Cytochrome *bo* from *Escherichia coli*: Binding of Azide to Cu_B[†]

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ABSTRACT: Azide binds to *fast* cytochrome *bo* with a stoichiometry of 1:1, the dissociation constant for this reaction being approximately 2×10^{-5} M. The changes induced in the electronic absorption are very slight and are consistent with heme *o* remaining hexacoordinate high-spin, an observation confirmed by room temperature MCD spectroscopy in the region 350−2000 nm. X-band EPR spectroscopy of the azide-bound form shows heme *o* remains coupled to Cu_B, but that the integer-spin signal ($g = 3.7$) that we have previously reported to be associated with the binuclear center of *fast* cytochrome *bo* [Watmough et al. (1993) *FEBS Lett. 319*, 151−154]. is shifted to higher field. The kinetics of azide binding are an order of magnitude faster than those observed for the binding of cyanide. Unlike cyanide, the observed rate constants do not saturate in the range 0 .05−25 mM. The value of K_{on} shows a marked dependence on pH, indicating that the active species is hydrazoic acid. It is argued that these data are consistent with the binding of azide ion as a terminal ligand to Cu_B yielding a binuclear center in the form Fe^{III}-OH_2:: Cu_B^{II}-N_3. The binding of azide in heme−copper oxidases may cause displacement of another nitrogenous ligand from Cu_B which might explain the absence of electron density associated with histidine-325 in the structure of the *Paracoccus denitrificans* CCO [Iwata et al. (1995) *Nature 376*, 660−669]. Formate appears to act as a bidentate ligand to the binuclear center, blocking not only the binding of azide to Cu_B but also the binding of cyanide to heme *o*.

Cytochrome *bo*, a quinol oxidase from *Escherichia coli*, is a member of a conserved superfamily of protonmotive heme−copper terminal oxidases which includes mitochondrial cytochrome *c* oxidase (CCO)[1] (van der Oost et al., 1994). Although quinol oxidases lack the dinuclear copper center, Cu_A (Hosler et al., 1993), they share with CCO three highly conserved subunits: subunit I, subunit II, and subunit III (Saraste, 1990). Subunit I consists of a minimum of 12 transmembrane helices and contains 3 metal centers, 2 hemes, and copper(II) ion known as Cu_B. Structural studies of the bovine mitochondrial CCO (Tsukihara et al., 1995) and of a CCO from the soil bacterium *Paracoccus denitrificans* (Iwata et al., 1995) are consistent with mutagenesis (Lemieux et al., 1992) and spectroscopic (Cheesman et al., 1993) studies of cytochrome *bo*, which show heme *b* to be a magnetically isolated low-spin ferric species with bishistidine ligation, and heme *o* is a histidine-coordinated high-spin ferric species. Cu_B has three histidine ligands and is magnetically coupled to heme *o* to form a binuclear center which is the site of oxygen reduction and the presumed site of proton translocation.

Because vectorial proton movements may be associated with ligand rearrangements around Cu_B (Gelles et al., 1986; Mitchell, 1987; Wikström, 1989), there is considerable interest in clarifying to what extent this center can expand or alter its coordination sphere. One approach to this problem is by studying the coordination of small ligands to the binuclear center, in particular those that induce spectral changes in response to binding to Cu_B.

It is established that the binuclear center of oxidized bovine mitochondrial CCO can bind fluoride, formate, and azide at heme a_3 which remains high-spin (Baker & Palmer, 1987; Brudvig et al., 1981; Moody et al., 1991; Palmer et al., 1988; Young, 1988). Cyanide binding to the same heme causes a transition to a low-spin species (Thomson et al., 1977; Van Buuren et al., 1972). Unequivocal identification of the ligand binding site is difficult for several reasons. Recognizable optical fingerprints for ligand-bound A-type hemes are not available as these hemes are not found in other proteins. Although heme A has unusual optical properties, it gives rise to EPR spectra which are almost identical to those of heme B with comparable ligation. However the spin−spin interaction between heme a_3 and Cu_B results either in no EPR from the binuclear site, as for the fast form of CCO, or in complicated broad spectral features as is the case for formate and fluoride-bound CCO (Brudvig et al., 1981).

A low-temperature MCD study of azide-bound CCO found no evidence for a high- to low-spin conversion of the binuclear site heme, heme a_3. However, it was suggested that the azide binds as a nonlinear heme−copper bridging ligand in a manner which reduces its effective ligand field strength at heme a_3 (Thomson et al., 1985). A recognizable azide-bound species was obtained only following partial reduction of the azide-treated enzyme. One-electron reduction of Cu_B(II) to Cu_B(I) leaves this ion diamagnetic and

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[1] Abbreviations: CCO, cytochrome *c* oxidase; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; FTIR, Fourier transform infrared; K_D, dissociation constant; k_{obs}, observed pseudo-first-order rate constant; Mb-azide, metmyoglobin azide; MES, 2-(*N*-morpholino)ethanesulfonic acid; MCD, magnetic circular dichroism; TAPS, *N*-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid.

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removes the spin coupling to heme a_3. EPR and NIR MCD spectra comparable to those of metmyoglobin azide are then observed. This constitutes evidence that azide is bound directly to heme a_3 in the partially reduced form of CCO but leaves the site of azide binding to the fully oxidized state in some doubt. A nonlinear binding of azide to heme is in itself insufficient explanation for lowering the ligand strength. It has been shown that a bent binding conformation is in fact preferred (Korszun & Moffat, 1981). A weakening of the azide ligand strength by bridging to Cu_B, as occurs with cyanide (Thomson et al., 1982), is a possible explanation. This was suggested following a more recent *room temperature* MCD study of the binding of azide to fast CCO, a study which also concluded that heme a_3 remains high-spin ferric with azide binding as a bridging ligand in the binuclear site (Li & Palmer, 1993).

Cytochrome *bo* contains O- and B-type hemes, the optical properties of which are well understood (Cheesman et al., 1991; Wu et al., 1992). Consequently, its electronic absorption and MCD spectra contain recognizable features which can be unambiguously assigned to the individual hemes. The oxidized form of cytochrome *bo* prepared in this laboratory that we refer to as *fast*[2] is defined by characteristic electronic absorption bands at 406.5 nm (Soret) and 624 nm (ligand−metal charge transfer band), by a broad complex EPR signal with prominent features at $g = 9.1$, 3.7, and 2.68, and by a room temperature MCD spectrum that reveals two charge-transfer bands characteristic of high-spin ferric heme *o* at 650 nm and 1100 nm (Cheesman et al., 1993; Watmough et al., 1993).

Changes in the spectrum of *fast* cytochrome *bo* in response to ligand binding are due to fluoride, formate, and cyanide ions binding directly to heme *o* within the binuclear site. Of these, only cyanide causes a change in the spin state of heme *o* (Cheesman et al., 1994). The spectra also suggested that in the "as isolated" *fast* form of cytochrome *bo*, heme *o* is coordinated by a water ligand.

We have now examined the reaction of fast cytochrome *bo* with azide, and the results presented below strongly suggest that this ligand behaves differently to fluoride, formate, and cyanide in that it binds to Cu_B and not to heme *o* in the fully oxidized form of cytochrome *bo*.

MATERIALS AND METHODS

Reagents. Sodium azide, Hepes, and octyl β-D-glucopyranoside were purchased from Sigma. Deuterium oxide was from Fluorochem.

Cell Growth and Enzyme Preparation. The *Escherichia coli* strain RG145 which overexpresses cytochrome *bo* and lacks cytochrome *bd* (Au & Gennis, 1987) was used as the source of cytochrome *bo* for the spectroscopic experiments. Conditions for batch cell culture and subsequent purification of the cytochrome *bo* were as previously described (Cheesman et al., 1993). For the kinetic experiments, enzyme was prepared from the strain GO105/pJRhisA (J. N. Rumbley and R. B. Gennis, personal communication) in which the enzyme has a histidine tag engineered to the carboxyl terminus of subunit II which allows isolation of homogeneous cytochrome *bo* in a single step using metal chelate affinity chromatography (Morgan et al., 1995). The binuclear center in the enzyme from the two sources was spectroscopically identical and behaved identically toward azide.

Spectroscopy. Electronic absorption spectra were recorded either on an Aminco DW2000 or on a Hitatchi U4001 spectrophotometer. EPR spectra were recorded on an X-band ER-200D spectrometer (Bruker Spectrospin) interfaced to an ESP1600 computer and fitted with a liquid helium flow-cryostat (ESR-9; Oxford Instruments). MCD spectra were recorded on either a circular dichrograph, JASCO J-500D, for the wavelength range 280−1000 nm or a laboratory-built dichrograph (Gadsby & Thomson, 1990) for the range 800−2500 nm using an Oxford Instruments superconducting solenoid with a 25 mm room temperature bore and capable of generating magnetic fields up to 6 T. MCD spectral intensities are linear with magnetic field at room temperature and are expressed per unit magnetic field, as debye (D) (M^{-1} cm^{-1} T^{-1}).

Sample Preparation for MCD Spectroscopy. All protein samples were in D_2O buffers containing 50 mM Hepes and 0.2% octyl β-D-glucopyranoside at pH* 7.4. pH* is the apparent pH of these buffers in D_2O measured with a standard glass pH electrode. Enzyme concentrations were determined spectrophotometrically using $\epsilon_{Soret} = 182$ mM^{-1} cm^{-1} for the starting oxidized fast form of the enzyme (Cheesman et al., 1993).

In order to study the MCD of cytochrome *bo* across the 280−2500 nm range, two samples are required: one with a concentration in excess of 200 mM is required to measure the weaker signals at near-infrared wavelengths, and a second of lower concentration is used to allow sufficient light transmission in the Soret region. Azide-bound samples for MCD spectroscopy were prepared by addition of concentrated buffered solutions of sodium azide to the two protein samples used. Final concentrations of protein and ligand are given in the legends to the figures. The continuity of the two samples was cross-checked by comparison of the absorption and MCD spectra at visible wavelengths where both may be readily measured.

Treatment of Binding Data. The fractional saturation (Y) of cytochrome *bo* by azide is calculated from the relationship:

$$Y = \frac{\Delta A}{\Delta A_{max}}$$

where ΔA is the observed absorbance change at a specified wavelength in response to the addition of a known concentration of ligand and ΔA_{max} is the absorbance change observed upon saturation of the protein by that ligand. The dissociation constant K_d is defined by

$$K = \frac{[S](1 - Y)([L] - Y[S])}{Y[S]}$$

where [L] is the concentration of ligand added and [S] is the concentration of binding sites. Expansion of this expression gives

$$Y = \frac{[S] + [L] + K_d - \sqrt{([S] + [L] + K_d)^2 - 4[S][L]}}{[2S]}$$

[2] *Fast* cytochrome *bo*: As prepared in our laboratory, this is defined as having a Soret maximum of 406.5 nm, a charge transfer band centered upon 624 nm, broad EPR signals with distinctive features at $g = 7.3$, $g = 3.7$, and $g = 2.68$, and binding 10 mM cyanide monophasically with an observed rate constant of 0.27 s^{-1}.

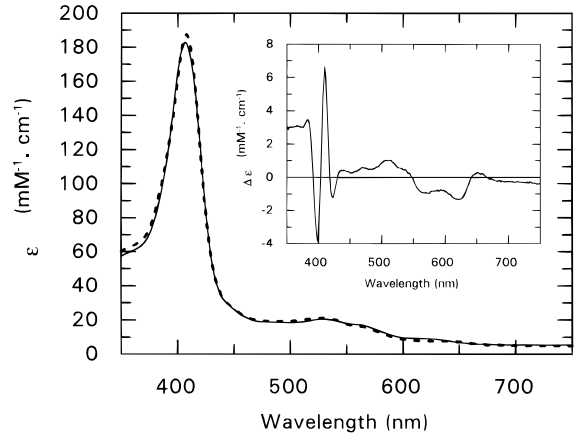

FIGURE 1: Electronic absorption spectra of *fast* cytochrome *bo* and its azide derivative. Cytochrome *bo* was made to a final concentration of 7.5 μM in 50 mM Bis-Tris propane, 0.1 mM EDTA, and 0.01% (w/v) dodecyl β-D-maltoside, pH 7.0. The spectrum shows *fast* cytochrome *bo* before (—) and after incubation with 1 mM sodium azide (- - -). The insert shows the difference spectrum of *fast* cytochrome *bo* + 1 mM sodium azide *minus* oxidized cytochrome *bo*.

The value of [S] was constrained to reflect a stoichiometry of azide binding of 1:1, and the value of K_d was calculated for each experiment using a nonlinear regression analysis done on a personal computer using Grafit v3.0 (Erithicus Software).

Kinetic Measurements. The binding of azide under pseudo-first-order conditions to cytochrome *bo* was measured in an Applied Photophysics Bio-Sequential DX.17MV stopped-flow spectrophotometer using a 1 cm path length cuvette. In each experiment, 500 data points were collected.

Treatment of Kinetic Data. The data were fitted to a single exponential process with software supplied by Applied Photophysics which uses a modernized and robust implementation of the Marquardt algorithm broadly based on the routine Curfit (Bevington, 1969). The dependence of the observed pseudo-first-order rate constants on azide concentration was analyzed using the linear regression routine found in the program Grafit v3.0 (Erithicus Software).

RESULTS

Electronic Absorption Spectroscopy. The changes in the room temperature electronic absorbance spectrum of fast cytochrome *bo* on addition of an excess of sodium azide are very slight (Figure 1). The difference spectrum, azide-bound cytochrome *bo* minus fast cytochrome *bo* (Figure 1; inset), shows there to be a shift in the Soret maximum from 406.5 to 407.0 nm. In the visible region of the spectrum, there is a small loss of intensity centered upon 560 nm and an increase in absorbance centered upon 500 nm. The shoulder near 625 nm arising from the high-spin ferric heme *o* charge transfer band is red-shifted to 634 nm. This manifests itself in the difference spectrum as a derivative-shaped feature. These small shifts and the retention of the charge transfer band indicate that heme *o* is still *high*-spin following azide binding.

Titration of cytochrome *bo* with small increments of azide monitored by ΔA_{412} (Figure 2) is consistent with a single site of azide binding and an observed dissociation constant at pH 7.5 of 17 ± 2.6 μM (n = 3).

EPR Spectroscopy. The lower panel of Figure 3 shows the X-band perpendicular-mode EPR spectra of azide-bound

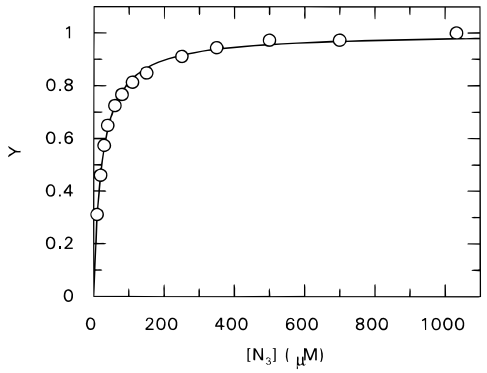

FIGURE 2: Titration of fast cytochrome *bo* with sodium azide. Cytochrome *bo* was made 6.6 μM in a final volume of 6 mL in 50 mM Bis-Tris propane, 0.1 mM EDTA, and 0.01% (w/v) dodecyl β-D-maltoside, pH 7.0. The sample was incubated with 50 units of catalase for 1 h prior to use. To both sample and reference cuvettes was added 3 mL of enzyme solution and a base line acquired. Sodium azide was added incrementally to the stirred sample cuvette from a concentrated stock solution. Twenty minutes after each addition, a new difference spectrum was acquired, and the change in absorption at 412 nm was plotted as a function of the total azide concentration. Open circles denote experimental data; the solid line is the best fit as described under Materials and Methods.

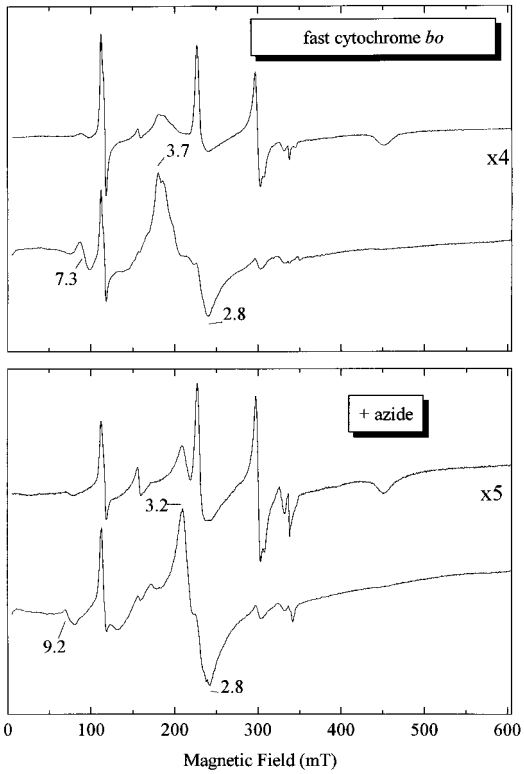

FIGURE 3: X-band EPR spectra of the fast and azide-bound forms of cytochrome *bo*. Protein concentrations were 640 μM for the fast form and 370 μM for the azide-bound form. For each sample, conditions were (upper spectrum) temperature, 10 K; microwave power, 2.03 mW; and (lower spectrum) temperature, 5 K; microwave power, 203 mW. All spectra: modulation amplitude, 1 mT.

cytochrome *bo*. At 10 K and with 2.02 mW of microwave power (upper trace), several sets of signals are observed. Those at g = 2.98, 2.26, and 1.50 are due to low-spin ferric heme *b* and are unchanged from the unliganded enzyme (Cheesman et al., 1993). In addition to signals near g = 4.3, g = 2.05, and g = 2.0 from adventitious ferric ion, cupric ion, and radicals, respectively, there is a feature near g = 6 from high-spin ferric ($S = {}^5/_2$) heme *o* which is uncoupled from Cu_B. This signal represents a few percent of possibly

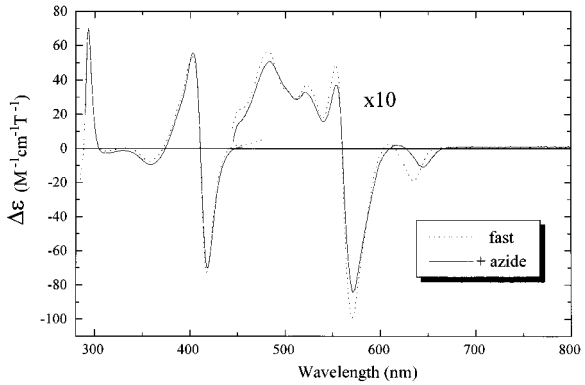

FIGURE 4: UV−visible room temperature MCD spectrum of the azide-bound form of cytochrome *bo*. Protein concentrations used were 55 μM (280−460 nm) and 370 μM (440−800 nm). Magnetic field, 5 T. The spectra of *fast* cytochrome *bo* are taken from Cheesman et al. (1994).

damaged protein and is unaltered by ligand additions (Cheesman et al., 1993). The important signals in this spectrum are a small derivative feature near $g = 9.3$ and the broad derivative with extrema at $g = 3.2$ and $g = 2.8$. As is seen in the lower trace, these features dominate the spectrum at high power and lower temperature (100 mW and 5 K). A similar pattern of one broad derivative feature below 1000 G with a second in the 1500−2500 G region is also observed for fluoride-bound and formate-bound cytochrome *bo*. These signals arise from the spin−spin interaction between heme *o* and Cu_B in the binuclear site and importantly show that heme o remains in the high-spin ferric state at low temperature.

The corresponding signals from the starting material, *fast* cytochrome *bo*, are illustrated in the upper panel of Figure 3. In this case, the important features are the broad derivative with extrema at $g = 3.7$ and 2.8 and the small derivative near $g = 7.3$ (Watmough et al., 1993).[3] However, this common pattern of EPR features is not evidence for a common site of ligand binding. We believe it indicates that the magnitude of the heme−copper spin coupling is not substantially altered between these ligand-bound forms. The significance of this may not become clear until the magnitude and mechanism of this coupling are firmly established.

MCD Spectroscopy. The MCD spectrum can give specific information as to the nature of the ligands bound to heme *o*. The UV−visible region spectrum is shown for azide-bound cytochrome *bo* in Figure 4. The spectrum is comparable in the pattern of bands observed to those reported for the fluoride-bound and formate-bound forms (Cheesman et al., 1994). Compared to the MCD spectrum of *fast* cytochrome *bo*, the Soret peak-to-trough intensity is unaltered, indicating

[3] In our original report of the broad signals arising from the binuclear center of *fast* cytochrome *bo* (Watmough et al., 1993), we noted that the low-field component of the signal had a minimum at $g = 9.1$ whereas the spectrum shown here has a derivative-shaped feature at $g = 7.3$. The precise line shape of the EPR signals arising from integer-spin transitions is very hard to determine under the conditions of low temperature and high power used, not least because the signals are sensitive to the enzyme concentration and the geometry of the EPR cuvettes. However, this particular discrepancy probably arises from the fact that while the *fast* conformer predominated in the preparation of cytochrome *bo* used in the previous study, other forms of the binuclear center were almost certainly present. In contrast, the spectrum presented here is typical of over 10 preparations from our laboratory and does not change after pulsing the enzyme according to the prescription of Moody and Rich (1994).

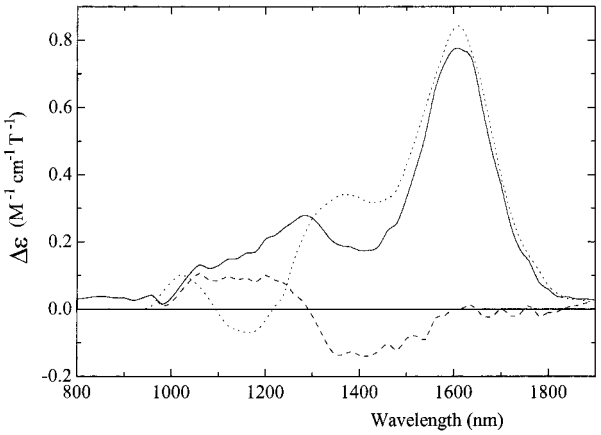

FIGURE 5: Near-infrared room temperature MCD spectrum of the azide-bound form of cytochrome *bo* (—). Protein concentration was 370 μM. Magnetic field, 5 T. The spectrum of fast cytochrome *bo* (•••) is taken from Cheesman et al. (1994). The azide−cytochrome *bo* minus heme *b* spectrum was obtained by subtraction as described in the text (- - -).

that there remains only one *low-spin* ferric heme in the enzyme. The same is true for the features in the region 400−600 nm. However, the weak negative band characteristic of high-spin ferric heme has moved from 635 nm to 645 nm. It is the energy of this band which correlates with the ligand distal to the histidine for a variety of high-spin ferric hemes. This particular wavelength would be consistent with two different modes of ligation involving histidine. For histidine/carboxylate coordination, the band has been observed between 634 and 645 nm (Dawson & Dooley, 1989; Kobayashi et al., 1977; Sievers et al., 1983). The binding of formate to heme *o* moves this band to 634 nm (Cheesman et al., 1994). This explanation is unlikely: no exogenous carboxylate has been added, and there is only limited evidence that an endogenous carboxylate ligand can with some difficulty coordinate heme *o* (Moody et al., 1995). However, the shift on azide binding still leaves the band at a wavelength which is also consistent with histidine/water ligation. The same feature is also observed at 645 nm for metmyoglobin (Vickery et al., 1976).

Figure 5 shows the near-infrared (NIR) MCD spectrum of azide-bound cytochrome *bo*. The only discernible features are a peak at 1610 nm and a sideband to higher energy. This is the characteristic form of a porphyrin (π)−ferric (d) charge-transfer band found between 800 and 2500 nm for low-spin ferric hemes (Gadsby & Thomson, 1990). The 1610 nm peak wavelength is diagnostic of bishistidine coordination, and the band, originating from heme *b*, is observed for all the oxidized cytochrome *bo* derivatives so far examined. Heme *b* is unchanged by the addition of ligands which bind to the binuclear site. However, in the high-spin ferric heme *o* derivatives of cytochrome *bo* (fast, F^-, $HCOO^-$), an additional derivative-shaped charge-transfer band from heme *o* was observed in the range 800−1300 nm (Cheesman et al., 1994). For comparison, the fast cytochrome *bo* spectrum is included in Figure 5. The characteristic high-spin ferric derivative feature is not obvious in the cytochrome *bo*−azide spectrum. It is possible that it has moved to longer wavelength and may be responsible for the apparent loss of heme *b* CT intensity around 1400 nm. This possibility is tested by subtracting the contribution of heme *b* from the spectrum of the azide-bound enzyme. The heme *b* contribution can be approximated by using the room

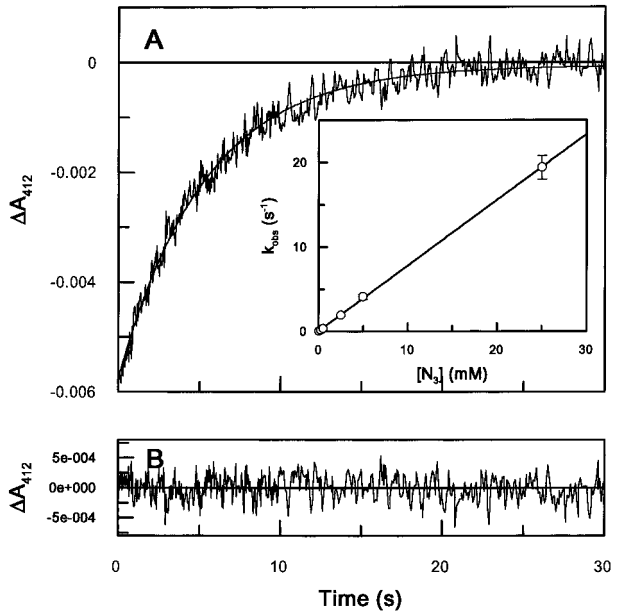

FIGURE 6: Kinetics of the reaction of fast cytochrome *bo* with sodium azide. The reaction was monitored at 412 nm in a stopped-flow spectrophotometer as described under Materials and Methods. Enzyme and ligand solutions were made in buffer containing 50 mM Bis-Tris propane, 0.1 mM EDTA, and 0.01% (w/v) dodecyl β-D-maltoside, pH 7.0. The concentration of enzyme after mixing was 1 μM. Panel A: Profile of the progress of the reaction at an azide concentration (after mixing) of 0.25 mM. The data are fitted to a single exponential process (k_{obs} = 0.1874 s^{-1}). Panel B: A plot of the residuals for this fit. Panel A (inset): Dependence of k_{obs} on azide concentration in the range 0.05−25 mM.

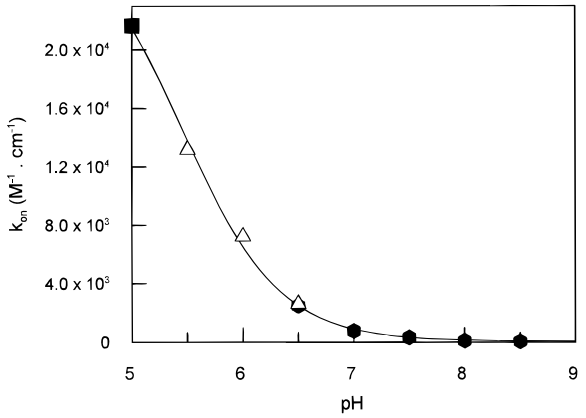

FIGURE 7: Dependence upon pH of the reaction of *fast* cytochrome *bo* with sodium azide. The value of k_{on} was determined in a number of different buffers and is plotted as a function of pH: 50 mM potassium phosphate/0.1 mM EDTA/0.01% (w/v) dodecyl β-d-maltoside (■); 50 mM MES/0.1 mM EDTA/0.01% (w/v) dodecyl β-D-maltoside (△); 50 mM Bis-Tris propane/0.1 mM EDTA/0.01% (w/v) dodecyl β-D-maltoside (⬢). The solid line is a fit to a single titratable group of pK_a = 5.2.

temperature NIR-MCD spectrum of fluoride-bound cytochrome *bo* (Cheesman et al., 1994). In this particular derivative, the CT band of high-spin ferric heme *o* is blue-shifted to ~900 nm, and the spectrum to longer wavelengths is that of low-spin ferric heme *b*. The spectrum obtained by this subtraction (Figure 5) contains a derivative feature centered near 1300 nm which we assign as the CT transition of high-spin ferric heme *o*. As was observed for the heme *o* CT transition near 630 nm (Figure 4), this band is significantly red-shifted compared to the corresponding features for fast cytochrome *bo*. Again, this is in contrast to the properties of Mb azide which exists at room temperature as a spin mixture, the minority high-spin population of which gives rise to a dispersion-shaped NIR-CT band centered near 900 nm (Nozawa et al., 1976). It is also important that no new *low-spin* ferric heme NIR-CT intensity is observed here, reinforcing the notion that azide binding does not effect a spin state change at heme *o*.

Kinetics of Azide Binding. The binding of azide to cytochrome *bo* under *pseudo*-first-order conditions followed simple monophasic kinetics under all conditions tested. The observed rate constants showed a linear dependence on apparent azide concentration from 0.05 to 25 mM (Figure 6). This contrasts with the case of cyanide binding to *fast* cytochrome *bo* where the value of k_{obs} is rate-limited at higher concentrations (R. H. Little and N. J. Watmough, unpublished data). The equilibrium constant at pH 7.5 was estimated from the ratio of k_{off}/k_{on} and is of the order of 8×10^{-5} M which is of the same order of magnitude as the value of 1.7×10^{-5} M obtained from equilibrium titrations. The second-order association constant (k_{on}) showed a strong dependence on pH (Figure 7) with an observed pK_a of 5.2. This is close to the pK_a[25] of hydrazoic acid of 4.75 (Cotton & Wilkinson, 1972), which argues for the active species being hydrozoic acid rather than azide ion. Since it is azide ion rather than hydrozoic acid which binds to the Cu_B (Yoshikawa & Caughey, 1992), then to maintain electrical neutrality within the binuclear center (Mitchell & Rich, 1994) the proton must bind at a second site close to Cu_B.

Effect of Formate on Azide Binding. The binding of formate to cytochrome *bo* is very slow compared with azide; at 2.5 mM formate, k_{obs} = 0.015 s^{-1} (Figure 8, panel A). The product of this reaction is a "slow" form of cytochrome *bo*, which is unreactive toward other anionic ligands and characterized by a blue-shifted Soret, a red-shifted charge-transfer band, and broad EPR signals with distinctive features at g = 13, g = 3.2, and g = 2.6 (Cheesman et al., 1994; Moody et al., 1995; Watmough et al., 1993). Incubation of fast CCO with formate gives a slow form of that enzyme (Moody et al., 1991) and also decreases the extent of the rapid phase of cyanide binding in a time-dependent manner, but not k_{obs} (Moody et al., 1991). This suggests that formate blocks the site of cyanide binding in CCO, explaining why the slow forms of the enzyme exhibit sluggish cyanide binding kinetics (Baker et al., 1987; Moody et al., 1991). In the case of cytochrome *bo*, formate decreases the extent of azide binding (Figure 8, panel B) and cyanide binding (Figure 8, panel C) in a time-dependent manner. Again the observed rate constants are unaffected. This suggests not only that formate also binds to Cu_B and blocks the binding of azide but also that it blocks the binding of cyanide [a ligand of heme *o* (Cheesman et al., 1994)]. The rate constants calculated by plotting the *extent* of ligand binding as a function of time are 0.019 and 0.016 s^{-1} for azide (Figure 8, panel B) and cyanide (Figure 8, panel C), respectively. Since both processes show identical kinetics to binding of formate to fast cytochrome *bo* as monitored by changes in the Soret difference spectrum (Figure 8A), it is inferred that formate is probably a bidentate ligand to both heme *o* and Cu_B.

DISCUSSION

The azide anion has been used as a probe of both heme and copper centers in a number of metalloproteins. When

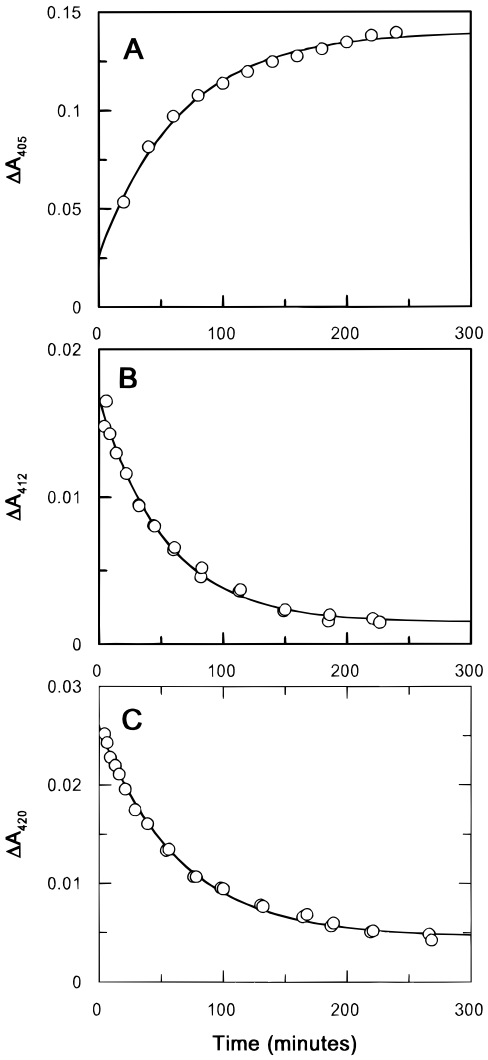

FIGURE 8: Reaction of fast cytochrome *bo* with sodium formate. (A) Change in absorbance at 405 nm: Cytochrome *bo* was made 7.4 μM in a final volume of 2.5 mL in a 1 cm path length stirred cuvette. Formate was added to a final concentration of 2.5 mM, and spectra were recorded every 20 min. The line is a fit to a single exponential process ($k_{obs} = 0.015$ s^{-1}). (B) Decrease in extent of azide binding: Cytochrome *bo* was made 2.6 μM in 50 mM TAPS/0.1 mm EDTA/0.01% dodecyl β-D-maltoside, pH 8.0. Formate was added to a final concentration of 2.5 mM, and the rate and extent of azide binding were determined in a stopped-flow spectrophotometer as described in the legend to Figure 6. The extent of azide binding monitored at 412 nm (○) is plotted as a function of time after the addition of formate. The line is a fit to a single exponential process ($k_{obs} = 0.019$ s^{-1}). (C) Decrease in the extent of cyanide binding: Cytochrome *bo* was made 1.1 mM in 50 mM TAPS/0.1 mM EDTA/0.01% dodecyl β-D-maltoside, pH 8.0. Formate was added to a final concentration of 2.5 mM, and the rate and extent of cyanide binding were determined. The extent of cyanide binding monitored at 420 nm (○) is plotted as a function of time after the addition of formate. The line is a fit to a single exponential process ($k_{obs} = 0.016$ s^{-1}).

azide binds to the sixth coordination site of the heme of metmyoglobin, it acts as a relatively strong-field ligand resulting in a thermal mixture of the high-spin ($S = {}^5/_2$) and low-spin ($S = {}^1/_2$) states at room temperature. Magnetic susceptibility, MCD, and EPR studies of Mb azide all confirm that $S = {}^1/_2$ is the ground state (Eglinton et al., 1983; Hori et al., 1989; Iizuka, I., & Kotani, 1968; Iizuki, T., & Kotani, 1968). Absorption and MCD studies show the system to be still ~80% in the low-spin form at room temperature (Smith & Williams, 1968; Vickery et al., 1976). In contrast, these data clearly show that no spin change occurs at either heme on binding of azide to cytochrome *bo*. In the RT MCD spectrum of Mb azide, the Soret and α bands have peak-to-trough intensities of 84 and 14.5 M^{-1} cm^{-1} T^{-1}, respectively (Vickery et al., 1976). Such intensities would be easily detected here. However, the data do suggest that heme *b* is largely unaffected by azide binding whereas the contributions to the spectra from heme *o* are altered.

The small spectral changes and the magnitude of the dissociation constant for azide binding to cytochrome *bo* suggest that this corresponds to the high-affinity phase ($K_D = 20-64$ μM) of azide binding to CCO (Li & Palmer, 1993; Wever et al., 1973b). In addition, CCO exhibits a second low-affinity phase of azide binding ($K_D = 20$ mM) in which the azide ion displaces one of the axial histidine ligands of heme *a* (Li & Palmer, 1993).

After the high-affinity phase of azide binding in CCO is complete, there are two notable features in the room temperature MCD spectrum reported by Li and Palmer (1993). First of all, there is no change in the intensity of the Soret MCD. This implies after azide is bound heme a_3 remains high-spin. This is in contrast to the case of cyanide binding to CCO in which the intensity of this transition doubles in response to the change of spin-state of heme a_3 (Thomson et al., 1985). Second, the position of the CT band centered upon 655 nm which is the heme a_3 analog of the 630 nm CT band of heme *o* changes. These observations are consistent with our own. However, the different nature of the hemes in cytochrome *bo* allows further conclusions to be drawn from the position of heme *o* CT bands, and this in conjunction with the EPR data leads us to the interpretation outlined below.

In contrast to CCO, the binuclear site of fast cytochrome *bo* gives rise to broad feature EPR features. These features underwent only minor shifts following the binding of azide. Although the same two-derivative pattern is maintained, more substantial changes in the EPR occur on binding formate or fluoride ion. Notably, the low-field derivative feature is markedly weaker for the fast and azide derivatives than for the fluoride and formate derivatives (Watmough et al., 1993). A similar division into two types of behavior occurs for CCO which binds formate and fluoride to give EPR spectra comparable to those of the same derivatives of cytochrome *bo* but has an EPR-silent binuclear site in the fast and azide-bound forms (Brudvig et al., 1981; Schoonover & Palmer, 1991; Van Gelder & Beinert, 1969).

Second, the MCD spectra of azide-bound cytochrome *bo*, specifically the 645 nm trough, suggest that the immediate ligands of heme *o* have not changed although small energy shifts are detected. This is strong evidence that the azide ion binds to Cu_B and not to heme *o* and raises the possibility that the differences in the EPR properties of the fast and azide-bound binuclear sites of CCO and cytochrome *bo* are to some extent due to differences in the heme ligand which is distal to histidine and therefore between the heme and Cu_B. This ligand could remain unchanged on azide binding, but when formate or fluoride is bound to either enzyme, this ligand is replaced by the exogenous anion giving common structures and therefore comparable EPR spectra from the two enzymes.

The binding of azide to Cu_B is supported by studies of azide binding to CCO monitored by infrared spectroscopies.

When azide binds to the binuclear center of CCO, new stretches centered at 2051 and 2041 cm^{-1} appear which were assigned to the nonspecific interaction of azide with protein and a Cu^{II}-N_3 species, respectively (Caughey et al., 1993; Yoshikawa & Caughey, 1992). This interpretation was challenged by Li and Palmer (1993), who using data obtained from FTIR spectroscopy argued that the 2051 cm^{-1} stretch is associated with the high-affinity phase of azide binding and is in fact the Cu^{II}-N_3 species. The same authors suggest the 2041 cm^{-1} stretch arises from the interaction of azide with oxidized heme *a*, an assignment cautiously endorsed by Caughey et al. (1993). These two studies fail to agree whether azide binds terminally to Cu_B or bridges between that center and heme a_3. The case for azide being a bridging ligand largely rests on the observation that cyanide is able to block the subsequent binding of azide (Li & Palmer, 1993).

We would suggest that the reason that the IR signatures associated with azide binding to Cu_B disappear if azido-CCO is subsequently incubated with cyanide is because the dissociation constant of cyanide binding to the binuclear center of heme−copper oxidases is approximately 1 mM (Van Buuren et al., 1972; R. H. Little and N. J. Watmough, unpublished data). This taken with evidence from MCD spectroscopy that cyanide is able to form a stable bridging ligand between heme a_3 and Cu_B (Thomson et al., 1982) suggests that it is not surprising that azide is unable to displace cyanide from the binuclear center.

Our interpretation that azide ion binds as a terminal ligand to Cu_B would not be inconsistent with recent structural information about the binuclear center of heme−copper oxidases. Crystals of a complex formed by a CCO from the soil bacterium *Paracoccus denitrificans* and the Fv fragment of a monoclonal antibody were obtained in the presence of 0.1% sodium azide (Iwata et al., 1995). This concentration of azide would saturate Cu_B, while a solvent molecule would remain bound to heme a_3 which might account for the unassigned electron density between the two metal centers observed by these authors. Interestingly, the structural model of the bovine enzyme based upon crystals obtained in the absence of inhibitors (Tsukihara et al., 1995) shows there to be electron density associated with all three nitrogenous ligands of Cu_B, while the *Paracoccus* structure shows no electron density as histidine-325 (H333 in the cytochrome *bo* sequence). These observations taken with our spectroscopic data might suggest that binding of azide to Cu_B causes one of the nitrogenous ligands to be displaced. The displacement of a protein ligand from a copper upon binding of azide has been observed in cuprozinc−superoxidase dismutase and galactose oxidase (Banci et al., 1989; Whittaker & Whittaker, 1993).

The calculated second-order rate constant for the binding of azide to cytochrome *bo* is about 800 M^{-1} s^{-1} at pH 7.5; this compares with a reported value of 6.5×10^3 M^{-1} s^{-1} for the rapid phase of the reaction of CCO at pH 7.3 with azide monitored at 558 nm (Wever et al., 1973a). However, unlike the reaction of azide with CCO, the rapid bimolecular reaction of cytochrome *bo* with azide is not followed by a slow process which is independent of azide concentration. This slower process in CCO has been attributed to a conformational rearrangement (Wever et al., 1973a), but perhaps is more likely accounted for by some heterogeneity in the enzyme preparation which resulted in a blue-shift in the Soret maximum (424−421 nm) upon addition of azide, an observation not reproduced by other workers.

The rapid bimolecular phase of azide binding to CCO exhibits a similar dependence upon pH (Wever et al., 1973a). This was also ascribed to hydrazoic acid being the active species. Unfortunately, the instability of the CCO preparation used in this study at low pH did not allow the determination of K_{on} below pH 6.2. However, based upon the reaction at pH 7.3, Wever et al. (1973b) calculated a value of K_{on} with respect to hydrozoic acid of 2.5×10^6 M^{-1} s^{-1}. This is about 2 orders of magnitude less than the limiting value at low pH we calculate for the reaction of hydrozoic acid at low pH (Figure 7). This difference is in part due to the fact that we use an experimentally determined value for the pK_a. Remaining differences may well be due to intrinsic differences between CCO and cytochrome *bo* as well as choice of experimental conditions.

That hydrozoic acid is the active species binding to the binuclear center of heme−copper oxidases is perhaps not surprising. A number of authors have emphasized the importance of maintaining electrical neutrality in catalytic centers of low dielectric constant (Millar et al., 1981; Mitchell & Rich, 1994). In order to achieve this, either proton(s) must be taken to compensate for reduction of the metal centers or anion binding, or, alternatively, the charge can be compensated by the loss of hydroxide ion(s). In this case, it would appear that a water molecule remains bound to heme *o* after azide binds to Cu_B. We suggest that charge compensation could be effected at Cu_B by the protonation of a bound hydroxide ion upon azide binding. The possibility that the charge is compensated in this way would mean that the ligands to Cu_B do not change their total charge, which may be important in retaining similar electronic properties of the cupric ion.

The observation that formate binding blocks both azide and cyanide binding is interesting. Previously it has been shown that formate binding to fast CCO causes changes in the EPR spectrum and reactivity toward other anionic ligands that are similar to those caused by the fast to slow transition. In particular, the extent of the rapid phase of cyanide (a bridging ligand) is decreased. Cytochrome *bo* does not readily undergo an endogenous fast to slow transition (Moody et al., 1995). However, the binding of formate to *fast* cytochrome *bo* produces a species that has EPR signatures similar to slow CCO (Moody et al., 1995; Watmough et al., 1993). Further, the magnitude of these EPR signals is directly proportional to the extent of the reaction of hydrogen peroxide with heme *o* (Moody et al., 1995). Here we demonstrate that formate ligation causes a decrease in the extent of cyanide (a bridging ligand) binding and azide (a terminal ligand to Cu_B) binding. This suggests that formate must bind to the binuclear center in a manner that makes both metal centers inaccessible to other ligands. Spectroscopic evidence would be consistent with direct formate binding to heme *o* (Cheesman et al., 1994), which taken together with the data presented here infers that formate binds as a bidentate ligand to both metal centers in cytochrome *bo*. A similar mode of ligation has been proposed for the binding of formate to CCO (Baker & Gullo, 1994). Such a ligation scheme might mediate the magnetic coupling between heme *o* and Cu_B which gives rise to the characteristic broad EPR signals of formate-liganded cytochrome *bo*.

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