Biochimica et Biophysica Acta xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

The quinone-binding sites of the cytochrome bo_3 ubiquinol oxidase from *Escherichia coli*

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ARTICLE INFO

Article history:
Received 14 December 2009
Received in revised form 4 March 2010
Accepted 13 April 2010
Available online xxxx

Keywords:
Ubiquinone
Oxidase
E. coli
Respiration
EPR

ABSTRACT

Cytochrome bo_3 is the major respiratory oxidase located in the cytoplasmic membrane of *Escherichia coli* when grown under high oxygen tension. The enzyme catalyzes the 2-electron oxidation of ubiquinol-8 and the 4-electron reduction of dioxygen to water. When solubilized and isolated using dodecylmaltoside, the enzyme contains one equivalent of ubiquinone-8, bound at a high affinity site (Q_H). The quinone bound at the Q_H site can form a stable semiquinone, and the amino acid residues which hydrogen bond to the semiquinone have been identified. In the current work, it is shown that the tightly bound ubiquinone-8 at the Q_H site is not displaced by ubiquinol-1 even during enzyme turnover. Furthermore, the presence of high affinity inhibitors, HQNO and aurachin C1–10, do not displace ubiquinone-8 from the Q_H site. The data clearly support the existence of a second binding site for ubiquinone, the Q_L site, which can rapidly exchange with the substrate pool. HQNO is shown to bind to a single site on the enzyme and to prevent formation of the stable ubisemiquinone, though without displacing the bound quinone. Inhibition of the steady state kinetics of the enzyme indicates that aurachin C1–10 may compete for binding with quinol at the Q_L site while, at the same time, preventing formation of the ubisemiquinone at the Q_H site. It is suggested that the two quinone binding sites may be adjacent to each other or partially overlap.

1. Introduction

Cytochrome bo_3 is the predominant respiratory oxidase present in *Escherichia coli* when grown under conditions of high aeration [1–3]. This enzyme is a member of the heme-copper superfamily [4,5], which includes both oxygen reductases as well as NO reductases. The oxygen reductases are of particular interest because these enzymes couple the redox chemistry to pumping protons across the membrane bilayer and generate a proton motive force.

Cytochrome bo_3 is in a sub-family of respiratory oxidases that directly catalyze the 2-electron oxidation of quinol within the membrane instead of the 1-electron oxidation of a soluble or membrane-bound cytochrome *c*. Cytochrome bo_3 has one copy each of 4 subunits. Subunits I, II and III are homologous to the mitochondrial encoded subunits of the eukaryotic (mitochondrial) cytochrome *c* oxidase [6]. Whereas, in the mitochondrial oxidase subunit II contains the docking site of the cytochrome *c* substrate and the Cu_A redox center, neither of these features is present in the homologous subunit II of the quinol oxidases [7,8]. The absence of the residues required for copper ligation to Cu_A is diagnostic of quinol oxidases and allows one to identify quinol oxidases in genome sequences. Phylogenetic analysis (manuscript in preparation) shows that cytochrome bo_3 and related quinol oxidases are derived from the cytochrome *c* oxidases, so the Cu_A site can be considered to have been "lost." In place of the Cu_A site, the quinol oxidases have acquired one or more sites for quinol oxidation. In the case of cytochrome bo_3 from *E. coli*, the substrate is ubiquinol-8, which is confined to the cytoplasmic membrane. Closely related enzymes include the aa_3-600 menaquinol oxidase from *Bacillus subtilis* [9,10].

Purified cytochrome bo_3 can be isolated with 0, 1 or 2 ubiquinones co-purifying with the enzyme, depending upon the detergent used and the preparative protocol [11,12]. This was the first indication that the enzyme may have more than one Q-binding site [11]. The effects of various inhibitors on the steady state kinetics of the enzyme have provided further evidence that there must be more than one Q-binding site [11,13,14], and the data have been interpreted in terms of two sites (Q_L or Q_H) with the inhibitors binding to one or the other Q-binding sites. The reactivity of the enzyme with synthetic quinone analogues has also been examined, showing the roles of the

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doi:10.1016/j.bbabio.2010.04.011

ring substituents and the hydrophobic side chain in determining the kinetic parameters [15,16].

Cytochrome bo_3 that is co-purified with at least one equivalent of ubiquinone exhibits a ubisemiquinone EPR signal when the solution potential is poised near 0 mV at pH 8, or when the enzyme is partially reduced [17,18]. At pH 8.2, the 1-electron reduction of the oxidized ubiquinone has an $E_{m2} = 15$ mV and the reduction of the semiquinone to the fully reduced dihydroubiquinol has an $E_{m1} = 2.5$ mV [18]. At pH 9, the stability constant for the semiquinone species is ~10 [18]. The protein binding site which stabilizes the semiquinone is referred to as the high affinity Q-binding site, or Q_H. Stabilizing the 1-electron reduced form of the quinone is consistent with the quinone bound at this site acting as a "converter," which can be reduced by two electrons and then transfer the electrons one-at-a-time to a 1-electron acceptor, heme b [19,20]. The rate of formation of the semiquinone upon mixing reduced ubiquinol-2 with the enzyme has been monitored by rapid freeze-quench EPR spectroscopy, and the results indicate that the semiquinone formed at the Q_H-site is an intermediate in the catalytic mechanism [21]. Transient formation of the semiquinone has also been examined by pulsed radiolysis, in which N-methylnicotinamide is used as a mediator for the one-electron reduction of the oxidized enzyme. The semiquinone at the Q_H-site is formed within 10 μs after pulsed radiolysis, and electron transfer from the semiquinone to heme b follows, with a first order rate constant of $1.5 \times 10^3\ s^{-1}$ [22]. Studies of the reaction of the fully reduced enzyme with O_2 have shown that electron transfer from heme b to the heme o_3/Cu_B binuclear center occurs with a rate of about $10^4\ s^{-1}$ [19,23]. A comparison of the intramolecular electron transfer events for enzyme containing the quinone bound at the Q_H-site with enzyme from which the quinone has been removed (prepared in Triton X-100) also supports the model that the tightly bound reduced quinone is capable of rapid electron transfer to heme b [12].

The intrinsic rate of "pure" electron transfer between the two hemes in cytochrome bo_3, independent of coupled (slower) processes, is extremely fast ($8 \times 10^8\ s^{-1}$) [24]. The slower rate ($10^4\ s^{-1}$) observed in the reaction of the reduced enzyme with O_2 is due to coupled proton transfers and/or conformational changes.

The X-ray structure of cytochrome bo_3 has been reported, but the detergent used (octylglucoside) resulted in stripping away the bound quinone, and substantial portions of the protein are not resolved [25]. Nevertheless, this structure was used to identify a reasonable candidate for a Q-binding site [25]. Subsequent EPR studies of site-directed mutants provided convincing evidence that this proposed site is the Q_H-site which stabilizes the observed semiquinone [26]. The residues implicated are R71, D75, H98 and Q101 [25,26].

The identity of the low affinity, or Q_L site, has been problematic to the extent that the existence of this site has been questioned [25]. Both the use of photo-reactive Q analogues designed to covalently attach to the protein at the Q_L site [27,28], as well as the location of inhibitor-resistant mutations [29], have been used to identify the Q_L site. In each case, subunit II was implicated, which was satisfying since the Cu_A site and docking site for cytochrome *c* in the related cytochrome *c* oxidases are located within subunit II. Mutations in one residue in subunit II, $W^{II}136A$, were shown to perturb the apparent K_m of ubiquinol-1 [30]. However, closer examination of $W^{II}136A$ and other specific residues suggested to be at or near the Q_L site [29,30] show that they are not near the membrane interface in the structure [25] and, therefore, are not likely to be part of a quinone binding site. Furthermore, sequence alignments of the many quinol oxidases (>400) presently available shows that $W^{II}136$ is not fully conserved. The residues identified by the photolabeling [28] and inhibitor-resistance [28,29] are not near the site proposed to be the Q_H site nor to any reasonable location for binding ubiquinol-8 from within the membrane bilayer.

In the current work, new evidence is presented to confirm that cytochrome bo_3 must contain two functionally distinct Q-sites. It is also shown that the inhibitor HQNO binds stoichiometrically to the enzyme and prevents formation of the ubisemiquinone at the Q_H-site, but does not displace the ubiquinone-8 bound at the Q_H-site. The inhibitor aurachin C1–10 also prevents formation of the ubisemiquinone at the Q_H-site, but appears to compete for quinol binding at the Q_L-site. The possibility is considered that perhaps the Q_H-, Q_L- and HQNO binding sites are adjacent or partially overlap, but that binding of ubiquinone-8 at the Q_H-site is not eliminated by simultaneous occupation of either the Q_L-site or the HQNO binding site.

2. Materials and methods

2.1. Expression and purification of cytochrome bo_3

Wild type and mutants of cytochrome bo_3 were expressed and the proteins were purified using the detergent dodecylmaltoside, as previously described [31].

2.2. Quinone extraction and analysis

The amount of ubiquinone-8 in the purified cytochrome bo_3 was determined by quinone extraction followed by reversed phase HPLC analysis. 10 nmol of purified enzyme, together with 10 nmol of ubiquinone-10 (Q_{10}), were diluted in 50 mM K_2HPO_4, 0.1% dodecylmaltoside, pH 8.3 buffer, and extracted with 3 mL of 6:4 methanol: petroleum ether by vortexing vigorously for 1 min (3 times). The mixture was allowed to sit for 10 min before vortexing again for an additional min, followed by centrifuging at 1,000 rpm for 10 min. The upper, organic layer was removed to a fresh tube, and the remaining mixture was subjected to further extraction two more times. The organic phase was then evaporated under a nitrogen stream, and the dried, oily residue was re-dissolved in ethanol.

The extracted quinones were analyzed using a reversed phase Microsorb-MV 100-5 C18 HPLC column (Varian, Palo Alto, CA) on a Beckman System Gold HPLC system fitted with a diode array detector, and monitored at 278 nm. The mobile phase was 4:3:3 ethanol: methanol:acetonitrile. The extracted ubiquinone-8 was quantified from the ratio of the peak areas of ubiquinone-8 to an internal standard, ubiquinone-10.

2.3. Preparation of quinone-free cytochrome bo_3

Purified cytochrome bo_3 in dodecylmaltoside (0.1%) was diluted at least 50-fold in buffer containing 50 mM K_2HPO_4, 0.1% Triton X-100 (Anatrace), pH 8.3, and then incubated for several hours with Ni-NTA resin with gentle stirring at 4 °C. The resin (with bound enzyme) was then loaded onto a column and washed with several column volumes of 50 mM K_2HPO_4, 0.1% Triton X-100, pH 8.3, followed by 50 mM K_2HPO_4, 0.1% dodecylmaltoside, pH 8.3. The protein was then eluted with the same buffer plus 100 mM imidazole. The eluted protein was pooled, concentrated, and dialyzed against 50 mM K_2HPO_4, 10 mM EDTA, 5% glycerol, 0.1% dodecylmaltoside, pH 8.3, and then flash-frozen in liquid nitrogen and stored at −80 °C.

2.4. Steady-state enzymatic activity assays

The steady state oxygen reductase activity of cytochrome bo_3 was measured using two different methods.

2.4.1. Method I: oxygen electrode

Oxygen concentration was monitored using a YSI model 53 oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH) equipped with a temperature-controlled 1.8-mL electrode chamber at 37 °C. The reaction mixture for this assay consisted of 50 mM K_2HPO_4, 0.1% DM, 2 mM dithiothreitol and 200 μM ubiquinone-1, pH 7.0. The concentration of oxygen in the air-saturated buffer at this

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temperature was assumed to be 250 μM, and the reaction was initiated by injecting a few microliters of diluted enzyme.

2.4.2. Method II: coupled enzyme assay

To determine the enzyme velocity as a function of the concentration of ubiquinol-1, it was advantageous to use a coupled enzyme assay that maintained the concentration of ubiquinol-1 constant. This was accomplished by using rat liver DT-diaphorase (kindly provided by Professor Gary Cecchini at the University of California, San Francisco), with NADH as the reductant. The reaction mixture for this assay consisted of 160 μM NADH and 20–80 μg/mL DT-diaphorase, and the reaction was followed by the decrease in absorption at 340 nm (oxidation of NADH) after the addition of cytochrome bo_3 at 25 °C.

2.5. Re-isolation of cytochrome bo_3 after turnover with ubiquinol-1

In order to determine if ubiquinol-8 bound at the Q_H-site was displaced during enzyme turnover with ubiquinol-1 the following procedure was used. Purified cytochrome bo_3 (5 mg), isolated in dodecylmaltoside, was diluted into 5 mL of 50 mM K_2HPO_4 buffer, pH 8.3, containing 0.1% dodecylmaltoside, 10.5 mM dithiothreitol and 1.05 mM ubiquinol-1 at 37 °C. The reaction mixture was incubated at 37 °C for 10 min, and then loaded onto a column containing the Ni-NTA resin (QIAGEN) pre-equilibrated with 50 mM K_2HPO_4, 0.1% dodecylmaltoside, pH 8.3. The Ni-NTA resin with bound cytochrome bo_3 was washed with buffer containing 50 mM K_2HPO_4, 0.1% dodecylmaltoside at pH 8.3, until the excess ubiquinone-1 was removed (monitored by the absorbance at 275 nm). The bound cytochrome bo_3 was eluted with the same buffer plus 100 mM imidazole, and then concentrated and dialyzed to remove the imidazole. The content of ubiquinone-8 was then determined as described above.

An alternative procedure was also used in which the enzyme was first bound to the Ni-NTA column and was washed by buffer containing dithiothreitol and ubiquinol-1. Any displaced ubiquinol-8 would be expected to be swept away by the buffer. Both procedures yielded the same results.

Variations of the same procedure were used to quantify the displacement of ubiquinone-8 after incubation in the presence of the inhibitors HQNO and AC1-10.

2.6. EPR spectroscopy

The CW EPR measurements were performed on an X-band Varian EPR-E122 spectrometer. Methods used for preparing samples and obtaining EPR spectra have been previously described [31,32].

2.7. HQNO binding monitored by fluorescence anisotropy

HQNO in water fluoresces with an emission maximum at 480 nm, but when bound to proteins the fluorescence is often completely quenched. Examples are the cytochrome bc_1 complex [33], fumarate reductase [34–36], nitrate reductase [37,38] and dimethylsulfoxide reductase [39]. However, attempts to monitor HQNO by changes in the fluorescence intensity revealed that the fluorescence of HQNO is not quenched upon binding to cytochrome bo_3. For this reason, the binding of HQNO to cytochrome bo_3 was monitored by the change in the fluorescence anisotropy of the emission from HQNO, using a Varian Cary Eclipse™ fluorescence spectrophotometer (Varian, Palo Alto, CA) fitted with automated polarizers. The anisotropy is defined in terms of the ratio of the intensity of light emitted parallel and perpendicular to the plane of polarization of the incident beam.

$$\text{anisotropy} = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

The titration was performed by increasing the enzyme concentration with a fixed concentration of HQNO to avoid problems of HQNO aggregation and to limit the amount of ethanol (used to prepare the stock solution of HQNO) which caused the protein to precipitate. Cytochrome bo_3 was titrated in 1–4 μL aliquots from a stock solution into 50 mM K_2HPO_4, 0.1% dodecylmaltoside, pH 8.3 buffer containing 1 μM HQNO. The excitation wavelength was 341 nm and the emission wavelength was 480 nm. As a control, enzyme was titrated into buffer in the absence of HQNO. The data were fit with a model assuming independent binding sites.

2.8. Synthesis of aurachin C1–10

The inhibitor aurachin C1–10 was synthesized based on a protocol described previously [40]. The most important modification was that the order of the first two steps of the 7-step synthesis was reversed. The methylation of crotonate was followed by the condensation of aniline and 3-methylcrotonate instead of carrying out the condensation of aniline and crotonate followed by methylation of 3-anilinocrotonate.

3. Results

3.1. One or two Q-binding sites?

The main argument supporting a two-site model is that the quinone at the Q_H-site does not exchange with exogenous ubiquinone-8 and, once removed, is difficult to replace the ubiquinone-8 [11,12]. This is consistent with the idea that the Q_H site cannot function as a catalytic site, which demands rapid exchange during catalytic turnover. It is noted that the bc_1 complex is isolated with tightly bound quinones at the Q_o and Q_i sites but, under turnover conditions, both sites are involved in catalysis with rapid binding of substrate and release of products [41].

To provide a more convincing test for the existence of the Q_L-site, the ability to exchange the bound ubiquinone-8 for exogenous quinone was examined under turnover conditions with ubiquinol-1. Fig. 1 illustrates the two models to be distinguished. The one-site model assumes that the quinone bound at the high affinity site can readily exchange under conditions of catalytic turnover and, therefore, is the only site required. The two-site model assumes that

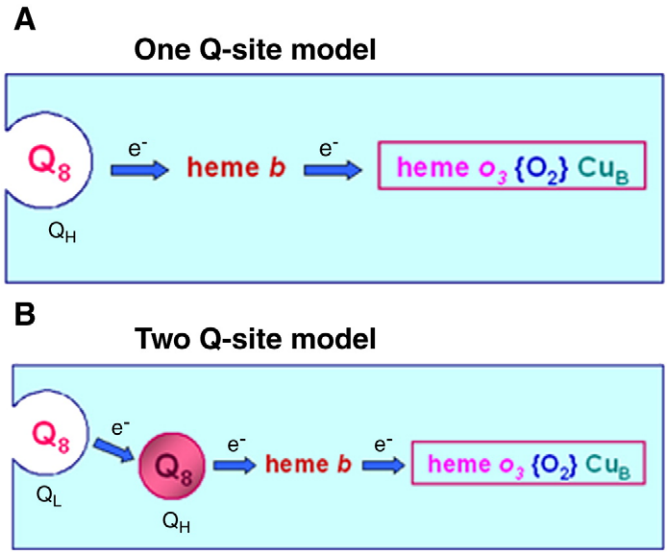

Fig. 1. Schematic illustrating the 1-site and 2-site models for quinone binding to cytochrome bo_3.

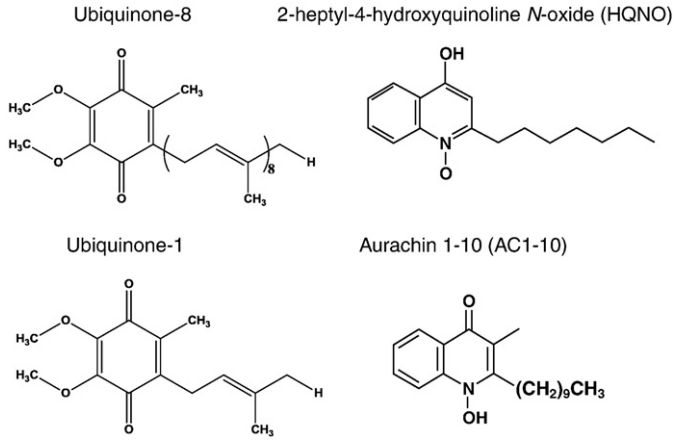

Fig. 2. Structures of quinone substrates and quinone-like inhibitors referred to in the text.

oxidation of the substrate occurs at the Q_L-site and that electrons are transferred, presumably in a two-electron transfer to the quinone at the Q_H-site. In order to distinguish between these two alternatives, the enzyme with one equivalent of bound ubiquinone-8 was allowed to incubate under aerobic conditions with reduced ubiquinol-1, a soluble analogue of the natural substrate, ubiquinol-8 (see Fig. 2). The incubation time was sufficient to allow multiple turnovers of ubiquinol-1. If the one-site model is correct, then multiple turnovers with ubiquinol-1 must displace the initially bound ubiquinol-8, which would become diluted into the detergent micelles. To determine whether the bound ubiquinone-8 had been displaced, the His-tagged enzyme was bound to the Ni-NTA column and extensively washed with buffer to remove free or weakly bound quinone. After elution of the enzyme, the quinone was extracted and quantified by HPLC analysis. The results (Fig. 3B) show that the ubiquinone-8 remained bound to the enzyme with the same stoichiometry as measured prior to the turnover with ubiquinol-1. The same experiment was done by allowing the enzyme to turnover with ubiquinol-1 while bound to the Ni-NTA column, during which buffer was continuously flowing. The results were identical. As a control, the enzyme was washed with Triton X-100 detergent to remove the quinone, and the quinone content determined by HPLC analysis (Fig. 3A). The data support the two Q-site model.

3.2. Binding of HQNO to cytochrome bo_3

It has been shown previously that HQNO (Fig. 2) is an effective inhibitor of cytochrome bo_3 [13,20]. Fig. 4A shows the inhibition of cytochrome bo_3 (with ubiquinone-8 bound to the Q_H site) by HQNO using the coupled assay to maintain the ubiquinol-1 substrate reduced. The K_i is 0.74 μM. Fig. 4B shows a double-reciprocal plot, indicating a pattern of inhibition that is noncompetitive, consistent with previous reports [13,20]. The noncompetitive inhibition pattern suggests that HQNO does not bind to the substrate (Q_L) binding site.

To extend these observations, the stoichiometry and binding affinity of HQNO to the enzyme were measured directly by fluorescence spectroscopy. HQNO is itself fluorescent, and the fluorescence is often quenched upon binding to proteins. However, upon binding to cytochrome bo_3, the fluorescence intensity is not substantially perturbed. Therefore, binding was monitored by measuring the fluorescence anisotropy, which increases substantially when HQNO binds to the enzyme. In order to avoid artifacts due to the aggregation of HQNO at high concentrations, the titration was performed by maintaining the total concentration of HQNO constant while increasing the amount of the protein. A representative binding isotherm is shown in Fig. 5. Fitting the data to a model, assuming independent sites, yields a best fit of 1.1 sites and a K_d of 4.0 μM. The reason why the kinetic inhibition constant and the dissociation constant are different by about a factor of 5 is not clear, but may be due to differences in assay conditions or enzyme preparation. The main point is that the data show that HQNO binds to a single site on cytochrome bo_3. The experiment was repeated in the presence of a large excess of oxidized ubiquinol-1 (Table 1) and the same result was obtained, suggesting that at least the oxidized form of ubiquinol-1 does not compete for binding at the same site.

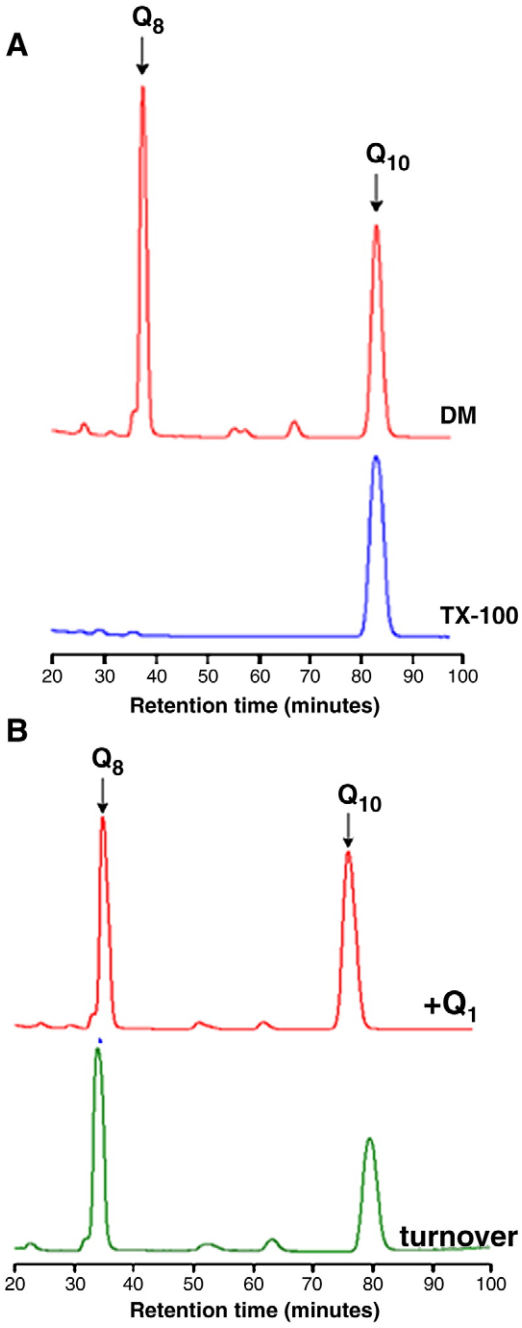

Fig. 3. HPLC elution profiles following extraction of ubiquinone-8 from purified cytochrome bo_3. A known amount of ubiquinone-10 is included in each sample as an internal standard. Panel A: enzyme isolated in dodecylmaltoside (DM) contains 1 equivalent of ubiquinone-8, whereas enzyme washed with Triton X-100 has no bound ubiquinone-8. Panel B: enzyme incubated with oxidized ubiquinone-1 (500 μM) retains bound ubiquinone-8 (top), as does enzyme which has been incubated under conditions of catalytic turnover with ubiquinol-1 (see text).

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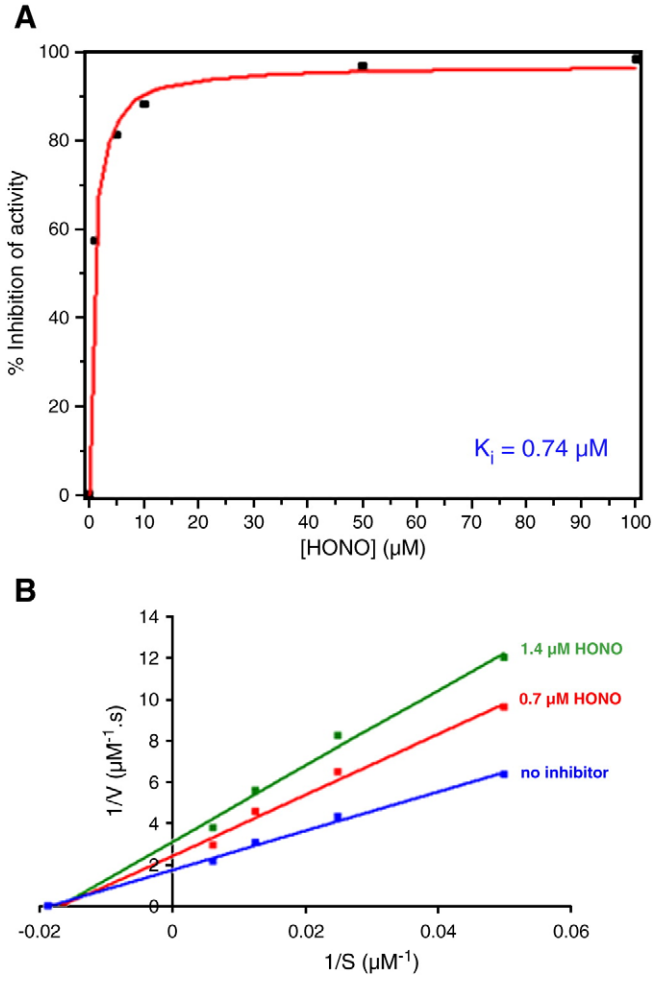

Fig. 4. Inhibition of steady state ubiquinol-1 oxidase activity by HQNO. Panel (A) is a direct plot of the data, and Panel (B) is a Lineweaver–Burke plot showing non-competitive inhibition.

Table 1
Characterization of wild type and mutants of cytochrome bo_3. Shown are the steady state kinetic parameters for ubiquinol-1 oxidase activity (V_{max} and K_M); the dissociation constant for HQNO determined by fluorescence anisotropy titration; and the concentration of AC1–10 required for 50% inhibition of ubiquinol-1 oxidase activity (K_i).

Cytochrome bo_3	Relative V_{max} (%)	K_M UQ_1H_2 (μM)	K_d HQNO (μM)	K_i AC1–10 (nM)
wild type	100	53	4.0	15
wild type + 300 μM Q_1	–	–	4.1	–
wild type + 1500 μM Q_1	–	–	6.8	–
wild type (no bound Q_8)	–	–	5.8	–
D188A	69	56	5.0	25
D188N	71	52	4.0	ND
R257Q	47	60	4.0	12
$W^{II}136A$	75	63	4.1	23
H98S	-	-	3.6	-

It has been previously reported that upon the addition of an excess of HQNO, the EPR signal of the pre-formed semiquinone at the Q_H-site is inhibited [18]. Fig. 6A shows that the presence of an excess of HQNO prevents formation of the semiquinone. If HQNO were binding at the Q_H site, it would displace the tightly bound ubiquinone-8. However, this does not occur. The enzyme was bound to the Ni-NTA column in the presence of HQNO and then washed with several column volumes of buffer containing HQNO. Following this, the enzyme was eluted and the quinone was extracted and quantified. Cytochrome bo_3 retains one equivalent of ubiquinone-8 in the presence of HQNO (Fig. 6B).

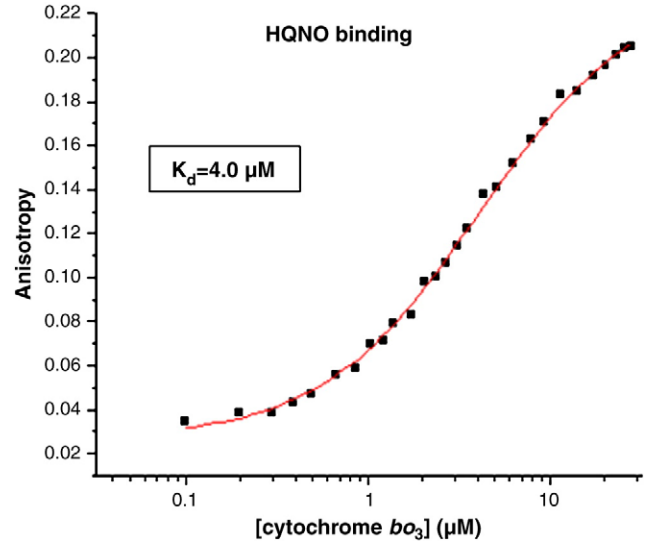

Fig. 5. HQNO binding to cytochrome bo_3 which has been isolated with one equivalent of ubiquinone-8 bound to the Q_H-site. Binding is monitored by fluorescence anisotropy, titrating the enzyme into a solution containing 1 μM HQNO. The points are experimental data and the line is a fit to 1 binding site with a K_d of 4 μM.

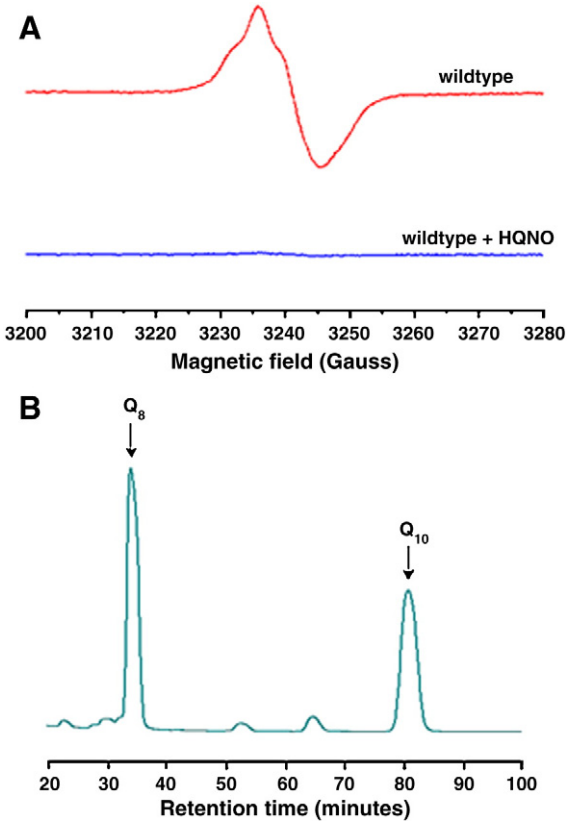

Fig. 6. (A) EPR spectrum of the semiquinone formed by ubiquinone-8 bound at the Q_H-site of wild type cytochrome bo_3. The presence of a 5-fold excess of HQNO prevents formation of the semiquinone. (B) HPLC elution profile of the ubiquinone-8 extracted from enzyme incubated under the same conditions as used for the EPR experiment in the top panel. The data show approximately 1 equivalent of ubiquinone-8 remains bound to the enzyme, unchanged from prior to the addition of HQNO.

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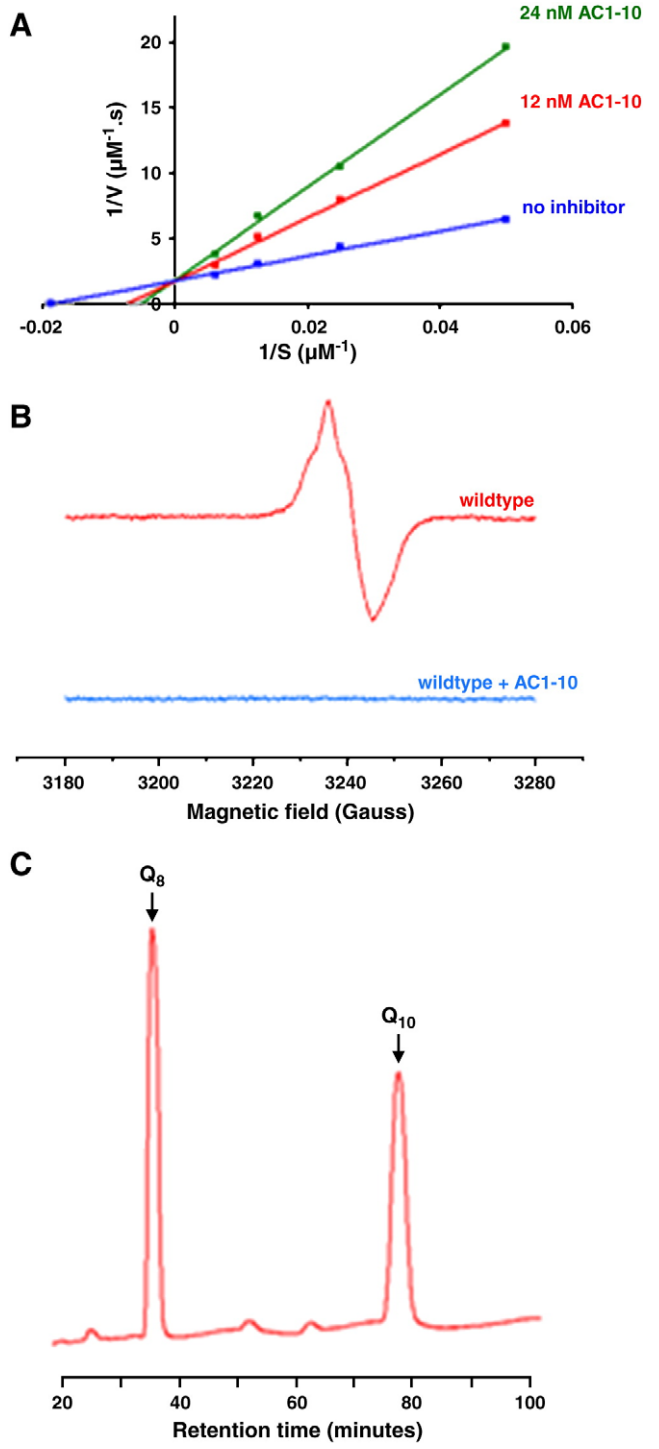

Fig. 7. Inhibition of cytochrome bo_3 by AC1-10. (A) A double reciprocal plot of (enzyme velocity)$^{-1}$ vs. (ubiquinol − 1 concentration)$^{-1}$ showing competitive inhibition. (B) EPR spectrum of the semiquinone formed by ubiquinone-8 bound at the Q_H-site of wild type cytochrome bo_3. The presence of a 5-fold excess of AC1–10 prevents formation of the semiquinone. (C) HPLC profile showing the ubiquinone-8 remains bound to cytochrome bo_3 in the presence of AC1–10. The ubiquinone-10 is an internal standard used to quantify the amount of ubiquinone-8 extracted from the enzyme.

The binding of HQNO to enzyme from which the tightly bound ubiquinone-8 had been removed was also examined. The results (Table 1) show one HQNO binds with a $K_d = 5.8$ μM, similar to the data obtained with enzyme containing ubiquinone-8 bound at the Q_H site.

3.3. The binding of AC1-10 to cytochrome bo_3

Previous work has shown that aurachin C and analogues are potent inhibitors of cytochrome bo_3 [40,42]. The inhibition of ubiquinol-1 oxidase activity by AC1–10 [40] is shown in Fig. 7. The K_i was determined to be about 15 nM, and the pattern of inhibition (Fig. 7A) indicates that AC1–10 is a competitive inhibitor. Previous work concluded that aurachin C binds to one site on cytochrome bo_3 [42]. Incubation of a 50 μM solution of cytochrome bo_3 with a 5-fold excess of AC1–10 prevents formation of the semiquinone EPR signal (Fig. 7B) but, as was the case with HQNO, the tightly bound ubiquinone-8 is not displaced from the Q_H site (Fig. 7C).

3.3.1. Site-directed mutagenesis of D188, R257 and $W^{II}136$

The location of the Q_L-site is not known. It is a reasonable assumption that residues that directly interact with ubiquinol at the Q_L-site will be conserved among the many ubiquinol oxidases. Residues that are conserved include R71, D75, M78 and H98, which are proposed to constitute the Q_H-site. Two additional residues in subunit I that are conserved in all the quinol oxidases are D188 and R257. These residues are located near each other on the surface of subunit I at a level expected to be within the membrane bilayer and reasonably close to the Q_H-site. Hence, mutants were made to test whether these residues might be part of the Q_L site: D188A, D188N and R257Q. The results are summarized in Table 1, along with data from $W^{II}136A$. In each case, the enzymes were purified and examined using (1) ubiquinol-1 oxidase activity (V_{max}), including the K_M of ubiquinol-1; (2) HQNO binding, measured by fluorescence anisotropy; and (3) inhibition of ubiquinol-1 oxidase activity by AC1–10. Although there is a modest loss of activity due to each mutation, there is no indication that either the Q_L quinol binding site, or the HQNO binding site, or the AC1–10 binding site involves these residues. It is noted that both D188 and R257 are also present in many cytochrome *c* oxidases, indicating a function other than quinol binding. One unusual observation is that neither the D188A nor R257Q mutant oxidases were able to support aerobic growth when expressed in a strain of *E. coli* without a genomically encoded respiratory oxidase. However, the isolated proteins were active (Table 1).

3.4. Additional mutations at the Q_H-site

A number of mutations at the Q_H-site (R71, D75, H98 and Q101) have been described [25,26]. In those cases where measurements were made, it was shown that these mutations do not reduce the amount of bound ubiquinone [25]. Table 2 shows result obtained with a number of additional mutations at the Q_H-site. The EPR spectroscopy of the D75E and D75H mutants has been previously reported [32]. Besides the conservative replacement D75E, all of the other mutants have very low or no quinol oxidase activity. Apart from D75E, D75H and D75N, the semiquinone is also not formed. Table 2 shows that none of these mutations result in loss of the tightly bound ubiquinone-8 from the Q_H-site. This is also true for the double mutation, R71D/D75R, in which the aspartate and arginine have been switched.

For one mutation at the Q_H-site, H98S, the binding of HQNO was also examined. The results (Table 1) show that the dissociation constant is essentially the same as for the wild type enzyme, indicating that this residue is not important for the affinity of HQNO to the enzyme.

3.5. Generating the semiquinone radical at the Q_H site using ascorbate

The semiquinone form of ubiquinone-8 bound at the Q_H site can be generated by using ascorbate as a reducing agent and, after an empirically determined time, freezing the partially reduced (mixed valence) enzyme [31,32,43–45]. The continuous wave X-band EPR

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Table 2
Characterization of mutants at the Q_H-site of cytochrome bo_3. Shown are the relative ubiquinol-1 oxidase activity, the content of ubiquinol-8 and whether the semiquinone is formed upon partial reduction by ascorbate.

Cytochrome bo_3	Relative activity (%)	Quinone content (Q_8/enzyme)	Semiquinone formation
wild type	100	1.1	Yes
D75E	135	1.2	Yes
D75H	4	0.9	Yes
D75N	~0	1.3	Yes
D75R	~0	1.2	No
R71D	~0	1.4	No
R71K	~0	1.3	No
R71Q	~0	1.3	No
H98N	1	1.4	No
H98S	2	1.4	No
H98T	1	1.5	No
Q101N	5	1.3	No
R71D/D75R	~0	1.3	No

spectra of the oxidized and the partially reduced forms of wild type enzyme are shown in Fig. 8A. The spectra show features previously

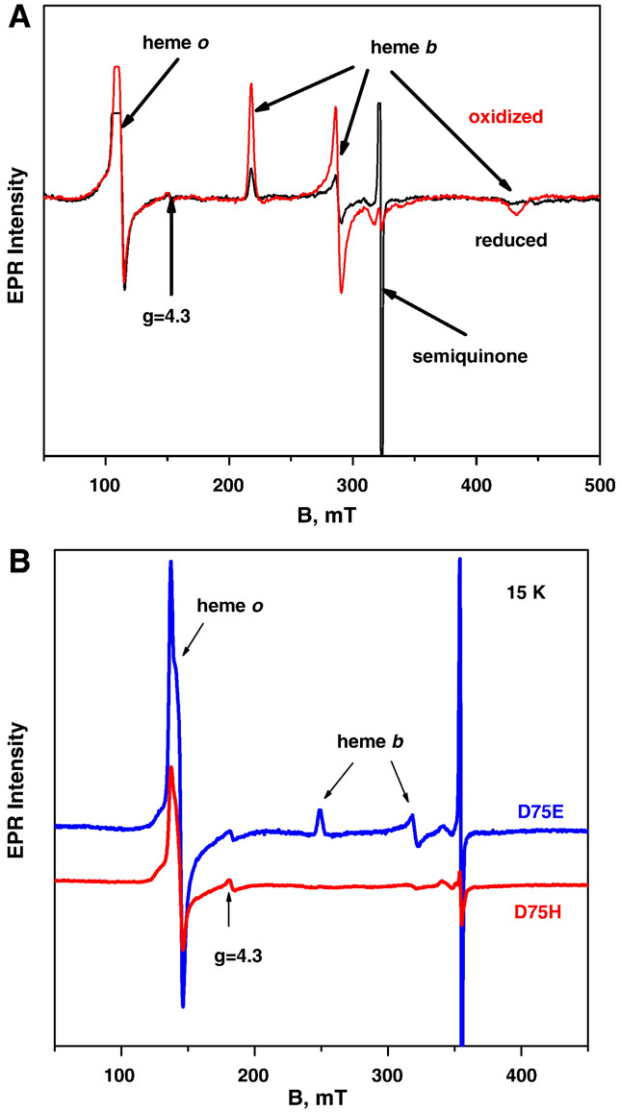

Fig. 8. (A) Continuous-wave X-band EPR spectrum of oxidized (red) and reduced (black) cytochrome bo_3 (see text for details). (B) EPR spectra of the reduced D75E and D75H mutants. Temperature 15 K, microwave power ~10 mW, modulation amplitude 0.5 mT, reduction time 6 h.

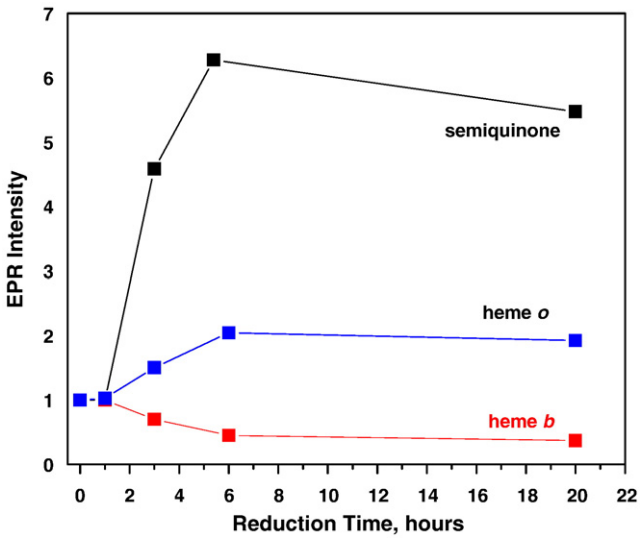

Fig. 9. Behavior of the EPR intensities of the semiquinone, heme o_3 and heme b as a function of reduction time. The peak height of each sample in the spectrum recorded at 1 h is arbitrarily set to a value of 1, and relative intensities are plotted as a function of time. The amplitudes of the signals due to ferric heme o_3 ($g=5.81$) and ferric heme b ($g=2.26$) were measured from the spectra recorded at 15 K. The amplitude of the semiquinone signal was measured at 90 K with low microwave power to avoid saturation conditions and significant overlap with other signals around $g\sim2$. The relative intensities of the heme b, heme o_3 and semiquinone signals are not necessarily proportional to the relative concentrations of the three species.

assigned [46,47] to high spin heme o_3 ($g=5.81$) and low spin heme b ($g=2.98$, 2.26 and 1.45) as well as the signal from the semiquinone radical ($g=2$). As previously discussed, the signal from the high spin heme o_3 ($g=5.81$) is due to a small fraction of the heme ($\approx$ 5% to 10%) which is no longer coupled to Cu_B [46,47]. The spectra of the partially reduced forms of two mutants in the Q_H site, D75E and D75H, are also shown in Fig. 8B. The D75E mutant is catalytically active and the EPR spectrum is similar to that of the wild type oxidase. The D75H mutant is catalytically inactive, and the EPR spectrum of the partially reduced enzyme lacks the signals from the low spin heme b, indicating that heme b in this mutant is completely reduced by the ascorbate.

The peak intensities of the EPR features for the wild type oxidase as a function of time after the addition of ascorbate are shown in Fig. 9. The intensity of the low spin heme b signal decreases with time. In contrast, the amplitude of the high spin heme o_3 signal as well as the amplitude of the semiquinone signal increase in parallel, reaching a maximum at about 6 hr. The simplest explanation is that the amount of reduced Cu_B (revealing the EPR signal from the oxidized heme o_3) varies in proportion to the amount of semiquinone present. In the D75H mutant, the reduction of heme b occurs substantially more rapidly than in either the wild type or D75E mutant oxidase. As a result, after 6 hours, the EPR signal from heme b is not observed.

4. Discussion

The motivation of the current work was primarily to test the validity of the two-site model of ubiquinone binding to cytochrome bo_3. It was previously shown that a large excess of ubiquinone-1 does not displace the bound ubiquinone-8 under conditions when all the species remain oxidized [11], and this has been repeated (Fig. 3). The critical test, however, is to examine displacement under conditions when the enzyme is turning over ubiquinol-1. The fact that ubiquinol-1 does not displace ubiquinol-8 at the Q_H site under turnover conditions clearly shows that ubiquinone-8 at this site does not readily exchange. These new data definitively demonstrate that there

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must be more than one quinone binding site, thus supporting the two-site model (Fig. 1) [11].

Arguments for the two-site model based on the effect of inhibitors on steady state kinetics [13,14] have assumed that some of the inhibitors, including HQNO, can bind to the Q_H-site. However, data presented in this paper as well as previously published data [11] show that HQNO does not displace the ubiquinone-8 at the Q_H-site. New data in the current work show, in addition, that only one equivalent of HQNO binds to the enzyme, and that the affinity is not significantly altered by removing the tightly bound ubiquinone-8 at the Q_H-site.

Most of the information about the interaction of ubiquinone at the Q_H-site is from EPR studies of the stabilized ubisemiquinone that forms at this site [26,31,32,45]. Data in Figs. 8 and 9 show the full EPR spectrum of the enzyme and the changes that occur during the partial reduction by ascorbate. Under the conditions utilized, the reduction of the wild type enzyme by ascorbate is slow (hours) and, in addition to formation of the ubisemiquinone, heme b and Cu_B are partially reduced in the final product.

HQNO also prevents the formation of the ubisemiquinone at the Q_H-site when the enzyme is partially reduced. This is shown in the present work, and was reported previously [18]. The addition of HQNO to the fully reduced enzyme also prevents the oxidation of ubiquinol-8 at the Q_H-site by O_2 [48]. Hence, HQNO functionally disrupts the reactions of the ubiquinone at the Q_H-site, though this quinone is not displaced from the enzyme. One possibility, previously discussed [13], is that the ubiquinone-8 bound at the Q_H-site is held in place primarily by interactions with the hydrophobic isoprene side chain. Therefore, inhibitors such as HQNO can displace the quinone ring without displacing the molecule as a whole. This would be consistent with the observation that the mutations at the Q_H-site which eliminate catalytic activity as well as prevent formation of the semiquinone species, retain the bound ubiquinone-8, shown in Table 2 as well as in previous work [26,48]. Arguing against this interpretation is the fact that the dissociation constant of HQNO to the enzyme is only slightly increased (4.0 μM to 5.8 μM) by removal of the ubiquinone-8 from the Q_H-site (Table 1). If HQNO were competing for the same site with a locally tethered head group of ubiquinone-8, one would expect a larger effect on the K_d of HQNO by the presence of the bound ubiquinone-8. Nevertheless, the competition between HQNO and ubiquinone-8 for binding to the Q_H-site needs to be examined under conditions where the enzyme is partially or fully reduced to further test this model. What can definitely be concluded at this point is that H98, although part of the Q_H-site, does not interact with HQNO, as shown by the fact that the H98S mutant has the same affinity for HQNO as does the wild type (Table 2).

The current work strongly supports the model that cytochrome bo_3 contains two quinone binding sites, Q_H and Q_L. The interactions critical for the high affinity of the tightly bound quinone at the Q_H-site remain unknown, since mutations of residues at the Q_H-site which are known to interact with the ubisemiquinone ring [31,32,45] do not physically eliminate the bound quinone (Table 2).

The location of the Q_L site is also not known. It is likely that the Q_L-site is adjacent to the Q_H-site. This would be consistent with the fact that AC1–10, which is a competitive inhibitor for the Q_L-site, also perturbs the Q_H-site, evidenced by blocking the formation of the ubisemiquinone at the Q_H-site upon reduction by ascorbate. It cannot be excluded that some of the residues involved at the Q_H-site are also involved in the Q_L-site.

Further examination of HQNO binding to mutants should be helpful to identify the HQNO binding site, which appears to be separate from both the Q_H-site and the Q_L-site. The possibility that the ubiquinone-8 bound at the Q_H-site is tethered by the hydrophobic side chain and has several modes of interaction between the quinone ring and the protein, depending on the redox state, should be seriously considered. In this model, HQNO could displace the ubiquinone head group at the Q_H-site without actually displacing the bound quinone. This would be consistent with the fact that no mutants have been found yet which increase the K_M for ubiquinol-1, since such mutations would also alter the Q_H-site and eliminate catalytic function. Clearly, further work is required.

5. Conclusions

It is definitively demonstrated that cytochrome bo_3 must have two ubiquinone binding sites. Ubiquinone-8 bound at the Q_H-site acts as a cofactor and is not displaced during enzyme turnover, nor in the presence of the inhibitors HQNO or AC1–10. AC1–10 appears to compete for ubiquinol-1 binding at the Q_L-site, which is the substrate binding site. HQNO binds stoichiometrically to one site, but is a noncompetitive inhibitor at the Q_L site. The Q_H-site, Q_L-site and the HQNO binding site are likely to be adjacent and may be partially overlapping.

Acknowledgments

We would like to that Dr. James Hemp for many helpful discussions and for pointing out the extent of conservation of residues in the quinol oxidases. This investigation was supported by Grants from Chemical Sciences, Geosciences and Biosciences Division, Office of Basic Energy Sciences, Office of Sciences, US DOE: DE-FG02-87ER13716 (to R.B.G.) and DE-FG02-08ER15960 (to S.A.D.), and the National Institutes of Health GM062954 Grant (to S.A.D.) and NIH/Fogarty Grant RP3 TW01495 (to R.I.S.).

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