Articles

Cyanide and Carbon Monoxide Binding to the Reduced Form of Cytochrome *bo* from *Escherichia coli*†

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ABSTRACT: Cyanide binds to fully reduced cytochrome *bo* and induces a blue shift of the Soret absorption band of the high-spin heme *o* and a change in the visible region spectrum consistent with the expected conversion to a low-spin state. The dissociation constant, determined by titration of the extent of the binding spectrum, is 7.0 ± 0.6 mM at pH 7.0. In contrast, cyanide does not bind significantly in this concentration range to the reduced form of cytochrome *bd*. The reduced cyanide compound of cytochrome *bo* can be laser photolyzed. Typically, less than 20% photolysis was attained with conditions that give essentially full photolysis of the carbon monoxide compound. The subsequent monophasic kinetics of recombination of cyanide at varying cyanide concentrations were used to determine k_{on}, k_{off}, and dissociation constant values at pH 7.0 of 572 ± 43 M^{-1} s^{-1}, 4.2 ± 0.7 s^{-1}, and 7.3 ± 1.3 mM, respectively. The dissociation constant changes very little in the pH range 6−8, indicating that a proton is bound together with the cyanide anion, as predicted by our recent proposal of a requirement for electroneutrality in the binuclear center [Mitchell, R., & Rich, P. R. (1994) *Biochim. Biophys. Acta* 1186, 19−26]. Competition studies confirm that cyanide and carbon monoxide cannot bind simultaneously, so that their binding sites must overlap. A small fraction of the reduced unliganded enzyme appears to have a distinct photolysis spectrum in the absence of added ligands, and this is transformed into a typical ferrous cyanide compound only at very high cyanide concentrations. Cyanide binding and photolysis were also examined in a number of mutant forms of cytochrome *bo*, and in a wild-type form which was partially depleted in Cu_B. Dramatic changes in rate constants and binding constants were found in several cases. Data from several mutants were compared with analogous data on the binding and photolysis of carbon monoxide, and the effects of mutation were quite different with the two ligands. A model is developed to explain the observed effects of point mutations on the recombination kinetics of both carbon monoxide and cyanide. Overall, the results indicate that the Cu_B site is required for binding of cyanide, but not carbon monoxide, to the reduced enzyme, possibly by providing the site for binding of the associated proton.

Bacterial cytochrome *bo* is a member of a superfamily of homologous protonmotive oxidases which includes the more intensively studied mitochondrial cytochrome *c* oxidase (Saraste, 1990). It has become evident that the copper−heme binuclear center is very similar in all of these oxidases even though the heme type varies. This binuclear center is the site of oxygen binding and reduction to water, and the notion that local ligand movements in the binuclear center drive proton translocation between proton channels in the protein structure (Mitchell, 1988) has become widely adopted as the most likely working model for the primary chemistry which drives vectorial proton translocation [for detailed variants on this theme, see Rich, (1991), Oliveberg and Malmström (1992), Woodruff (1993), Rousseau et al. (1993), and Babcock and Wikström (1992)]. The importance of understanding the protonation states of the stable intermediates and the requirement of electroneutrality in terms of elucidating the proton/electron coupling mechanism has been stressed recently (Rich, 1995).

Site-directed mutagenesis in bacterial systems of conserved possible metal ligands has allowed fairly reliable assignment of ligands to the three metal centers (two heme groups and one copper, Cu_B) in the critical subunit I of these oxidases, and has led to a consensus model of the possible folded protein structure around the metal centers (Lemieux et al., 1992; Minagawa et al., 1992; Brown et al., 1994; Calhoun et al., 1993a,b; Hosler et al., 1993). One useful technique for the characterization of the mutant forms of these oxidases has been the observation of the laser photolysis and recombination of carbon monoxide. It has already been reported that mutation of specific residues can lead to acceleration or retardation of the rate of recombination of carbon monoxide with the binuclear center (Brown et al., 1994; Lemon et al., 1993). Acceleration of recombination was also observed if the wild-type enzyme was grown in copper-limited conditions so that Cu_B is absent (Ciccognani et al., 1992; Moody et al., 1993). Since Cu_B is likely to act as an intermediate binding site as carbon monoxide moves

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from the aqueous phase onto the high-spin heme, it seems likely that acceleration of binding in some mutants is due to the loss of Cu_B, so that binding occurs directly onto the heme without prebinding to the copper. Evidence in support of this notion has come from the lack of saturation of the recombination rate with carbon monoxide in two mutants which show such accelerated recombination (Lemon et al., 1993). We have suggested that the retardation of carbon monoxide recombination in other mutants may arise from a Cu_B site in which the metal is retained but with a lower affinity for the carbon monoxide (Mitchell & Rich, 1994a).

We have now extended our studies by analysis of the laser photolysis and recombination of cyanide with the fully reduced form of cytochrome *bo*. It has already been shown that cyanide can bind to the reduced form of cytochrome *c* oxidase (van Buuren et al., 1972; Antonini et al., 1971), and photolysis and monophasic recombination have been observed (Hill & Marmor, 1991). In this paper, we extend our preliminary report that the same phenomena occur in cytochrome *bo* (Mitchell & Rich, 1994a), and show that information from cyanide photolysis studies can complement that obtained from studies of the photolysis and recombination of carbon monoxide. Data are compared with information available for binding of these ligands to other ferrous hemoproteins.

MATERIALS AND METHODS

Cell Growth and Membrane and Enzyme Preparation. The *Escherichia coli* strain RG145, which overexpresses cytochrome *bo* and lacks cytochrome *bd* (Au & Gennis, 1987), was used in the studies of the wild-type enzyme. Growth conditions and the methods used for isolation of membranes and purification of cytochrome *bo* were as described in Moody and Rich (1994).

The membranes from *E. coli* strains that express forms of cytochrome *bo* containing point mutations were supplied by Prof. R. B. Gennis (University of Illinois). In these strains, plasmids coding for the various point-mutated forms of cytochrome *bo* had been used to transform the host strain GL101, which expresses cytochrome *bd*, but lacks cytochrome *bo* (Lemieux et al., 1992). These strains were grown and membranes were isolated according to Lemieux et al. (1992), except that the growth was supplemented with 100 μg/mL $CuSO_4$.

Photolysis of Fully Reduced, Cyanide-Ligated Enzyme. Fully reduced cytochrome *bo* was formed by incubation of membranes or enzyme with solid sodium dithionite for several minutes in a buffer of 50 mM potassium phosphate and 2 mM ethylenediaminetetraacetic acid (EDTA)[1] at pH 7.0, until no further development of the reduced Soret band could be detected. Cyanide was added from a stock 2 M solution which had been neutralized before use. The cuvette was sealed to avoid loss of HCN. Room temperature photolysis was achieved with a flash from a frequency-doubled Nd-YAG laser (Spectron Ltd., Rugby, U.K.). This produced 10 ns pulses of light at 532 nm with energy in excess of 100 mJ/pulse. The photomultiplier was protected with appropriate filters to avoid flash artifacts. Transients were recorded separately at several wavelengths and signal-averaged if necessary. Kinetic spectra at various times after the flash were reconstructed from these transients as described previously (Brown et al., 1994).

Photolysis of Fully Reduced, Carbon Monoxide-Ligated Enzyme. The sample was treated as for cyanide above, but the cyanide addition was replaced by gassing of the samples for several minutes with mixtures of argon and carbon monoxide. Data were collected as for cyanide photolysis.

Preparation of Cu_B-Depleted Cytochrome bo. For the experiments comparing *E. coli* RG145 cells or membranes grown in the presence or absence of copper supplementation, the growth conditions and membrane preparation were as described in Moody and Rich (1994), with the 10 μM copper sulfate supplement to the medium being omitted as appropriate.

The degree to which the cytochrome *bo* lacked the Cu_B center could be monitored in intact cells during growth by laser flash photolysis of the carbon monoxide compound of fully reduced cytochrome *bo*. *E. coli* RG145 cells grown with a copper supplement (10 μM copper sulfate) show monophasic CO recombination kinetics [$k_{obs} \approx 50\ s^{-1}$ with 1 atm CO; see Brown et al. (1994)], indicative of a full complement of Cu_B. However, the cells grow equally well with only the trace copper levels in the medium (Figure 1A, compare ● and ▼), and the quantity of cytochrome *bo* is the same, as judged by the total extent of $\Delta A_{416-430nm}$ after photolysis of the carbon monoxide compound (Figure 1A, compare ○ and △). In the cells grown without copper supplementation, however, recombination of carbon monoxide is biphasic (Figure 1B), consistent with there being two populations of cytochrome *bo*, one containing Cu_B, for which $k_{obs} \approx 50\ s^{-1}$, and the other lacking Cu_B, for which $k_{obs} \approx 400\ s^{-1}$. The relative extent of the fast phase of carbon monoxide recombination increases as the cell density increases, reaching >70% at stationary phase in some cases.

RESULTS

Spectral Changes Induced by Binding of Cyanide to Reduced Cytochrome bo. Figure 2 illustrates the binding spectrum induced by the addition of 20 mM cyanide to a preparation of cytochrome *bo* which had been pre-reduced with sodium dithionite. In comparison to the carbon monoxide compound (also shown in Figure 2), a relatively small shift of the Soret region was observed, resulting in an asymmetric peak and trough at 424 and 434 nm, respectively. In the visible region, however, prominent spectral features were observed with peaks at 530 and 559 nm and troughs at 546 and 574 nm. Extinction coefficients of the cyanide compound of the reduced enzyme were calculated and compared to those of the better studied carbon monoxide compound. These are tabulated in Table 1.

The extent of the spectral shift in the visible region at 530−546 nm was titrated by stepwise addition of cyanide to a dithionite-reduced sample at pH 7.0 (Figure 2, inset). A curve fitted to these points by linear regression gives a value for the dissociation constant of 7.0 ± 0.6 mM.

We also tested whether a cyanide compound could be formed with the reduced form of the second terminal oxidase of *Escherichia coli*, cytochrome *bd*. In a membrane sample from an *E. coli* strain (GL101) that produces cytochrome *bd*, but not cytochrome *bo*, at concentrations of cyanide up

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; K_D, dissociation constant; k_{obs}, observed pseudo-first-order rate constant; numbering of amino acids refers to the sequence of subunit I of the wild-type enzyme from *E. coli* (Chepuri et al., 1990).

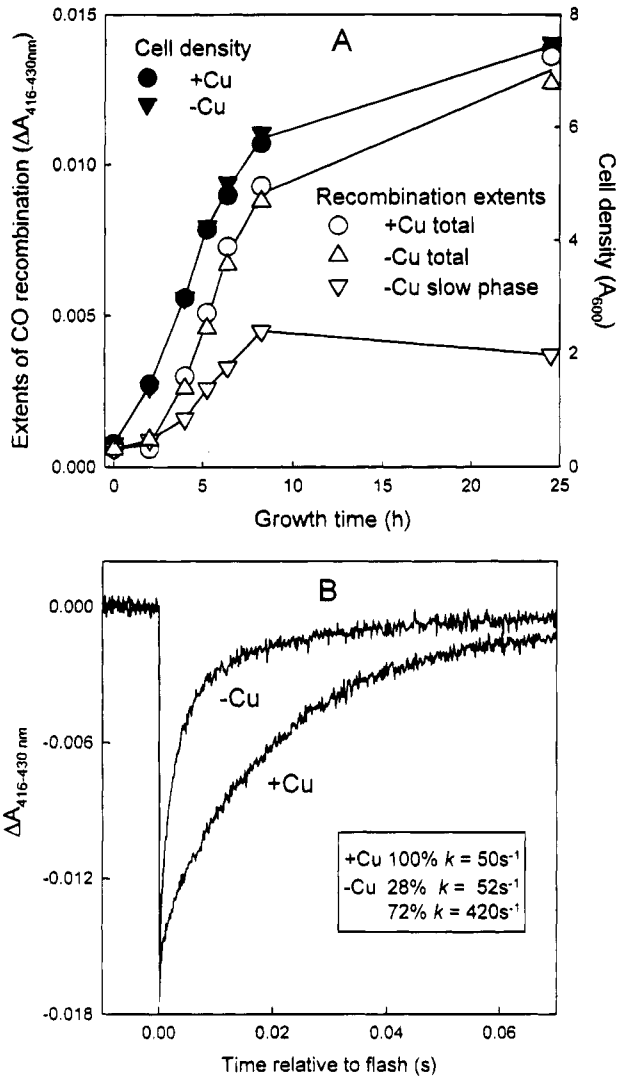

FIGURE 1: Effects on CO recombination kinetics of growth of *E. coli* RG145 in the presence and absence of 10 μM copper sulfate. Panel A shows the time courses of A_{600} for parallel growths with (●) and without (▼) copper supplementation (10 μM copper sulfate). Also shown are extents of the slow ($k_{obs} \approx 50\ s^{-1}$) and fast ($k_{obs} \approx 400\ s^{-1}$) phases of $\Delta A_{416-430nm}$ of the recombination of carbon monoxide after photolysis at 20 °C and 1 atm CO (see Materials and Methods and panel B). The combined extents of the two phases (○, △), and the extent of the slow phase only (▽) are shown. These were obtained by curve-fitting using the Marquardt algorithm (Press et al., 1992) to data such as those in panel B. Panel B shows the CO photolysis and recombination transients for *E. coli* RG145 grown for 24.5 h with (+Cu) and without (−Cu) copper supplementation. Samples of cells (200 μL) were diluted to 1.6 mL with 50 mM potassium phosphate and 0.5 mM K-EDTA at pH 7.0. Solid sodium dithionite was then added, and the sample was bubbled vigorously with carbon monoxide for 30 s. The time courses shown are derived from the average of 50 transients taken at each wavelength, with 1 s of dark adaptation between flashes.

to 200 mM spectral changes were negligible, so that there was no evidence for the existence of such a compound. Hence, measurement of the extent of the cyanide compound offers a possible method to quantitate the level of cytochrome *bo* in membrane samples containing both oxidases, and has proved useful at Glynn for estimating levels of oxidase in the presence of other hemoproteins (B. Meunier unpublished).

Photolysis of the Cyanide Compound of Reduced Cytochrome bo. Figure 3A illustrates the transient spectra

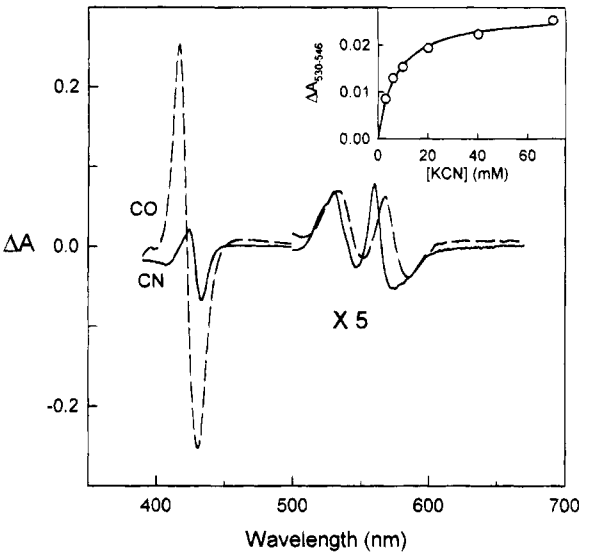

FIGURE 2: Comparison of the binding spectra of cyanide and carbon monoxide with reduced cytochrome *bo*. Purified cytochrome *bo* was dissolved to around 2.6 μM (based on the carbon monoxide compound) in 50 mM potassium phosphate and 2 mM EDTA at pH 7.0, and the sample was fully reduced by addition of a small amount of solid sodium dithionite. Spectra are the difference spectra, relative to the reduced enzyme, induced by addition of 20 mM cyanide (solid line) or saturating carbon monoxide (dashed line). The inset shows the extent of formation of the cyanide compound at 530−546 nm on the titration of an identical sample with aliquots of neutralized potassium cyanide.

Table 1: Extinction Coefficients of Binding of Cyanide and Carbon Monoxide to Purified Cytochrome *bo*

spectrum	wavelength pair (nm)	extinction coefficient ($mM^{-1}\ cm^{-1}$)[a]
reduced minus oxidized	427−460	235
	560−580	20.5
reduced/HCN minus reduced	424−434	56
	530−546	11.3
	560−575	16.6
reduced/CO minus reduced	416−430	196
	534−552	6.5
	568−586	7.8

[a] All values are quantified relative to the reduced minus oxidized spectrum at 560−580 nm, which is assumed to have an extinction coefficient of 20.5 mM^{-1} (Minghetti et al., 1992).

observed in the Soret region at various times after laser photolysis of a sample of reduced, cyanide-ligated cytochrome *bo*. The constancy of peaks, troughs, and isosbestic points during decay, together with the monophasic decay kinetics, indicates that a simple one-step recombination process is occurring.

In Figure 3B, this transient photolysis spectrum is compared with the static spectrum elicited by binding of 20 mM cyanide to the reduced enzyme. An enzyme sample was reduced with dithionite, and the reduced *minus* oxidized spectrum was recorded (solid line). 20 mM cyanide was then added, and the static cyanide binding spectrum was obtained (dashed line). The photolysis data taken 1.8 ms after the laser flash were then inverted, normalized to the static spectrum, and plotted as filled circles over the data in Figure 3B. It is clear that the static (reduced *plus* cyanide) *minus* (reduced) difference spectrum is essentially the inverse of the photolysis spectrum, with a peak and trough at 424 and 434 nm, respectively, again indicative of a simple

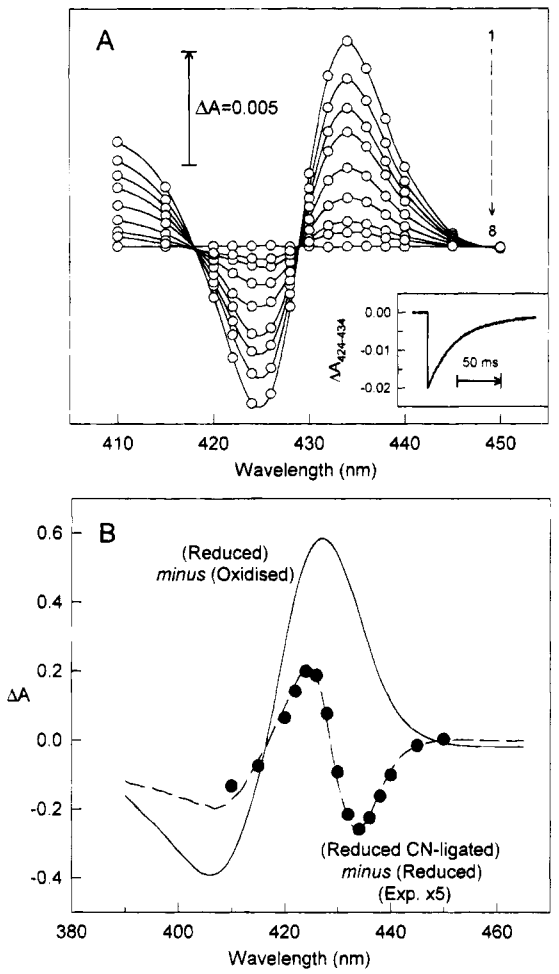

FIGURE 3: Transient photolysis spectra of the cyanide compound of cytochrome *bo* and comparison with a static cyanide binding spectrum. In panel A, purified cytochrome *bo* was reduced in Figure 2 and treated with 75 mM potassium cyanide. Transients induced by the laser flash were recorded at 15 wavelengths, and each was signal-averaged 10 times with 1 s dark adaptation between flashes. A typical transient response is shown in the inset. Spectra were calculated from individual transients at 1.8, 5.6, 9.4, 13, 21, 32, 52, and 71 ms after the laser flash. In panel B, an identical enzyme sample was used. Solid sodium dithionite was added, and the reduced *minus* oxidized spectrum was recorded when the sample had stabilized (solid line). 20 mM potassium cyanide was then added. The small blue shift induced by cyanide is shown as the difference spectrum (reduced *plus* 20 mM cyanide) *minus* (reduced), and is plotted (dashed line) at a 5-fold expansion compared to the reduced *minus* oxidized spectrum. For comparison, the 1.8 ms transient spectrum from panel A was inverted and normalized to the static cyanide binding spectrum, and is plotted as the filled circles over the cyanide binding spectrum.

photolysis and recombination process. However, the transient spectrum was only around one-fifth of the size of the static spectrum (with different enzyme batches, we found that the photolysis yield ranged between 7 and 24%). A low photolysis yield is also reported with the cyanide compound of reduced cytochrome *c* oxidase (Hill & Marmor, 1991).

Figure 4 illustrates the transient kinetic response to a laser flash of a sample of purified, reduced, wild-type cytochrome *bo* in the presence of increasing concentrations of cyanide at pH 7. The size of the transient increased as the concentration of cyanide was increased, consistent with the titration of the formation of the cyanide compound over this concentration range (Figure 2, inset). A plot of observed

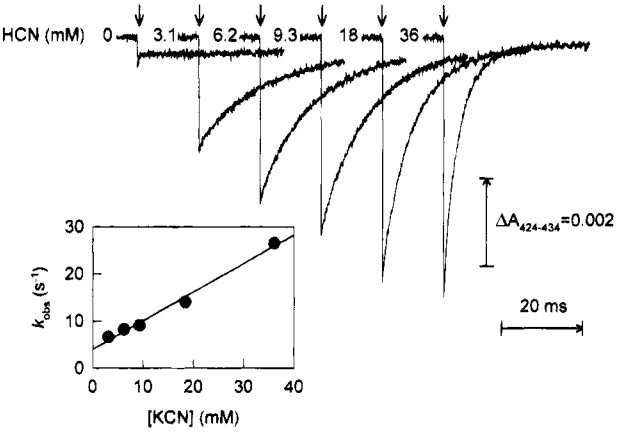

FIGURE 4: Photolysis of the cyanide compound of cytochrome *bo* at various concentrations of cyanide. Purified cytochrome *bo* was dissolved in a buffer of 50 mM potassium phosphate and 2 mM EDTA at pH 7.0 to a final concentration of 1.4 μM. The sample was incubated with a small amount of solid dithionite until the enzyme had become fully reduced. Ten transients were signal-averaged at 424 and 434 nm. These measurements were repeated in the presence of increasing concentrations of cyanide as indicated. The plot (inset) is derived from these data by fitting an exponential decay with rate constant k_{obs} to each 424−434 nm decay. The *y*-axis intercept indicates k_{off}, and the slope is a measure of k_{on}.

rate constant, k_{obs}, *versus* ligand concentration gave a straight line plot from which k_{on}, k_{off}, and k_D could be determined (Figure 4, inset). For the wild-type enzyme, these values at pH 7.0 were determined by linear regression to be the following: k_{on}, 570 M^{-1} s^{-1}; k_{off}, 4.2 s^{-1}; and K_D, 7.3 mM.

It can be seen that a small photochemical response of the fully reduced enzyme occurs even in the absence of any added ligand. Its spectrum is different from that of the cyanide compound, having peak, isosbestic, and trough positions at 418, 425, and 432 nm, respectively. Furthermore, its recombination rate of 1 s^{-1} is cyanide concentration-independent. The amount varies between preparations, but is typically less than 5% of the cyanide compound signal when measured at 424−434 nm. We have previously noted such small ligand-independent changes in cytochrome *c* oxidase (unpublished). Since the photolysis yield of the cyanide compound is low in comparison to that of the carbon monoxide compound, this background photolysis of the unliganded enzyme is particularly noticeable in Figure 4. The signal size appears to remain the same at cyanide concentrations sufficient to generate most of the cyanide compound and, therefore, most likely represents a very small fraction of enzyme as prepared in an unidentified ligand-bound state. It can be displaced by cyanide only with high (≥0.1 M) cyanide concentrations.

The experiment of Figure 4 was repeated at pH values of 6 and 8, and plots of k_{obs} *versus* cyanide concentration gave almost identical results for rate and binding constants (data not shown). Since the predominant form of cyanide is HCN at these pH values (pK_a of HCN = 9.31 at 25 °C; *CRC Handbook of Chemistry and Physics*, 1987), this pH-independence of the binding constant and binding rate demonstrates that binding of cyanide to the reduced enzyme must also result in binding of a proton; i.e., HCN, not CN^-, is overall taken up from solution. This finding extends the data in support of our previous generalization from experiments with cytochrome *c* oxidase that all binuclear center ligand changes are electroneutral (Rich, 1995; Mitchell et al., 1992; Mitchell & Rich, 1994b).

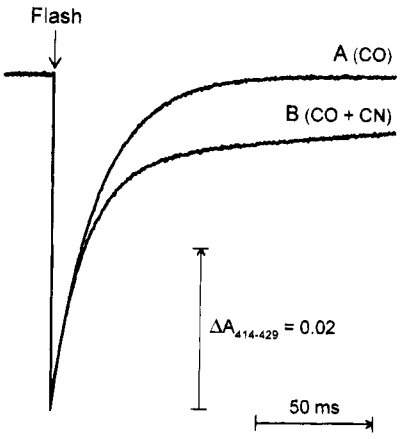

FIGURE 5: Photolysis behavior of reduced cytochrome *bo* in the presence of cyanide and carbon monoxide. Purified cytochrome *bo* was dissolved in a buffer of 50 mM potassium phosphate and 2 mM EDTA at pH 7.0 to a final concentration of 0.8 μM and reduced with sodium dithionite. The sample was saturated with carbon monoxide (approximately 1 mM). Five transients were recorded at each wavelength with 1 s of dark adaptation between flashes, and the averaged transient at 414−429 nm was plotted (trace A). Neutralized potassium cyanide was added under a CO atmosphere to the same sample to a final concentration of 40 mM, and the measurement was repeated (trace B). The wavelength pair was chosen in order to exclude any cyanide binding contribution to the signal.

Competitive Binding of Carbon Monoxide and Cyanide to the Reduced Enzyme. It has already been reported that the binding of cyanide and carbon monoxide to reduced cytochrome oxidase are mutually exclusive (Orii, 1985), although this conclusion has been challenged recently (Tsubaki & Yoshikawa, 1993; Hill, 1994). We investigated this point in cytochrome *bo* in the experiment illustrated in Figure 5. Reduced cytochrome *bo* was dark-adapted in the presence of 1 mM carbon monoxide, and the control photolysis and recombination kinetics were recorded (trace A). Cyanide (40 mM) was then added to the same sample. Because of the much higher affinity of the ferroheme *o* for carbon monoxide (K_D = 1.4 μM; Mitchell & Rich, 1994a) than for cyanide (K_D = 7−8 mM), 99% of the enzyme will be in the carbon monoxide-ligated form at equilibrium. If cyanide can bind simultaneously and independently, then all enzymes will also be almost saturated with bound cyanide. Photolysis of the sample in the presence of both ligands again resulted in a full dissociation of the carbon monoxide, as judged from the size of the laser-induced transient (Figure 5, trace B), and the photolysis spectrum (not shown) was consistent with photolysis of the carbon monoxide adduct. However, in contrast to photolysis in the presence of 1 mM carbon monoxide alone, where all of the enzyme recombines with a k_{obs} of 52 s^{-1} (Figure 5, trace A), recombination of carbon monoxide when 40 mM cyanide was also present occurred heterogeneously (Figure 5, trace B). Around 75% of the population recombined with a rate constant close to the cyanide-free control, but the remainder recombined much more slowly. The slow fraction increased as the ratio of cyanide to carbon monoxide in the medium was increased.

These kinetics of recombination in the presence of both ligands can be simulated in a model in which all enzymes have only carbon monoxide bound before the flash, all of which photodissociate into the unliganded state on laser activation. Carbon monoxide and cyanide then compete for a single stable binding site according to their known binding and dissociation rate constants. A best fit was obtained for this simple competitive model using values of k_{on} for CO = 54 s^{-1} at 1 mM CO, k_{off} for heme O^{2+}−CO = 0.077 s^{-1} (see Table 3), k_{on} for cyanide at 40 mM cyanide = 17.6 s^{-1}, and k_{off} for heme o^{2+}−HCN = 4.4 s^{-1}. Those enzymes which first bind cyanide can then bind carbon monoxide only slowly, since the cyanide must first dissociate from the common binding site.

In contrast to the close fit between data and simulation with the above model, simulation with any model in which both ligands can bind simultaneously was not possible. Hence, the data in Figure 5 definitively confirm the competitive nature of binding of cyanide and carbon monoxide to a single site in the reduced enzyme.

Carbon Monoxide and Cyanide Binding to Copper-Depleted Cytochrome bo. Figure 6A shows a comparison between the static binding spectra in the visible region for two membrane samples derived from *E. coli* RG145 cells, one batch grown with copper supplementation (+Cu membranes, solid lines), and the other batch grown without copper supplementation (−Cu membranes, dashed lines). The carbon monoxide binding spectra in the visible region of both enzymes are similar, both qualitatively and quantitatively.

The recombination kinetics after flash photolysis of carbon monoxide from the fully reduced forms of these enzymes are shown in Figure 6B. The biphasic kinetics of the −Cu membranes are identical to those of the cells from which they were derived (data not shown). The relative extents of the two phases can be equated directly with the relative levels of enzyme that has or has not got Cu_B (see Materials and Methods section, and Figure 1), in this case 55% and 45%, respectively.

Figure 6A also shows a comparison between the static cyanide binding spectra for the same two dithionite-reduced membrane samples. The size of the cyanide binding spectrum of the +Cu membranes is consistent with the expected (≈85%) extent of formation of the cyanide adduct at the concentration of cyanide used. However, although qualitatively the same, the binding spectrum of the −Cu membranes had only 51% of the predicted amplitude. This is consistent with cyanide binding only to the fraction (55%) of the enzyme that has Cu_B present, with no significant binding at all to the fraction that lacks Cu_B.

Cyanide Binding to Mutant Forms of Cytochrome bo. We have also studied cyanide binding and photolysis in mutant forms of cytochrome *bo*, and these results are summarized in Table 2. Several mutant forms displayed no signal with 20 mM cyanide that could be attributed to the formation of a reduced heme *o*−cyanide complex. Included in this class are H106L, Y288C, Y288F, H333L, H334L, K362L, K362M, H419L, H419N, and H421L. In several of these (H106L, Y288C, and H419L/N), there was also no carbon monoxide compound, indicating that the high-spin heme is absent. For the mutant H411L, a signal could be observed immediately after cyanide addition, but this slowly disappeared ($t_{1/2}$ ≈ 10 min), indicating an unstable structure in this case.

In all other mutant forms tested in Table 2, binding and photolysis spectra characteristic of a cyanide compound were detected. However, quite large effects on the k_{on} and k_{off} rate constants, and therefore on the binding constants, were commonly observed.

Comparison of Mutation Effects on Cyanide and Carbon Monoxide Photolysis Parameters. We chose to further

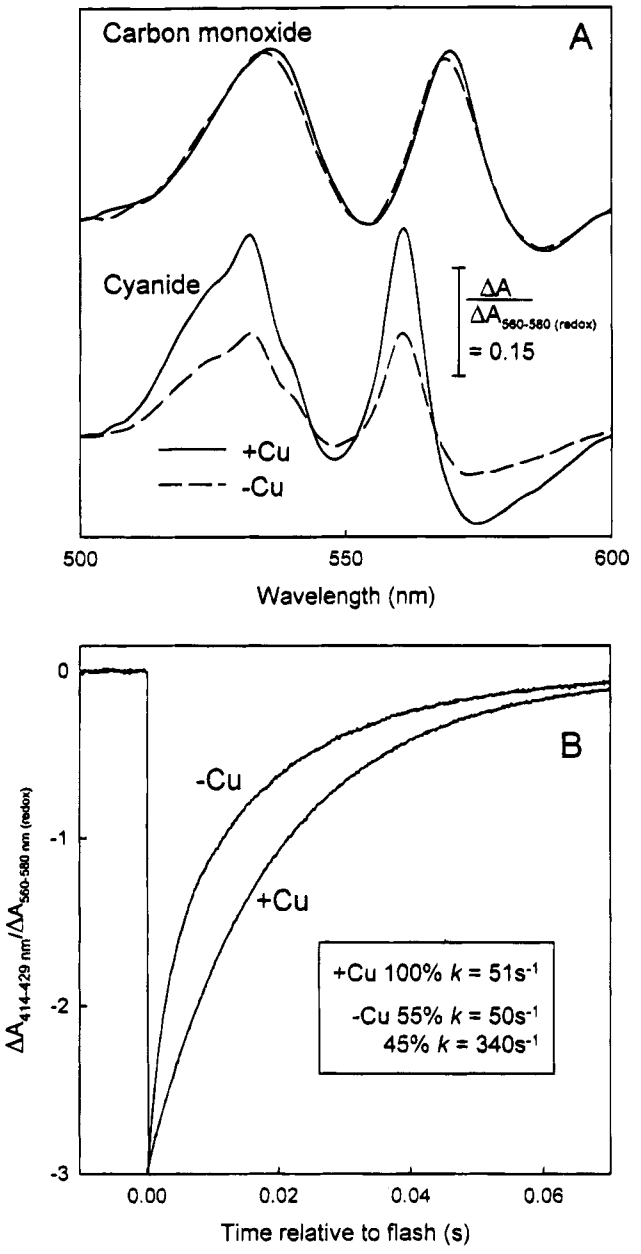

FIGURE 6: Comparison of static binding spectra and recombination kinetics of carbon monoxide of cytochrome *bo* in membrane samples derived from *E. coli* RG145 cells grown with or without copper supplementation. Membranes were diluted in 50 mM potassium phosphate and 0.5 mM K-EDTA at pH 7.0 to a final cytochrome *bo* concentration of approximately 2 μM and reduced by incubation with solid sodium dithionite for 5 min. Either saturating carbon monoxide or 40 mM potassium cyanide was added to produce the difference spectra shown in panel A. The spectra are normalized relative to $\Delta A_{560-580}$ obtained from a dithionite-reduced *minus* air-oxidized spectrum, and then corrected for light-scattering using the quadratic-base line-compensation method (Brown et al., 1993; using approximate isosbestic points at 500, 552, and 624 nm in all cases). The slight distortion of the spectra caused by the latter procedure is negligible in the current context. A comparison of the recombination kinetics in the presence of saturating carbon monoxide at 20 °C is shown in panel B. The copper-supplemented membrane sample exhibited monophasic recombination with a rate constant of 51 s^{-1}, typical of the Cu_B-containing enzyme. In contrast, the copper-depleted membrane sample shows biphasic kinetics with 55% at 50 s^{-1} and 45% at 340 s^{-1}.

examine the mutants H284L, H333L, and H334L in order to try to understand better the possible origin of the mutation effects on the cyanide binding parameters. Of particular interest was a comparison of the effects of point mutations on cyanide kinetics with their effects on carbon monoxide recombination behavior. Our previous observations on these mutants (Brown et al., 1994) were extended to include carbon monoxide concentration dependency of rates, as already described by Lemon et al. (1993), so that k_{on}, k_{off}, and K_D values could also be determined. Typical data are illustrated in Figure 7.

Table 2: Comparison of Cyanide Binding Parameters[a] of Wild-Type and Mutant Forms of Cytochrome *bo*

mutation	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (mM)
purified *bo*	572 ± 43	4.2 ± 0.7	7.3 ± 1.3
membrane *bo*	294 ± 45	3.2 ± 1.3	11 ± 5
H106L	− (no *bo*)[b]		
Y288C	− (no *bo*)[b]		
H419L	− (no *bo*)[b]		
H419N	− (no *bo*)[b]		
Y288F	− (no CN signal)[c]		
H333L	− (no CN signal)[c]		
H334L	− (no CN signal)[c]		
K362L	− (no CN signal)[c]		
K362M	− (no CN signal)[c]		
H421L	− (no CN signal)[c]		
D135N	182 ± 31	5.2 ± 3.1	29 ± 7
H284L	631 ± 103	15 ± 2	24 ± 5
H284Q	3542 ± 166	5.1 ± 2.2	1.4 ± 0.6
E286A	225 ± 25	3.3 ± 0.7	14 ± 4
E286Q	496 ± 31	3.3 ± 0.5	6.6 ± 1.1
Y288H	4147 ± 265	119 ± 6	29 ± 2
Y288S	27433 ± 592	98 ± 14	3.6 ± 0.5
T352S	94 ± 9	0.85 ± 0.16	9.0 ± 1.9
M353A	108 ± 5	2.6 ± 2	24 ± 2
P358A	1108 ± 134	7.7 ± 3.7	6.9 ± 2.6
T359S	195 ± 19	2.7 ± 0.4	14 ± 2
H411G	665 ± 45	1.4 ± 0.6	2.1 ± 0.9
H411L	− (unstable cyanide compound)[d]		
R481Q	1687 ± 33	4.0 ± 0.6	2.4 ± 0.4
R482Q	251 ± 20	2.4 ± 0.5	9.6 ± 2.1

[a] Kinetic parameters of cyanide binding to reduced cytochrome *bo* were determined by the titration of photolysis behavior with cyanide, as shown in Figure 4. Photolysis yield varied between 3 and 30% in different mutant forms. [b] These membranes failed to show a carbon monoxide compound characteristic of the presence of the ferrous heme *o* of cytochrome *bo*. [c] These membranes displayed carbon monoxide compounds characteristic of the ferrous heme *o* of cytochrome *bo*, but did not bind cyanide. [d] In this case, the cyanide compound was too unstable to allow accurate determination of kinetic parameters.

For the H284L form, the kinetic parameters could be accurately determined from the plot of observed rate constant *versus* carbon monoxide concentration. In the case of the wild-type, however, the k_{off} value could not be accurately measured by this method owing to the much lower K_D, and an alternative procedure was used. Cytochrome *bo* was incubated with a slight excess of sodium dithionite and bubbled with carbon monoxide so that the reduced CO compound was formed. The sample was then aerated with a stream of air so that any excess dithionite was consumed and the sample became aerobic. Formation of the oxidized enzyme was monitored at 422−406 nm. We assume that this reaction is rate-limited by the dissociation rate constant of carbon monoxide from the reduced enzyme, as is the case for wild-type cytochrome oxidase where the rate is a direct measure of the dissociation rate constant of 0.023 s^{-1} (Gibson & Greenwood, 1963).

In addition to these measurements, the degree of cyanide binding to the oxidized forms of these mutant enzymes was

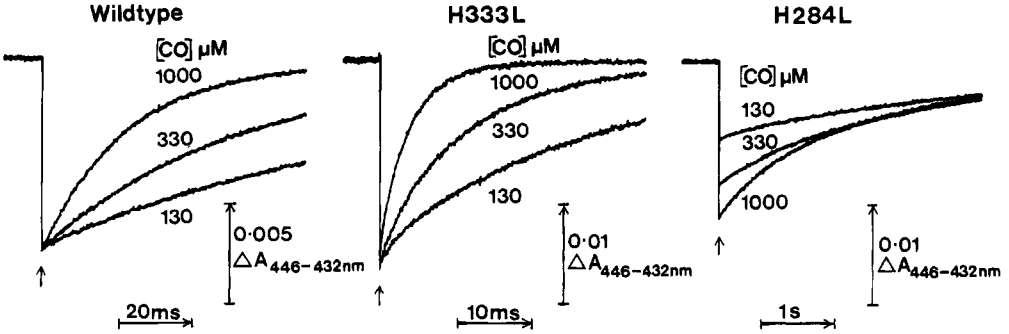

FIGURE 7: Carbon monoxide concentration dependency of recombination rates in wild-type and mutant forms of cytochrome *bo*. Purified cytochrome *bo* or membrane samples derived from the H333L or H284L mutant strains of *E. coli* were dissolved to a final enzyme concentration of around 0.25 μM in 50 mM potassium phosphate and 2 mM EDTA at pH 7.0. Samples were then reduced with solid sodium dithionite, gassed for 3 min with mixtures of carbon monoxide/argon, and sealed to prevent gas exchange. For each concentration of carbon monoxide, four transients were signal-averaged at 432 and 446 nm, with a dark time of 10 s between flashes. Data are plotted as transients at 446−432 nm, a wavelength pair which minimizes any interference from the background cytochrome *bd* which is also present in the membrane samples.

Table 3: CO and Cyanide Binding Characteristics of Reduced Membrane-Bound Cytochrome *bo* from Wild-Type and Selected Mutant Strains of *E. coli*, and Cyanide Binding Characteristics of Oxidized Samples[a]

	CO binding (reduced)			CN binding (reduced)			CN Binding (oxidized)
membrane type	$\Delta A_{416-430}$	k_{on} (s^{-1})	k_{off} (s^{-1})	$\Delta A_{530-546}$	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	$\Delta A_{420-402}$
wild-type (RG145)	0.139	50	0.08	0.0052	460	4.5	0.067
H284L	0.082	0.6	0.3	0.0019	630	15	0.045
H333L	0.100	250	*b*	≤0.0003			0.046
H334L	0.041	450	*b*	≤0.0003			0.046

[a] In parallel experiments, samples were either dithionite-reduced and treated with saturating CO (1 mM) or 20 mM KCN, or else preoxidized 10 min with 0.2 mM potassium ferricyanide and then allowed to react to completion with 20 mM KCN (5−10 min). See text and Figure 4 for details of measurement of kinetic constants. [b] Not determined.

measured. For these experiments, membrane samples were preincubated with 0.2 mM potassium ferricyanide for 10 min before two sequential additions each of 10 mM neutralized potassium cyanide. In all mutants tested (H284L, Y288F, Y288H, H333L, H334L), the spectral change at 420−402 nm induced by cyanide addition was at least as large as that induced in a membrane sample containing wild-type enzyme.

Table 3 summarizes the ligand binding data obtained by the above methods with the wild-type and selected mutant enzymes.

DISCUSSION

The cyanide anion is a well-known ligand of pentacoordinated heme iron. It binds more strongly to ferric than to ferrous heme because of the higher net positive charge of the former, and is a particularly strong ligand to ferric heme in accordance with its position in the spectrochemical series. However, because ferrous heme is uncharged overall, cyanide binding to this form is generally much weaker.

In contrast, uncharged ligands such as carbon monoxide bind more strongly to ferrous than to ferric heme. This arises from the additional electron on ferrous heme which is available for orbital interactions with the ligand. In the case of carbon monoxide, this electron provides additional bonding energy through interaction of the iron d orbitals with the antibonding p orbital of the carbon atom of the ligand.

Cyanide Binding in Relation to Other Reduced Hemoproteins. Cyanide binding to the reduced forms of a variety of high-spin hemoproteins has been studied, including ferrous myoglobin (Bellelli et al., 1990), ferrous hemoglobin (Brunori et al., 1992), and horseradish peroxidase (Phelps et al., 1971). In several cases, for example in hemoglobin and myoglobin, the affinity for cyanide is extremely weak [dissociation constants are of the order of 1 M (Brunori et al., 1992) and 0.4 M (Bellelli et al., 1990), respectively] so that stable compounds can only be observed at very high cyanide concentrations. We have recently found (Meunier and Rich, unpublished) that the flavohemoglobins found in *Escherichia coli* (Orii et al., 1992; Ioannidis et al., 1992; Andrews et al., 1992) and in yeast (Oshino et al., 1973; Zhu & Riggs, 1992) also have large dissociation constants for cyanide from their reduced forms of ≥0.5 M. In contrast, the affinity of ferrous horseradish peroxidase for cyanide is much greater. In this case, a dissociation constant in the millimolar range has been determined, and it has been established that HCN, rather than the cyanide anion, is the net species bound (Phelps et al., 1971). Cytochrome *c* peroxidase shows a similar behavior to horseradish peroxidase (Meunier and Rich, unpublished).

The binding spectra induced by cyanide in the visible region in all of these ferroheme B complexes are smaller and spectrally quite distinct from the cyanide binding spectra induced in reduced bovine and yeast cytochrome *c* oxidase, where it has been shown that cyanide binds with a submillimolar K_D and induces a prominent spectral change in the visible region (van Buuren et al., 1972; Antonini et al., 1971).

The cyanide compound of reduced cytochrome *bo* is optically rather similar to the ferroheme B−cyanide compound of the peroxidases, as expected from the similarity of the porphyrin ring structures of hemes B and O (Wu et al., 1992). This similarity extends to the similar relative extinction coefficients in the visible band region of the cyanide and carbon monoxide compounds, and the size and

symmetry of the Soret band cyanide difference spectrum (Figure 2). However, although it is spectrally quite different from the ferroheme A−cyanide compound of cytochrome oxidase, it resembles it in terms of its relatively low dissociation constant.

Protonation Associated with Cyanide Binding. We have already demonstrated with bovine cytochrome *c* oxidase that cyanide binding to both oxidized and reduced enzymes involves the net uptake of a proton and a cyanide anion from the medium; i.e., HCN rather than CN^- is bound. Indeed, we have found that all ligand state changes of the binuclear center measured to date are electroneutral (Mitchell & Rich, 1994b) and have proposed that this principle of electroneutrality has a central importance in proton/electron coupling (Rich, 1995). The pH-independence of the dissociation constant of the cyanide compound of reduced cytochrome *bo* confirms that HCN overall is bound in this case also, consistent with this electroneutrality requirement.

Data on the net species bound in other reduced hemoproteins are rather sparse. In the case of myoglobin and hemoglobin, the very poor affinities of the ferrous forms for cyanide make this determination difficult. Perhaps the most complete data come from studies with the ferrous form of horseradish peroxidase, where it has been shown from the pH-dependency of the binding constant that HCN is the species bound overall (Phelps et al., 1971). One further example where charge compensation has been indicated comes from the X-ray crystal structure of the fluoride form of ferric cytochrome *c* peroxidase, where evidence of protonation associated with the fluoride binding has been discussed (Edwards et al., 1984). It would be of interest to know whether the strength of binding of anionic ligands to hemoproteins in general was most influenced by the availability of appropriate sites for charge compensation.

Site of Protonation in the Reduced Binuclear Center. Although the species bound overall is HCN, it is likely from considerations of the possible chemistry of metal−cyanide bonding orbitals that cyanide is bound to the ferrous heme *via* its carbon atom, so that the proton would be detached from the anion. Experimental support for this bonding comes indirectly from the fact that alkyl isocyanides are well-known heme ligands (Reisberg & Olson, 1980). In the case of ferric forms of cytochrome *c* oxidase, direct evidence for a carbon−iron bond comes from infrared spectroscopy (Yoshikawa et al., 1985). It therefore seems inevitable that the proton must dissociate from the carbon atom of HCN and bind at another location in the cyanide−protein complex.

In the case of the peroxidases, it seems likely that such a protonatable site is provided by a conserved histidine residue (His_{52} in cytochrome *c* peroxidase; His_{42} in horseradish peroxidase) that is appropriately placed in the distal heme pocket. Unfortunately, adequate structural data on the oxidases are not yet available. However, our data show that the affinity of reduced cytochrome *bo* for cyanide is radically decreased with wild-type enzyme depleted of Cu_B (Figure 6), and with the two mutants H333L and H334L that are also likely to be devoid of Cu_B (Minagawa et al., 1992), to such an extent that no cyanide compound could be observed at all at concentrations of cyanide up to 200 mM. We propose that the most likely reason for this is that the ligand environment around Cu_B, or Cu_B itself, provides the site for binding of the associated proton. Disruption of this site would then prevent the obligatory proton binding and therefore prevent binding of cyanide to the heme group.

Comparison with Cyanide Binding to the Oxidized Binuclear Center. Although cyanide is unable to bind to the reduced forms of H333L and H334L, presumed to be because of the loss of Cu_B and hence of an associated protonation site in these mutants, this was not the case when cyanide was added to the oxidized forms of these mutants (Table 3). Indeed, the addition of 20 mM cyanide to the ferric enzymes induced binding spectra measured at 420−402 nm at least as large as expected from the amount of high-spin heme *o* estimated from the size of the carbon monoxide compound. Since cyanide binding to the oxidized binuclear center is also associated with the binding of a proton (Mitchell & Rich, 1994b), it appears that a protonatable group for this purpose is available in the oxidized forms of H333L and H334L, but not in their reduced forms. If the total number of protonatable groups has been diminished in these mutants so that redox-linked protonation uses all available charge-compensating groups, then none remain available in the reduced enzyme for binding of the cyanide-linked proton.

Comparison of Cyanide and Carbon Monoxide Binding to the Reduced Enzyme. The experiment illustrated in Figure 5 shows definitively that cyanide and carbon monoxide compete for a single binding site in the reduced enzyme, in agreement with previous conclusions for cytochrome oxidase (Orii, 1985). A small discrepancy may be noted in the best-fit value of the cyanide binding constant required for the simulation (equivalent to a second-order rate constant of 440 M^{-1} s^{-1}) in comparison to that found when only cyanide is present (572 ± 43 M^{-1} s^{-1}, see Table 1). However, this is likely to arise from an optically invisible prebinding stage of carbon monoxide on Cu_B which precludes cyanide binding. The "K_m" for this prebinding state has been estimated at 2.4 mM by Lemon et al. (1993), a value which would give around 29% occupancy within 2 ms of photolysis in the presence of 1 mM carbon monoxide. This is sufficient to account for the smaller simulated value of k_{on} for cyanide in the presence of carbon monoxide. It might also be noted that there can be little or no optically-invisible prebinding of cyanide under these conditions, since cyanide does not significantly affect the fast phase of carbon monoxide binding.

During the documentation of these data, a paper appeared which reported an experiment with bovine oxidase which is in total disagreement with our findings and from which the opposite conclusion was reached, i.e., that cyanide binds in the presence of carbon monoxide and hinders CO photodissociation (Hill, 1994). However, it seems certain that this result is an artifact, arising because insufficient dark relaxation time had been allowed between flashes for the slow exchange of ligands back to the equilibrium position of almost full occupancy by carbon monoxide [this rate is governed by the k_{off} for cyanide which has been reported to be 0.015−0.05 s^{-1} (van Buuren et al., 1972; Hill & Marmor, 1991)]. We have carried out an experiment identical to that in Hill (1994) with the same result when flashes are given too fast at 2 Hz. However, a decrease of the flash frequency to less than 0.2 Hz changes the behavior (data not shown) so that it resembles that of cytochrome *bo* in Figure 5, showing that cyanide does *not* bind to the CO-ligated bovine enzyme.

The monophasic nature of cyanide recombination also strongly supports a substantial body of data indicative of a single binding site for cyanide. In contrast, data obtained from infrared measurements have been interpreted in terms of several cyanide binding sites (Yoshikawa et al., 1985). It seems likely that the multiple signals seen in such infrared studies arise from multiple enzyme forms, rather than from multiple binding sites for cyanide or for concurrent binding of cyanide and carbon monoxide within a single enzyme.

Although cyanide was unable to bind to the reduced forms of the Cu_B-depleted enzyme or the H333L or H334L mutants, a high-spin heme is present, and this can still bind carbon monoxide (Brown et al., 1994; Calhoun et al., 1993a; and Table 3). Indeed, the rate of carbon monoxide binding after photolysis is very much accelerated in these enzyme forms. We have deduced in previous work (Brown et al., 1994) that an acceleration of recombination rate of 5-fold or more can be taken as a strong indication that Cu_B is absent. We have often observed heterogeneity of CO recombination in mutant forms of cytochrome *bo* [for example, in H411L (Brown et al., 1994), but also in many other mutants], with the more rapid forms being prevalent under copper-limited conditions. Since even the wild-type enzyme has a propensity to be assembled without Cu_B under copper-limited conditions, it is likely that many mutant forms have a binding site for Cu_B with altered affinity for the metal, so that a major cause of heterogeneity is a variable occupancy of the site with a metal. Observation of a drastically accelerated recombination rate of carbon monoxide, or of a low yield of the ferroheme *o*−cyanide complex, can be taken as a good indication of the presence of the Cu_B-deficient form.

Provided that copper concentrations during growth are sufficient, some mutations (e.g., H284L, Y288H, Y288S) have resulted in enzymes in which the recombination rate of carbon monoxide is significantly slowed down (Brown et al., 1994). Our provisional explanation for this effect has been in terms of an alteration in the properties of Cu_B, which is likely to be retained in these mutants, so that it has a lower affinity for carbon monoxide. Since CO recombination with the heme involves a step of prebinding to Cu_B (Lemon et al., 1993; Einarsdóttir et al., 1993), such a change would result in a slower observed rate of recombination with the heme group.

In Table 2, it can be seen that mutants that exhibit slow rates of CO recombination are still able to bind cyanide in their reduced forms, in marked contrast to those forms that have lost Cu_B. In all examples studied to date (H284L, Y288H, Y288S), their behavior with cyanide has differed from that of the wild-type form, however, in that the binding and dissociation rate constants, k_{on} and k_{off}, are faster.

Model for the Effects of Mutation or Loss of Cu_B on Ligand Binding Reactions. These comparative results lead to a general working model for binding of cyanide and CO to the fully reduced enzyme. In both cases, Cu_B is involved in the binding mechanism. In the case of CO, it is a transient site only, before the CO is transferred onto the reduced heme, as already proposed (Lemon et al., 1993; Einarsdóttir et al., 1993; Woodruff et al., 1991). In the case of cyanide binding, however, Cu_B remains a major binding component of the final stable cyanide complex, possibly by providing a site for the proton which is bound with the cyanide anion. Hence, in the reduced enzyme, loss of the Cu_B allows faster direct binding of CO to the heme, but prevents cyanide binding completely. This behavior is confirmed in the wild-type enzyme that has lost Cu_B and in mutants H333L and H334L (and possibly K362M) that are also likely to have lost Cu_B.

By the same criteria, mutations H284L or Y288H are proposed to result in retention of Cu_B, so that the capability of binding of both carbon monoxide and cyanide is retained by the reduced forms. However, in these mutants the rate of recombination of carbon monoxide is slower than the wild-type. This behavior could arise either because of a higher dissociation constant at the prebinding site on Cu_B or because of a decreased transfer rate from Cu_B to heme. These can be distinguished by measurement of the dependency of recombination rate on carbon monoxide concentration: the former predicts no saturation of rate (at least up to 1 atm) whereas saturation should be observed in the latter case. Figure 7 shows the results of such titrations. For the wild-type and H333L forms, the overall dissociation constants for CO were very low (<100 μM), and recombination rates were proportional to carbon monoxide concentrations of up to 1 atm, as already reported by Lemon et al. (1993). In the case of H284L, the rate was also dependent on carbon monoxide concentration so that a plot of k_{obs} *versus* [CO] was also linear, showing that no saturation occurred, in agreement with the model in which the prebinding site for Cu_B has a lower affinity for the carbon monoxide. In this case, the equilibrium dissociation constant of carbon monoxide on the heme *o* was also much higher than the wild-type, and a value of 0.6 mM could be directly estimated from the plot of k_{obs} *versus* [CO].

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