Stabilization of a semiquinone radical at the high-affinity quinone-binding site (Q_H) of the *Escherichia coli bo*-type ubiquinol oxidase**

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Abstract Reaction of ubiquinone in the high-affinity quinone-binding site (Q_H) in *bo*-type ubiquinol oxidase from *Escherichia coli* was revealed by EPR and optical studies. In the Q_H site, ubiquinol was shown to be oxidized to ubisemiquinone and to ubiquinone, while no semiquinone signal was detected in the oxidase isolated from mutant cells that cannot synthesize ubiquinone. The Q_H site highly stabilized ubisemiquinone radical with a stability constant of 1–4 at pH 8.5 and the stability became lower at the lower pH. Midpoint potential of QH_2/Q couple was −2 mV at pH 8.5 and showed −60 mV/pH dependence indicative of $2H^+/2e^-$ reaction. The E_m was more negative than that of low-spin heme *b* above pH 7.0. We conclude that the Q_H mediates intramolecular electron transfer from ubiquinol in the low-affinity quinol oxidation site (Q_L) to low-spin heme *b*. Unique roles of the quinone-binding sites in the bacterial ubiquinol oxidase are discussed.

Key words: E. coli bo-type ubiquinol oxidase; Quinol oxidation; Semiquinone radical; EPR; Potentiometric titration; Intramolecular electron transfer

1. Introduction

Cytochrome *bo*-type ubiquinol oxidase in *Escherichia coli* was first identified as cytochrome *o* in 1959 [1] and belongs to the heme-copper respiratory oxidase superfamily [2]. It catalyzes the two-electron oxidation of ubiquinol-8 (Q_8H_2) at the periplasmic side of the cytoplasmic membrane and the four-electron reduction of dioxygen at the cytoplasmic side [3]. It has been shown that not only protolytic scalar reactions but also redox-coupled proton pumping establish an electrochemical proton gradient across the membrane [4,5].

Subunit I of the oxidase binds low-spin heme *b*, high-spin heme *o* and Cu_B and plays a central role of dioxygen reduction and proton pumping [6,7]. Recent site-directed mutagenesis studies revealed a molecular structure of the heme *o*-Cu_B binuclear center [6,7] and a possible mechanism for the facilitated heme *b*-to-heme *o* electron transfer [6,8]. Based on photoaffinity cross-linking experiments, a quinone-binding site(s) has been assigned to reside in subunit II [9]. Therefore, electrons seem to travel from the quinol oxidation site in subunit II to the metal centers in subunit I as in cytochrome *c* oxidase. Since quinols are two-electron redox components while the metal centers of terminal oxidases are one-electron transfer systems, there must be a specific mechanism for gating electron flux, which, however, is yet unknown.

Recently, we demonstrated the presence and functional importance of a novel high-affinity quinone-binding site (Q_H) in the *E. coli bo*-type ubiquinol oxidase [10]. The Q_H site is distinct from a low-affinity quinol oxidation site (Q_L [11]) and is close to both the Q_L site and low-spin heme *b* [10]. Based on these observations, we postulated that the Q_H mediates electron transfer from the Q_L to the metal centers, like the Q_A of the photosynthetic reaction center [10]. In this article, we present evidence for involvement of the Q_H in electron transfer from the Q_L to heme *b*. It is also suggested that a mechanism of electron transfer in the Q_H site is different from that in the Q_A site that stabilizes semiquinone but destabilizes a fully reduced form, since the Q_H site can tightly bind ubiquinol and stabilize semiquinone radical.

2. Materials and methods

2.1. Purification of bo-type ubiquinol oxidase

The wild-type and the bound Q_8-free oxidases were isolated from the *E. coli* strains GO103/pMFO2 (*cyo*$^+$*Δcyd*/*cyo*$^+$) and MU1227/pMFO2 (*cyo*$^+$*cyd*$^+$*Δ ubiA*/*cyo*$^+$), which is defective in ubiquinone biosynthesis, respectively [10,12]. The purified enzymes in 50 mM Tris-HCl (pH 7.4) containing 0.1% sucrose monolaurate SM-1200 (Mitsubishi Kasei Food, Tokyo, Japan) were stored at −80°C until use. By the polyethylene glycol treatment [10], any loosely bound quinones were removed from the wild-type enzyme preparation. Thus, the wild-type enzyme used in this study contains only a single tightly bound Q_8 molecule [10].

2.2. EPR redox titration experiments

The stock enzyme was diluted to 10 μM with 50 mM Tris-HCl at the indicated pH containing 0.1% sucrose monolaurate and 100 mM KCl. The redox mediators used were 40 μM phenazine ethosulfate, 20 μM 2,3,5,6-tetramethyl phenylenediamine, 40 μM *N,N,N′,N′*-tetramethyl

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Abbreviations: Q_8H_2, ubiquinol-8; Q_H site, a high-affinity quinone-binding site; Q_L site, a low-affinity quinol oxidation site; Q_H, ubiquinone-8 bound at the Q_H site; Q_n, ubiquinone-n; $Q^{\bullet-}/QH_2$, semiquinone/quinol; $Q/Q^{\bullet-}$, quinone/semiquinone; Q/QH_2, quinone/quinol.

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phenylenediamine and 40 μM ferric ethylendiaminetetraacetic acid. Any quinone-like mediators were avoided for the titration. Redox titrations were performed at 25°C in oxidative direction by adding a small aliquot of the sodium ferricyanide solution to the dithionite-reduced enzyme. Redox potential of the reaction medium, monitored by a Pt electrode with an Ag/AgCl reference electrode, was expressed in mV unit referred to the normal hydrogen electrode. Samples were poised at every 25–35 mV change, transferred to EPR tubes and quickly frozen in liquid N_2. The sample and tubes were flushed with water-saturated Ar gas for 1 h before and during the titration. EPR spectra were recorded at 20 K with an X-band Bruker ER 200D spectrometer equipped with a liquid-helium cryostat (ESR-900, Oxford Instruments) in the Analytical Center of National Institute of Basic Biology as described by Hata et al. [13]. EPR conditions were as follows: temperature, 20 K; modulation frequency, 100 kHz; microwave frequency, 9.56 GHz. Microwave power and modulation amplitude were 0.2 mW and 0.5 mT, respectively, in the measurements of semiquinone and 10 mW and 2 mT, respectively, in the measurements of the high- and low-spin hemes. The spin concentration of semiquinone was determined with an oxidized P700 cation spin in the chloroplast photosystem I particles as a standard at the lower microwave power conditions.

2.3. Optical redox titration experiments

The enzyme was diluted to 1 μM with 50 mM Tris-HCl (pH 7.5) containing 0.1% sucrose monolaurate and 100 mM KCl and introduced into an anaerobic glass cuvette with ~1 cm path length and 10 ml working volume. Redox titrations were carried out at room temperature with redox mediators as follows: 5 μM pyocyanine, 50 μM phenazine methosulfate, 20 μM 2,3,5,6-tetramethyl phenylenediamine and 50 μM ferric ethylendiaminetetraacetic acid. The sample and cuvette were flushed with water-saturated N_2 gas. Difference spectra were recorded with a Shimadzu UV-3000 spectrophotometer (Shimadzu, Kyoto, Japan) at 10–20 mV intervals upon addition of a small aliquot of the ferricyanide solution to the dithionite-reduced enzyme.

2.4. Miscellaneous

Protein concentration and heme content were determined as described previously [12]. The concentration of the purified enzyme was estimated from the total heme content, assuming that it contians 1 mol each of hemes B and O [12]. The syntheses of 2,6-dichloro-4-nitrophenol and 2,6-dichloro-4-dicyanovinylphenol were as described previously [11]. Other chemicals were commercial products of analytical grade.

3. Results

3.1. Stabilization of ubisemiquinone radical at Q_H site

Our previous studies suggested that the Q_H site can stabilize ubisemiquinone radical during the oxidation of substrate to ensure sequential one-electron transfer to the metal centers [10]. To verify this postulate, we determined EPR spectra of the wild-type and the bound Q_8-free enzymes. The titration experiments with the wild-type enzyme were carried out at pH 8.5. At E_h = 0 mV where semiquinone radical was stabilized, we observed a typical ubisemiquinone radical signal at g = 2.0 with partially resolved splittings [14] (Fig. 1a). Addition of 2,6-dichloro-4-dicyanovinylphenol, a potent competitive inhibitor for the Q_L site (K_i = 3.1 μM [11]), to a final concentration of 100 μM did not affect the intensity of the g = 2 signal (Fig. 1b). In contrast, this signal was totally absent in the bound Q_8-free oxidase (Fig. 1c) and not observed in a free Q_1 solution containing 50 mM Tris-HCl (pH 7.4) and 0.1% sucrose monolaurate (data not shown). When the reduced wild-type sample, which was anaerobically poised at −25 mV and frozen to 77 K, was subjected to several cycles of freeze-thaw under aerobic conditions to slowly elevate dioxygen level, the g = 2 semiquinone signal increased its intensity until the 3rd run and became negligible after the 7th run (data not shown). This suggests electron transfer from the Q_H site to dioxygen via the metal centers. These observations strongly suggest that the Q_H site can stabilize ubisemiquinone radical during the oxidation of Q_8H_2 supplied from a quinol pool in the cytoplasmic membrane.

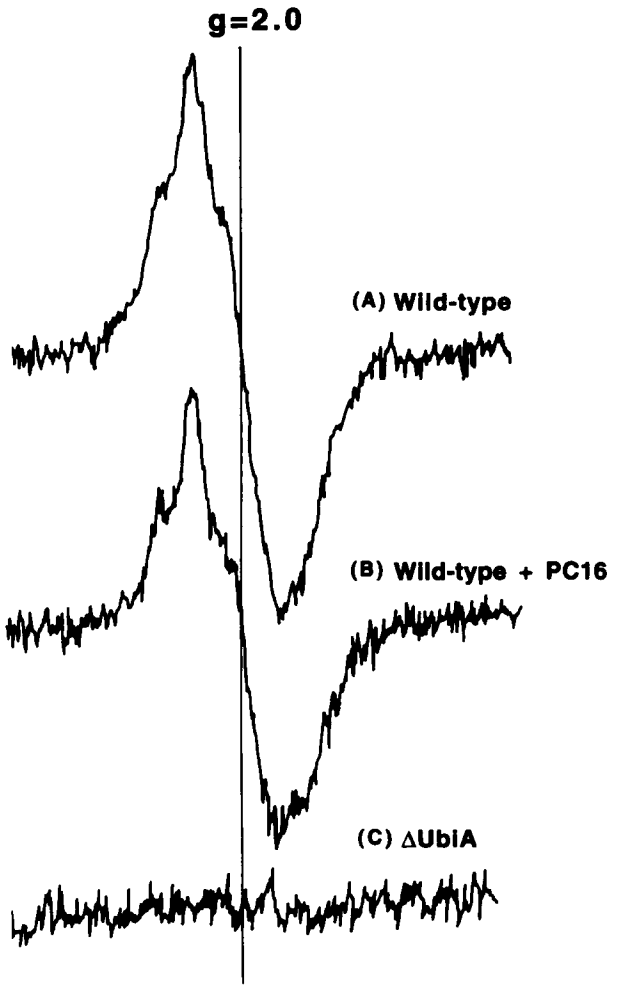

Fig. 1. EPR spectra of the wild-type oxidase in the absence (A) and presence (B) of 2,6-dichloro-4-dicyanovinylphenol (PC16) and of the bound Q_8-free oxidase (C). Measurements were done at pH 8.5 with or without preincubation with 100 μM 2,6-dichloro-4-dicyanovinylphenol for 15 min on ice.

3.2. Potentiometric EPR analysis of bound Q_8 and metal centers

The g = 2 semiquinone radical was further analysed potentiometrically with the wild-type enzyme. The redox titrations revealed that Q_8 bound at the Q_H site gives a bell-shaped curve with a peak at −2 mV. This suggests that Q_8 can accept two electrons to form Q_8H_2 (Fig. 2). The titration curve was analysed using the Nernst's equation as described in figure legend. The midpoint potential values for the $Q^{\bullet-}/QH_2$ and $Q/Q^{\bullet-}$ couples (E_2 and E_1) were determined to be +10 and −13 mV, respectively. The value for a two-electron couple, Q/QH_2 (E_m), was obtained as an average of those of one-electron couples (−1.5 mV). The concentration of g = 2 semiquinone spin at E_h = 0 mV peak was estimated to be 0.4 ± 0.1/oxidase from the

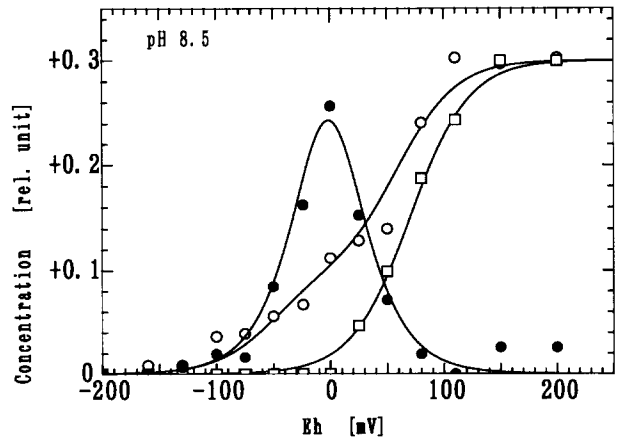

Fig. 2. Potentiometric titrations of the $g = 2$ semiquinone signal (closed circles), the $g = 3$ low-spin signal (open squares) and the $g = 6$ high-spin signal (open circles) at pH 8.5. The titration curve of semiquinone was fitted by a function; [signal intensity] = $C/(1 + 10^{(E_h - E_1)/59} + 10^{(E_2 - E_h)/59})$ with E_1 and E_2 values of −13 and +10 mV, respectively, where C is a optimal concentration of semiquinone at the Q_H site. The curve of heme titration was fitted by a function; [signal intensity] = $C/(1 + 10^{(E_m - E_h)/59}$ with $E_m = 70$ mV in the case of $g = 3$ low-spin heme and by a sum of two set of this function with E_m value of −50 and 60 mV, respectively, in the case of $g = 6$ high-spin heme. The constant 59 (mV) comes from RT/F where R is the gas constant, F is the Faraday constant and T is absolute temperature.

measurement of spin densituy. The stability constant of the semiquinone (= $[Q^{\bullet-}]/[Q]\,[QH_2]$) is, then, calculated to be 1–4 at pH 8.5 indicative of the high stabilization of semiquinone in the Q_H site.

The $g = 3$ low-spin heme signal was titrated as a single $n = 1$ component with a midpoint potential of +70 mV. This value is compatible with the idea that ubisemiquinone radical in the Q_H site donates an electron to ferric low-spin heme *b*. The $g = 6$ high-spin signal of heme *o*, on the other hand, showed a titration curve that can be fitted by a 2:1 mixture of two components with midpoint potential values of 60 and −50 mV, respectively, although the data quality was insufficient to determine these values correctly. The amount of the −50 mV fraction roughly corresponds to the amount of semiquinone radical in the oxidase and suggests their interaction.

3.3. *Effect of bound Q_8 on redox potentials of metal centers*

Previously, we found that the absence of Q_8 at the Q_H site results in a 3-nm red shift of the Soret peak of the air-oxidized enzyme to 412 nm [10]. This shift is likely to be associated with a change in the redox potential of low-spin heme *b* [10]. To examine the effect of the bound Q_8 on the metal centers, optical redox titration analyses were carried out with the wild-type and bound Q_8-free oxidases by monitoring the α and Soret peaks in redox difference spectra.

It is known that low-spin heme *b* contributes to most of the α absorption while the Soret absorption is a sum of equal contributions of low- and high-spin hemes [15]. The titration curves for both bands at pH 7.5, therefore, can be fitted by a two-component model with similar E_m values, as shown in Fig. 3. The composite curves can be attribed to heme *b*-heme *o* interactions in the oxidase molecule as has been suggested by [16]. The E_m values for the wild-type and bound Q_8-free oxidases were calculated to be 67 and 123 mV and 92 and 143 mV, respectively. This indicates that binding of a Q_8 molecule to the Q_H site resulted in 20–25 mV reduction of the E_m value for low-spin heme *b* and possibly for heme *o* through the heme *b*-heme *o* interactions. As discussed previously [10], the E_m shift could occur via direct electrostatic interactions between the Q_H and heme *b* or conformational change of the enzyme upon binding of Q_8 to the Q_H site. Similar findings were reported for interactions between the Q_i site and cytochrome b_{562} of cytochrome bc_1 complex [17]. In addition to the main components, the titration curve in the Soret region of the wild-type enzyme indicated a presence of a small fraction with a midpoint potential of about −50 mV. This fraction seems to correspond to the fraction of high-spin heme *o* titrated with an estimated midpoint potential of −50 mV in Fig. 2. The titration in the bound Q_8-free oxidase does not show such a low potential fraction (this was confirmed by EPR) so that the high-spin heme with this negative potential may reflect some kind of interaction between $Q^{\bullet-}$ and the heme.

3.4. *pH dependence of midpoint potentials of ubisemiquinone and of metal centers*

We examined the pH dependence of the midpoint potentials of the $g = 2$ semiquinone signal and the $g = 3$ low-spin heme signal. The peak intensities of the semiquinone signal at pH 7.6 and 6.2 in the titrations were ~20% that at pH 8.5 (not shown)

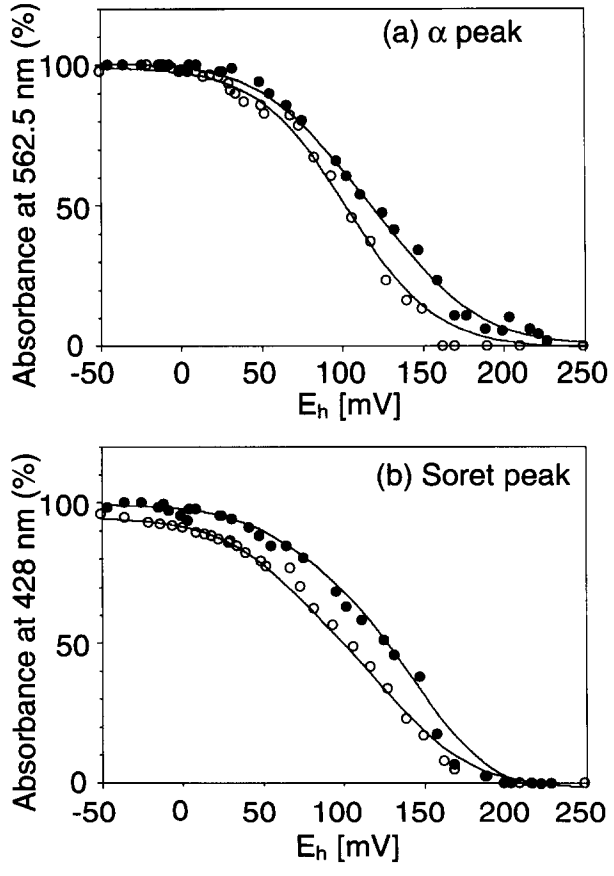

Fig. 3. Potentiometric titrations of the α (A) and Soret (B) peaks of the wild-type (open circles) and the bound Q_8-free (closed circles) oxidases at pH 7.5. The data for the α and Soret bands are best fitted by a two-component model with similar E_m values.

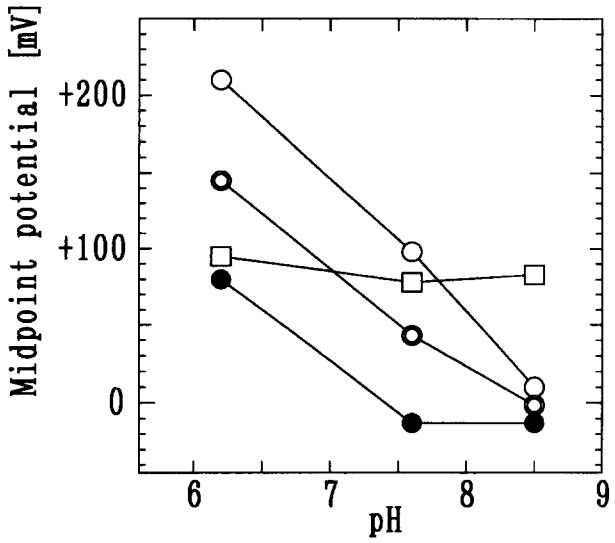

Fig. 4. pH dependence of the midpoint potential of the $Q^{\bullet-}/QH_2$ (E_2, ○), $Q/Q^{\bullet-}$ (E_1, ●), Q/QH_2 (E_m, ◎) and the $g = 3$ low-spin signal (□). Midpoint potential values were calculated as described in Fig. 2.

indicating the destabilization of semiquinone at the lower pH. With these peak heights, and the positions and shapes of titration curves, the values of E_1, E_2 and $E_m = (E_1 + E_2)/2$ were estimated and plotted in Fig. 4. The E_m value decreased sharply with increasing the medium pH with a slope of approximately −60 mV/pH unit indicative of $2H^+/2e^-$ ratio of the quinol oxidation at the Q_H site. E_2 decreased with a slope of approximately −120 mV/pH unit around pH 8. E_1 showed a pK at around pH 7.5 and increased below this pH with a dependency of −60 mV/pH unit. E_2 decreased with a steeper slope. Above pH 7.5, the deprotonation seems to occur mainly in the oxidation step from QH_2 to $Q^{\bullet-}$.

In contrast, the low-spin heme signal showed almost no pH dependence at the pH values examined. The E_m of the heme was more positive than the E_m of QH_2/Q couple above pH 7 indicative of electron transfer from QH_2 to low-spin heme *b*.

4. Discussion

Cytochrome *bo*-type ubiquinol oxidase in *E. coli* contains the high-affinity quinone-binding site (Q_H [10]) and the low-affinity quinol oxidation site (Q_L [11]). It was postulated that the Q_H site functions as a molecular gate which regulates electron flux from the two-electron redox component (i.e. quinols) to one-electron carriers (i.e. heme irons) and mediates electron transfer from the Q_L site in subunit II to low-spin heme *b* in subunit I [10]. Thus, the Q_H site is expected to stabilize transiently ubisemiquinone radical during the catalytic cycle, in a manner similar to the Q_A site of the photosynthetic reaction center. In the present study, we carefully prepared the wild-type oxidase with and without a single bound Q_8 molecule at the Q_H site and carried out detailed potentiometric analyses of ubisemiquinone and the low-spin heme signals. We obtained clear evidence for stabilization of ubisemiquinone radical in the oxidase by monitoring the $g = 2$ signal associated with the bound Q_8 at the Q_H site. A loss of this signal in the bound Q8-free enzyme supports this conclusion. Since the Q_H can be doubly reduced, redox chemistry in the Q_H site (i.e. two one-electron transfer reactions and the addition of two protons) resembles that of the Q_B site rather than the Q_A site of the photosynthetic reaction center although the tight-binding of quinone in the Q_H site resembles that in the Q_A site. It seems that the Q_H site functions as a transient electron reservoir that facilitates the two-electron oxidation of Q_8H_2 that is bound to the Q_L site. Subsequently, the Q_8 at the Q_L site will be replaced by another Q_8H_2 molecule in the membrane pool and a next redox cycle of quinone will be started. To ensure the succesive four-electron reduction of molecular oxygen at the heme-copper binucear center, the Q_L site may have a low affinity for Q_8 to achieve rapid dynamic equilibrium with the Q_8H_2 pool.

Very recently, Ingledew et al. [14] have reported EPR studies on the purified *E. coli bo*-type ubiquinol oxidase in the presence of 20-fold excess amount of Q_1. Based on detailed theoretical considerations, they concluded that ubisemiquinone radical can be stabilized at the quinol oxidation site although it is unclear whether this semiquinone signal was originated from either the Q_L site or the Q_H site. Their EPR spectra and titration curve quite resemble to ours. The result in the present study suggests that their signals are also originated from the Q_H site rather than the Q_L site.

We also tried to demonstrate stabilization of semiquinone radical at the Q_L site, as postulated previously [11]. The Q_H site of the bound Q_8-free oxidase was first reconstituted with 2,6-dichloro-4-nitrophenol, a high-affinity quinone analog for the Q_H site ($K_d = 1.8$ μM) (M. Sato-Watanabe, T. Mogi, H. Miyoshi and Y. Anraku, unpubl. data), and then we observed a similar semiquinone radical signal in the EPR spectrum of the reconstituted enzyme in the presence of 1 mM Q_1 (data not shown). However, due to a slow exchange of the bound 2,6-dichloro-4-nitrophenol at the Q_H site with free Q_1 molecules [10], the assignment of observed semiquinone signal was difficult.

The Q_H site affected the absorption spectrum and the redox behavior of heme *b*. The redox potential of heme *b* was decreased by 20 mV by the presence of the Q_H quinone. This may facilitate electron transfer from ubiquinol to the heme by increasing the motive force (energy gap) between them. This effect might be structural or electrostatic since the absorption and Raman spectra of heme *b* were altered as well [10]. The interaction with high-spin heme *o* was also suggested since the redox midpoint potential of the latter became more negative in the presence of $Q^{\bullet-}$ as seen in Fig. 2. The effect may be caused by the electrostatic interaction between the charges on the semiquinone and heme *o* or may be due to some structural effect. The details of this effect are now being analysed.

In conclusion, we have demonstrated that the Q_H site has molecular properties favorable for mediating electron transfer between the Q_L site and low-spin heme *b*. Recent time-resolved spectroscopic studies on *bo*-type ubiquinol oxidase provided a support for the involvement of the Q_H site in intramolecular electron transfer processes (M. Sato-Watanabe, T. Mogi, Y. Anraku and Y. Orii, unpubl. data). Identification of the Q_L and Q_H sites will provide a clue for structure and mechanism of the quinol oxidation site.

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