Three molecules of ubiquinone bind specifically to mitochondrial cytochrome $bc_1$ complex

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Running title: Ubiquinone Binding Sites in Cytochrome $bc_1$ Complex
Summary

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 unfavourable 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.
Introduction

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 proton-motive 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 $Q_P$) 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. 1B). 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 and colleagues proposed that the different line shapes may also reflect different states of the complex or different positions of the ubiquinone headgroup (10). Resolving this issue will be a prerequisite to understand the chemistry of this unique reaction.

Experimental procedures

Synthesis of $^{13}$C-labelled ubiquinone

$^{13}$C-ubiquinone was synthesized anaerobically according to the method described for the synthesis of ethoxy-ubiquinone 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 sodium methoxide was placed in the bottom of the tube. 10 mg of $Q_{10}$ (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 $Q_{10}$ solution was then carefully tipped into $^{13}$C-methanol/methoxide solution. This mixture was incubated at room temperature for 2 hours in the dark with constant shaking. At 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 (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 minutes 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 NaN₃, 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 50000 x gav; the red pellet was resuspended in 50 ml dialysis buffer made with D₂O, and the liposomes were sedimented again by overnight centrifugation at 50000 x gav. The concentration of cytochrome bc₁ complex was determined spectroscopically using $\varepsilon_{562-575} = 28.5$ cm⁻¹mM⁻¹ for the sum of two heme b groups per monomer of cytochrome bc₁ 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 heterogenous system composed of water, liposomes containing cytochrome bc₁ complex and ubiquinone to determine the binding stoichiometry of $^{13}$C-6-n-decyl-ubiquinone to membrane bound cytochrome bc₁ complex. We used a mixture of unlabeled and labeled 6-n-decyl-ubiquinone ($^{13}$C-Q₀C₁₀, 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 linewidth of 7 Hz) under HR-MAS conditions (Fig. 2). Solid state MAS on frozen liposomes was not chosen for this investigation since double labelled 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 bc₁ bound ubiquinones assume the correlation time of the membrane protein in the liposome which is of the order of μs. Thus, by displacing the cytochrome bc₁ 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 dimmer (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$_0$C$_{10}$ 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.

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 non-specific 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 the approach: We
added $^{13}$C-Q$_{0}$C$_{10}$ (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 moles 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/VIS 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 mole ubiquinone per mole 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-ß-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-ß-methoxyacrylate inhibitors have shown to be inhibitors of the plastoquinone reduction site of plastidial cytochrome $b_{6}f$ 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 of 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 ubiquinone 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$_{ow}$ (cf. Fig. 1) was reported to have the highest affinity (11).

Our results clearly indicate that a total of three ubiquinones binds specifically to mitochondrial cytochrome $bc_{1}$ complex: One binds at the ubiquinone reduction center and is displaced by antimycin, 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 bc₁ 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). Figure 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 bc₁ 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 Qₐ site model” by Ding et al. suggesting a functional role for two ubiquinones (11;27). 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 bc₁ 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 bc₁ 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, also quantitative binding studies with no need to use inhibitors will be possible.

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Figure Legends

**Fig. 1:** Involvement of one or two ubiquinones in bifurcated electron flow at the ubiquinol oxidation site of the cytochrome bc₁ complex. The mechanism of ubiquinol oxidation has to ensure the divergent reduction of the high potential ‘Riske’ iron-sulfur cluster (Fe₂S₂) and the low potential heme b₅. 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 towards heme b₅; (B) Two quinone model involving a tightly bound ubiquinone (QoS) and a weakly bound ubiquinol (Qow) that cooperate by charge-transfer (red arrow) during catalysis (11;12).

**Fig. 2:** HR-MAS-NMR-measurements of free ubiquinone in proteoliposomes. Two-dimensional [H]⁻¹⁻³C heteronuclear single-quantum correlated (HSQC) NMR-spectrum of a 60 µl suspension of membrane bound cytochrome bc₁ complex (0.23 mM) in D₂O (16-22) The spectrum was acquired at 298 K with a 1.5 s relaxation delay (total experimental time of 4 hours) on a Bruker DRX600 instrument equipped with a [H]/⁻¹⁻³C⁻¹⁵N-HR-MAS probe and HR-MAS control unit. The spinning rate was 5 kHz. Na⁻¹⁻³C-acetate was added as standard at a concentration of 1.5 mM. The well resolved signals (boxed) of [C]⁻¹⁻³C⁻⁻₀C₁₀ (3.9 ppm / 61 ppm) and Na⁻¹⁻³C-acetate (1.8 ppm / 24 ppm) were referenced to the Na⁻¹⁻³C-acetate signal and integrated by means of the XWINNMR software (Bruker). All other signals are due to the natural abundance of 1.1% ¹³C in the proteoliposomes. Characteristic traces along ω₂ of the ¹³C labelled methyl groups of acetate (a,b) and ubiquinone (c,d) are shown as insets. (a,c) without inhibitors; (b,d) with added antimycin and stigmatellin. The concentration of [C]⁻⁻₀C₁₀ was 2.1 mM, as evaluated by integration to determine the volume under its cross peak relative to that under the cross peak from Na 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⁻ᵀ₁ / ∆) with T₁ 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 ∆.
Fig. 3: Stoichiometry of specific $^{13}$C-6–n-decyl-ubiquinone binding to cytochrome bc$_1$ complex as determined by inhibitor displacement. As an internal standard, 1.5 mM $^{13}$C sodium-acetate in D$_2$O was added to freshly prepared proteoliposomes. $^{13}$C-Q$_{0}$C$_{10}$ was added from a stock solution in dimethylsulfoxide. 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 brackets): 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 standard deviation 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 C$\alpha$ 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 oxodation (Q$_o$) center. A possible hydrogen bond is indicated by a purple 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 300K using the program CNS (33).
A

Movement of single quinone

~ 0 mV

~ +300 mV

B

Charge-transfer between two quinones

$Q_{os}$ $Q_{ow}$
**Supplementary Data**

Individual data used for Fig. 3:

| $^{13}$C-$Q_0C_{10}$ | bc$_1$ complex | Antimycin | Myxothiazol | Stigmatellin | Displaced Q |
|-----------------------|----------------|-----------|-------------|--------------|-------------|
| mM                    | mol / mol      |            |             |              |             |
| 1.6                   | 0.23           | 0.3       | -           | -            | 0.78        |
| 2.1                   | 0.23           | 0.3       | -           | -            | 1.16        |
| 1.2                   | 0.15           | 0.8       | -           | -            | 1.14        |
| 1.2                   | 0.15           | 0.8       | -           | -            | 1.09        |
| 3.6                   | 0.29           | 1.5       | -           | -            | 1.02        |
| 2.1                   | 0.23           | -         | 0.3         | -            | 2.04        |
| 2.1                   | 0.23           | -         | 0.3         | -            | 2.31        |
| 3.6                   | 0.29           | -         | 1.5         | -            | 2.36        |
| 3.6                   | 0.29           | -         | 1.5         | -            | 2.40        |
| 1.6                   | 0.23           | -         | -           | 0.3*         | 1.91        |
| 2.1                   | 0.23           | -         | -           | 0.3*         | 1.90        |
| 2.1                   | 0.23           | -         | -           | 0.3          | 1.79        |
| 2.1                   | 0.23           | 0.3       | 0.3         | -            | 3.16        |
| 2.1                   | 0.23           | 0.3       | 0.3         | -            | 3.25        |
| 3.6                   | 0.29           | 1.5       | 1.5         | -            | 2.78        |
| 3.6                   | 0.29           | 1.5       | 1.5         | -            | 3.08        |
| 1.6                   | 0.23           | 0.3       | -           | 0.3*         | 2.85        |
| 2.1                   | 0.23           | 0.3       | -           | 0.3*         | 3.06        |
| 2.1                   | 0.23           | 0.7       | -           | 0.7*         | 2.92        |

*in the presence of 1 mM Na-ascorbate.
Three molecules of ubiquinone bind specifically to mitochondrial cytochrome bc1 complex
Stefan Bartoschek, Malin Johansson, Bernhard H. Geierstanger, Jürgen G. Okun, C. Roy D. Lancaster, Eberhard Humpfer, Linda Yu, Chang-An Yu, Christian Griesinger and Ulrich Brandt

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