The Binding of Cyanide to Ferroperoxidase

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1. The equilibrium and kinetics of cyanide binding to ferroperoxidase were investigated. At pH 9.1 the equilibrium and kinetic measurements agree closely and disclose a single process with an affinity constant of $1.1 \times 10^3 \text{M}^{-1}$ and combination and dissociation velocity constants of $29 \text{M}^{-1} \cdot \text{s}^{-1}$ and $2.5 \times 10^{-2} \text{s}^{-1}$ respectively. 2. At pH values below 8 the affinity constant falls until at pH 6.0 the ferroperoxidase·cyanide complex is no longer formed. This is shown to be associated with the formation of ferriperoxidase·cyanide complex in the mixture even in the presence of excess of sodium dithionite. 3. Rapid-pH-jump experiments show a fast pseudo-first-order interconversion between ferroperoxidase·cyanide complex at pH 9.1 and ferriperoxidase·cyanide complex at pH 6.0. 4. The kinetics of binding of cyanide to dithionite-reduced peroxidase at pH 6.0 are complicated and radically different from those observed at pH 9.1. 5. Above pH 8 the change of affinity constant with pH is consistent with the undissociated species, HCN, being bound by the ferroperoxidase. The enthalpy for this process measured both by equilibrium and kinetic methods is about $-8 \text{kcal/mol}$. 6. The binding of cyanide to reconstituted peroxidases, proto, meso and deuto, was investigated. 7. The results are discussed in relation to known data on cyanide binding to other haemoproteins.

Horseradish peroxidase contains one protohaem IX group/protein molecule and in its naturally occurring ferric state it combines with ligands such as cyanide, F⁻, N₃⁻, NO, NH₂OH and H₂S, and in the ferrous form it combines with another series of ligands, namely CO, cyanide, RNC, NO and O₂. The spectra of these various liganded forms are each similar to those of myoglobin and haemoglobin (Keilin & Hartree, 1951). The equilibria and kinetics of these reactions of ferroperoxidase with ligands have not been extensively investigated except for CO and O₂ (Kertesz, Antonini, Brunori, Wyman & Zito, 1965; Wittenberg et al. 1967; Brunori, Antonini, Phelps & Amiconi, 1969).

Keilin & Hartree (1955) showed that ferriperoxidase combines reversibly with cyanide over a wide range of pH, forming a stable ferriperoxidase·cyanide complex with an absorption spectrum very similar to that of metmyoglobin cyanide. The kinetics of cyanide binding by ferriperoxidase have been explored by Ellis & Dunford (1968).

On the other hand ferroperoxidase, though combining reversibly with cyanide, was shown by Keilin & Hartree (1955) to do so within a much more limited range of pH, forming a less stable ferroperoxidase·cyanide complex with an affinity two or three orders of magnitude less than that observed for ferriperoxidase·cyanide complex. The system also offers a unique opportunity for the study of the binding of cyanide to a ferrous haemoprotein, a study that is not possible in myoglobin or haemoglobin since those proteins possess extremely low affinities for this ligand.

This paper reports experiments on the equilibria and kinetics of binding of cyanide to the ferrous form of horseradish peroxidase isoenzyme C (Shannon, Kay & Lew, 1966).

EXPERIMENTAL

Materials

Horseradish peroxidase. This was purified as described by Phelps & Antonini (1969).

Ferroperoxidase solutions were made by adding a slight excess of Na₂S₄O₄ to stoppered tubes of ferroperoxidase solution. Deuteroperoxidase was prepared by coupling the apoperoxidase prepared as described by Phelps & Antonini (1969) with a slight excess of deuterohaem dissolved in the minimum amount of 10mM NaOH and diluted into the protein solution in 50mM-sodium borate buffer, pH 9.1. The deuterohaem was prepared by hydrolysis withaq. 25% (w/v) HCl of the dimethyl ester of the porphyrin purchased from Koch–Light Laboratories Ltd. (Colnbrook, Bucks., U.K.), and subsequent insertion of iron by adding aqueous FeSO₄ to the solution of porphyrin in pyridine–acetic acid (1:50, v/v) and

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extracting the pigments with ether, washing with 25% (w/v) HCl and evaporating to dryness.

Mesoperoxidase was prepared similarly except that the dimethyl ester of mesoporphyrin (Koch-Light Laboratories Ltd.) was used.

Protoperoxidase was prepared by titration of apoperoxidase with haematin solution and was used as a control of the reconstitution procedure.

All reconstituted peroxidases were chromatographed free of excess of haem by adsorption on and elution from a column of CM-cellulose (previously equilibrated with 5 mM-sodium acetate buffer, pH 4.5) with 100 mM-sodium acetate buffer, pH 4.5. The resultant solutions were dialysed extensively against 50 mM-sodium borate buffer, pH 9.1.

Buffer solutions. These were prepared from stock solutions of 1 mM-NaH₂PO₄ and 1 mM-K₂HPO₄ in the range pH 5.0–8.5 and were 200 mM; to extend the range to pH 8.5–11.0 50 mM-sodium borate buffers were used.

Reagents. These were of analytical quality where possible and all water used was double glass-distilled.

Methods

Spectra were measured with a Cary model 14 recording spectrophotometer with 1 cm-light-path cells in a thermostatically controlled cuvette holder.

Stopped-flow kinetic determinations were carried out with a Gibson–Durrum apparatus (Gibson & Milnes, 1964) with a 2 cm observation tube. Bandwidths were 1–2 nm and the dead-time of the instrument was 3–4 ms.

Kinetic difference spectra were measured on the same instrument by varying the spectral wavelengths at 4 nm intervals over the range 404–432 nm.

Equilibrium titrations were measured on 2.0 ml of 5 μM-ferreroxidase dissolved in the appropriate buffer, reduced with 1–2 mg of Na₂S₂O₄, with additions of 0.1 mM-KCN (25, 50, 100 and 200 μl). KCN solutions were standardized by titration against 0.1 mM-AgNO₃ and were used immediately. For experiments involving the determination of the enthalpy change the temperature of the water circulating in the thermostatically controlled cuvette holder was varied in 5°C increments from 20°C to 40°C. The pH of the cyanide solutions was corrected to the required value by careful additions of 1 mM-HCl to the initially extremely alkaline solution of KCN in water.

RESULTS AND DISCUSSION

Keilin & Hartree (1955) demonstrated that the affinity of ferroperoxidase for cyanide varied markedly with the pH at which combination occurred. This was confirmed in the present work, where the equilibrium and kinetic properties of the system were studied across the whole pH range. In the alkaline range, from pH 7.0–10.0, the absorption maximum of the Soret band of ferroperoxidase at 437 nm shifts on binding cyanide to produce the ferroperoxidase-cyanide complex with a spectral maximum at 432 nm. However, below pH 6.0, even in the presence of excess of dithionite, ferroperoxidase-cyanide complex is formed with totally different spectral properties (λmax, 423 nm).

Fig. 1 shows the spectral characteristics of ferro- and ferri-peroxidase and their cyanide derivatives together with that of the ferroperoxidase-CO complex.

Affinity constant. The affinity constant was determined by recording the spectra of 5 μM-ferroperoxidase in the requisite buffer containing 2 mg of sodium dithionite/ml after successive additions of 0.1 mM-potassium cyanide solution, which had previously been corrected to the appropriate pH by careful titration with 1 mM-hydrochloric acid. The results of such a series of experiments in buffers over the range pH 6.0–10.0 were expressed as the reciprocal of the observed E₄₃₂ values and were plotted against the reciprocals of the cyanide concentrations. Straight lines resulted in all cases. The concentration of total cyanide required for 50% formation of the ferroperoxidase-cyanide complex gave the affinity constants shown in Table 1. The affinity constant rises progressively from very low values at pH 6.0 to a maximum at pH 8–9, whereafter a decline occurs at higher pH values. Below pH 6 only the spectra of ferroperoxidase-cyanide complex could be distinguished. Thus, as the species of cyanide compound formed depends on the pH range used, the binding in the acid range is below treated separately from that in the alkaline range.

Binding of cyanide to ferroperoxidase. (a) Acid
**Table 1. Affinity constants of ferroperoxidase for cyanide as a function of pH**

The values are spectrophotometrically obtained and refer to the equilibrium: ferroperoxidase$+$cyanide $\rightleftharpoons$ ferroperoxidase$\cdot$cyanide complex.

$$
K_{\text{affine}} = \frac{[\text{ferroperoxidase}]\cdot[\text{cyanide}]}{[\text{ferroperoxidase} \cdot \text{cyanide complex}]}
$$

<table>
<thead>
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<th>Temp. (°C)</th>
<th>pH 7.5</th>
<th>8.0</th>
<th>8.5</th>
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<td>240</td>
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range. Two simple experiments serve to demonstrate the behaviour of the haemoproteins towards cyanide in this range.

(i) A solution of ferriperoxidase at pH 5.0, when reduced with dithionite, binds carbon monoxide to produce the ferriperoxidase$\cdot$CO complex, though the same solution binds cyanide to produce a ferriperoxidase$\cdot$cyanide complex. When this latter solution is treated with carbon monoxide the ferriperoxidase$\cdot$cyanide complex is totally replaced by that of ferriperoxidase$\cdot$CO complex.

(ii) A solution of ferroperoxidase$\cdot$cyanide complex at pH 8 titrated with acid to pH 6–5.5 shows the clear replacement of the initial complex by that of the ferriperoxidase$\cdot$cyanide complex.

Keilin & Hartree (1955) showed that the absorption spectra of peroxidase containing cyanide and dithionite in different buffers between pH 6.2 and 10.3 exhibited four distinct isosbestic points, which lay on the absorption trace of the ferriperoxidase$\cdot$cyanide complex and not on that of ferriperoxidase. They concluded that, despite the presence of dithionite, the two components present were the ferriperoxidase$\cdot$cyanide complex and the ferriperoxidase$\cdot$cyanide complex within the pH range studied.

The clearest indication of the correctness of this postulate is shown in Fig. 2, which records the kinetic difference spectrum observed when the ferriperoxidase$\cdot$cyanide complex in dilute borate buffer at pH 9.1 is rapidly mixed in the stopped-flow machine with 0.2M-phosphate buffer, pH 6.0, containing 25mM-cyanide and dithionite so as to create a pH jump. Fig. 2 also records the static difference spectrum of the samples as obtained from Fig. 1. By comparison with the recorded spectra of ferroperoxidase$\cdot$cyanide and ferriperoxidase$\cdot$cyanide complexes in Fig. 1, it is clear that the interconversion from the ferroperoxidase$\cdot$cyanide derivative into the ferriperoxidase$\cdot$cyanide derivative takes place directly without the appearance of an intermediate state within the dead-time of the instrument. The time-course of this process corresponds to a pseudo-first-order constant of 0.5s$^{-1}$.

On the other hand, if ferroperoxidase is allowed to react with cyanide so that the final reaction mixture contains both ferroperoxidase$\cdot$cyanide and ferriperoxidase$\cdot$cyanide complexes, a condition fulfilled below pH 7.0, the time-course of the reaction is complicated. Some of the complexity of the kinetics is indicated in Fig. 3 where the
The results of stopped-flow experiments at two pH values are compared, in which a solution of ferroperoxidase is rapidly mixed with a solution of cyanide containing dithionite while the observation wavelength is altered. By reference to Fig. 1 the results collected at pH 9.1 all substantiate the disappearance of ferroperoxidase and the concomitant appearance of ferroperoxidase-cyanide complex. This aspect is treated more fully below. The results at pH 6.0 display a more complex pattern, which depends on the observation wavelength employed. At 432 nm, an initial fast reaction which would correspond to the formation of ferroperoxidase-cyanide complex from ferriperoxidase is followed by a slower process, which would then reflect the conversion of ferroperoxidase-cyanide complex into the ferric derivative of the haemoprotein. Even more clearly at 440 nm, a wavelength that is isosbestic with the ferriperoxidase-ferroperoxidase-cyanide system, a lag precedes the slow decrease in extinction observed, which would be in agreement with the same proposal. At 425 nm, however, a wavelength that is isosbestic with ferro- and ferri-peroxidase-cyanide complexes, the only observable component is a fast one corresponding to the process ferroperoxidase $\rightarrow$ ferriperoxidase-cyanide complex.

The variation in ligand affinity over the range pH 6–8 requires some explanation. Ellis & Dunford (1968) determined the dissociation constant for ferriperoxidase-cyanide complex and their values, expressed as affinity constants, vary between $6 \times 10^5$ M$^{-1}$ at pH 6 and $3 \times 10^5$ M$^{-1}$ at pH 8. The corresponding value at pH 8 for the ferriperoxidase-cyanide complex is $1.1 \times 10^5$ M$^{-1}$ and the value at pH 6 is not measurable. It is evident that the pH-dependence of the affinity constant for ferriperoxidase for cyanide is too small to account for the observed change in the ratio between the ferrous and ferri-peroxidase-cyanide complexes, since the former changes only twofold over this pH range. The 300-fold higher affinity of cyanide for the ferric species over that for the ferrous implies that, at equilibrium, the presence of the ligand would favour the formation of the ferriperoxidase-cyanide complex. So the root cause of the acid-binding anomalies with ferriperoxidase would seem to lie in the inability of dithionite to maintain the system in a reduced state in the presence of cyanide at pH values lower than 7. This must have its explanation in the progressive overlapping of the redox potentials of the system ferriperoxidase/ferroperoxidase with that of the products of dithionite action (Clark, 1960). Since the midpoint potential of the oxidation-reduction for the peroxidase system becomes less negative with a decrease in pH.
(Harbury, 1957) the effect must be attributed to changes in the redox system involving dithionite and its by-products with pH.

(b) Alkaline range. In contrast with the complexities encountered in the acid region, the behaviour of ferroperoxidase above pH 8 is more tractable.

The decline in the affinity constant from pH 8 to pH 10.5 may be explained on the basis that the undissociated species, HCN, and not CN\(^-\), binds to ferroperoxidase. Izatt, Christensen, Pack & Bench (1962) reported the pK values for the dissociation of hydrocyanic acid at J 0 to be 9.36 at 20°C, 9.11 at 30°C and 8.88 at 40°C, and gave a \(\Delta H^0\) value of 10.4 kcal/mol at 25°C.

The theoretical behaviour for the variation of affinity constant with pH and temperature may then be derived from simple mass-law considerations.

Let \(\alpha\) be the fraction of the total cyanide species present as CN\(^-\) so that \((1-\alpha)\) represents the fraction present as undissociated HCN. Then:

\[
\frac{[H^+][CN^-]}{[HCN]} = K
\]

where \(pK = 9.2\), and:

\[
\frac{[HCN][\text{peroxidase}^{2+}]}{[\text{peroxidase}^{2+}\cdot\text{HCN}]} = K'
\]

where \(K'\) is the true dissociation constant of ferroperoxidase-HCN complex. Then:

\[
\text{pH} - pK = \log \left( \frac{\alpha}{1-\alpha} \right)
\]

or:

\[
\frac{\alpha}{1-\alpha} = K \cdot [H^+]
\]

Whence:

\[
\alpha = \frac{K \cdot [H^+]}{1 + K [H^+]}
\]

and the apparent affinity constant \(K''\) of ferroperoxidase for HCN is then given by:

\[
K'' = K' \cdot \left( \frac{K \cdot [H^+]}{1 + K [H^+]} \right)
\]

The theoretical curves agree well with the experimental points, as shown in Fig. 4, and suggest an affinity constant of \(1.4 \times 10^3\) M\(^{-1}\) at pH 8 and 20°C. The effect of temperature on the system is discussed below.

**Kinetics of the binding of cyanide to ferroperoxidase.** In the reaction:

\[
\text{Peroxidase}^{2+} + \text{HCN} \xrightleftharpoons[k_{-1}]{k_{+1}} \text{peroxidase}^{2+}\cdot\text{HCN}
\]

the rate equation is:

\[-\frac{d}{dt}[\text{peroxidase}^{2+}] = k_{+1} \cdot [\text{peroxidase}^{2+}][\text{HCN}] - k_{-1} \cdot [\text{peroxidase}^{2+}\cdot\text{HCN}]
\]

The rate of approach to equilibrium under conditions where [HCN] is much greater than [peroxidase\(^{2+}\)] is \(k' = k_{+1} + k_{+1} \cdot [\text{HCN}]\), where \(k'\) is the pseudo-first-order rate constant measured under such conditions. Thus a plot of \(k'\) against [HCN] should give a straight line with intercept equal to \(k_{-1}\) and a slope equal to \(k_{+1}\). Fig. 5 shows the graphical summary of three series of experiments in which 2 \(\mu\)M-peroxidase was mixed rapidly at pH 9.1 and 20°C with various concentrations of cyanide, the concentration of the latter being expressed as the total cyanide species present. The results indicated:

\[
k_{+1} = 29 M^{-1}\cdot s^{-1} \text{ and } k_{-1} = 2.5 \times 10^{-2} s^{-1}
\]

The affinity constant \(K\), i.e. \(k_{+1}/k_{-1}\), is 1.16 \(\times 10^3\) M\(^{-1}\), which agrees well with the statically determined value of \(1.05 \times 10^3\) M\(^{-1}\) from titration at the same pH.

As a further confirmation of the validity of these values, the total extinction change that occurs during the rapid-mixing experiments can be plotted to
Fig. 5. Reaction of $5\mu$m-ferroperoxidase with cyanide in 50mm-borate buffer, pH 9.1, at 20°C. The pseudo-first-order rate constant, $k'$, is plotted against the concentration of NaCN.

Fig. 6. Extinction changes derived from rapid-mixing experiments when $5\mu$m-ferroperoxidase was mixed with various concentrations of NaCN. The total extinction change occurring is plotted against the concentration of NaCN after mixing.

give equilibrium-titration curves, as shown in Fig. 6. From the formation of 50% ferroperoxidase-cyanide complex at 0.9mm total cyanide concentration, the affinity constant of $1.11 \times 10^3$ M$^{-1}$ can be derived. Thus the results obtained by three independent methods agree well and give support to the postulate that the single-process scheme outlined above adequately represents the binding of cyanide to ferroperoxidase.

(c) Effect of temperature on ligand binding. Fig. 4 shows the results obtained in experiments where the affinity constant of cyanide for ferroperoxidase was determined in the temperature range 20–40°C. The logarithm of the affinity constant is plotted against the pH values at a series of three temperatures. The theoretical curves computed for the variation of the affinity constant with pH on the basis of the undissociated species, HCN, binding to the protein are shown in the same figure. The curves correspond to the change in affinity with pH for a process with $\Delta H^\circ = -8$ kcal/mol involving a linked ionization with $pK_9.6$ at 20°C and $\Delta H$ ionization = $-12$ kcal/mol. The results agree fairly closely with the theoretical curves supporting the contention that only the undissociated species HCN binds to the protein.

Confirmation of these findings was obtained from a series of kinetic experiments in which $3\mu$m-peroxidase in 50mm-borate buffer containing a few crumbs of dithionite was rapidly mixed with cyanide solutions also containing a trace of dithionite at the same pH at three different temperatures: 19.6, 28.8 and 38.9°C (Fig. 7). As described above, under pseudo-first-order conditions this enables $k_{on}$ and $k_{off}$ constants to be evaluated. From the temperature-dependence of the logarithm of the rate constants, the activation energies $E$ for the ‘on’ and ‘off’ reactions was determined as 10±1.5 and 19.2±0.5 kcal/mol respectively. Since $E = \Delta H^\circ + RT$ the kinetically determined heats of activation correspond to 9.4±1.5 and 18.6±0.5 kcal/mol respectively. Thus the kinetically observed heat of binding, $-9.2$ kcal/mol, is in excellent agreement with the value determined by equilibrium methods.

Cyanide binding to peroxidase reconstituted with unnatural haems. Brunori, Antonini, Phelps & Amiconi (1969) demonstrated very large changes in the affinity rate constants for binding of CO to ferroperoxidase reconstituted from deuterohaem whereas proteins reconstituted with mesohaem or protohaem had similar rates to that of naturally occurring peroxidase. One series of equilibrium experiments showed that with such reconstituted peroxidases proto- and deuto-peroxidase differed insignificantly in the affinity constant for cyanide at pH 9.1 and 20°C whereas that for mesoperoxidase was one-quarter of that of naturally occurring peroxidase.

The small amount of material available limited kinetic experiments to one set of conditions. The results are shown in Table 2, where the combination
and dissociation velocity constants for reaction with cyanide are given. It appears likely that the change in affinity constant reflects a change uniquely in the combination velocity constant. Again the agreement between the statically determined and the kinetically derived affinity constant is good.

**General discussion.** The reaction of ferroperoxidase with cyanide in the range pH 8–10 corresponds to a simple process. This is evidenced by the fact that the time-course of the reaction corresponds to that provided in the scheme:

\[
\text{Ferroperoxidase} + \text{HCN} \xrightarrow{k_{+1}} \text{ferroperoxidase}-\text{HCN} \xrightarrow{k_{-1}} \text{ferroperoxidase} + \text{CN}^-
\]

![Graph showing effect of temperature on pseudo-first-order rate constant](image)

**Fig. 7.** Effect of temperature on the pseudo-first-order rate constant. The ferroperoxidase was 3.0 μM before mixing. The buffer was 50 mM borate, pH 9.1, and the temperature as indicated. The values obtained were: at 19.6°C, \(k_{\text{on}} = 18 \text{M}^{-1} \cdot \text{s}^{-1}, k_{\text{off}} = 0.05 \text{s}^{-1}; \) at 28.8°C, \(k_{\text{on}} = 33 \text{M}^{-1} \cdot \text{s}^{-1}, k_{\text{off}} = 0.134 \text{s}^{-1}; \) at 38.9°C, \(k_{\text{on}} = 51.5 \text{M}^{-1} \cdot \text{s}^{-1}, k_{\text{off}} = 0.040 \text{s}^{-1}.\) These values were used to calculate the values for \(\Delta E_{\text{on}}\) and \(\Delta E_{\text{off}},\) and \(\Delta H_{\text{on}}^*\) and \(\Delta H_{\text{off}}^*\) as described in the text. and is further substantiated by the agreement between \(k_{+1}/k_{-1}\) and \(K\) as determined kinetically and statically.

It seems likely that the undissociated species of cyanide is bound to the ferroperoxidase as the affinity constant varies in the pH range 8–10 in a manner expected by simple dissociation theory with an acid species of pK 9.4–9.6 at 20°C. The measurements of the enthalpy changes occurring in the reaction obtained both kinetically and by equilibrium methods substantiate the same picture.

Keilin & Hartree (1955) first showed that in the acid pH range ferroperoxidase is in equilibrium with a small amount of ferriperoxidase even when the solution contains excess of dithionite. Thus cyanide and CO, which have great affinities for the tervalent and bivalent iron respectively, shift the equilibrium either towards ferriperoxidase-CN complex or towards ferroperoxidase-CO complex. As a corollary to this, it is found in solutions of peroxidase treated with cyanide and dithionite at pH values lower than 7.0 that the two compounds present are ferro- and ferri-peroxidase-cyanide complexes. The kinetics of reaction of cyanide with dithionite-reduced peroxidase at pH 6.0 are complex and suggest a reaction scheme such as that given in Scheme 1; thus any rigorous analysis must take account of eight rate constants. Further difficulties arise since the products of dithionite action themselves depend on the pH. The affinity of cyanide for the ferriperoxidase is 200 times that for ferroperoxidase. This, coupled with the fact that the pH-dependence of the affinity constant of cyanide for the tervalent form does not change significantly (twofold) from pH 6 to pH 8 (Ellis & Dunford, 1968), suggests that the pH-dependent ligand uptake of ferroperoxidase in this pH range may have its explanation in the progressive overlapping of the redox potential of the ferro-/ferri-peroxidase system with that of dithionite acid products.

Ferroperoxidase binds cyanide with a relatively high affinity, in contrast with other proteins containing protohaem such as myoglobin and haemoglobin. Thus haemoglobin-cyanide complex can be obtained only as a very unstable complex which rapidly liberates haemoglobin; Keilin & Hartree (1955) showed that myoglobin-cyanide complex

<table>
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<tr>
<th>Ferroperoxidase</th>
<th>(K) (static) (M(^{-1}))</th>
<th>(k_{\text{on}}) (M(^{-1})·s(^{-1}))</th>
<th>(k_{\text{off}}) (s(^{-1}))</th>
<th>(K) (kinetic) (M(^{-1}))</th>
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<td>Reconstituted protohaem</td>
<td>1.2 × 10(^7)</td>
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<td>2.5 × 10(^{-2})</td>
<td>1.16 × 10(^3)</td>
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<td>Reconstituted mesohaem</td>
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<td>4.6 × 10(^{-2})</td>
<td>0.18 × 10(^3)</td>
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<tr>
<td>Reconstituted deuterohaem</td>
<td>1.4 × 10(^3)</td>
<td>25</td>
<td>3.5 × 10(^{-2})</td>
<td>1.0 × 10(^3)</td>
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was sufficiently stable for studies to be undertaken, but that a very high cyanide concentration and a fairly high pH (above 9) were needed to complete formation of this complex. Thus ferroperoxidase offers the most tractable system in which cyanide binding to proteins containing protohaem can be investigated, and is probably the only one in which it can be established which of the species, CN- or HCN, is the entity that binds.

The relative affinity for ligands varies in different haemoproteins. This can be demonstrated by considering the partition constant between CO and HCN. The observed affinity constant of ferroperoxidase for CO is quoted by Kertesz et al. (1965) to be $4.5 \times 10^6 \text{M}^{-1}$. The present work gives the affinity constant of the same protein under the same conditions for HCN as $1.1 \times 10^3 \text{M}^{-1}$. Thus the partition constant between CO and HCN is $4.0 \times 10^3$. For myoglobin Antonini (1965) gives the value of the affinity constant for CO binding as $3.9 \times 10^6 \text{M}^{-1}$; the calculated affinity constant for cyanide derived from the work of Keilin & Hartree (1955) is approx. $2.5 \text{M}^{-1}$, leading to a partition constant between CO and HCN of $1.6 \times 10^6$.

Table 3 summarizes the main findings of the present work and, for comparison, data on O$_2$ and CO binding to both myoglobin and ferroperoxidase are included. It is worth while to compare the affinity and rates of reaction of horseradish peroxidase with ligands to those of other haem compounds. Though the information on ligands for peroxidase is scanty at present, it does underline the fact that the environment of the binding site plays a very large role in determining the reactivity of the haem group towards the same ligand in different proteins. In general peroxidase has a much smaller $k_{on}$ for ligands such as O$_2$ or CO than has myoglobin. The reactions described in this paper with HCN are therefore surprising in that they offer the only documentation of the equilibrium and kinetics of cyanide binding to a haemoprotein.

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REFERENCES


