

Kinetics and Equilibria of Cyanide Binding to *Coprinus cinereus* Peroxidase

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Equilibria and kinetics of cyanide binding to *Coprinus cinereus* peroxidase have been studied. Spectral results show the presence of one heme binding site per molecule of enzyme. Isosbestic points between the native enzyme and the enzyme–cyanide complex are 349, 413.5, 492, 521 and 640 nm. The dissociation constant is $(0.52 \pm 0.03) \mu\text{M}$ at 25°C in phosphate buffer of 0.1 M ionic strength at pH 6.98. The pH dependence of the apparent second-order rate constant indicates the presence of an acidic heme-linked group in the enzyme with a $\text{p}K_a$ of 5.1 ± 0.2 . The protonated form of cyanide binds to the basic form of the enzyme with a second-order association rate constant of $(7.7 \pm 0.2) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$.

Coprinus cinereus peroxidase (donor: H_2O_2 oxidoreductase [EC 1.11.1.7]), like other peroxidases, catalyses the oxidation of a number of substrates by reduction of hydrogen peroxide. *Coprinus cinereus* peroxidase (CiP) is isolated from the inkcap Basidiomycete *Coprinus cinereus*, which has been suggested as a new practical source of peroxidase for clinical analyses, in addition to the horseradish root. CiP is an acidic 38 000-dalton single polypeptide protein with a pI value of 3.5, containing one iron protoporphyrin IX prosthetic group.^{1,2} Structural, functional and enzymatic properties, such as guaiacol and hydrogen peroxide reactivities, amino acid and sugar composition, circular dichroism, magnetic circular dichroism, nuclear magnetic and electron paramagnetic resonance spectra have been reported.^{1,2} Transient state kinetic studies of ligand binding to CiP are described here for the first time for this enzyme.

Peroxidases, belonging to the same class of enzymes (oxidoreductases), catalyse similar types of reaction. Different peroxidases modulate reactivity and specificity by changes in molecular and electronic structures and characteristics of the active site play a major role in the understanding of this modulation. In this paper, we report the results of an equilibrium and kinetic study of cyanide binding to CiP that exploits the spectral differences between the native enzyme and its cyanide-bound complex.

Experimental

CiP was cultured and provided in electrophoretically homogeneous form by Novo Nordisk A/S, Denmark. Samples

were dialyzed extensively against deionized water (Milli-Q system) before use. The enzyme preparation used in this study exhibited an RZ value (A_{405}/A_{280}) of 2.49. The enzyme concentration was calculated from the absorbance at 405 nm using a molar absorptivity (ϵ_{405}) of $109 \text{ mM}^{-1} \text{ cm}^{-1}$. This value was determined by means of the pyridine hemochrome method.³ Enzyme samples were stable for at least one month when kept at 5°C, as seen by monitoring the UV–VIS spectra.

The dissociation constant of the enzyme–cyanide complex, K_d was determined by titrating the enzyme solution with a cyanide solution. The absorbance change was recorded after every addition and analyzed as a function of the ligand concentration using the appropriate equations.⁴ Corrections for dilution were made only when the added volume exceeded 2% of the initial volume. Absorption measurements were performed with a Cary 219 spectrophotometer.

The cyanide-binding process was also measured kinetically by monitoring the absorbance change at 405 nm under pseudo-first-order conditions with cyanide in excess. Kinetic measurements were conducted using a Photal (formerly Union Giken) RA 601 Rapid Reaction Analyzer and interfaced with a digital computer system, Alps Electric Co., model No. CP 10A, and an Epson VP-550 recorder. One reservoir contained the enzyme in buffer, at concentrations of 0.8 to 2.5 μM after mixing, while the other reservoir contained potassium cyanide in buffer. The cyanide concentrations were in at least a tenfold excess to maintain pseudo-first-order kinetic. The pseudo-first-order rate constants, k_{obs} , were determined by means of a curve-fitting computer analysis of the exponential traces. These

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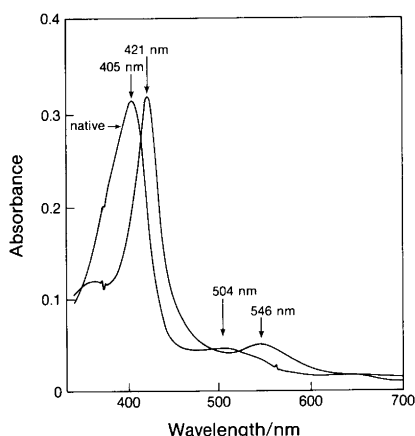


Fig. 1. Visible spectra of native CiP and CiP-cyanide complex. Native enzyme (2.7 μM) in phosphate buffer (pH 6.98, $\mu = 0.1$ M). CiP-cyanide complex (2.7 μM) after addition of 8 μM KCN.

were converted into apparent second-order rate constants, k_{app} , by taking the slope of plots of k_{obs} versus total cyanide concentrations. The second-order rate constant independent of pH, k_1 , the acid dissociation constant of the enzyme, K_1 , and the acid dissociation constant of hydrocyanic acid, K_2 , were determined by means of a non-linear least-squares computer analysis of plots of k_{app} versus pH.

All experiments were performed at 25 $^{\circ}\text{C}$ and at an ionic strength (μ) of 0.1 M with addition of potassium sulfate to keep the ionic strength constant. The pH was varied from 3.66 to 9.97. All reagents were of the highest grade available. Potassium cyanide was from Fisher Scientific Company, and used without further purification. The concentration was determined by weight. Fresh solutions were made before every experiment. The quality of the solution was checked by titration with a standard solution of silver nitrate. The following buffers were used in 0.01 M concentrations: citric acid-sodium citrate (pH 3.66 to 6.03), potassium phosphate (6.98), Tris-hydrochloric acid (pH 8.20 and 8.78) and sodium carbonate-sodium hydrogen carbonate (pH 9.37 and 9.97). The pH was measured with a Fisher microprobe electrode and a Fisher digital pH meter.

Results

Cyanide binding to CiP results in a rapid spectral shift in λ_{max} from 405 to 421 nm with an isosbestic point at 413.5 nm. Fig. 1 shows the visible spectra of the native enzyme and its cyanide-bound complex. Other differences in the spectra include the disappearance of the peak at 504 nm for the native enzyme and the appearance of a peak at 546 nm for the cyanide-bound complex. Isosbestic points were observed at 349, 493, 413.5, 521 and 640 nm.

Titration experiments were performed to determine the number of binding sites and the dissociation constant. The absorbance change at 405 nm was measured over a range of cyanide concentrations until further addition of cyanide

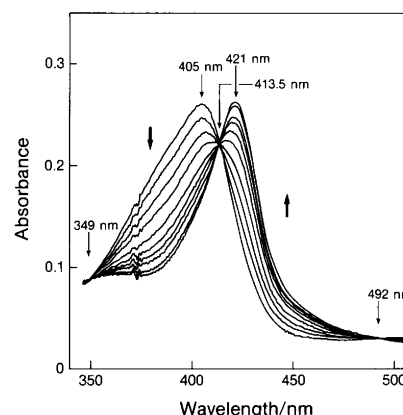


Fig. 2. Titration of CiP with cyanide. The reference cuvette contained phosphate buffer while the sample cuvette contained 2.4 μM CiP in the same buffer (pH 6.98, $\mu = 0.1$ M). Microliters of 500 μM KCN were successively added to both cuvettes and spectra scanned from 345–600 nm. Arrows show direction of change in absorbance.

caused no further spectral change. Fig. 2 shows the appearance of the peak at 421 nm, characteristic of the cyanide-bound complex. The number of binding sites was determined from a plot of the absorbance change at 405 nm against total concentration of cyanide (Fig. 3). Extrapolation of the graph shows that (3.6 ± 0.1) μM of enzyme binds (4.0 ± 0.1) μM of cyanide, indicating a 1:1 complex, to within experimental error.

The changes in absorbance at 405 nm with increasing concentrations of cyanide were exploited to determine the dissociation constant for the enzyme-cyanide complex, K_d by means of a Scatchard plot (Fig. 4), according to the Scatchard equation⁵ [eqn. (1)]. A_0 is the absorbance of the

$$(A_0 - A)/[E]_t[L]_f = \Delta\epsilon/K_d - (A_0 - A)/[E]_t K_d \quad (1)$$

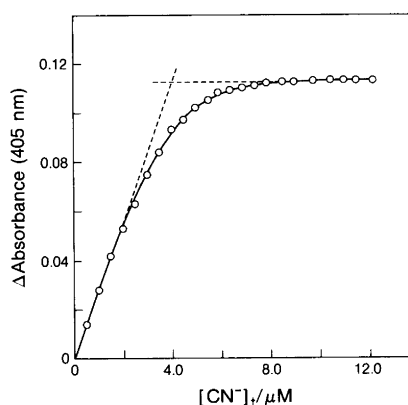


Fig. 3. Number of binding sites of CiP. Conditions as in Fig. 2, except the enzyme concentration (3.6 ± 0.1) μM . Absorbance changes were followed at 405 nm, as a function of total cyanide concentration, $[\text{CN}^-]_t$. Extrapolation of the graph shows that (3.6 ± 0.1) μM CiP binds (4.0 ± 0.1) μM of cyanide suggesting one binding site per enzyme molecule.

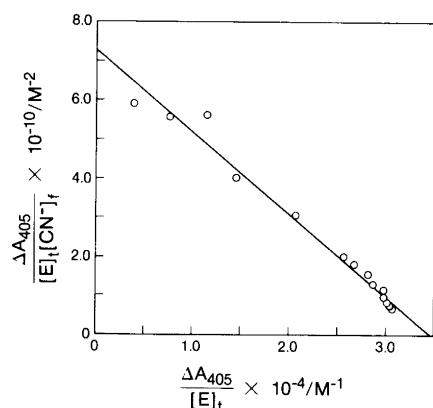


Fig. 4. Scatchard plot obtained from absorbance changes at 405 nm with increasing concentrations of cyanide. Conditions as in Fig. 2, except the enzyme concentration, $(3.6 \pm 0.1) \mu\text{M}$. $K_d = -1/\text{slope} = (0.49 \pm 0.01) \mu\text{M}$.

enzyme at a particular wavelength in the absence of ligand, A the absorbance in the presence of ligand, $[E]_t$ the total enzyme concentration, $\Delta\epsilon$ the difference in molar absorptivity between the native enzyme and the complex, $[L]_f$ the free ligand concentration at equilibrium, and K_d is the dissociation constant of the complex. The dissociation constant can be determined from the slope of the plot. The value for the dissociation constant was determined to be $(0.49 \pm 0.01) \mu\text{M}$. In Fig. 5 a second method was used, the Bjerrum semilog plot,⁴ and a K_d value of $(0.54 \pm 0.03) \mu\text{M}$ was obtained from a non-linear least-squares data analysis, fitting the data to the Bjerrum plot. Therefore the mean value of K_d is $(0.52 \pm 0.03) \mu\text{M}$.

Pseudo-first-order kinetics of cyanide binding to CiP were also investigated. Fig. 6 shows a typical plot of absorbance at 405 nm versus time for the reaction of enzyme with potassium cyanide. The pseudo-first-order rate constant, k_{obs} was evaluated from a curve-fit of the experimental curve. The k_{obs} values were measured as a function of cyanide concentration, $[\text{CN}^-]$, and were shown to increase linearly with the concentration of cyanide, fitting eqn. (2).

$$k_{\text{obs}} = k_{\text{app}}[\text{CN}^-] + k_{-\text{app}} \quad (2)$$

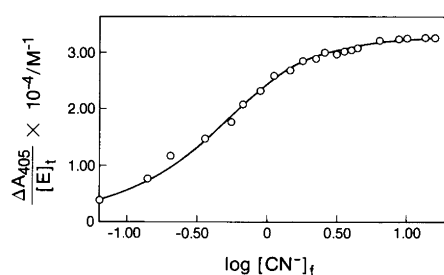


Fig. 5. Bjerrum (semilog) plot. Conditions as in Fig. 2, except the enzyme concentration, $(3.6 \pm 0.1) \mu\text{M}$. K_d determined from curve-fitting analysis is $(0.54 \pm 0.03) \mu\text{M}$.

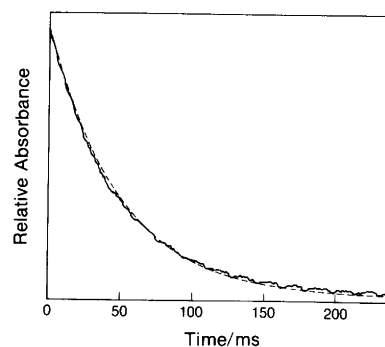


Fig. 6. Plot of relative absorbance at 405 nm versus time for the reaction of $1.8 \mu\text{M}$ CiP with $27 \mu\text{M}$ KCN in phosphate buffer (pH 6.98, $\mu = 0.1 \text{ M}$). Dashed line is the computed first-order curve fit. The ordinate is given without values because of the relative behavior of the exponential function.

A typical example is shown in Fig. 7 for data obtained at pH 6.98. From the slope, the value of the apparent second-order association rate constant, $k_{-\text{app}}$ is $(7.4 \pm 0.3) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and from the intercept, the apparent first-order dissociation rate constant, $k_{\text{app}} = (3.4 \pm 3.0) \text{ s}^{-1}$, is essentially zero to within experimental error. Because of the large error in the kinetically determined value of $k_{-\text{app}}$, typical of values obtained by extrapolation, we determined $k_{-\text{app}}$ from the product of k_{app} and K_d , both of which were determined with a much smaller error. The result is $k_{-\text{app}} = (0.52 \pm 0.03) \mu\text{M} \times (7.4 \pm 0.3) \times 10^5 \text{ M}^{-1} \text{ s}^{-1} = (0.38 \pm 0.04) \text{ s}^{-1}$.

Pseudo-first-order conditions were again used to determine the pH dependence of k_{app} . Fig. 8 shows that the apparent second-order association rate constant was at its maximum from pH 6.03 to 8.20 and decreased at high and low pH values. The data points were fitted to eqn. (3) using a non-linear least-squares analysis.

$$k_{\text{app}} = k_1 / (1 + [\text{H}^+]/K_1)(1 + K_2/[\text{H}^+]) \quad (3)$$

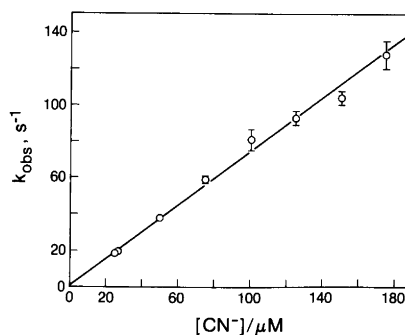


Fig. 7. Pseudo-first-order rate constants of cyanide binding to CiP plotted as a function of $[\text{CN}^-]$ (total cyanide concentration during this kinetic experiment). CiP, $(1.5\text{--}2.5) \mu\text{M}$ in phosphate buffer (pH 6.98, $\mu = 0.1 \text{ M}$). The error bars show the standard deviation calculated from at least seven measurements for each cyanide concentration.

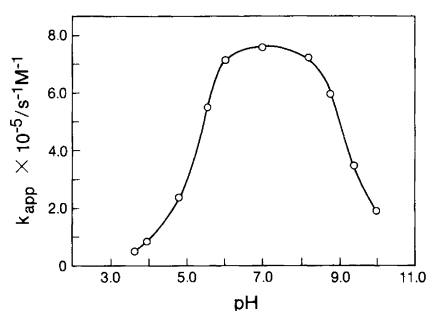
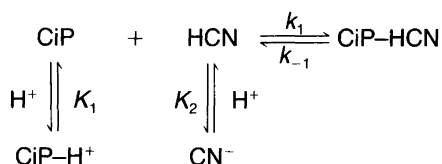


Fig. 8. pH dependence of the apparent second-order association rate constant of cyanide binding. Conditions as in Fig. 7, except that the pH was varied from 3.66 to 9.97 and therefore various buffers were used.

The constant k_1 is the second-order pH-independent association rate constant, K_1 the acid dissociation constant in the active site of the enzyme, and K_2 the acid dissociation constant for hydrocyanic acid. Eqn. (3) was derived based on the assumption that an acid dissociation in the active site influences the binding of cyanide to the enzyme. From the fit the following parameters were determined: $k_1 = (7.7 \pm 0.2) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, $K_1 = (7.9 \pm 1.1) \times 10^{-6} \text{ M}$ and $K_2 = (4.5 \pm 0.6) \times 10^{-10} \text{ M}$. Thus, an acid group with $pK_1 = 5.1 \pm 0.2$ is involved in cyanide binding. The sequence of events that accounts for these parameters is given in Scheme 1.



Scheme 1.

Discussion

Absorption maxima for the native enzyme occur at 405, 504 and 645 nm. For the enzyme–cyanide complex they occur at 421 and 546 nm. In addition, five isosbestic points (349, 413.5, 492, 521 and 640 nm) have been determined for the enzyme–cyanide complex in the wavelength range 345–700 nm. Differences of up to 5 nm in the absorption maxima occur between our results and those reported earlier.^{1,2} These differences can be interpreted in terms of experimental error for the different measurements in different laboratories or in terms of different treatments given to the three enzyme preparations. A different approach for converting the enzyme from a mixture of high- and low-spin forms to the high-spin form (cyanide free) has been taken for each of the three preparations. Lukat *et al.* used sodium dithionite reduction² followed by air oxidation, whereas Morita *et al.* treated their preparation with *p*-chloromercuribenzoate.¹ In this work extensive dialysis (60 h) was

used for the removal of bound cyanide instead of the possibly less mild methods: sodium dithionite and *p*-chloromercuribenzoate.^{6–8} In fact, comparison of spectra for the dialyzed and undialyzed enzyme (data not shown) used in this work show the same spectral changes as for the high- and low-spin spectra of the enzyme¹ obtained with the *p*-chloromercuribenzoate treatment. Spectrophotometrically, any bound cyanide is without significance. The absorption maxima changed from 406, 499 and 644 nm (undialyzed CiP) to 405, 504 and 645 nm (dialyzed CiP), respectively. Evaluation of any differences between the dialyzed and undialyzed enzyme based on absolute absorbances cannot be made due to dilution during dialysis.

Generally, peroxidases of different sources show low K_d values for cyanide complex formation,¹ indicating very tight cyanide binding. The K_d values reported earlier^{1,2} are different (1.6 μM , 14 μM , respectively) compared with the one in this work, $(0.52 \pm 0.03) \mu\text{M}$. These differences may reflect differences in the methods of calculation of K_d or differences in enzyme production and purification or treatment of the enzyme, all of which may change the ligand-binding affinity. In the case that a given enzyme contains some bound cyanide, a higher K_d value will be obtained after cyanide titration compared with the value of a cyanide-free enzyme. As the K_d value obtained here is the lowest of the values reported, the enzyme preparation used in this work is cyanide-free after dialysis.

On the other hand, the absorption maximum for the native enzyme (405 nm) compared with the absorption maximum for the cyanide-saturated complex (421 nm), in comparison with similar values² (403 and 420 nm), indicates that if any bound cyanide is present, it makes an insignificant contribution to the change in absorption maxima in the cyanide titration. The K_d values^{1,2} could be determined for enzyme preparations containing cyanide, or otherwise changed ligand-binding affinity.

In Table 1 kinetic and spectral constants are summarized for a number of various peroxidases and their cyanide complexes. Large differences in ϵ_{max} and λ_{max} are seen, probably based on differences in the nature of the prosthetic group and its surroundings. The rate of association of cyanide and CiP is fast and of the same order of magnitude as that of horseradish peroxidase. The values listed for these two peroxidases are similar, which is expected since it has been found that they are very similar with respect to structural and functional properties^{1,2} although differences within the heme site have been noted.²

The pH profile of the apparent second-order rate constant for cyanide binding shows a bell-shaped curve corresponding to a two-step (enzyme and hydrocyanic acid) combined titration of an acid and a base in agreement with the proposed reaction scheme. The experimental values fit well to eqn. (3) for the apparent second-order association rate constant derived for the reaction scheme. A pK_1 value

Table 1. Data for native peroxidases and their cyanide complexes: Soret region maximum molar absorptivity, ϵ_{\max} ; wavelength of absorption band peak, λ_{\max} ; cyanide association rate constant, k_1 ; and cyanide dissociation constant, K_d .

Peroxidase	$\epsilon_{\max}/\text{mM}^{-1} \text{ cm}^{-1} (\lambda_{\max}/\text{nm})$		$k_1/10^5 \text{ M}^{-1} \text{ s}^{-1}$	$K_d/10^{-6} \text{ M}$
	Native	Cyanide complex		
Horseradish ⁹⁻¹¹	102 (403)	— (420)	1.19±0.02	2.0±0.3
Lignin ¹²	133 (407.6)	—	—	—
Manganese ¹³	129 (406)	—	—	—
<i>Coprinus cinereus</i>	109 (405)	101 (421)	7.7±0.2	0.52±0.03
Lacto- ^{14,15}	112 (412)	100.1 (430)	13±2	0.42
Myelo- ¹⁶	178 (430)	— (454)	43±3	0.53±0.01
Peanut ¹⁷	112 (405)	103 (421)	1.9±0.1	0.30±0.05
Turnip ^{18,19}	115 (403)	— (421)	0.9	—
Chloro- ²⁰	91.2 (400)	78 (430)	0.52±0.05	95±10
Prostaglandin H synthase ^{21,22}	123 (410)	— (424)	0.028±0.002	65±10
<i>Pseudomonas</i> cytochrome c ^{23,24}	237 (407)	— (418)	0.65±0.02	—
Yeast cytochrome c ^{25,26}	92 (408)	103 (426)	1.2±0.1	2.0±0.6

of 5.1 ± 0.2 indicates that an acidic group is present in or close to the active site of the enzyme which, when protonated, prevents binding of cyanide. Thus, the protonated form of cyanide binds to the unprotonated form of the enzyme. This agrees well with related studies,^{10,27,28} which show that weakly acidic ligands bind to the enzyme in their protonated form. As the pH is lowered to acidic values, the group is protonated and the affinity of the enzyme for cyanide is reduced and k_{app} decreases. Our value, $\text{p}K_2 = 9.3 \pm 0.1$, is, to within experimental error, identical with the literature value,²⁹ of 9.31. Therefore, the decrease in the apparent second-order association rate constant in the alkaline region can be attributed to the ionization of the hydrocyanic acid. A group with a $\text{p}K_a$ value of 5.1 ± 0.2 involved in cyanide binding can be assigned to three amino acids: aspartate, glutamate and histidine which, in proteins, have $\text{p}K_a$ range values³⁰ covering the obtained $\text{p}K_a$ value.

The data in Fig. 8 can also be fitted by a reaction between cyanide anion and a protonated enzyme. However the resultant rate constant is $1.4 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ which exceeds the diffusion-controlled limit. Therefore this alternative mechanism is physically impossible.

In summary, the results show that cyanide and *Coprinus cinereus* peroxidase form a complex with the simplest binding ratio of 1:1. The complex formation is fast and of the same order of magnitude as that for similar peroxidases. In this respect, the K_d value is also comparable, giving very tight cyanide binding. The cyanide binding is a function of pH and is at its maximum for neutral pH, decreasing in acidic and alkaline regions. An acidic heme-linked group ($\text{p}K_a = 5.1 \pm 0.2$) regulates the mechanism of cyanide binding and gives information about the characteristics of the enzyme's active site.

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