Cyanide Binding to Cytochrome *c* Peroxidase (H52L)†

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ABSTRACT: Cyanide binding to a cytochrome *c* peroxidase (CcP) variant in which the distal histidine has been replaced by a leucine residue, CcP(H52L), has been investigated as a function of pH using spectroscopic, equilibrium, and kinetic methods. Between pH 4 and 8, the apparent equilibrium dissociation constant for the CcP(H52L)/cyanide complex varies by a factor of 60, from 135 μM at pH 4.7 to 2.2 μM at pH 8.0. The binding kinetics are biphasic, involving bimolecular association of the two reactants, followed by an isomerization of the enzyme/cyanide complex. The association rate constant could be determined up to pH 8.9 using pH-jump techniques. The association rate constant increases by almost 4 orders of magnitude over the pH range investigated, from 1.8×10^2 M^{-1} s^{-1} at pH 4 to 9.2×10^5 M^{-1} s^{-1} at pH 8.6. In contrast to wild-type CcP, where the binding of HCN is the dominant binding pathway, CcP(H52L) preferentially binds the cyanide anion. Above pH 8, cyanide binding to CcP(H52L) is faster than cyanide binding to wild-type CcP. Cyanide dissociates 4 times slower from the mutant protein although the pH dependence of the dissociation rate constant is essentially identical for CcP(H52L) and CcP. Isomerization of the CcP(H52L)/cyanide complex is observed between pH 4 and 8 and stabilizes the complex. The isomerization rate constant has a similar magnitude and pH dependence as the cyanide dissociation rate constant, and the two reactions are coupled at low cyanide concentrations. This isomerization has no counterpart in the wild-type CcP/cyanide complex.

Heme peroxidases are distinguished from other major classes of heme proteins by their rapid reaction with hydrogen peroxide. Hydrogen peroxide oxidizes the ferric heme in peroxidases with rate constants of about 10^7-10^8 M^{-1} s^{-1} while the ferric heme groups in oxygen transport proteins such as metmyoglobin are oxidized by hydrogen peroxide with rate constants of about 10^2-10^3 M^{-1} s^{-1} (*1*). It has been shown that the distal histidine residue in the peroxidases is a critical component in accelerating the rate of the reaction with hydrogen peroxide. Replacement of the distal histidine residue in the peroxidases with a nonpolar residue decreases the rate of reaction with hydrogen peroxide by 5 orders of magnitude (*2*, *3*); these rates are similar to those of metmyoglobin. The mere presence of a distal histidine in a heme protein cannot be the only factor accelerating the peroxide reaction since metmyoglobin has a distal histidine but reacts slowly with hydrogen peroxide. Other factors such as the steric relationship between the distal histidine and the heme iron, the presence of water and the hydrogen-bonding pattern within the distal pocket, the conformational dynamics of the heme pocket, and heme electronic features may also be important.

The differential reactivity of the ferric heme group in the peroxidases and the globins extends to other reactions in addition to that with hydrogen peroxide. Cytochrome *c* peroxidase (CcP)[1] binds cyanide with a rate constant of 1.1×10^5 M^{-1} s^{-1} (*4*) between pH 6 and 8, while metmyoglobin has a maximum reported rate of 8.9×10^2 M^{-1} s^{-1} at pH 8.2 (*5*), about 2 orders of magnitude slower. The generally accepted mechanism for cyanide binding to heme proteins is that hydrocyanic acid, HCN, diffuses into the distal heme pocket and binds to the heme iron. The proton associated with HCN may either dissociate or be retained within the complex. It appears that the proton dissociates from the metmyoglobin/cyanide complex but is retained in peroxidase/cyanide complexes (*6*). The pH dependence of the reaction between CcP and both hydrocyanic acid and hydrogen peroxide is essentially identical, indicating that the neutral forms of the two reactants react with CcP when the distal histidine is unprotonated (*4*, *7*). The similarity in the reactions can be attributed to the observation that the rate-limiting step in the oxidation of CcP to compound I by hydrogen peroxide is the binding of the peroxide to the heme iron (*1*). The similarities of the reactions of both HCN and hydrogen peroxide with CcP suggest that the binding of HCN should be a good surrogate for the binding of hydrogen peroxide. Investigating the cyanide reaction has two major advantages

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[1] Abbreviations: CcP, cytochrome *c* peroxidase; CcP(MI), recombinant CcP with Met and Ile at positions −2 and −1, respectively, and T53I and D152G substitutions compared to bakers' yeast CcP; rCcP, recombinant CcP with the exact amino acid sequence as bakers' yeast CcP; CcP(H52L), cytochrome *c* peroxidase in which the distal histidine, His-52, has been replaced by a leucine residue; CcP(MI,H52L), CcP(MI) with the H52L mutation; rCcP(H52L), rCcP with H52L substitution; CcPCN, the cyanide complex of CcP; CcP(H52L)CN, the cyanide complex of CcP(H52L).

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over the hydrogen peroxide reaction. First, cyanide binding is reversible, and one can obtain two additional parameters, the dissociation rate constant and the equilibrium binding constant. In addition, the reaction with cyanide is not complicated by the redox reactions, both specific and nonspecific, that accompany the reaction between hydrogen peroxide and heme proteins. Because of these considerations, we have initiated kinetic and NMR studies of cyanide binding to CcP and CcP mutants in order to gain further insight into the control of heme group reactivity by the protein matrix. In this paper we describe the binding of cyanide to CcP(H52L) to further elucidate the role of the distal histidine in modulating the reactions of the Fe(III) heme in CcP. The accompanying paper (*8*) describes the results of proton NMR investigations of rCcP(H52L) and rCcP(H52L)CN.

MATERIALS AND METHODS

CcP(H52L) from two different clones was used in this work. The first clone is CcP(MI) (*9*). CcP(MI) has an N-terminal methionine and isoleucine added to the mature protein, positions −2 and −1, respectively. In addition, CcP(MI) differs from the wild-type bakers' yeast CcP at positions 53 and 152. Bakers' yeast CcP has threonine and aspartate residues at positions 53 and 152, respectively, while CcP(MI) has isoleucine and glycine at the analogous positions. The H52L mutant of this clone is designated CcP(MI,H52L). The second clone has been engineered to be a true replica of wild-type bakers' yeast CcP with no N-terminal extensions and with threonine and aspartate residues at position 53 and 152, respectively (*10*, *11*). The H52L mutant of this clone is designated rCcP(H52L).

Concentrations of enzyme were determined spectroscopically at pH 6.0 in potassium phosphate buffers, 0.1 M ionic strength. In this buffer, the absorptivity of CcP(H52L) at the Soret maximum, 404 nm, is 97 mM^{-1} cm^{-1} (*12*). The concentration of potassium cyanide solutions was determined from weight/volume measurements. The ionic strength of the cyanide solutions was adjusted to 0.1 M ionic strength with potassium phosphate if necessary. All buffers had an ionic strength of 0.10 M. Between pH 4.0 and 5.5, buffers were 10 mM in acetate with potassium phosphate added to adjust the ionic strength. Potassium phosphate buffers were used between pH 5.5 and 8.0.

Spectra were obtained with either a Cary Model 3 double-beam UV-vis spectrophotometer or a Hewlett-Packard 8452A diode array spectrophotometer. Stopped-flow measurements were made with either a Hi-Tech Scientific Model PQ/SF-53 instrument or an Applied Photophysics Ltd. Model DX.17MV stopped-flow instrument. All measurements were carried out at 25 °C. Between pH 4 and 8, the cyanide binding reaction was observed by mixing equal volumes of buffered enzyme and cyanide solutions and monitoring the change in absorbance at 428 nm. The experiments were done under pseudo-first-order conditions with excess cyanide. To extend the pH range to higher pH values, pH-jump experiments were performed. Enzyme at either pH 6 or pH 8 was rapidly mixed with alkaline solutions of potassium cyanide in a stopped-flow instrument, the reaction monitored, and the pH of the resulting solutions determined after the experiment. The alkaline solutions of potassium cyanide were adjusted to 0.1 M ionic strength with K_2HPO_4 and/or K_3PO_4.

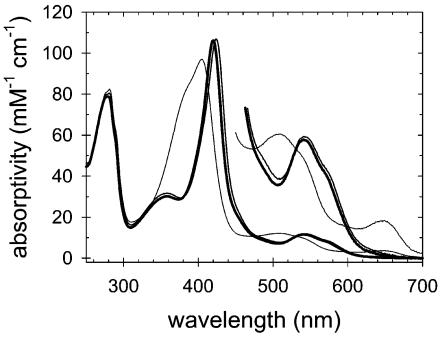

FIGURE 1: Spectra of CcP(H52L) and the CcP(H52L)/cyanide complex: thin solid line, CcP(H52L) at pH 6.0, Soret maximum 404 nm; medium solid line, the CcP(H52L)/cyanide complex at pH 6.0, Soret maximum 424 nm; thick solid line, the CcP(H52L)/cyanide complex at 8.0, Soret maximum 420 nm. The visible region of the spectrum is shown expanded 5-fold.

RESULTS

Preliminary studies on the binding of cyanide to CcP(H52L) were carried out using CcP(MI,H52L). The final results presented here are those obtained using rCcP(H52L). No significant differences were observed for data obtained with CcP(MI,H52L) and with rCcP(H52L).

S*pectroscopic Properties of CcP(H52L) and Its Cyanide Complex.* The spectra of rCcP(H52L) and its cyanide complex at pH 6.0 are shown in Figure 1. The spectrum of rCcP(H52L) is characteristic of a pentacoordinate, high-spin ferric heme protein with a Soret maximum at 404 ± 1 nm, a distinct shoulder with high absorptivity at 380 nm, and broad charge-transfer bands in the visible region of the spectrum at 510 ± 2 and 649 ± 2 nm. This is consistent with NMR results (*8*). The spectrum of CcP(H52L) depends to some extent on the nature of the buffer and the pH (*2*). The pH dependence of the spectrum is due to formation of low-spin forms in the alkaline region (*13*). The alkaline transitions are complex with at least four kinetic processes detectable between pH 8 and 11.6 (*13*).

The spectrum of the cyanide complex of CcP(H52L) is also dependent upon the pH of the solution. At pH 6, the spectrum of CcP(H52L)CN is characteristic of a hexacoordinate, low-spin ferric heme protein with the Soret maximum at 424 nm (absorptivity 107 mM^{-1} cm^{-1}), a distinct β band at 541 ± 2 nm, and an α band appearing as a shoulder near 575 nm (Figure 1). The spectrum of the cyanide complex is independent of pH between pH 4 and 6.5. Above pH 6.5, the Soret maximum shifts to shorter wavelength, reaching a value of 420 nm at pH 8 with an absorptivity of 106 mM^{-1} cm^{-1} (Figure 1). The apparent pK_A for the acid/alkaline transition in the cyanide complex of CcP(H52L) is estimated to be 7.5 ± 0.2.

The spectrum of the cyanide complex of wild-type bakers' yeast CcP also shows a similar acid/alkaline transition (*4*). The acid and alkaline forms of wild-type CcPCN have Soret maxima at 426 and 421 nm at pH 6 and 9, respectively. The absorptivity at the Soret maximum of wild-type CcPCN is 103 mM^{-1} cm^{-1} for both the acidic and alkaline forms. The shift in the absorbance spectrum of wild-type CcPCN is associated with two isomerization reactions observed above

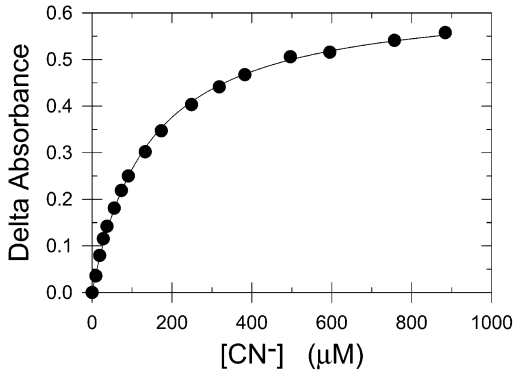

FIGURE 2: Spectrophotometric titration of CcP(H52L) with cyanide. The absorbance of a 10 μM solution of CcP(H52L) at pH 4.7 was monitored at 428 nm as a function of increasing concentrations of cyanide. Solid circles represent experimental data. The solid line was calculated using eq 1 in the text with the equilibrium dissociation constant, K_D^{spec}, equal to 135 μM.

pH 7. The origin of the 4−5 nm blue shifts in the Soret band for both CcPCN and CcP(H52L)CN at alkaline pH is probably associated with changes in solvation of the heme group.

Equilibrium Titration of CcP(H52L) with Cyanide. The very large changes in the absorption spectrum of CcP(H52L) upon cyanide binding provide an easy method to follow formation of the CcP(H52L)CN complex. Equilibrium dissociation constants were determined by titrating the enzyme with increasing concentrations of cyanide while monitoring the absorption of the solution. The binding of cyanide is quite strong, and absorbance changes as a function of the total cyanide concentration were fit to the equation:

$$\Delta A_{obs} = \Delta A_{max}([[B] - ([B]^2 - 4[P][L])^{1/2}]/2[P]) \quad (1)$$

[P] and [L] represent the total protein and total cyanide concentrations, respectively, and [B] is equal to ([P] + [L] + K_D). Equation 1 was derived assuming one-to-one complex formation and solving the equilibrium expression to determine the fraction of protein converted to the complex in terms of the total enzyme and cyanide concentrations. A typical titration is shown in Figure 2. The absorbance change as a function of the total enzyme and cyanide concentrations was fit to eq 1 using two parameters: ΔA_{max}, the maximum absorbance change of the enzyme in the presence of infinite concentrations of cyanide, and the equilibrium dissociation constant, K_D. Values of K_D determined by spectrophotometric titrations are designated K_D^{spec}. Values of K_D^{spec} were determined as a function of pH between pH 4.2 and 8.0 and are collected in Table 1.

Values of K_D^{spec} for the CcP(H52L)CN complex are shown as a function of pH in Figure 3. Published data for the wild-type yeast CcPCN complex are also shown in Figure 3 (*4*). The data for wild-type CcPCN were determined in buffers containing potassium nitrate to adjust the ionic strength. Potassium nitrate binds to CcP and CcP(H52L) and influences the pH dependence of the association rate constant for hydrogen peroxide binding and, by inference, the rate constant for cyanide binding (*2*, *7*, *14*). Under the experimental conditions used to determine the equilibrium binding constants for the two proteins, wild-type CcP binds cyanide more tightly that CcP(H52L). Cyanide binding becomes stronger as the pH increases for both CcP and CcP(H52L).

Table 1: pH Dependence of the Equilibrium and Rate Constants for Cyanide Binding to CcP(H52L)[a]

pH	k_a (M^{-1} s^{-1})	k_d (s^{-1})	$k_i + k_{-i}$ (s^{-1})	K_D^{spec} (μM)
4.0	$(1.8 \pm 0.1) \times 10^2$	0.04 ± 0.02	0.13 ± 0.03	
4.2				55 ± 2
4.3				65 ± 2
4.5	$(2.4 \pm 0.1) \times 10^2$	0.05 ± 0.03	0.13 ± 0.04	
4.7				135 ± 4
5.0	$(2.8 \pm 0.2) \times 10^2$	0.07 ± 0.05	0.14 ± 0.02	
5.2				131 ± 5
5.5	$(5.3 \pm 0.1) \times 10^2$	0.05 ± 0.03	0.18 ± 0.05	
5.7				80 ± 3
6.0	$(1.6 \pm 0.1) \times 10^3$	0.10 ± 0.08	0.18 ± 0.07	
6.1				40 ± 3
6.5	$(3.0 \pm 0.1) \times 10^3$	0.28 ± 0.04	0.18 ± 0.03	18 ± 1
7.0	$(7.0 \pm 0.5) \times 10^3$	0.15 ± 0.07	0.10 ± 0.02	6.3 ± 0.5
7.5	$(2.8 \pm 0.4) \times 10^4$	0.13 ± 0.03	0.09 ± 0.02	3.3 ± 1.2
8.0	$(7.6 \pm 0.1) \times 10^4$	0.02 ± 0.02	0.06 ± 0.01	2.2 ± 0.6
8.0[b]	$(1.8 \pm 0.1) \times 10^5$			
8.6[b]	$(9.2 \pm 1.2) \times 10^5$			
8.9[b]	$(6.2 \pm 0.6) \times 10^5$			

[a] All data collected in 0.10 M ionic strength buffers, 25 °C. [b] Data acquired by pH-jump techniques.

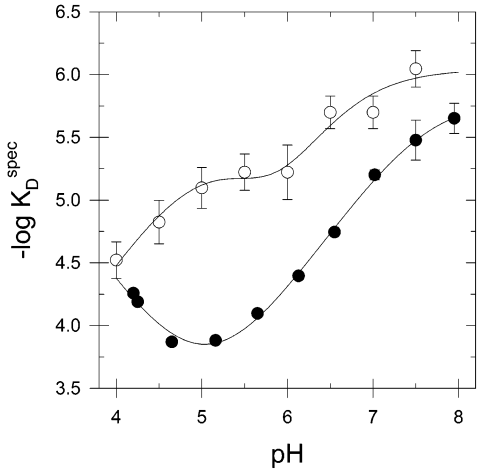

FIGURE 3: pH dependence of the logarithm of the equilibrium dissociation constant, K_D^{spec}, for the CcP(H52L)/cyanide complex (solid circles). Open circles represent the logarithm of the equilibrium dissociation constant, K_D, for formation of the wild-type CcP/cyanide complex (*4*).

Kinetic Studies of Cyanide Binding to CcP(H52L). The binding of cyanide to CcP(H52L) is sufficiently fast that stopped-flow studies were used to monitor the reaction. The reaction of cyanide with CcP(H52L) is biphasic. Under pseudo-first-order conditions with cyanide in excess, the time dependence of the absorbance change was fit to the sum of two exponentials:

$$\Delta A = \Delta A_f \exp(-k_f t) + \Delta A_s \exp(-k_s t) \quad (2)$$

The subscripts f and s represent the fast and slow reaction. At low concentrations of cyanide, the values of k_f and k_s are not significantly different, and the two reaction phases are coupled. The concentration dependence of the observed rate constants and amplitudes at pH 4.5 is shown in Figure 4.

The concentration dependence of the two pseudo-first-order rate constants (Figure 4A) indicates that the faster reaction is due to the binding of cyanide and the slower reaction is due to an isomerization reaction. The simplest mechanism to account for the kinetic data involves binding

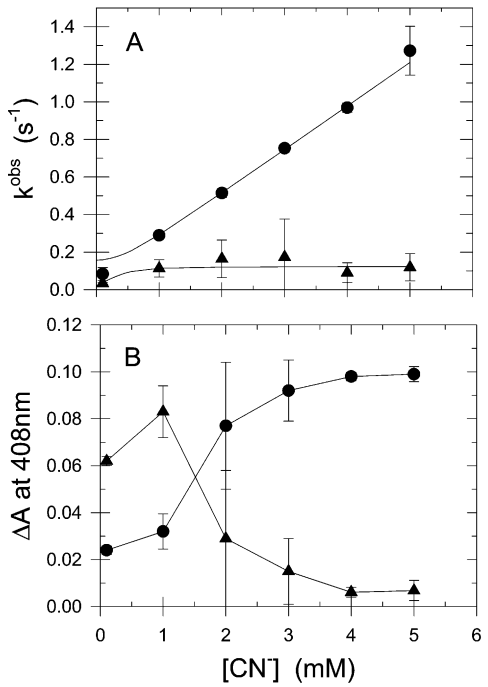

FIGURE 4: (A) Dependence of the two observed pseudo-first-order rate constants on the cyanide concentration for the interaction of cyanide with CcP(H52L) at pH 4.5: solid circles, rate constant for the fast phase, k_f; solid triangles, rate constant, k_s, for the slow phase. The data were fit to a mechanism involving initial formation of a CcP(H52L)/cyanide complex, followed by an isomerization. At low concentrations of cyanide, the association and isomerization reactions are coupled. Equations 5−8 in the text were used to calculate the solid lines through the data. (B) Amplitudes of the two kinetic phases observed as changes in absorbance at 428 nm: solid circles, amplitude of the fast phase; solid triangles, amplitude of the slow phase.

of the ligand to the enzyme to form an initial enzyme/ligand complex (eq 3), followed by isomerization of the initial complex to a second, more stable complex (eq 4). The

$$E + L \underset{k_d}{\overset{k_a}{\rightleftharpoons}} EL' \tag{3}$$

$$EL' \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} EL \tag{4}$$

observed rate constants for the mechanism shown in eqs 3 and 4 are given by eqs 5−8. Fitting the data to eqs 5−8 is

$$k_f = {}^1/_2[b + (b^2 - 4c)^{1/2}] \tag{5}$$

$$k_s = {}^1/_2[b - (b^2 - 4c)^{1/2}] \tag{6}$$

$$b = k_a[L] + k_d + k_i + k_{-i} \tag{7}$$

$$c = k_a[L](k_i + k_{-i}) + k_i k_d \tag{8}$$

a challenge. First, the initial determination of k_f and k_s at low cyanide concentrations is subject to significant error due to the similar magnitude of k_f and k_s, and second, both observed rate constants depend on four fitting parameters. An alternative analysis is to use the data at high concentrations of cyanide where the two reaction phases uncouple.

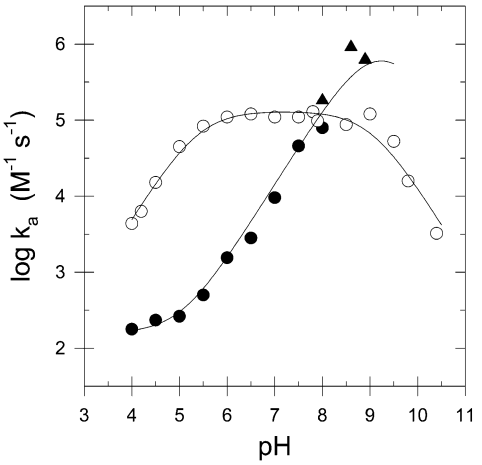

FIGURE 5: pH dependence of the logarithm of the association rate constant, k_a, for formation of the initial CcP(H52L)/cyanide complex (solid circles and solid triangles). The solid triangles represent data acquired by pH-jump techniques. Open circles represent the logarithm of the association rate constant, k_a, for formation of the wild-type CcP/cyanide complex (*4*). The solid lines are calculated according to mechanisms discussed in the text.

At sufficiently high cyanide concentrations, the two rate constants are given by eqs 9 and 10. The values of k_a and k_i

$$k_f = k_a[L] + k_d \tag{9}$$

$$k_s = k_i + k_{-i} \tag{10}$$

$+ k_{-i}$ can be determined with accuracy by fitting the high cyanide concentration data to eqs 9 and 10 (see Figure 4A). However, the value of k_d is subject to considerable error. If eq 9 is used to obtain k_d by extrapolation to zero ligand concentration, the extrapolation is quite large, giving values of k_d close to zero and with substantial error. The average estimated error for k_d is 80% of the mean. Values of k_a, k_d, and $k_i + k_{-i}$ are collected in Table 1.

The amplitudes of the two reaction phases (Figure 4B) are complex functions of the cyanide concentration. At low cyanide concentration (0.1−2 mM), the kinetic phases are coupled, leading to a complex dependence of the amplitudes on all four rate constants defined in eqs 3 and 4, the cyanide concentration, and the relative extinction coefficients of the native enzyme and the two enzyme/cyanide complexes. At the highest cyanide concentration, the reactions are uncoupled, and the amplitude of the slow phase is related primarily to the isomerization of the enzyme/cyanide complexes. The amplitude of the slow phase is quite small at 428 nm, averaging 7 ± 4% of the total reaction amplitude between pH 4 and 7 but increasing to 30% at pH 8.

pH Dependence of the Association Rate Constant. The association rate constant, k_a, for the binding of cyanide to CcP(H52L) varies almost 4 orders of magnitude between pH 4 and 8.6, from 1.8×10^2 M^{-1} s^{-1} at pH 4 to 9.2×10^5 M^{-1} s^{-1} at pH 8.6 (Table 1). The logarithm of the association rate constant for cyanide binding to CcP(H52L) is shown in Figure 5 along with published data for the binding of cyanide to wild-type bakers' yeast CcP. The difference in the pH dependence of cyanide binding to CcP and CcP(H52L) is remarkable. Even more remarkable is that, above pH 8, cyanide binds more rapidly to CcP(H52L) than to wild-type CcP.

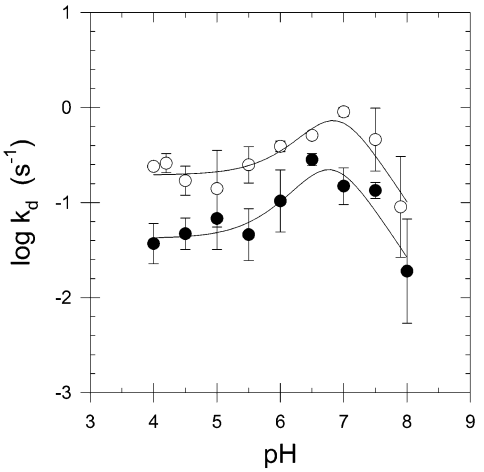

FIGURE 6: pH dependence of the logarithm of the dissociation rate constant, k_d, for the initial CcP(H52L)/cyanide complex formed (solid circles). Open circles represent the logarithm of the dissociation rate constant, k_d, for the wild-type CcP/cyanide complex (*4*). The solid lines are calculated according to mechanisms discussed in the text.

pH Dependence of the Dissociation Rate Constant. The dissociation rate constants for the CcPCN and CcP(H52L)-CN complexes as a function of pH are shown in Figure 6. Interestingly, the pH dependence of the dissociation rate constant is essentially identical for the cyanide complexes of CcP and CcP(H52L), suggesting that His-52 is not involved in modulating the pH dependence of the dissociation; i.e., the pK_A of His-52 in the CcPCN complex must be outside the pH range 4−8. The NMR data in Figure 9 of the companion paper (*8*) indicate that His-52 titrates above 8.7 (pH′ 8.3) in the CcPCN complex. However, His-52 does influence the absolute rate of cyanide dissociation, and the dissociation rate constant for CcP(H52L)CN is 4 times slower, on average, than the dissociation rate constant for the CcPCN complex. To explain the small increase and decrease of the observed dissociation rate constant as the pH is increased between 4 and 8 (Figure 6), a mechanism involving two ionizations in the enzyme/cyanide complex was used. To obtain the maximum dissociation rate, one ionizable group must be in its basic (unprotonated) form and the other ionizable group must be in its acidic (protonated) form. The pK_A of the basic group is designated pK_{C1}, and that of the acidic group is designated pK_{C2}. Nonlinear regression was used to determine best-fit values for the apparent pK_A values. The best-fit values for pK_{C1} are 8.6 and 8.0 for the CcP and CcP(H52L) cyanide complexes, respectively. The best-fit values for pK_{C2} are 5.3 and 5.5 for the CcP and CcP(H52L) cyanide complexes, respectively. An unusual aspect of the two pK_A values is that the basic group has the higher pK_A, near 8, while the acidic group has the lower pK_A, near pH 5.5. The apparent pK_A values are not well defined due to the relatively large errors in determining the dissociation rate constant and the very small pH dependence of the dissociation rate constant. At best, the two-ionizable group mechanism is used to establish an empirical equation for fitting the data, and no attempt has been made to try to associate the apparent pK_A values with particular groups within the protein. The solid lines in Figure 6 were calculated using the two-ionizable group mechanism for cyanide dissociation.

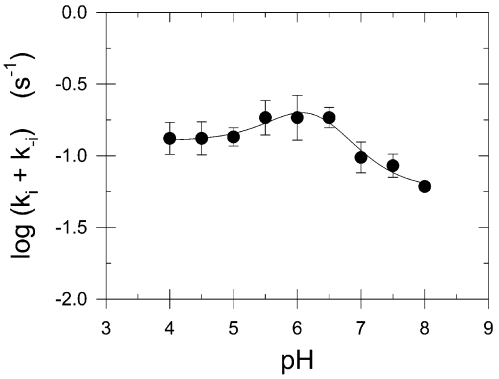

FIGURE 7: pH dependence of the logarithm of the sum of the two isomerization rate constants, $k_i + k_{-i}$, for conversion of the initial CcP(H52L)/cyanide complex to the stabilized complex (solid circles). There is no counterpart of this isomerization reaction in the wild-type CcP/cyanide complex (*4*). The solid line was calculated according to a mechanism discussed in the text.

pH Dependence of the Isomerization Rate Constant. The pH dependence of the isomerization rate, $k_i + k_{-i}$, is shown in Figure 7. The isomerization rate is similar to the rate of cyanide dissociation from the CcP(H52L)CN complex, averaging about a factor of 2 times faster than the dissociation rate over the pH range 4−8. The similar magnitude of these two rates causes strong kinetic coupling between binding and isomerization. The pH dependence of the isomerization rate was fit to a mechanism involving two apparent ionizations in the enzyme/cyanide complex. Just as with the dissociation rate constant, this mechanism serves only to provide an empirical equation with which to fit the data. Nonlinear regression gave best-fit values of 6.2 and 6.3 for the two ionizations. The equation derived from the two-ionization mechanism was used to calculate the solid line in Figure 7.

The amplitude of the isomerization reaction is quite small, averaging 7 ± 4% of the total absorbance change at 428 nm upon cyanide binding between pH 4 and 7. The isomerization process in the CcP(H52L)CN complex characterized by the data shown in Figure 7 has no counterpart in the wild-type CcPCN complex although the wild-type CcPCN complex does show a much slower isomerization between pH 7 and 8.5 and a somewhat faster isomerization process between pH 8 and 11.

Comparison of the Equilibrium Dissociation Constant and Kinetic Rate Constants. The isomerization equilibrium constant can be determined from a comparison of the equilibrium dissociation constant determined from the spectroscopic titrations (Figures 2 and 3) and the kinetic rate constants. For the mechanism shown in eqs 3 and 4, two equilibrium constants can be defined as follows: $K_D^{kin} = k_d/k_a$ and $K_I = k_i/k_{-i}$. The equilibrium dissociation constant determined by the spectrophotometric titration, K_D^{spec}, is given by eq 11. Using eq 11, the value of K_I can be

$$K_D^{spec} = K_D^{kin}/(1 + K_I) \qquad (11)$$

determined from K_D^{spec} and K_D^{kin}. Even though K_D^{kin} has a relatively large error due to the error in determining the dissociation rate constant, it can be shown that K_D^{kin} averages 2.4 ± 2.0 times larger than K_D^{spec} over the pH range 4−8, 25 °C. The value of K_I is approximately unity. This indicates that the initial and final CcP(H52L)CN complexes (EL′ and

EL in eq 4) are approximately equal in concentration and equilibrate at a rate of 0.06−0.18 s^{-1}, depending upon pH (Table 1).

DISCUSSION

The most striking finding in this study is the very large difference in the pH dependence of the association rate constant for cyanide binding to CcP(H52L) compared to the pH dependence of cyanide binding to wild-type CcP (Figure 5).

Mechanism of Cyanide Binding to Wild-Type CcP. The pH dependence of cyanide binding to wild-type CcP (Figure 5) is influenced by three acid/base reactions. HCN ionizes to the cyanide anion with an apparent pK_A of 9.0 under the conditions in which the data shown in Figure 5 were collected (*4*). Two ionizations in wild-type CcP also influence the observed rate of cyanide binding. The distal histidine, His-52, ionizes with an apparent pK_A of 5.5 ± 0.1 (*4*, *7*), and the pentacoordinate form of CcP existing at neutral pH is converted to a hydroxy-ligated form with an apparent pK_A of 9.7 ± 0.1 (*7*, *13*, *15*).

Between pH 4 and 9, the dominant pathway for binding of cyanide to CcP is the binding of HCN to the pentacoordinate form of CcP when His-52 is unprotonated. The unprotonated distal histidine residue promotes the binding of HCN by acting as a base catalyst, assisting in the removal of the proton from HCN as cyanide binds to the heme iron. The association rate constant for this reaction is 1.4×10^5 M^{-1} s^{-1} (Figure 5). The decrease in the observed rate below pH 6 is due to protonation of His-52 giving an unreactive form of CcP since the protonated form of His-52 cannot function as a base catalyst. It is possible that HCN reacts slowly with CcP when His-52 is protonated, and an upper limit for this uncatalyzed rate can be estimated as 1.8×10^3 M^{-1} s^{-1} from the data in Figure 5 (*4*).

The mechanism of cyanide binding to wild-type CcP above pH 9 is ambiguous. HCN ionizes to the cyanide anion, and CcP undergoes a complex series of alkaline transitions above pH 8. There is rapid conversion of pentacoordinated CcP to hydroxy-ligated CcP with an apparent pK_A of 9.7 (*13*, *15*). Either ionization of HCN, formation of hydroxy-ligated CcP, or both could be responsible for the decrease in the observed cyanide-binding rate above pH 9 (Figure 5). Two previous studies shed some light on the two possibilities (*7*, *13*). The hydroxy-ligated form of CcP is at least a factor of 10 times less reactive toward hydrogen peroxide than the pentacoordinated form (*7*). Since the reaction of HCN with CcP mimics the reaction of hydrogen peroxide with CcP in many respects, it is reasonable to expect that HCN will react with hydroxy-ligated CcP with a rate constant that is at least 10 times smaller than the reaction of HCN with pentacoordinate CcP; i.e., $k \leq 1.4 \times 10^4$ M^{-1} s^{-1}. Support for direct anion binding to the heme iron is provided by results obtained with *p*-nitroperoxybenzoic acid. *p*-Nitroperoxybenzoic acid ionizes to form the *p*-nitroperoxybenzoate anion with an apparent pK_A of 7.1 (*13*). Both the neutral acid and the negatively charged *p*-nitroperoxybenzoate anion react rapidly with wild-type CcP, with the anion reacting about 10 times slower than the neutral acid (*13*). Diffusion of the reactants through the protein matrix into the distal heme pocket appears to be the rate-limiting step for the reaction of the neutral acid and the anion with the heme iron (*16*). A 10-fold decrease in diffusion rate through the protein matrix for the *p*-nitroperoxybenzoate anion relative to the neutral acid is reasonable (*17*, *18*).

Although the cyanide anion is expected to diffuse through the protein matrix more slowly than HCN, this does not appear to be the rate-limiting step for cyanide complex formation. The maximum association rate constant for cyanide binding to CcP is about 2 orders of magnitude slower than the reaction between CcP and either hydrogen peroxide or *p*-nitroperoxybenzoic acid. The rate-limiting step for cyanide complex formation is the actual rate of bond formation between ligand contained in the heme pocket and the heme iron. The apparent bimolecular rate constant can be envisioned as being the product of an equilibrium constant describing the concentration of free ligand in the heme pocket of unliganded enzyme, K_P, and the rate constant for bond formation between the free ligand in the heme pocket and the heme iron, k_{bond} (*19*, *20*):

$$k_a = K_P k_{bond} \quad (12)$$

A detailed analysis of the data for binding of cyanide to wild-type CcP, based on the above considerations, indicates that the pH dependence of the reaction can be described by both HCN and the cyanide anion binding to the pentacoordinated form of CcP. Binding of either HCN or cyanide ion to the alkaline form of CcP does not contribute to the observed rate up to pH 10.4. The second-order rate constants for binding of HCN and cyanide ion to pentacoordinated CcP are 1.4×10^5 M^{-1} s^{-1} and 2.0×10^4 M^{-1} s^{-1}, respectively. The solid line through the data for the binding of cyanide to wild-type CcP in Figure 5 was calculated for a mechanism in which both HCN and the cyanide anion bind to pentacoordinated CcP. There is no detectable binding of HCN to wild-type CcP when the distal histidine is protonated.

In terms of eq 12, partitioning of HCN into the distal heme pocket should be more favorable than partitioning of the cyanide anion, and K_P will be larger for HCN than for the cyanide ion. On the other hand, cyanide ion does not require the assistance of the distal histidine to bind to the heme iron, and k_{bond} for the cyanide ion is expected to be larger than k_{bond} for HCN. The combination of these two factors results in a k_a value that is 7 times larger for HCN than for the cyanide anion in wild-type CcP.

Mechanism of Cyanide Binding to CcP(H52L). Hydroxide ion binds to CcP(H52L) at alkaline pH in a manner similar to that of wild-type CcP (*12*). The apparent pK_A for conversion of the pentacoordinate heme group in CcP(H52L) to the hydroxy-ligated form is 9.4 (*13*). The rate of cyanide binding to CcP(H52L) increases dramatically with pH, increasing from an observed value of 180 M^{-1} s^{-1} at pH 4 to a value of 9.2×10^5 M^{-1} s^{-1} at pH 8.6 (Figure 5).

At low pH, HCN binds to pentacoordinate CcP(H52L) with a calculated rate constant of 160 ± 50 M^{-1} s^{-1}. The low pH data are important since they allow determination of the effect of His-52 on the binding of HCN to the pentacoordinated heme group in CcP. The basic form of His-52 accelerates the rate of HCN binding to CcP by about 3 orders of magnitude, from 160 M^{-1} s^{-1} for the mutant lacking His-52 to 1.4×10^5 M^{-1} s^{-1} for wild-type CcP. In terms of

eq 12, this is primarily due to a large decrease in k_{bond} in the absence of His-52.

As the pH increases from 4 to 8.9, the concentrations of both the cyanide anion and hydroxy-ligated CcP(H52L) increase, and the rate of complex formation also increases. The increase in the observed rate constant can be modeled if the binding of HCN to hydroxy-ligated CcP(H52L) has a rate constant of 3.0×10^6 M^{-1} s^{-1} or if the binding of cyanide anion to the pentacoordinate form of CcP(H52L) has a rate constant of 1.7×10^6 M^{-1} s^{-1} or some combination of the two pathways. Binding of HCN to hydroxy-ligated CcP(H52L) with a rate constant of 3.0×10^6 M^{-1} s^{-1} does not seem plausible when HCN binds to the pentacoordinate form of CcP(H52L) with a rate constant of 160 M^{-1} s^{-1}. The preferred interpretation is that the increase in rate for cyanide binding to CcP(H52L) at high pH is due to cyanide ion binding to the pentacoordinated form of CcP(H52L). This is reasonable since binding of cyanide ion does not require the assistance of His-52 for rapid reaction. One unusual aspect of the binding of the cyanide anion to CcP(H52L) is that the observed rate, 1.7×10^6 M^{-1} s^{-1}, is 85 times larger than the rate of cyanide anion binding to wild-type CcP. This can be explained by eq 12. The value of k_{bond} should be essentially the same in CcP and CcP(H52L), and the 85-fold difference must be due to a larger value of K_P for CcP-(H52L) compared to CcP. Replacing His-52 with a leucine residue makes the heme pocket less polar and alters the distribution of water molecules and hydrogen bonds within the heme pocket (*2*). With the heme pocket more hydrophobic, the positive charges on the pentacoordinate Fe(III) heme and Arg-48 are destabilized. A lower energy state can be achieved by having the negatively charged cyanide anion residing in the heme pocket to help neutralize the excess positive charge. This will lead to an increase in the partition coefficient, K_P, for cyanide anion in the heme pocket of CcP-(H52L) compared to wild-type CcP and could account for the larger association rate constant for binding cyanide anion to CcP(H52L).

Correlation of the Cyanide Binding Studies with NMR Studies. NMR studies presented in a companion paper (*8*) demonstrate that there are at least two forms of CcP(H52L) present in solution above pH 6. The two forms are in slow to intermediate exchange on the NMR time scale. The NMR studies also show that there are at least four spectroscopically distinct forms of the CcP(H52L)CN complex. The most plausible mechanism to reconcile the NMR data with the observation of only two kinetic phases in the stopped-flow studies is to assume that the interconversion of the two enzyme forms, E and E′, although slow on the NMR time scale, is fast compared to the rate of cyanide binding under the conditions of the stopped-flow experiments. Under these conditions only a single reaction phase would be observed for the binding of cyanide. The observed rate would be the average rate of cyanide binding for the two enzyme forms, weighted according to the concentration of the two forms. The two forms of the enzyme would each form their own initial cyanide complex (eq 4), and these initial cyanide complexes would isomerize to form the more stable, final complexes. If the interconversion of the two initial cyanide complexes is in rapid equilibrium with each other on the stopped-flow time scale and the two final cyanide complexes are also in rapid equilibrium with each other on this time scale, then only a single isomerization rate would be observed. The observed isomerization rate would be a complex function of the equilibrium concentrations of the four CcP(H52L)CN complexes and their rates of interconversion. The amplitude of the isomerization reaction would also be small, since the electronic absorption spectrum of the various cyanide complexes must be very similar (Figure 1). There is evidence via NMR observations in the companion paper (Figure 6 in ref *8*) for chemical exchange between two of the four rCcP(H52L) forms, which supports this mechanism.

The above mechanism is not unique. There are at least two other reasonable explanations for the observation of a single binding rate when two forms of the resting-state enzyme exist. (1) The NMR-detectable forms may be very similar in terms of the heme pocket structure, and cyanide could bind to both forms with equal rates. (2) At the opposite extreme is a mechanism in which only one of the two forms binds cyanide while the second is completely inactive. Since the NMR studies indicate that all of the resting-state enzyme is converted to the cyanide complex, the inactive form must isomerize to the reactive form. The rate-limiting step for conversion of the inactive enzyme to the cyanide complex would be the isomerization step.

If the observed isomerization step (Figure 4) is actually the isomerization of the two resting-state enzyme forms, as suggested by the second mechanism above, then isomerization between the multiple forms of the CcP(H52L)CN complex was not observed. This is also entirely plausible if the electronic absorption spectra of the different cyanide complexes are identical rather than similar or if the rate of cyanide complex isomerization is faster that the rate of cyanide binding.

Conclusions. Although a variety of mechanisms are consistent with the kinetic data, several important conclusions can be made concerning binding of cyanide to wild-type CcP and the CcP(H52L) mutant. The major difference in cyanide binding to CcP and CcP(H52L) occurs at low pH where rapid binding of HCN requires base catalysis by His-52. This type of base catalysis occurs in CcP but not in CcP(H52L). Base catalysis by His-52 increases the rate of HCN binding to CcP by about 3 orders of magnitude, somewhat less than the 5 order of magnitude increase in the rate of the hydrogen peroxide/CcP reaction (*2*).

At alkaline pH, the cyanide anion binds rapidly to both CcP and CcP(H52L), with CcP(H52L) actually binding cyanide about 85 times faster than wild-type CcP. The effect of replacing the distal histidine by a leucine residue appears to enhance the partitioning of the cyanide anion into the distal heme pocket to compensate for the positive charges on the pentacoordinate Fe(III) heme and Arg-48.

The similarity in the pH dependence of cyanide dissociation from CcP and CcP(H52L) indicates that the small pH dependence is not associated with His-52. However, His-52 does influence the dissociation rate since the dissociation rate is 4 times slower from the CcP(H52L)CN complex than from CcPCN. NMR studies indicate that a hydrogen bond exists between the distal histidine and iron-bound cyanide in the peroxidases (*6*), and the accompanying paper documents its titration at high pH (*8*). The availability of the hydrogen-bonded proton in the CcPCN complex permits a more rapid dissociation of HCN from the CcPCN complex

than from the CcP(H52L)CN complex where a comparable hydrogen bond is not possible. In this case, the hydrogen bond between the distal histidine and the bound ligand does not stabilize the complex but facilitates ligand dissociation.

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