivity were measured for each fraction.

Amino Acid and Sequence Determination. Amino acids were analyzed by the methods of Heinrikson and Meredith (1984) and Bidlingmeyer et al. (1984) on an HPLC reversed-phase column after derivatization with phenyl isothiocyanate (PITC) to phenylthiocarbamoyl amino acids (PTC-amino acid). The isolated peptide from HPLC was hydrolyzed in 6 N HCl at 110 °C for 24 h. The hydrolysate was reacted with PITC, and PTC-amino acids were quantitated by HPLC with external amino acid standards, using an Ultrosphere-ODS column (0.46 × 25 cm) in a gradient formed with solvents A (50 mM ammonium acetate, pH 6.0) and B [44% (v/v) acetonitrile–10% (v/v) methanol–46% (v/v) 0.22 M ammonium acetate, pH 6.0].

The amino acid sequence analysis was performed by automated Edman degradation using a Model 470A gas-phase protein sequencer with on-line detection of the released amino acid phenylthiohydantoin derivatives (PTH-amino acids) by a Model 120A PTH-amino acid analyzer (Applied Biosystems, Foster City, CA). The isolated QPc-9.5 kDa suspended in water was extensively dialyzed against water, lyophilized, and then dissolved in 20% acetonitrile containing 0.1% trifluoroacetic acid. The QPc-9.5 kDa solution and the Q-binding peptide obtained from HPLC were absorbed into a polybrene-coated glass microfiber filter. The analyses were done at the Molecular Biology Resource Facility, Saint Francis Hospital of Tulsa Medical Research Institute, University of Oklahoma Health Sciences Center, under the supervision of Dr. Ken Jackson.

Production and Purification of Antibody against QPc-9.5 kDa. The isolated QPc-9.5 kDa (1 mg) was suspended 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 then injected into a rabbit subcutaneously. Boosters, 0.5 mg of QPc-9.5 kDa, were given weekly for 1 month, and sera were collected by cardiac puncture.

Purification of antibody 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, pH 8.0, containing 50 mM NaCl and dialyzed against the same buffer. The dialyzed solution containing IgG was applied to a DEAE Affi-Gel blue column (5 times the serum volume) equilibrated with 20 mM Tris-HCl, 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 antibody was prepared in the same way with serum from the same rabbit taken prior to immunization.

$F(ab')_2$ fragment and Fab′ fragment were prepared by pepsin digestion of IgG according to the methods of Stanworth and Turner (1978) and Taniguchi et al. (1978), respectively. Fab′ fragment–horseradish peroxidase conjugate (Fab′–HRP) was prepared by the reported method (Ishikawa et al., 1983) with some modifications. Two milligrams of horseradish peroxidase (type II, Sigma) dissolved in 0.3 mL of 0.1 M sodium phosphate, pH 7.0, was mixed with 20 μL of a coupling reagent, 0.1 mM 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylic acid *N*-succinimido ester in *N*,*N*-dimethylformamide, and incubated at 30 °C for 45 min. After centrifu-

gation, the supernatant solution was applied to a Sephadex G-25 column equilibrated with 0.1 M sodium phosphate, pH 6.0, to remove any excess coupling reagent. Peroxidase with maleimide groups was collected and concentrated to a protein concentration of 4 mg/mL. Fab′ fragment was prepared by reducing the cystine groups of the $F(ab')_2$ fragment to cysteine groups with a 100 times molar excess of 2-mercaptoethanol. The mixture was applied to a Sephadex G-25 column equilibrated with 0.1 M sodium phosphate, pH 6.0, containing 5 mM EDTA to remove the 2-mercaptoethanol. Fab′ was collected and adjusted to a protein concentration of 5 mg/mL. The freshly prepared Fab′ fragment (2.5 mg) was mixed with the derivatized peroxidase (2 mg) and incubated at 4 °C for 20 h. Fab′–HRP conjugate was separated on a Sephacryl S-200 HR column in 0.1 M sodium phosphate, pH 6.5. The horseradish peroxidase activity in the purified conjugate was assayed by the reported method (Chance & Maehly, 1955) using pyrogallol as substrate.

Immunoinhibition of Ubiquinol-Cytochrome c Reductase and Succinate-Cytochrome c Reductase Activities. Forty micrograms of intact ubiquinol-cytochrome *c* reductase and intact succinate-cytochrome *c* reductase was mixed with various amounts of anti-QPc-9.5 kDa and preimmune IgG fractions in 40 μL of 50 mM PBS containing 0.1% sodium cholate and 5% glycerol. The antibody–antigen mixtures were incubated at room temperature for 10 min and then at 4 °C for 12 h before the activities were assayed. When delipidated ubiquinol-cytochrome *c* reductase and delipidated succinate-cytochrome *c* reductase were used, the activity was measured after antigen–antibody mixtures were reconstituted with 1 nmol of Q_2 and 30 μg of micelle asolectin (10 mg/mL). The reductase activities were assayed as reported (Yu & Yu, 1980).

Topological Study of Ubiquinol-Cytochrome c Reductase. Digitonin-treated intact mitochondria (Greenawalt, 1974) and electron-transport particles (ETP) were mixed with 10 milliunits of anti-QPc-9.5 kDa Fab′–HRP conjugate (approximately 5.2 units/mg of protein) and preimmune Fab′–HRP conjugate (approximately 4.9 units/mg of protein) in 50 mM sodium phosphate, pH 7.4, containing 0.25 M sucrose and incubated at 4 °C for 3 h. The mixtures were centrifuged at 30000*g* for 15 min, and the collected precipitate was suspended in 50 mM sodium phosphate, pH 7.4, containing 0.25 M sucrose. This procedure was repeated three more times before aliquots of the suspension were taken, and the horseradish peroxidase activity was assayed with a horseradish peroxidase substrate kit (Bio-Rad) according to the manufacturer's instructions.

Results and Discussion

Electrophoretic Behavior, Molecular Mass, Isolation, and Purity of the Small Molecular Mass Ubiquinone-Binding Protein (QPc-9.5 kDa). It was reported that when ubiquinol-cytochrome *c* reductase was labeled with [^{3}H]azido-QH_2 upon illumination and subjected to SDS–PAGE analysis, two proteins, which correspond to subunits III and VI (Yu & Yu, 1982; Yu et al., 1985) of the seven subunits observed in the Weber–Osborn gel system or to subunits III and VII (Yu & Yu, 1982b) of the nine subunits resolved in the Swank–Munkres gel system, were heavily labeled. Subunit III in both gel systems is identified as cytochrome *b*. Subunit VI of the Weber–Osborn system and subunit VII of the Swank–Munkres system, which have relative electrophoretic mobilities of 0.89 and 1.2, respectively, in reference to cytochrome *c*, are identified as the small molecular weight Q-binding protein on the basis of the observation that the amount of radioactive labeling on these subunits is proportional to the extent of

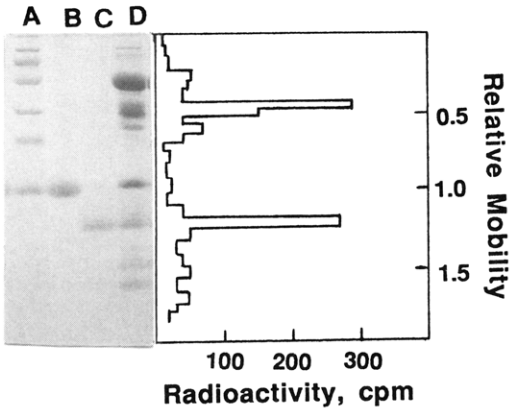

FIGURE 1: SDS–PAGE of isolated QPc-9.5 kDa and radioactivity distribution among subunits of ubiquinol-cytochrome *c* reductase. The photoaffinity-labeled delipidated ubiquinol-cytochrome *c* reductase, isolated QPc-9.5 kDa, and molecular weight standards were dissolved in 50 mM Tris-HCl, pH 6.8, containing 4 M urea, 5% β-mercaptoethanol, and 2% SDS to a protein concentration of about 1 mg/mL and incubated at 100 °C for 5 min before being subjected to SDS–PAGE analysis. (Lane A) The molecular mass standards: phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). (Lane B) Cytochrome *c*. (Lane C) Purified QPc-9.5 kDa. (Lane D) [^{3}H]Azido-QH_2-labeled ubiquinol-cytochrome *c* reductase. For determining the distribution of the radioactivity, the gel in lane D was sliced according to the stained protein bands after the staining and destaining process. The gel slices were hydrolyzed with 2 N NH_4OH as previously described (Yu & Yu, 1982), and the radioactivity was determined.

inactivation upon illumination. The different band position (number) observed for the azido-Q-labeled small molecular weight subunit in these two SDS–PAGE systems was attributed to this protein having different electrophoretic behavior under different SDS–PAGE conditions and not to the nonspecific association of azido-Q to different subunits under different SDS–PAGE systems. To confirm this, proteins of these two bands were eluted from their respective SDS–PAGE gels and analyzed for N-terminal amino acid sequences. Both proteins have partial N-terminal amino acid sequences of Gly-Arg-Gln-, indicating that they are indeed the same protein with different electrophoretic mobility under different SDS–PAGE conditions. This N-terminal amino acid sequence is the same as that of subunit VII of the reductase resolved in the gel system of Schägger et al. (1986). Since this SDS–PAGE system gives the best resolution of the subunit structure of ubiquinol-cytochrome *c* reductase and the amino acid sequences of subunits VI–XI of this system are available, it is of interest to confirm the azido-Q labeling pattern in this system. Eleven protein subunits are resolvable from the purified ubiquinol-cytochrome *c* reductase. The smallest subunit, however, shows up well only when large amounts of protein are used. Under the described electrophoresis conditions, it appears as a very faint band and is not reproduced well in the photograph of the gel as indicated in Figure 1. The radioactivity distribution among the subunits of ubiquinol-cytochrome *c* reductase resolved in the SDS–PAGE system of Schägger et al. is shown in the right panel of Figure 1. Two proteins corresponding to subunits III and VII are heavily labeled, confirming that the small molecular weight Q-binding protein is subunit VII in the Schägger et al. system.

The apparent molecular mass of the small Q-binding protein, as estimated from the SDS–PAGE systems of Weber–Osborn, Swank–Munkres, and Schägger et al., is 17 000, 11 000, and 11 000 daltons, respectively. The true molecular mass of this protein as estimated from amino acid sequencing is 9507 daltons (Borchart et al., 1986). Therefore, we des-

Table I: Summary of Purification Data

treatment	vol (mL)	protein (mg)	recovery (%)
delipidated ubiquinol-cytochrome *c* reductase	18.6	130.00	100
first calcium phosphate column effluent	25.5	76.50	59
second calcium phosphate column eluate	10.3	22.00	17
first Sephacryl S-200 HR	12.8	2.34	1.8
second Sephacryl S-200 HR	4.6	1.07	0.8[a]

[a] Assuming the molecular mass of ubiquinol-cytochrome *c* reductase is 250 000 daltons, the protein recovery of QPc-9.5 kDa is 21.4%.

ignate this Q-binding protein as QPc-9.5 kDa.

The key steps involved in isolation of QPc-9.5 kDa are Triton X-100 and urea treatment, calcium phosphate–cellulose column chromatography at different pHs, and molecular sieve chromatography. Purification data for QPc-9.5 kDa are summarized in Table I. About 21% of the QPc-9.5 kDa present in ubiquinol-cytochrome *c* reductase is recovered in the final step, assuming that the molecular mass of the ubiquinol-cytochrome *c* reductase complex is 250 000 daltons and that it contains one QPc-9.5 kDa per complex. The isolated protein shows only one band, which corresponds to subunit VII of the reductase in the SDS–PAGE system of Schägger et al. (see Figure 1), indicating that it is homogeneous and is indeed the one identified as the small molecular weight Q-binding protein by photoaffinity-labeling techniques. Isolated QPc-9.5 kDa contains about 0.3 mol of azido-Q per mol of protein when photolyzed, [^{3}H]azido-Q-treated, PL- and Q-depleted reductase (which lost 95% of the reconstitutive activity compared to unilluminated sample) is used as the starting material. The substoichiometry of azido-Q with respect to protein in the isolated QPc-9.5 kDa is probably due to the partial release of bound azido-Q from the protein during purification. The starting material contained about 2 mol of azido-Q/mol of reductase protein. The quantification was done by spotting the photolyzed sample on a piece of Whatman filter paper followed by developing the paper with a chloroform–methanol (2:1) mixture, and thus, the radioactivity and protein concentration on the original spot were determined. It is likely that part of the bound azido-Q may result from the nonspecific hydrophobic binding of azido-Q by protein. The majority of the bound azido-Q should result from the covalent linkage of azido-Q to the Q-binding site because the amount of azido-Q uptake by protein correlates to the extent of inactivation upon illumination. Although the nature of the chemical bond between the photoactivated azido group and protein is unknown, the *N*-oxide bond has been reported to be unstable and may be cleaved during purification. The presence of unstable *N*-oxide bonds in azido-Q-labeled protein has been indirectly implicated by the increased release of radioactivity upon acidification of the labeled protein.

The N-terminal amino acid sequence of the isolated protein was determined to be Gly-Arg-Gln-Phe-Gly-His-Leu-Thr-Arg-Val-Arg-His-. This sequence is identical with the N-terminal sequence reported for a protein with a molecular mass of 9507 daltons (Borchart et al., 1986) in ubiquinol-cytochrome *c* reductase. It is, however, different from the N-terminal sequence of the M_r = 13 400 protein (Wakabayashi et al., 1985) isolated from ubiquinol-cytochrome *c* reductase and reported to be a Q-binding protein (QP-C) by Wang and King (1982). This protein corresponds to subunit VI of the reductase resolved in the SDS–PAGE system of Schägger et al. The 13.4-kDa protein was isolated as a Q–protein–phospholipid mixture, but it was not labeled by [^{3}H]azido-Q after illumination. A brief account of this protein has been reported (Yu et al., 1989), and a more detailed discussion of this protein will be presented elsewhere.[2]

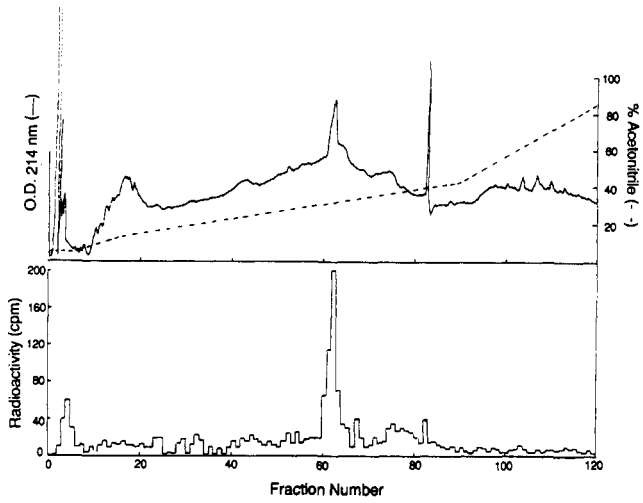

FIGURE 2: ^{3}H radioactivity distribution on HPLC chromatogram of trypsin-digested, [^{3}H]azido-QH_2-labeled QPc-9.5 kDa. The isolated, [^{3}H]azido-QH_2-labeled QPc-9.5 kDa (1 mg/mL, 2 × 10^5 cpm/mg) in 0.1 M ammonium bicarbonate, pH 8.5, containing 0.1% SDS and 2 M urea was digested with 20 μg of trypsin at 37 °C for 16 h, and 50-μL aliquots were subjected to HPLC separation as described under Experimental Procedures. 100-μL aliquots were withdrawn for radioactivity determination in a Packard Tri-Carb 1900CA liquid scintillation analyzer.

Table II: Amino Acid Composition of Tryptic Azido-Q-Labeled Peptide

amino acid	no. of residues[a]	amino acid	no. of residues[a]
Asx	0.1 (0)	Ile	0.3 (0)
Thr	1.9 (2)	Leu	1.1 (1)
Ser	0.1 (0)	Tyr	2.0 (2)
Glx	2.8 (3)	Phe	1.9 (3)
Pro	2.3 (2)	Lys	1.3 (1)
Gly	1.4 (1)	His	0.1 (0)
Ala	2.0 (2)	Arg	0.2 (0)
Val	3.0 (3)	Cys	ND (1)

[a] The numbers of amino acids presented were normalized to three valines. The numbers given in parentheses are the numbers of the amino acids present in the segment between residues 48 and 68 of the published sequence data (Borchart et al., 1986) for the M_r = 9507 protein.

Isolation and Characterization of Ubiquinone-Binding Peptide. Figure 2 shows the radioactivity distribution among the tryptic peptides of QPc-9.5 kDa separated by HPLC. When isolated, [^{3}H]azido-Q-labeled QPc-9.5 kDa is digested with trypsin (2% by weight) at 37 °C for 16 h followed by HPLC separation using a Synchropak RP-8 column eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid, a majority of the radioactivity is found in fraction 63. When the amino acid composition of this fraction is analyzed (see Table II), it closely resembles amino acid residues 48–68 in the reported protein sequence for the 9.5-kDa protein (Borchart et al., 1986), indicating that this segment of the protein is responsible for the Q-binding domain. Some radioactivity is also found in fractions 5 and 83. However, no amino acids are detected in these two fractions, indicating that they are decomposed 3-azido-Q or azido-Q adducts of detergent or phospholipid.

The partial N-terminal amino acid sequence of fraction 63 was determined to be Val-Ala-Pro-Pro-Phe-Val-Ala-Phe-Tyr-Leu-. This sequence is identical with amino acid residues 48–57 of the 9.5 kDa protein. If we adopt the structural model

[2] To be published.

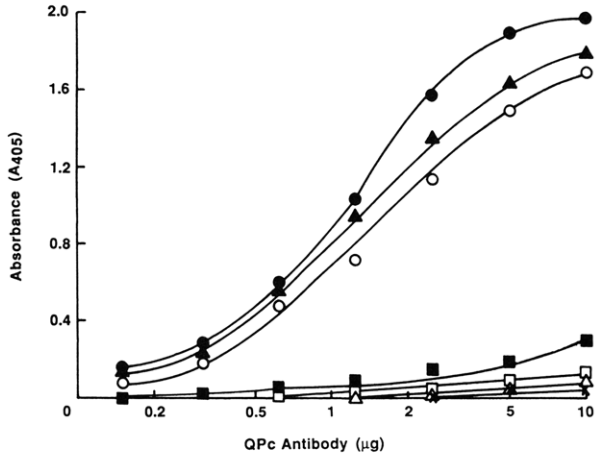

FIGURE 3: Immunological specificity of antibodies against QPc-9.5 kDa analyzed by ELISA. The antigens, 1 μg of QPc-9.5 kDa (●), 10 μg of succinate-cytochrome *c* reductase (○), 10 μg of ubiquinol-cytochrome *c* reductase (▲), 10 μg of succinate-ubiquinone reductase (△), 1 μg of 13.4-kDa protein (■), and 1 μg of QPs (□), were coated on the ELISA plate and then reacted with various amounts of purified anti-QPc-9.5 kDa. Meanwhile, the preimmune IgG (×) was reacted with 1 μg of QPc-9.5 kDa. 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.

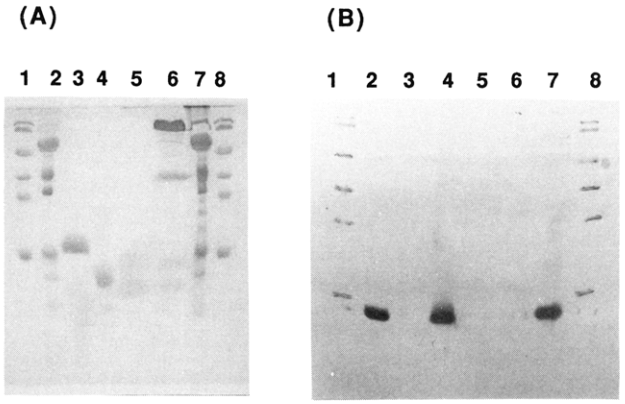

FIGURE 4: Western blot analysis of antibodies against QPc-9.5 kDa. (A) SDS–PAGE of ubiquinol-cytochrome *c* reductase (50 μg, lane 2), the M_r = 13 400 protein (8 μg, lane 3), QPc-9.5 kDa protein (5 μg, lane 4), QPs (4 μg, lane 5), succinate-Q reductase (20 μg, lane 6), succinate-cytochrome *c* reductase (50 μg, lane 7), and the biotinylated molecular weight standard (lanes 1 and 8). SDS–PAGE was carried out according to the method of Laemmli (1970) using 16% gel containing 8 M urea (B); the proteins on the gel of (A) were electrophoretically transferred to a nitrocellulose membrane without staining and then reacted with anti-QPc-9.5 kDa. Protein A–horseradish peroxidase conjugate and avidin–horseradish peroxidase conjugate were then reacted with the membrane. The detection of peroxidase activity was performed with a horseradish peroxidase color development kit (Bio-Rad).

proposed for the M_r = 9500 protein by Borchart et al. (1986), the isolated peptide that is labeled with azido-Q is located within the membrane toward the surface of the matrix side. Although the photoaffinity-labeling technique does not permit us to specifically identify the amino acid residue or residues that are directly involved in Q binding because of the rather long life and nonspecific nature of the activated nitrene radical, it is tempting to suggest that the hydroxyl groups of two threonine and two tyrosine residues may hydrogen bond with the carbonyl groups in the quinone ring of the Q molecule. The involvement of hydrogen bonding between protein and the two carbonyl oxygens of quinone has been suggested in Q_A and Q_B binding in the photosynthetic reaction center (Deisenhofer & Michel, 1989). The quantitative recovery of threonine or tyrosine residues in the amino acid composition analysis of fraction 63 (Table II) should not be used as evidence to negate the possibility of the formation of an azido-Q–threonine or azido-Q–tyrosine adduct because the chemical bond formed between activated nitrene and the oxygen of the hydroxy group of threonine or tyrosine is rather labile; it is hydrolyzed during acid hydrolysis. The recovery of phenylalanine, on the other hand, was lower than the calculated value, indicating that some azido-Q may be covalently linked to a phenylalanine residue upon illumination. This result can be used to indicate that one of the phenylalanine residues may be involved in or near the Q-binding site. Phenylalanine has been shown to be a significant part of the Q_B-binding pocket in the reaction center (Deisenhofer & Michel, 1989).

Although identification of a Q-binding peptide from QPc-9.5 kDa clearly indicates the involvement of a portion of QPc-9.5 kDa in Q binding, it does not preclude the involvement of other segments of QPc-9.5 kDa or other subunits of ubiquinol-cytochrome *c* reductase. It would not be a surprise if the Q_i-binding site is a pocket formed from more than one segment of molecules of different subunits of reductase complex, as in the case of Q_A- or Q_B-binding pockets in the reaction center complex (Deisenhofer & Michel, 1989). The participation of a portion of QPc–cytochrome *b* protein in the Q_i site is very likely, because QPc–cytochrome *b* is also highly labeled with azido-Q in the photoaffinity-labeling study. Identification of Q-binding peptides of QPc–cytochrome *b* and their topological locations will provide useful information leading to the understanding of the Q_i site. Work on these aspects is currently in progress in our laboratory.

Immunological Specificity of Antibodies against QPc-9.5 kDa. Figure 3 shows the antigen–antibody binding data from ELISA. For each antigen, succinate-cytochrome *c*, ubiquinol-cytochrome *c*, and succinate-Q reductases, QPc-9.5 kDa, QPs, and M_r = 13 400 protein (subunit VI), a fixed protein concentration is titrated with varying amounts of antibodies specific for QPc-9.5 kDa and preimmune IgG preparations. The protein concentrations of succinate-cytochrome *c* and ubiquinol-cytochrome *c* reductase used were 10 times higher than that of isolated QPc-9.5 kDa on ELISA because the amount of QPc-9.5 kDa present in these reductases is less than 10%. Antibodies against QPc-9.5 kDa react with a high titer to antigens that contain QPc-9.5 kDa, such as isolated QPc-9.5 kDa, succinate-cytochrome *c*, and ubiquinol-cytochrome *c* reductases. No binding is observed with QPs, M_r = 13 400 protein, and succinate-Q reductase, indicating that QPc-9.5 kDa is immunologically different from the other proteins in succinate-cytochrome *c* reductase. This is to be expected because QPc-9.5 kDa differs from the other proteins in molecular weight and amino acid composition and sequence (Borchart et al., 1986; Wakabayashi et al., 1985).

The immunological specificity of antibodies against QPc-9.5 kDa is further characterized by a Western blot using succinate-cytochrome *c*, ubiquinol-cytochrome *c*, and succinate-Q reductases and isolated QPc-9.5 kDa, QPs, and the M_r = 13 400 protein as antigens (Figure 4). Antibodies against QPc-9.5 kDa bind specifically to subunit VII of ubiquinol-cytochrome *c* reductase and to isolated QPc-9.5 kDa. As expected, no binding is observed with succinate-Q reductase, QPs, or the M_r = 13 400 protein.

Immunoinhibition of Ubiquinol-Cytochrome c Reductase by Antibodies against QPc-9.5 kDa. Since a QPc-9.5 kDa deficient (or depleted) ubiquinol-cytochrome *c* reductase is not yet available, investigation of the biological function of QPc-9.5 kDa through reconstitution studies is impossible at the present

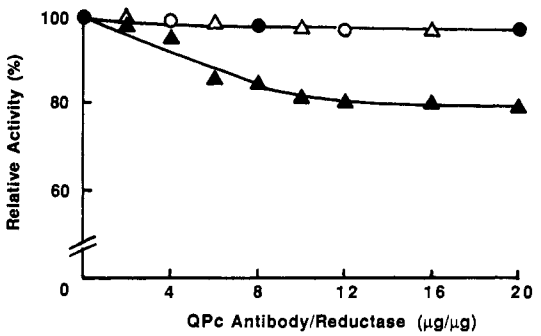

FIGURE 5: Immunoinhibition of ubiquinol-cytochrome *c* reductase activity by anti-QPc-9.5 kDa. Forty micrograms of intact (○, ●) and delipidated (△, ▲) ubiquinol-cytochrome *c* reductases was incubated with various amounts of anti-QPc-9.5 kDa (●, ▲) and preimmune IgG (○, △). The ubiquinol-cytochrome *c* reductase activity of the treated intact and delipidated (after reconstitution of Q_2 and asolectin) reductases was assayed. 100% activity for intact and reconstituted reductases is 103 and 88 μmol of cytochrome *c* reduced min^{-1} (mg of protein)$^{-1}$ at 23 °C, respectively.

time. However, the availability of antibodies against QPc-9.5 kDa enables us to take an immunological approach to study the functional role of this Q-binding protein. Figure 5 shows the effect of antibodies against QPc-9.5 kDa on intact and delipidated ubiquinol-cytochrome *c* reductases. With intact ubiquinol-cytochrome *c* reductase no inhibition is observed. There are two possible explanations for this lack of immunoinhibition: (i) QPc-9.5 kDa is not involved in the catalytic cycle of ubiquinol-cytochrome *c* reductase; (ii) QPc-9.5 kDa is involved in the catalytic cycle, but the epitope(s) at the catalytic site of QPc-9.5 kDa is (are) buried in the phospholipid environment and is (are) inaccessible to antibodies. To test these possibilities, a reconstitutively active, Q- and PL-depleted ubiquinol-cytochrome *c* reductase (Yu & Yu, 1980) prepared by repeated ammonium sulfate fractionation in the presence of 1% sodium cholate and 10% glycerol was used. When delipidated reductase is incubated with various amounts of antibodies against QPc-9.5 kDa for 20 min at room temperature followed by reconstitution with Q and phospholipid, a 20% decrease in the restoration of ubiquinol-cytochrome *c* reductase is observed. However, when the delipidated reductase is reconstituted with phospholipid prior to incubation with antibodies against QPc-9.5 kDa, no decrease in the restoration of ubiquinol-cytochrome *c* reductase is observed. These results indicate that epitopes at the catalytic site(s) of QPc-9.5 kDa are not exposed when sufficient phospholipid is present, as in the case of intact reductase. The intact reductase is known to contain approximately 40 mol of phospholipid/mol of protein (based on cytochrome c_1) (Yu & Yu, 1980). Epitopes at the catalytic site(s) of bovine heart cytochrome *b* have also been reported to be buried in the phospholipid environment (Haley et al., 1986). These results contrast with those of cytochrome *b* (Sidhu & Beattie, 1982) and subunit VII protein (Japa et al., 1987) of the yeast mitochondrial system in which epitopes at catalytic sites are apparently exposed and accessible to their respective antibodies in the phospholipid sufficient state. Antibodies against these two proteins inhibit intact yeast mitochondrial ubiquinol-cytochrome *c* reductase (Sidhu & Beattie, 1982; Japa et al., 1987). Although it is quite unlikely, the possibility exists that the inhibition observed in the antibody-treated delipidated reductase results from a decrease in the efficiency of reconstitution between phospholipids and protein in the presence of antibodies. This speculation, however, was negated by observations that the Fab fragment and whole antibodies cause the same degree of inhibition and that inhibition was QH_2 concentration dependent.

It should be noted that in the ELISA assay antibodies against QPc-9.5 kDa have the same titer against intact and delipidated ubiquinol-cytochrome *c* reductases. This is because the phospholipids of the intact reductase are removed by the repeated washing with 0.05% Tween-20 employed in the ELISA assay procedure.

Effect on Apparent K_m for Ubiquinol in Ubiquinol-Cytochrome c Reductase by Antibodies against QPc-9.5 kDa. The nature of the immunoinhibition of ubiquinol-cytochrome *c* reductase by antibodies against QPc-9.5 kDa can be studied by examining the kinetic parameters of the treated reductase. The ubiquinol-cytochrome *c* reductase activity is assayed after the delipidated reductase is incubated with antibodies and then is reconstituted with asolectin. The apparent K_m for Q_2H_2, calculated from Lineweaver–Burk plots, for ubiquinol-cytochrome *c* reductases treated with preimmune IgG or antibodies against QPc-9.5 kDa, increases from 3.8 to 6.9 μM, with no change in V_{max} when antibodies against QPc-9.5 kDa are at the level that gives maximum inhibition (12 μg of antibody/μg of complex). It should be emphasized that the observed increase in apparent K_m for Q_2H_2 is not due to nonspecific interference of antibodies on the Q-binding site because the same K_m value for Q_2H_2 is obtained for reductase with or without incubation with preimmune IgG. It was reported by Fato et al. (1988) that the true K_m for Q_2H_2 in ubiquinol-cytochrome *c* reductase is quite different from that of the apparent K_m. Since apparent K_m's depend heavily on the assay conditions, comparison of K_m's for Q_2H_2 is valid only when assays are carried out under identical conditions, as was the case in the present study.

Since the assay mixture for ubiquinol-cytochrome *c* reductase contains 25 μM Q_2H_2, which is much greater than the determined apparent K_m value for antibody-treated ubiquinol-cytochrome *c* reductase, it is quite possible that the low level of immunoinhibition observed results from a partial override of immunoinhibition by Q_2H_2 present in the assay mixture. To test this possibility, immunoinhibition of succinate-cytochrome *c* reductase by antibodies against QPc-9.5 kDa was examined, and the results are described in the following section.

Immunoinhibition of Succinate-Cytochrome c Reductase by Antibodies against QPc-9.5 kDa. Figure 6 shows immunoinhibition of intact and delipidated succinate-cytochrome *c* reductases by antibodies against QPc-9.5 kDa. Four kinds of electron-transferring activities were examined: succinate-cytochrome *c*, succinate-plastoquinone, ubiquinol-cytochrome *c*, and succinate-Q reductases. The succinate-plastoquinone reductase activity detected in succinate-cytochrome *c* reductase is antimycin sensitive while succinate-Q reductase is not. Like intact ubiquinol-cytochrome *c* reductase, intact succinate-cytochrome *c* reductase is not inhibited by antibodies against QPc-9.5 kDa. However, when delipidated succinate-cytochrome *c* reductase is incubated with various amounts of antibodies against QPc-9.5 kDa followed by reconstitution with Q and phospholipids, a maximum decrease of 85%, 75%, and 35%, respectively, in restoration of succinate-cytochrome *c*, succinate-plastoquinone, and ubiquinol-cytochrome *c* reductase activities is observed when 12 μg of antibody is used per microgram of reductase protein. Since succinate-cytochrome *c* reductase is composed of succinate-Q and ubiquinol-cytochrome *c* reductases and antibodies against QPc-9.5 kDa do not react with succinate-Q reductase when analyzed by ELISA and Western blot and shown no immunoinhibition against isolated succinate-Q reductase, the 85% inhibition observed for succinate-cytochrome *c* reductase activity must result from

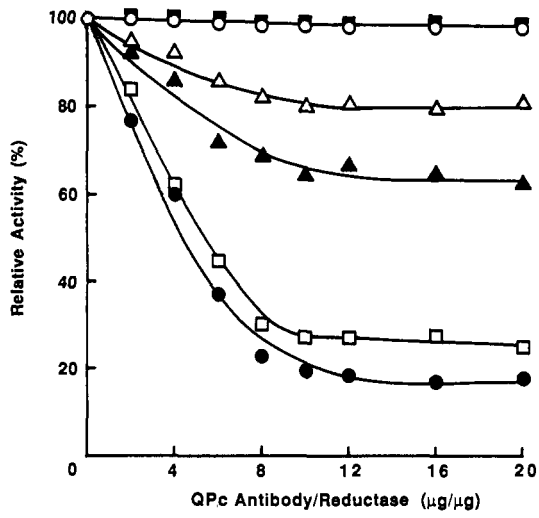

FIGURE 6: Immunoinhibition of succinate-cytochrome *c* reductase by anti-QPc-9.5 kDa. Forty micrograms of intact and delipidated succinate-cytochrome *c* reductases was treated with various amounts of anti-QPc-9.5 kDa and preimmune IgG. After the delipidated succinate-cytochrome *c* reductase was reconstituted with 1.0 nmol of Q_2 and 20 μg of asolectin, the succinate-cytochrome *c* reductase activity was determined in anti-QPc-9.5 kDa treated intact (○) and reconstituted (●) reductases and in preimmune IgG-treated, reconstituted reductase (■). The activities of succinate-Q reductase (△), ubiquinol-cytochrome *c* reductase (▲), and succinate-plastoquinone reductase (□) in anti-QPc-9.5 kDa treated, reconstituted succinate-cytochrome *c* reductase were also measured. 100% activity for the intact succinate-cytochrome *c* reductase and the succinate-cytochrome *c*, succinate-Q, succinate-plastoquinone, and ubiquinol-cytochrome *c* reductases in reconstituted succinate-cytochrome *c* reductase is 5.4, 3.7, 4.4, and 0.2 μmol of succinate and 30.8 μmol of ubiquinol oxidized min^{-1} (mg of protein)$^{-1}$ at 23 °C, respectively.

inhibition of ubiquinol-cytochrome *c* reductase. This result seems to favor our explanation that the low degree of inhibition of ubiquinol-cytochrome *c* reductase results from the high concentration of Q_2H_2 in the assay mixture.

Since antimycin-sensitive plastoquinone reduction is believed to involve both succinate-Q reductase and the Q_i site of the cytochrome b–c_1 complex (Weiss & Wingfield, 1979; Chen et al., 1986), the strong inhibition of plastoquinone reduction by antibodies against QPc-9.5 kDa suggests that QPc-9.5 kDa is involved in or serves as a part of the Q_i site. This result is in contrast to the report that subunit VII of yeast ubiquinol-cytochrome *c* reductase is involved in the Q_o site and is located on the face of the matrix side (Japa et al., 1987). The observation that antibodies against QPc-9.5 kDa increase the K_m for Q_2H_2 of ubiquinol-cytochrome *c* reductase may be due to their binding at or in the vicinity of the Q_i site and may indirectly cause the conformational change at the Q_o site where Q_2H_2 binds.

The observation that antibodies inhibit the ubiquinol-cytochrome *c* reductase activity of reconstituted succinate-cytochrome *c* reductase more than ubiquinol-cytochrome *c* reductase itself suggests that the presence of succinate-Q reductase facilitates the binding of antibody to epitopes at the catalytic site of QPc-9.5 kDa. Alternatively, two nonidentical ubiquinol-cytochrome *c* reductases may be present in isolated succinate-cytochrome *c* reductase; one accepts electrons from endogenous QH_2 or ($QH^•$), and the other accepts electrons directly from exogenous QH_2. This is plausible since isolated succinate-cytochrome *c* reductase contains 1 mol of succinate-Q reductase and 2 mol of ubiquinol-cytochrome *c* reductase. The presence of a high concentration of Q_2H_2 in the ubiquinol-cytochrome *c* reductase assay mixture may have decreased the inhibition of the ubiquinol-cytochrome *c* reductase, which accepts electrons directly from Q_2H_2, more than the inhibition of the ubiquinol-cytochrome *c* reductase, which accepts electrons from endogenous Q reduced by succinate-Q reductase.

The observation that succinate-Q reductase activity in reconstituted succinate-cytochrome *c* reductase is slightly (20%) inhibited by antibodies against QPc-9.5 kDa is rather surprising because no inhibition is observed with isolated succinate-Q reductase or intact succinate-cytochrome *c* reductase. The inhibition cannot be due to the direct binding of antibody to the succinate-Q reductase moiety because no binding between antibody and succinate-Q reductase is detected by ELISA and Western blot (Figures 3 and 4). Perhaps the binding of antibodies to the catalytic site of QPc-9.5 kDa interfers with Q_2 from the succinate-Q reductase assay mixture gaining access to the QPs of succinate-Q reductase in succinate-cytochrome *c* reductase. This explanation is valid only if succinate-cytochrome *c* reductase is indeed a supercomplex of succinate-Q and ubiquinol-cytochrome *c* reductases (Gwak et al., 1986).

Topology of QPc-9.5 kDa in the Mitochondrial Membrane. To obtain clues concerning the quaternary structure and membrane topology of ubiquinol-cytochrome *c* reductase in the inner mitochondrial membrane, the topological arrangement of QPc-9.5 kDa was examined immunologically with Fab′ fragment–horseradish peroxidase (Fab′–HRP) conjugate prepared from antibodies against QPc-9.5 kDa in bovine heart mitoplasts (digitonin-treated intact mitochondria) and electron-transport particles (ETP, reverse orientation). The advantages of using Fab′–HRP conjugate are 2-fold: (i) the conjugate has easy access to the epitopes of the antigen because of its smaller molecular size; (ii) the amount of conjugate bound to the antigen can be estimated by assaying the horseradish peroxidase activity. When anti-QPc–Fab′–HRP is reacted with mitoplasts and ETP, no inhibition of succinate-oxidase activity is observed, indicating that epitopes at the catalytic site of QPc-9.5 kDa are inaccessible from either side of the membrane. The lack of immunoinhibition by anti-QPc–Fab′–HRP in both ETP and mitoplasts however, does not rule out the possibility that QPc-9.5 kDa is transmembranous and has portions of its mass extending into the hydrophilic environment. To test this, the peroxidase activity was assayed on ETP and mitoplasts that have been reacted with anti-QPc–Fab′–HRP. As shown in Figure 7, peroxide activity is observed with both mitoplasts and ETP preparations but more with the ETP. When preimmune Fab′–HRP is used, very low peroxide activity is observed in both ETP and mitoplasts. This low activity is probably due to nonspecific binding. After the amount of nonspecific binding is subtracted from that of anti-QPc-9.5 kDa–Fab′–HRP conjugate bound to mitoplasts and ETP, the peroxidase activity in ETP is about twice that in mitoplasts. Since the Fab′–HRP used is derived from polyclonal antibodies, the results obtained would suggest that QPc-9.5 kDa is a transmembranous protein, possibly with more mass on the matrix side. This is consistent with the topological structure model of the cytochrome b–c_1 complex recently proposed by Gonzalez-Halphen et al. (1988). According to their model, subunit VII protein, which has the same N-terminal sequence as QPc-9.5 kDa, is transmembranous. It should be mentioned that, in rare cases, the observed difference in peroxidase activity between mitoplasts and ETP does not indicate uneven distribution of the mass of the protein on different sides of the membrane; rather, it is a result of epitopes exposed on both sides of the membrane having different binding affinities to Fab′–HRP. Probing with monoclonal

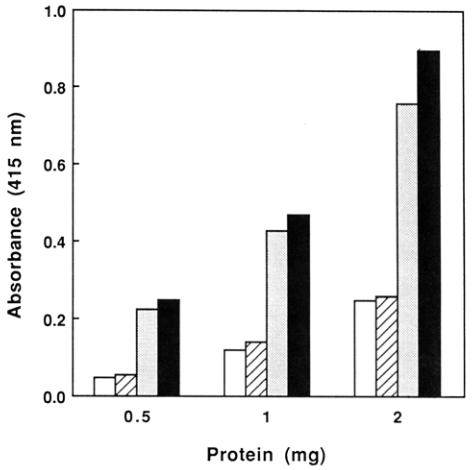

FIGURE 7: Binding of anti-QPc-9.5 kDa Fab′–horseradish peroxidase conjugate to mitoplasts and electron-transport particles. The indicated amounts of mitoplasts (open and stippled bars) and electron-transport particles (ETP) (hatched and solid bars) were treated with 2 μg of anti-QPc-9.5 kDa–Fab′ fragment–horseradish peroxidase conjugate (stippled and solid bars) and preimmune Fab′ fragment–horseradish peroxidase conjugate (open and hatched bars). After the excess conjugates were washed by centrifugation, the peroxidase activity was assayed.

antibodies or monospecific polyclonal antibodies should provide a better idea about the topological arrangement of this Q-binding protein. Investigation toward this end is currently in progress.

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