Resonance Raman Studies of *Escherichia coli* Cytochrome *bd* Oxidase. Selective Enhancement of the Three Heme Chromophores of the "As-Isolated" Enzyme and Characterization of the Cyanide Adduct†

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Received May 23, 1995⊗

ABSTRACT: Cytochrome *bd* oxidase is a terminal bacterial oxidase containing 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 at a dinuclear b_{595}/d site, whereas b_{558} is mainly involved in transferring electrons from ubiquinone. One of the unique functional features of this enzyme is its resistance to high concentrations of cyanide (K_i in the millimolar range). With the appropriate selection of laser lines, the ligation and spin states of the b_{558}, b_{595}, and *d* hemes can be probed selectively by resonance Raman (rR) spectroscopy. Wavelengths between 400 and 500 nm predominantly excite the rR spectra of the b_{558} and b_{595} chromophores. Spectra obtained within this interval show a mixed population of spin and ligation states arising from b_{558} and b_{595}, with the former more strongly enhanced at higher energy. Red excitation wavelengths (590−650 nm) generate rR spectra characteristic of chlorins, indicating the selective enhancement of the *d* heme. These rR results reveal that cytochrome *bd* oxidase "as isolated" contains the b_{558} heme in a six-coordinate low-spin ferric state, the b_{595} heme in a five-coordinate high-spin (5cHS) ferric state, and the *d* heme in a mixture of oxygenated ($Fe^{II}O_2 \leftrightarrow Fe^{III}O_2^-$; d_{650}) and ferryl-oxo ($Fe^{IV}=O$; d_{680}) states. However, the rR spectra of these two chlorin species indicate that they are both in the 5cHS state, suggesting that the *d* heme is lacking a strongly coordinated sixth ligand. Addition of inhibitory concentrations of CN^- (10−40 mM) to the as-isolated enzyme leads to loss of the d_{650} absorbance of the oxygenated species. Resonance Raman spectra of these cyanide-containing samples have been monitored by 413.1-, 514.5-, and 647.1-nm laser excitation. The data show that b_{558} experiences little or no change, whereas b_{595} is partially autoreduced to a 5cHS ferrous species. However, neither the remainder of the 5cHS ferric b_{595} nor the autoreduced b_{595} gives any indication of cyanide binding as judged by rR spectroscopy, even in the presence of 40 mM KCN. Loss of O_2 and electron transfer from the resultant heme d^{2+} to $b_{595}{}^{3+}$ would generate oxidized heme *d* and autoreduced b_{595}. We propose that the resultant heme *d* is present in a mixture of states, including residual oxygenated (Fe—O_2 stretch at 568 cm^{-1}) and ferryl-oxo (Fe=O stretch at 815 cm^{-1}) states as well as a cyanide-reacted, oxidized state. The combined spectra, including that of oxidized heme *d*, indicate the presence of predominantly high-spin, pentacoordinate species. The observation of a cyanide complex that is only five coordinate is consistent with the proposed absence or loss of an endogenous protein ligand to the chlorin cofactor.

Cytochrome *bd* oxidase is a bacterial terminal oxidase that catalyzes the four-electron reduction of dioxygen to water using ubiquinol as an electron donor (Anraku, 1988; Anraku & Gennis, 1987; Gennis, 1987; Kranz & Gennis, 1985; Poole, 1983). The enzyme is fundamentally different from the oxidase superfamily, containing cytochrome *c* oxidase or cytochrome *bo* oxidase; for example, it contains no additional cooper cofactors (Trumpower & Gennis, 1994). It catalyzes the transmembrane separation of protons and electrons, but does not function as a proton pump (Puustinen et al., 1991). 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 cofactor, chlorin *d* (Lorence et al., 1986). The heme b_{558} functions by transferring electrons from ubiquinol (Dueweke & Gennis, 1990, 1991). The b_{595} heme and chlorin *d* appear to be closely associated at the dioxygen reductase site (Hill et al., 1993; Meinhardt et al., 1989; Rothery & Ingledew, 1989; Hata-Tanaka et al., 1987; Hata et al., 1985). Cytochrome *bd* oxidase provides an interesting alternative to the oxidase superfamily for the study of terminal oxidase reaction mechanisms. Unexpectedly, cytochrome *bd* oxidase is highly resistant to cyanide.

Understanding of the structural composition of cytochrome *bd* oxidase is still very limited in comparison with the well-studied cytochrome *c* oxidase. The chlorin *d* is postulated

† This work was supported by grants from the U.S. Public Health Service, National Institutes of Health [GM34468 (T.M.L.) and HL16101 (R.B.G.)]. Instrumentation in the laser Raman spectroscopy laboratory at OGI was purchased with funds from shared instrumentation grants from the NIH (RR02676) and the National Science Foundation (BIR-9216592).

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⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1995.

0006-2960/95/0434-12144$09.00/0

to be a dihydroxyprotochlorin (Sotiriou & Chang, 1988; Andersson et al., 1987; Vavra et al., 1986; Timkovich et al., 1985), which is very similar to the prosthetic groups of *Escherichia coli* catalase HPII (Loewen et al., 1993; Chiu et al., 1989) and *Neurospora crassa* chlorin catalase (Andersson, 1989; Jacob & Orme-Johnson, 1979). However, the axial ligands of all heme cofactors in cytochrome *bd* oxidase are still uncertain. An oxygenated *d* chromophore 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(*d*)−O_2 vibrational stretching mode at 568 cm^{-1} and confirmed by $^{18}O_2$ substitution (Kahlow et al., 1993). Earlier, a ferryl-oxo species was identified in the *d* heme from the unique Fe^{IV}=O vibration and confirmed with $^{18}O_2$ and mixed-isotope $^{16}O/^{18}O$ labeling (Kahlow et al., 1991). More recently it has been proposed that the b_{595} and *d* hemes are in close proximity, constituting a dinuclear center as the O_2 reduction site (Hill et al., 1993), similar to the heme−copper center of the oxidase superfamily.

Cytochrome *bd* oxidase has an unusually low sensitivity to cyanide. Pudek and Bragg (1974), using an unpurified suspension of respiratory particles from *E. coli*, reported a K_i value of 7.8 mM. 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 even in the presence of 10 mM cyanide. In contrast, cytochrome *c* oxidase and many heme proteins are exceedingly sensitive to cyanide (Fabian & Palmer, 1995; Bosshard et al., 1991; Jones et al., 1984; Palcic et al., 1981). The interaction between cyanide and cytochrome *bd* oxidase has been studied by electronic spectroscopy. The decrease in the 645-nm peak attributed to the oxy-*d* species[1] in the native enzyme has been correlated with direct binding of cyanide at the *d* site (Kauffman & Van Gelder, 1974). However, absorbance and MCD red shifts in the Soret region have been interpreted as a high-spin to low-spin transition of the oxidized b_{595} (Krasnoselskaya et al., 1993). The resonance Raman (rR)[2] spectral data presented here are not consistent with CN^- ligation at b_{595} because we find it to be reduced and high spin, thereby favoring CN^- binding to heme *d*.

Resonance Raman spectroscopy offers a powerful structural probe in the study of heme proteins (Spiro, 1988). A further feature of rR spectroscopy is its ability to observe individual sites in a protein having multiple chromophores by using selective enhancement at different excitation wavelengths (Babcock et al., 1981). This behavior has allowed us to selectively monitor changes in the individual b_{558}, b_{595}, and *d* moieties of cytochrome *bd* oxidase in its "as-isolated" form and in the presence of exogenous ligands.

MATERIALS AND METHODS

The *E. coli* enzyme has been sequenced, cloned, and overexpressed (Fang et al., 1989; Green et al., 1984, 1988). Protein samples were prepared according to standard procedures (Miller & Gennis, 1983) and stored under liquid nitrogen. Typical enzyme concentrations were 24.3 μM in a pH 7.5 buffer solution of 50 mM sodium phosphate with 0.02% or 0.05% *N*-lauroylsarcosine (sodium salt). Samples for rR studies were used directly or, in some cases, further concentrated through ultracentrifugation (Centricon). Samples with cyanide were prepared by adding aliquots of 100 mM KCN solution to aerobic or anaerobic (argon-purged) solutions of the as-isolated enzyme. Azide samples were made by mixing appropriate amounts of 40 mM sodium azide solution with 24.3 μM enzyme solution. Change of pH to 9.5 was achieved by two cycles of 10× dilution with 20 mM CAPS buffer (pH 10.0) and reconcentration.

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. Rayleigh scattering was attenuated by use of Kaiser Optical (Ann Arbor, MI) notch or supernotch filters. For some experiments, a Dilor (E.G.&G./PAR, Princeton, NJ) Z-24 scanning Raman spectrophotometer was used. Excitation sources consisted of a Coherent (Santa Clara, CA) Innova 90-6 argon laser (488.0 and 514.5 nm) and an Innova 302 krypton laser (406.7, 413.1, and 647.1 nm). In earlier studies, a Spectra-Physics (Mt. View, CA) Model 2025-11 krypton laser was used. A Liconix 4240NB He/Cd laser was the source of the 441.6-nm line. Excitation at 572 and 595 nm was from an argon-laser-pumped Coherent 599-01 dye laser using rhodamine 6G. All laser lines were filtered through Applied Photophysics (Leatherhead, UK) optical glass (vis) or quartz (NUV) prism monochromators to remove plasma emissions. Incident power at the sample was held at ~0.15 mW (slightly defocused) for Soret excitations (406.7−441.6 nm) due to severe photoreduction observed at higher incident power. At this low value, however, no photoreduction was observed in repetitively recorded spectra with 406.7- and 413.1-nm excitation. The power of the 488.0- and 514.5-nm laser lines was ~20 mW at the sample, and power for longer wavelengths (572−647.1 nm) was ≤50 mW. Spectra were collected in a 90° scattering geometry from solution samples contained in capillary tubes in contact with a copper cold-finger immersed in an ice−water bath (some samples were at room temperature). Spectral resolution was ~4.5 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 with the Dilor spectrophotometer. Optical absorption spectra of the Raman samples were obtained on a Perkin-Elmer Lambda 9 spectrophotometer at a spectral band width of 2 nm, and the sample capillary tubes were placed in a black Delrin cell holder (Loehr & Sanders-Loehr, 1993). In this way, sample integrity was monitored before and after laser exposure on each sample in its capillary tube. Other optical absorption spectra were performed using an SLM-AMINCO DW2000 spectrophotometer with a spectral bandwidth of 3 nm.

RESULTS AND DISCUSSION

Selective Enhancement of Heme Cofactors in the As-Isolated Enzyme. The electronic absorption bands of the enzyme are at 413, ~530, and 645 nm with a prominent shoulder at ~680 nm. The 645- and 680-nm features have been assigned to the oxygenated and ferryl-oxo *d* chromophores, respectively (Lorence & Gennis, 1989; Poole et

[1] The oxygenated heme *d* has a prominent absorption at ~650 nm and is referred to in the literature as the d_{650} species. We measure its λ_{max} at ~645 nm.

[2] Abbreviations: EPR, electron paramagnetic resonance; ImH, imidazole; MCD, magnetic circular dichroism; PP, protoporphyrin IX; rR, resonance Raman. Designations for heme coordination and spin states: 5cHS, five-coordinate high-spin; 6cHS, six-coordinate high-spin; 6cLS, six-coordinate low-spin.

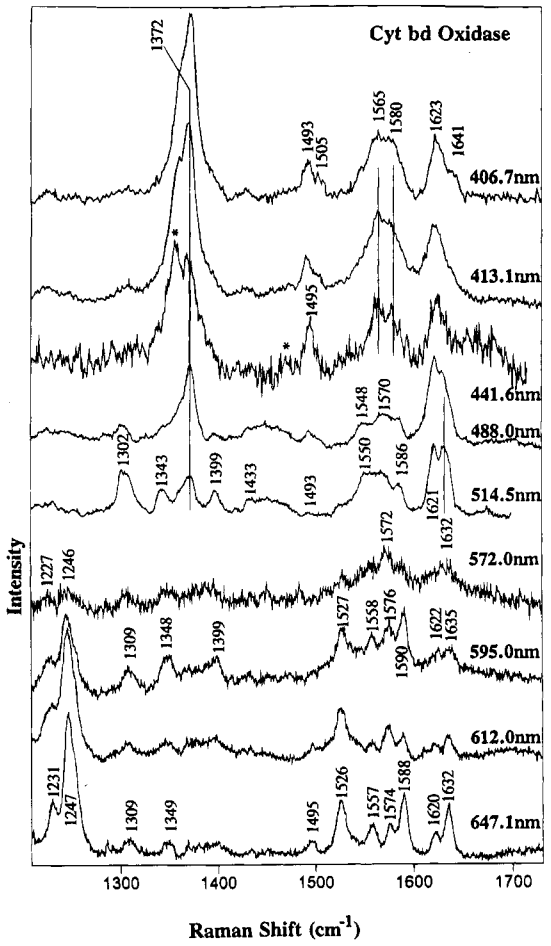

FIGURE 1: Resonance Raman spectra of the enzyme as isolated. The spectra obtained with 406.7-, 413.1-, 441.6-, 488.0-, and 514.5-nm excitation used the CCD spectrograph and an enzyme concentration of 24.3 μM. The spectra obtained with 572.0-, 595.0-, 612.0-, and 647.1-nm excitation were recorded on the scanning Dilor Z-24 Raman spectrophotometer (enzyme at 200 μM).

al., 1983a; Kahlow et al., 1991). The bands at 413 and 530 nm can be ascribed to the b_{595} and b_{558} chromophores, because rR spectra obtained within these absorptions are characteristic of ferric *b* hemes (see the following). Although b_{558} and b_{595} do not exhibit distinct, resolved bands in optical spectra, rR peaks of these two chromophores can be selectively enhanced by excitation within these bands.

(a) Soret Excitation. Resonance Raman spectra in the 1200−1750-cm^{-1} region of the as-isolated enzyme as a function of excitation wavelength are shown in Figure 1. The spectra obtained with 406.7- or 413.1-nm excitation are dominated by ν_4 at 1372 cm^{-1}. The frequency and intensity of this band are characteristic of ferric *b* hemes [for rR reviews, see Wang and Van Wart (1993), Spiro (1983, 1988), and Spiro et al. (1979)]. The remaining rR bands can also be assigned to porphyrin skeletal modes of ferric *b* hemes; however, they indicate the presence of a spin-state mixture. For example, the band at ~1494 cm^{-1} is characteristic of ν_3 of a 5cHS ferric *b* heme (b_{595}), whereas the bands at 1505, 1580, and 1641 cm^{-1} are the ν_3, ν_2, and ν_{10} modes, respectively, of a 6cLS ferric *b* heme (b_{558}). Since both sets of these frequencies are observed in 406.7- and 413.1-nm excitation, the 1372-cm^{-1} ν_4 band in these rR spectra must have contributions from both b_{595} and b_{558}. Similarly, the band at 1623 cm^{-1} is very likely a mixture of the C=C$_{vinyl}$ stretching vibrations of the hemes of both b_{595} and b_{558}. Porphyrin skeletal modes ν_3 and ν_{10} are reliable indicators

Table 1: Raman Frequencies of the *b* Hemes of Cytochrome *bd* Oxidase[a] Compared with Model Systems[b]

	5cHS ferric hemes		6cLS ferric hemes	
mode	b_{595}	Fe^{III}(PP)Cl	b_{558}	[FeIII(PP)(ImH)$_2$]$^+$
ν_2	1565	1570	1580	1579
ν_3	1493	1491	1505	1502
ν_4	1372	1373	1372	1373
ν_{10}	1632	1626	1641	1640
ν_{11}	1549	1553		1562
ν_{19}	1570	1571	1586	1586

[a] This work. [b] Choi et al., 1982.

of spin and coordination states of heme proteins. The band at ~1494 cm^{-1} cannot be mistaken for anything other than ν_3 of the 5cHS b_{595} chromophore. Additionally, the observation of a strong band at 1632 cm^{-1} (ν_{10}) that is preferentially enhanced with Q-band excitation at 488.0 and 514.5 nm provides further evidence for the 5cHS ferric b_{595} (see the following). Given these unambiguous assignments, we attribute the band at 1565 cm^{-1} (Soret excitation) to the ν_2 mode of the 5cHS b_{595} (Sun et al., 1994; Adachi et al., 1993; Palaniappan & Terner, 1989; Smulevich et al., 1988; Evangelista-Kirkup et al., 1985; Asher & Schuster, 1979).

The 413.1-nm excitation used closely matches the Soret maximum of the enzyme. However, the relative intensities of the 6cLS rR bands at 1505, 1580, and 1641 cm^{-1} *increase* vs the 1494- and 1565-cm^{-1} bands of the 5cHS heme upon 406.7-nm excitation, but are further decreased with 441.6-nm excitation. This enhancement behavior indicates that the absorption maximum of cytochrome b_{595} is ≥413 nm and that of b_{558} is <413 nm. It is worth noting that the exceedingly low power and defocused laser beam, together with the high collection efficiency of the CCD spectrometer, were absolutely necessary to record the Soret rR spectra shown in Figure 1 with minimal photoreduction. At higher laser power (>1 mW), photoreduction of the ferric hemes occurs as evidenced by the shift in ν_4 to 1358 cm^{-1}; repetitively recorded spectra show a change with time to give a final spectrum identical to that of cytochrome *bd* oxidase chemically reduced by sodium dithionite (data not shown). However, for the data shown with 406.7- and 413.1-nm excitation, no time-dependent frequency shifts occurred. Photoreduction was even more severe with 441.6-nm excitation, as signaled by the increased intensity of ν_4 and ν_3 of Fe^{II} *b* hemes at 1358 and 1473 cm^{-1}, respectively (* in Figure 1).

(b) Blue/Green Excitation. When cytochrome *bd* oxidase is studied with 488.0- and 514.5-nm excitation, the observed rR spectra are typical of ferric *b* hemes obtained with Q-band excitation (Figure 1). Typically, the totally symmetric modes ν_2, ν_3, and ν_4 decrease markedly in intensity, and nonsymmetric modes ν_i with $i \geq 10$ increase in intensity, often occurring near the same frequencies. However, the modes generally can be distinguished by their polarization behavior. Among these, the bands at 1632, 1570, and 1549 cm^{-1} may be assigned to ν_{10}, ν_{19}, and ν_{11}, respectively, of the 5cHS ferric b_{595}. The rR peak at 1586 cm^{-1} is assigned to ν_{19} of the 6cLS b_{558}. Observed porphyrin skeletal mode frequencies of b_{595} and b_{558} in cytochrome *bd* oxidase are listed in Table 1 and compared with rR frequencies of ferric heme model systems: 5cHS hemin chloride and 6cLS (ImH)$_2$Fe^{III}PP (Choi et al., 1982).

(c) Red Excitation. By moving the excitation wavelength further toward the red, rR features belonging to the *d*

Table 2: Porphyrin-Equivalent Modes (cm^{-1}) of Iron−Chlorin Complexes Observed in Resonance Raman Spectra[a]

	ν_4	ν_3	ν_{38}	ν_{11}	ν_{19}	ν_2	ν_{37}	ν_{10}
four-coordinate, intermediate-spin Fe(II)								
Fe(OEC)[b]	1370	1498	1539	1571	1589	1577		1628
five-coordinate, high-spin Fe(II)								
(1,2-diMeIm)Fe(OEC)[c]	1366			1536				1608
(2-MeIm)Fe(OEC)[c]	1365	1477		1535	1559	1579		1608
sulfMb (reduced)[d]	1344	1473	1515	1545	1561	1582		1606
six-coordinate, low-spin Fe(II)								
$(Im)_2$Fe(OEC)[c]	1367	1494		1545	1583	1601		1628
$(4\text{-CHO-py})_2$Fe(OEC)[c]	1369	1495		1548	1583	1600		1629
$(py)_2$Fe(OEC)[c]	1368	1494		1548	1584	1598		1629
$[(n\text{-BuNH}_2)_2$Fe(OEC)$]^+$ [c]	1367	1492		1540	1582	1600		1626
five-coordinate, high-spin Fe(III)								
FFe(OEC)[c]	1373	1498	1531		1573		1590	1633
ClFe(OEC)[e]		1492		1557			1586	1626
BrFe(OEC)[c]	1371	1494	1530		1570		1589	1630
IFe(OEC)[c]	1371	1492	1527		1570		1589	1629
[2-MeImFe(OEC)]$^+$ [c]	1371	1497		1540	1569		1589	1628
[(1,2-diMeIm)Fe(OEC)]$^+$ [c]	1373	1497		1540	1570		1590	1630
ClFe(pPP)[f]	1369	1492	1533	1553	1558	1571	1586	1625
ClFe(DC)[f]	1370	1489	1534	1553	1570	1577	1588	1631
cytochrome *bd* oxidase, as isolated[g(a)]		1495	1526	1557	1574		1588	1632
cytochrome *bd* oxidase + H_2O_2 (ferryl)[g(b)]		1496	1529	1560	1574		1587	1632
cytochrome *bd* oxidase, oxidized[g(c)]		1496	1528	1556	1572		1587	1634
six-coordinate, high-spin Fe(III)								
$[(DMSO)_2$Fe(DC)$]^+$ [f]	1367	1477	1508	1550	1563	1563	1574	1608
			1520				1597	
$[(DMSO)_2$Fe(pPP)$]^+$ [f]	1368	1479	1505		1552	1558	1568	1608
			1516				1578	
$[(CH_3OH)_2$Fe(OEC)$]^+$ [c]	1372	1489		1522	1566		1579	1618
$[(DMSO)_2$Fe(OEC)$]^+$ [c]	1371	1486		1521	1564		1576	1613
sulfMb (oxidized)[d]	1369	1479	1514	1546	1563	1563	1583	1612
six-coordinate, low-spin Fe(III)								
$[(Im)_2$Fe(OEC)$]^+$ [c]	1372	1508		1550	1579	1601		1640
$[(n\text{-BuNH}_2)_2$Fe(OEC)$]^+$ [c]	1372	1506		1548	1576	1600		1639

[a] Porphyrin-equivalent modes are features in the chlorin rR spectra of similar frequency and behavior to the porphyrin spin- and coordination-state marker bands; given the altered mode compositions of chlorin normal modes, this identification is made for convenience at the expense of accuracy. Abbreviations: OEC, octaethylchlorin; pPP, photoprotoporphyrin; DC, deuterochlorin. [b] Mylrajan et al., 1995; data obtained with Soret and Q excitation. [c] Ozaki et al., 1986a; 488.0-nm excitation only. [d] Andersson et al., 1984; Soret and Q excitation. [e] Ozaki et al., 1979; 488.0-nm excitation only. [f] Andersson et al., 1985; Soret and Q excitation. [g] Cytochrome *bd* oxidase (a) as isolated, (b) after the addition of 90× H_2O_2, conditions that maximize the ferryl-oxo species, and (c) the fully oxidized enzyme: this work and Kahlow et al., 1993; the 647.1-nm excitation used for all three spectra probes only the *d* hemes. The band at ~1621 cm^{-1} assigned to the $\nu(C{=}C)_{vinyl}$ of the protochlorin macrocycle is omitted from the table.

chromophore emerge. The appearance of a prominent band at ~1247 cm^{-1} is most characteristic of chlorin spectra (Kahlow et al., 1993; Andersson et al., 1985, 1992; Procyk et al., 1992; Prendergast & Spiro, 1991; Fonda et al., 1990; Schick & Bocian, 1987; Ozaki et al., 1986b). Already at 572 nm, a weak peak at ~1247 cm^{-1} is apparent that becomes the dominant feature with 647.1-nm excitation (Figure 1). We believe that the chlorin is responsible for most, if not all, of the bands in the rR spectra obtained with excitation ≥595 nm. Due to the reduced molecular symmetry of the chlorin relative to the heme, the rR spectral assignments are considerably more complex (Procyk & Bocian, 1992; Boldt et al., 1987; Schick & Bocian, 1987). In addition, many chlorin rR bands are polarized, and hence, the powerful method for identifying vibrational modes from depolarization ratios (that is well established for porphyrins rich in anomalously polarized and depolarized modes) is far less useful in the study of chlorins (Andersson et al., 1985). The limited number of metallochlorin, oxychlorin, and ferryl-oxo chlorin model studies has also made assignment of these rR bands far more difficult. Nevertheless, comparisons of rR spectra of chlorin complexes reveal distinct categories for different spin and coordination states (Table 2). Thus, the *d* heme in as-isolated cytochrome *bd* oxidase has spectral signatures that correlate best with those of pentacoordinate, high-spin ferric species.

The as-isolated enzyme has been estimated to be a mixture of oxygenated (70%) and ferryl-oxo (30%) heme *d* species on the basis of its absorption spectrum (Kahlow et al., 1993). The rR spectrum shows distinctive peaks for $\nu(Fe{-}O_2)$ at 568 cm^{-1} and for $\nu(Fe{=}O)$ at 815 cm^{-1} for the oxygenated and ferryl species, respectively, with the latter being considerably more intense (Figure 2). However, the majority of the vibrational features are seemingly unaffected by changes in ligation. Thus, the same set of chlorin rR modes (Table 2) is observed for the oxidized ($Fe^{III}OH$?), oxygenated ($Fe^{II}O_2 \leftrightarrow Fe^{III}O_2^-$), and ferryl-oxo ($Fe^{IV}{=}O$) forms of heme *d* in cytochrome *bd* oxidase (Kahlow et al., 1991, 1993). The rR spectra also indicate that these chlorin *d* species are all 5cHS; they are inconsistent with either 6cHS or 6cLS ferric species. The alternative of a four-coordinate species appears unlikely at the ferric oxidation level. These findings lead us to suggest that the *d* heme has no endogenous ligand or, at most, a very weakly interacting ligand.

The proposed absence of a strong endogenous ligand for heme *d* is supported by the observation of a $g = 6$ EPR signal attributed to high-spin ferric heme *d* in the as-isolated enzyme (Meinhardt et al., 1989; Rothery & Ingledew, 1989). Another line of evidence is the anomalously high Fe=O stretching frequency of 815 cm^{-1} for cytochrome *bd* oxidase compared to values of 745−788 cm^{-1} for ferryl vibrations in other heme enzymes [Kahlow et al. (1991) and references

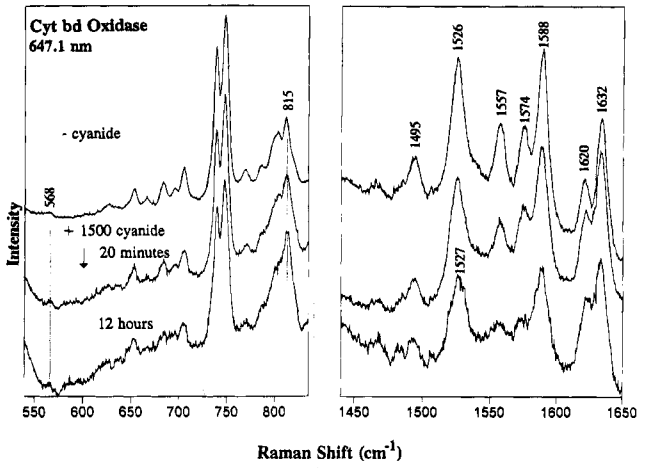

FIGURE 2: Resonance Raman spectra of as-isolated cytochrome *bd* oxidase (24.3 μM) in the absence (−cyanide) and presence of 36 mM (1500× molar excess) cyanide (647.1-nm excitation with 14 mW of power; CCD spectrometer).

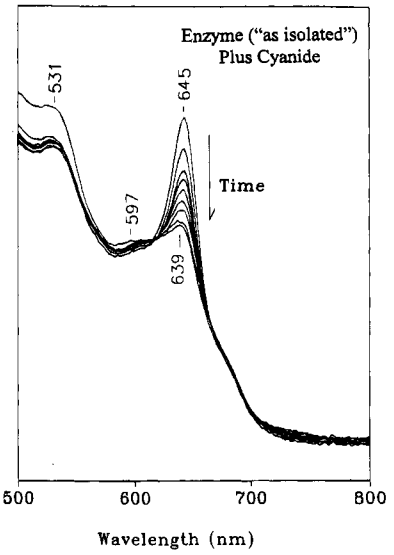

FIGURE 3: Absolute visible absorption spectra of as-isolated cytochrome *bd* oxidase (~350 μM) and the effect of addition of 40-fold excess cyanide. Time intervals for the nine scans from top to bottom at 645 nm are as follows: before the addition of CN^-, immediately after addition, 10, 15, 20, 30, 40, and 60 min, and 14 h. The spectrum after 36 h is identical to that after 14 h. The first trace, before CN^- addition, has been rescaled to correct for the dilution.

therein]. Comparisons of chlorin and porphyrin model compounds reveal that their ferryl species have similar ν-(Fe=O) frequencies (Ozawa et al., 1994). Thus, the 815-cm^{-1} value for cytochrome *bd* oxidase is not a consequence of its being a chlorin. Rather, it is best matched by ferryl porphyrin complexes with weak or absent sixth ligands (Oertling et al., 1990).

Reactions with Cyanide. (a) UV/Vis Spectral Changes. The enzyme as isolated has absorption bands at 413, 530, and 645 nm and a shoulder 680 nm. After the addition of cyanide, the absorbance at 645 nm decreases markedly, suggesting the loss of the oxy-*d* species. A sequence of absolute spectra over a period of minutes to hours is shown in Figure 3. Between 1 and 14 h further changes are slight, as shown in the [as isolated + CN^-] − [as isolated] difference spectrum (Figure 4). The slight loss of absorbance at 680 nm is probably due to endogenous electron sources, which cause turnover of the ferryl species. At much higher

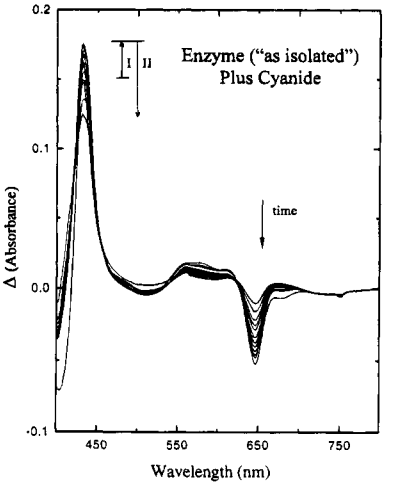

FIGURE 4: UV/vis difference spectra (enzyme plus cyanide *minus* enzyme without cyanide) of as-isolated cytochrome *bd* oxidase (11.5 μM) showing the effect of 50 mM CN^-. The subtrahend spectrum was rescaled for the difference spectra to correct for dilution. Spectra were recorded at 2, 4, 7, 9, 11, 15, 20, 26, 34, 45, and 60 min and 14 h after CN^- addition. In the Soret region, there is an initial increase in intensity after the addition of CN^- during the first 10 min (phase I), followed by a small red shift and a gradual decrease (phase II).

concentrations of enzyme this decrease is not noticeable (Figure 3). The changes observed in the Soret region are an initial increase at ~432 nm at the expense of absorbance at ~413 nm (Figure 4, phase I), followed by a reversal, i.e., decrease in A_{432} and increase in A_{413} (Figure 4, phase II). Similar findings have been reported previously and were interpreted in terms of a high-spin to low-spin transition caused by CN^- binding to ferric b_{595} (Krasnoselskaya et al., 1993). However, *ferric* cyano-b_{595} would not be expected to lose this ligand and, hence, should not show the phase II reversal with time. An alternative explanation is a fast *reduction* of b_{595} followed by a slow aerobic *reoxidation*. This view is reasonable because ferrous b_{595} has a Soret absorption at ~429 nm as observed in the fully reduced enzyme (Sun et al., manuscript submitted for publication). Furthermore, the loss of A_{645} with cyanide signals that the reduction of b_{595} is correlated with the loss of the oxygenated fraction of heme *d* in the as-isolated enzyme. No autoreduction is observed upon cyanide treatment of the fully oxidized cytochrome *bd* oxidase. Finally, the rR data presented in the following provide further compelling evidence for the autoreduction of the as-isolated b_{595} in the presence of cyanide.

(b) Soret Excitation rR Spectra. The resonance Raman spectrum obtained 20 min after the addition of a 1500-fold excess of cyanide excited at 413.1 nm is dominated by a ν_4 peak at 1358 cm^{-1} which, together with peaks at 1473 (ν_3) and 1602 cm^{-1} (ν_{10}), indicates a 5cHS ferrous heme (Sun et al., 1993; Mylrajan et al., 1990; Palaniappan & Terner, 1989; Dasgupta et al., 1989; Mino et al., 1988; Spiro, 1983; Choi et al., 1982; Spiro et al., 1979; Spiro & Strekas, 1974), most probably reduced b_{595} (Figure 5). These new features are in addition to the peaks described earlier, such as ν_4 at 1372 cm^{-1} of the oxidized *b* hemes. This latter ν_4 peak, together with the pair at 1493 (ν_3) and 1565 (ν_2) cm^{-1}, indicates the 5cHS ferric b_{595}; however, the pair at 1505 (ν_3) and 1581 (ν_2) cm^{-1} belongs to the 6cLS ferric b_{558}. Hence, the cyanide system contains at least three species of *b* hemes: ferrous and ferric 5cHS b_{595} and ferric 6cLS b_{558}. It should be

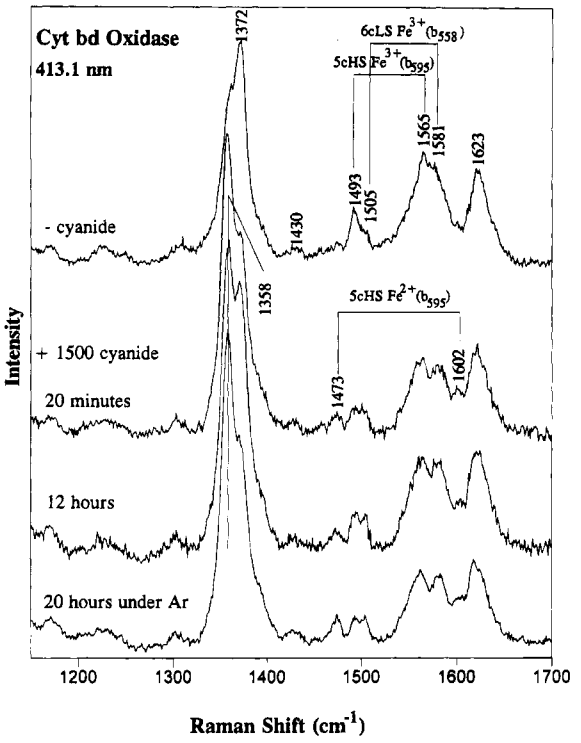

FIGURE 5: Resonance Raman spectra of as-isolated cytochrome *bd* oxidase (24.3 μM) and the effect of 36 mM (1500× molar excess) cyanide (413.1-nm excitation with 0.15 mW of cw power; CCD spectrometer). Top trace: Before the addition of CN^-. Lower traces: After the addition of CN^- at various incubation times. Each spectrum is the average of nine separate 3-min acquisitions, and no spectral changes were apparent between individual sets.

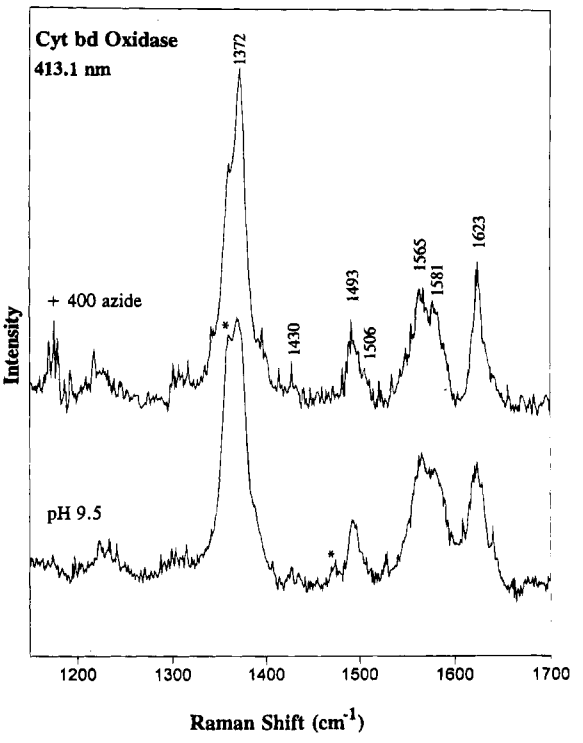

FIGURE 6: Resonance Raman spectra of as-isolated cytochrome *bd* oxidase (24.3 μM) in the presence of 10 mM (400× molar excess) azide (upper trace) and at pH 9.5 without added anions (lower trace) (413.1-nm excitation with 0.30 (upper) and 0.15 mW (lower) of cw power; CCD spectrometer). The upper spectrum is the average of four separate 3-min acquisitions, whereas the lower spectrum is the average of three separate 5-min acquisitions. The peaks marked with an asterisk at 1358 and 1474 cm^{-1} in the pH 9.5 spectrum arise from partial photoreduction and were noted to grow with time and/or higher laser power.

emphasized that the bands due to the ferrous species are *not* produced by photoreduction. The laser beam is defocused at low power (~0.15 mW), conditions that were shown not to cause photoreduction. In addition, repetitively recorded spectra are identical, and the intensities of ferric species bands remain constant during long exposure times. Moreover, the relative intensity of the ferrous heme vs the ferric heme (1358 vs 1372 cm^{-1}) is higher for the sample reacted with KCN for ~20 min than that incubated overnight open to air (~12 h). This observation and the UV/vis results described earlier provide evidence that b_{595} is autoreduced upon cyanide addition and then slowly reoxidized in air. If CN^- is incubated in the cytochrome *bd* oxidase sample under an oxygen-free atmosphere, reoxidation does not occur even after ~20 h, as shown in the bottom trace of Figure 5. The autoreduction of b_{595} upon cyanide addition is verified in a rR spectrum obtained with 514.5-nm excitation, in which the 1602-cm^{-1} band, assigned to the 5cHS ferrous ν_{10} mode of b_{595}, is observed with greatly enhanced intensity, as expected for Q excitation (spectrum not shown).

Although the addition of KCN to cytochrome *bd* oxidase causes autoreduction of b_{595}, the reaction is difficult to quantitate. The percent reduction cannot be easily evaluated from relative Raman intensities because of the markedly greater scattering efficiency of ferrous than ferric hemes in the oxidase (Sun et al., unpublished results). For example, the 1358 cm^{-1} (ν_4) band overwhelmingly dominates the rR spectra of ferrous hemes, whereas the corresponding ferric band at 1372 cm^{-1} is only 2−3 times as intense as other strong rR bands. Thus, the extent of reduction is far less than the 1358/1372 cm^{-1} intensity ratio indicates.

The spectra in Figure 5 also indicate that the remaining ferric b_{595} does not bind cyanide, as shown by the persistence of the 5cHS ferric heme marker bands, ν_3 and ν_2, at 1493 and 1565 cm^{-1}, respectively, in the presence of 1500-fold cyanide. The undiminished intensities of these bands over many hours indicate a very low affinity of ferric b_{595} for cyanide. *If* CN^- *were* bound to b_{595}, it would be expected to produce a hexacoordinate, low-spin heme with (i) an intense ν_{10} at ~1640 cm^{-1} with 514.5-nm excitation and (ii) complete loss of the ν_3 (1495 cm^{-1}) and ν_2 (1565 cm^{-1}) 5cHS ferric bands; neither is observed. Although the rR bands at 1505 and 1581 cm^{-1} in Figure 5 are from a 6cLS ferric *b* heme, we attribute these to b_{558} since they are identical in frequency and relative intensity to the same features in the sample without cyanide (trace 1, Figure 5). Moreover, the ν_3 and ν_{10} bands at 1473 and 1602 cm^{-1} of the ferrous heme also exclude the possibility that cyanide binds to ferrous b_{595} because these values are characteristic for 5cHS ferrous and not 6cLS ferrous hemes (Mino et al., 1988; Spiro et al., 1979).

The resistance of b_{595} to bind cyanide is further exemplified by its lack of interaction with other anions. As shown in Figure 6, cytochrome *bd* oxidase in the presence of 400-fold molar excess azide gives the rR spectrum identical to that without azide (413.1-nm excitation). Furthermore, a change in solution pH to 9.5 also produces no significant alterations in the rR spectrum, thus indicating a low affinity of b_{595} for OH^- as well (Figure 6).

(c) Red Excitation rR Spectra. The effect of cyanide addition on the rR spectrum of the *d* heme is shown for two regions of interest: (a) 550−850 cm^{-1} and (b) 1450−1650 cm^{-1} (Figure 2). One apparent consequence of CN^- addition

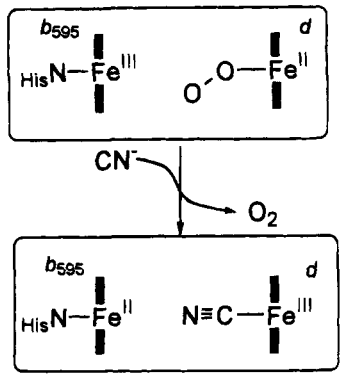

FIGURE 7: Proposed model of the reaction of cyanide with the oxygenated form of cytochrome *bd* oxidase. Heme b_{595} with an axial histidine ligand and heme *d* with an axial O_2 are both in five-coordinate, high-spin states. Cyanide displaces O_2, leading to autoreduction of b_{595} and a new 5cHS species of heme *d* with CN^-. Heme b_{558} (not shown) remains in the ferric 6cLS state. The model suggests that heme *d* has either no endogenous protein ligand or a weakly coordinated one that is readily displaced when exogenous ligands bind.

is a loss in overall spectral quality, as indicated by the greater noise level of the 20-min and 12-h traces. This decrease in spectral quality is directly linked to the diminished resonance enhancement as a result of the reduced absorptivity in this region (Figure 3). Nonetheless, the pattern of the Raman bands remains the same in both regions, indicating that the species responsible for the rR spectra are similar *before* and *after* cyanide addition and that they are still five coordinate, high spin. This is in agreement with EPR studies of the anaerobically oxidized enzyme, which show that the *d* heme remains high spin after the addition of cyanide (Hata et al., 1985; Gennis et al., unpublished results).

Two bands of particular interest in the low-frequency region are the ν(Fe=O) at 815 cm^{-1} (Kahlow et al., 1991) and the ν(Fe—O_2) at 568 cm^{-1} (Kahlow et al., 1993). The 815-cm^{-1} feature appears to be fully intact even after overnight incubation in KCN, suggesting that ferryl-oxo *d* heme species remains in the presence of 1500-fold excess cyanide (Figure 2). The sample treated for 12 h with cyanide shows a residual feature at 568 cm^{-1} arising from ν(Fe—O_2) (although a chlorin mode at this frequency cannot be ruled out), but its extremely low intensity makes quantitation difficult. Because the 645-nm absorption band is responsible for the resonance enhancement of the 568-cm^{-1} Fe—O_2 mode, the marked decrease in absorbance at 645 nm upon the addition of cyanide provides indirect evidence that a significant fraction of the oxygenated species has lost its O_2.

CONCLUSIONS

The observations from the electronic and rR spectroscopic studies of cytochrome *bd* oxidase reported here may be summarized by a working model that is illustrated in Figure 7 and is described as follows.

(1) The predominant heme *d* species in the as-isolated enzyme are the oxygenated and ferryl-oxo species. Interpretation of the high-frequency rR bands suggests that both of these are in pentacoordinate, high-spin states (Table 2). Ferrous heme *d*, with its ν_{10} mode at 1609 cm^{-1}, also looks to be five coordinate and high spin (J. Sun et al., manuscript submitted for publication). Thus, the ferrous enzyme appears to have an endogenous or aqua ligand to heme *d* that is either weakened or displaced upon binding exogenous ligands.

(2) Addition of cyanide in the millimolar range to the as-isolated enzyme causes decay of the d_{650} band that is associated with the oxygenate form. This spectral change suggests the displacement of O_2 from the *d* heme.

(3) Autoreduction of b_{595} is seen, and we propose that the source of the reducing equivalents is electron transfer from the Fe^{II}—O_2 form of heme *d* concomitant with the loss of O_2. Thus, the products are reduced heme b_{595} and oxidized heme *d*.

(4) Unlike the typical behavior of heme proteins in the presence of the strong cyanide ligand, cytochrome *bd* oxidase forms no new hexacoordinate low-spin species, as judged by rR spectroscopy. There are no rR features characteristic of either (His)N—Fe^{II}—CN or (His)N—Fe^{III}—CN. We conclude that b_{595} remains as a 5cHS species. The rR spectra of cytochrome *bd* oxidase obtained with red excitation appear to be insensitive to CN^-, and no new low-spin *d* species are apparent. Nevertheless, the loss of O_2 makes it likely that cyanide has become an axial ligand to the chlorin. The fact that the rR spectrum of the resultant 5cHS ferric cyanide complex is apparently indistinguishable from that of the oxygenated or oxidized heme *d* is consistent with the insensitivity of the chlorin vibrational modes to the nature of the fifth ligand in this enzyme. The observed autoreduction upon addition of cyanide to the as-isolated enzyme is also consistent with the proposed dinuclear site (Hill et al., 1993), in that electron transfer from *d* to b_{595} would be facile, as sketched in Figure 7.

It is interesting to compare the cyanide reactions of several terminal oxidases. The oxidized bovine enzyme (aa_3 type) reacts with cyanide to produce a low-spin Fe^{III}(a_3)—CN^-—Cu_B^{2+} (Fee et al., 1993). In contrast, the addition of cyanide to *Thermus thermophilus* cytochrome *c* oxidase (ba_3 type) results in autoreduction of heme a_3 (Oertling et al., 1994), behavior similar to that we observed with cytochrome *bd* oxidase. The reduced a_3 in the ba_3 oxidase produces a ferrous low-spin species with CN^-, and Cu_B^{2+} can bind another CN^- in the dinuclear site (Oertling et al., 1994). The ability of both metal centers in ba_3 oxidase to bind exogenous ligands is also reminiscent of the *bd* oxidase. In the latter enzyme, the b_{595} is also capable of binding CO (Hill et al., 1993) and O_2 at high pressure (J. Sun et al., unpublished results), in addition to the primary reaction at heme *d*. Similarly, mutants have been isolated in which b_{595} appears to be six coordinate, but that still allow O_2 binding to the *d* heme (R. B. Gennis et al., unpublished results). Thus, in this dinuclear center, heme *d* provides the site for O_2 reduction, and heme b_{595} serves primarily as an electron donor.

ACKNOWLEDGMENT

We thank Professors Joann Sanders-Loehr, Chi K. Chang, and James O. Alben for helpful discussions and suggestions.

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BI9511550