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A Kinetic Study of the Binding of Carbon Monoxide to Ferrous Chloroperoxidase†

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ABSTRACT: The binding of carbon monoxide to ferrous chloroperoxidase in the pH range 4–6.5 is influenced by a titratable group on the enzyme having a pK_A of 5.5 ± 0.2 at 20 °C. The basic form of the enzyme reacts much faster with carbon monoxide than does the protonated form of the enzyme. The $\Delta H°$ for the ionization of the functional group in the enzyme involved in carbon monoxide binding is about 8 kcal mol^{-1}, and the $\Delta S°$ is approximately 1 cal mol^{-1} K^{-1}. These pK_A and $\Delta H°$ values suggest that this functional group is an imidazole ring associated with a histidine residue situated at the active site of the enzyme. The rates of the reaction for the formation and dissociation of the complex suggest that this histidine residue is not directly liganded to the iron atom of the heme prosthetic group. The relatively good agreement between the various kinetic approaches with several methods of experimentation, data collection, and data analysis lends strength to a proposed model in which the histidine occupies a distal site close to the sixth axial ligand position of the heme iron atom.

Chloroperoxidase, produced by *Caldariomyces fumago*, is a hemoprotein capable of catalyzing several different types of peroxidative reactions. These reactions include the halogenation of metabolic products, the oxidation of hydrogen donors, and the dismutation of hydrogen peroxide (Thomas et al., 1970). Chloroperoxidase also resembles cytochrome P-450$_{cam}$ in terms of its spectral and chemical properties. The close similarities between chloroperoxidase and cytochrome P-450$_{cam}$ in terms of optical absorption (Hollenberg & Hager, 1973), electron spin resonance (Chiang et al., 1975), Mössbauer properties (Champion et al., 1975), and resonance Raman spectra (Champion et al., 1976) suggest similar environments for the heme prosthetic groups in these two enzymes. A comparison of the ferrous enzyme–carbon monoxide complexes of the two enzymes is especially striking. Both enzymes form complexes with carbon monoxide that have the long-wavelength absorption band in the 446–450-nm range,

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characteristic of the P-450 cytochromes. Our interest in the nature of these complexes and in the heme environment that produces them prompted us to explore the kinetics of the binding of carbon monoxide to ferrous chloroperoxidase. Two different approaches have been used for measuring the rate of carbon monoxide binding to the reduced enzyme. In one study utilizing a stopped-flow instrument, the rate of complex formation was determined by measuring the increase in absorption at 446 nm after mixing the enzyme with saturated solutions of carbon monoxide. The second study involved flash photolysis of the preformed complex, followed by measuring the rate of re-formation of the complex at either 450 or 420 nm.

Experimental Procedures

Materials. Several batches of purified chloroperoxidase were used during the course of the experiments reported in this paper. All enzyme samples were isolated and purified by the previously reported methods (Morris & Hager, 1966) with only slight modification. Enzyme preparations used in the stopped-flow experiments had R_z values of 1.25–1.35. The enzyme preparation used in the flash photolysis work had an R_z value of 1.46. The R_z value is a measure of enzyme purity and is defined as the ratio of the absorbance at 398 nm to the absorbance at 280 nm. The enzyme preparations used in the stopped-flow experiments were obtained from cells grown on the regular glucose–malt extract media (Morris & Hager, 1966). The more highly purified enzyme preparation used in the flash photolysis experiments was isolated from culture media that contained fructose as its sole carbon source (Pickard, 1981). The sodium phosphate and citrate buffers employed in these experiments were obtained at various times from Baker, Fisher, or Mallinckrodt Chemical Co. The sodium dithionite used in all of the experiments was from a single bottle purchased from Matheson Coleman and Bell.

Stopped-Flow Experiments. A Durrum stopped-flow instrument was used for measuring the rate of complex formation. Enzyme and carbon monoxide buffer solutions were prepared separately for each stopped-flow experiment. The buffer solution, adjusted to the appropriate pH value, was made oxygen free and saturated with carbon monoxide by bubbling carbon monoxide through the buffer in a closed (with serum cap and hypodermic needles) container for 2–4 h. A concentration value of 1.0 mM was used for the buffer solutions saturated with carbon monoxide at room temperature (*Handbook of Chemistry and Physics*, 1960). The enzyme solution was prepared in a small three-neck flask equipped with two serum caps each penetrated with a hypodermic needle. A powder-addition tube was fitted into the third neck of the flask. Enzyme dissolved in the appropriate buffer was placed in the flask, and sodium dithionite was placed in the addition tube. The apparatus was then flushed with a gentle stream of nitrogen for 2–3 h. Just before use sodium dithionite was added to the enzyme solution to reduce the hemoprotein. The resulting ferrous chloroperoxidase solution was forced directly from the flask into a stopped-flow drive syringe (N_2 pressure) without exposure to air. The buffer saturated with carbon monoxide was withdrawn directly into a drive syringe with a minimum of exposure to air. The stopped-flow apparatus was checked for air (oxygen) leaks by injecting reduced methylviologen into the instrument and monitoring its stability. The dead time of the stopped-flow apparatus was approximately 4–5 ms.

The data from the stopped-flow experiments were collected and processed on a DEC LSI-11 minicomputer. The program for data collection and treatment of the experimental data involved a printout of the digitized absorbance values from the photomultiplier tube output voltage. A simple least-squares program then was used to calculate the first-order rate constant for any segment of the time curves. Most of the observed rate constants were obtained in this manner. In some instances, the data were plotted by hand, and k_{obsd} was calculated from the observed half-life. Ten to fifteen separate determinations were carried out for each experimental point involving a given pH value. A comparison of the k_{obsd} values obtained in each set of these 10–15 separate determinations indicated that the experimental error in the stopped-flow measurements was less than 5%.

The two reactions involved in the overall reaction are

$$4(Fe^{3+})enz + 6H_2O + S_2O_4^{2-} \rightarrow 4(Fe^{2+})enz + S_2O_6^{2-} + 9H_3O^+ \quad (1)$$

$$(Fe^{2+})enz + CO \rightleftharpoons (Fe^{2+}\text{–}CO)enz \quad (2)$$

Typical concentrations used in the stopped-flow experiments were 6 mM sodium dithionite and 6 μM reduced enzyme. An excess of dithionite was used to prevent enzyme reoxidation during the hour needed to complete the serial determinations run in each experiment.

An approximate 100-fold excess of CO was used in order to assure pseudo-first-order kinetics for the formation of the enzyme–carbon monoxide complex. The buffer used most often was 0.05 M sodium citrate. Occasionally 0.01 or 0.02 M sodium or potassium phosphate buffers were employed. No systematic differences were detected when different buffers were employed.

Stopped-Flow Experiments with Varied Carbon Monoxide Concentrations. Three stopped-flow experiments were done at pH 6.3 at less than saturating concentrations of carbon monoxide. For these experiments the enzyme solution was prepared and introduced into the stopped-flow apparatus in the usual manner. The desired concentration of the carbon monoxide was obtained by dilution of carbon monoxide saturated buffer with oxygen-free buffer in an anerobic mixing system. This system was designed with two supply syringes connected through an eight-jet mixer to a receiver syringe. Mixing was carried out in the three-syringe system in order to minimize contact of the solutions with air. The diluted carbon monoxide solution was then introduced directly from this receiver syringe into the drive syringe of the stopped-flow apparatus.

Flash Photolysis Experiments with Temperature Control. The basic instrumentation used in the flash photolysis experiments has been previously described by Austin et al. (1975) and Beece et al. (1981). Preparation of the reduced enzyme–CO complex was carried out by using equipment similar to that used in the stopped-flow experiments. For the flash photolysis experiments, native chloroperoxidase was dissolved in a buffer solution that had been adjusted to the appropriate pH value, and carbon monoxide was bubbled through the oxidized enzyme–buffer solution for 3–3.5 h. Just before the start of the flash photolysis experiment, the enzyme was reduced with the dithionite. The concentration of the enzyme in the flash photolysis experiments was 3.4 μM, and the concentration of carbon monoxide was 1 mM. The buffer solutions used for these experiments were either 0.25 M sodium citrate or 0.12 M sodium phosphate. For experimental observations, a small portion of the ferrous chloroperoxidase–CO complex was transferred to a 1-mm cell that was sealed with stopcock grease. This cell was then placed in the temperature-controlled compartment of the flash photolysis apparatus and aligned with the optical windows of both the laser and the

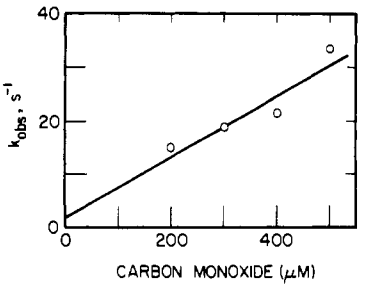


FIGURE 1: Effect of carbon monoxide concentration on rate of complex formation. Observed pseudo-first-order rate constants for the formation of the ferrous chloroperoxidase–CO complex are plotted as a function of the concentration of CO. The experiments were carried out at pH 6.3 and 23 °C according to the stopped-flow method described under Experimental Procedures.

photomultiplier units. Temperature control was effected by liquid nitrogen cooling offset by electrical heaters. The temperature usually did not vary by more than ±0.035 °C during the course of the experiment. Light transmission was monitored at either 450 or 420 nm with band-pass filters (4-nm FWHM). Photodissociation was initiated with a 530-nm flash of 30-ns duration from a frequency-doubled neodymium-glass laser. The photomultiplier voltage output was recorded on a 256-channel analyzer and a logarithmic time-base transient digitizer that permitted signal measurement continuously from 2 μs to 1 ks (Austin et al., 1976). The data from five or six flashes were recorded for each experiment. The data for all the points were analyzed by a least-squares program with plotting capability on the cyber computer.

Results

Kinetics of Complex Formation As Measured by Stopped-Flow Techniques. All of the studies on the rate of complex formation between ferrous chloroperoxidase and carbon monoxide were carried out in the presence of a relatively large excess of carbon monoxide. Preliminary experiments with different concentrations of carbon monoxide showed that, under these conditions, all of the reactions obeyed pseudo-first-order kinetics. The results from stopped-flow experiments at pH 6.3 on the kinetics of formation of the ferrous chloroperoxidase–carbon monoxide complex at different concentrations of carbon monoxide are shown in Figure 1. These results show a linear relationship between the observed rate constant, k_{obsd}, and the concentration of carbon monoxide. Thus, the rate data for the reaction of the ferrous enzyme with carbon monoxide can be analyzed by eq 3–5. It

$$\text{enz} + \text{CO} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{enz–CO} \tag{3}$$

$$\text{rate} = \frac{\text{d[complex]}}{dt} = k_{on}[\text{CO}][\text{enz}] - k_{off}[\text{enz–CO}] \tag{4}$$

$$\text{rate} = (k_{on}[\text{CO}] + k_{off})[\text{enz}] - k_{off}[\text{enz}]_{total} \tag{5}$$

should be noted that the last term in eq 5 is a constant. Thus, when the concentration of carbon monoxide remains essentially constant (carbon monoxide in large excess), the observed rate constant, k_{obsd}, may be expressed in terms of k_{on} and k_{off} according to eq 6. These two rate constants were calculated

$$k_{obsd} = k_{on}[\text{CO}] + k_{off} \tag{6}$$

from the slope and intercept of Figure 1 and found to be approximately $k_{on} = 6 \times 10^4$ M^{-1} s^{-1} and $k_{off} = 2$ s^{-1}. The dissociation constant, K_D, for the ferrous enzyme–carbon monoxide complex is equal to k_{off}/k_{on}. With the above calculated rate constants for k_{on} and k_{off}, the value of K_D was calculated to be about 3×10^{-5} M at pH 6.3. This value for K_D is in good agreement with a previous determined value that was based on direct equilibrium measurements for the reaction (Makino et al., 1976). These workers determined the value of K_D to be 3.8×10^{-5} M at pH 6.3.

Table I: Summary of Stopped-Flow Results[a]

pH	k_{obsd} (s^{-1})	pH	k_{obsd} (s^{-1})
4.35	8.2	5.65	23.5
4.71	10.4	5.79	26.1
4.84	12.2	5.93	26.7
4.99	12.0	6.12	27.9
5.09	14.0	6.25	34.9
5.19	15.2	6.31	33.4
5.34	19.0	6.44	36.1
5.42	21.0	6.50	44.3
5.50	20.0	6.62	33.7
5.59	23.2	6.78	40.1

[a] [CO] = 0.5 mM.

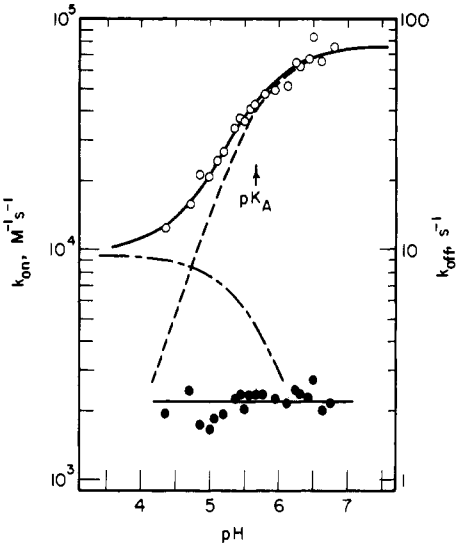


FIGURE 2: Effect of pH on rate of formation and dissociation of carbon monoxide–ferrous chloroperoxidase complex. The formation [k_{on} (○)] and dissociation [k_{off} (●)] experimental rate constants for the ferrous chloroperoxidase–CO complex were measured as a function of pH. The experimental methods are described under Experimental Procedures. The solid line curve represents the theoretical curve for k_{on} based on formulations given in eq 10. The other two theoretical curves indicate the contributions of unprotonated (---) and protonated (– – –) forms of the enzyme to the overall rate of complex formation. The experimental data plotted in this figure were taken from Table I. The arrow indicates the pK_A value for the interconversion of the neutral and acid forms of the enzyme.

Effect of pH on Rate of Complex Formation. Since the results in Figure 1 indicate that 500 μM concentrations of carbon monoxide are satisfactory for the reaction to obey eq 6, only two concentrations of carbon monoxide (500 and 1000 μM) were used in the subsequent studies on the effect of pH on the rates of reaction. When the relationship between K_D and k_{off}/k_{on} is combined with eq 6, k_{on} and k_{off} can be expressed in terms of k_{obsd}, K_D, and the concentration of carbon monoxide as shown in eq 7 and 8. The results (k_{obsd}) from

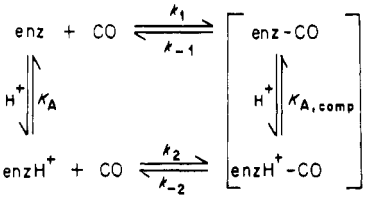
$$k_{on} = k_{obsd}/([\text{CO}] + K_D) \tag{7}$$

$$k_{off} = K_D k_{obsd}/([\text{CO}] + K_D) \tag{8}$$

the stopped-flow experiments at various pH values are given in Table I. The pK_D values were deduced from the results of Makino et al. (1976). The values of k_{on} and k_{off} were

calculated by using eq 7 and 8. Figure 2 shows a semilogarithmic plot of k_{on} and k_{off} vs. pH.

If the formation of the enzyme–carbon monoxide complex is influenced by one or more ionizing groups on the enzyme, the concentration of the active enzyme species will be pH dependent. When the equilibrium reaction forming the acidic and basic forms of the enzyme is fast compared to the reaction of the enzyme with other ligands, the rate constant for the formation of the enzyme–carbon monoxide complex will obey the Henderson–Hasselbalch expression. Thus, a semilogarithmic plot of the rate constant as a function of pH permits an exploration of this relationship. If both the protonated and unprotonated forms are equally reactive with carbon monoxide, a horizontal plot would result. If only one form of the enzyme is reactive for complex formation, there would be a horizontal portion in the pH rate profile where all or most of the enzyme is reactive. This flat portion of the pH rate profile would bend to a curve having a slope of ±1 for intermediate and very low concentrations of the active form of the enzyme. If both protonated and unprotonated forms of the enzyme react with carbon monoxide but at different rates, a composite curve of sigmoidal shape would be obtained. Theoretical curves can be constructed based on the estimated concentrations of the two species of enzyme at different pH values. The experimental pH–rate profile data in Figure 2 suggest that both a protonated and unprotonated species of the enzyme react with carbon monoxide. A simple reaction scheme to account for the experimental observations for the reaction of chloroperoxidase with carbon monoxide would be



(9)

where the unprotonated enzyme–CO and the protonated enzyme–CO complexes in the brackets are spectroscopically indistinguishable. With this scheme, the values for k_{on} and k_{off} can be evaluated according to eq 10 and 11. On the basis

$$k_{on} = \frac{k_1}{1 + [H^+]/K_A} + \frac{k_2}{1 + K_A/[H^+]} \quad (10)$$

$$k_{off} = \frac{k_{-1}}{1 + [H^+]/K_{A,comp}} + \frac{k_{-2}}{1 + K_{A,comp}/[H^+]} \quad (11)$$

of eq 10, a theoretical curve for the pH profile of k_{on} (solid line in Figure 2) was constructed to obtain a best fit curve with the following values for the rate and dissociation constants in eq 10: $k_1 = 7.6 \times 10^4$ M^{-1} s^{-1}; $k_2 = 9.4 \times 10^3$ M^{-1} s^{-1}; $K_A = 2.2 \times 10^{-6}$ ($pK_A = 5.65$, shown by the arrow in Figure 2). Since the experimental points (open circles) fit very close to the theoretical curve, it appears that this model is quite feasible. This model predicts that the unprotonated enzyme reacts about 8 times more rapidly with carbon monoxide than does the protonated form ($k_1/k_2 \simeq 8/1$). Since k_{off} values (closed circles, Figure 2) do not show significant variation in this pH range, it would appear that k_{-1} and k_{-2} are very similar. Alternatively, it could be possible that $K_{A,comp}$ is quite different from K_A in this pH range as reported for horseradish peroxidase (Hayashi et al., 1976).

Flash Photolysis and Effect of pH and Temperature on Complex Formation. The results from the temperature-controlled flash photolysis experiments are plotted in Figures 3 and 4. Since the flash photolysis experiments were carried out at high carbon monoxide concentrations (1000 μM), the value of K_D in eq 7 is negligibly small compared to concentration of carbon monoxide. Thus, it could be assumed that k_{on} would be given from the ratio of k_{obsd} to the concentration of carbon monoxide. Preliminary flash photolysis experiments performed at room temperature, with a different enzyme preparation and more data points, especially at lower pH values (B. N. Campbell, unpublished results), again suggested that the ratio of k_1 to k_2 (see eq 9) was close to 8. Thus the same reaction scheme (eq 9) used for the stopped-flow experiment was used for a study of the effect of pH and temperature in the flash photolysis experiments. Even though it was difficult to determine precisely the value of k_2 with the limited number of points shown in Figure 3, we could calculate the values of k_1 and K_A within a small error range since the contribution of k_2 would be very small in the pH range above 5. The values

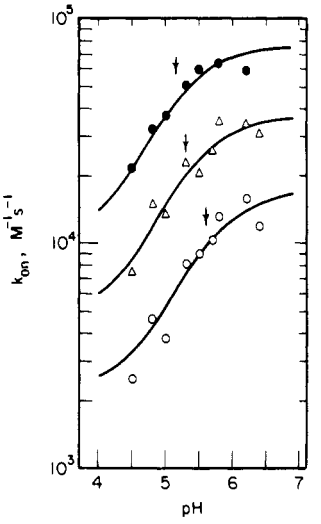


FIGURE 3: Effect of temperature and pH on rate of formation of ferrous chloroperoxidase–CO complex measured at 450 nm. The data were obtained from temperature-controlled flash photolysis experiments according to the methods described under Experimental Procedures. Data were collected at 5 (○), 25 (△), and 35 (●) °C. The solid lines are theoretical best fit curves based on the formulations presented in eq 10. The arrows indicate pK_A values for the interconversion of the neutral and acid forms at the various temperatures.

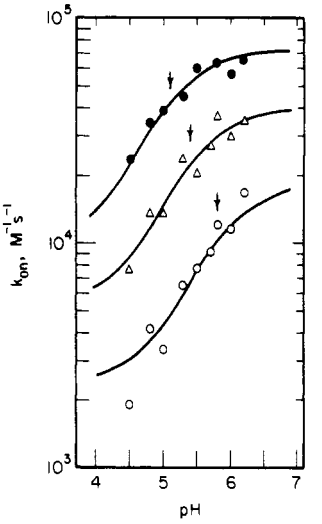


FIGURE 4: Effect of temperature and pH on rate of formation of ferrous chloroperoxidase–CO complex measured at 420 nm. The conditions are the same as described in the legend to Figure 3. In this case, the rate of complex formation was measured by the change in absorbance at 420 nm.

Table II: Rate Constant for CO Complex Formation and Ionization Constant for Acid Form of Ferrous Chloroperoxidase[a]

temp (°C)	$k_1 \times 10^{-4}$ (M^{-1} s^{-1})	$k_2 \times 10^{-3}$ (M^{-1} s^{-1})	pK_A	method
room temp (23 ± 1)	7.6	9.4	5.65	stopped flow
5	2.1 ± 0.4	2 ± 1	5.9 ± 0.1	flash photolysis
20	4.2 ± 0.4	5 ± 2	5.6 ± 0.1	flash photolysis
35	7.4 ± 0.5	15 ± 5	5.3 ± 0.1	flash photolysis

[a] The enzyme used for stopped-flow and flash photolysis measurements was obtained by a different method (see Experimental Procedures).

of k_1, k_2, and K_A at various temperatures are summarized in Table II.

In Figure 5 the pK_A values are plotted as a function of $1/T$. The equation being plotted in Figure 5 is

$$\log K_A \text{ (or } -pK_A) = \frac{-\Delta H^\circ}{2.303R}\left(\frac{1}{T}\right) + \frac{\Delta S^\circ}{2.303R} \quad (12)$$

Thus, a value of $\Delta H^\circ = 8 \pm 2$ kcal mol^{-1} and $\Delta S^\circ = 1 \pm 4$ cal mol^{-1} K^{-1} for the ionization of the enzyme can be calculated from the slope and intercept of the curve in Figure 5.

Similarly, an Arrhenius plot of the log of k_1 against $1/T$ for a given pH can be made (Figure 5). The equation is

$$\log k_1 = -E_a/(2.303RT) + \log A \quad (13)$$

With the data from the curved portion of the plots in Figures 3 and 4, the values of $E_{a,obsd}$ represent a mixture of E_a for the kinetic process and ΔH° for the ionization equilibrium. Analysis of this plot with the determined values for k_1 suggests an energy of activation of about 7.3 ± 0.7 kcal/mol and a value of $2 \pm 1.5 \times 10^{10}$ M^{-1} s^{-1} of the Arrhenius frequency factor for the reaction of the unprotonated enzyme with carbon monoxide.

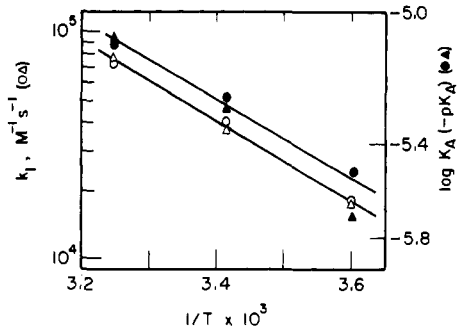


FIGURE 5: Effect of temperature on experimentally observed pK_A values and an Arrhenius plot for the formation of ferrous chloroperoxidase–CO complex. The log K_A (or $-pK_A$) values (open circles and triangles) for the ionization of the neutral and acid forms of ferrous chloroperoxidase are plotted as a function of $1/T$. The experimental data were obtained from Figure 3 [(●) measured at 450 nm] and from Figure 4 [(▲) measured at 420 nm]. The log of k_1 (the formation rate constant of the ferrous chloroperoxidase–CO complex for the reaction of the unprotonated form of chloroperoxidase with CO) is plotted as a function of $1/T$ (closed circles and triangles). The values of k_1 were obtained from the best fit parameters from the k_{on}–pH profiles shown in Figure 3 [(○) 450 nm] and in Figure 4 [(△) 420 nm].

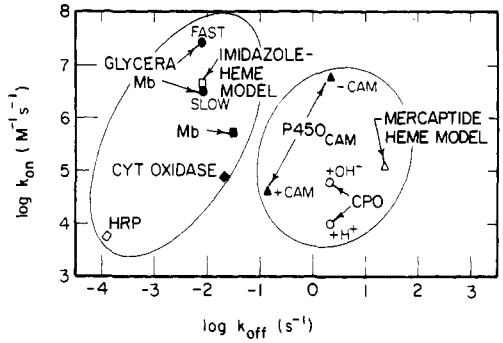


FIGURE 6: Comparison of kinetic properties for reaction of carbon monoxide with various hemoproteins and related model compounds.

Discussion

Kinetic studies of the reaction of carbon monoxide with various hemoproteins and related heme model compounds have been the subject of several investigations. Thus, individual rate constants for the formation (k_{on}) and dissolution (k_{off}) of the carbon monoxide complexes are known in several cases. Some of these rate constants are summarized in Table III. In Figure 6 the kinetic properties of several hemeproteins are compared on the basis of their k_{on} and k_{off} values for carbon monoxide complex formation. Figure 6 shows that the hemoprotein family can be roughly divided into two broad groups depending on the *off* rate for CO binding. Since the spectral properties of chloroperoxidase and cytochrome P-450 can be duplicated by various mercaptide–heme model compounds, it has been postulated that both chloroperoxidase and the P-450 cytochromes have a thiolate group serving as a fifth ligand

Table III: Comparison of Kinetic Constants for Reaction of Carbon Monoxide with Various Hemoproteins and Related Model Compounds

compound	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	pH	temp (°C)
myoglobin[a]	5.1×10^5	2.3×10^{-2}	7.0	20
glycera myoglobin				
fast phase[b]	2.5×10^7	8.6×10^{-3}	6.0	20
slow phase[b]	3.0×10^6		6.0	20
horseradish peroxidase (isozyme C)	5.7×10^3 [c]	1.1×10^{-4} [d]	7.0	20
chloroperoxidase				
neutral form	7.2×10^4	2.2	4.5-6.5	23
acidic form	9.6×10^3	~2	4.5-6.5	23
P-450$_{cam}$[e]				
minus camphor	5.1×10^6	2.3	7.4	4
plus camphor	3.7×10^4	0.14	7.4	4
chelated imidazole-heme[f]	3.6×10^6	9×10^{-3}	7.3	20
imidazole-heme[g]				
Im-heme	5.7×10^6		(toluene)	21
Im-heme	3.4×10^4			
mercaptide-heme[h]	1.1×10^5	18	(DMA)	23

[a] Sono et al. (1976). [b] Seamonds et al. (1976). [c] Brunori et al. (1969). [d] Tamura & Yonetani (1973). [e] Peterson & Griffin (1972). [f] Traylor & Berzinis (1980). [g] Stanford et al. (1980). [h] Chang & Dolphin (1976).

to the heme iron prosthetic group (Chang & Dolphin, 1976). It is readily apparent from the plots in Figure 6 that chloroperoxidase, P-450$_{cam}$, and a mercaptide–heme model compound show similar kinetic behavior in terms of CO binding. Figure 6 also shows that chloroperoxidase, P-450$_{cam}$, and the mercaptide–heme model can be distinguished from other heme compounds and enzymes that are known to have an imidazole as a fifth ligand on the basis of their *on* and *off* rates for carbon monoxide complex formation. Thus, the kinetic properties of the reaction of carbon monoxide with chloroperoxidase and P-450$_{cam}$ are in accord with the assumption that chloroperoxidase and P-450$_{cam}$ differ from most other hemoproteins in terms of the chemical nature of their fifth axial ligands.

Both the stopped-flow results and the flash photolysis data presented in this paper indicate that the unprotonated form of chloroperoxidase reacts with carbon monoxide approximately 8 times faster than does the protonated form. It has been reported that the rate of carbon monoxide binding to an imidazole–heme model compound is about 200 times faster than the reaction of CO with an imidazolate–heme model compound (Stanford et al., 1980) caused by a change of electric charge on the group from −1 to 0. By analogy to these models, the decrease in the rate of CO binding associated with the protonated form of chloroperoxidase could be attributed to the protonation of an amino acid residue of the peptide chain directly bound to the iron atom of the heme group of chloroperoxidase as a fifth axial ligand (proximal site). However, this model seems to be a poor analogy because there are no marked pH-dependent differences in the k_{off} values for the breakdown of the chloroperoxidase–CO complex, especially around pH 5.5, the measured pK_A for the process. If an axial ligand to the heme iron of chloroperoxidase was being protonated at pH values below pH 5.5, the values of k_{off} for the complex should be dramatically changed at acidic pH values. However, ionization of an axial ligand should not be substantially affected by carbon monoxide binding. In addition, no marked spectral differences between the protonated and unprotonated forms of the enzyme have been detected. If the acidic and neutral forms of the ferrous chloroperoxidase–carbon monoxide complex involved protonation of an axial ligand, we would expect to detect spectral differences in the two complexes. As would be expected, the protonated and unprotonated model compound–carbon monoxide complexes do show distinct spectral differences (Stanford et al., 1980).

A better analogy for explaining the pH dependence of the binding of CO to chloroperoxidase appears to be the effect of camphor on the binding of CO to P-450$_{cam}$. P-450$_{cam}$ is thought to have a binding site for camphor at a distal position near the sixth coordination site of the heme iron (Peterson & Griffin, 1972). The rate of formation of the P-450$_{cam}$–carbon monoxide complex decreases 140-fold in the presence of bound camphor. However, the change in k_{off} in the presence of camphor shows only a 16-fold decrease. We interpret these observations to mean that steric hinderance at a distal site has a much more pronounced effect on the *on* rate of formation rather than on the *off* rate for the dissociation of the enzyme–CO complex. Thus, by analogy to P-450$_{cam}$, the decrease in the rate of reaction of the protonated form of chloroperoxidase with CO can be attributed to steric hindrance caused by protonation of a distal base. This conclusion is also supported by analogy with model compound studies. Traylor (1981) has concluded from his heme model studies that proximal strain, resulting from axial ligands, will be reflected in about equal changes in both the *on* and *off* rates for carbon monoxide binding. However, in the model studies, distal-side steric effects show a major effect only on the k_{on} for binding. These arguments in favor of steric effects by a distal base are also consistent with studies on the effect of pH on the *on* rate of binding of carbon monoxide to ferrous horseradish peroxidase (Kertesz et al., 1965). In alkaline solution the *on* rate for the binding of carbon monoxide to horseradish peroxidase is approximately twice the *on* rate in acidic solution. Although a pK_A value for the horseradish peroxidase functional group involved in carbon monoxide binding was not precisely determined, a plot of the experiment results indicates a pK_A value between 7 and 8 for this group. This pK_A value thus correlates with the measured ionization constant (pK_A = 7.2) of the distal base in ferrous horseradish peroxidase (Yamada & Yamazaki, 1974; Yamada et al., 1975). In summary, steric hindrance by a distal base at the chloroperoxidase active site, in both the protonated and unprotonated forms, provides an adequate explanation for the effect of pH on the binding of carbon monoxide to ferrous chloroperoxidase.

Table IV: Ionization Constants for Conversion of Acid-Neutral Forms of Ferrous Hemoproteins (pK_A) and Respective CO Complexes (p$K_{A,comp}$)

	pK_A	p$K_{A,comp}$
myoglobin[a]	5.57	5.67
horseradish peroxidase		
isozyme A	5.8[b]	6.7[c]
isozyme C	7.25[a]	8.25,[a] 8.80[c]
chloroperoxidase	5.5	

[a] Hayashi et al. (1976). [b] Yamada et al. (1975). [c] Barlow et al. (1976).

The chemical nature of the putative distal base in chloroperoxidase that is involved in the carbon monoxide reaction can be approached by comparing the pK_A and $\Delta H°$ data for the process with established values. Although the pK_A values for amino acid residues in proteins can shift by 2–3 pK units according to their electronic environment, the values of $\Delta H°$ for the ionization of an amino acid functional group in a protein are not expected to show such great variation (Christensen & Izatt, 1976). The range in values for the pK_A and $\Delta H°$ for ionization of an amino acid carboxylic acid group in a protein are approximately 1.5–6.0 (pK_A) and ±1.5 kcal/mol ($\Delta H°$). For an amino acid thiolate group, the pK_A varies between 7.5 and 10.3, while $\Delta H°$ is in the range of 6–7 kcal/mol (Cecil, 1963). For the imidazole group of a histidine residue, the pK_A shows a variation between 5.6 and 7.0, and the $\Delta H°$ values are in the range of 6.9–7.5 kcal/mol (Cohn & Edsall, 1943). The pK_A values of 5.5 and the $\Delta H°$ value of 8 kcal/mol for the reaction of chloroperoxidase with carbon monoxide suggest that the proposed distal base in chloroperoxidase associated with CO binding is most likely a histidine imidazole group.

The pK_A values for the interconversion of the neutral and acid forms of ferrous myoglobin, horseradish peroxidase, and their carbon monoxide complexes have been measured and are recorded in Table IV. These ionizations have been interpreted as protonation and deprotonation of distal bases and are attributed to the ionization of the imidazole ring of a histidine residue (Stryer et al., 1964; Yamada & Yamazaki, 1974, 1975; Welinder & Mazza, 1977). It has been suggested that the interaction between the distal imidazole and the outer ligand at the sixth coordination position is greater in horseradish peroxidase than in myoglobin. This argument is based on the magnitude of the shift in the pK_A for the interconversion of the acid and neutral forms of the carbon monoxide complexes of these proteins (Table IV) and the pH dependence of the IR spectra of these complexes (Barlow et al., 1976). The same

kind of interaction appears to be strong in chloroperoxidase, presumably because of the existence of a strong steric effect for carbon monoxide binding. Thus, the three-dimensional structural relation between the distal base and the heme prosthetic group undoubtedly plays an important role in the peroxidatic activity of hemoproteins. It has been suggested that general acid–base catalysis should be involved in the mechanism of peroxidase compound I formation for horseradish peroxidase (Schonbaum & Lo, 1972; Dunford & Araiso, 1979). The proposed distal base imidazole in chloroperoxidase could also play an important role in general acid–base catalysis for the formation of compound I in the peroxidatic reactions catalyzed by this halogenating enzyme.

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