Resonance Raman Spectroscopic Identification of a Histidine Ligand of b_{595} and the Nature of the Ligation of Chlorin *d* in the Fully Reduced *Escherichia coli* Cytochrome *bd* Oxidase†

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ABSTRACT: Cytochrome *bd* oxidase is a bacterial terminal oxidase that contains three cofactors: a low-spin heme (b_{558}), a high-spin heme (b_{595}), and a chlorin *d*. The center of dioxygen reduction has been proposed to be a binuclear b_{595}/*d* site, whereas b_{558} is mainly involved in transferring electrons from ubiquinol to the oxidase. Information on the nature of the axial ligands of the three heme centers has come from site-directed mutagenesis and spectroscopy, which have implicated a His/Met coordination for b_{558} (Spinner, F., Cheesman, M. R., Thomson, A. J., Kaysser, T., Gennis, R. B., Peng, Q., & Peterson, J. (1995) *Biochem.* J. 308, 641−644; Kaysser, T. M., Ghaim, J. B., Georgiou, C., & Gennis, R. B. (1995) *Biochemistry 34*, 13491−13501), but the ligands to b_{595} and *d* are not known with certainty. In this work, the three heme chromophores of the *fully reduced* cytochrome *bd* oxidase are studied individually by selective enhancement of their resonance Raman (rR) spectra at particular excitation wavelengths. The rR spectrum obtained with 413.1-nm excitation is dominated by the bands of the 5cHS b_{595}^{2+} cofactor. Excitation close to 560 nm yields a rR spectrum dominated by the 6cLS b_{558}^{2+} heme. Wavelengths between these values enhance contributions from both b_{595}^{2+} and b_{558}^{2+} chromophores. The rR bands of the ferrous chlorin become the major features with red laser excitation (595−650 nm). The rR data indicate that d^{2+} is a 5cHS system whose axial ligand is either a weakly coordinating protein donor or a water molecule. In the low-frequency region of the 441.6-nm spectrum, we assign a rR band at 225 cm^{-1} to the (b_{595})Fe^{II}−N(His) stretching vibration, based on its 1.2-cm^{-1} upshift in the ^{54}Fe-labeled enzyme. This observation provides the first physical evidence that the proximal ligand of b_{595} is a histidine. Site-directed mutagenesis had suggested that His 19 is associated with either b_{595} or *d* (Fang, H., Lin, R.-J., & Gennis, R. B. (1989) *J.* Biol. Chem. *264*, 8026−8032). On the basis of the present study, we propose that the proximal ligand of b_{595} is His 19. We have also studied the reaction of cyanide with the fully reduced cytochrome *bd* oxidase. In ∼700-fold excess cyanide (∼35 mM), the 629-nm UV/vis band of d^{2+} is blue-shifted to 625 nm and diminished in intensity. However, the rR spectra at each of three different λ_0 (413.1, 514.5, and 647.1 nm) are identical *with or without cyanide*, thus indicating that both b_{595} and *d* remain as 5cHS species in the presence of CN^-. This observation leads to the proposal that a native ligand of ferrous chlorin *d* is replaced by CN^- to form the 5cHS d^{2+} cyano adduct. These findings corroborate our companion study of the "as-isolated" enzyme in which we proposed a 5cHS d^{3+} cyano adduct (Sun, J., Osborne, J. P., Kahlow, M. A., Kaysser, T. M., Hill, J. J., Gennis, R. B., & Loehr, T. M. (1995) *Biochemistry 34*, 12144−12151). To further characterize the unusual and unexpected nature of these proposed high-spin cyanide adducts, we have obtained EPR spectral evidence that binding of cyanide to fully oxidized cytochrome *bd* oxidase perturbs a spin-state equilibrium in the chlorin d^{3+} to yield entirely the high-spin form of the cofactor.

Cytochrome *bd* oxidase is a bacterial terminal oxidase that catalyzes the four-electron reduction of O_2 to H_2O using ubiquinol as an electron donor (Poole, 1983; Anraku & Gennis, 1987; Anraku, 1988). Unlike members of the oxidase superfamily, such as cytochrome *c* oxidase and cytochrome *bo* oxidase, cytochrome *bd* oxidase contains no additional copper cofactors (Trumpower & Gennis, 1994).

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It catalyzes transmembrane separation of protons and electrons but does not function as a proton pump (Puustinen et al., 1991). Native cytochrome *bd* oxidase contains two *b* hemes (one low-spin, designated as b_{558}, and one-high spin, designated as b_{595}) and a chlorin *d* cofactor (Lorence et al., 1986). The heme b_{558} serves to transfer electrons from ubiquinol (Dueweke & Gennis, 1990, 1991). The b_{595} heme and chlorin *d* appear to be closely associated at the O_2-reduction site (Hata-Tanaka et al., 1987; Meinhardt et al., 1989; Rothery & Ingledew, 1989), and recent CO- and CO/CN^--binding studies, respectively, suggested that these two cofactors behave as a binuclear center, perhaps similar to the heme a_3/Cu_B pair of the oxidase superfamily (Hill et al., 1993; Tsubaki et al., 1995). A similar cytochrome *bd* oxidase occurs in *Azotobacter vinelandii*, where it is thought to confer aerotolerance. Photodissociation studies of this enzyme show reversible CO binding to both b_{595} and *d* and suggest O_2 migration from *d* to b_{595} in a binuclear center (D'mello et al., 1994). However, more recent ligand-binding studies on the *A. vinelandii* enzyme suggest that their b_{595} and *d* cytochromes are not sufficiently close to qualify as an interacting binuclear center (Jünemann & Wrigglesworth, 1995).

Understanding of the structural composition of cytochrome *bd* oxidase is still very limited in comparison with the other well-studied oxidases. The chlorin *d* is postulated to be a dihydroxyprotochlorin (Sotiriou & Chang, 1988; Andersson et al., 1987; Vavra et al., 1986; Timkovich et al., 1985), similar to the prosthetic groups of *Escherichia coli* catalase HPII and *Neurospora crassa* chlorin catalase (Loewen et al., 1993; Andersson, 1989; Chiu et al., 1989; Jacob & Orme-Johnson, 1979). An oxygenated *d* chromophore (d_{650}) has been inferred from UV/vis spectroscopy (Lorence & Gennis 1989; Poole et al., 1983a,b) and has been verified by direct observation of the Fe−O_2 stretching vibration at 568 cm^{-1} that shifts to 542 cm^{-1} with $^{18}O_2$ substitution (Kahlow et al., 1993). Earlier, a ferryl−oxo species (d_{680}) was discovered from its unique Fe^{IV}═O stretching vibration at 815 cm^{-1}, supported by $^{18}O_2$- and mixed-isotope $^{16}O^{18}O$-labeling experiments (Kahlow et al., 1991). We have further corroborated this assignment by noting an ^{54}Fe-dependent frequency shift (J. Sun, T. M. Loehr, T. M. Kaysser, and R. B. Gennis, manuscript in preparation).

Regarding the nature of the axial ligands of the three heme cofactors in cytochrome *bd* oxidase, only those of b_{558} are known with some certainty. Initially, site-directed mutagenesis was carried out to identify these ligands (Fang et al., 1989; Zuberi, 1993; Kaysser et al., 1995). Of the 10 conserved histidines, only two, His 19 and His 186, were shown to affect heme ligation. A H186→Leu mutation eliminated the low-spin UV/vis absorption peaks, and, on this basis, His 186 was proposed to be one of the two axial ligands for b_{558}. The second b_{558} ligand has been shown to be a methionine since a M393→Leu mutation converted b_{558} into a pentacoordinate, high-spin (5cHS)[1] heme (Kaysser et al., 1995). Recent MCD spectroscopic studies are fully consistent with such a His/Met ligation of the low-spin b_{558} (Spinner et al., 1995). Mutation of His19→Leu affected the b_{595} and *d* hemes, suggesting its role as a ligand to one or the other cofactor (Fang et al., 1989). However, an ENDOR study ruled out a strongly coordinating nitrogenous ligand to chlorin *d* (Jiang et al., 1993). The results of the present study provide evidence for a histidine ligand to b_{595}, and we suggest that His 19 is, indeed, the proximal ligand of b_{595}.

An unusual feature of cytochrome *bd* oxidase is its low sensitivity toward cyanide, as indicated by the high K_i value of 7.8 mM in unpurified respiratory particles (Pudek & Bragg, 1974). As a result, bacteria containing cytochrome *bd* oxidase have shown cyanide resistance (Henry, 1981). Meunier et al. (1995) reported that purified cytochrome *bd* oxidase had 6% activity in the presence of 10 mM of cyanide. In contrast, cytochrome *c* oxidase and many heme proteins are exceedingly sensitive to cyanide, with micromolar K_i values (Bosshard et al., 1991; Jones et al., 1984; Palcic & Dunford, 1981). The interaction between cyanide and cytochrome *bd* oxidase has been studied by electronic spectroscopy (Kauffman & Van Gelder, 1973, 1974; Krasnoselskaya et al., 1993). For the native enzyme, the decrease of the 645-nm peak (attributed to the oxygenated chlorin cofactor, d_{650}) has been correlated with a direct binding of CN^- at the *d* site (Kauffman & Van Gelder, 1974). However, absorbance and MCD red shifts in the Soret region have been interpreted as CN^- binding to b_{595}^{3+} with a concomitant high- to low-spin conversion (Krasnoselskaya et al., 1993). Our recent rR study, on the other hand, led to a reinterpretation of the optical changes as (i) the displacement of the O_2 ligand in the "as-isolated" enzyme by CN^-, (ii) the concurrent autoreduction of b_{595}^{3+} to b_{595}^{2+} (this b_{595} site appears inert to cyanide in both its ferric and ferrous states), and (iii) the formation of an atypical *high-spin* cyano adduct of chlorin d^{3+} (Sun et al., 1995).

In this paper, we have used rR spectroscopy to characterize the ligation/spin states of the heme cofactors in the *fully reduced* cytochrome *bd* oxidase in the absence and presence of cyanide. In addition, we have used EPR spectroscopy to identify these states in the *fully oxidized* enzyme to further corroborate the formation of high-spin cyano species of chlorin *d*. Resonance Raman spectroscopy is a powerful tool for the study of heme proteins (Spiro, 1988). If proteins contain multiple chromophores, rR spectroscopy has the ability to observe individual chromophores by using different excitation wavelengths for their selective enhancement (Babcock et al., 1981). Thus, coordination and spin states of the b_{558}, b_{595}, and *d* moieties can be determined individually with limited interference from one another.

MATERIALS AND METHODS

The *E.* coli enzyme has been sequenced, cloned, and overexpressed (Fang et al., 1989; Green et al., 1988, 1984). Purification of the wild-type enzyme has been described previously (Miller & Gennis, 1986); however, minor modifications were made as follows. The bacterial cells used for purification purposes were GO105/pTK1 which overexpress the cytochrome *bd* complex (Kaysser et al., 1995). After washing, the cells are frozen at −70 °C until use. In a blender, 75 g of frozen cells are suspended in 300 mL of a pH 8.3 buffer consisting of 100 mM Tris-HCl, 15 mM

[1] Abbreviations: Designations for heme coordination and spin states: 5cHS, five-coordinate high-spin; 4cIS, four-coordinate intermediate-spin; 6cLS, six-coordinate low-spin. EPR, electron spin resonance; MCD, magnetic circular dichroism; rR, resonance Raman. Degree of polarization (qualitative) of Raman lines: ap, anomalously polarized; dp, depolarized; p, polarized. DC, deuterochlorin; ImH, imidazole ($C_3H_4N_2$); OEC, octaethylchlorin; PP, protoporphyrin; THF, tetrahydrofuran; 2-MeIm, 2-methylimidazole.

EDTA, 15 mM benzamidine, and 1 mM phenylmethanesulfonylfluoride (PMSF). The suspension is then passed through a Microfluidizer (Microfluidics) 6 times to ensure thorough breakage of cells. Unbroken cells are pelleted by centrifugation for 20 min at 10000*g*. The supernatant is then centrifuged at 200 000*g* for 2 h to pellet membranes. Subsequent membrane solubilization and fractionation of protein on DEAE and hydroxyapatite columns are as described by Miller and Gennis (1986), except for the DEAE column, which had a slower flow rate of 75 mL/h and a slightly larger elution gradient volume of 2 L. The "as-isolated" enzyme samples were stored under liquid nitrogen until their use.

To prepare ^{54}Fe-labeled enzyme, 1 mL of *E.* coli culture was used to inoculate 100 mL of minimal medium containing 6 mg/L $^{54}FeSO_4$ (made from 99.8% ^{54}Fe metal and H_2SO_4). After 12 h growth, 10 mL each was withdrawn to further inoculate 8 1-L cultures of ^{54}Fe minimal medium/ampicillin. The same enzyme purification procedures were followed. Typical enzyme concentrations for ^{NA}Fe preparations were 24.3 μM in a pH 7.5 buffer solution of 50 mM sodium phosphate with 0.02% or 0.05% of *N*-lauroylsarcosine (sodium salt). For the ^{54}Fe preparation, the enzyme concentration was 100 μM in a pH 7.5 buffer solution of 20 mM sodium phosphate with 0.02% or 0.05% of *N*-lauroylsarcosine (sodium salt). Samples for rR studies were used directly or, in some cases, further concentrated to 100−200 μM through ultracentrifugation (Centricon). The "as-isolated" enzyme samples were reduced by addition of a minimum amount of freshly prepared 20 mM sodium dithionite solution (in 20 mM phosphate buffer, pH 7.5) after purging the enzyme with Ar for 5−10 min. The reduction was monitored by UV/vis spectroscopy. Samples with cyanide were prepared by adding appropriate amounts from a 100 mM KCN anaerobic stock solution to the reduced enzyme. Fully oxidized (triferric) enzyme was prepared for EPR studies from the fully reduced form by reaction with $Fe(CN)_6^{3-}$ in the presence of mediator dyes (10 nM each of phenazine methosulfate (80 mV), ferricenium hexasulfate (380 mV), hydroxyethyl ferrocene (402 mV), and 1,4-napthoquinone (60 mV)), followed by G-25 chromatography to remove excess reagents.

Resonance Raman spectra were obtained on a custom spectrometer consisting of a McPherson (Acton, MA) Model 2061/207 single monochromator operated at a focal length of 0.67 m and a Princeton Instruments (Trenton, NJ) LN1100 CCD detector with a Model ST-130 controller. Rayleigh scattering was attenuated by use of Kaiser Optical (Ann Arbor, MI) notch or super-notch filters. For some experiments, a DILOR (Instruments SA, Edison, NJ) Z-24 scanning Raman spectrophotometer was used. Excitation sources consisted of Coherent (Santa Clara, CA) Innova 90-6 Ar (457.9, 488.0, and 514.5 nm) and Innova 302 Kr (413.1 and 647.1 nm) lasers. In earlier studies, a Spectra-Physics (Mt. View, CA) 2025-11 Kr laser was used. A Liconix 4240NB He/Cd laser was the source of the 441.6-nm line. Excitations at 562.0, 595.0, and 629.0 nm were from an Ar laser pumped Coherent 599-01 dye laser using rhodamine 6G. All laser lines were filtered through Applied Photophysics (Leatherhead, U.K.) optical glass or quartz prism monochromators to remove plasma emissions. Incident powers were ~20 mW (413.1 and 441.6 nm), ~40 mW (457.9, 488.0, and 514.5 nm), and ~60 mW for longer wavelengths (562−647.1 nm). Spectra were collected in a 90°-scattering geometry from solution samples contained in glass capillary tubes in contact with a copper cold-finger immersed in an ice−water bath, although some spectra were recorded at room temperature. Spectral resolution was ~5.0 cm^{-1}. CCl_4 was used as a standard for polarization. Indene was used as the calibrant for the frequency shifts with the CCD spectrograph, whereas CCl_4 was used for this purpose on the DILOR spectrophotometer. Optical absorption spectra of the Raman samples were obtained on a Perkin-Elmer Lambda 9 spectrophotometer set to a spectral band width of 2 nm. The sample capillary tubes were supported and masked by a black Delrin cell holder (Loehr & Sanders-Loehr, 1993).

X-band EPR spectra were obtained with a Bruker ER 300 spectrometer equipped with an ER035M NMR gaussmeter and a Hewlett-Packard 5352B microwave frequency counter. Low temperatures were maintained using an Oxford Instruments continuous-flow liquid helium cryostat. All samples were prepared anaerobically unless otherwise stated. A titration tonometer attached to a quartz optical cell was used in conjunction with UV/vis spectrophotometry to prepare all adducts of the protein. Samples of the various adducts were removed from the titration cell using a gas-tight syringe and added to anaerobic EPR tubes that had been flushed with Ar for at least 15 min. The samples were then immediately frozen in liquid nitrogen.

RESULTS AND DISCUSSION

(A) Selective rR Enhancement of Heme Cofactors. (1) Porphyrin Skeletal Modes. The fully reduced cytochrome *bd* oxidase has a Soret absorption peak at 429 nm, a slight shoulder at 436 nm, and Q-bands at 530, 558, 595, and 629 nm (Figure 1). Raman scattering from a particular chromophore is maximally enhanced when the excitation wavelength matches its absorption energy. The most intense bands in heme rR spectra are the in-plane porphyrin skeletal vibrational modes with frequencies >1200 cm^{-1} which include many reliable markers for spin, ligation, and oxidation states of the heme iron (Wang & van Wart, 1993; Spiro, 1988, 1983). Resonance Raman spectra of the fully reduced enzyme obtained with laser lines covering the whole electronic absorption range are shown in Figure 2. Frequencies and proposed assignments for all three cofactors are given in Table 1.

(a) Soret Excitation. As shown in Figure 2, excitation at 413.1 nm yields a rR spectrum dominated by ν_4 at 1360 cm^{-1} (p).[1] This frequency and its high intensity are indicative of ferrous *b* hemes, as expected for this chemically reduced enzyme system. Most of the other rR bands obtained with Soret excitation can also be assigned to the porphyrin skeletal modes of a 5cHS ferrous *b* heme. For example, the band at 1474 cm^{-1} (p) is characteristic of ν_3 whereas bands at 1391 (dp), 1556 (p), and 1603 cm^{-1} (dp) are appropriate for ν_{29}, ν_2, and ν_{10}, respectively, of a 5cHS ferrous *b* heme (Wang & van Wart, 1993; Spiro, 1988, 1983) and, hence, must arise from b_{595}^{2+}. However, the weaker band at 1493 cm^{-1} (p) is probably ν_3 of a 6cLS ferrous heme, i.e., b_{558}^{2+}. The peak at 1581 cm^{-1} (p) is very likely a mixture of ν_2 of b_{558}^{2+} and ν_{37} of b_{595}^{2+}. Similarly, the bands at 1428 (dp) and 1617 cm^{-1} (p) are assigned to vinyl modes of these two ferrous *b* hemes.

With longer excitation wavelengths (near-Soret: 441.6 and 457.9 nm), the rR spectra are quite similar to that obtained

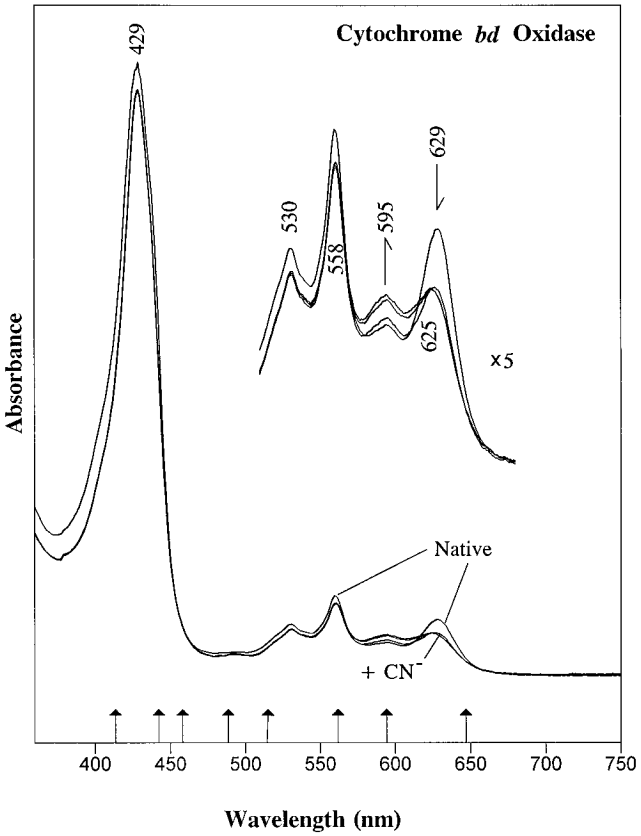

FIGURE 1: UV/vis spectra of the fully reduced cytochrome *bd* oxidase in the presence of ~700-fold (35 mM) cyanide. Time intervals between recordings are as follows: (i) before cyanide addition (scaled to the dilution factor), (ii) immediately after addition, and (iii) 5 min and (iv) 15 min after addition. The arrows along the abscissa indicate the laser wavelengths used for the resonance Raman spectra.

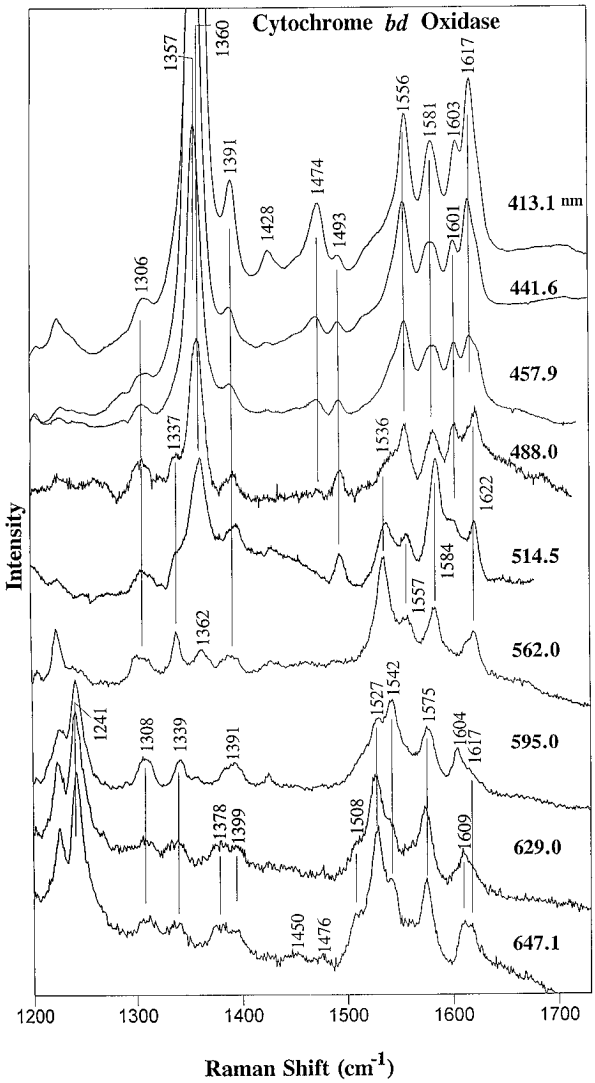

FIGURE 2: Resonance Raman spectra of the fully reduced cytochrome *bd* oxidase. The spectra obtained with 413.1-, 441.6-, 457.9-, 488.0-, and 514.5-nm excitation are from the CCD spectrometer (~100 μM enzyme). Laser power at the sample is 20, 15, 40, 35, and 45 mW, respectively. The spectra obtained at 562.0, 595.0, 629.0, and 647.1 nm were recorded with the DILOR Z-24 Raman spectrophotometer (~100 μM enzyme). Laser power is ~50−60 mW.

with 413.1 nm; however, relative intensities of Raman bands change as the relative contributions of the low-spin and high-spin *b* hemes change. The most prominent band is now at 1357 cm^{-1} (p), assigned to the ν_4 mode of b_{558}^{2+}. The 1581-cm^{-1} band in these two spectra is probably more characteristic of ν_2 of b_{558}^{2+}, compared with the same band in the 413.1-nm spectrum. Yet, these two spectra indicate a mixture of ferrous b_{595} and ferrous b_{558} species as revealed by the coexistence of intensities at 1474/1494 cm^{-1} (ν_3) and 1556/1581 cm^{-1} (ν_2). The intensity changes observed in the rR data presented here suggest that the 429-nm UV/vis absorption is chiefly due to b_{595}^{2+}, whereas b_{558}^{2+} contributes predominately to the shoulder at 436 nm.

The contribution of chlorin *d* to the Soret-excited rR spectra is believed to be negligible, based on the following observations. First, no multiplet structure is seen on the ν_4 bands at 1360 or 1357 cm^{-1}. Chlorin compounds show a generally richer Raman spectrum, including apparent splittings of ν_4 as the result of the lowered symmetry of the hydroporphyrin macrocycle (Mylrajan et al., 1995; Ozaki et al., 1986). Second, the ν_2- and ν_{10}-equivalent frequencies of d^{2+} are determined to be at 1575 and 1609 cm^{-1} (see "(c) Red Excitation"); there is no hint of these bands with Soret excitation (413.1−457.9 nm).

(b) Q-Band Excitation. Whereas Soret-excited rR spectra as shown above are dominated by totally symmetric porphyrin modes (ν_2, ν_3, and ν_4), their intensities decrease with Q-excitation to yield a new set of porphyrin skeletal bands (ν_{10}, ν_{11}, and ν_{19}) for *b* hemes (Wang & van Wart, 1993; Spiro, 1988, 1983). The rR spectra obtained at 488.0, 514.5, and 562.0 nm show some new features not yet described. Most notable is a new band at 1622 cm^{-1} (dp), already apparent as a shoulder in the 457.9-nm spectrum, and is assigned to ν_{10} of b_{558}^{2+}. We assign the band at 1584 cm^{-1} (514.5- and 562.0-nm spectra) to ν_{19} of b_{558}^{2+} based on its frequency and intensity pattern. Although this band is close to that of ν_2 (1581 cm^{-1} with Soret excitation), one does not expect to observe ν_2 with Q-band excitation. As further proof, a depolarization ratio analysis using 562.0-nm excitation showed that the 1584-cm^{-1} band is inversely polarized, a necessary requirement for ν_{19}. In the 488.0-nm spectrum, the peak at this position may be an overlap of ν_2 and ν_{19} of b_{558}^{2+}. In addition to the bands at 1622 (dp) and 1584 cm^{-1} (ap), two other intense bands are noted at 1557 (dp) and 1536 cm^{-1} (dp). The 1557-cm^{-1} band may be distinguished from ν_2 of b_{595}^{2+} at 1556 cm^{-1} (Figure 2, 413.1 nm) because the latter is polarized. Although the bands at 1557 and 1536 cm^{-1} are both good candidates for the ν_{11} mode of b_{558}^{2+},

Table 1: Raman Frequencies (cm^{-1}) of Ferrous b_{595}, Ferrous b_{558}, and Ferrous *d* in the Fully Reduced Cytochrome *bd* Oxidase[a]

compounds	ligand(s)	ν_4	ν_3	ν_{11}	ν_{19}	ν_2	ν_{10}	refs
b_{595} of cyt bd	**His**	**1360**	**1474**	**1542**	**nd**[c]	**1556**	**1602**	**this work**
b_{558} of cyt bd	**His/Met**	**1357**	**1493**	**1558**	**1584**	**1581**	**1622**	**this work**
b_{562}	His/Met	1362	1493	1548	~1588	~1586	1621	Bullock & Myer, 1978; Kitagawa et al., 1975
cytochrome *c*	His/Met	1364	1496	1551	1587	*b*	1626	Hu et al., 1993
cytochrome b_5	His/His	1364	1494	1538	1587	nd	1619	Adar & Erecinska, 1974
$(PP)Fe^{II(Im)}_2$	His/His	1359	1493	1539	1583	1584	1617	Choi et al., 1982
alkaline cyt *c*	His/Lys	1359	1489	1533	1583	nd	nd	Kitagawa et al., 1975
turnip cyt *f*	His/H_2N_{term}[d]	1361	1490	1532	1581	nd	~1616	Davis et al., 1988
HRP/cyanide	His/CN^-	1362	1494	1564	1584	1584	1625	Palaniappan & Terner, 1989
d of cyt bd	**?**	**nd**	**1475**	**1527**	**nd**	**1575**	**1609**	**this work**

[a] The frequencies of b_{558} are compared with other 6cLS ferrous *b* heme model compounds and proteins. Raman frequencies of 5cHS ferrous *b* hemes and 5cHS ferrous chlorins have been compiled previously (Sun et al., 1993, 1995). [b] The frequency is quite different due to the presence of thioether groups instead of vinyl groups. [c] n.d. = not determined or not observed. [d] Martinez et al., 1994.

we prefer to assign the 1557-cm^{-1} band to ν_{11}. The 1536-cm^{-1} band is also depolarized, but its frequency is too far from ν_{11} values assigned for *E. coli* b_{562}^{2+} (1548 cm^{-1}; Bullock & Myer, 1978; Kitagawa et al., 1975) and cytochrome c^{2+} (1551 cm^{-1}; Hu et al., 1993), both of which are known to have His/Met axial ligation. Hence, we assign the 1536-cm^{-1} band to ν_{38} of b_{558}^{2+}. By comparison, the ν_{38} mode of the cyanide adduct of ferrous horseradish peroxidase has been reported at 1541 cm^{-1} (Palaniappan & Terner, 1989).

(c) Red Excitation. Upon further decreases in the excitation energy (595.0−647.1 nm), the chlorin *d* bands become dominant as characterized by a strong band at ~1241 cm^{-1}. This polarized feature arises from a porphyrin-equivalent E_u mode (ν_{42A}). Andersson et al. (1985) have shown that this mode is strongly enhanced in deuterochlorins with Q-band excitation, although it is forbidden for *b* hemes. Its appearance thus signals the preferential rR enhancement of the reduced symmetry chlorin cofactor. With 595.0-nm excitation, some ferrous b_{595} bands are still observed. For example, the peak at 1542 cm^{-1} is depolarized and is assigned to the ν_{11} mode of b_{595}^{2+}, whereas the band at the same frequency observed in the 629.0-nm spectrum belongs to the chlorin since it is polarized. The band at 1604 cm^{-1} in the 595.0-nm spectrum is probably a mixture of ν_{10} of b_{595}^{2+} (1602 cm^{-1}) and the chlorin's ν_{10}-equivalent band at 1609 cm^{-1}. With deeper red excitation at 629.0 and 647.1 nm, all the observed bands can be attributed to the chlorin, because all are polarized with the exception of the band at 1308 cm^{-1} (dp). All A_{1g} and B_{1g} porphyrin modes (ν_1 to ν_{18}) become polarized in chlorins owing to their reduced symmetry. In contrast, Q-band excitation of *b* hemes and metalloporphyrins usually gives rise only to depolarized and anomalously polarized bands. Here, the band at 1609 cm^{-1} can be assigned as the ν_{10}-like mode of a 5cHS ferrous chlorin. We prefer to assign the 1609- rather than the 1617-cm^{-1} band to "ν_{10}" because the former is a more satisfactory value for a 5cHS species (Mylrajan et al., 1995). If the chlorin cofactor were a 6cLS or a 4cIS ferrous species, "ν_{10}" would have been seen at ~1629 cm^{-1} (Mylrajan et al., 1995). The 1617-cm^{-1} band may be assigned to the $\nu(C{=}C)_{vinyl}$ mode of chlorin *d* (Sotiriou & Chang, 1988). Although vinyl modes are *not* strongly enhanced by Q-excitation for *b* hemes, rR spectra of vinyl-bearing chlorins have not been explored systematically with red excitation.

Model studies greatly assist in the identification of the coordination state of the chlorin cofactor. Kitagawa and coworkers (Ozaki et al., 1986) have assigned frequencies of the pentacoordinate Fe^{II}(OEC)(2-MeIm)[1] complex using 488.0-nm excitation as follows: 1365 cm^{-1} (ν_4), 1477 (ν_3), 1535 (ν_{11}), 1559 (ν_{19}), 1579 (ν_2), and 1608 cm^{-1} (ν_{10}). We have studied this same compound with red excitation (612 nm) and located the following frequencies (with proposed porphyrin-equivalent assignments): 1366 (p, ν_4), 1394 (p), [1474 (p, ν_3) is only observed with Soret excitation], 1534 (p, ν_{11}), 1558 (dp, ν_{19}), 1579 (p, ν_2), and 1606 cm^{-1} (p, ν_{10}) [J. Sun, C. K. Chang, and T. M. Loehr, unpublished observations; data for Fe(OEC)(THF) and Fe(DC)(2-MeIm) are very similar]. Two interesting facts emerge from these studies. First, the intensity patterns of the rR spectra above 1200 cm^{-1} resemble those of the chlorin *d* spectra (Figure 2). In all cases, the most intense bands are those at ~1240 and ~1530 cm^{-1}. Second, red excitation of the chlorins appears to enhance the spin- and coordination-state sensitive marker bands that are familiar from the Soret and Q-excitation data of hemes. Thus, these model studies provide a good basis for the identification of the ferrous chlorin *d* as a 5cHS species (Table 1). The axial ligand of the reduced *d* heme could, thus, be an endogenous amino acid residue, or even a water molecule, which is lost or replaced in the oxygenated, ferryl−oxo, and oxidized forms of the chlorin since the rR spectra of those adducts are also most consistent with those of 5cHS chlorins (Sun et al., 1995).

To summarize, all of the high-frequency marker bands of the three ferrous cytochrome *bd* oxidase cofactors are collected in Table 1. The frequencies of b_{595}^{2+} fit those of well-characterized 5cHS ferrous hemes (Sun et al., 1993). Combining the results of mutagenesis and spectroscopic studies suggests that His 19 is probably the proximal ligand of b_{595}, as will be supported further by direct rR evidence from the low-frequency region described below. The frequencies of b_{558}^{2+} are compared with other 6cLS ferrous hemes having known axial ligands, and these provide compelling support for the presence of a 6cLS *b* heme in the fully reduced cytochrome *bd* oxidase. Finally, the frequencies of the chlorin moiety indicate that it exists as a 5cHS ferrous *d* species (Sun et al., 1995).

(2) Axial-Ligand Stretching-Frequency Region. The low-frequency regions (180−450 cm^{-1}) of the rR spectra of the fully reduced cytochrome *bd* oxidase and those of the ^{54}Fe-labeled enzyme obtained at two different excitation lines are shown in Figure 3. In the 441.6-nm spectrum, the low-frequency modes are strongly enhanced. Here, we have obtained high-quality (unsmoothed) data from the spectrograph with its CCD detector. We assign the band at 225 cm^{-1} (p) to the (b_{595}^{2+})Fe−N(His) stretching mode on the

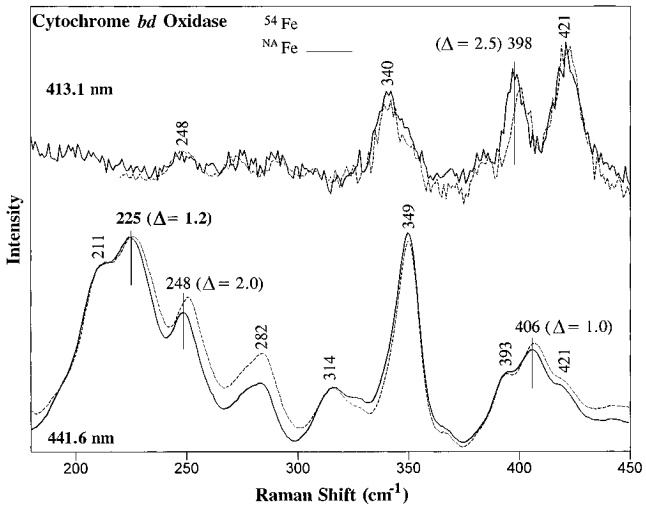

FIGURE 3: Low-frequency region of the resonance Raman spectra of the fully reduced cytochrome *bd* oxidase (solid lines: ^{NA}Fe, and dashed lines: ^{54}Fe). The spectrum with 413.1 nm (25 mW) was collected on the DILOR Z-24 Raman spectrophotometer (~200 μM oxidase); the 441.6-nm (15 mW) spectrum was collected with the CCD Raman spectrograph (~100 μM).

basis of its 1.2-cm^{-1} *up*shift in the ^{54}Fe-labeled enzyme. For histidine-bound, 5cHS ferrous *b* hemes, the Fe^{II}−N(His) stretching mode is known to be maximally enhanced with excitations at ~436 nm (Wells et al., 1991). Furthermore, the only ^{54}Fe-sensitive band in the frequency range ~200−245 cm^{-1} thus far discovered in 5cHS ferrous *b* hemes is the Fe^{II}−N(His) stretching mode. Although bands at similar frequency have been found for 6cLS ferrous protoporphyrin model compounds (237 cm^{-1} for Fe^{II}(PP)(ImH)$_2$ and 228 cm^{-1} for [Fe^{II}(PP)(CN)$_2$]$^{2-}$), these were shown not to be ^{54}Fe sensitive (Choi & Spiro, 1983).

Because the high-frequency region of the 441.6-nm rR spectrum (Figure 2) indicated a mixture of 5cHS b_{595}^{2+} and 6cLS b_{558}^{2+} bands, the accompanying low-frequency region (Figure 3) is also expected to show bands from both of these chromophores. However, iron isotope-sensitive Fe−N(His) modes are not documented for six-coordinate species. With 457.9-nm excitation, a +1.2-cm^{-1} ^{54}Fe/^{NA}Fe shift was also detected at 225 cm^{-1} (data not shown). We are thus very confident that the band at 225 cm^{-1} is the (b_{595}^{2+})Fe−N(His) stretching mode, even though it may have a contribution from a porphyrin band of b_{558}^{2+} at this frequency. The identification of a His ligand in b_{595} is entirely consistent with mutagenesis, where the H19L product was shown to contain only b_{558} and a residual amount of chlorin *d*, but no heme b_{595} (Fang et al., 1989).

The magnitude of the ^{54}Fe/^{NA}Fe isotope shift can be estimated from two different models proposed to account for the Fe−N(His) vibration in myoglobin (Wells et al., 1991). The first is described by an imidazole−iron(porphyrin) oscillator with "effective masses" of 88 and 92 amu, respectively. The second treats FeN_{Im} (70 amu) moving as a unit against the remainder of the imidazole ring (54 amu). The ^{54}Fe/^{NA}Fe isotopic shifts calculated from these two models are 1.1 and 1.3 cm^{-1}, respectively, values which exactly bracket the observed value. In ferrous myoglobin, the Fe−N(His) stretching vibration is seen at 220 cm^{-1} (Kitagawa et al., 1979), and this proximal histidine ligand is weakly hydrogen bonded to the protein backbone. The higher frequency of 225 cm^{-1} for b_{595} in cytochrome *bd* oxidase implies that its proximal histidine is more strongly H-bonded. By comparison, the Fe−N(His) stretching frequencies for strongly H-bonded and fully ionized imidazoles of histidine are at ~235 and ~245 cm^{-1}, respectively (Stein et al., 1980; Smulevich et al., 1988).

A second ^{54}Fe-sensitive band in the 441.6-nm spectrum is located at 248 cm^{-1} (p) (Figure 3). This band must be excluded from consideration as the (b_{595}^{2+})Fe−N(His) stretching mode based on the following arguments: (i) The frequency is somewhat too high for an Fe−N(His) stretching vibration. (ii) The ^{54}Fe-isotope shifts estimated from the two models described above are 1.2 and 1.45 cm^{-1}, respectively; these values are smaller than the observed value of 2.0 cm^{-1}. (iii) An iron-sensitive pyrrole-tilting mode is expected at this frequency for both b_{595} and b_{558}; ^{54}Fe/^{NA}Fe isotope shifts of 2−3 cm^{-1} have been observed in model compound studies (Choi & Spiro, 1983).

The 413.1-nm data (Figure 3) are suspected to be largely due to b_{595}^{2+} since the corresponding high-frequency region (Figure 2) shows predominately rR bands of the 5cHS ferrous *b* heme. Here, the band at 398 cm^{-1} shifts up ~2.5 cm^{-1} with ^{54}Fe substitution and may be assigned to an out-of-plane mode corresponding to an ^{54}Fe-sensitive band of deoxymyoglobin at 373 cm^{-1} (Choi & Spiro, 1983). (Apparently, a residual of the iron-sensitive 398-cm^{-1} band may still be observed at 406 cm^{-1} in the 441.6-nm excitation spectrum.) Although the frequencies of these peaks in myoglobin and cytochrome *bd* oxidase differ, out-of-plane vibrations are more influenced by the protein environment than the in-plane modes. For example, another strong b_{595} out-of-plane band at 421 cm^{-1} (λ_0 = 413.1 nm) is also at a higher frequency than that of the corresponding myoglobin band at 408 cm^{-1} assigned to pyrrole folding. The higher frequencies of the out-of-plane modes in the oxidase may be related to the unique, O_2-activating environment of the b_{595}/*d* binuclear center.

(B) Reactions with Cyanide. (1) UV/Vis Changes. Upon addition of a large excess of cyanide (~35 mM) to the fully reduced enzyme, the UV/vis absorption band at 629 nm decreases in intensity and is blue-shifted to 625 nm (Figure 1). The band at 595 nm shows a small intensity increase. These changes are complete within 5 min, much faster than the reaction between cyanide and the "as-isolated" cytochrome *bd* oxidase (Sun et al., 1995). Other UV/vis spectral bands show only insignificant changes in intensity and energy. Interestingly, the notable decrease of the 629-nm band of the reduced oxidase is similar to the loss in intensity of the 645-nm peak of the "as-isolated" form when treated with CN^-. In the latter case, the spectral change was attributed to the displacement of the bound O_2 by CN^- (Sun et al., 1995). The parallel behavior in the reduced form of the oxidase suggests displacement of an axial ligand from the chlorin *d* cofactor by cyanide.

(2) Resonance Raman Spectra. Resonance Raman spectra of the fully reduced cytochrome *bd* oxidase with and without CN^- excited at 413.1 nm (giving maximal enhancement of cytochrome b_{595}), 514.5 nm (maximizing the contribution from b_{558}), and 647.1 nm (cytochrome *d*) are shown in Figure 4. Remarkably, *addition of cyanide causes no significant changes in their rR spectra*. The lack of any changes in the 413.1- and 514.5-nm excited rR spectra is consistent with a similar insensitivity to cyanide as shown in the UV/vis spectra (Figure 1). In our earlier study of the "as-isolated"

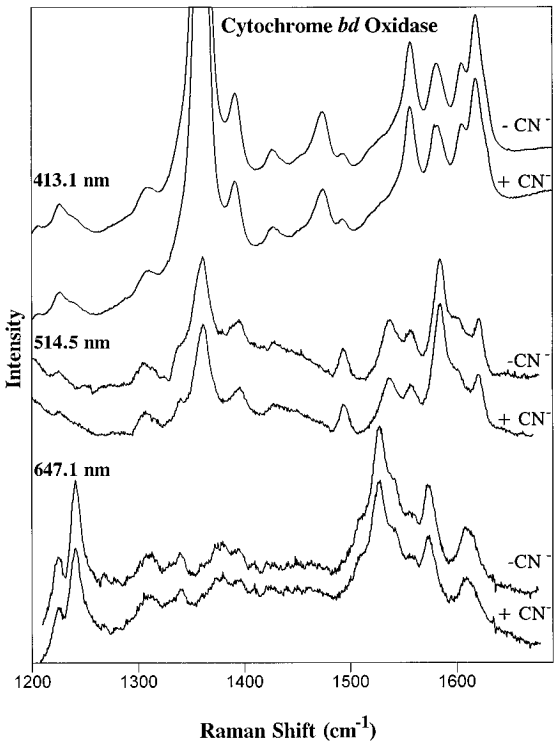

FIGURE 4: Resonance Raman spectra of the fully reduced cytochrome *bd* oxidase in the absence (~100 μM) and presence (~50 μM) of ~700-fold (35 mM) cyanide. The spectra were collected with the CCD Raman spectrograph. 413.1 nm (20 mW), 514.5 nm (45 mW), and 647.1 nm (60 mW) excitation wavelengths. Frequencies of individual Raman peaks can be obtained from Figure 2.

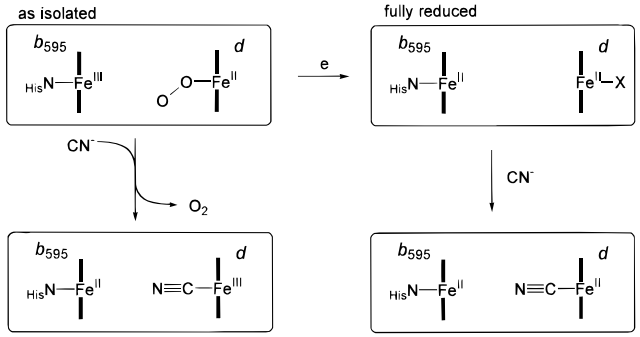

FIGURE 5: Proposed model of the reaction of cyanide with the "as-isolated" and fully reduced cytochrome *bd* oxidase. The heme b_{558} cofactor, which has a His/Met axial ligation and is not affected by cyanide, is not shown. The side of attachment of cyanide or X has not been established.

enzyme, we noted that ferric b_{595} is partially autoreduced on addition of CN^-, but that neither the remaining ferric nor the autoreduced b_{595}^{2+} bind CN^- because no hexacoordinate species are formed (Sun et al., 1995). In the present case, it is also evident that the reduced b_{595}^{2+} has an extremely low affinity for cyanide. Although the d^{2+} heme is perturbed in the presence of CN^- as seen in the absorption spectrum, it also *does not appear to bind* cyanide directly because it does not form a hexacoordinate species. The chlorin *d* rR spectrum maintains the character of a 5cHS species, and no new 6cLS chlorin bands emerge in the 647.1-nm spectrum (Figure 4). In parallel to the behavior of the "as-isolated" cytochrome *bd* oxidase reported by us previously (Sun et al., 1995), we propose that the native proximal d^{2+} ligand (X in Figure 5) dissociates and is replaced by cyanide. A scheme of the proposed axial ligands of b_{595} and *d* that is consistent with the spectroscopic data is shown in Figure 5. Attempts to verify cyanide ligation to chlorin d^{2+} by direct observation of rR Fe−CN stretching and bending modes with $^{12}CN^-$ and $^{13}CN^-$ (λ_0 = 630 nm) were unsuccessful in our hands. We suspect that the extent of resonance enhancement of these Fe−CN modes with red light is too low to be observable; even Soret-excited Fe−CN vibrational intensities are extremely low in adducts of catalases and cytochrome P450 (Simianu & Kincaid, 1995; Al-Mustafa et al., 1995). However, in a recent FTIR study of the *E. coli* cytochrome *bd* oxidase, ν(CN) modes were reported at 2161 and 2138 cm^{-1} (Tsubaki et al., 1995), thus verifying the formation of a $d{\cdot}CN^-$ complex. While these authors suggest that the 2161-cm^{-1} peak represents a bridged CN^- ($b_{595}^{3+}-NC-d^{3+}$) in a binuclear center, the NO and CN^- binding studies on the *A. vinelandii* cytochrome *bd* oxidase showed that cooperative interactions between these metal centers were weak and, hence, argued *against* the presence of a binuclear center and would call a bridging structure into question (Jünemann & Wrigglesworth, 1995). On the other hand, the photodissociation studies of D'mello et al. (1994) support a binuclear site.

However, our observed autoreduction of b_{595}^{3+} by CN^- from the "as-isolated" level and the lack of rR evidence for a new hexacoordinate b_{595} species (Sun et al., 1995) do not appear to be consistent with a bridging cyanide system. In this view, would an alternate interpretation be possible for the FTIR results of Tsubaki et al. (1995)? Addition of CN^- to the CO saturated system, $b_{595}^{3+}/d^{2+}{\cdot}CO$, could give $b_{595}^{2+}/d^{3+}{\cdot}CN^-$, with the cyano−chlorin complex exhibiting ν(CN) at 2161 cm^{-1}. The ferric−chlorin cyano complex lacking a sixth ligand could have a higher frequency than, for example, a six-coordinate system like horseradish peroxidase with (His)N−Fe−CN ligation and a ν(CN) of 2131 cm^{-1} (Yoshikawa et al., 1985). Subsequent reduction of the $b_{595}^{2+}/d^{3+}{\cdot}CN^-$ complex would be expected to give initially some $b_{595}^{2+}/d^{2+}{\cdot}CN^-$, and hence a second ν(CN) may be observed at 2138 cm^{-1} from the ferrous−chlorin cyano complex, while some of the d^{2+} is recombining with CO to regenerate ν(CO) at 1981 cm^{-1}. With time, all of the CN^- will be displaced by the more stable $d^{2+}{\cdot}CO$ with its 1981-cm^{-1} peak. In all traces, the small shoulder at ~1975 cm^{-1} may represent a small population of $b_{595}^{2+}{\cdot}CO$, as had been shown in the flash studies of Hill et al. (1993). Many data seem to suggest that the two centers, b_{595} and *d*, are close, but whether they are within bridging distance of a diatomic ligand remains to be established through additional studies.

(3) EPR Spectra. The EPR spectrum for fully oxidized cytochrome *bd* oxidase at 5 K is shown in Figure 6 (solid line) and is identical to that previously reported for purified enzyme (Meinhardt et al., 1989). The strong positive feature at g = 6.0 is due to high-spin chlorin *d*, and the features at g = 2.5, 2.3, and 1.85 to $g_{z,y,z}$ of a minor low-spin form of this same site. The shoulders at g = 6.3 and 5.5 have been assigned previously to the g_z and g_y features of high-spin cytochrome b_{595}, which gives an EPR signal having an integrated signal intensity that is comparable to that for the chlorin *d*. Both high-spin hemes are presumed to give overlapping g_x features at g = 1.99. The only clearly assigned portion of the EPR signal of the low-spin cytochrome b_{558} is its g_z feature at g = 3.3. We find that addition of cyanide to the fully oxidized enzyme results in a perturbation and net increase in intensity in the g = 6 region

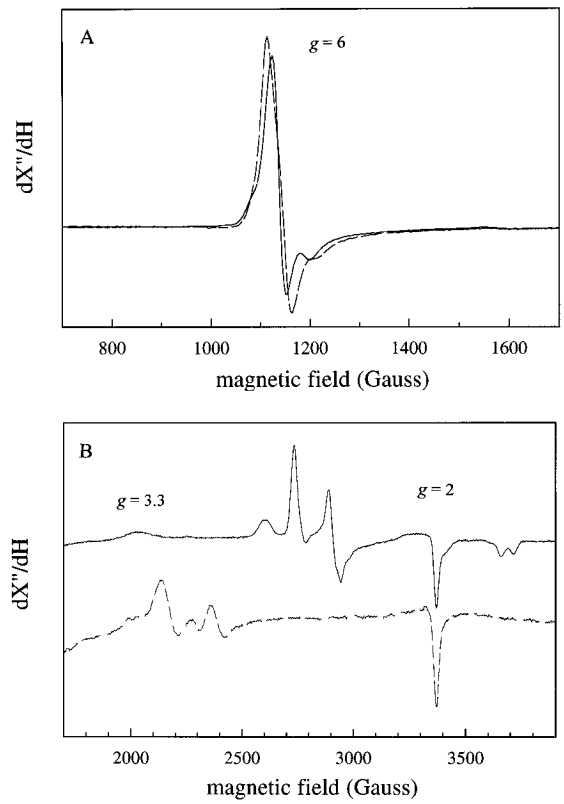

FIGURE 6: The EPR spectra of cytochrome *bd* oxidase in the absence (solid) and presence (dashed) of 50 mM potassium cyanide. Panel A, low-field region, and Panel B, high-field region (at 10× greater sensitivity). Instrument settings were 9.455 GHz microwave frequency, 11 mW microwave power, 7 G modulation amplitude, and 100 kHz modulation frequency with sample $T = 5$ K.

of the spectrum, a modest increase in the $g = 1.99$ feature of the high-spin heme(s), and loss of the features attributable to the low-spin component of chlorin *d* in the $g = 1.85-2.5$ region of the spectrum (Figure 6, dashed line). It is clear from these results that cyanide binding to the chlorin *d* results in a shift in the spin-state equilibrium of this site *toward high-spin*. It should be noted that the features of both chlorin *d* and heme b_{595} in the $g = 6$ region are perturbed when cyanide binds to *d*, suggesting that the two sites are in close proximity to one another. In addition to these spectral changes in the high-field region of the EPR spectrum, weak features become more evident in the $g = 3.2-2.8$ region. Although this signal has been previously assigned to a low-spin chlorin d^{3+}·CN complex, we consider it most likely that these features arise from heme $b_{558}{}^{3+}$(see below). It is evident from the spectra shown in Figure 6 that if the former interpretation were correct, the low-spin chlorin must be a very minor species: cyanide-complexed chlorin *d* is high-spin to an even greater extent than is the uncomplexed center.

The above interpretation contrasts with the conclusions of cyanide binding studies with membrane-bound cytochrome *bd* oxidase (Rothery & Ingledew, 1989) and with the more recent results of both Jünemann and Wrigglesworth (1995) and Tsubaki et al. (1995) using the purified enzyme. Working with the closely related cytochrome *bd* oxidase from *A. vinelandii*, Jünemann and Wrigglesworth (1995) found that binding of cyanide to the "as-isolated" enzyme results in a slight decrease in signal intensity in the $g \approx 6$ region and appearance of a signal in the high-field region of the spectrum ($g \approx 2.97$) that qualitatively resembles that seen in the present work. The high-field region of the EPR spectra of the "as-isolated" *A. vinelandii* oxidase gives no evidence for a low-spin population. In this experiment, loss of intensity in the high-spin EPR signal presumably is due to the autoreduction of heme b_{595} on addition of cyanide, i.e., displacement of O_2 from d·O_2 (d_{650}), present in the "as-isolated" enzyme, with subsequent electron transfer from chlorin d^{2+} to heme $b_{595}{}^{3+}$, as documented by Sun et al. (1995). It has been suggested that the $g \approx 2.97$ signal is due to a low-spin chlorin d^{3+}·CN complex (Rothery & Ingledew, 1989; Jünemann & Wrigglesworth, 1995). Such an assignment is not consistent with the present observation of a net increase in intensity of the high-spin EPR signal of the oxidase upon cyanide binding (at the expense of the unliganded d^{3+} signal), or with the observation that the intensity of the $g \approx 2.97$ signal is significantly reduced when cyanide binds to the one-electron-reduced enzyme, conditions under which the d^{3+}·CN complex is fully formed as shown in the near-IR spectrum (J. J. Hill, R. J. Rohlfs, C. F. Hemann, R. Hille, and R. B. Gennis, manuscript in preparation). Instead, it appears likely that the signal arises from the 6cLS $b_{558}{}^{3+}$ rather than a low-spin cyanide complex of chlorin *d*. In the work of Tsubaki et al. (1995) with the *E. coli* cytochrome *bd* oxidase, loss of the low-spin component of the chlorin d^{3+} EPR signal is observed upon cyanide binding and is consistent with the present results. The features in the $g = 3.2-2.8$ region do not appear immediately upon addition of cyanide (presumably due to extensive reduction of heme b_{558} upon binding cyanide to the chlorin *d*), but grow in over a period of days as the enzyme slowly reoxidizes. Again, this latter signal was ascribed to a low-spin d^{3+}·CN complex and is inconsistent with the present results. In both of these recent studies, the cyanide-induced increase in integrated spin intensity of the high-spin component of cytochrome *bd* oxidase is masked by the reduction of heme b_{595} that occurs upon CN^- displacement of O_2 from the d·O_2 complex when "as-isolated" rather than fully oxidized enzyme is used.

CONCLUSIONS

Our rR results establish that the *fully reduced* cytochrome *bd* oxidase contains a 6cLS ferrous b_{558}, a 5cHS ferrous b_{595}, and a 5cHS ferrous chlorin. Identification of an $Fe^{II}-N$(His) stretching vibration at 225 cm^{-1} provides the first physical evidence that the proximal ligand of b_{595} is indeed a histidine. This band shifts by $+1.2$ cm^{-1} in the ^{54}Fe-labeled enzyme. Given that the His19Leu mutation caused the loss of the b_{595} heme and lowered the binding affinity for the *d* heme at the binuclear site, we conclude that the proximal ligand of b_{595} is His 19. The rR frequencies of ferrous b_{558} are consistent with a His/Met axial ligation (H186 and M393). The chlorin *d* cofactor in the reduced cytochrome *bd* oxidase is also in a 5cHS state; however, the identity of its axial ligand X remains unknown. It may be an endogenous amino acid residue or a water molecule. This fifth ligand apparently dissociates from heme *d* upon O_2 binding to form the oxygenated d_{650} species and remains off in the subsequent formation of the ferryl−oxo d_{680} state. If X is an endogenous protein donor, then it is highly unlikely to be a histidine; His was ruled out by both mutagenesis (Fang et al., 1989) and ENDOR studies (Jiang et al., 1993). In fact, the ENDOR study eliminated any strongly bound nitrogenous ligand. There is no spectral evidence that X could be a cysteine since no P450- or P420-like behavior has ever been documented.

Tyrosinate (as in catalase) would not be a good ligand for an O_2-binding Fe(II) center. These considerations reduce the list of possible protein ligands to methionine, carboxylates, alcohols, or peptide carbonyls. However, as mentioned above, a water molecule would satisfy the available spectral criteria.

Addition of cyanide to the fully reduced enzyme causes no structural changes in either the b_{558} or b_{595} site. The affinity of 5cHS ferrous b_{595} for cyanide is very low, and there is no spectroscopic evidence for cyanide binding. On the other hand, cyanide does perturb the 5cHS ferrous chlorin site as seen by UV/vis spectral changes. The resultant chlorin moiety still appears to exist as a 5cHS species, thus suggesting that the native chlorin d^{2+} ligand is replaced by a cyanide to form a pentacoordinate cyano−*d* adduct (Figure 5). The EPR results presented here clearly demonstrate that binding of CN^- to the *oxidized* chlorin *d* site also results in a *high-spin* chlorin species. The present paper augments our previous characterization of the "as-isolated" enzyme (Sun et al., 1995), and together with the new EPR data, both of these studies support the existence of high-spin cyano adducts of chlorin *d* in its oxidized and reduced states.

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