The Binding of Ethyl Isocyanide to Ferroperoxidase

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The equilibrium and kinetics of ethyl isocyanide binding to ferroperoxidase were studied. At pH 9.1 the results of both studies are consistent with a single-process model with an affinity constant of 95 M^{-1} and combination and dissociation constants of $2.2 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ and 23 s^{-1} respectively. Ethyl isocyanide is not bound significantly at pH values lower than 6.0, and in this behaviour and the pH-dependence of the affinity constant, similarities exist between isocyanide and cyanide binding. The enthalpy of the process measured by equilibrium methods is -59 kJ/mol (-14 kcal/mol). At pH values below 9, the ethyl isocyanide adduct changes in a slow time-dependent manner, giving rise to a new species. These changes are reversible on increasing the pH. The results are discussed in relation to other known information about ligand binding to ferroperoxidase and to myoglobin.

The great similarity in the types of ligand bound by horseradish peroxidase and by haemoglobin and myoglobin was originally emphasized by Keilin & Hartree (1951), and is shown clearly in the absorption spectra of the ferro and ferri species. One reason for this similarity may be that the haem site of horseradish peroxidase includes a histidine residue that binds to the proximal side of the haem group, whereas the distal position may involve a tyrosine residue (Phelps *et al.*, 1971b).

Previous studies on ligand binding to peroxidase have involved small gaseous ligands such as CO and O_2 (Kertesz *et al.*, 1965; Wittenberg *et al.*, 1967a,b), and this work has been extended to include the equilibria and kinetics of binding of cyanide to ferroperoxidase (Phelps *et al.*, 1971a).

No reports have been made of the equilibria and kinetics of the binding of alkyl isocyanides to ferroperoxidase. These ligands possess two advantages: first, the size of the alkyl group can be varied whilst the isocyanide-iron bond remains unchanged thus acting as steric probes for the size of the haem site, and secondly, they are water soluble, which enables simple anaerobic experiments to be done without the difficult anaerobic handling of gaseous ligands.

In the present paper we record the binding of ethyl isocyanide to the ferro form of horseradish peroxidase (isoenzyme B; Shannon *et al.*, 1966).

Experimental

Materials

Horseradish peroxidase. This enzyme was purified as described by Phelps & Antonini (1969). Ferroperoxidase solutions were made by adding a slight

excess of $\text{Na}_2\text{S}_2\text{O}_4$ to stoppered tubes of ferri-peroxidase solution.

Ethyl isocyanide. This compound was synthesized by the base-catalysed dehydration of *N*-ethyl-formamide by the toluene-*p*-sulphonyl chloride-quinoline method of Schuster *et al.* (1966), except that preparations were performed on a vacuum-line system. The crude material was redistilled through a 15 cm Vigreux column at atmospheric pressure and the liquid distilling at $76\text{--}80^\circ\text{C}$ was collected. I.r. spectroscopy revealed that the material was pure. However, on storage at -18°C for long periods, the solutions became coloured, possibly through polymer formation, and each batch of isocyanides prepared was stored in multiple small phials and the contents of a phial were distilled in a microdistillation apparatus before use. Only material with the correct boiling point was collected and used.

Buffer solutions. These were prepared from stock solutions of $1 \text{ M-NaH}_2\text{PO}_4$ and $1 \text{ M-K}_2\text{HPO}_4$ in the range pH 5.5–8.0 and had concentrations of 100 mM. To extend the range to pH 8.5–11, 50 mM-sodium borate buffers were used.

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 about 4 ms. Kinetic difference spectra were measured on the same instrument by varying the

spectral wavelengths in 4 nm intervals over the range 420–450 nm.

Equilibrium titrations were measured in 2.0 ml of 5 μ M-ferriperoxidase in the appropriate buffer, reduced with 1–2 mg of $\text{Na}_2\text{S}_2\text{O}_4$, with additions of 25, 50, 100 and 200 μ l of standardized ethyl isocyanide. This latter was measured by stoichiometric titration with haemoglobin α -chains. For other details see Talbot *et al.* (1971).

Results

Spectra

Keilin & Hartree (1955) demonstrated that methyl isocyanide was bound to ferriperoxidase and they reported some spectral values. Fig. 1 shows the spectra of ferriperoxidase and its complex at saturation with ethyl isocyanide at pH 9.1 in 50 mM-sodium borate buffer. The spectra at pH 9.1 were stable for periods of up to several hours. However at lower pH values, 'slow' spectral changes occurred after the initial rapid reactions. The initial spectrum observed at these lower pH values was similar to that observed at pH 9.1.

Fig. 1. Absolute absorption spectra of ferriperoxidase (—) and its complex at saturation with ethyl isocyanide (----)

For details see the text. The numbers in parentheses are the ϵ_{max} for each peak.

Equilibrium measurements

Measurement of the affinity constant. This was measured by recording the spectra of 5 μ M-ferriperoxidase in the appropriate buffer containing 2 mg of $\text{Na}_2\text{S}_2\text{O}_4$ /ml after successive additions of standardized ethyl isocyanide (2.7 mM). The reciprocals of the observed ΔE_{433} values, corrected for dilution, were plotted against the reciprocals of the ligand concentration. Straight lines were obtained in all cases. The concentration of ligand required to achieve 50% formation of the ferriperoxidase-ethyl isocyanide complex gave the affinity constants shown in Fig. 2. It is seen that the affinity constant increases from pH 6.0 to pH 8.0 and then there is a small but noticeable decrease. Below pH 6.0 it is impossible to demonstrate the binding of ethyl isocyanide to ferriperoxidase. This pH-dependent behaviour of ligand binding is similar to the phenomenon of cyanide binding to ferriperoxidase reported by Phelps *et al.* (1971a), where the inability to bind cyanide below pH 6.0 was thought to be primarily because of the progressive overlapping of the redox potentials of the ferro-/ferri-peroxidase system with that of the dithionite products.

Effect of temperature on ligand binding. Fig. 3 shows the results of experiments where the affinity constant was measured by titration at 15°C and

Fig. 2. $\log$ (Affinity constants) of ferriperoxidase for ethyl isocyanide as a function of pH

The values were obtained spectrophotometrically (see the Experimental section) at 20°C and refer to the equilibrium:

Fig. 3. pH-dependence of the affinity constant of ferropoxidase for ethyl isocyanide at two temperatures

The values were spectrophotometrically obtained (see the Experimental section) at 15°C (○) and 26°C (●).

26°C. The value for the activation energy ΔH^0 for the process of binding ethyl isocyanide to ferropoxidase is derived by using the Arrhenius relationship:

$$\frac{d \log k}{dT} = \frac{-\Delta H^0}{2.303RT^2}$$

This gives $\Delta H^0 = -59 \pm 5.5 \text{ kJ/mol}$ ($-14 \pm 1.3 \text{ kcal/mol}$) over the pH range 8–10 shown in Fig. 3.

Kinetics of the binding process

The simplest scheme is:

where EIC denotes ethyl isocyanide.

Under pseudo-first-order conditions where $[\text{EIC}]$ is much greater than $[\text{peroxidase}^{2+}]$ the observed rate constant $k' = k_{-1} + (k_{+1} \cdot [\text{EIC}])$. Thus by plotting k' against $[\text{EIC}]$, both k_1 and k_{-1} may be evaluated. Fig. 4 shows the results of two different experiments when $1.77 \mu\text{M}$ -peroxidase was mixed with 75 mM -ethyl isocyanide at 20°C in buffers of pH 7.0 and 9.1, respectively. The results indicated that at pH 7.0, $k_{+1} = 2.3 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k_{-1} = 28 \text{ s}^{-1}$, and at pH 9.1, $k_{+1} = 2.2 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k_{-1} = 23 \text{ s}^{-1}$. The affinity constant K (i.e. k_{+1}/k_{-1}) is 80 M^{-1} at pH 7.0 and 95 M^{-1} at pH 9.1, which is in fairly good agreement with the values obtained from static titration measurements at the same pH.

A similar value of the equilibrium constant was obtained when values of the change in extinction occurring in the rapid-mixing experiments were

Fig. 4. Reaction of $1.8 \mu\text{M}$ -ferropoxidase with ethyl isocyanide in 100 mM -sodium phosphate buffer, pH 7.0, (○) and 50 mM -sodium borate buffer, pH 9.1 (●)

For details see the text. The pseudo-first-order rate constant, k' , is plotted against the concentration of ethyl isocyanide.

Fig. 5. Changes in extinction derived from rapid mixing experiments when $1.8 \mu\text{M}$ -ferropoxidase was mixed with various concentrations of ethyl isocyanide in 50 mM -sodium borate, pH 9.1

The total change in extinction is plotted against the concentration of ethyl isocyanide after mixing. The arrow indicates the value of the affinity constant.

plotted against the isocyanide concentration (Fig. 5). The effect of varying the wavelength of the monitoring beam was also studied. The wavelength was varied over the range $420\text{--}350 \text{ nm}$ at approx. 4 nm intervals and the total change in extinction occurring

after mixing $1.6\mu\text{M}$ -protein with 25mM -ethyl isocyanide was measured. The value of the pseudo-first-order constant was independent of the wavelength used to monitor the reaction. Fig. 6 shows the results obtained. By comparing these results with those in Fig. 1, it can be concluded that the kinetic difference spectra are best interpreted as a disappearance of the ferro species and a simultaneous appearance of the ethyl isocyanide adduct, and the magnitude of the change in extinction accounts for 95% of the absorbance of the reaction observed statically by spectrophotometry.

Long-term changes

A typical static-titration curve took 3–5 min to produce. The spectra obtained were stable at 20°C for at least 15 min. However, if the same solutions were remeasured after longer intervals (30 min), significant changes were seen, which were dependent on the pH at which the measurements were made. These effects were more pronounced at 35°C and low pH values and Fig. 7(a) shows the time-course of

change in extinction when $4\mu\text{M}$ -protein was mixed with 20mM -ethyl isocyanide at 35°C at different pH values. It is noteworthy that the extrapolated zero-time absorbance produced identical values for E_{432} . The time-course of the change in extinction at pH 7.5 in 200mM -sodium phosphate buffer as a function of concentration of ligand is plotted against isocyanide concentration in Fig. 7(b), and the linear relationship shows that the decay process is related to isocyanide concentration. Fig. 7(c) shows the spectra of the stable final products of this reaction. It is noticeable that a new species is formed with a peak at 465nm . In contrast, the binding of ethyl isocyanide to ferroperoxidase at pH 9.5 yields a final species with spectral characteristics identical with those recorded in Fig. 1. The anomalous complex at pH 7.5 can be converted into the true complex by changing the pH of the medium to above 9.0. This behaviour is reversible and is confirmed both by spectrophotometry and by measurement of circular-dichroism spectra (C. Phelps, P. Fasella & E. Antonini, unpublished work).

Discussion

The results of both kinetic and static experiments at pH 9.1 are in sufficient agreement with each other to suggest that the process of binding of ethyl isocyanide to ferroperoxidase is essentially simple and can be described by a scheme:

There are many similarities in the behaviour of this protein with ethyl isocyanide and cyanide as ligands. In particular the pH-dependence of the process suggests involvement of the protein portion of the molecule, which is not seen in reactions with simple gaseous ligands. Thus for both cyanide and ethyl isocyanide, no binding can be observed below pH 6 and the variation in affinity with pH in the range 6–9 is similar for both ligands.

One central problem in haemprotein reactivity is the understanding of the role exercised by the protein in controlling the response of the protohaem centre to various ligands. This can be illustrated by reference to Table 1, where the kinetic data and affinity constants of a variety of ligands are compared for both horseradish peroxidase and horse heart myoglobin. The situation is exemplified by a comparison of the behaviour of the two proteins with carbon monoxide and ethyl isocyanide. For carbon monoxide the k_{off} for peroxidase is an order of magnitude less than that observed for myoglobin, whereas with ethyl isocyanide the k_{off} for peroxidase is 50 times greater than that for myoglobin.

With the exception of cyanide binding, it would

Fig. 6. Difference spectra determined kinetically (○) and statically (—) when $1.6\mu\text{M}$ -ferroperoxidase is mixed with 25mM -ethyl isocyanide in 50mM -sodium borate buffer, pH 9.1

Sodium dithionite was present (2mg/ml).

Fig. 7. Time-courses and the final product of the anomalous reaction between ferropoxidase and ethyl isocyanide (a) Time dependence of the change in extinction at 35°C when 4 μ M-ferropoxidase is mixed with 20 mM-ethyl isocyanide, as a function of the reaction. (b) Rate of change in extinction at pH 7.5 and 35°C when 4 μ M-ferropoxidase is mixed with various concentrations of ethyl isocyanide. (c) Absorption spectra of the final products formed when 4 μ M-ferropoxidase is mixed with (—) 5 mM-, (----) 10 mM-, (·····) 20 mM- and (— · —) 27 mM-ethyl isocyanide.

Table 1. Binding of ligands to ferropoxidase and myoglobin

This table compares the known binding constants for horseradish ferropoxidases at pH 9.1 in 50 mM-sodium borate buffer with those of horse heart myoglobin in 100 mM-sodium phosphate buffer, pH 7.1, both at 20°C.

Ligand	Horseradish ferropoxidase				Horse heart myoglobin			
	K (M^{-1})	$k_{(on)}$ ($M^{-1} \cdot s^{-1}$)	$k_{(off)}$ (s^{-1})	Reference	K (M^{-1})	$k_{(on)}$ ($M^{-1} \cdot s^{-1}$)	$k_{(off)}$ (s^{-1})	Reference
O ₂	—	5.8×10^4	—	Wittenberg <i>et al.</i> (1967b)	0.8×10^6	1.4×10^7	11	Antonini & Brunori (1971)
CO	4.5×10^6	3.4×10^3	0.001	Kertesz <i>et al.</i> (1965)	3.9×10^7	5.0×10^5	0.017	Antonini & Brunori (1971)
Cyanide	1.2×10^3	29	0.025	Phelps <i>et al.</i> (1971a)	2.5	—	—	Keilin & Hartree (1955)
Ethyl isocyanide	95	2.2×10^3	23	The present paper	1.7×10^5	7×10^4	0.41	Talbot <i>et al.</i> (1971)

seem that the reactivity of the protohaem centre in ferropoxidase is controlled more by the ‘off’ reaction than by the combination process. The value of $k_{(on)}$ for O₂, CO and ethyl isocyanide differs 25-fold, whereas the ‘off’ constants differ 23 000-fold. The value of combination rate constant of ferropoxidase with its ligands is also much smaller than the corresponding value for myoglobin. Thus oxygen combination is 200 times slower for ferropoxidase than for myoglobin; carbon monoxide combinations

is similarly 150 times slower; and ethyl isocyanide combination is 30 times slower. This work was supported by a Research Grant no. 482 from the North Atlantic Treaty Organization.

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