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Fluoride-Binding to the *Escherichia coli bd*-Type Ubiquinol Oxidase Studied by Visible Absorption and EPR Spectroscopies[1]

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Cytochrome *bd*-type ubiquinol oxidase in the aerobic respiratory chain of *Escherichia coli* contains two hemes *b* (b_{558} and b_{595}) and one heme *d* as redox metal centers. To clarify the structure of the reaction center, we analyzed the fully oxidized enzyme by visible and EPR spectroscopies using fluoride ion as a monitoring probe. The visible spectral changes upon fluoride-binding were typical of ferric iron-chlorine species, indicating heme *d* as a primary binding site. The negative peak at 645 nm in the difference spectrum indicates that heme b_{595} also provides the low-affinity fluoride-binding site. Fluoride-binding caused a complete disappearance from the EPR spectra of the low-spin signals ascribable to heme *d* and spectral changes in both rhombic and axial high-spin signals. After fluoride-binding, each component of the rhombic high-spin signal showed superhyperfine splitting arising from the interaction of the unpaired spin of the heme *d* iron with the nuclear magnetic moment of ^{19}F. The axial high-spin species was converted to a new rhombic high-spin species assignable to heme b_{595}-fluoride. The $g=2$ component of this new species also gave ^{19}F-superhyperfine splitting. These results indicate that both heme *d* and heme b_{595} can coordinate with a fluoride ion with different affinities in the fully oxidized state.

Key words: cytochrome *bd*, EPR, fluoride, superhyperfine splitting, ubiquinol oxidase.

Two structurally unrelated terminal oxidases are present in the aerobic respiratory chain of *Escherichia coli* (*1, 2*). Cytochrome *bo*, a heme-copper oxidase, is expressed under highly aerated growth conditions while an alternative oxidase, cytochrome *bd*, predominates under microaerobic conditions. The affinity of cytochrome *bd* for dioxygen is 1-2 orders of magnitude greater than that of cytochrome *bo* (*3*). Thus, cytochrome *bd* is suitable for respiration under oxygen-limited growth conditions such as the stationary phase of growth. Cytochrome *bd* is a two-subunit ubiquinol oxidase that catalyzes the two-electron oxidation of ubiquinol-8 on the periplasmic side of the cytoplasmic membrane and the four-electron reduction of dioxygen on the cytoplasmic side, thus generating an electrochemical proton gradient across the membrane (*1, 2*). It is believed that the *bd* type ubiquinol oxidase contains two hemes *b* (heme b_{558} and heme b_{595}) and one heme *d* as the redox metal centers (*1, 2*).

Heme *d* is structurally unique in that it is actually an iron-chlorine derived from an iron-protoporphyrin IX. The pyrrole ring C of the macrocycle is saturated by introducing two hydroxyl groups at the C5 and C6 positions in the *trans* configuration (*4, 5*). These alterations give heme *d* a green color and a very distinct visible absorption spectrum. Heme *d* is the primary active site where dioxygen binds and is to be reduced (*1, 2*). It is likely that the chemistry of the four-electron reduction of dioxygen to water is essentially the same as that of the heme-copper oxidase, since four intermediate forms (reduced, oxygenated, oxoferryl, and oxidized) in the catalytic cycle, differing in the state of heme *d*, have been found (*1, 2*). But the precise mechanism of dioxygen activation is not well understood and may be different. In the air-oxidized state, the enzyme exists as an approximately 70:30 mixture of two stable oxy-forms, the ferrous oxygenated (Fe^{2+}-O_2) (*6, 7*) and oxoferryl (Fe^{4+}=O) species (*1, 8*). Only a portion (~10%) of the heme *d* exists in the ferric state in the air-oxidized enzyme (*9, 10*). It is thought that heme *d* and heme b_{595} are located fairly close to each other (*11*) and may form a binuclear reaction center, in which the role of Cu_B in the heme-copper oxidase may be mimicked by heme b_{595} (*12-14*). Thus, clarification of the coordination structure around heme *d* and heme b_{595} becomes increasingly important to understand the mechanism of the dioxygen activation by this unique terminal oxidase.

Among various spectroscopic techniques, EPR spectroscopy is particularly suitable for the analysis of the coordination structure of heme iron when various heme ligands bind (*15-18*). In the present article, we apply this method, along with visible absorption spectroscopy, utilizing fluo-

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ride ion as a probe to analyze the active site structure of the *bd*-type ubiquinol oxidase from *E. coli*.

MATERIALS AND METHODS

Preparation of bd-Type Ubiquinol Oxidases—Cytochrome *bd* was purified from the overproducing strain GR84N/pNG2 (*cyo*$^+$ *cydA2/cyd*$^+$), a generous gift from R.B. Gennis, as described previously (*14*), and stored at −80°C in 50 mM Na-phosphate buffer (pH 7.4) containing 0.1% sucrose monolaurate 1200 (Mitsubishi-Kagaku Foods, Tokyo). The concentration of the enzyme was calculated from the extinction coefficient in the air-oxidized state at 414 nm ($\varepsilon = 223.0$ mM^{-1}·cm^{-1}), based on the heme content as determined by the pyridine ferrohemochromogen method (*14*).

Cytochrome *bd* was oxidized as follows. The air-oxidized dioxygen-bound cytochrome *bd* in a small tube with a rubber septum was deoxygenated under flowing pure argon gas passed through two needles (one for inlet and the other for outlet). Then, solid sodium dithionite was added anaerobically to obviate the protective effect of the bound dioxygen (or oxoferryl species) against oxidants, and the solution was incubated on ice for 30 min. Then, solid ferricyanide (in large excess) was added anaerobically and the sample was kept on ice for an additional 30 min. The fully oxidized sample was subjected to gel filtration on an Ampure SA cartidge column (Amersham) in 50 mM Na-phosphate buffer (pH 7.4) containing 0.1% sucrose monolaurate 1200. The visible absorption spectra were then analyzed. Solid NaF (Nacalai Tesque, Kyoto) was added directly to the air-oxidized and fully oxidized enzymes.

Visible and EPR Spectroscopies—Visible absorption spectra were measured with a Shimadzu UV-2400PC spectrophotometer (Shimadzu, Kyoto). EPR measurements were carried out at X-band (9.23 GHz) microwave frequency with a Varian E-12 EPR spectrometer equipped with an Oxford flow cryostat (ESR-900) as described previously (*19*).

RESULTS AND DISCUSSION

Visible Absorption Spectroscopic Study—To clarify fluoride-binding to cytochrome *bd*, we conducted oxidation of the air-oxidized enzyme, which exists as a mixture of three forms. The visible absorption spectrum of the fully oxidized enzyme, prepared according to the procedure described in "MATERIALS AND METHODS," is characterized by an unresolved Soret band centered at 413 nm (not shown) and weaker bands at 535, 595, 645, and 743 nm (Fig. 1A). The 743 nm band ($\varepsilon = 2.6$ mM^{-1}·cm^{-1}) can be ascribed to the ferric heme *d* high-spin charge-transfer band, based on the similarity of the peak location to those of various iron-chlorine containing hemoproteins and iron-chlorine model complexes (*20-22*). The 595 nm band is also likely due to ferric heme *d*, since a corresponding band is always associated with the ~740 nm band (with a spacing of 120-160 nm) for these iron-chlorine complexes in the ferric high-spin state (*20-22*). The 645 nm band ($\varepsilon = 9.8$ mM^{-1}·cm^{-1}) in the spectrum of the fully oxidized enzyme is not due to the residual ferrous heme *d*-dioxygen species, but rather it can be attributed to heme b_{595} in the ferric high-spin state. The spectrum of the anaerobically oxidized enzyme during redox titration retains a band at around 645 nm (*23*). The nature of the 535 nm band is not clear at this stage, but is likely due to ferric heme b_{558}. It should be noted that the 680 nm band characteristic of oxoferryl species (*1, 8*) is eliminated completely by full oxidation.

The stepwise additions of sodium fluoride to the fully oxidized enzyme caused a gradual change in the visible spectrum as shown in Fig. 1A. The absorption bands at 743 and 595 nm were replaced by two new bands at 708 ($\varepsilon = 6.9$ mM^{-1}·cm^{-1}) and 575 nm, and the 645 nm band underwent a decrease in intensity. In contrast, the 535 nm band showed no appreciable change. For clarification, the difference spectrum (fluoride-bound oxidized *minus* oxidized) is shown in Fig. 1B. The observed spectral changes upon fluoride-binding (blue shifts of high-spin charge-transfer

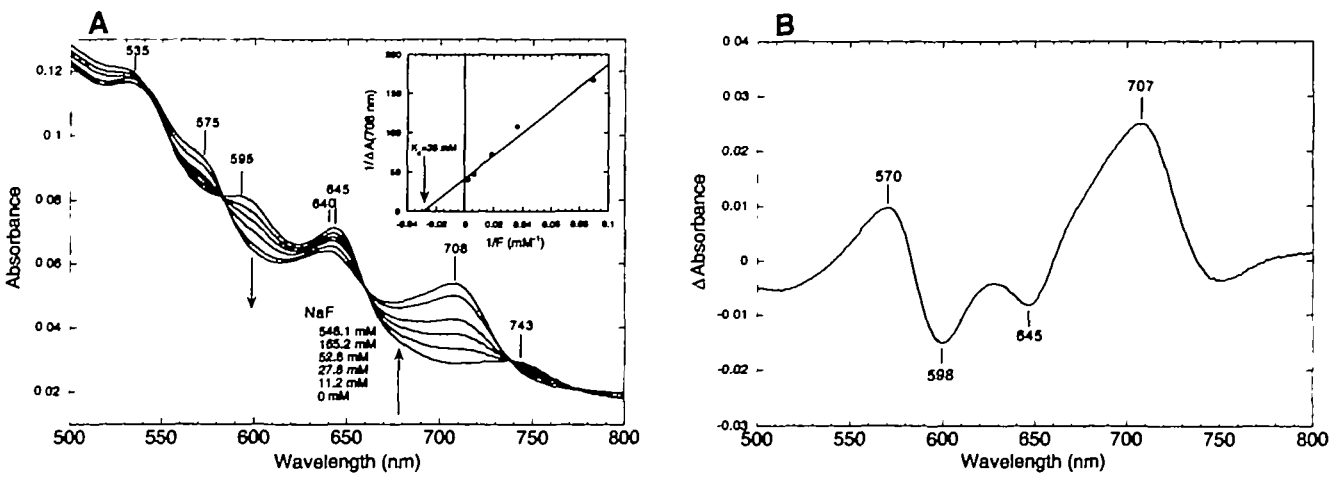


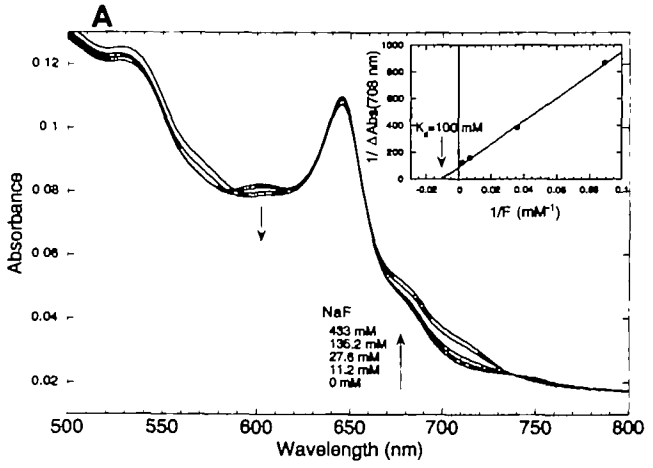
Fig. 1. **Visible absorption spectral changes in fully oxidized cytochrome *bd* upon the addition of fluoride (A) and the difference spectrum of the fluoride-bound oxidized state *minus* the oxidized state (B).** Inset in panel A shows a double reciprocal plot of the absorbance change at 708 nm upon the addition of fluoride ions. Conditions of measurements are as follows: temperature, 20°C; sample concentration, 4.18 μM in 50 mM Na-phosphate buffer (pH 7.4) containing 0.1% sucrose monolaurate 1200. Fluoride ions were added to the oxidized enzyme as in sodium fluoride with final concentrations of 11.2, 27.6, 52.8, 165.2, and 656.1 mM. In the difference spectrum (panel B), the concentration of fluoride ions in the sample cell was 656.1 mM.

bands from 743 and 595 nm to 708 and 575 nm, respectively) are typical of those observed for other ferric iron-chlorine species, suggesting that the fluoride ion binds primarily at ferric heme *d*. The K_d-value for fluoride binding to ferric heme *d* was estimated from the absorbance change at 708 nm to be 36 mM at pH 7.4 (Fig. 1A, inset). The binding affinity, however, shows a considerable pH dependency; at pH 6.0 the K_d value is ~20 mM, whereas, at pH 8.4, it increases to ~500 mM.

The clear negative peak at 645 nm in the difference spectrum (Fig. 1B) suggests that ferric heme b_{595} also participates in fluoride-binding. The binding affinity of ferric heme b_{595} for fluoride ion could not be estimated accurately due to the overlapping spectral changes caused by fluoride binding to ferric heme *d*. The K_d value seemes to be several hundred mM. The pH dependence of the binding affinity is not so marked as for ferric heme *d*, but is apparently weaker at higher pH.

The addition of fluoride ion to the air-oxidized enzyme at pH 7.4 caused slight but clear spectral changes in the 700 nm region (Fig. 2A). The difference spectrum (Fig. 2B) shows a pattern very similar to that obtained for the oxidized enzyme (Fig. 1B), positive peaks at 707 and 570 nm, and negative peaks at 645 and 598 nm, respectively. These results indicate that fluoride ion does not bind to the ferrous-oxygenated or oxoferryl species of heme *d*. An apparent K_d value for fluoride-binding to the ferric heme *d* of the air-oxidized enzyme was estimated similarly to be 100 mM at pH 7.4 (Fig. 2A, inset), 3-fold larger than that of the fully oxidized enzyme.

EPR Spectroscopic Study—The addition of fluoride ion (250 mM) to the air-oxidized enzyme caused a complete



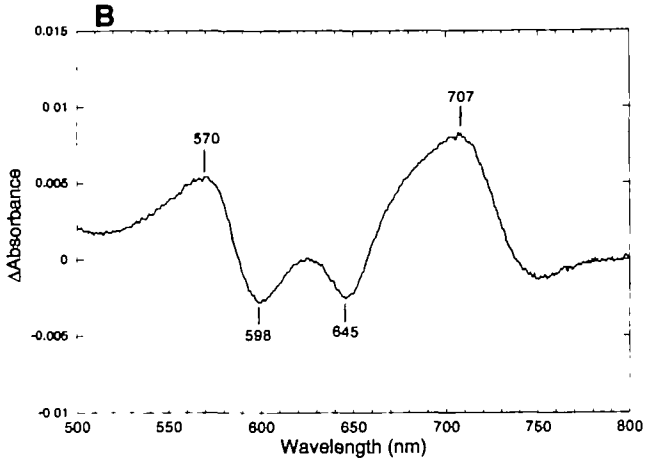
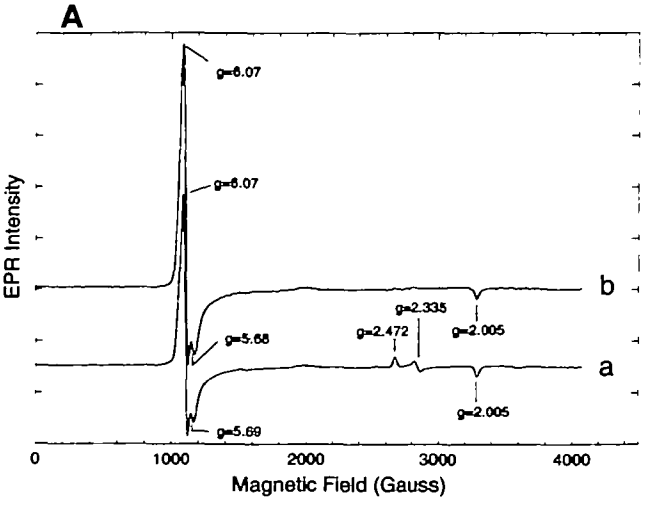


Fig. 2. **Changes in the visible absorption spectra of air-oxidized cytochrome *bd* upon the addition of fluoride (A) and the difference spectrum of the fluoride-bound air-oxidized state *minus* the air-oxidized state (B).** Fluoride ions were added to the oxidized enzyme as in the case of sodium fluoride at final concentrations of 11.2, 27.6, 135.2, and 433 mM. In the difference spectrum (panel B), the concentration of fluoride ions in the sample cell was 433 mM. Other details are the same as in the legend to Fig. 1.



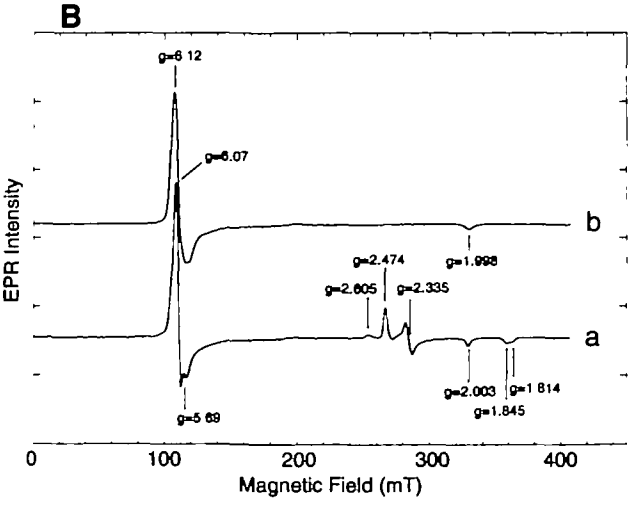


Fig. 3. **EPR spectra of cytochrome *bd* in the air-oxidized state (A) and in the fully oxidized state (B) before (a) and after (b) the addition of 250 mM fluoride.** Conditions of measurements are as follows: microwave frequency, 9.22 GHz; incident microwave power, 5 mW; 100-kHz field modulation width, 0.5 mT; temperature, 15 K. Sample concentration was 0.415 mM in 50 mM Na-phosphate buffer (pH 7.4) containing 0.1% sucrose monolaurate 1200.

disappearance of the low-spin EPR signals ($g_z = 2.47$ species) and slight changes in both the $g = 6$ axial and rhombic high-spin signals (Fig. 3A-b). The low-spin EPR signals have been assigned to ferric heme *d* (*9, 24*) and, therefore, the disappearance of these signals and the slight intensifying of the high-spin signals each be explained by the formation of the ferric heme *d*-fluoride high-spin species (see next section).

The oxidation of the air-oxidized enzyme caused a significant increase in the intensity of the ferric heme *d* low-spin signals (Fig. 3B-a). However, the intensities of both the $g = 6$ axial and rhombic high-spin signals did not increase so much, indicating that the major portion of ferric heme *d* adopts a low-spin state at 15 K.

Upon the addition of fluoride ion (250 mM) to the fully oxidized enzyme, significant spectral changes occurred (Fig. 3B-b). In the lower magnetic field region, both of the two components of the *g*6 rhombic high-spin signal showed clear doublets with splittings of 3.5 and 3.0 mT, respectively (Fig. 4A-b). In addition, a considerable decrease in the intensity of the axial high-spin signal ($g = 6.03$) and an increase in intensity around $g \sim 6.15$ suggest the appearance of another type of g6 rhombic signal. The latter spectral changes can be interpreted as the binding of a fluoride ion to the ferric axial high-spin heme to form a new rhombic high-spin species. On the other hand, in the higher magnetic field region, we observed that the ferric heme *d* low-spin signals ($g_z = 2.61$ and $g_z = 2.47$ species) (*9, 24*) disappear completely (Fig. 3B-b). Further, two types of high-spin $g = 2$ signals are newly formed (Fig. 4B-b), showing splittings of 11.2 and 1.5 mT, respectively. At 2.5 mM fluoride, only the $g = 2$ high-spin signal with a larger splitting and the rhombic high-spin signal with clear doublet structures remained in the EPR spectra (spectra not shown). At 25 mM fluoride, on the other hand, the EPR spectra in both the g6 and g2 regions (spectra not shown) were almost the same as those measured at 250 mM fluoride.

These observations led us to following conclusions. First, the $g = 2$ signal with a larger splitting must originate from the rhombic high-spin species with clear doublet structures. Second, this rhombic high-spin species has a higher binding affinity for fluoride than the other high-spin species. Third, accordingly, the rhombic high-spin species with clear doublet structures can be assigned to the ferric heme *d*-fluoride.[3] Fourth, the other rhombic high-spin species with a smaller splitting for the $g = 2$ component, which is converted from the axial high-spin species upon fluoride binding, must arise from the heme b_{595}-fluoride.[4]

The observed doublets for both the heme *d* and heme b_{595} high-spin signals provide conclusive evidence for the hyperfine interaction of the unpaired electron spin of heme iron with the nuclear magnetic moment of ^{19}F, which has a spin of one-half (*25, 26*). Thus, the present results establish that both heme *d* and heme b_{595} can coordinate fluoride ions simultaneously in the fully oxidized state, but with different binding affinities. This conclusion is consistent with our previous observation that NO binds primarily to ferrous heme *d*, but ferrous heme b_{595} also provides the low-affinity binding site for NO (*19*). Although in conflict with previous assignments of the EPR signals, in which the rhombic and axial high-spin signals have been assigned to ferric heme b_{595} and ferric heme *d*, respectively (*24, 27*), we are confident of our new assignments.

Structural Implications for the Active Site—Although ferric heme *d* represents only a minor part (~10%) of the total heme *d* population in the air-oxidized enzyme, we could detect the binding of fluoride ion to ferric heme *d* by both EPR (disappearance of the low-spin EPR signals (Fig. 3A) and appearance of the split $g = 2$ signal (not shown)) and visible absorption [two positive peaks at 707 and 570 nm in the difference spectrum (Fig. 2B)] spectroscopies.

[3] The binding interactions of both hemes *d* and b_{595} with fluoride ion seem to become stronger at lower temperature, as evidenced by the EPR spectra at 25 mM NaF. The interaction of heme b_{595} with fluoride, however, was still much weaker than that of heme *d*.

[4] The EPR signal of the heme b_{595}-fluoride species in the g6 region overlapped significantly with the heme *d* rhombic high-spin signal (Fig. 4A) and, therefore, we could not ascertain whether these was a doublet structure or not.

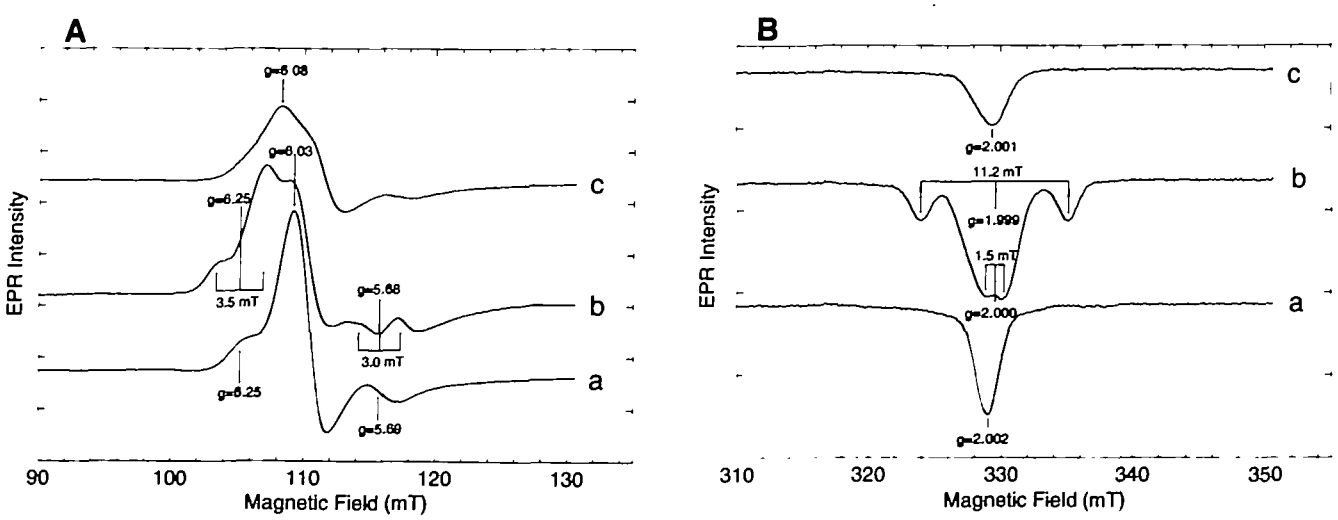


Fig. 4. **High-spin EPR spectra in the g6 (A) and in g2 (B) regions of cytochrome *bd* in the oxidized state before (a) and after (b) the addition of 250 mM fluoride, and after the further addition of 10 mM of cyanide (c).** Temperature, 5 K. Other conditions for samples and measurements are the same as described in the legend to Fig. 3.

However, the hyperfine splittings of the g6 rhombic high-spin signal were obscured by an overlapping g6 rhombic signal with no hyperfine structure, which is clearly different from that of the heme b_{595}-fluoride species. This indicates that frozen samples at low temperature contain a fair amount of rhombic high-spin heme *d* species that does not bind fluoride ion. These altered ferric heme *d* species might be due to the partially denatured structure at the active site or produced upon freezing (*i.e.*, rupture of the ferric heme *d* iron-fluoride bond) (*11, 28*).

On the other hand, a major part of the ferric heme b_{595} in the air-oxidized enzyme, where heme *d* is mostly in either the ferrous-dioxygen or oxoferryl state while heme b_{595} is fully in the oxidized state (*6-8*), shows no reactivity towards fluoride ion. Thus, the binding of fluoride ion to ferric heme b_{595} seems to require the oxidized state of heme *d*. This may be due to either steric hindrance from the heme *d*-coordinated dioxygen (or oxoferryl ligand) and/or to some kind of redox-dependent structural change. Since heme b_{595} and heme *d* are likely to be located in close proximity to each other, as previously suggested based on various spectroscopic studies (*12, 14*), the former possibility seems very likely. Indeed, we found that the addition of cyanide to the fluoride-coordinated fully oxidized enzyme simultaneoulsy caused a complete disappearance of both types of rhombic high-spin signals (Fig. 4, A-c and B-c). The addition of azide also caused the simultaneous disappearance of both types of rhombic high-spin signals (spectra not shown). These results indicate that the two fluoride binding sites and the cyanide (or azide) binding sites overlap at least partially.

The unusually large hyperfine splittings (3.5, 3.0, and 11.2 mT) observed for the ferric heme *d*-fluoride high-spin EPR signal are noteworthy. For ferrimyoglobin-fluoride in frozen solution, it has been reported that the separation of the $g=2$ signal is about 4.4 mT (*26*). For ferrimyoglobin-fluoride in a single crystal, the two components of $g \sim 6$ rhombic signals have splittings of 2.35 and 2.15 mT (*25*). For the ferrimyeloperoxidase-fluoride complex, the splitting of the $g=2$ signal is 3.5 mT (*29*). Ferrisulfmyoglobin, which contains an iron-chlorine-type prosthetic group, also shows a splitting of 4.8 mT for the $g=2$ signal upon fluoride binding (*21*). Thus, the large hyperfine splitting of the $g=2$ signal cannot be ascribed to the chlorine macrocycle alone, but is more likely to be due to the protein tertiary structure around the heme *d* moiety. Since the chlorine macrocycle has a saturated pyrrole ring, it is, therefore, more susceptible to the influence of the protein moiety. This may give the heme *d* moiety an unusual geometry such as a ruffled or a nonplanar macrocycle structure (*30*). This a flexible nature of the macrocycle may explain why cyanide ion forms a bridging structure between heme *d* and heme b_{595} in the oxidized state (*14*).

It is very interesting to note that both the heme-copper oxidase and the *bd*-type ubiquinol oxidase form a "resting state" at the binuclear metal center when the supply of electron equivalents and/or dioxygen is shut off. Although the exact nature of the "resting state" seems very different for these two types of terminal oxidase, each "resting state" seems to have a common physiological role, *i.e.*, protection of the binuclear metal center from access by inhibitory exogenous ligands such as cyanide, azide, and fluoride ions. To maintain the physiological protective ligands at the primary binding site, the presence of an auxiliary metal center (*i.e.*, Cu_B or heme b_{595}) may be essential. On the other hand, the protective ligands (dioxygen or intermediates in the catalytic cycle) can be easily removed upon replenishment of the electron.

In conclusion, using visible absorption and EPR spectroscopies, we have found that both heme *d* and heme b_{595} can coordinate with a fluoride ion with different affinities in fully oxidized state. The inaccessibility of the ferric heme b_{595} center for fluoride ions in air-oxidized state suggests the close proximity of heme b_{595} to heme *d*, or the existence of a strong redox-dependent interaction between these two hemes.

REFERENCES

1. Jünemann, S. (1997) Cytochrome *bd* terminal oxidase. *Biochim. Biophys. Acta* **1321**, 107-127
2. Mogi, T., Tsubaki, M., Hori, H., Miyoshi, H., Nakamura, H., and Anraku, Y. (1998) Two terminal quinol oxidase families in *Escherichia coli*: Variations on molecular machinery for dioxygen reduction. *J. Biochem. Mol. Biol. Biophys.* **2**, 79-110
3. D'mello, R., Hill, S., and Poole, R.K. (1996) The cytochrome *bd* quinol oxidase in *Escherichia coli* has an extremely high oxygen affinity and two oxygen-binding haems: Implications for regulation of activity in vivo by oxygen inhibition. *Microbiology* **142**, 755-763
4. Vavra, M.R., Timkovich, R., Yap, F., and Gennis, R.B. (1986) Spectroscopic studies on heme *d* in the visible and infrared. *Arch. Biochem. Biophys.* **250**, 461-468
5. Sotiriou, C. and Chang, C.K. (1988) Synthesis of the heme *d* prosthetic group of bacterial terminal oxidase. *J. Am. Chem. Soc.* **110**, 2264-2270
6. Lorence, R.M. and Gennis, R.B. (1989) Spectroscopic and quantitative analysis of the oxygenated and peroxy states of the purified cytochrome *d* complex of *Escherichia coli*. *J. Biol. Chem.* **264**, 7135-7140
7. Kahlow, M.A., Loehr, T.M., Zuberi, T.M., and Gennis, R.B. (1993) The oxygenated complex of cytochrome *d* terminal oxidase: Direct evidence for Fe-O_2 coordination in a chlorin-containing enzyme by resonance Raman spectroscopy. *J. Am. Chem. Soc.* **115**, 5845-5846
8. Kahlow, M.A., Zuberi, T.M., Gennis, R.B., and Loehr, T.M. (1991) Identification of a ferryl intermediate of *Escherichia coli* cytochrome *d* terminal oxidase by resonance Raman spectroscopy. *Biochemistry* **30**, 11485-11489
9. Hata, A., Kirino, Y., Matsuura, K., Itoh, S., Hiyama, T., Konishi, K., Kita, K., and Anraku, Y. (1985) Assignment of ESR signals of *Escherichia coli* terminal oxidase complexes. *Biochim. Biophys. Acta* **810**, 62-72
10. Krasnoselskaya, I., Arutjunjan, A.M., Smirnova, I., Gennis, R., and Konstantinov, A.A. (1993) Cyanide-reactive sites in cytochrome *bd* complex from *E. coli*. *FEBS Lett.* **327**, 279-283
11. Hata-Tanaka, A., Matsuura, K., Itoh, S., and Anraku, Y. (1987) Electron flow and heme-heme interaction between cytochromes b_{558}, b_{595} and *d* in a terminal oxidase of *Escherichia coli*. *Biochim. Biophys. Acta* **893**, 289-295
12. Hill, J.J., Alben, J.O., and Gennis, R.B. (1993) Spectroscopic evidence for a heme-heme binuclear center in the cytochrome *bd* ubiquinol oxidase from *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **90**, 5863-5867
13. D'mello, R., Palmer, S., Hill, S., and Poole, R.K. (1994) The cytochrome *bd* terminal oxidase of *Azotobacter vinelandii*: Low temperature photodissociation spectrometry reveals reactivity of cytochromes b_{595} and *d* with both carbon monoxide and oxygen. *FEMS Microbiol. Lett.* **121**, 115-120
14. Tsubaki, M., Hori, H., Mogi, T., and Anraku, Y. (1995) Cyanide-binding site of *bd*-type ubiquinol oxidase from *Escherichia coli*. *J. Biol. Chem.* **270**, 28565-28569
15. Tsubaki, M., Hiwatashi, A., Ichikawa, Y., and Hori, H. (1987)

Electron paramagnetic resonance study of ferrous cytochrome P-450$_{scc}$-nitric oxide complexes: Effects of cholesterol and its analogues. *Biochemistry* **26**, 4527-4534
16. Hori, H., Masuya, F., Tsubaki, M., Yoshikawa, S., and Ichikawa, Y. (1992) Electronic and stereochemical characterization of intermediates in the photolysis of ferric cytochrome P450scc nitrosyl complexes. Effects of cholesterol and its analogues on ligand binding structures. *J. Biol. Chem.* **267**, 18377-18381
17. Tsubaki, M., Uno, T., Hori, H., Mogi, T., Nishimura, Y., and Anraku, Y. (1993) Cytochrome *d* axial ligand of the *bd*-type terminal quinol oxidase from *Escherichia coli*. *FEBS Lett.* **335**, 13-17
18. Tsubaki, M., Mogi, T., Anraku, Y., and Hori, H. (1993) Structure of the heme-copper binuclear center of the cytochrome *bo* complex of *Escherichia coli*: EPR and Fourier-transform infrared spectroscopic studies. *Biochemistry* **32**, 6065-6072
19. Hori, H., Tsubaki, M., Mogi, T., and Anraku, Y. (1996) EPR study of NO complex of *bd*-type ubiquinol oxidase from *Escherichia coli*: The proximal axial ligand of heme *d* is nitrogenous amino acid residue. *J. Biol. Chem.* **271**, 9254-9258
20. Stolzenberg, A.M., Strauss, S.H., and Holm, R.H. (1981) Iron(II, III)-chlorin and -isobacteriochlorin complexes. Models of the heme prosthetic groups in nitrite and sulfite reductases: Means of formation and spectroscopic and redox properties. *J. Am. Chem. Soc.* **103**, 4763-4778
21. Berzofsky, J.A., Peisach, J., and Blumberg, W.E. (1971) Sulfheme proteins I. Optical and magnetic properties of sulfmyoglobin and its derivatives. *J. Biol. Chem.* **246**, 3367-3377
22. Bracete, A.M., Kadkhodayan, S., Sono, M., Huff, A.M., Zhuang, C., Cooper, D.K., Smith, K.M., Chang, C.K., and Dawson, J.H. (1994) Iron chlorin-reconstituted histidine-ligated heme proteins as models for naturally occurring iron chlorin proteins: Magnetic circular dichroism spectroscopy as a probe of iron chlorin coordination structure. *Inorg. Chem.* **33**, 5042-5049
23. Koland, J.G., Miller, M.J., and Gennis, R.B. (1984) Potentiometric analysis of the purified cytochrome *d* terminal oxidase complex from *Escherichia coli*. *J. Biol. Chem.* **23**, 1051-1056
24. Meinhardt, S.W., Gennis, R.B., and Ohnishi, T. (1989) EPR studies of the cytochrome-*d* complex of *Escherichia coli*. *Biochim. Biophys. Acta* **975**, 175-184
25. Morimoto, H. and Kotani, M. (1966) Fluorine superhyperfine structure in EPR spectra of the single crystal of the myoglobin fluoride. *Biochim. Biophys. Acta* **126**, 176-178
26. Peisach, J., Blumberg, W.E., Ogawa, S., Rachmilewitz, E.A., and Oltzik, R. (1971) The effects of protein conformation on the heme symmetry in high spin heme proteins as studied by electron paramagnetic resonance. *J. Biol. Chem.* **246**, 3342-3355
27. Rothery, R.A. and Ingledew, W.J. (1989) The cytochromes of anaerobically grown *Escherichia coli*. An electron-paramagnetic-resonance study of the cytochrome *bd* complex *in situ*. *Biochem. J.* **261**, 437-443
28. Spinner, F., Cheesman, M.R., Thomson, A.J., Kaysser, T., Gennis, R.B., Peng, Q., and Peterson, J. (1995) The haem b_{558} component of the cytochrome *bd* quinol oxidase complex from *Escherichia coli* has histidine-methionine axial ligation. *Biochem. J.* **308**, 641-644
29. Wever, R. and Bakkenist, A.R.J. (1980) The interaction of myeloperoxidase with ligands as studied by EPR. *Biochim. Biophys. Acta* **612**, 178-184
30. Crane, B.R., Siegel, L.M., and Getzoff, E.D. (1997) Structure of the siroheme- and Fe_4S_4-containing active center of sulfite reductase in different states of oxidation: Heme activation via reduction-gated exogenous ligand exchange. *Biochemistry* **36**, 12101-12119