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Three Molecules of Ubiquinone Bind Specifically to Mitochondrial Cytochrome bc_1 Complex*[S]

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Bifurcated electron flow to high potential "Rieske" iron-sulfur cluster and low potential heme b_L is crucial for respiratory energy conservation by the cytochrome bc_1 complex. The chemistry of ubiquinol oxidation has to ensure the thermodynamically unfavorable electron transfer to heme b_L. To resolve a central controversy about the number of ubiquinol molecules involved in this reaction, we used high resolution magic-angle-spinning nuclear magnetic resonance experiments to show that two out of three *n*-decyl-ubiquinones bind at the ubiquinol oxidation center of the complex. This substantiates a proposed mechanism in which a charge transfer between a ubiquinol/ubiquinone pair explains the bifurcation of electron flow.

A central question in membrane biochemistry is how cofactors interact with membrane proteins. Here we introduce a general NMR-based method to quantify the stoichiometry of binding of hydrophobic cofactors to membrane proteins to resolve a central controversy about the number of ubiquinone binding sites in mitochondrial cytochrome bc_1 complex. The cytochrome bc_1 complex plays a crucial role in oxidative phosphorylation, a universal process that converts most of the energy provided by foodstuffs into the general energy source adenosine 5′-triphosphate (ATP). Within this process, the cytochrome bc_1 complex connects hydrophobic ubiquinol and water-soluble cytochrome *c*, transferring electrons between these two freely diffusible intermediates and thereby linking the exergonic reaction to a vectorial proton translocation across the inner mitochondrial membrane. Molecular structures of the cytochrome bc_1 complex from different sources (1–4) are fully consistent with the electron transfer scheme of the protonmotive ubiquinone cycle proposed earlier (5–7). The reaction most critical for energy conservation is an obligatory bifurcation of the electron path linked to the two-electron oxidation of ubiquinol (Fig. 1). Molecular structures indicate that this unique reaction occurs in a rather spacious (Q_o or Q_P)[1] pocket formed mostly by transmembrane cytochrome *b* and the tip of the mobile hydrophilic domain of the "Rieske" iron-sulfur protein (1–4). As predicted by enzymological studies (8), methoxyacrylate-type inhibitors like myxothiazol and the chromone-type inhibitor stigmatellin were found to bind with very high affinity to different but overlapping sites within this pocket (9). In crystal structures, bound ubiquinone could only be seen in the ubiquinone reduction (Q_i or Q_N) center facing the opposite side of the inner mitochondrial membrane (2, 4). Presumably because of very weak binding of the substrate, no corresponding electron density could be identified in the ubiquinol oxidation pocket. It is still a controversial issue whether ubiquinol oxidation in the cytochrome bc_1 complex involves just a single quinone that may have to move to transfer the second electron (6, 10) or whether two quinone molecules occupy this binding pocket simultaneously (11) and facilitate bifurcated electron flow (12) (Fig. 1*B*). Double occupancy of the ubiquinol oxidation pocket was proposed by Ding *et al.* (11) based on specific line shape changes in the EPR spectrum of the reduced Rieske iron-sulfur cluster of bacterial cytochrome bc_1 complex. However, Crofts *et al.* (10) proposed that the different line shapes may also reflect different states of the complex or different positions of the ubiquinone headgroup. Resolving this issue will be a prerequisite to understand the chemistry of this unique reaction.

EXPERIMENTAL PROCEDURES

Synthesis of ^{13}C-Labeled Ubiquinone—[^{13}C]Ubiquinone was synthesized anaerobically according to the method described for the synthesis of ethoxyubiquinone derivatives (13). The reaction was carried out in a Thunberg tube with a two-arm stopper. 1.5 ml of hexane solution containing 100 μl of [^{13}C]methanol and 1 mg of sodium methoxide was placed in the bottom of the tube. 10 mg of Q_0C_{10} (2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone) in 0.5 ml of hexane was placed in one arm of the stopper. 20 μl of 10 N acetic acid was placed in the other arm of the stopper. The assembly was then subjected three times to evacuation and argon flushing. The Q_0C_{10} solution was then carefully tipped into [^{13}C]methanol/methoxide solution. This mixture was incubated at room temperature for 2 h in the dark with constant shaking. At

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[S] The on-line version of this article (http://www.jbc.org) contains a table.

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[1] The abbreviations used are: Q, ubiquinone; Q_0C_{10}, 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone; Mops, 4-morpholinepropanesulfonic acid; HR-MAS, high resolution magic angle spinning; HSQC, heteronuclear single quantum correlation.

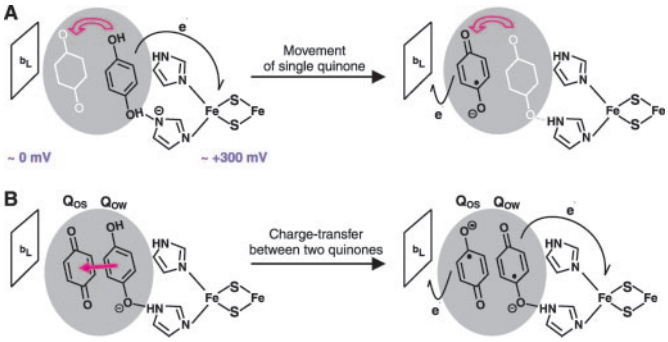

FIG. 1. **Involvement of one or two ubiquinones in bifurcated electron flow at the ubiquinol oxidation site of the cytochrome bc_1 complex.** The mechanism of ubiquinol oxidation has to ensure the divergent reduction of the high potential Riske iron-sulfur cluster (Fe_2S_2) and the low potential heme b_L. *Curved black arrows* indicate electron transfer. *A*, single quinone model, involving one ubiquinol molecule and a mobile semiquinone intermediate (10) with the unoccupied alternate binding site highlighted in *white* and a *curved red arrow*, indicating the direction of the movement from the Riske cluster toward heme b_L. *B*, two-quinone model involving a tightly bound ubiquinone (Q_{os}) and a weakly bound ubiquinol (Q_{ow}) that cooperate by charge transfer (*red arrow*) during catalysis (11, 12).

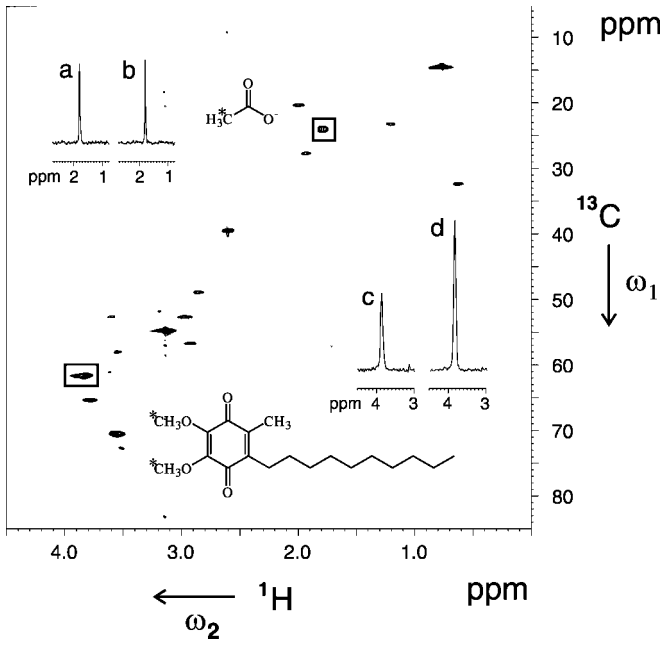

FIG. 2. **HR-MAS-NMR measurements of free ubiquinone in proteoliposomes.** Two-dimensional ^{1}H-^{13}C HSQC NMR spectrum of a 60-μl suspension of membrane-bound cytochrome bc_1 complex (0.23 mM) in D_2O (16–22). The spectrum was acquired at 298 K with a 1.5-s relaxation delay (total experimental time of 4 h) on a Bruker DRX600 instrument equipped with a ^{1}H/^{13}C/^{15}N HR-MAS probe and HR-MAS control unit. The spinning rate was 5 kHz. Sodium [^{13}C]acetate was added as standard at a concentration of 1.5 mM. The well resolved signals (*boxed*) of [^{13}C]Q_0C_{10} (3.9 ppm/61 ppm) and [^{13}C]acetate (1.8 ppm/24 ppm) were referenced to the [^{13}C]acetate signal and integrated by means of the XWINNMR software (Bruker). All other signals are due to the natural abundance of 1.1% ^{13}C in the proteoliposomes. Characteristic traces along ω_2 of the ^{13}C-labeled methyl groups of acetate (*a*, *b*) and ubiquinone (*c*, *d*) are shown as *insets*. *a* and *c*, without inhibitors; *b* and *d*, with added antimycin and stigmatellin. The concentration of [^{13}C]Q_0C_{10} was 2.1 mM, as evaluated by integration to determine the volume under its cross-peak relative to that under the cross-peak from sodium acetate present at known concentration. Corrected integrals $I^{corrected}$ were obtained from the measured integrals $I^{measured}$ according to $I^{corrected} = I^{measured}/(1 - e^{-\Delta/T_1})$ with T_1 values for ubiquinone of 0.75 s and acetate of 3.43 s (determined by using the inversion recovery method (29) and the repetition delay of the HSQC pulse sequence including the acquisition time Δ).

the end of incubation the mixture was acidified by tipping in the acidic acid. The acidified mixture was concentrated under vacuum, redissolved in 0.3 ml of hexane, and subjected to thin-layer chromatography (TLC) separation. The TLC plate was developed with a mixture of hexane:ether (3.5:1.0). The yield of the synthesis was 75%. The pattern of ^{13}C labeling was analyzed by mass spectroscopy. 22.6% of the ubiquinone molecules were found to carry two, 57.9% one, and 19.5% no ^{13}C-methoxy group, corresponding to an average of 1.0 ^{13}C-atom per molecule.

Preparation of Cytochrome bc_1 Complex—Cytochrome bc_1 complex proteoliposomes were prepared by the cholate dialysis method essentially as described in Ref. 15. 1 g of a mixture of 75% phosphatidylcholine (99% Sigma Type III-E), 20% phosphatidylethanolamine (98% Sigma Type IV-S), and 5% cardiolipin (>80% bovine heart) was dissolved in 26 ml of 3% sodium cholate, 1% octyl glucoside, 100 mM KCl, 2 mM EDTA, 2 mM NaN_3, 20 mM K^+/Mops, pH 7.2, by sonication. 40–50 ml of a 20 μM solution of cytochrome bc_1 complex in 10% glycerol was added to the dissolved lipids. After stirring for 15 min on ice, the mixture was placed into a dialysis tube and dialyzed overnight against a 100-fold volume of 100 mM KCl, 2 mM EDTA, 2 mM NaN_3, 20 mM K^+/Mops, pH 7.2. The dialysis buffer was changed once after 4 h. The proteoliposomes were sedimented by centrifugation for 5–6 h at 50,000 $\times$ g_{av}; the red pellet was resuspended in 50 ml of dialysis buffer made with D_2O, and the liposomes were sedimented again by overnight centrifugation at 50,000 $\times$ g_{av}. The concentration of cytochrome bc_1 complex was determined spectroscopically using $\epsilon_{562-575}$ = 28.5 cm^{-1} mM^{-1} for the sum of two heme *b* groups per monomer of cytochrome bc_1 complex.

Experimental Set-up for HR-MAS Measurements—As a novel approach to directly measure the binding of an extremely hydrophobic, but weakly bound ligand to a membrane protein complex, we have used high resolution magic-angle-spinning nuclear magnetic resonance (HR-MAS NMR) spectroscopy of the liquid heterogeneous system composed of water, liposomes containing cytochrome bc_1 complex, and ubiquinone to determine the binding stoichiometry of [^{13}C]6-*n*-decylubiquinone to membrane-bound cytochrome bc_1 complex. We used a mixture of unlabeled and labeled 6-*n*-decylubiquinone ([^{13}C]Q_0C_{10}, Fig. 2) carrying one ^{13}C-methoxy group at C-2 or C-3 or two ^{13}C-methoxy groups at C-2 and C-3 of the quinone ring (13). Since the ubiquinone diffused freely in the phospholipid bilayer that was not immobilized, *e.g.* by freezing or orientation between glass plates as used in solid state NMR investigations, the labeled methyl groups not only had identical ^{1}H and ^{13}C chemical shifts but also gave rise to sharp resonances (proton line width of 7 Hz) under HR-MAS conditions (Fig. 2). Solid state MAS on frozen liposomes was not chosen for this investigation, since double labeled ubiquinone would have been necessary for the suppression of the protein background, and the experiments would have suffered from 10 times lower sensitivity. In the HR-MAS measurements, the enzyme-bound ubiquinones did not contribute to the NMR signal, because immobilization by the large integral membrane protein complex caused the signal to broaden beyond detection, since cytochrome bc_1-bound ubiquinones assume the correlation time of the membrane protein in the liposome, which is of the order of microseconds. Thus, by displacing the cytochrome bc_1 complex-bound ubiquinones with specific inhibitors, the number of bound ubiquinones can be inferred from the increase of the NMR signal.

Cytochrome bc_1 complex isolated from bovine heart mitochondria (14) was reconstituted into unilamellar proteoliposomes at a molar ratio of 3000–4000 lipids per cytochrome bc_1 complex dimer (15). Signals were calibrated using [^{13}C]acetate as an internal standard (*cf.* Fig. 2) that remains in the water phase and neither interacts with the lipid membrane nor with the cytochrome bc_1 complex. Calculated concentrations took into account corrections for differences in the NMR T_1 relaxation times of the nuclei giving rise to the signals from ubiquinone and acetate (see Fig. 2). Controls using liposomes without protein (not shown) confirmed that it was possible to calculate the concentration of the mobile, unbound species from the integral of the [^{13}C]Q_0C_{10} signal in a two-dimensional HR-MAS heteronuclear single-quantum correlation (HSQC) experiment (16–22). To assess unspecific binding, we measured immobilization of ubiquinone by reconstituted bovine heart cytochrome *c* oxidase (23), a membrane-bound complex of similar size but not containing a ubiquinone binding site. Unspecific binding was significant in the millimolar range and increased with the concentration of ubiquinone added to the sample. However, the extent of unspecific binding was very similar for cytochrome *c* oxidase and cytochrome bc_1 complex (with the specific sites blocked, see below), suggesting that it was largely due to weak association of ubiquinone molecules with the membrane domain of the complexes.

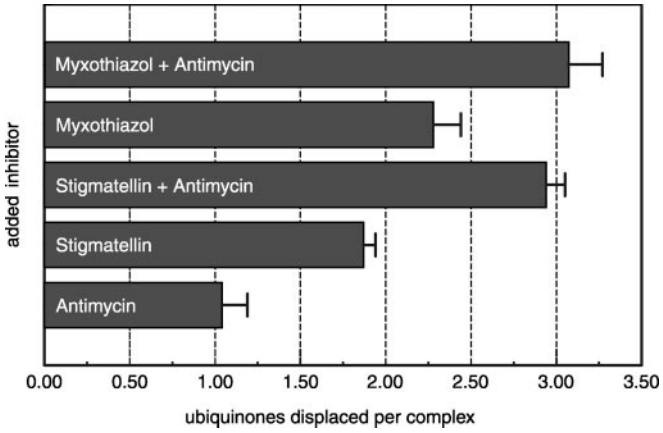

FIG. 3. **Stoichiometry of specific [^{13}C]6-*n*-decylubiquinone binding to cytochrome bc_1 complex as determined by inhibitor displacement.** As an internal standard, 1.5 mM ^{13}C-labeled sodium acetate in D_2O was added to freshly prepared proteoliposomes. [^{13}C]Q_0C_{10} was added from a stock solution in dimethyl sulfoxide. This solvent was found not to affect the NMR measurement or binding at the level added. The desired amount of the inhibitor(s) was placed as an ethanolic stock solution into a dry tube. The solvent was evaporated before [^{13}C]ubiquinone (1.2–3.6 mM), and acetate-containing proteoliposomes (0.15–0.29 mM cytochrome bc_1 complex) were added. The final sample volume was adjusted to 85 μl, of which 60 μl were used in the HR-MAS NMR measurements (see Fig. 2). Inhibitors were added at the following final concentrations (the number of measured samples is given in *parentheses*): antimycin, 0.3 mM (2), 0.8 mM (2), 1.5 mM (1); stigmatellin, 0.3 mM (1), 0.3 mM + 1 mM sodium ascorbate (2), 0.8 mM; myxothiazol, 0.3 mM (2), 1.5 mM (2); antimycin + stigmatellin (each), 0.3 mM + 1 mM sodium ascorbate (2), 0.7 mM + 1 mM sodium ascorbate (1), 0.8 mM; antimycin + myxothiazol (each), 0.3 mM (2), 1.5 mM (2). Variation in inhibitor concentrations within these ranges and addition of sodium ascorbate in the case of stigmatellin had no effect on the stoichiometry of ubiquinone release from the cytochrome bc_1 complex. The *error bars* give the S.D. of all measurements listed for a given inhibitor or combination of inhibitors.

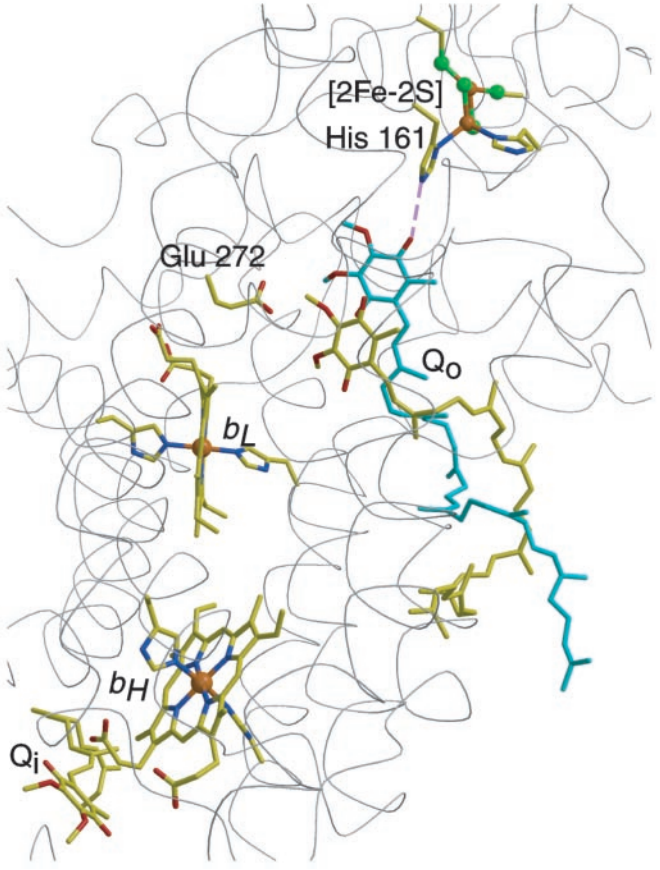

FIG. 4. **Two ubiquinone molecules fit into the ubiquinol oxidation pocket of the cytochrome bc_1 complex.** Shown are the Cα traces of the cytochrome *b* and the Rieske subunits, the [2Fe-2S] cluster, the cytochrome *b* heme groups b_L and b_H, and their ligands as well as the ubiquinone molecule at the reduction (Q_i) center and the ubiquinone-7 models at the ubiquinol oxidation (Q_o) center. A possible hydrogen bond is indicated by a *violet line*. According to Crofts *et al.* (10) (*cf.* Fig. 1), glutamine 272 changes its conformation upon movement of the ubiquinone. The figure was prepared with a version of MolScript (30) modified to enable color ramping (31). PDB entry 2BCC (32) was used as the starting structure for modeling of the chicken enzyme. At the ubiquinol oxidation pocket, the inhibitor stigmatellin was replaced by a pair of ubiquinone-7 molecules, and the system was subjected to energy minimization and molecular dynamics simulations at 300 K using the program CNS (33).

RESULTS AND DISCUSSION

To determine the number of specific ubiquinone binding sites of cytochrome bc_1 complex, we used high affinity inhibitors (24) with precisely known binding sites determined by x-ray crystallography (9) as well defined competitors. Counting the number of ubiquinone molecules per cytochrome bc_1 complex displaced by these highly specific inhibitors in the presence of saturating concentrations of ubiquinone avoided interference by nonspecific immobilization of ubiquinone by the cytochrome bc_1 complex. Unspecific binding was always observed at the high concentrations of ubiquinone necessary to saturate the rather weak ubiquinone binding sites of the cytochrome bc_1 complex under conditions of the NMR experiment and made direct quantitative binding studies impossible (not shown).

For the ubiquinone reduction site, x-ray structures show that a single ubiquinone shares a common binding pocket with the inhibitor antimycin (2, 4). Competition was used to validate our approach. We added [^{13}C]Q_0C_{10} (1.2–3.6 mM) to cytochrome bc_1 complex (0.15–0.29 mM) in proteoliposomes; antimycin (0.3–1.5 mM) displaced 1.04 ± 0.15 mol of ubiquinone from each mole of cytochrome bc_1 complex as expected from the molecular structure (Fig. 3). It should be stressed that this ratio was calculated using independently determined concentrations in the sample for the cytochrome bc_1 complex via UV-visible spectroscopy and for ubiquinone via referencing of the NMR integral to the NMR integral of acetate. Stigmatellin, a chromone-type inhibitor of the ubiquinol oxidation site, was found to displace 1.87 ± 0.07 mol of ubiquinone/mol of cytochrome bc_1 complex. If both antimycin and stigmatellin where added to the cytochrome bc_1 complex proteoliposomes, 2.94 ± 0.11 mol/mol were released. Stigmatellin is known to bind with much higher affinity to the ubiquinol oxidation pocket when the Rieske iron-sulfur protein is reduced (24). However, reducing this redox center by sodium ascorbate prior to the competition experiment had no effect on the displacement stoichiometries. In the presence of the *E*-β-methoxyacrylate inhibitor myxothiazol, a somewhat higher displacement ratio of 2.28 ± 0.16 mol/mol was measured, but antimycin plus myxothiazol again displaced only 3.07 ± 0.20 mol/mol. A possible explanation for the slight difference between the stoichiometries with stigmatellin and myxothiazol alone is that in contrast to stigmatellin, myxothiazol may have a weak affinity for the ubiquinone reduction site and could therefore partially displace ubiquinone in competition with antimycin. This seems feasible as other *E*-β-methoxyacrylate inhibitors have been shown to be inhibitors of the plastoquinone reduction site of plastidial cytochrome b_6f complex (25). None of the found stoichiometries was affected upon variation of the ubiquinone concentrations from 1.6 up to 3.6 mM or upon variation of the inhibitor concentrations from 0.33 up to 1.5 mM (see Fig. 3 for details). When less than 1.6 mM ubiquinone was present, lower stoichiometries were observed for stigmatellin and myxothiazol, but not for antimycin (not shown), indicating incomplete saturation of the ubiquinol oxidation (Q_o or Q_P) site

and somewhat tighter binding of ubiquinone to its reduction (Q_i or Q_N) center. This is in agreement with ubquinone occupancy in molecular structures (2) and seems characteristic for mitochondrial cytochrome bc_1 complex. It should be noted however that in the bacterial enzyme Q_{oS} (*cf.* Fig. 1) was reported to have the highest affinity (11).

Our results clearly indicate that a total of three ubiquinones bind specifically to mitochondrial cytochrome bc_1 complex: one binds at the ubiquinone reduction center and is displaced by antimycin, and two bind at the ubiquinol oxidation center and are displaced by stigmatellin and myxothiazol. To test whether this finding is in accordance with structural data obtained by x-ray crystallography, we modeled two ubiquinone molecules carrying a long isoprenoid side chain that for technical reasons could not be used in the experiments into the ubiquinol oxidation pocket of the cytochrome bc_1 complex (Fig. 4). Two ubiquinone molecules could be accommodated by changes of the order of 1.5 Å in the atomic positions of a few neighboring amino acid residues. Movements on this scale are only slightly larger than those that have been observed experimentally for the removal of ubiquinone from the reaction center from *Rhodopseudomonas viridis* (26). Fig. 4 shows one set of possible conformations of the two quinones in the ubiquinol oxidation site; other conformations are possible.

Our equilibrium binding approach, using high concentrations of ubiquinone and cytochrome bc_1 complex, inherently provides no information on the functional meaning of the binding of two ubiquinone molecules at the ubiquinol oxidation center. However, our finding is in perfect agreement with the "double occupancy Q_o site model" by Ding *et al.* (11, 27), suggesting a functional role for two ubiquinones. In this complementary study, specific line shape changes in the EPR spectrum of the reduced Rieske iron-sulfur cluster were interpreted as reflecting the presence of two functionally interacting ubiquinone species in the ubiquinol oxidation pocket of bacterial cytochrome bc_1 complex. However, the indirect way in which ubiquinone binding was monitored in this approach allowed alternative interpretations of the data. Crofts and colleagues (10) proposed that the different line shapes may also reflect different states of the complex or different positions of the ubiquinone headgroup. However, our compelling result that two ubiquinone molecules bind to the ubiquinol oxidation pocket and are specifically displaced by inhibitors of this site corroborates the interpretation of Ding and colleagues. Together, both approaches provide strong support for the functional implications that have been based on the "double occupancy model" (11, 28). In particular, charge transfer between ubiquinone and ubiquinol molecules, as implemented in the "proton-gated charge-transfer" mechanism (28), appears as a chemically attractive paradigm for the role of a ubiquinone pair in bifurcated electron flow at the ubiquinol oxidation center of the cytochrome bc_1 complex.

The HR-MAS approach presented may be widely employed to study binding of hydrophobic cofactors to membrane proteins. It should be useful for the analysis of ubiquinone binding to other respiratory chain complexes such as complex I and ubiquinol oxidase, for which specific inhibitors are available but the binding stoichiometries are uncertain. The method may develop into a general procedure for analyzing the binding of hydrophobic ligands to membrane bound proteins. If tighter binding reduces the problem of unspecific binding, quantitative binding studies with no need to use inhibitors will also be possible.

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