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Minireview

Mitochondrial Ubiquinol-Cytochrome *c* Reductase Complex: Crystallization and Protein: Ubiquinone Interaction

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The ubiquinol-cytochrome *c* reductase complex was crystallized in a thin plate form, which diffracts X-rays to 7 Å resolution in the presence of mother liquor. This crystalline complex contains ten protein subunits and 140 nmol phospholipid per milligram protein. Over 90% of the phospholipid and ubiquinone in the reductase can be removed by repeated ammonium sulfate precipitation in the presence of 0.5% sodium cholate. The delipidated complex has no enzymatic activity and shows significant changes in the circular dichroism spectrum in the near UV region and in the EPR characteristics of both cytochromes *b*. Enzyme activity and spectral characteristics can be restored by replenishing the phospholipid and ubiquinone. The structural requirements of ubiquinone for electron transport were studied by measuring the ability of a variety of synthetic ubiquinone derivatives to restore the enzymatic activity and native spectroscopic signatures to the delipidated complex. Q-binding proteins and binding domains were identified using photoaffinity labeled Q-derivatives and HPLC separation of photolabeled peptides. Interaction between ubiquinol-cytochrome *c* reductase and succinate-Q reductase was established by differential scanning calorimetry and saturation transfer EPR using spin-labeled ubiquinol-cytochrome *c* reductase. Involvement of iron-sulfur protein in proton translocation by ubiquinol-cytochrome *c* reductase was investigated by hematorporphyrin-promoted photoinactivation of the complex. The *c*DNAs encoding the Rieske iron–sulfur protein and a small molecular mass Q-binding protein (QPc-9.5 kDa) were isolated and their nucleotide sequences determined. These will be useful in future structural and mechanistic studies of ubiquinol-cytochrome *c* reductase via *in vitro* reconstitution between an over-expressed, mutated subunit and a specific subunit-depleted reductase.

KEY WORDS: Mitochondrial ubiquinol-cytochrome *c* reductase; electron transport; synthetic ubiquinone derivatives; ubiquinone binding sites; proton pumping, protein crystallization.

INTRODUCTION

Ubiquinol-cytochrome *c* reductase (commonly known as Complex III or the cytochrome bc_1 complex) is a segment of the mitochondrial respiratory chain which catalyzes antimycin-sensitive electron transfer from ubiquinol to cytochrome *c* (Hatefi *et al.* 1962; Rieske, 1976; Hatefi, 1985). The reaction is coupled to the translocation of protons across the mitochondrial inner membrane to generate a proton gradient and membrane potential for ATP synthesis. Bovine heart mitochondrial ubiquinol-cytochrome *c* reductase was first isolated in 1962 (Hatefi *et al.* 1962); since then, several purification methods have been introduced (Hatefi, 1978; Yu and Yu, 1980a; Engel *et al.*, 1980; Ljungdahl *et al.*, 1987). The purified reductase appears to contain 7–11 protein subunits depending on the sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) system used (Yu *et al.*, 1974; Bell and Capaldi, 1976; Gellerfors and Nelson, 1977; Gonzalez-Halphen *et al.*, 1988). The amino acid sequences of all but two of the core proteins are known (Wakabayashi *et al.*, 1982; Schägger *et al.*, 1985; Borchart *et al.*, 1986). The

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amino acid sequences of core I and II of human ubiquinol-cytochrome *c* reductase have been deduced by nucleotide sequencing (Hosokawa *et al.*, 1989). The essential redox components are: two *b* cytochromes (b_{565} and b_{562}, one *c*-type cytochrome (c_1), one high-potential iron-sulfur cluster (2Fe-2S Rieske center), and a ubiquinone. Results of recent intensive investigations on the electron transfer and proton translocation mechanisms have generated a general enthusiasm for the protonmotive Q-cycle hypothesis (Mitchell, 1976; Trumpower, 1990). Chemical labeling and proteolytic enzyme digestion studies have provided substantial information concerning the spatial arrangement of this enzyme complex in the membrane (Gonzalez-Halphen *et al.*, 1988). However, structural and mechanistic studies of mitochondrial ubiquinol-cytochrome *c* reductase have been somewhat hindered by its structural complexity and by a lack of knowledge about its three-dimensional structure. The recent success in protein crystallization of this complex (Yue *et al.*, 1991; Kubota *et al*; 1991; Berry *et al.*, 1992) should speed up structural studies and enhance mechanistic investigations.

Our laboratory has had a long-term interest in ubiquinol-cytochrome *c* reductase, particularly in certain aspects of structure–function relationships and in protein:ubiquinone interactions. Over the past two decades, we have used multiple approaches to investigate this membrane protein complex. In this review we will summarize our work on this complex, emphasizing protein:ubiquinone interactions and identification of ubiquinone binding sites. For other work on this and related complexes, excellent reviews are available (Wikström *et al.*, 1981; Cramer and Crofts, 1982, Rieske, 1986; Hauska *et al.*, 1983, 1988).

PREPARATION AND SUBUNIT STRUCTURE OF CRYSTALLINE UBIQUINOL-CYTOCHROME *c* REDUCTASE

Preparation of Ubiquinol-Cytochrome *c* Reductase

Bovine heart mitochondrial ubiquinol-cytochrome *c* reductase (or Complex III) was first isolated from NADH-cytochrome *c* reductase in 1962 (Hatefi *et al.*, 1962). The purification procedure was subsequently improved (Rieske, 1967) with respect to purity, yield, and activity using a supernatant solution obtained during the preparation of NADH-cytochrome *c* reductase. This method is still used by many investigators. In contrast, the ubiquinol-cytochrome *c* reductase preparation used in our laboratory starts with highly purified succinate-cytochrome *c* reductase (Yu and Yu, 1980a). Succinate dehydrogenase in extensively dialyzed succinate-cytochrome *c* reductase is solubilized and removed upon alkalization (to pH 10) under anaerobic conditions. The resulting residue is termed the cytochrome bc_1 particle, which contains both cytochrome bc_1 complex (ubiquinol-cytochrome *c* reductase) and a protein fraction that converts succinate dehydrogenase into succinate-Q reductase (QPs). The cytochrome bc_1 particle is capable of reconstituting with pure succinate dehydrogenase to form succinate-cytochrome *c* reductase. A highly purified ubiquinol-cytochrome *c* reductase is obtained upon solubalization of bc_1 particle by deoxycholate and removal of QPs and other contaminants by ammonium acetate fractionation (Yu and Yu, 1980a). The purified reductase contains 8.3 nmol cytochrome *b*, 4.7 nmol cytochrome c_1 and 3.5 nmol ubiquinone per mg protein. The specific activity of the reductase is 11 μmol cytochrome *c* reduced per min per nmol cytochrome *b* at 23°C. When the purified reductase is subjected to high resolution SDS-PAGE, eleven protein bands are observed (Schägger *et al.*, 1986; Gonzales-Halphen *et al.*, 1988).

Crystallization of Ubiquinol-Cytochrome *c* Reductase

Purified ubiquinol-cytochrome *c* reductase in 50 mM Tris-Cl buffer, pH 7.8, containing 0.67 M sucrose, was first crystallized in 6% polyethylene glycol-4000 (PEG) in the presence of 0.25 M sodium chloride, 1.8% heptanetriol, and 0.04% decanoyl-*N*-methyl-glucamide. Good sized crystals formed within 2–4 weeks. The largest one had dimensions of $4 \times 2 \times 1$ mm. Although the crystals show a high degree of birefringence under polarized light, they diffract X-rays only at low resolution (Yu *et al.*, 1991). Crystalline ubiquinol-cytochrome *c* reductase is composed of ten protein subunits. It contains 2.5 nmol ubiquinone, 8.4 nmol cytochrome *b*, 4.2 nmol cytochrome c_1, and 140 nmol phospholipid per mg protein. Of the latter 36% is diphosphatidylglycerol. These crystals are very stable in the cold and show full enzymatic activity when redissolved in aqueous solution. Absorption spectra of the redissolved crystals show a Soret to UV ratio of 0.88 and 1.01 in the oxidized and the reduced forms, respectively.

When crystals were grown in X-ray capillary

tubes in 50 mM MES buffer, pH 7.0, instead of 50 mM Tris-Cl buffer, pH 8.0, they diffract X-rays to 7 Å resolution in the presence of mother liquor. Preliminary experiments on a Xuong/Hamin area detector indicate that they belong to a tetragonal system; the space group is probably $P4_122$ (or $P4_322$) with cell constants $a = b = 158.7$ Å, $C = 593$ Å, and $\gamma = 90°$ (Yu *et al.*, 1993). Assuming one cytochrome bc_1 complex dimer per asymmetric unit, the crystals would have a solvent content of 70%. Removal of the mother liquor from the crystals causes severe loss of diffraction quality. Moreover, the tendency of the crystals to move in a liquid-filled capillary tube makes data collection difficult. To circumvent these difficulties, we recently developed a method to crystallize ubiquinol-cytochrome *c* reductase in the gel state. Purified ubiquinol-cytochrome *c* reductase, 20 mg/ml, in 50 mM MES buffer, pH 7.0, containing 0.67 M sucrose was mixed, at 18°C, with an equal volume of precipitating solution containing 0.08% decanoyl-*N*-methylglucamide, 3.6% heptanetriol, 0.5 M sodium chloride, 12% polyethylene glycol, and 0.8–0.4% low-gelling-temperature agarose. The mixture was placed in capillary tubes, cooled to 4°C, overlaid with equilibrating solution, and incubated in a shock-free environment at 4°C. Under these conditions, ubiquinol-cytochrome *c* reductase crystals formed within 2–4 weeks. The size, shape, and diffraction quality of these crystals approach those obtained from the liquid state. These crystals will facilitate future data collection using a high-energy synchrotron source. Figure 1 shows crystals of ubiquinol-cytochrome *c* reductase grown under different conditions.

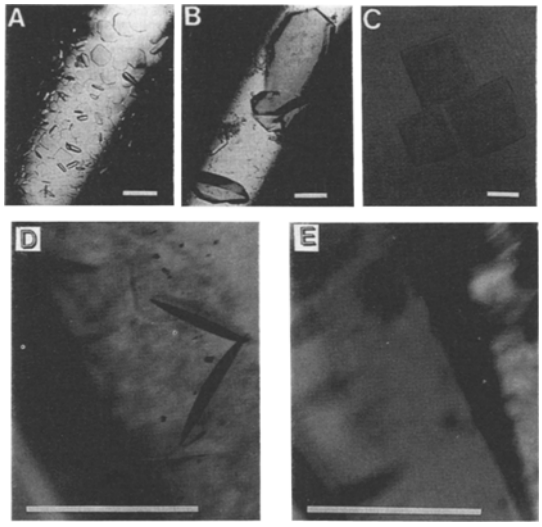

Fig. 1. Crystals of ubiquinol-cytochrome *c* reductase grown under different conditions. Panel A, B, and C are crystals grown in 50 mM Tris-Cl buffer, pH 8.0: A, early stage in Pasteur pipet; B, later stage in Pasteur pipet, C, in test tube. Panels D and E are crystals grown in 50 mM MES buffer, pH 7.0, in X-ray capillary tubes at early and later stages, respectively. The bars indicate 1 mm in length.

INTERACTION OF UBIQUINOL-CYTOCHROME *c* REDUCTASE WITH PHOSPHOLIPIDS

Purified ubiquinol-cytochrome *c* reductase contains 140–220 nmol phospholipids (PL) and 3.5 nmol ubiquinone (Q) per mg protein. Over 90% of the PL and Q in ubiquinol-cytochrome *c* reductase can be removed by repeated precipitation with ammonium sulfate in the presence of 0.5% sodium cholate (Yu and Yu, 1980b). Maximal removal of PL requires two cycles of ammonium sulfate/cholate precipitations, whereas maximal removal of Q requires five. The delipidated enzyme complex is inactive. Removal of PL from ubiquinol-cytochrome *c* reductase results in an immediate decrease of approximately 15% in molar ellipticities in the far-UV and the Soret regions. A further decrease in ellipiticities is observed upon incubation of the delipidated enzyme at 0°C in 50 mM phosphate buffer, pH 7.4. Delipidation also causes a significant alteration of the EPR characteristics of both cytochromes *b*, but has little effect on the signals of cytochrome c_1 and the iron–sulfur cluster (Yu and Yu, 1987a). Replenishing the delipidated enzyme complex with PL and Q restores the molar ellipticity in both regions and the EPR signals of the cytochromes *b*. Full restoration of enzymatic activity can only be achieved with a freshly prepared delipidated enzyme complex, made in the presence of 20% glycerol. The amount of activity restored decreases, and the incubation time required to reach maximal activity increases, as the age of the delipidated enzyme increases (see Fig. 2). The absolute requirement for PL in ubiquinol-cytochrome *c* reductase is also demonstrated by treatment of the enzyme with purified phospholipase A_2. Inactivation of ubiquinol-cytochrome *c* reductase by phospholipase A_2 is not prevented by the presence of excess exogenous ubiquinone. The enzymatic activity of the phospholipase A_2-treated complex is fully restored upon the addition of EDTA and PL (asolectin).

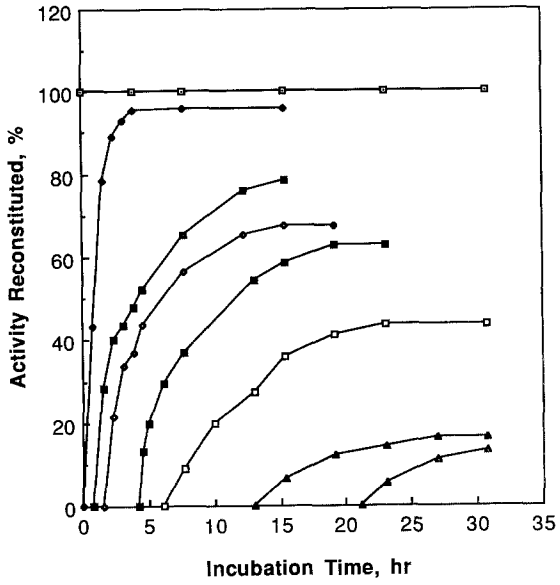

Fig. 2. The delipidated enzyme age and incubation-time-dependent maximal activity restoration: PL- and Q-depleted ubiquinol-cytochrome *c* reductase, 10 mg/ml in 50 mM phosphate buffer, pH 7.4, containing 0.5% sodium cholate and 20% glycerol was incubated at 0°C. At indicated times, aliquots of complex were diluted to 1 mg/ml with phosphate buffer and mixed with Q_2 (30 μM) and asolectin (1 mg/ml). The mixtures were incubated at 0°C and the restored activity assayed at various times.

INTERACTION OF UBIQUINOL-CYTOCHROME *c* REDUCTASE WITH SYNTHETIC QUINONE DERIVATIVES AND INHIBITORS

The availability of a reconstitutively active PL- and Q-depleted ubiquinol-cytochrome *c* reductase has provided us the opportunity to study the role of ubiquinone in this complex using synthetic Q analogs and quinone-like inhibitors. Success in identifying Q-binding proteins or binding domains depends on the success of the reconstitution between the photoactivatable Q derivatives and the PL- and Q-depleted complex. Only synthetic quinone analogs which are capable of restoring enzymatic activity to Q- and PL-depleted ubiquinol-cytochrome *c* reductase can be used for studying Q:protein interactions or identifying Q-binding proteins (domains). Over the past 15 years, we have systematically synthesized various Q derivatives (see Fig. 3) and determined their activities as electron acceptors, donors, and mediators in an attempt to understand the structural requirements for the Q function in the mitochondrial electron transfer chain.

Q-Derivatives with Different Alkyl Groups at the 6-Position

To study the effect of variation in the alkyl side chain on the electron-transfer activity of Q in mitochondrial succinate-cytochrome *c* reductase, we have synthesized, through a radical coupling reaction between Q_2 and alkanoyl peroxides, a series of derivatives that possess the basic ubiquinone structure of 2,3-dimethoxy-5-methyl-1,4-benzoquinone with different alkyl side chains at the 6-position. These side chains vary in chain length, degree of saturation, and location of double bonds (Yu *et al.*, 1985a). Reduction of quinone derivatives by succinate is measured with succinate-Q reductase and with succinate-cytochrome *c* reductase. Oxidation of quinol derivatives is measured with ubiquinol-cytochrome *c* reductase. The electron-transfer efficacy of Q derivatives is compared to that of 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone. When a Q derivative is used as an electron acceptor for succinate-Q reductase, an alkyl

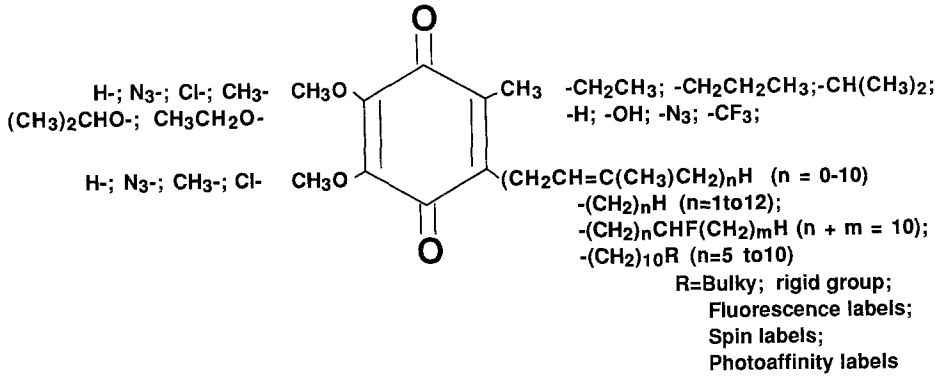

Fig. 3. Structures of various synthetic ubiquinone derivatives.

side chain of 6 carbons is needed to obtain maximum activity. However, when it serves as an electron donor for ubiquinol-cytochrome *c* reductase or as a mediator in succinate-cytochrome *c* reductase, an alkyl side chain of 10 carbons is required for maximum efficacy. Q derivatives with alkyl side chains significantly longer than 10 carbons have less activity. This is due mainly to the lack of a proper dispersion condition for these highly hydrophobic derivatives. For instance, when Q_{10} is dispersed in 1% sodium cholate solution, it restores only about 20% of the original enzymatic activity to the delipidated ubiquinol-cytochrome *c* reductase, but it restores full activity if Q_{10} is codispersed with asolectin in 0.1% cholate and 0.5% decanoyl-*N*-methylglucamide (Gu *et al.*, 1990).

Introduction of one or two isolated double bonds into the alkyl side chain of Q has little effect on electron-transfer activity. *Trans* derivatives have slightly higher activity than their *cis*-counterpart. However, introduction of a conjugated double bond system drastically reduces electron-transfer efficiency. The effect of the conjugated double bond system on the electron-transferring efficiency depends on its location in the alkyl side chain. When located far from the benzoquinone ring, the effect is minimal. When conjugated double bonds are adjacent to the benzoquinone ring, a drastic reduction in the electron transfer activity is observed. These observations show that flexibility in the portion of the alkyl side chain immediately adjacent to the benzoquinone ring is required for efficient electron-transfer activity of Q.

Derivatives with Different Substitutions on Benzoquinone Ring

The effect of substituents on the 1,4-benzoquinone ring of Q on its electron-transfer activity was studied by using synthetic derivatives that have a decyl (or geranyl) side-chain at the 6-position and various arrangements of methyl, methoxy, and hydrogen at the 2-, 3- and 5-positions of the benzoquinone ring (Gu *et al.*, 1990). When quinone derivatives are used as the electron acceptor for succinate-Q reductase, the methyl group at the 5-position is less important than the methoxy groups at the 2- and 3-positions. Replacing the 5-methyl group with hydrogen causes a slight increase in activity. However, replacing one or both of the 2- and 3-methoxy groups with a methyl completely abolishes electron-acceptor activity. Replacing the 3-methoxy group with hydrogen results in a complete loss of electron-acceptor activity, while replacing the 2-methoxy with hydrogen results in an activity decrease of 70%, suggesting that the 3-methoxy group is more critical than the methoxy group at the 2-position.

The structural requirements for quinol derivatives to be oxidized by ubiquinol-cytochrome *c* reductase are less strict. All 1,4-benzoquinol derivatives examined show partial activity when used as electron donors. Derivatives that possess one unsubstituted position, at 2-, 3- or 5-, with a decyl group at the 6-position, show substrate inhibition at high concentrations, something not observed with fully substituted derivatives.

The structural requirements for quinone derivatives to be reduced by succinate-cytochrome *c* reductase are also less specific than those for succinate-Q reductase. Replacing one or both of the 2- and 3-methoxy groups with a methyl and keeping the 5-position unsubstituted (plastoquinone derivatives) yield derivatives with no acceptor activity for succinate-Q reductase. However, these derivatives are reducible by succinate in the presence of succinate-cytochrome *c* reductase. This reduction is antimycin-sensitive and requires endogenous Q, suggesting that these (plastoquinone) derivatives can only accept electrons from the ubisemiquinone radical at the Q_i site of ubiquinol-cytochrome *c* reductase, and cannot accept electrons from the QPs of succinate-ubiquinone reductase.

Derivatives with Ethoxy Groups

The necessity of methoxy substituents at the 2- and 3-positions of the benzoquinone ring in electron transfer catalyzed by succinate-cytochrome *c* reductase was also investigated. Two ethoxy-substituted Q-derivatives, 2 (or 3)-ethoxy- or 3 (or 2)-methoxy-5-methyl-6-decyl-1,4-benzoquinone (EtO-Q_0C_{10}), and 2,3-diethoxy-5-methyl-6-decyl-1,4-benzoquinone [(EtO-Q_0C_{10}] were synthesized and characterized (He *et al.*, 1992). They were synthesized from 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (Q_0C_{10} and (ETO)2-Q_2C_{10}) by reaction with sodium ethoxide/ethanol in hexane under anaerobic conditions. The products, EtO-Q_0C_{10}, are separated by TLC using hexane:ether (3.5:1) as developing solvent. The R_f values for diethoxy and monoethoxy derivatives are 0.7 and 0.6, respectively. The spectral and redox properties of EtO-Q_0C_{10} and $(EtO)_2$-Q_0C_{10} are very similar to those of Q_0C_{10}. Ethoxy-Q derivatives exhibit concentration-dependent inhibition of

both Q-simulated and unstimulated succinate-Q reductase activities. $(EtO)_2$-Q_0C_{10} is the more potent inhibitor. These derivatives do not inhibit ubiquinol-cytochrome *c* reductase activity. When used as a substrate for ubiquinol-cytochrome *c* reductase, EtO-$Q_0C_{10}H_2$ has about 55%, and $(ETO)_2$-$Q_0C_{10}H_2$ has about 15%, of the activity of $Q_0C_{10}H_2$, but with higher K_m. Since the titration of these ethoxy ubiquinol derivatives with ubiquinol-cytochrome *c* reductase shows a saturation behavior, the low efficiency of these compounds as electron donors is apparently not due to weak binding to ubiquinol-cytochrome *c* reductase but rather to their intrinsic properties. These results indicate that the binding environment of the benzoquinone ring in succinate-Q reductase is very specific and is different from that of ubiquinol-cytochrome *c* reductase.

Derivatives with Various 5-Alkyl Groups

To investigate the role of substituents at the 5-position, a series of 5-alkyl-ubiquinone derivatives were synthesized through a radical coupling reaction between 2,3-dimethoxy-6-decyl-1,4-benzoquinone (5-H-Q_0C_{10}) and alkanoyl peroxides (Wan *et al.*, 1975). The products, 5-ethyl-Q_0C_{10}, 5-propyl-Q_0C_{10}, 5-isopropyl-Q_0C_{10}, and 5-butyl-Q_0C_{10}, are separated by TLC using hexane:ether (3.5:1) as developing solvent. The R_f values for 5-alkyl derivatives are 0.47, 0.50, 0.55, and 0.52 respectively. The reducibility and oxidizability of these derivatives, measured with succinate-Q reductase and ubiquinol-cytochrome *c* reductase, decreases as the size of the alkyl group increases. As an electron acceptor for succinate-Q reductase, 5-ethyl-Q_0C_{10} has about 50% of the activity of Q_0C_{10}. 5-Alkyl groups with three or more carbon atoms are totally ineffective as electron acceptors for succinate-Q reductase. In contrast to the lack of reducibility of 5-butyl-Q_0C_{10} by succinate-Q reductase, the reduced form of 5-butyl-Q_0C_{10} is partially oxidized by ubiquinol-cytochrome *c* reductase in a concentration-dependent manner. These results confirm that the binding environment of the benzoquinone ring in succinate-Q reductase is more specific than that of ubiquinol-cytochrome *c* reductase.

Derivatives with Hydroxyl or Chlorine Substituents on Benzoquinone Ring

Substitution of methoxy or methyl groups with a hydroxyl group or chlorine atom has a profound effect on electron transfer activity. A ubiquinone derivative, 3-chloro-5-hydroxyl-2-methyl-6-decyl-1,4-benzoquinone (3-CHMDB), was synthesized and characterized (Gu *et al.*, 1989). This derivative inhibits mitochondrial ubiquinol-cytochrome *c* reductase but activates the cytochrome b_6-*f* complex of chloroplasts. When ubiquinol-cytochrome *c* reductase is treated with varying concentrations of 3-CHMDB and assayed at constant substrate (Q_2H_2) concentration, a 50% inhibition is observed when 2 mol of 3-CHMDB per mol of enzyme are used. The degree of inhibition is dependent on the substrate concentration. When ubiquinol-cytochrome *c* reductase is treated with 2 mol of 3-CHMDB per mol of enzyme, less inhibition is observed with lower substrate concentration, suggesting the existence of two forms of reductase: one with a high affinity for ubiquinone and another with a low affinity, and 3-CHMDB competes more effectively with the latter. 2-Chloro-5-hydroxyl-3-methyl-6-decyl-1,4-benzoquinone (2-CHMDB), an isomer of 3-CHMDB, is a much less potent inhibitor of mitochondrial ubiquinol-cytochrome *c* reductase, suggesting a highly specific quinone binding site. In contrast to its inhibition of ubiquinol-cytochrome *c* reductase, 3-CHMDB does not inhibit plastoquinol-plastocyanin reductase of chloroplast cytochrome b_6-*f* complex, regardless of whether plastoquinol-2 or ubiquinol-2 is used as substrate. 3-CHMDB restores the dibromothymoquinone-altered EPR spectrum of the iron-sulfur protein in both complexes. With the cytochrome b_6-*f* complex, 3-CHMDB also partially restores the dibromothymoquinone-inhibited activity. The reduced form of 3- or 2-CHMDB is oxidized by the cytochrome b_6-*f* complex, but not by the cytochrome bc_1 complex. These results suggest that the quinol oxidizing sites in the cytochrome b_6-*f* complex, differ from those in mitochondrial cytochrome bc_1 complex, and the electron transfer mechanisms in these two complexes may differ substantially (Cramer *et al.*, 1987).

Quinone-like Inhibitors

Quinone-like inhibitors (Von Jagow and Link, 1986) have been extensively used in the study of Q:protein interactions. It is generally assumed that these types of compounds behave like ubiquinone in ubiquinol-cytochrome *c* reductase. However, data supporting this assumption are not available. To investigate the inhibitory action of a quinone-like

molecule, 5-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) (Trumpower and Haggerty, 1980) and its binding site, a series of 4,7-dioxobenzothiazole derivatives were synthesised and their inhibitor efficiencies studied (Yang *et al.*, 1989). Replacing the 6-hydroxyl or 2-hydrogen of UHDBT with a bromo or methoxy group causes only a slight decrease in inhibitory efficacy, indicating that the 6-hydroxy or the 2-hydrogen of UHDBT is not a structural requirement for inhibition. 5-Undecyl-6-bromo (or methoxy)-4,7-dioxobenzothiazole shows a pH-dependent inhibition similar to that observed with UHDBT, suggesting that the pH dependence is due to the presence of a dissociable group in the protein complex and not to deprotonation of the hydroxyl group of UHDBT.

Replacement of the 6-hydroxyl group with an azido group gives a molecule whose effects on ubiquinol-cytochrome *c* reductase are similar to those observed with UHDBT; this inhibition is accompanied by alteration of the EPR characteristics of the reduced iron-sulfur protein inubiquinol-cytochrome *c* reductase. The extent of inhibition is not changed upon illumination of the treated reductase. When 6-azido-5-{1′,2′-[^{3}H]undecyl}-4,7-dioxobenzothiazole ([^{3}H]azido-UDBT)-treated reductase is illuminated and then subjected to organic solvent extraction, no radioactivity is found in the reductase protein. Rather, the radioactivity is located in the phospholipid fraction. A [^{3}H]azido-UDBT-cardiolipin adduct, identified after separation of the phospholipid fraction by high-performance liquid chromatography, has 6-azido-UDBT linked to an acyl group, not to the head group of the cardiolipin molecule. These results suggest that inhibition by UHDBT is due to perturbation of specific cardiolipin molecules in ubiquinol-cytochrome *c* reductase. Since UHDBT and 6-azido-UDBT also inhibit the ubiquinol-cytochrome *c* reductase activity of delipidated reductase (10% of the original lipid remaining) assayed after reconstitution with ubiquinone and phospholipid, and, again, the [^{3}H]azido–UBDT–cardiolipin adduct is found in the delipidated reductase, the UHDBT-perturbed cardiolipin molecule appears to be structurally indispensable and tightly bound to reductase protein, most likely to the quinone binding proteins.

Derivatives with Various Reporting Groups

Various 6-alkylubiquinone or 6-(ω-haloalkyl)-ubiquinone derivatives were synthesized through a radical coupling reaction between alkanoyl or ω-haloalkanoyl peroxides and Q_0 (Yu and Yu, 1982a). Q_0 was synthesized from 2-methoxy-4-methylphenol via nitration, methylation, reduction, and oxidation by modifications of the reported methods. 6-(ω-haloalkyl)-ubiquinones were converted to 6-(ω-hydroxylalkyl)-ubiquinones by a mercuric-assisted solvolysis technique (McKillop and Ford, 1974). The 6-(ω-hydroxyalkyl)-ubiquinones were then esterified with carboxylic acid anhydrides or carboxylic acid-bearing reporting groups, such as a photoaffinity label, *n*-(4-azido-2-nitrophenyl)-β-alanine (NAPA), a spin-label, 3-carboxy-2,2,5,5-tetramethyl-3-pyrrolinyl-1-oxy (TMPOC), or a fluorescence label, 6-(7-nitrobenz-oxa-1,3-diazo-4-yl)-aminohexanoic acid (NBDHA). Esterification was catalyzed by dicyclohexylcarbodiimide, and the esters were purified by preparative silica gel TLC, developed with 3% ethanol in benzene. The spectral properties and biological functions of the synthesized ubiquinone derivatives (Q_0C_{10}NAPA, Q_0C_{10}TMPOC, Q_0C_{10}NBDHA) were studied. The concentration dependencies of these ubiquinone derivatives on succinate-cytochrome *c* reductase, succinate-Q reductase and ubiquinol-cytochrome *c* reductase activities were compared with that of Q_0C_{10}.

Q_0C_{10}NAPA

In the dark, Q_0C_{10}NAPA partially restores ubiquinol-cytochrome *c* reductase activity to the delipidated reductase upon reconstitution with PL. This restored activity is abolished upon illumination. The activity of illuminated Q_0C_{10}NAPA-treated, delipidated reductase is only partially restored by addition of excess Q, suggesting that the photoactivated Q derivative is covalently bound to a specific Q-binding protein in this segment of the respiratory chain.

Q_0C_{10}TMPOC

Specific interaction between Q and protein is suggested by the immobilization of the spin-label of a synthesized spin-labeled ubiquinone derivative. When Q_0C_{10}TMPOC was incubated with Q- and PL-depleted ubiquinol-cytochrome *c* reductase, immobilization of spin-label was observed. Immobilization was indicated by the broadened spectrum and the appearance of low- and high-field signals. Titration of Q- and PL-depleted reductase with Q_0C_{10}TMPOC,

based on the appearance of a low-field signal, shows that depleted reductase binds 1 mole Q-derivative per mole of cytochrome *b* (Yu and Yu, 1982a).

Q_0C_{10}NBDHA

Binding of Q_0C_{10}NBDHA to succinate-cytochrome *c* reductase results in an enhancement of fluorescence and causes a blue shift of the emission maximum of about 10 nm. The emission maximum is at 540 nm when the sample is excited at 463 nm. Titration of intact or Q- and PL-depleted succinate-cytochrome *c* reductase with Q_0C_{10}NBDHA shows that maximal fluorescence is obtained when one mole of Q_0C_{10}NBDHA is bound per mole of cytochrome *b* (Yu and Yu, 1984). This simple binding stoichiometry further support the idea of a specific interaction between Q and reductase protein.

Fluorine-labelled Q Derivatives

Most recently we have used ^{19}F NMR to investigate the protein:Q interaction in succinate-cytochrome *c* reductase region. Three fluorine-substituted ubiquinone derivatives, 2,3-dimethoxy-6-(9′-fluorodecyl)-1,4-benzoquinone (9FQ), 2-methoxy-5-trifluoromethyl-6-decyl-1,4-benzoquinone (TFQ) and 2-methoxy-5-trifluoromethyl-6-(9′-fluorodecyl)-1,4-benzoquinone (9FTFQ), were synthesized (Yang *et al.*, 1991). 9FQ was synthesized by radical coupling of Q_0 and bis(10-fluoroundecanoyl)peroxide. The latter was prepared by fluorination of undecylenic acid followed by thionyl chloride treatment and peroxidation. TFQ was synthesized from 2,2,2-trifluoro-*p*-cresol by methylation, nitration, reduction, acetylation, nitration, reduction, oxidation, and radical alkylation. 9FTFQ was prepared by the radical alkylation of 2-methoxy-5-trifluoromethyl-1,4-benzoquinone with bis(10-fluoroundecanoyl)peroxide. All three fluoro-Q derivatives are active (>50% of the activity of Q_0C_{10}) when used as electron acceptors for succinate-Q reductase. However, only 9FQ is active when used as an electron donor for ubiquinol-cytochrome *c* reductase or as an electron mediator for succinate-cytochrome reductase. Since both TFQ and 9FTFQ are competitive inhibitors of ubiquinol-cytochrome *c* reductase, it is expected that these derivatives bind to the Q binding site. A ^{19}F NMR peak-broadening effect is observed with 9FQ when it is reconstituted with ubiquinone-depleted ubiquinol-cytochrome *c* reductase. A drastic up-field chemical shift is observed with TFQ when it is reconstituted with the ubiquinone-depleted reductase. These results indicate that the binding environments of the benzoquinone ring and the alkyl side chain of the Q molecule are different. The strong up-field chemical shift for TFQ, and the lack of significant chemical shift for 9FQ, suggest that the benzoquinone ring is bound near a paramagnetic cytochrome *b* heme. Table I summarizes various synthetic ubiquinone derivatives and their electron transfer activity.

IDENTIFICATION OF UBIQUINONE-BINDING PROTEINS (SUBUNITS)

By Arylazido-Q

An arylazido ubiquinone derivative, 2,3-dimethoxy-5-methyl-6{10-[4-(azido-2-nitroanilinopropionoxy)]-decyl}-1,4-benzoquinone (Q_0C_{10}NAPA) (Yu and Yu, 1982b), restores about 60% of the electron transfer activity to Q- and PL-depleted ubiquinol-cytochrome *c* reductase, compared to that restored by 2,3-dimethoxy-5-methyl-6-(10-bromodecyl)-1,4-benzoquinone (Q_0C_{10}Br). The restored activity is fully sensitive to illumination with long-wavelength UV light, suggesting that this arylazidoubiquinone derivative is bound to the ubiquinone site. Inhibition change during illumination parallels the amount of arylazido-Q derivative incorporated into the protein. The enzymatic activity of the illuminated reductase is restored to a maximum of 40%, by treatment with excess Q_0C_{10}Br. When the Q present in the reductase is not removed before the addition of Q_0C_{10}NAPA, very little inhibition is observed upon illumination, indicating that very little exchange takes place between Q_0C_{10}NAPA and the intrinsic Q. By reacting [^3H]-labeled Q_0C_{10}NAPA with depleted reductase, and performing sodium dodecyl sulfate–polyacrylamide gel electrophoresis after illumination, the radioactivity distribution among the subunits of ubiquinol-cytochrome *c* reductase is obtained. The most heavily labeled subunits are those with mobilities, relative to cytochrome *c*, of 0.475 and 0.841 in the Weber and Osborn system (Weber and Osborne, 1969). These were previously identified as the 37-kDa (42 kDa based on amino acid sequence) (cytochrome *b*) and 17-kDa (9.5 kDa based on amino acid sequence) proteins. Addition of phospholipids before illumination had little effect on the radioactivity distribution pattern. Treatment of the system with antimycin A or UHDBT, which inhibits the restored activ-

ity, also produced no significant effect on the labeling pattern, suggesting that these inhibitors are not bound to the same site as Q.

By Azido-Q

Although the use of [^{3}H]-arylazido Q has identified Q-binding proteins, the location of the photoactivatable group on the alkyl side chain of the Q-molecule and the partial restoration of activity of treated reductase by excess $Q_0C_{10}Br$ raise questions concerning the specific site on the protein that is covalently linked to the photoactivated Q. To address this shortcoming, we have synthesized and characterized a series of azido-ubiquinone derivatives with an azido group located at various positions on the benzoquinone ring (Yu *et al.*, 1985b). 3-Azido-2-methyl-5-methoxy-6-(3,7-dimethyloctyl)-1,4-benzoquinone (azido-Q) was found to be suitable for studying the specific interaction between Q and protein. It was synthesized with high specific radioactivity and used to identify the Q-binding proteins in purified ubiquinol-cytochrome *c* reductase. The azido-Q derivative restores activity to the Q- and PL-depleted ubiquinol-cytochrome *c* reductase in the dark. Azido-Q treated samples are completely inactivated by illumination, and the inactivation is not reversed by addition of Q_0C_{10} or other Q derivatives. This indicates that photoactivated azido-Q is covalently linked to the Q-binding site. Two protein subunits with MW = 42 kDa and 9.5 kDa were found to be heavily labeled when depleted ubiquinol-cytochrome *c* reductase was treated with the [^{3}H]azido-Q derivative followed by illumination and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Yu *et al.*, 1985b). These two proteins are referred to as QPc-cytochrome *b* and QPc-9.5 kDa. These two proteins are the same as those identified by Q_0C_{10}NAPA. The redox state of the azido-Q derivative has little effect on the distribution of radioactivity. The amount of radioactive labeling of QPc-9.5 kDa, but not of QPc-cytochrome *b*, is proportional to the degree of inactivation and is affected by the presence of phospholipids. Since the amount of radioactive labeling at QPc-9.5 kDa is affected by phospholipids and correlates with the enzymatic activity, this subunit is probably the specific Q-binding protein of the complex. The distribution of radioactivity among the subunits of ubiquinol-cytochrome *c* reductase is not affected by the presence of antimycin A or UHDBT, suggesting that the binding site(s) of these inhibitors are not primary Q-binding sites. The azido-Q derivative was also successfully used to identify Q-binding proteins in cytochrome bc_1 complexes from other sources (Yu *et al.*, 1986; Yang *et al.*, 1986, Yu and Yu, 1987b; Kriauciunas *et al.*, 1989).

IDENTIFICATION OF UBIQUINONE-BINDING DOMAINS (SITES):

Q-Binding Site (domain) on QPc-9.5 kDa

The smaller molecular mass ubiquinone-binding protein (QPc-9.5 kDa) was purified to homogeneity from [^{3}H]-azido-QH_2 labeled ubiquinol-cytochrome *c* reductase. The N-terminal amino acid sequence of the isolated protein is GRQFGHLTRVRH-, which is identical to that of a Mr = 9.5 kDa protein in the reductase (Borchart *et al.*, 1986). A tryptic ubiquinone-binding peptide was isolated, from trypsin digested labeled protein, by HPLC. The partial N-terminal amino acid sequence of this peptide, VAPPF-VAFYL-, corresponds to amino acid residues 48–57 in the Mr = 9.5 kDa protein (Usui *et al.*, 1990b). According to the proposed structural model for this protein, this azido-Q-labeled peptide is located in the membrane on the matrix side.

To study the topological arrangement of this Q-binding protein, antibodies against QPc-9.5 kDa protein was raised in rabbits. Purified antibodies against QPc-9.5 kDa have a high titer with isolated QPc-9.5 kDa protein and its complexes. Although antibodies against QPc-9.5 kDa do not inhibit intact succinate-cytochrome *c* reductase and ubiquinol-cytochrome *c* reductase, a decrease of 85% and 20% in the restoration of succinate-cytochrome *c* reductase and ubiquinol-cytochrome *c* reductase, respectively, is observed when delipidated succinate-cytochrome *c* reductase or ubiquinol-cytochrome *c* reductase are incubated with antibodies prior to reconstitution with Q and PL, 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 reconstituted succi-

Table I. Comparison of Electron Transfer Activity of Q-Derivatives

Derivative	Structure				E_m (mV)	Activities (%)		Reference
	R2	R3	R5	R6		As Acceptor for SQR	As Donor for QCR	
Q_0C_{10}	MeO	MeO	Me	Decyl	100	100	100	Yu and Yu, 1982
Q_0	H	H	H	H		0	0	Yu and Yu, 1982a
2-Me-Q_0C_{10}	Me	MeO	Me	Decyl		0	15	Gu *et al.*, 1990
3-Me-Q_0C_{10}	MeO	Me	Me	Decyl		0	14	Gu *et al.*, 1990
5-Me-PQ_0C_{10}	Me	Me	Me	Decyl		0	20	Gu *et al.*, 1990
5-MeO-PQ_0C_{10}	Me	Me	MeO	Decyl		0	23	Gu *et al.*, 1990
5-H-Q_0C_{10}	MeO	MeO	H	Decyl	67	114	90	Gu *et al.*, 1990
2-H-Q_0C_{10}	H	MeO	Me	Decyl	41	29	56	Gu *et al.*, 1990
3-H-Q_0C_{10}	MeO	H	Me	Decyl	40	0	26	Gu *et al.*, 1990
2-H-5-Me-PQ_0C_{10}	H	Me	Me	Decyl	41	32	22	Gu *et al.*, 1990
3-H-5-Me-PQ_0C_{10}	Me	H	Me	Decyl	68	0	20	Gu *et al.*, 1990
2-Me-5-H-Q_0C_{10}	Me	MeO	H	Decyl		18	25	Gu *et al.*, 1990
3-Me-5-H-Q_0C_{10}	MeO	Me	H	Decyl		9	23	Gu *et al.*, 1990
PQ_0C_{10}	Me	Me	H	Decyl	85	0	35	Gu *et al.*, 1990
3,5-di-H-Q_0C_{10}	MeO	H	H	Decyl		0	16	Gu *et al.*, 1990
2,5-di-H-Q_0C_{10}	H	MeO	H	Decyl		30	11	Gu *et al.*, 1990
5-H-2Me-Q_0C_{10}	Me	MeO	H	Decyl		53		Gu *et al.*, 1990
3-Cl-2-Me-5-OH-Q_0C_{10}	Me	Cl	OH	Decyl	47	–	–	Gu *et al.*, 1989
2-Cl-3-Me-5-OH-Q_0C_{10}	Cl	Me	OH	Decyl	63	–	±	Gu *et al.*, 1989
TFQ	MeO	MeO	CF_3	Decyl	200	58	0	Yang *et al.*, 1991
9GQ	MeO	MeO	Me	9′F-Decyl	100	95	98	Yang *et al.*, 1991
TF9FQ	MeO	MeO	CF_3	9′F-Decyl	200	55	0	Yang *et al.*, 1991
Ethoxy-Q	EtO	MeO	Me	Decyl	103	–	55	He *et al.*, 1992
Diethoxy-Q	EtO	EtO	Me	Decyl	105	–	15	He *et al.*, 1992
5-Ethyl-Q_0C_{10}	MeO	MeO	Ethyl	Decyl		49	52	He *et al.*, 1992
5-Propyl-Q_0C_{10}	MeO	MeO	Propyl	Decyl		9	32	Yu and Yu, 1982
5-Butyl	MeO	MeO	Butyl	Decyl		6	26	Yu and Yu, 1982
3-N_3-Q_0C_{10}	MeO	N_3	Me	Decyl	67	23		Yu *et al.*, 1985a
2-N_3-Q_2	N_3	MeO	Me	Geranyl	65	22		Yu *et al.*, 1985a
3-N_3-Q_2	MeO	N_3	Me	Geranyl	68	18		Yu *et al.*, 1985a
5-N_3-Q_2	MeO	MeO	N_3	Geranyl	113	39		Yu *et al.*, 1985a
2-N_3-3-Me-5-MeO-Q_2	N_3	Me	MeO	Geranyl	84	12	99	Yu *et al.*, 1985a
3-N_3-2-Me-5-MeO-Q_2	Me	N_3	MeO	Geranyl		12	99	Yu *et al.*, 1985a
5-N_3-PQ_2	Me	Me	N_3	Geranyl		5		Yu *et al.*, 1985a
3-NH_2-2-Me-5-MeO-Q_2	Me	NH_2	MeO	Geranyl		0		Yu *et al.*, 1985a
Q_0C_1	MeO	MeO	Me	Methyl		1	1	Yu *et al.*, 1985a
Q_0C_2	MeO	MeO	Me	Ethyl		7	2	Yu *et al.*, 1985a
Q_0C_3	MeO	MeO	Me	Propyl		12	4	Yu *et al.*, 1985a
Q_0C_4	MeO	MeO	Me	Butyl		30	12	Yu *et al.*, 1985a

Table I. Continued.

Derivative	Structure				E_m (mV)	Activities (%)		Reference
	R2	R3	R5	R6		As Acceptor for SQR	As Donor for QCR	
Q_0C_6	MeO	MeO	Me	Hexyl		98	40	Yu *et al.*, 1985a
$Q_0C_{2'4'}$	MeO	MeO	Me	Hexa-2′4′-dienyl		60	13	Yu *et al.*, 1985a
$1985Q_0C_7$	MeO	MeO	Me	Heptyl		100	62	Yu *et al.*, 1985a
$Q_0C_{72'4'}$	MeO	MeO	Me	Hepta-2′4′-dienyl		62	30	Yu *et al.*, 1985a
Q_0C_9	MeO	MeO	Me	Nonyl		100	96	Yu *et al.*, 1985a
$Q_0C_{92'4'}$	MeO	MeO	Me	Nona-2′4′-dienyl		62	85	Yu *et al.*, 1985a
Q_2	MeO	MeO	Me	Geranyl		100	100	Yu *et al.*, 1985a
Q_2s	MeO	MeO	Me	3′7′-Dimethyl-octyl		100	100	Yu *et al.*, 1985a
Q_2 triene	MeO	MeO	Me	3′7′-Dimethyl-octa-2′4′6′-trienyl		65	45	Yu *et al.*, 1985a
Qret-s	MeO	MeO	Me	Decahydroretinyl		52	79	Yu *et al.*, 1985a
Q_2ret	MeO	MeO	Me	Retinyl		17	9	Yu *et al.*, 1985a
Q_0C_10ret	MeO	MeO	Me	10′-Retinoylonyloxydecyl		35	75	Yu *et al.*, 1985a
Q_0C_{10}NAPA	MeO	MeO	Me	C_{10}NAPA		90	90	Yu and Yu, 1982a
Q_0C_{10}Br	MeO	MeO	Me	C_{10}Br		100	100	Yu and Yu, 1982a
Q_0C_{10}OH	MeO	MeO	Me	C_{10}OH		80	20	Yu and Yu, 1982a
Q_0C_{10}TMPOC	MeO	MeO	Me	C_{10}TMPOC		91	100	Yu and Yu, 1982a

[a] SQR, succinate-Q reductase; QCR, ubiquinol-cytochrome *c* reductase. The activities given are the maximum activities of each compound obtained through the concentration titration. The maximum activity was used as 100% in calculation. The symbols − and ± indicate inhibition and no effect, respectively.

nate-cytochrome *c* reductase, QPc-9.5 kDa may be involved at 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.

Q-Binding Sites (Domains) in the Cytochrome *b* Protein

To identify the Q-binding domain in cytochrome *b*, [^{3}H]-azido-Q-labeled cytochrome *b* protein was purified from a photoaffinity-labeled reductase complex by a combination of column chromatographic methods and preparative electrophoresis. SDS present in the electrophoretically eluted pure cytochrome *b* protein was removed by cold acetone precipitation. This pure cytochrome *b* protein was completely digested by chymotrypsin after reductive alkylation and succinylation. The chymotrypic peptides were separated by HPLC. The two peptides with the highest radioactivity were collected and subjected to the amino acid sequence analysis. One peptide (CP-142) has the amino acid sequence of GATVITNLLSAIPY, and the other (CP-336) has a sequence of ALVADLLTLTW. These two peptides correspond to amino acid residues 142 to 155 and 336 to 346 of cytochrome *b*. Monospecific polyclonal antibodies against these two peptides have been raised in rabbits. Both antibodies inhibit ubiquinol-cytochrome *c* reductase activity when incubated with the PL-depleted reductase prior to reconstitution with PL. These antibodies do not inhibit intact, lipid-sufficient ubiquinol-cytochrome *c* reductase. This suggests that these two Q-binding domains are buried or shielded by phospholipid. At present we do not have enough information to establish whether these two Q-binding peptides form a single binding domain or two separate domains, with or without help from other proteins. It would not be surprising if peptides other than the two analyzed, are involved in Q-binding.

INTERACTION OF UBIQUINOL-CYTOCHROME *c* REDUCTASE WITH OTHER COMPLEXES

Differential Scanning Calorimetry

In addition to Q:protein interaction in ubiquinol-cytochrome *c* reductase, interactions between this reductase and its neighboring complexes are also of interest. The interaction between succinate-Q reductase and ubiquinol-cytochrome *c* reductase in the purified, detergent-dispersed state and in embedded phospholipid vesicles was studied by differential scanning calorimetry and by electron paramagnetic resonance (Gwak *et al.*, 1986). When purified, detergent-dispersed succinate-Q reductase, ubiquinol-cytochrome *c* reductase, NADH-Q reductase, and cytochrome *c* oxidase undergo thermodenaturation, they show an endothermic transition. If an interaction between two complexes occurs, one expects that the enthalpy change of a mixture of two complexes differs from the sum of the enthalpy changes of the individual complexes upon thermodenaturation. This is indeed the case for the mixture of succinate-Q reductase and ubiquinol-cytochrome *c* reductase, but not for mixtures of ubiquinol-cytochrome *c* reductase and cytochrome *c* oxidase or ubiquinol-cytochrome *c* reductase and NADH-Q reductase. This suggests that succinate-Q reductase and ubiquinol-cytochrome *c* reductase form a supercomplex (Gwak *et al.*, 1986). To avoid possible complications resulting from the presence of detergent in the isolated complexes, the interaction study was repeated with reconstituted phospholipid vesicles. When these isolated electron-transfer complexes are embedded in phospholipid vesicles, they undergo exothermodenaturation. The energy released may result from the collapse of the strained interactions between unsaturated fatty acyl groups of phospholipids and exposed areas of the complex formed by the removal of interacting proteins. The exothermic enthalpy change of thermodenaturation of a protein-phospholipid vesicle containing both succinate-Q reductase and ubiquinol-cytochrome *c* reductase was smaller than that of a mixture of protein-PL vesicles formed from the individual electron-transfer complexes. Other combinations of electron transfer complexes did not show such a difference in the enthalpy change. This suggests specific interaction between succinate-Q reductase and ubiquinol-cytochrome *c* reductase in the membrane.

Saturation Transfer EPR of Spin-Labeled Ubiquinol-Cytochrome *c* Reductase

The interaction between succinate-Q and ubiquinol-cytochrome *c* reductase in phospholipid vesicles was further studied by saturation transfer electron paramagnetic resonance (ST-EPR) (Thomas *et al.*, 1976). Purified, detergent-dispersed ubiquinol-cytochrome *c* reductase is labeled with 3-maleimide-2,2,5,5-tetramethy-1-pyrrolidinyloxy prior to incorporation into phospholipid vesicles alone, or in combination with other complexes, and used for ST-EPR measurements. ST-EPR studies show that the rotational correlation time (τ_2) of spin-labeled ubiquinol-cytochrome *c* reductase increases when it is mixed with succinate-Q reductase prior to embedding in phospholipid vesicles indicating that these two complexes are indeed present in the membrane as a supercomplex. To ensure that the observed τ_2 increase, upon mixing succinate-Q reductase with spin-labeled ubiquinol-cytochrome *c* reductase, is indeed due to specific interaction between these two complexes and formation of a supercomplex, succinate-cytochrome *c* reductase, and is not due to a change of protein concentration or self-aggregation, a titration of spin-labeled ubiquinol-cytochrome *c* reductase with succinate-Q reductase was carried out. If a specific interaction between these two complexes exists, it is expected that a break point in the τ_2 will be obtained when the ratio of succinate-Q reductase to ubiquinol-cytochrome *c* reductase approaches 0.5, because an isolated succinate-cytochrome *c* reductase and in the mitochondrial inner membrane the ratio between succinate-Q reductase and ubiquinol-cytochrome *c* reductase has been shown to be 0.5. The titration data are exactly as predicted. This confirms that succinate-Q reductase and ubiquinol-cytochrome *c* reductase indeed form a supercomplex in the membrane.

No such supercomplex is detected between NADH-Q reductase and ubiquinol-cytochrome *c* reductase or between succinate-Q reductase and NADH-Q reductase. The status of interaction between ubiquinol-cytochrome *c* reductase and cytochrome *c* oxidase is rather unclear since the exothermic enthalpy change during thermodenaturation of a PL-vesicle containing both complexes was only slightly smaller than that of a mixture of vesicles formed from individual complexes. In a separate study, formation of a supercomplex between cytochrome *c* oxidase and ATP synthase was clearly demonstrated (Qiu *et al.*, 1992).

THE ROLE OF UBIQUINONE AND IRON–SULFUR CLUSTER IN PROTON TRANSLOCATION

Proton Pumping

Although the popular Q-cycle mechanism of ubiquinol-cytochrome *c* reductase supports the direct ligand conduct ion mechanism of proton translocation and predicts a fixed proton/electron ratio of 2, ratios different from 2 have been reported by many laboratories. The deviation of the H^+/e^- ratio from that predicted by the Q-cycle mechanism questions the direct ligand conduction mechanism of proton translocation and favors the redox-linked proton pumping mechanism. The pH dependent redox potential of cytochrome *b* and the iron–sulfur cluster are consistent with the proton pumping hypothesis (Papa *et al.*, 1982).

The Role of the Iron–Sulfur Proton Translocation

Purified ubiquinol-cytochrome *c* reductase is very stable in aqueous solution; it suffers little damage upon illumination with visible light under aerobic or anaerobic conditions. However, it is rapidly inactivated when the photosensitizer, hematoporphyrin, is present during illumination (Miki *et al.*, 1991). The hematoporphyrin-promoted photoinactivation is dependent on sensitizer dose, illumination time, and oxygen. Singlet oxygen has been shown to be the destructive agent in this system. Photoinactivation of ubiquinol-cytochrome *c* reductase is prevented by excess exogenous ubiquinone, regardless of its redox state. This protective effective is not due to protein-Q interactions but to the singlet oxygen scavenger property of Q. Q also protects against hematoporphyrin-promoted photoinactivation of succinate-Q reductase and cytochrome *c* oxidase. The photoactivation site in ubiquinol-cytochrome *c* reductase is the iron–sulfur cluster of Rieske's protein. Two histidine residues, presumably serving as two ligands for the iron–sulfur cluster (Gurbiel *et al.*, 1989), are destroyed. No polypeptide bond cleavage is detected. Photoinactivation has little effect on the spectral properties of cytochromes *b* and c_1 but alters their reduction rates substantially. Also, it causes the formation of proton-

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                                                  GAATTCGGGGGTGACCTCGAGGCTGCGGTGACC ATG
                                                                                     met
1                                                 11
GGC CGC CAG TTT GGG CAT CTG ACA CGG GTG CGG CAT GTG ATC ACC TAC AGC TTG TCG CCC
gly arg gln phe gly his leu thr arg val arg his val ile thr tyr ser leu ser pro
21                                                31
TTC GAG CAG CGC GCC TTC CCG CAC TAC TTC AGC AAG GGC ATC CCC AAC GTT CTG CGC CGA
phe glu gln arg ala phe pro his tyr phe ser lys gly ile pro asn val leu arg arg
41                                                51
ACT CGG GCG TGC ATC CTT CGC GTC GCG CCG CCG TTC GTA GCG TTT TAT CTT GTC TAC ACA
thr arg ala cys ile leu arg val ala pro pro phe val ala phe tyr leu val tyr thr
61                                                71
TGG GGA ACG CAG GAG TTT GAG AAA TCG AAG AGG AAG AAT CCA GCT GCC TAT GAA AAT GAC
trp gly thr gln glu phe glu lys ser lys arg lys asn pro ala ala tyr glu asn asp
81
AGA TAA GCAGCTCATCTGGATAATGGTTCCTTGTCTCTGAAAGACCCTTCTCTGGGAGAGGAGTGTATATTGTATGG.
arg  *
TCTTGAAGACACAATAAACTTATGGGCTTCACTGTTGTGATTTTGTGTGTTGTGTAAGCAGAGTCCCTTGGAATGTGAC
TGCCAGTTTCAAGTTCATAAACTCCCCTCCCCAGCATGATTGCTGAACCATTTTGGCTAGTCTGAGTTTGGGAAGGAGT.
GTGGCTCACCCCTTGTTCTGTAATCTACAAGACTGTAGCTGCAACTGAACAGTTGTGGGTGGCATCAACTGTAGGGAAT.
GTTAACTCCCTGCCCACTGCTTCCATATTGACAATAAAATCTAGTAATTACTGTGTCAGGTAAAAAAAAAAAAAAAAAA.
AAAAACCCGAAT
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Fig. 4. The nucleotide and deduced amino acid sequences of QPc-9.5 kDa.

leaking channels in the complex. When the photoinactivated reductase is co-inlaid with intact ubiquinol-cytochrome *c* reductase or cytochrome *c* oxidase in a phospholipid vesicle, no proton ejection can be detected during the oxidation of their respective substrates. These results suggest that the Rieske iron–sulfur protein plays an important role in the proton pumping of ubiquinol-cytochrome *c* reductase. Whether or not the iron–sulfur cluster is involved directly remains to be investigated.

MOLECULAR CLONING AND AMINO ACID SEQUENCING

Amino acid sequences of all the subunits of ubiquinol-cytochrome *c* reductase have been determined, mostly by amino acid sequencing. The sequences of cytochrome *b* and two core proteins were deduced from nucleotide sequencing. For further structure and function studies of ubiquinol-cytochrome *c* reductase, we have recently cloned the Rieske iron–sulfur protein and the low molecular-weight Q-binding protein by screening a bovine heart *c*DNA expression library in λgt11 (from Promega) with antisera directed against the isolated proteins. The mature iron–sulfur protein is composed of 196 amino acid residues, and there is a presence of 73 residues. The sequence deduced from the *c*DNA sequence is identical to that determined chemically, with the exception of Ser-72 and Asp-191 (Usui et al., 1990a).

The *c*DNA insert in the QPc-9.5 kDa clone was 683 base pairs with an open reading frame of 246 base pairs encoding for an 82-amino acid protein. The *c*DNA sequence and deduced amino acid sequence of QPc-9.5 kDa are shown in Fig. 4. This sequence of QPc-9.5 kDa, deduced from nucleotide sequencing, is the same as that obtained by protein sequencing (Borchart *et al.*, 1986) except for residue No. 6, which is tryptophan instead of cysteine. This protein has no presequence.

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Note added in proof: In collaboration with Drs. Di Xia and Johan Deisenhofer, recently we have examined ubiquinol-cytochrome *c* reductase crystals using rotation camera at Stanford Synchrotron Radiation Laboratory. Crystals grown in 50 mM MES buffer diffract x-ray to 4 Å resolution at 4°C. Crystals grown in agarose gel show the same x-ray diffractibility.