Tight Binding of Inhibitors to Bovine $bc_1$ Complex Is Independent of the Rieske Protein Redox State

CONSEQUENCES FOR SEMIQUINONE STABILIZATION IN THE QUINOL OXIDATION SITE*

Received for publication, August 7, 2002, and in revised form, September 29, 2002
Published, JBC Papers in Press, October 2, 2002, DOI 10.1074/jbc.M208060200

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To determine the effect of the redox state of the Rieske protein on ligand binding to the quinol oxidation site of the $bc_1$ complex, we measured the binding rate constants ($k_1$) for stigmatellin and myxothiazol, at different concentrations of decylbenzozquinone or decylbenzozquinol, in the bovine $bc_1$ complex with the Rieske protein in the oxidized or reduced state. Stigmatellin and myxothiazol bound tightly and competitively with respect to quinol or quinol, independently of the redox state of the protein. In the oxidized $bc_1$ complex, the $k_1$ values for stigmatellin ($-2.6 \times 10^8 \text{M}^{-1}\text{s}^{-1}$) and myxothiazol ($-8 \times 10^8 \text{M}^{-1}\text{s}^{-1}$), and the dissociation constant ($K_d$) for quinol, were similar between pH 6.5 and 9, indicating that ligand binding is independent of the protonation state of histidine 161 of the Rieske protein (pK$_a$ ~7.6). Reduction of the Rieske protein increased the $k_1$ value for stigmatellin and decreased the $K_d$ value for quinol by 50%, without modifying the $k_1$ for myxothiazol. These results indicate that reduction of the Rieske protein and protonation of histidine 161 do not induce a strong stabilization of ligand binding to the quinol oxidation site, as assumed in models that propose the existence of a highly stabilized semiquinone as a reaction intermediate during quinol oxidation.

The key reaction that allows energy conservation in ubiquinol-cytochrome $c$ oxidoreductase ($bc_1$ complex) is the bifurcation of electrons from quinol toward the iron-sulfur cluster of the Rieske protein and heme $b_1$ of cytochrome $b$ (1, 2). Several models have been proposed to explain the mechanism that prevents the thermodynamically favorable (but energetically wasteful) reduction of the iron-sulfur cluster by both electrons from quinol. These proposals differ, among other aspects, in the stability constant and the role assigned to semiquinone as a reaction intermediate in the quinol oxidation site ($Q_0$ site). Crofts and Wang (3) originally proposed a highly unstable semiquinone, which according to recent modifications of this mechanism (4, 5), would move from the $Q_0$ site subdomain occupied in crystal structures by inhibitors such as stigmatellin to the pocket occupied by myxothiazol and methoxyacrylate inhibitors. Because no electron paramagnetic resonance-detectable semiquinone is observed in the $Q_0$ site (6), and the redox potentials of the iron-sulfur cluster and the $b$ hemes exert a reciprocal control on their reduction rates by quinol, Snyder et al. (7) proposed a concerted mechanism in which the rapid oxidation of semiquinone by heme $b_1$ of cytochrome $b$ drives the thermodynamically unfavorable one-electron reduction of the iron-sulfur cluster by quinol.

In contrast to these models, Link (8) suggested that reduction of the iron-sulfur cluster by quinol leads to a stabilization of semiquinone through a strong hydrogen bond between this intermediate and one of the histidines that binds to the redox cluster (histidine 161 in the bovine $bc_1$ complex). This unusually strong interaction between semiquinone and the reduced Rieske protein would restrain the soluble domain of this subunit from diffusing away from the interface with cytochrome $b$ toward cytochrome $c_1$ until semiquinone reduces heme $b_1$ (9). This would ensure the recycling of the second electron from quinol through cytochrome $b$. The stable semiquinone intermediate would not be detected by electron paramagnetic resonance because of magnetic coupling between the reduced iron-sulfur cluster and semiquinone (8). The basis for assuming the existence of this stable semiquinone is the 250-mV shift in the redox potential of the iron-sulfur cluster induced by binding of stigmatellin (considered as an analogue of semiquinone) to the $bc_1$ complex (10). This effect has been interpreted as indicating an ~$10^3$ higher binding constant of the inhibitor to the iron-sulfur cluster-reduced $bc_1$ complex compared with the fully oxidized enzyme. In apparent agreement with this interpretation, removal of the Rieske protein from the $bc_1$ complex changes stigmatellin inhibition from tightly bound ($K_d < 0.1 \text{nM}$) to weak and reversible ($K_d~50 \mu\text{M}$) (11). However, there is no direct evidence that binding of stigmatellin or quinone is stabilized by reduction of the Rieske protein. Moreover, we have shown recently (12) that quinone binding is not stabilized by protonation of histidine 161 of the Rieske cluster, nor is quinol binding stabilized by deprotonation of this residue, suggesting that the putative hydrogen bond formed between histidine 161 and $Q_0$ site ligands is not essential for binding of substrate and product.

To obtain evidence on the stabilization of ligands at the $Q_0$ site upon reduction of the Rieske protein, we determined the binding rate constants of the tightly bound inhibitors stigmatellin and myxothiazol to the purified bovine heart $bc_1$ complex with the Rieske protein in the oxidized and reduced state and as a function of the protonation state of histidine 161, which has a pK$_a$ of ~7.6 (13). The affinity toward quinone or quinol in these conditions was also determined through competition with the inhibitors. Our results indicate that the effect of the reduction of the iron-sulfur cluster, or the protonation of histidine 161, on the binding of ligands to the $Q_0$ site is much more weaker than that required by models that consider sta-
bilization of semiquinone through a hydrogen bond to the reduced iron-sulfur cluster.

**EXPERIMENTAL PROCEDURES**

**Chemicals—**Decylbenzoquinone, horse heart cytochrome c, 1-ascorbic acid, and Tween 20 were purchased from Sigma. Stigmatellin was from Fluka, and myxothiazol was from ICN Biomedicals Co. All other reagents of analytical grade were from standard suppliers. Decylenzooquinol was prepared as described (14). UV-spectrophotometric quantification of decylbenzoquinone and decylbenzoquinol was performed in ethanol using extinction coefficients reported in Ref. 15, and solubility in the aqueous detergent-containing reaction medium was verified as described before (12). Stigmatellin and myxothiazol quantification was performed using wavelengths and extinction coefficients reported previously (16).

**Purification of Bovine Heart bc Complex—**The isolation procedure and quantification of heme content was done as described previously (12). The purified bc complex contained 7.1 and 3.6 nmol of hemes b and c1/mg of protein, respectively (no heme a was detected spectrophotometrically), and was stored at a concentration of 6–10 μM in 50% (v/v) glycerol and 0.01% β- n-laurylmaltoside at –20 °C and diluted to 1 μM before the assay.

**Determination of Inhibitor Rate Constants and Kd Values for Quinone and Quinol—**Rate constants were determined by using Equation 1,

\[
\frac{[E_i]}{[E_f]} = e^{-k't} \cdot k_1
\]

which describes the time-dependent binding of an inhibitor (I) to the enzyme (E), where [Ei] is the active enzyme remaining at time t, [Ef] is the active enzyme before the addition of inhibitor (time 0), k1 is the second order rate constant for the binding of the inhibitor to the enzyme, and k-1 is the first order rate constant for the dissociation of the enzyme-inhibitor complex. The complete derivation of Equation 1 can be found in Ref. 17. When [I] ≫ [Ei], the amount of free inhibitor can be considered constant throughout the time course of inhibitor binding. Under this condition, the initial rate of cytochrome c reduction is a measure of the amount of active enzyme. Therefore, the time-dependent decrease of the initial rate at each inhibitor concentration was fitted to a single exponential decay function to obtain a pseudo-first order rate constant (k’i) that depended only on the concentration of [I]. k’i is defined by Equation 2.

\[
k'_i = k_i[I] + k_{-1}
\]

According to Equation 2, the value of the second order rate constant (k1) for the binding of the inhibitor to the enzyme was obtained by plotting k’i as a function of [I] and fitting the data to a straight line, where the slope is k1, and the ordinate is k-1. During the fitting procedure, k-1 was constrained to positive values. The variation of the binding rate constants as a function of the concentration of either decylbenzoquinone or decylbenzoquinol (k1 and k2) was fitted to a competitive binding model according to Equation 3.

\[
k_{1,obs} = \frac{k_1}{[Q]} \cdot \frac{1}{K_d}
\]

where k1 is the second order rate constant describing the binding of the inhibitor to the enzyme in the absence of quinone or quinol (Q), and Kd is the dissociation constant of the bc1-Q complex. Mixed or non-competitive kinetic models resulted in poor fitting. All fitting procedures were performed using the Origin 5.0 program (OriginLab Corp.).

**Determination of Inhibitor Binding to the bc Complex with Oxidized or Reduced Rieske Protein—**The assay medium contained 0.01% Tween 20, 1 mM EGTA, and 20 mM of citric acid (pH 7.5), and Taps1 (pKb 8.4) to efficiently buffer the pH in the range of 6.5 to 9. All measurements were performed at 30 °C. No difference was found between the spectra of the air-oxidized complex at pH 7.5, where the Rieske protein is more oxidizing than cytochrome c1, and at pH 9, where the redox potential of the Rieske protein decreases below that of cytochrome c1 (13), indicating that the Rieske protein was oxidized fully (Fig. 1, traces a and b). Comparison with the ferricyanide-oxidized bc1 complex (Fig. 1, trace c) indicated that >97% of cytochrome c1 in the

1 The abbreviation used is: Taps, 3-[(2-hydroxy-1,1-bis(hydroxymethyl)amino)-1-propanesulfonic acid (systematic).

**FIG. 1. Spectra of purified bovine heart bc1 complex 1 μM with different reductants. a, air-oxidized (pH 7.5); b, air-oxidized (pH 9); c, potassium ferricyanide (1 mM)-oxidized; d, ascorbate (1 mM)-reduced; e, decylbenzoquinol (4 μM)-reduced; f, dithionite-reduced; g, dithionite minus ascorbate-reduced; h, dithionite minus decylbenzoquinol-reduced; i, decylbenzoquinol minus ascorbate-reduced. Spectra were carried out in the reaction medium described under "Experimental Procedures." All spectra, except g, were obtained at pH 7.5.**

**RESULTS**

**Binding and Dissociation Rate Constants for Stigmatellin and Myxothiazol in the Fully Oxidized bc1 Complex—**The time-

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2 The decylbenzoquinol minus ascorbate-reduced spectrum showed that the full reduction of cytochrome c1 (and therefore of the Rieske protein) was attained by adding ascorbate or decylbenzoquinol.
Stigmatellin Binds Tightly to the Oxidized bc₁ Complex

FIG. 2. Determination of kinetic rate constants for stigmatellin and myxothiazol binding to the oxidized bc₁ complex at pH 7.5. Pseudo-first order rate constants (k') for various concentrations of stigmatellin (A) and myxothiazol (B) in the air-oxidized bc₁ complex were obtained by fitting each time-dependent inhibition of the initial rate to a first-order decay function. The slopes (± S.E. of the fitting) obtained by linear fitting of the k’ versus inhibitor concentration data (C) correspond to the second order binding rate constants (kₐ) for stigmatellin in both air- and ferricyanide-oxidized conditions (values of −0.004 were obtained if the ordinates were not constrained to positive values) and 0.0037 ± 0.001 for myxothiazol.

**DISCUSSION**

Stigmatellin Binds Tightly to All Redox Forms of the bc₁ Complex—Our finding that stigmatellin binds tightly to the Q₁ site independently of the redox state of the Rieske protein (Table I) indicates that the 250-mV increase in the redox potential of the iron-sulfur cluster induced by this inhibitor (10) cannot be attributed to a 10⁵-fold tighter binding to the reduced Rieske protein (9). In consequence, the proposal of a stabilized, electron paramagnetic resonance invisible semiquinone as a reaction intermediate that prevents the transfer of the second electron of quinol to the iron-sulfur cluster is unsupported. The dissociation rate constant in all conditions was close to zero, indicating a Kᵣ value in the nanomolar range (Table I). The same results were obtained when the bc₁ complex was oxidized with ferricyanide prior to the binding assay and with this oxidant present during the incubation with stigmatellin (Fig. 2C), discarding the possibility of reduction of the Rieske protein by endogenous reductants when the enzyme was diluted to 1 nm.

Assuming a constant value for k₋₁ in all redox states of the enzyme, the 50% increase in k₁ upon reduction of the iron-sulfur cluster would only account for 10 mV of the 250-mV increase induced by stigmatellin, according to the equations relating the change in redox potential, ΔG, and Kᵣ (8).

We consider that the effect of stigmatellin on the redox
Stigmatellin Binds Tightly to the Oxidized bc₁ Complex

Table 1
Kinetic parameters of stigmatellin, myxothiazol, quinone, and quinol binding to fully oxidized and Rieske protein reduced bovine bc₁ complex

| Redox state       | Stigmatellin | Myxothiazol |
|-------------------|--------------|-------------|
|                   | $k_1$ (×10⁶ M⁻¹ s⁻¹) | $k_1$ (s⁻¹) | $K_d$ (µM) | $K_d$ (µM) |
| Oxidized pH 6.5   | 2.6 ± 0.2    | <0.005     | 36 ± 6    | 36 ± 6    |
| Oxidized pH 7.5   | 2.5 ± 0.1    | <0.001     | <0.4     | 46 ± 4    |
| Oxidized pH 9     | 2.2 ± 0.1    | <0.002     | <0.9     | 32 ± 3    |
| Ascorbate-reduced | 4 ± 0.14     | <0.004     | <1       | 16 ± 1    |
| Decylbenzoquinol-reduced | 3.5 ± 0.2 | <0.004     | <1      | 15 ± 2    |

$^a$ $K_d$ value for the inhibitor, corresponding to $k_1/k_2$.

Fig. 3. Binding rate constants for stigmatellin and myxothiazol binding to the oxidized bc₁ complex as a function of pH. Determination of binding rate constants at each pH value was determined as shown in Fig. 2. In the case of stigmatellin, filled symbols correspond to values obtained in the air-oxidized enzyme, and open symbols correspond to those determined in the ferricyanide-oxidized complex.

The 50% tighter binding of stigmatellin upon reduction of the Rieske protein found in the present work suggests a slight shift of the equilibrium between the different positions of the soluble domain of this subunit toward the cytochrome b interface. Thus, tight binding of stigmatellin to the Q₀ site occurs when the soluble domain of the Rieske protein (oxidized or reduced) is in contact with cytochrome b (5, 21). Therefore, an important conclusion derived from our results is that the redox potential of the oxidized iron-sulfur cluster of the wild-type Rieske protein increases 250 mV when this subunit is in the $b$ position, as has been observed in the Rieske protein mutants that constrain the movement of this subunit (18, 19).

By measuring the oxidation of cytochrome $c₁$ caused by the stigmatellin-induced increase in the redox potential of the Rieske protein at alkaline pH values, Zhang et al. (22) estimated binding rate constants that were almost 30 times lower than the values reported in the present work. These differences cannot be attributed to the different concentration and type of detergent used (which could in principle modify the effective concentration of inhibitor in the bc₁ complex micelles), because the $k_1$ value for stigmatellin assayed in 0.05% of lauryl maltoside (22) under our experimental conditions was 1.5 × 10⁶ M⁻¹ s⁻¹ (not shown), 15 times higher than the values calculated by Zhang et al. (22).

Two factors could explain the lower $k_1$ values obtained from cytochrome $c₁$ oxidation induced by stigmatellin. 1) Cytochrome $c₁$ oxidation induced by stigmatellin is only possible when the Rieske protein is oxidized. The bc₁ complex preparation used by Zhang et al. (22) contained only 17% of Rieske protein in the oxidized state. Thus, most of the stigmatellin binding occurred to the larger fraction of enzyme with the Rieske protein in the reduced state, which is undetected by the methodology used, resulting in underestimated $k_1$ values. 2) Electron transfer between heme $c₁$ and the iron-sulfur cluster is not possible when the Rieske protein is at the cytochrome b interface (21); therefore, the binding rate constants obtained from cytochrome $c₁$ oxidation measurements correspond to the rate of stigmatellin binding to bc₁ complex molecules where the Rieske protein is at a position in which its redox potential is already higher than that of cytochrome $c₁$ but still in a sufficiently close distance from heme $c₁$ to allow electron transfer. In contrast, the binding rate constants determined in the present work involve binding to all conformations of the bc₁ complex, including that where the Rieske protein is close to cytochrome b, a position in which the redox potential of the iron-sulfur cluster is 250 mV higher than at the interface with cytochrome $c₁$. This high redox potential conformation in the $b$ position can represent up to 50% of the total unbound enzyme, according to some crystal structures (20), and is more accessi-
ble to stigmatellin binding, because it is stabilized by this inhibitor (21).

**Hydrogen Bonding to Histidine 161 Is Not the Main Stabilizing Force for the Binding of Quinone, Quinol, and Inhibitors**—The protonation state of the histidine with pK<sub>a</sub> 7.5 that binds the iron-sulfur cluster did not affect the binding rate constant of stigmatellin in the oxidized bc<sub>1</sub> complex significantly (Fig. 3). Zhang et al. (22) also found little effect of pH between 7.5 and 9.2 on stigmatellin k<sub>1</sub> values, which are probably underestimated for the reasons discussed above. We have proposed that this pK<sub>a</sub> 7.5 residue corresponds to histidine 161 of the Rieske protein, because our previous kinetic analysis suggested that this group is a primary acceptor of a proton from quinol (12). Electron transfer calculations and simulations also point to deprotonated histidine 161 as the residue that receives a proton from quinol simultaneously with the iron-sulfur cluster reduction (23, 24). Even though this residue is in hydrogen-bonding distance of the carbonyl of stigmatellin (5), our obser-
vitation of pH- and redox-independent binding of stigmatellin suggests a more important role for the interactions between the hydrophobic side chain of the inhibitor and cytochrome b residues in determining the tight binding behavior of stigmatellin. In agreement with this last proposal, Ohnishi et al. (25) found that modifications of the side chain of stigmatellin (displacement or removal of methoxy groups and saturation of double bonds) decrease the affinity toward this inhibitor several orders of magnitude. Moreover, some of these derivatives, with a higher hydrophobicity than stigmatellin, induced a shift in the redox potential of the Rieske protein of only 80–90 mV, despite having the same carbonyl that is in hydrogen-bonding distance to histidine 161. Hydroxydioxobenzothiazoles (26) and hydroxynaphthoquinones (27) cause redox potential shifts similar to those induced by stigmatellin derivatives with modified side chains (−70 mV), even though they also possess the carbonyl group.

Genetic evidence also points to an important role of cytochrome b residues in determining the tight binding of stigmatellin (28). Several residues at which mutations confer resistance to this inhibitor, such as methionine 125, phenylalanine 129, isoleucine 147, and leucine 295, are within 3 to 4 angstroms from side chain atoms of stigmatellin (5). Glutamate 272 hydrogen bonds to a hydroxyl group from the chromone moiety of stigmatellin, whereas other residues, such as glycine 137, asparagine 256, and tryptophan 273 appear to be important for determining the overall conformation of the stigmatellin binding pocket. These findings indicate that tight binding of stigmatellin (and its associated conformational and redox potential changes in the Rieske protein) cannot be attributed to a single strong hydrogen bond between the inhibitor and histidine 161 of the Rieske protein. However, because crystal structures clearly show a hydrogen bond between stigmatellin and histidine 161 (4, 5), it is probable that the inhibitor recruits a proton to establish the hydrogen bond once it has bound to its site.

It was shown previously that the protonation state of the pK\textsubscript{a} ~7.5 residue, probably histidine 161 of the Rieske protein, affects the affinity of the bc\textsubscript{1} complex toward quinone slightly without affecting the K\textsubscript{m} value for quinol (12). Instead, quinone binding was modified by groups with pK\textsubscript{a} values of 5.7 and 9.2 (probably glutamate 272 of cytochrome b and histidine 141 of the Rieske protein). In agreement with previous work, the K\textsubscript{d} values obtained for quinone (see Fig. 4 and Table I) showed a maximum at pH 7.5, slightly decreasing toward more acidic and basic pH values, probably as a result of the influence of other protonatable groups. Furthermore, the K\textsubscript{d} value of 10–15 μM (Fig. 6) obtained for quinol with the reduced Rieske protein, where histidine 161 is expected to be protonated fully because of the shift in its pK\textsubscript{a} from 7.6 to ~11 (13), was similar to the K\textsubscript{m} values reported previously obtained from measuring the steady-state activity between pH 6.7 and 9 (12). Because these K\textsubscript{m} values are an approximation of the K\textsubscript{d} for quinol in the oxidized enzyme (where histidine 161 has a pK\textsubscript{a} of 7.6), it follows that the affinity toward the substrate is not affected by the redox state of the bc\textsubscript{1} complex. Therefore, our present results support the proposal that a particular protonation state of histidine 161 is not essential for ligand binding to the Q\textsubscript{o} site, even though ligands could recruit or eliminate a proton in this residue after binding.

The insensitivity of myxothiazol binding to both the redox state of Rieske protein and the protonation state of the pK\textsubscript{a} 7.5 histidine of the Rieske protein was expected, considering that crystal structures show no interaction between this inhibitor and any Rieske protein residues (5). In contrast, methoxyacrylate inhibitors, which bind to the Q\textsubscript{o} site subdomain proximal to heme b\textsubscript{1}, like myxothiazol (5), undergo a decrease in their K\textsubscript{d} values as the redox centers of the bc\textsubscript{1} complex are reduced sequentially (29). In addition, the binding of these reversible inhibitors is favored by the deprotonation of histidine 161 (29). Notably, we found that myxothiazol is a purely competitive inhibitor with respect to quinone and quinol, in contrast with the mixed-type inhibition pattern found for methoxyacrylates (30). These differences may be attributed to the smaller size of the methoxyacrylate compounds, which allows the simultaneous binding of quinone and methoxyacrylate to the Q\textsubscript{o} site. Moreover, the weaker interaction of methoxyacrylates with cytochrome b might additionally cause their binding to be more sensitive to subtle conformational changes derived from either the reduction of redox centers or the deprotonation of residues in the Rieske protein. According to mutational studies (28) and crystal structures (5), some residues that also interact with stigmatellin, such as methionine 125, phenylalanine 129, and leucine 295, are within 3 to 4 angstroms of myxothiazol side chain atoms. Other residues that specifically confer sensitivity to myxothiazol, such as alanine 126 and glycine 143, are also at close distance to side chain atoms. Two other residues (tyrosine 132 and phenylalanine 275) surround the amide group of the methoxyacrylate moiety (5), and their mutation confers resistance to both myxothiazol and methoxyacrylates (28). Therefore, as is the case with stigmatellin, the tightly bound nature of myxothiazol inhibition results mainly from interactions between cytochrome b residues and the inhibitor side chain.
Stigmatellin Binds Tightly to the Oxidized bc₁ Complex

Implications for the Mechanism of Quinol Oxidation—Contrary to proposals that question the binding of quinone to the Qo site (31), our kinetic analysis demonstrates directly that quinone and quinol, despite being undetectable in crystal structures, bind to the Qo site in competition with stigmatellin and myxothiazol. However, our results indicate that there is no basis for the assumption that the hydrogen bond between histidine 161 and the Qo site ligand is involved in a strong ligand stabilization upon reduction of the iron-sulfur cluster (8). Furthermore, tight binding of inhibitors to the Qo site should be attributed to the structure of the hydrophilic side chains of stigmatellin and myxothiazol, which are considerably different from the isoprenoid chain of ubiquinone or the aliphatic tail of decylbenzoquinone. Thus, stigmatellin and myxothiazol should not be considered as analogues of a highly stabilized semiquinone with respect to their tightly bound nature. Our results are consistent with models of quinol oxidation that propose a highly unstable semiquinone (3,7), which is oxidized rapidly by heme b₁, thereby ensuring the bifurcation of electrons required to translocate protons in the bc₁ complex. If a single quinol molecule binds to the Qo site (4), the unstable semiquinone formed after Rieske protein reduction could rapidly move to the Qo site subdomain proximal to heme b₁ to be oxidized. It is likely that such movement also occurs with quinol and quinone, as suggested by the finding that substrate and product binding is not dependent on hydrogen bonding to histidine 161 of the Rieske protein (see Ref. 12 and Table 1), explaining why quinine is not detected in the Qo site of crystallized bc₁ complexes (5). Moreover, in the light of mutational studies that constrain the Rieske protein to the cytochrome b interface (18,19), the tight binding of stigmatellin to the bc₁ complex, independently of the redox state of the Rieske protein, indicates that the redox potential of the iron-sulfur cluster is 250 mV more oxidant in the b position as compared with the freely moving subunit. This implies that the Rieske protein facilitates the first oxidation of quinol by becoming more oxidant when in contact with the Qo site and becomes more reducing at the cytochrome c₁ interface to transfer electrons to this subunit efficiently.

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