Uncompetitive Substrate Inhibition and Noncompetitive Inhibition by 5-*n*-Undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) and 2-*n*-Nonyl-4-hydroxyquinoline-*N*-oxide (NQNO) is Observed for the Cytochrome bo_3 Complex: Implications for a Q(H_2)-Loop Proton Translocation Mechanism†

Siegfried M. Musser,‡,§ Michael H. B. Stowell,‡ Hung Kay Lee,‡ Jon N. Rumbley,‖,⊥ and Sunney I. Chan*,‡

Division of Chemistry and Chemical Engineering, Arthur Amos Noyes Laboratory of Chemical Physics, California Institute of Technology, Pasadena, California 91125, and School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801

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ABSTRACT: The cytochrome bo_3 ubiquinol oxidase complex from *Escherichia coli* contains two binding sites for ubiquinone(ol) (UQ(H_2)). One of these binding sites, the ubiquinol oxidation site, is clearly in dynamic equilibrium with the UQ(H_2) pool in the membrane. The second site has a high affinity for ubiquinone (UQ), stabilizes a semiquinone species, and is located physically close to the low-spin heme *b* component of the enzyme. The UQ molecule in this site has been proposed to remain strongly bound to the enzyme during enzyme turnover and to act as a cofactor facilitating the transfer of electrons from the substrate ubiquinol to heme *b* [Sato-Watanabe et al. (1994) *J. Biol. Chem. 269*, 28908−28912]. In this paper, the steady-state turnover of the enzyme is examined in the presence and absence of inhibitors (UHDBT and NQNO) that appear to be recognized as ubisemiquinone analogs. It is found that the kinetics are accounted for best by a noncompetitive inhibitor binding model. Furthermore, at high concentrations, the substrates ubiquinol-1 and ubiquinol-2 inhibit turnover in an uncompetitive fashion. Together, these observations strongly suggest that there must be at least two UQ(H_2) binding sites that are in rapid equilibrium with the UQ(H_2) pool under turnover conditions. Although these data do not rule out the possibility that a strongly bound UQ molecule functions to facilitate electron transfer to heme *b*, they are more consistent with the behavior expected if the two UQ(H_2) binding sites were to function in a Q(H_2)-loop mechanism (similar to that of the cytochrome bc_1 complex) as originally proposed by Musser and co-workers [(1993) *FEBS Lett. 327*, 131−136]. In this model, ubiquinol is oxidized at one site and ubiquinone is reduced at the second site. While the structural similarities of the heme-copper ubiquinol and cytochrome *c* oxidase complexes suggest the possibility that these two families of enzymes translocate protons by similar mechanisms, the current observations indicate that the Q(H_2)-loop proton translocation mechanism for the heme-copper ubiquinol oxidase complexes should be further investigated and experimentally tested.

The *Escherichia coli* cytochrome bo_3 ubiquinol oxidase (UQO)[1] complex is a member of the superfamily of respiratory terminal oxidase complexes. These enzymes are characterized by a binuclear heme-copper dioxygen activation and reduction center, a low-spin heme mediating electron flow, and the capability to translocate protons against transmembrane electrical and pH gradients. This superfamily consists of two families of enzymes: the quinol oxidase complexes and the cytochrome *c* oxidase complexes (Calhoun et al., 1994; García-Horsman et al., 1994). While the method of dioxygen activation and reduction is apparently quite similar in these two families on the basis of CO-flash studies (Puustinen et al., Svensson & Nilsson, 1993) as well as the similar behavior of mutants (Hosler et al., 1993), different electron and proton transfer mechanisms have been observed (Hallén et al., 1993; Wang et al., 1995). The different electron donors (ubiquinol and ferrocytochrome *c*) are expected to dictate alternate electron input mechanisms (Musser et al., 1993). The proton translocation mechanisms are usually *assumed* to be identical in the two families of enzymes based on the high structural similarities (Brunori & Wilson, 1995; Trumpower & Gennis, 1994; Wikström et al., 1994) but there are virtually no experimental data which address this issue. It is certainly possible that sequence differences may accommodate the different electron donors and couple the highly exergonic dioxygen reduction reactions

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* Author to whom correspondence should be sent. Tel: (818) 395-6508. FAX: (818) 578-0471. E-mail: ChanS@starbase1.caltech.edu.

‡ California Institute of Technology.

§ Present address: Division of Biological Sciences, Section of Plant Biology, University of California, Davis, CA 95616.

‖ University of Illinois.

⊥ Present address: Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA 19104.

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[1] Abbreviations: UQO, ubiquinol oxidase, specifically the *Escherichia coli* cytochrome bo_3 complex; UQ_n, ubiquinone-*n*; UQ_nH_2, ubiquinol-*n*; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole; Me-UHDBT, 5-*n*-undecyl-6-methoxy-4,7-dioxobenzothiazole; NQNO, 2-*n*-nonyl-4-hydroxyquinoline-*N*-oxide; HQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide; DDM, *n*-dodecyl β-D-maltoside; DMF, dimethylformamide; DTT, dithiothreitol; UQ(H_2), ubiquinone(ol); Na-phos, NaH_2PO_4/Na_2HPO_4 buffer.

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to similar proton translocation machineries. On the other hand, radically different proton translocation mechanisms are possible.

Quinone(ol) ($Q(H_2)$) binding proteins often have two $Q(H_2)$ binding sites in order to facilitate the coupling of one-electron redox chemistry to an two-electron acceptor/donor (Kotlyar et al., 1990; Miki et al., 1992; Suzuki & King, 1983; Trumpower, 1990; Warncke, et al. 1994; Westenberg et al., 1993). Typically, one of these binding sites is primarily responsible for stabilizing the semiquinone radical whereas the other acts as the electron input/output site. Based in part on this fact, Musser and co-workers (1993) suggested that the UQO complex has two ubiquinone(ol) ($UQ(H_2)$) binding sites. Subsequently, Sato-Watanabe and co-workers (1994b) reported a tightly-bound ubiquinone-8 (UQ_8) molecule in the as-isolated UQO complex that could not be removed by high concentrations of ubiquinol-1 (UQ_1H_2) or inhibitors. These investigators concluded that the UQ_8 binding site was not the ubiquinol oxidation site and that there were in fact two $UQ(H_2)$ binding sites on the UQO complex, in agreement with the proposal of Musser and co-workers (1993). These data were interpreted to imply that the tightly-bound ubiquinone acts as an intermediate electron acceptor from ubiquinol subsequently passing electrons on to heme *b*. According to this model, the electron input reactions of the UQO complex are similar to the electron output reactions of photosynthetic reaction centers. It has been found, however, that UQ_1, UQ_6, and UQ_8 as well as 2,6-dichloro-4-nitrophenol (an inhibitor) can be reconstituted into enzyme with an empty UQ_8 binding site (Puustinen et al., 1996; Sato-Watanabe et al., 1994b). These data suggest that the UQ_8 binding site is fairly accessible from the solvent. It is possible, therefore, that this ubiquinone binding site is accessible from the $UQ(H_2)$ pool on the time scale of enzyme turnover.

Clearly, the basic biochemistry of UQO turnover must be understood in greater detail before the proton translocation mechanism of this enzyme can be comprehended. In this study, UQO activity was investigated at high concentrations of ubiquinol and it was observed that ubiquinol acts as an uncompetitive inhibitor under these conditions. Furthermore, two common $UQ(H_2)$ analogs, 2-*n*-nonyl-4-hydroxyquinoline-*N*-oxide (NQNO) and 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) Figure 1), act as potent inhibitors in a noncompetitive fashion. These data indicate that there are two $UQ(H_2)$ binding sites in dynamic equilibrium with the $UQ(H_2)$ pool during enzyme turnover. One of these binding sites is clearly the ubiquinol oxidation site whereas the other site is likely to be the UQ_8 binding site found earlier (Puustinen et al., 1996; Sato-Watanabe et al., 1994b). The implications of these findings on the $Q(H_2)$-loop hypothesis initially proposed by Musser and co-workers (Musser et al., 1993) are discussed.

EXPERIMENTAL PROCEDURES

Reagents. UHDBT and 5-*n*-undecyl-6-methoxy-4,7-dioxobenzothiazole (Me-UHDBT) were synthesized as described by Selwood and Jandu (1988). The synthesis and characterization (NMR, high-resolution mass spectroscopy) of ubiquinone-1 (UQ_1) and ubiquinone-2 (UQ_2) will be described elsewhere. NQNO was kindly provided by Bernard Trumpower (Dartmouth Medical School). The detergents *n*-dodecyl β-D-maltoside (DDM) and *n*-octyl β-D-glucoside were purchased from Anatrace.

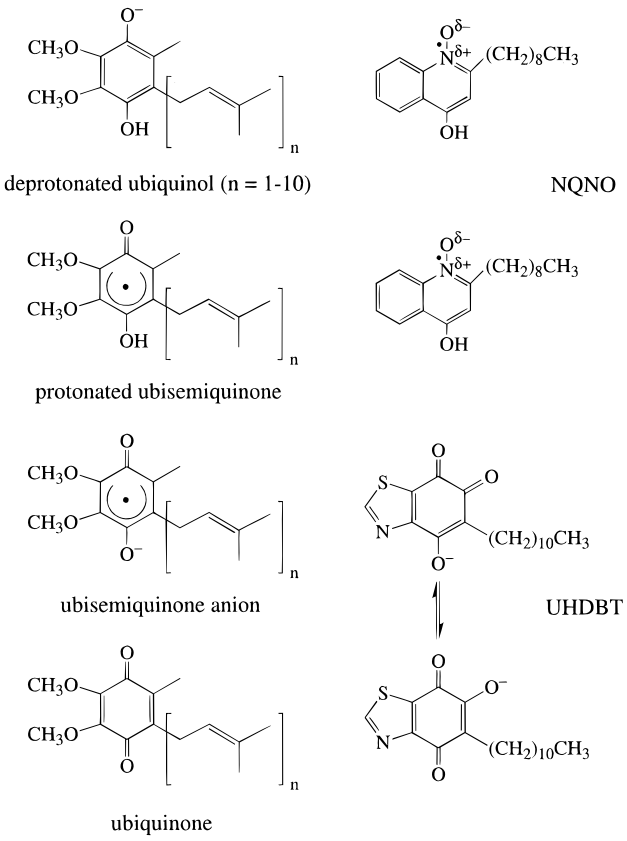


FIGURE 1: Chemical structures of ubiquinone in various redox states and of the inhibitors NQNO and UHDBT. For UQ_1 and UQ_2, *n* is 1 and 2, respectively. Note that UHDBT ($pK_a = 6.5$) (Trumpower & Haggerty, 1980) is ionized under the conditions of the turnover experiments (pH 7.4). Ubiquinol has a pK_a of about 10 (Ksenzhek et al., 1982). The phenolic oxygen on the bottom tautomer of UHDBT is esterified with a methyl group in Me-UHDBT. Thus, Me-UHDBT can act only as a ubiquinone analog.

Protein Purification. Growth of *E. coli* cells and isolation of the "His-tagged" UQO complex was accomplished essentially according to Morgan and co-workers (1995). The University of Illinois (Urbana—Champaign) fermentation facility was utilized for cell growth. Cells (200 g) were treated with 0.5 g of lysozyme in 2 L of 200 mM Tris, 2.5 mM EDTA, pH 7.5, for 15 min and centrifuged for 1 h at 13700*g*, 4 °C. The pellets were suspended in 1.1 L of osmotic lysis buffer (10 mM Tris, 2.5 mM EDTA, pH 7.5), stirred for 30 min, and centrifuged at 30100*g*, 4 °C, for 4 h. The membranes were resuspended with an equal volume of 100 mM NaCl, 5 mM imidazole, 25 mM Tris, pH 7.8. The UQO complex was solubilized from this suspension by addition of an eighth volume each of 10% DDM and 10% *n*-octyl β-D-glucoside (final concentration of each detergent was 1%) followed by stirring on ice for 1 h. After ultracentrifugation at 90700*g*, 4 °C, for 30 min, the red supernatant was diluted with an equal volume of distilled water and applied to a 100 mL Ni-NTA-agarose (Qiagen) column equilibrated with 25 mM Tris, 0.1% DDM, pH 7.8 (Buffer A). The column was washed with (1) 200 mL of Buffer A; (2) 200 mL of Buffer A supplemented with 300 mM NaCl; and (3) 200 mL of Buffer A supplemented with 20 mM imidazole. Pure four-subunit enzyme was eluted with Buffer A supplemented with 200 mM imidazole. Fractions containing pure enzyme were pooled and concen-

trated with a Filtron 100 kDa membrane. A 20-fold dilution with Buffer A and subsequent reconcentration cycle allowed removal of excess imidazole. The enzyme was aliquoted, frozen in liquid nitrogen, and stored at −80 °C until use.

Extinction Coefficients. UHDBT concentration was determined in 0.1 mM ethanolic NH_4OH using $\epsilon_{280} = 15.6$ mM^{-1} cm^{-1} (Trumpower & Haggerty, 1980). Me-UHDBT concentration was estimated in ethanol using $\epsilon_{288} = 12.2$ mM^{-1} cm^{-1}, the extinction coefficient for the protonated form of UHDBT (Trumpower & Haggerty, 1980). NQNO concentration was determined in 1 mM NaOH using $\epsilon_{348} = 9.45$ mM^{-1} cm^{-1} (van Ark & Berden, 1977). Ubiquinone (UQ_1 and UQ_2) and ubiquinol (UQ_1H_2 and UQ_2H_2) concentrations were determined in 80% ethanol using $\epsilon_{276} = 14.0$ mM^{-1} cm^{-1} and $\epsilon_{288} = 4.14$ mM^{-1} cm^{-1}, respectively (Rich, 1984). Enzyme concentration was determined by the pyridine hemochrome method using $\epsilon^{\text{red-ox}}_{566-588} = 68.4$ mM^{-1} cm^{-1} (Berry & Trumpower, 1987). The use of this value assumes that there are two hemes B in the cytochrome *bo*$_3$ complex. While it is true that the absorption spectra of the pyridine hemochrome of hemes B and O are slightly different, the pyridine hemochrome spectra of these two hemes are sufficiently similar (Puustinen & Wikström, 1991) that this extinction is an adequate approximation.

Activty Assay. Approximately 10 mg of ubiquinone was solubilized in about 200 μL of dimethylformamide (DMF) and added to an equal volume mixture of water and diethyl ether (total volume ≈ 3 mL). After the ubiquinone (yellow) was completely reduced to ubiquinol (colorless) by addition of excess solid sodium dithionite, the organic phase was washed once with an equal volume of water and evaporated completely under vacuum. The ubiquinol residue was dissolved under a nitrogen atmosphere in DMF. Enzyme catalyzed ubiquinol oxidation was monitored spectrophotometrically in freshly-made 100 mM Na-phos, 0.1% DDM, pH 7.4, using a Hewlett-Packard 8452 diode array UV/vis spectrophotometer. Ubiquinol was added directly to the assay reaction mixture from the anaerobic DMF stock solution, and the solution was allowed to stabilize (flat base line) before addition of enzyme (∼4 nM) to the stirred cuvette. For a given set of turnover measurements, the total DMF concentration was kept constant and was usually kept below 0.8% of the total assay volume. The oxidized minus reduced $\Delta\epsilon$ for ubiquinone was found to vary as a function of total ubiquinone concentration in the UV region of the optical spectrum (Figure 2). This observation is simply explained by aggregation−induced exciton interactions between ubiquinone molecules within the detergent micelles. For example, the $\Delta\epsilon$ at 292 nm for UQ_2 minus UQ_2H_2 *decreases* by approximately a factor of 2 from 0 to 400 μM. Conversely, the $\Delta\epsilon$ at 316 nm *increases* over the same concentration range. At 412 nm, however, the $\Delta\epsilon$ remains constant and is approximately 0.49 mM^{-1} cm^{-1} for UQ_2 minus UQ_2H_2 and 0.44 mM^{-1} cm^{-1} for UQ_1 minus UQ_1H_2. All turnover numbers reported here were calculated by estimating the initial slope of the kinetics monitored at 412 nm.

Kinetic Model. The kinetic model and notation used in describing the observed kinetics are shown in Figure 3. In this kinetic scheme, the symbol EOO is used to denote the enzyme without bound inhibitor or ubiquinol substrate; the first "O" represents the catalytic substrate binding site,

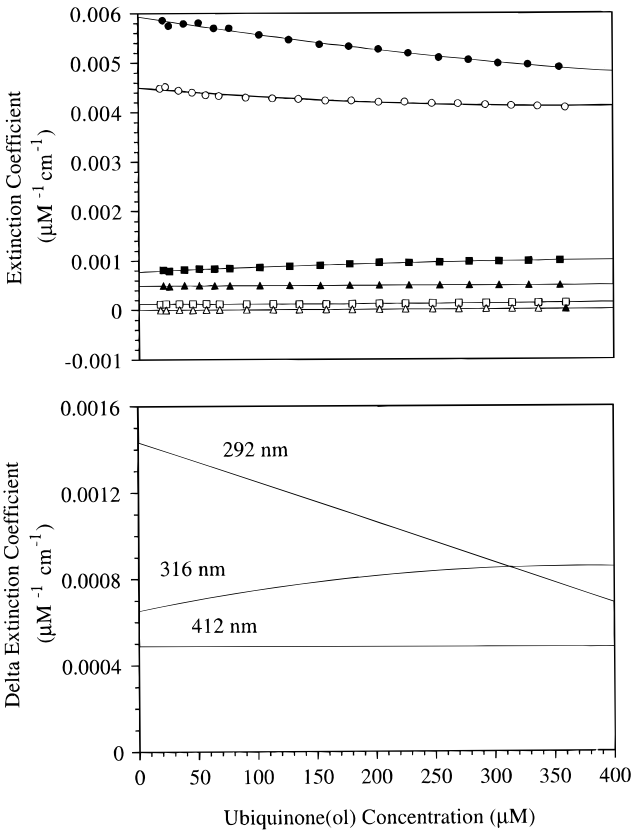
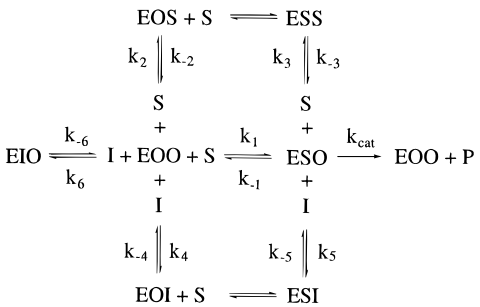


FIGURE 2: (Top) Extinction coefficients (ϵ) for UQ_2 (filled symbols) and UQ_2H_2 (open symbols) as a function of concentration in 100 mM Na-phos, 0.1% DDM, pH 7.4 at 292 nm (circles), 316 nm (squares), and 412 nm (triangles). (Bottom) $\Delta\epsilon$ for UQ_2 minus UQ_2H_2 at these same three wavelengths using the second-degree polynomial fits shown in the top panel.



$$K_M = \frac{k_{-1} + k_{cat}}{k_1} \approx K_D = \frac{[EOO][S]}{[ESO]} = \frac{k_{-1}}{k_1}$$

$$K_{Iu1} = \frac{[EOO][I]}{[EOI]} = \frac{k_{-4}}{k_4}$$

$$K_{S1} = \frac{[EOO][S]}{[EOS]} = \frac{k_{-2}}{k_2}$$

$$K_{Iu2} = \frac{[ESO][I]}{[ESI]} = \frac{k_{-5}}{k_5}$$

$$K_{S2} = \frac{[ESO][S]}{[ESS]} = \frac{k_{-3}}{k_3}$$

$$K_{Ic} = \frac{[EOO][I]}{[EIO]} = \frac{k_{-6}}{k_6}$$

FIGURE 3: Simplified kinetic scheme used for interpretation of the steady-fast turnover data. EOO denotes the enzyme without ubiquinol or inhibitor in either of the two binding sites; S, ubiquinol; P, ubiquinone; and I, an inhibitor. Using the experimental values obtained here (Table 1) and estimating k_1 as at least 1×10^8 M^{-1} s^{-1}, the assumption $K_m \approx K_D$ is accurate to within a factor of 2.

whereas the second "O" is the alternate substrate binding site. Thus, ESO and EIO denote substrate and inhibitor in the catalytic substrate binding site, respectively, whereas EOS and EOI denote substrate and inhibitor in the alternate substrate binding site, respectively. Typical Michaelis−Menten analysis of $v = d[P]/dt = k_{cat}[ESO]$ and the steady-state assumption $d[ESO]/dt \approx 0$ yields the following expression of the initial rate of ubiquinol oxidation:

$$\nu = (V_{max}[S])/\left[K_m\left(1 + \frac{[S]}{K_{S1}} + \frac{[I]}{K_{Ic}} + \frac{[I]}{K_{Iu1}}\right) + [S]\left(1 + \frac{[S]}{K_{S2}} + \frac{[I]}{K_{Iu2}}\right)\right] \quad (1)$$

where $V_{max} = k_{cat}[E_{total}]$. As there is no way to distinguish kinetically between EIO and EOI, the average binding constant $K_{ave} = (K_{Ic}K_{Iu1})/(K_{Ic} + K_{Iu1})$ is introduced to simplify the kinetic expression:

$$\nu = (V_{max}[S])/\left[K_m\left(1 + \frac{[S]}{K_{S1}} + \frac{[I]}{K_{ave}}\right) + [S]\left(1 + \frac{[S]}{K_{S2}} + \frac{[I]}{K_{Iu2}}\right)\right] \quad (2)$$

Also, the K_{S1} term was found to be kinetically unimportant for the UQO complex:

$$\nu = (V_{max}[S])/\left[K_m\left(1 + \frac{[I]}{K_{ave}}\right) + [S]\left(1 + \frac{[S]}{K_{S2}} + \frac{[I]}{K_{Iu2}}\right)\right] \quad (3)$$

Note that *competitive* inhibitors bind to the catalytic substrate binding site with a binding affinity described by the inhibition constant K_{Ic}. Kinetically, these inhibitors increase the effective K_m but do not affect V_{max} because the effect of the inhibitor can be obviated by high substrate concentration. *Uncompetitive* and *noncompetitive* inhibitors do not bind to the catalytic substrate binding site but rather decrease enzyme activity when bound to another site on the enzyme. Since these types of inhibitors can bind to both free enzyme, EOO, as well as the enzyme−substrate complex, ESO, two inhibition constants are required per inhibitor in the kinetic model (K_{Iu1} and K_{Iu2} for the inhibitor, I, and K_{S1} and K_{S2} for the substrate, S, where the subscripts 1 and 2 are used to denote the dissociation constant when there is not and when there is substrate in the catalytic site, respectively). Uncompetitive inhibitors are defined as those which bind much stronger to ESO than to EOO ($K_{Iu1} \gg K_{Iu2}$; $K_{S1} \gg K_{S2}$) while noncompetitive inhibitors bind to both ESO and EOO with approximately equal affinity (K_{Iu1} and K_{Iu2} are comparable; K_{S1} and K_{S2} are comparable). Note that if $K_{Iu1} \ll K_{Iu2}$, the inhibitor behaves competitively; it must be determined by other means whether the inhibitor binds to the substrate binding site or to an alternate site. Kinetically, uncompetitive inhibitors decrease V_{max} and K_m by the same factor ($1 + [I]/K_{Iu2}$). In contrast, noncompetitive inhibitors decrease V_{max} by the factor ($1 + [I]/K_{Iu2}$) but K_m remains the same since these inhibitors bind equally well to both EOO and ESO. The number of constants required to fit the data and where they are required allows assignment of the inhibitor type for a given inhibitor. Note that an inhibitor can behave noncompetitively and yet not bind to the enzyme according to the classical definition given above (i.e., that $K_{Iu1} = K_{Iu2}$). For example, an inhibitor that binds competitively (e.g., at the catalytic site) and also binds uncompetitively (e.g., to ESO) will behave kinetically as a noncompetitive inhibitor (Cleland's Rules). In this work, the data were fitted with Kaleidagraph using least-squares regression.

RESULTS AND DISCUSSION

Substrate Inhibition

Figure 4 shows that enzyme activity is inhibited by high concentrations of UQ_1H_2 or UQ_2H_2. For both ubiquinols, a good fit to the data was obtained using eq 3. When the constant K_{S1} was included in the fitting algorithm as in eq 2, the values obtained for this constant were highly variable and typically exceedingly large. The magnitude and high variability of K_{S1} imply that this constant is kinetically unimportant (i.e., the ubiquinol substrate acts as an uncompetitive inhibitor). The large difference between K_{S1} and K_{S2} indicates, however, that strong allosteric interactions exist between the two ubiquinol binding sites. The values for k_{cat}, K_m and K_{S2} found in this study are tabulated in Table 1.

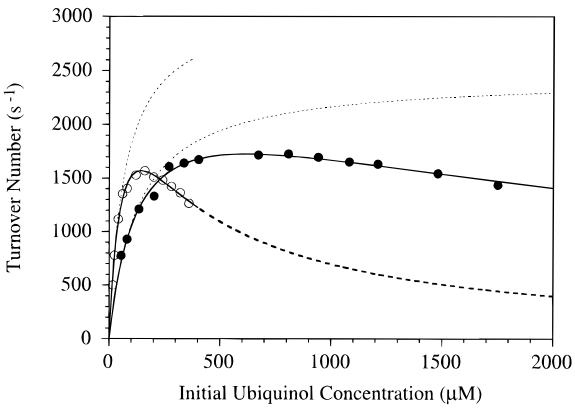


FIGURE 4: UQ_1H_2 (filled circles) and UQ_2H_2 (open circles) activity of the cytochrome bo_3 complex. The data were fitted using eq 3 to yield $k_{cat} = 2440$ s^{-1}, $K_m = 128$ μM, and $K_{S2} = 3000$ μM for UQ_1H_2 and $k_{cat} = 3090$ s^{-1}, $K_m = 70.1$ μM, and $K_{S2} = 296$ μM for UQ_2H_2. Turnover number $= \nu/[E_{total}]$ where ν is in units of μM e$^-$/s. The dashed curves describe the expected activity assuming no substrate inhibition occurs and were simulated using the k_{cat} and K_m values from the eq 3 fit.

Table 1: Summary of Kinetic Parameters[a]

	UQ_2H_2	UQ_1H_2
k_{cat}	3030 ± 370 (6)	2450 ± 260 (3)
K_m	65.4 ± 13.6 (6)	172 ± 52 (3)
K_{S2}	320 ± 132 (6)	2970 ± 790 (3)
	NQNO	UHDBT
K_{ave}	0.270 ± 0.075 (4)	2.78 ± 0.26 (4)
K_{Iu2}	1.58 ± 0.69 (4)	6.70 ± 2.26 (4)

[a] Values are given as average ± standard deviation (number of independent data sets). Units for k_{cat} are s^{-1}. All other values are given in units of μM. Each of the values is averaged over data from two different preparations of enzyme. The inhibitor binding constants were measured with UQ_2H_2 as the substrate.

The shapes of the best-fit curves for UQ_1H_2 and UQ_2H_2 are clearly similar yet they differ dramatically in scale along the ubiquinol concentration axis. This difference results from the difference in partition coefficients describing the distribution between the aqueous and micellar phases for the two ubiquinols due to the extra isoprene moiety of UQ_2H_2 (Rich & Harper, 1990). Under the conditions of the turnover experiments, the reduction rates of UQ_1 and UQ_2 by dithiothreitol (DTT) differ by about an order of magnitude (data not shown). As DTT partitions preferentially into the aqueous phase and this is where ubiquinone reduction occurs, UQ_1 clearly is more soluble in the aqueous phase. The solubility limit of UQ_2H_2 under the experimental conditions was found to be about 400 μM whereas that for UQ_1H_2 is greater than 2 mM. The difference in partition coefficients for the two ubiquinones also explains the difference in their

oxidized minus reduced $\Delta\epsilon$ at 412 nm (see Experimental Procedures). The more hydrophilic environment experienced on average by UQ_1 and UQ_1H_2 lowers the absorption extinction coefficients of these molecules relative to UQ_2 and UQ_2H_2, respectively.

The high concentrations of ubiquinol used in these experiments are expected to lead to nonideal solution behavior. One might question, therefore, whether the kinetic effect observed arise at least in part from nonideal behavior of the $UQ(H_2)$ in solution. The hyperchromic and hypochromic interactions observed in the UV region of the optical spectrum (Figure 2) indicate that the $UQ(H_2)$ molecules interact electronically with each other. This interaction occurs most likely through π-stacking of the chromophores which in turn can decrease the activity of the ubiquinol in the buffer solution. However, since the extinction coefficients for the ubiquinones vary by at most about 20%, the activity coefficients are not expected to deviate from unity by greater than 20−30% (Ts'o & Chan, 1964). In any case, the values for K_{S2} determined here are upper limits for this constant.

Figure 4 also shows the simulated Michaelis−Menten curves (dashed lines) assuming no substrate inhibition occurs using the k_{cat} and K_m values determined from the eq 3 fit of the data. The difference between the observed and the expected enzyme activity at relatively low ubiquinol concentrations (e.g., 100 μM UQ_2H_2 and 400 μM UQ_1H_2) dramatically demonstrates that substrate inhibition occurs even at these low ubiquinol concentrations. The 15−20% variation in the UQ_2 and UQ_2H_2 extinction coefficients (Figure 2) is insufficient to explain the approximate 2-fold difference between the observed and expected enzyme activity at the higher initial UQ_2H_2 concentrations (e.g., 350 μM).

Inhibition by NQNO and UHDBT

The inhibition of UQO activity by NQNO and UHDBT is demonstrated in Figure 5. The kinetic constants k_{cat}, K_m, and K_{S2} for UQ_2H_2 determined in the absence of inhibitor were used to fit the data obtained in the presence of inhibitor. Importantly, for both inhibitors, the inclusion of both K_{ave} and K_{Iu2} was required to fit the data adequately. This is dramatically shown in Figure 6 where the best fit to the data with either one of these constants alone is shown. The values for K_{ave} and K_{Iu2} found in this study are also tabulated in Table 1. The turnover measurements conducted in the absence of an inhibitor can be considered as control experiments for the same measurements conducted in the presence of an inhibitor. The ubiquinol self-interactions present in solution are expected to be present to approximately the same extent in both situations at the low concentrations of inhibitor examined relative to the ubiquinol concentrations used here.

Ubiquinol Binding Sites

The substrate inhibition of UQO activity at high concentrations of UQ_1H_2 and UQ_2H_2 implies that ubiquinol can bind two different locations on the enzyme complex. One of these sites is clearly the ubiquinol electron input site (high-affinity ubiquinol binding site) while the other must be a distinctly different site (low-affinity ubiquinol binding site). The fact that two inhibition constants are required to fit the inhibitor

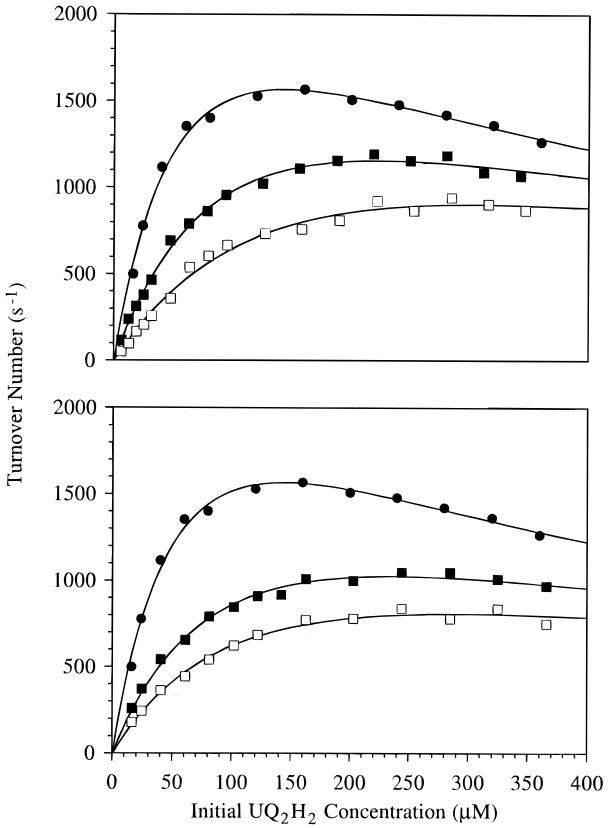


FIGURE 5: NQNO and UHDBT inhibition of UQ_2H_2 activity. (Top) No inhibitor (filled circles), 0.5 μM NQNO (filled squares), and 1.0 μM NQNO (open squares). (Bottom) No inhibitor (filled circles), 4.1 μM UHDBT (filled squares), and 8.2 μM UHDBT (open squares). The data were fitted using eq 3.

data indicates that NQNO and UHDBT must bind to a site on the enzyme distinct from the ubiquinol oxidation site. Since these two inhibitors are $UQ(H_2)$ analogs and, as such, are expected to bind at $UQ(H_2)$ binding sites, it is reasonable to conclude that these inhibitors bind to the low-affinity ubiquinol binding site. Sato-Watanabe and co-workers found a UQ_8 molecule strongly bound to the wild type UQO complex as isolated that could not be removed by either excess UQ_1H_2 or inhibitors (Sato-Watanabe et al., 1994b). When the UQO complex is isolated using DDM for membrane solubilization according to the procedure used in this study, the enzyme obtained contains this UQ_8 molecule (Morgan et al., 1995) as we have independently confirmed (data now shown). If the binding site for the UQ_8 molecule corresponds to the inhibitor binding site (or the low-affinity ubiquinol binding site), then the strongly bound UQ_8 molecule must exchange with the $UQ(H_2)$ pool under turnover conditions. This UQ_8 binding site has been previously shown to be accessible in the oxidized enzyme, though not in rapid exchange with species in solution (Puustinen et al., 1996; Sato-Watanabe et al., 1994b). Unfortunately, at this juncture, we cannot rule out the possibility that there are three $UQ(H_2)$ binding site (the UQ_8 binding site plus the two sites found here under turnover conditions). However, we favor the simpler two-binding-site scenario. It is quite possible that the long isoprene tail of UQ_8 is wrapped tightly around the hydrophobic transmembrane domain of the enzyme (as is found in photosynthetic reaction center structures). The head group of such a strongly-bound ubiquinone molecule could potentially move

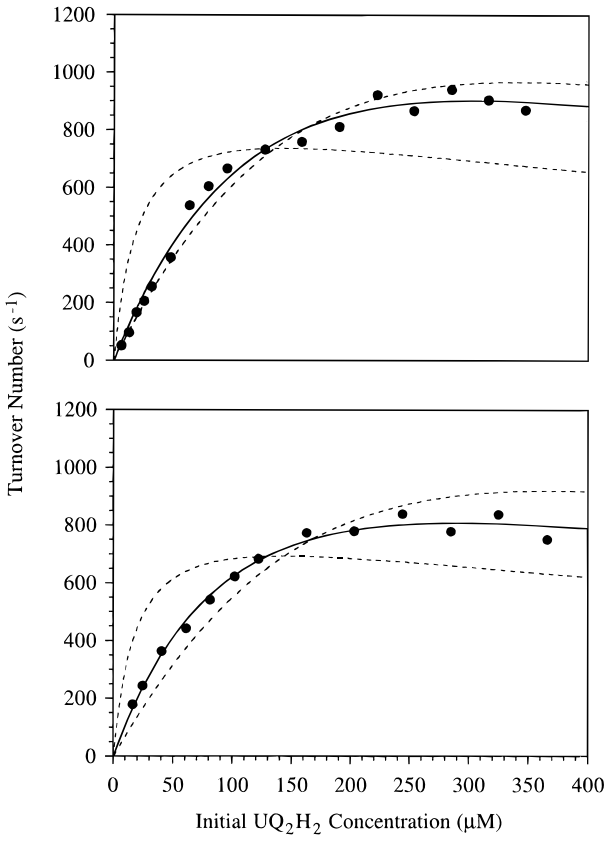


FIGURE 6: UQ_2H_2 activity in the presence of 1.0 μM NQNO (top) and 8.2 μM UHDBT (bottom). The solid lines are the best fit to eq 3 with both K_{ave} and K_{Iu2} included. The dashed curves are the best fits when only one of these constants is included. Same data as shown in Figure 5.

relatively freely into and out of its binding pocket but exchange between detergent micelles is expected to be severely inhibited due to the extreme hydrophobicity and large size of the UQ_8 molecule. Therefore, it is not surprising that tightly-bound UQ_8 cannot be removed from the UQO complex by simply washing the enzyme with detergent buffer.

The chemical nature of NQNO and UHDBT offers some insight into the structural features of the low-affinity ubiquinol binding site. The chemical structures of these inhibitors and the various oxidation states of ubiquinone are shown in Figure 1. UHDBT has a phenolic proton with a pK_a of 6.5 (Trumpower & Haggerty, 1980). Thus, under the conditions of the inhibition experiments, UHDBT is ionized and two tautomers are present in solution. One of these tautomers behaves as an ubiquinone analog, and the other behaves as a ubisemiquinone anion analog. NQNO can behave as a deprotonated ubiquinol molecule or a protonated ubisemiquinone analog. Therefore, three scenarios explaining the observed inhibition can be distinguished: (1) UHDBT is recognized as a ubiquinone analog; (2) NQNO is recognized as a deprotonated ubiquinol analog; and (3) both inhibitors are recognized as stable ubisemiquinone analogs. In each of these scenarios, the presence of an inhibitor in the low-affinity ubiquinol binding site slows enzyme turnover [slow reduction of UHDBT may occur under these conditions as its $E_{m,7} = -40$ mV (Trumpower & Haggerty, 1980)]. High concentrations of ubiquinone (at least 100 μM UQ_2) are required in order to inhibit UQ_2H_2 oxidation (data not shown). Further, Me-UHDBT, a form of UHDBT which can only act as a ubiquinone analog, is less than 10% as effective as UHDBT as an inhibitor (data not shown).[2] These observations rule out scenario (1) leading us to surmise that the UHDBT tautomer that is chemically similar to ubisemiquinone is the inhibitory form of this inhibitor. As the noncompetitive nature of UHDBT inhibition indicates that UHDBT binds to the low-affinity ubiquinol binding site, this site must therefore stabilize ubisemiquinone. Note that the enzyme's affinity for both NQNO and UHDBT is stronger than that for the ubiquinol substrates (by at least 10-fold) and both act noncompetitively. It is quite likely, then, that NQNO is recognized by the enzyme as a ubisemiquinone analog as well. As the pK_a of ubiquinol is about 10 (Ksenzhek et al., 1982) and thus ubiquinol is fully protonated under the turnover conditions utilized here, it is unlikely that scenario 2 is a valid explanation of the NQNO inhibition data. Thus, scenario 3 alone appears to be the most reasonable explanation of the inhibition data. Whether UHDBT and NQNO bind both competitively (e.g., to the catalytic site) and uncompetitively (e.g., to ESO) and therefore yield a noncompetitive binding signature or whether they bind to EOO and ESO with approximately equal affinity (classical noncompetitive inhibitor) cannot be determined from the data here. However, it is clear that both inhibitors must bind to a site that is distinct from the ubiquinol oxidation site. The above analysis suggests that this alternate binding site recognizes these inhibitors as stable ubisemiquinone analogs.

Matsushita and co-workers (1984) and Sato-Watanabe and co-workers (1994a) observed that HQNO (same as NQNO but with a shorter hydrocarbon tail) noncompetitively inhibits UQO turnover in agreement with the studies reported here. The latter workers tested the inhibitory effects of an extensive series of benzophenols and found that the inhibition kinetics varies dramatically. For example, changing both of the bromine substituents of 2,6-dibromo-4-dicyanovinylphenol to iodine alters the observed inhibitory mechanism from noncompetitive to competitive; changing a single bromine substituent to a methoxy group results in an uncompetitive inhibitor. All three of these benzophenol inhibitors have a p$I_{50} = 4.1-4.2$ (pI_{50} = log of the reciprocal of the molar concentrations of the inhibitors required to halve the full enzyme activity). Noncompetitive and uncompetitive inhibitors clearly must bind to a site different from the ubiquinol oxidation site. Also, an inhibitor can behave competitively and yet not bind to the substrate binding site (e.g., $K_{Iu1} \ll K_{Iu2}$); for example, 2,6-dichloro-4-nitrophenol behaves as a competitive inhibitor and yet binds to the UQ_8 binding site (Sato-Watanabe et al., 1994a,b). As phenols are chemically similar to ubisemiquinones, it is reasonable to assume that these benzophenol inhibitors bind to the low-affinity ubiquinol binding site found here. Thus, the three benzophenol inhibitors mentioned above likely all inhibit turnover by binding to the low-affinity ubiquinol binding site but the variation in substituents alters the relationship between K_{Iu1} and K_{Iu2} dramatically. Note that a difference in K_{Iu1} and K_{Iu2} requires allosteric interactions between the two ubiquinol binding sites.

[2] Attempts to determine accurately the inhibition constants of UQ_2 and Me-UHDBT were unsuccessful because the high concentrations that are required perturb the activity coefficient of ubiquinol in an unknown manner.

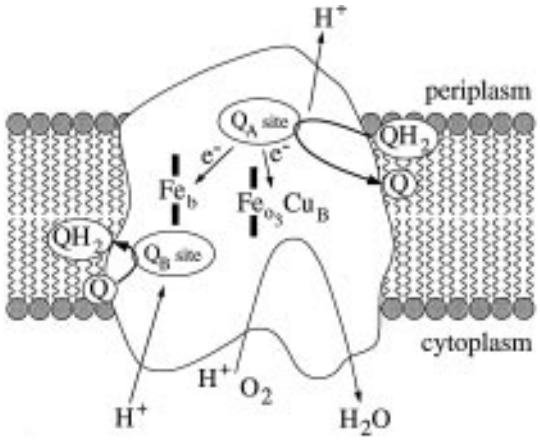
Mechanism of Proton Translocation

The structural similarities of the ubiquinol and cytochrome *c* oxidase complexes lead naturally to the hypothesis that both families of enzymes catalyze proton translocation via similar mechanisms. The observed overall H^+/e^- stoichiometry for the UQO complex has been found to be 2 (Puustinen et al., 1989). Half of the protons translocated most certainly arise from the scalar release of protons from the ubiquinol oxidation chemistry and the uptake of protons from the opposite side of the membrane to electrically balance the dioxygen reduction chemistry. A cytochrome *c* oxidase-type proton pump mechanism may account for the other protons:

$$2QH_2 + 4H^+ \text{(inside-scalar)} + O_2 + 4H^+ \text{(inside-pump)} \rightarrow 2Q + 4H^+ \text{(outside-scalar)} + 2H_2O + 4H^+ \text{(outside-pump)} \quad (4)$$

While only one UQ(H_2) binding site is required to satisfy the chemistry of this turnover cycle, the finding that there are two UQ(H_2) binding sites on the UQO complex does not imply that this proton translocation mechanism is incorrect. For example, Sato-Watanabe and co-workers (1994b) postulated that a strongly bound ubiquinone molecule (at the Q_H site) mediates electron input from ubiquinol (at the Q_L site). The ubiquinone binding site (Q_H site) could certainly, in fact is expected to, stabilize a ubisemiquinone species. However, this ubiquinone binding site is *not* expected to be in dynamic equilibrium with the UQ(H_2)-pool as it is unreasonable to postulate that a UQ_8 molecule acting simply as an electron-accepting prosthetic group would exchange with the UQ(H_2)-pool during turnover. Such an hypothesis is tantamount to postulating that a heme or copper ion diffuses in and out of a binding pocket during turnover. Note that the Q_A quinone in bacterial reaction centers, which functions as a transient electron acceptor, remains tightly bound throughout enzyme turnover and is actually quite difficult to remove or reconstitute (Warncke et al., 1994). Thus, this model does not predict uncompetitive substrate inhibition or noncompetitive inhibition by UQ(H_2) analogs. A third UQ(H_2) binding site could be involved in regulating enzyme turnover. There would then be two UQ(H_2) binding sites in dynamic equilibrium with the UQ(H_2)-pool in addition to the site that strongly binds ubiquinone. According to the data presented here, however, such a regulatory UQ(H_2) binding site would have to strongly stabilize ubisemiquinone. Such a scenario would be unprecedented.

Musser and co-workers (1993) postulated that a Q(H_2)-loop operates during turnover of the UQO complex. According to this Q(H_2)-loop model, the two Q(H_2) binding sites are termed the Q_A site and the Q_B site (Figure 7). Ubiquinol oxidation occurs at the Q_A site, formed at least in part by the transmembrane segments of subunit II, by analogy with the Cu_A electron input site of the cytochrome *c* oxidase complexes. Studies with a photoreactive azidoubiquinone derivative support this hypothesis (Welter et al., 1994). Ubiquinone reduction occurs at the Q_B site which is located near cytochrome *b*, by analogy with the Q_N site of the cytochrome bc_1 complex (Trumpower, 1990). The blue-shift of the Soret absorption band and the perturbations to resonance Raman lines for heme *b* but not for heme o_3 upon



	Q_B site	Q_A site
$K_D(UQ_1)$	0.0025 mM[a]	> 10 mM[b]
$K_D(UQ_1H_2)$	2.97 mM[c]	0.172 mM[c]
location	subunit I	subunit II

FIGURE 7: Schematic of the proposed Q(H_2)-loop proton translocation mechanism of the cytochrome bo_3 complex. See text for details. Notes: (a) from Sato-Watanabe and co−workers (Sato-Watanabe et al., 1994b). This value is certainly much lower for UQ_8; (b) on the basis of the difference in UQ(H_2) binding affinities of the Q_B site and the apparent absence of ubiquinone inhibition, this value is likely to be greater than 10 mM; and (c) this work (assuming $K_m \approx K_D$). All of these binding constants are lower for UQ_2H_2 (this work) and UQ_2 (expected).

addition of UQ_1 to the ubiquinone-free enzyme (Sato-Watanabe et al., 1994b) support this postulate. The two ubiquinol electrons split at the Q_A site. Under steady-state conditions, one electron is tranferred to the dioxygen binding and reduction site, whereas the other is donated to the cytochrome *b*/ubiquinone shunt. This model predicts that the Q_B site stabilizes a ubisemiquinone intermediate and therefore agrees well with the stable ubisemiquinone species found by a number of investigators (Ingledew et al., 1995; Sato-Watanabe et al., 1995). In order for net proton translocation to occur during enzyme turnover, all protons released from the Q_A site must exit on the periplasmic side of the membrane whereas the protons required at the Q_B site must originate on the cytoplasmic side of the membrane. The experimentally observed $2H^+/e^-$ stoichiometry (Puustinen et al. 1989) is thus fully accounted for by the Q(H_2)-loop hypothesis:

$$4QH_2\ (Q_A \text{ site}) + 4H^+ \text{(scalar)} + O_2 + 4H^+\ (Q_B \text{ site}) + 2Q\ (Q_B \text{ site}) \rightarrow 4Q\ (Q_A \text{ site}) + 8H^+\ (Q_A \text{ site}) + 2H_2O + 2QH_2\ (Q_B \text{ site}) \quad (5)$$

Both of these UQ(H_2) binding sites are necessarily in dynamic equilibrium with the UQ(H_2) pool and the binding of ubiquinol or inhibitors to the Q_B site are expected to perturb the oxidation kinetics of ubiquinol at the Q_A site. It is therefore unnecessary to postulate a third UQ(H_2) binding site to explain the data reported here: the high-affinity ubiquinol binding site is equivalent to the Q_A site, whereas the low-affinity ubiquinol binding site is equivalent to the Q_B site. In this scenario, the Michaelis constants (K_m) for

UQ_1H_2 and UQ_2H_2 determined here (Table 1) are good estimates of the Q_A site binding affinities for UQ_1H_2 and UQ_2H_2, respectively, whereas the uncompetitive substrate inhibition constants (K_{S2}) are a measure of the binding affinity of the Q_B site for UQ_1H_2 and UQ_2H_2 (Figure 7). Sato-Watanabe and co-workers (1994b) obtained a dissociation constant of 2.5 μM for UQ_1 bound to the UQ_8 binding site; according to this Q(H_2)-loop model, then, this dissociation constant describes the affinity of the Q_B site for UQ_1. The large difference in the Q_B site binding affinities for UQ_1 and UQ_1H_2, as well as the lack of ubiquinone inhibition (except at, perhaps, very high ubiquinone concentrations), suggests that the dissociation constant for UQ_1 in the Q_A site is quite large (e.g., >10 mM) (Figure 7). While from a structural standpoint there is good reason to presume a cytochrome *c* oxidase-type proton pump mechanism for the ubiquinol oxidase complexes, this Q(H_2)-loop proton translocation mechanism offers an alternative model that is consistent with the experimental data.

Energetics of the Turnover Cycle

It is imperative to question whether it is thermodynamically feasible for the UQO complex to catalyze proton translocation for every electron input to the enzyme according to the above Q(H_2)-loop mechanism. The cytochrome *c* oxidase complexes pump protons only during the second half of the dioxygen reduction cycle (Wikström, 1989). This feature of cytochrome *c* oxidase activity is explained by the thermodynamics of the chemistry: the H_2O_2/H_2O redox couple is much more exergonic than the O_2/H_2O_2 redox couple ($\geq$1 V vs $\geq$500 mV, respectively) (Musser & Chan, 1995). However, whereas cytochrome *c* has a reduction potential of about 250 mV (Rodkey & Ball, 1950), ubiquinone has a reduction potential of about 70 mV (Rich, 1984). Assuming a similar potential for the O_2/H_2O_2 redox couple in the ubiquinol and cytochrome *c* oxidase complexes ($\geq$500 mV) (Musser & Chan, 1995), more than 400 meV of redox free energy is available to translocate $2H^+/e^-$ against a typical 200 mV proton motive force (Rottenberg, 1979; Wikström & Saraste, 1984) for the first two electrons of the dioxygen reduction cycle. This analysis implies that essentially all of the redox free energy would be conserved ($2H^+ \times 200$ meV = 400 meV) by the proton translocation reactions. Energy to *drive* this process may ensue from a high ubiquinol:ubiquinone ratio and/or from a greater functional potential of the O_2/H_2O_2 redox couple. Note that for the exergonic dioxygen reduction reactions to be coupled to the proton translocation reactions of the proposed Q(H_2)-loop mechanism, proton uptake at the Q_B site must occur subsequent to dioxygen binding, that is, after two-electron reduction of the heme o_3-Cu_B binuclear center. Cytochrome *b* may be utilized to temporarily store an electron so that the two-electron reduction of the Q_B site ubiquinone in the absence of proton uptake is not required. Thus, it is thermodynamically reasonable that all four electrons of the UQO dioxygen reduction cycle are coupled to proton translocation reactions.

Since the dioxygen chemistry of the ubiquinol and cytochrome *c* oxidase complexes is so similar, it is certainly expected that the third and fourth electrons input to the UQO complex are energetic enough to drive the translocation of $3H^+/e^-$ (one of which is a scalar proton; the other two could arise from a cytochrome *c* oxidase-type proton pump mechanism). This is the electron-dependent stoichiometry inherent in eq 4. If it is assumed that a cytochrome *c* oxidase-type proton pump mechanism is responsible for the translocation of $2H^+/e^-$ for the third and fourth electrons of the dioxygen chemistry, then there is no thermodynamic reason why $1H^+/e^-$ cannot be pumped for the first two electrons via partially uncoupling the same translocation mechanism. If protons are pumped for the first two electrons of the catalytic cycle in this manner, the overall number of protons released on the periplasmic side of the membrane would be $10H^+/4e^-$ (4 scalar protons and 6 pumped protons, two for the first two electrons, four for the last two electrons). The fact that the cytochrome bc_1 and cytochrome *c* oxidase complexes acting in tandem translocate $12H^+/4e^-$ indicates from a *thermodynamic* standpoint the UQO complex could certainly translocate the same number of protons. However, the observed stoichiometry is $8H^+/4e^-$ (Puustinen et al., 1989). Note that the proton translocation efficiency of the proposed Q(H_2)-loop cannot be *increased* for the more energetic third and fourth electrons. Thus, this Q(H_2)-loop proton translocation mechanism rationally explains why the UQO complex translocates $8H^+/4e^-$ despite the thermodynamic possibility of translocating $10H^+/4e^-$ or even $12H^+/4e^-$. It is certainly possible, however, that mechanistic limitations preclude coupling the first two electrons of the catalytic cycle to proton translocation.

Physical Basis of Substrate Inhibition

In the experiments reported here, enzyme turnover is monitored after mixing of enzyme and ubiquinol. There is no exogenous ubiquinone (there are approximately 2 equiv of UQ_8 bound to the enzyme, however, at time $t = 0$). Considering that the observed enzyme turnover is at least 500 s^{-1} and the enzyme concentration is about 4 nM, micromolar concentrations of ubiquinone are expected to arise within the 2−3 s mixing time in the stirred cuvette. This concentration of ubiquinone is sufficient for coupled turnover (i.e., the proton translocation mechanisms are operational) according to the Q(H_2)-loop model presented here. At low initial ubiquinol concentrations, turnover proceeds as expected: ubiquinol binds preferentially to the Q_A site and ubiquinone binds preferentially to the Q_B site. It is thus apparent that in the kinetic model described here, ESO must denote the enzyme species with a ubiquinone (or ubisemiquinone) molecule in the Q_B site and a ubiquinol molecule in the Q_A site. However, the Q_B site's affinity for ubiquinone is much higher than the Q_A site's affinity for ubiquinol (ensuring that a ubiquinone molecule is present at the Q_B site when a ubiquinol molecule binds to the Q_A site) (Figure 7). As a consequence, the Q_B site's affinity for ubiquinol is higher than the Q_A site's affinity for ubiquinone. At a UQ_2H_2 concentration of 200 μM, then, UQ_2H_2 molecules can effectively compete with micromolecular concentrations of UQ_2 for the Q_B site. Enzyme with ubiquinol molecules in both the Q_A and Q_B sites cannot turnover because the Q_A-Q_B electron transfer path is inhibited (the Q_B−heme *b* site cannot accept another electron). Thus, self-inhibition by UQH_2 is observed at high initial UQH_2 concentrations; any slower turnover that does occur when UQH_2 is bound to the Q_B site is most likely to result of electron leakage. In contrast, 100 μM UQ_2 cannot effectively compete with 100 μM UQ_2H_2 for the Q_A site; the UQ_2/UQ_2H_2 ratio must be much higher before ubiquinone can inhibit electron input at the Q_A site. The Q(H_2)-loop model

presented here therefore explains both why substrate inhibition *is* observed as well as why product inhibition is *not* observed.

CONCLUDING REMARKS

The turnover kinetics of the cytochrome bo_3 complex are clearly more complicated than originally envisioned. The k_{cat} and K_m determined from the experimental data are significantly higher (2−3-fold) when it is recognized that substrate inhibition occurs and the data are fitted with a model that takes into account this fact. The inhibition of ubiquinol oxidase activity by high concentrations of ubiquinol and by the inhibitors NQNO and UHDBT indicates that there are two UQ(H_2) binding sites in dynamic equilibrium with the UQ(H_2) pool during enzyme turnover. The uncompetitive nature of the observed substrate inhibition and the differences between K_{Iu1} and K_{Iu2} for a number of inhibitors indicates that there are allosteric interactions between the two UQ(H_2) binding sites. Since ubiquinone inhibition is not observed (except, perhaps, at very high concentrations), it is clear that the ubiquinol oxidation site (Q_A site) has a low affinity for ubiquinone. The low-affinity ubiquinol binding site (Q_B site) binds UQ_2H_2 with a dissociation constant of 320 μM and UQ_1H_2 with a dissociation constant of 2970 μM (K_{S2}). This Q_B site is likely to be the site occupied by a UQ_8 molecule in the DDM-isolated enzyme. The high affinity of the Q_B site for ubisemiquinone analogs indicates that this UQ(H_2) binding site strongly stabilizes ubisemiquinone, in agreement with previous EPR studies (Ingledew et al., 1995; Sato-Watanabe et al., 1995). A turnover cycle in which all protons are translocated via a Q(H_2)-loop mechanism is considered the most reasonable explanation for these data, although a cytochrome *c* oxidase-type proton pump mechanism (functioning to translocate perhaps only some protons) cannot be ruled out. The current work indicates the need for further experimentation to distinguish these proton-pumping mechanisms.

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