Identification of the Residues Involved in Stabilization of the Semiquinone Radical in the High-Affinity Ubiquinone Binding Site in Cytochrome bo_3 from *Escherichia coli* by Site-Directed Mutagenesis and EPR Spectroscopy†

Petra Hellwig,*,‡,§ Takahiro Yano,|| Tomoko Ohnishi,|| and Robert B. Gennis‡

Department of Biochemistry, University of Illinois, 600 South Mathews Street, Urbana, Illinois 61801, Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6059, and Institut für Biophysik, Universität Frankfurt, Theodor-Stern-Kai 7, Haus 74/75, 60590 Frankfurt am Main, Germany

Received December 11, 2001; Revised Manuscript Received June 27, 2002

ABSTRACT: During turnover of cytochrome bo_3 from *Escherichia coli*, a semiquinone radical is stabilized in a high-affinity binding site. To identify binding partners of this radical, site-directed mutants have been designed on the basis of a recently modeled quinone binding site (Abramson et al., 2000). The R71H, H98F, D75H, and I102W mutant enzymes were found to show very little or no quinol oxidase activity. The thermodynamic and EPR spectroscopic properties of semiquinone radicals in these mutants were characterized. For the H98F and the R71H mutants, no EPR signal of the semiquinone radical was observed in the redox potential range from −100 to 250 mV. During potentiometric titration of the D75H mutant enzyme, a semiquinone signal was detected in the same potential range as that of the wild-type enzyme. However, the EPR spectrum of the D75H mutant lacks the characteristic hyperfine structure of the semiquinone radical signal observed in the wild-type oxidase, indicating that D75 or the introduced His, interacts with the semiquinone radical. For the I102W mutant, a free radical signal was observed with a redox midpoint potential downshifted by about 200 mV. On the basis of these observations, it is suggested that R71, D75, and H98 residues are involved in the stabilization of the semiquinone state in the high-affinity binding site. Details of the possible binding motif and mechanistic implications are discussed.

Cytochrome bo_3 is a terminal oxidase in the aerobic respiratory chain of *Escherichia coli*. It catalyzes the two-electron oxidation of ubiquinol-8 and the four-electron reduction of dioxygen, and these reactions are coupled to the translocation of protons across the cytoplasmic membrane by a pumping mechanism (*1*−*3*). The presence of two ubiquinol-8 binding sites with distinct functional roles has been proposed for cytochrome bo_3 from *E. coli* (*3*). In one possible mechanism, ubiquinone bound at a high-affinity site, acting as a cofactor, mediates electron transfer from the ubiquinol substrate, which binds to a low-affinity binding site, to the low-spin heme *b* (*3*−*5*). Reduced heme *b* then provides electrons to the binuclear center, formed by heme o_3 and Cu_B, where oxygen binds and is reduced to water.

The locations of the two proposed quinone binding sites within cytochrome bo_3 are not clear, and indeed, direct unambiguous evidence for two different sites is lacking. The low-affinity site that binds the substrate ubiquinol was suggested to be located at least in part within subunit II of the enzyme on the basis of site-directed mutagenesis, photoaffinity labeling, and inhibitor-resistant mutants (*5*−*8*). Recently, a chimeric construct was investigated, in which parts of subunit II of cytochrome bo_3 from *E. coli* were replaced by their corresponding parts, the cytochrome *c* binding and/or Cu_A sites, of cytochrome *c* oxidase (*9*). This construct still exhibits ubiquinol oxidase activity, and this in fact would exclude these regions of subunit II of *E. coli* enzyme from being involved in ubiquinol binding.

The second binding site contains a tightly bound quinone, which can be retained in the purified enzyme and, significantly, forms a stabilized semiquinone (SQ) radical. This radical has been implicated as an intermediate during enzyme turnover (*10*). Previous spectroscopic studies of the tightly bound quinone by EPR[1] (*10*, *11*), FTIR (*12*), and ENDOR spectroscopy (*13*, *14*) as well as detailed inhibitor binding studies (*7*, *15*, *16*) have provided important information about the structure and orientation of the bound quinone and quinone analogue inhibitors. EPR spectroscopy studies have shown that the tightly bound quinone can be stabilized as a semiquinone anion radical in the protein, which displays an X-band EPR spectrum with characteristic hyperfine structure

† Financial support is gratefully acknowledged from DFG He-3150/1 to P.H. and NIH Grants GM30736 to T.O. and DEFG02-87ER13716 to R.B.G.

* To whom correspondence should be addressed: tel, 69-6301-4227; fax, 69-6301-5838; e-mail, hellwig@biophysik.uni-frankfurt.de.

‡ University of Illinois.

§ Universität Frankfurt.

|| University of Pennsylvania School of Medicine.

[1] Abbreviations: DM, *n*-dodecyl β-D-maltoside; UQ_8, ubiquinone-8; EPR, electron paramagnetic resonance, FTIR, Fourier transform infrared; ENDOR, combined electron spin and nuclear magnetic resonances; Q_H, high-affinity quinone binding site; Q_L, low-affinity quinol oxidation site; WT, wild type.

10.1021/bi012146w CCC: $22.00
Published on Web 08/02/2002

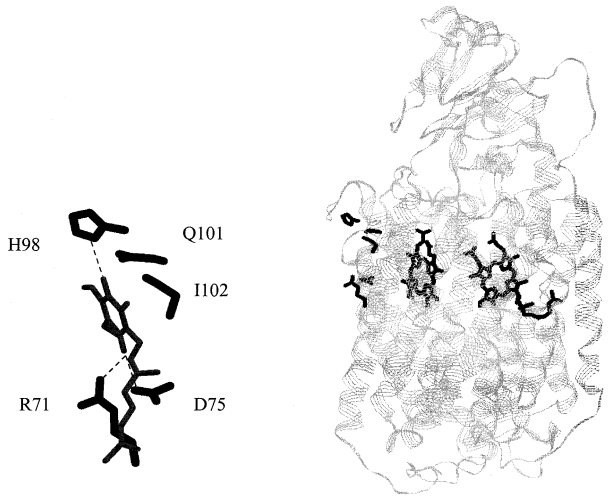

FIGURE 1: Structure of the high-affinity ubiquinone binding site in subunit I of cytochrome bo_3 from *E. coli*. A modeled quinone is displayed according to Abramson et al. (*17*).

(*10*, *11*). On the basis of a combined FTIR spectroscopy and ^{13}C labeling approach, it was demonstrated that the fully oxidized, bound quinone, as well as the reduced and protonated quinol, is hydrogen bonded to the protein matrix and that the hydrogen bonding is both weak and symmetric (*12*). FTIR studies as well as studies with inhibitors suggest that the 2-methoxy group of ubiquinone is sterically hindered, whereas the 3-methoxy group is free from steric constraints (*12*, *15*). Recent ENDOR work shows a strong interaction with the C1—O (*13*, *14*) and suggests a symmetrical pattern of hydrogen binding for both the C1—O and C4—O groups of the semiquinone anion radical. In recent work, the interaction of the free radical with a nitrogen nucleus from the backbone of the protein was suggested (*26*).

Recently, the crystal structure of cytochrome bo_3 from *E. coli* was solved to about 3.5 Å resolution (*17*). In the model of the enzyme, although bound quinone could not be resolved, amino acid residues forming a feature for quinone binding were recognized (*17*), and a quinone binding site within subunit I was postulated on the basis of the X-ray data. In the model, Q101 and H98 were proposed to interact with ubiquinone C4═O, whereas D75 and R71 were proposed as hydrogen bond partners to C1═O of the bound ubiquinone. This proposed binding site is located in subunit I close to heme *b* and is shown in Figure 1. Site-directed mutagenesis data have provided support for this model, insofar as several mutations result in strongly reduced quinol oxidase activity and increased K_m values for quinol oxidation (*17*). Recently, electrochemically induced FTIR difference spectra of the D75E mutant cytochrome bo_3 from *E. coli* show that D75 is protonated upon reduction of the protein and quinone (*24*). This work is, thus, consistent with the presence of D75 at the quinone binding site and suggests an important functional role for this residue.

In the present work, EPR spectroscopy was used to identify residues involved in the stabilization of the semiquinone anion radical. Site-directed mutants in this suggested binding site in subunit I were selected which have no or only residual quinol oxidation activity, and the radical signal was monitored in redox titrations.

MATERIALS AND METHODS

Site-directed mutagenesis on the pJRHisA plasmid (*19*) was performed according to the "quick change" protocol of Stratagene. Mutant DNA was transformed into GL101 (*18*). The mutations were confirmed by DNA sequencing (Biotechnology Center, University of Illinois).

Cytochrome bo_3 ubiquinol oxidase from *E. coli* containing 1 equiv of bound UQ_8 was purified in *n*-dodecyl β-D-maltoside (DM) according to the method described previously (*19*). The oxidase activity assay of the purified protein was performed at 37 °C as previously described (*8*) in Tris buffer, pH 7.4, and 0.1% DM. For control experiments, wild-type enzyme devoid of bound UQ_8 was obtained by solubilization of membranes with Triton X-100, followed by exchange of the detergent to DM. Further purification was performed with the same protocol as mentioned above. After solubilization with Triton X-100 as detergent, the tightly bound quinone is lost (*20*). The presence or absence of ubiquinone was confirmed by FTIR spectroscopy as reported (*12*) (data not shown).

Potentiometric redox titrations were carried out at 20 °C according to refs *10* and *21*). Cytochrome bo_3 (10−50 μM) in 100 mM phosphate buffer, pH 8, and 0.1% DM were used. Final concentrations for the titrations shown here were as follows: WT, 46 μM; D75H, 41 μM; R71H, 10 μM; H98F, 33 μM; I102W, 25 μM; and WT devoid of UQ, 15 μM. The measurements were reproduced with several different protein preparations. The following redox mediator dyes (40 μM of each) were added: 1,2-naphthoquinone-5-sulfonate, 1,4-naphthoquinone, 1,2-naphthoquinone-2-sulfonate, indigodisulfonate, indigotrisulfonate, indigocarmine, and duroquinone. Pyocyanine was added at a concentration of 1 μM. After samples were poised at appropriate redox potentials, they were frozen in an ethanol/dry ice freezing solution and stored in liquid nitrogen.

EPR spectra of the redox-poised samples were obtained using a Varian E109 spectrometer fitted with a TE102 rectangular cavity. The temperature of the sample was regulated to 123 K. Potentiometric titrations were analyzed and fitted with Microsoft Origin software. EPR spectra at 80 K and power saturation profiles were recorded by a Bruker ESP 300 spectrometer at X-band (9.2 GHz), using an Oxford Instrument ESR-9 helium flow cryostat to control the sample temperature. Power saturation data were analyzed by computer curve fitting as described previously (*22*, *23*).

RESULTS

Characterization of the Mutant Enzymes. Polarographic assays of the quinol oxidase activity with an oxygen electrode showed less than 1% of wild-type activity for the H98F mutant cytochrome bo_3 and a complete loss of activity for the D75H mutant (see also ref *24*), as well as for the I102W and the R71H mutant enzymes. UV/vis spectra (air oxidized/dithionite reduced) indicated no perturbation of the absorbance of the hemes due to the H98F mutation and a small shift of the Soret band from 409 to 406 nm for the oxidized form of the D75H mutant. In the case of the R71H mutant, a shift of the Soret band of the reduced form from 426 to 422 nm was observed. The visible spectra obtained from I102W samples after purification clearly indicate partial reduction of the hemes in the enzyme. Since the cofactors

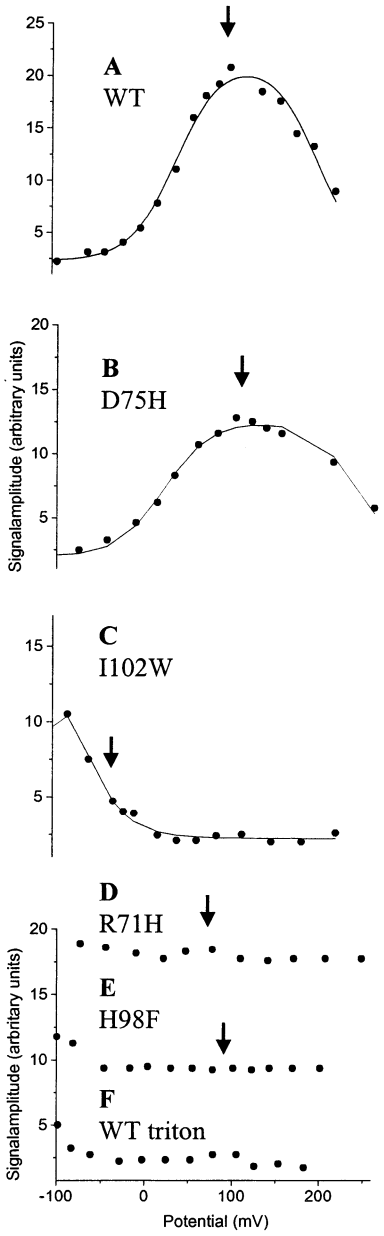

FIGURE 2: Potentiometric redox titrations for wild-type (A), D75H (B), I102W (C), H98F (D), and R71H (E) mutant cytochrome bo_3 from *E. coli* at pH 8 purified in DM and wild type purified in Triton (F). The full line displays the theoretical Nernst fit. The redox titrations were carried out according to ref *10*; for details see Materials and Methods.

in oxidases interact, an effect on both heme centers and the quinone as well (see below) is likely. UQ was present in all of the purified mutants, as determined by FTIR spectroscopy using the procedures described (*12*).

Potentiometric Titrations of the Ubisemiquinone. Figure 2 shows the potentiometric redox titrations of the ubisemiquinone species in the wild-type (A), D75H (B), I102W (C), H98F (D), and R71H (E) cytochrome bo_3 mutants from *E. coli* at pH 8. As a negative control, the wild-type enzyme prepared so as to be devoid of ubiquinone was also examined (Figure 2F). The redox titrations were carried out according to refs *10* and *21*.

The redox midpoint potential at pH 8.0 ($E_{m_{8.0}} = 120$ mV) for the $n = 2$ reaction (QH_2/Q) and for the $n = 1$ reaction ($E_{m_1} = 145$ mV and $E_{m_2} = 99$ mV)[2,3] and the stability constant ($K_s = 6.0$)[2] for the wild type can be determined. This is about a 100 mV positive shift from the previously reported values (*8*, *10*). This discrepancy may be attributed to differences in sample preparation and handling. Furthermore, an effect of the His tag (cf. Materials and Methods), present in this enzyme in contrast to the previously used enzyme, may account to this effect. The K_s values, obtained from the evaluation of the SQ spin concentration, are in the range of 1−6, indicating that this ubiquinone can function as a converter between two- and one-electron transfer systems, as discussed previously (*10*, *27*). Similar values ($E_{m_{8.0}} = 122$ mV and $K_s = 4.1$) were obtained for the D75H mutant. For this mutant enzyme, however, the bell-shaped titration curve is considerably broader than the theoretical single component curve. Perhaps the SQ species of this mutant is composed of a heterogeneous population of SQ species, which would not allow to extract single E_m or K_s values. We note that the data for the D75H have to be interpreted carefully, however, clearly allowing the main conclusion that the radical can still be stabilized in the enzyme.

For the H98F and R71H mutant enzymes, and for wild-type cytochrome bo_3 prepared in Triton X-100, no significant semiquinone signal was detected in the potential range from −100 to +250 mV. In the I102W mutant, a radical signal was observed but only at very low potentials, below −38 mV. The full EPR titration curve of the radical in I102W could not be deciphered due to the interference from free radical signals from mediator dyes which, according to control experiments, are present below −70 mV.

EPR Studies of the Stabilized Ubisemiquinone Species. Figure 3 presents EPR spectra of the stabilized ubisemiquinone free radical signals from the wild-type (A), D75H (B), I102W (C), H98F (D), and R71H (E) mutant cytochrome bo_3 from *E. coli*. The spectra were monitored at the maximum point of each titration set, except I102W, for which a sample at the shoulder of the redox titration peak was chosen to avoid contributions from the mediators (each chosen point is indicated with an arrow in Figure 2).

The stabilized ubisemiquinone spectrum observed in the wild-type enzyme shows a characteristic hyperfine structure resulting from magnetic interactions between the unpaired electron and protons at this site, as reported previously (*10*, *11*, *13*, *14*). Veselov et al. (*13*) conclude that there must be a strong hydrogen-bonding interaction between the protein and position C1—O of the semiquinone. In contrast, Grimaldi et al. (*26*) discuss the interaction with a protein backbone nitrogen. In the current work, EPR spectra of the wild-type enzyme at different points (E_h values) of the titration curve were compared, and all show an identical hyperfine structure (data not shown), confirming that the free radical signal is derived from a single, uniform species in the wild-type enzyme.

[2] The stability constant (K_s) for ubisemiquinone (SQ) at a given pH is defined by $K_s = [SQ]^2/[Q][QH_2]$ where the terms in brackets refer to the total concentration of each species, regardless of protonation state.

[3] The stability constant is also given in terms of $n = 1$ midpoint potentials, $E_{m_1}(Q/SQ)$ and $E_{m_2}(SQ/QH_2)$, by $E_{m_1}(Q/SQ) - E_{m_2}(SQ/QH_2) = 59 \log K_s$. An $n = 2$ midpoint potential, $E_m(Q/QH_2)$, is an average of $E_{m_1}(Q/SQ)$ and $E_{m_2}(SQ/QH_2)$ as follows: $E_m(Q/QH_2) = {}^1/_2[E_{m_1}(Q/SQ) + E_{m_2}(SQ/QH_2)]$.

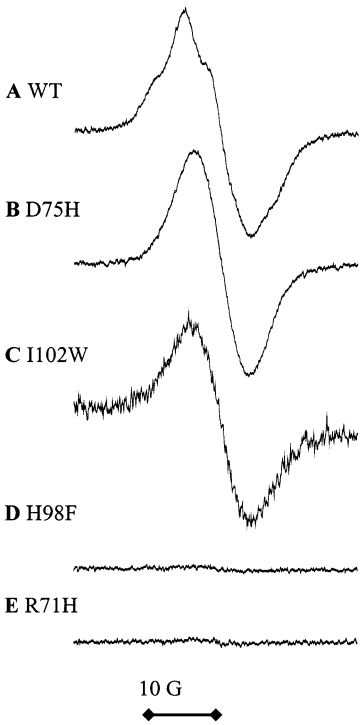

FIGURE 3: EPR spectra of wild-type (A), D75H (B), I102W (C), H98F (D), and R71H (E) mutant cytochrome bo_3 from *E. coli*. EPR conditions: sample temperature, 80 K; modulation frequency, 100 kHz; modulation amplitude, 2.548 G for spectra A and B and 5.085 G for spectra C−E; microwave power, 0.01 mW; microwave frequency, 9.44 GHz.

Notably, the spectrum of the D75H semiquinone radical (Figure 3B) displays no hyperfine coupling structure, indicating significant changes in the electronic/magnetic properties of the stabilized ubisemiquinone. The quinone in the D75H mutant has a potential similar to that of the wild-type enzyme. It is, therefore, unlikely that the spectroscopic changes of D75H semiquinone are due to a strong structural distortion of the protein caused by the mutation.

In the I102W mutant, on the other hand, a ubisemiquinone free radical was observed at a redox potential of −30 mV. In the EPR spectrum of I102W (Figure 3C), the hyperfine coupling cannot be discussed, since the modulation amplitude had to be increased to obtain a higher signal amplitude.

Power Saturation of the Semiquinone Free Radical EPR Signal. The data points and the best fit of the power saturation curves at 80 K with the wild-type (empty circles) and the D75H mutant enzyme (filled circles) are shown in Figure 4. Clearly, one homogeneous component is present in each curve. The radicals show power saturation of the signals with a $P_{1/2}$ value (half-saturation parameter) of 0.229 mW for wild type and of 0.302 mW for the D75H mutant. The differences in the $P_{1/2}$ parameters for the wild-type and D75H mutant enzymes are only minor. This indicates that the semiquinone species observed are localized and oriented in a similar way in both cases. The power saturation behavior of the wild-type cytochrome bo_3 is comparable to the previous measurement reported by Ingledew et al. (*10*).

DISCUSSION

On the basis of the experimental evidence shown here, it is suggested that H98 and R71 are crucial residues for the high-affinity binding site of cytochrome bo_3 from *E. coli* and are required for stabilization of the semiquinone radical formed during catalytic turnover. As exemplified by the well-characterized ubiquinone binding sites, Q_A and Q_B of the bacterial reaction center, reactive semiquinone radicals are generally considered to be confined and controlled in protein environments by specific interactions with the protein sites. Previous ENDOR spectroscopic studies clearly indicate mutual interactions between the stabilized semiquinone radical in cytochrome bo_3 and the surrounding protein. Specifically, interactions between ubiquinone oxygen atoms (C1—O, C4—O) and H-bond-donating amino acid residues have been suggested to be an important factor in determining the properties of the bound ubiquinone (*13*, *14*). The current work strongly supports the fact that R71 and H98 are interacting with the C—O groups of the semiquinone radical, consistent with the model depicted in Figure 1.

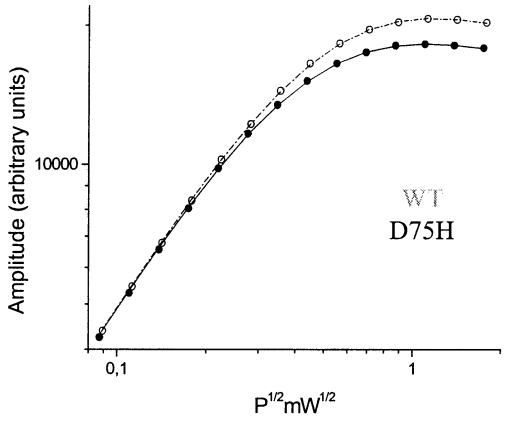

FIGURE 4: Power saturation of wild-type cytochrome bo_3 from *E. coli* (empty circles) in direct comparison to the D75H mutant (filled circles). A power saturation of the signal with a $P_{1/2}$ of 0.229 mW for wild type and of 0.302 mW for the D75H mutant was found.

Interestingly, the H98F mutant still exhibits a residual activity of 1%. This may suggest that some electron transfer can still take place without the quinone being stabilized as a radical. In a recent pulse radiolysis study, the transient formation of the ubisemiquinone radical in cytochrome bo_3 from *E. coli* was presented (*25*). In samples devoid of bound quinone, a direct reduction of the hemes was reported but with a low yield. This is in line with the observation presented here that some electron transfer can take place, albeit very small, even in the absence of a stabilized radical. The mechanism of the direct reduction is not clear, however.

In the I102W mutant enzyme, a free radical signal is observed, but the redox midpoint potential of the stabilized radical in the I102W mutant enzyme is strongly downshifted by more than 200 mV. This mutation places a tryptophan residue into the helix between the proposed quinone binding site and heme *b*. The lower potential required to form the stabilized semiquinone could be responsible for the loss of activity of the mutant. We note that also the possible potential shift of the hemes, as observed in the visible spectra of isolated enzyme, may be leading to the loss of activity. Since the radical is still formed in the I102W mutant, it is possible that I102 is not directly in contact with the quinone, which is then oriented more toward the protein surface, than suggested as one of the possibilities discussed by Abramson et al. (*17*).

The current work indicates that D75 is not directly involved in radical stabilization. However, it seems possible that D75 does directly interact with the radical, probably by hydrogen bond formation. The substitution of D75 by histidine diminishes the hyperfine coupling of the radical, suggesting that the H-bond provided by D75 is responsible for determining the electronic structure of the bound quinones. This view is consistent with previous ENDOR studies of semiquinone radicals stabilized in reconstituted cytochrome bo_3. In these studies, H−D exchange-sensitive hyperfine structures were found and attributed to O−H hydrogen bonds with the semiquinone. On the other hand, Grimaldi et al. (*26*) suggest the interaction of the semiquinone radical with a protein backbone nitrogen. In a possible explanation for the apparent discrepancy, the backbone interaction could be altered in the D75H mutant or a new interaction with the introduced His is present. To clarify the details of the interaction ENDOR studies on this mutant are in preparation.

The effect of the mutations on the radical signal presents clear evidence of the proximity of the discussed residues to the so-called high-affinity quinone binding site. Additionally, the protonation of D75 upon reduction of cytochrome bo_3 was directly observed in combined electrochemical and FTIR spectroscopic studies (*24*). These data suggest that D75 takes up protons from the bound quinol. D75 may play an important role in proton-coupled electron transfer reactions in the high-affinity quinone binding site of cytochrome bo_3. Based on the likely position of the residue near the heme ligands, an alternative possibility for the origin of the proton accepted by D75, including the possible equilibration with the outside, seems improbable. We note that in this part of the protein very few protonatable groups are present, namely, R71 (a possible ligand of the quinone, as presented above) and R80.

The bound quinone studied in this work is proposed to mediate electron transfer from the low-affinity quinol oxidation site to the low-spin heme *b* (see the introduction and citations therein). In direct comparison to the different types of semiquinone sites involved in mitochondrial electron transport, as reviewed by Dutton et al. (*27* and citations therein), a functional role of the high-affinity quinone (Q_H) in cytochrome bo_3 similar to that of Q_A in the bacterial reaction center may be suggested. Whereas the stability constant for the radical suggests a role as converter between $n = 1$ (in this case heme *b*) and $n = 2$ (Q-pool), the electron transfer reaction is too fast for this role. The electron transfer from the radical to heme *b* was determined to be 1.5×10^3 s^{-1} (*25*). The slow proton exchange rate of the Q_H with the Q-pool as well as the negligible effect of potent inhibitors on the spin coupling signal studied by EPR (*10*, *11*) provides additional arguments for a second binding site. Yet to be clearly defined is the location of the presumed low-affinity quinol binding site and the manner of the interactions between the two inferred Q sites.

ACKNOWLEDGMENT

We are indebted to Dr. Blanca Barquera for support and valuable discussions. We are grateful to Professor Dr. So Iwata (University of London) and colleagues for providing the coordinates of the cytochrome bo_3 structure prior to publication and to Dr. Anne Puustinen (University of Helsinki) for providing the Q101N and H98F mutant enzymes prior to publication.

REFERENCES

1. Calhoun, M. W., Thomas, J. W., and Gennis, R. B. (1994) *Trends Biochem. Sci. 19*, 325−330.
2. Garcia-Horsman, J. A., Barquera, B., Rumbley, J., Ma, J., and Gennis, R. B. (1994) *J. Bacteriol. 176*, 5587−5600.
3. Mogi, T., Tsubaki, M., Hori, H., Miyoshi, H., Nakamura, H., and Anraku, Y. (1998) *J. Biochem., Mol. Biol. Biophys. 2*, 79−110.
4. Sato-Watanabe, M., Mogi, T., Ogura, T., Kitagawa, T., Miyoshi, H., Iwamura, H., and Anraku, Y. (1994) *J. Biol. Chem. 269*, 28908−28912.
5. Sato-Watanabe, M., Mogi, T., Miyoshi, H., and Anraku, Y. (1998) *Biochemistry 37*, 5355−5361.
6. Tsatsos, P. H., Reynolds, K., Nickels, E. F., He, D.-Y., Yu, C.-A., and Gennis, R. B. (1998) *Biochemistry 37*, 9884−9888.
7. Ma, J., Puustinen, A., Wikström, M., and Gennis, R. B. (1998) *Biochemistry 37*, 11806−11811.
8. Sato-Watanabe, M., Mogi, T., Sakamoto, K., Miyoshi, H., and Anraku, Y. (1998) *Biochemistry 37*, 12744−12752.
9. Sakamoto, K., Mogi, T., Noguchi, S., and Sone, N. (2000) *J. Biochem. 126*, 934−939.
10. Ingledew, W. J., Ohnishi, T., and Salerno, J. C. (1995) *Eur. J. Biochem. 227*, 903−908.
11. Sato-Watanabe, M., Itoh, S., Mogi, T., Matsuura, K., Miyoshi, H., and Anraku, Y. (1995) *FEBS Lett. 374*, 265−269.
12. Hellwig, P., Mogi, T., Tomson, F. L., Gennis, R. B., Iwata, J., Miyoshi, H., and Mäntele, W. (1999) *Biochemistry 38*, 14683−14689.
13. Veselov, A. V., Osborne, J. P., Gennis, R. B., and Scholes, C. P. (2000) *Biochemistry 39*, 3169−3175.
14. Hastings, S., Heathcote, P., Ingledew, W. J., and Rigby, S. E. J. (2000) *Eur. J. Biochem. 267*, 5638−5645.
15. Sakamoto, K., Miyoshi, H., Takegami, K., Mogi, T., Anraku, Y., and Iwamura, H. (1996) *J. Biol Chem. 271*, 29897−29902.
16. Musser, S. M., Stowell, M. H., Lee, H. K., Rumbley, J. N., and Chan, S. I. (1997) *Biochemistry 36*, 894−902.
17. Abramson, J., Riistama, S., Larsson, G., Jasaitis, A., Svensson-Ek, M., Laakkonen, L., Puustinen, A., Iwata, S., and Wikström, M. (2000) *Nat. Struct. Biol. 7*, 910−917.
18. Lemieux, L. J., Calhoun, M. W., Thomas, J. W., Ingledew, W. J., and Gennis, R. B. (1992) *J. Biol. Chem. 267*, 2105−2113.
19. Rumbley, J. N., Nickels, E. F., and Gennis, R. B. (1997) *Biochim. Biophys. Acta 1340*, 131−142.
20. Puustinen, A., Verkhovsky, M. I., Morgan, J. E., Belevich, N. P., and Wikström, M. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 1545−1548.
21. Dutton, P. L. (1971) *Biochim. Biophys. Acta 226*, 63−80.
22. Vinogradov, A. D., Sled, V. D., Burbaev, D. S., Grivennikova, V. G., Moroz, I. A., and Ohnishi, T. (1995) *FEBS Lett. 370*, 83−87.
23. Rupp, H., Rao, K. K., Hall, D. O., and Cammack, R. (1978) *Biochim. Biophys. Acta 537*, 255−260.
24. Hellwig, P., Barquera, B., and Gennis, R. B. (2001) *Biochemistry 40*, 1077−1082.
25. Kobayashi, K., Tagawa, S., and Mogi, T. (2000) *Biochemistry 39*, 15620−15625.
26. Grimaldi, S., MacMillan, F., Ostermann, T., Ludwig, B., Michel, H., and Prisner, T. (2001) *Biochemistry 40*, 1037−1043.
27. Dutton, P. L., Ohnishi, T., Darrouzet, E., Leonard, M. A., Sharp, R. E., Gibney, B. R., Daldal, F., and Moser, C. C. (2000) *Coenzyme Q Oxidation Reduction Reactions in Mitochondrial Electron Transport*, Chapter 4, pp 65−81, CRC Press LLC, Boca Raton, FL.

BI012146W