Binding Equilibria and Catalase-like Reactivity of Deuteroferrihaem†–Poly-L-lysine Complexes

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At pH 10.5, 25 °C, and ionic strength 0.1 mol dm^{-3}, deuteroferrihaem binds to poly-L-lysine as a monomeric haem species with a single axial lysine ligand. The observed rate constant for catalytic decomposition of H_2O_2 by deuteroferrihaem increases initially with increasing [poly-L-lysine] but then falls rapidly at higher [poly-L-lysine]. The results imply that the catalytic rate constant for poly-L-lysine bound deuteroferrihaem monomer is closely similar to that of free deuteroferrihaem monomer at low [poly-L-lysine] but decreases sharply (by almost 10^3 fold) at higher [poly-L-lysine].

A wide range of Fe^{III} complexes display catalytic properties in the decomposition of hydrogen peroxide and in the coupled oxidation of donor substrates by hydrogen peroxide. They are therefore, at least in a phenomenological sense, model systems for the catalase and peroxidase enzymes. Some more complex systems, for example those consisting of $[Fe(terpy)(OH)_2]^+$ (terpy = 2,2′:6′,2″-terpyridyl) anchored to poly-L-glutamate or poly-D-glutamate possess more subtle 'enzyme-like' attributes, showing stereoselectivity in catalysing the hydrogen peroxide oxidation of optically-active substrates.[1] However, it is only with the Fe^{III} porphyrins (ferrihaems), some of which constitute the prosthetic groups of the enzymes, that catalytic mechanisms which resemble those of the hydroperoxidases occur.[2] In these systems the initial reaction of H_2O_2 upon the resting, Fe^{III}, catalyst is oxidative, resulting in the formation of catalytic intermediates which are, formally, Fe^{IV} and Fe^{V} species. The detailed structures of these unusual intermediates have been extensively studied.[3]

This paper reports studies of the binding of deuteroferrihaem† (dfh) to poly-L-lysine and the influence of binding to the polypeptide on the 'catalase-like' properties of the haem. The basis for the investigation was three-fold.

(*i*) Earlier binding studies of protoferrihaem‡ to poly-L-lysine showed that, at pH >11, when the polymer is uncharged and entirely in the helical form, the ferrihaem binds as a monomer to two lysine residues from different helices.[4] The resultant complex is a low-spin haemichrome species, which would not be expected to show peroxidatic properties. A similar situation arises in the peroxidase enzymes, where an 'alkaline transition', which probably involves a conformational change in the protein and binding of a protein residue in the sixth distal co-ordination position of the ferrihaem prosthetic group, results in loss of catalytic activity.[5] Our experiments with deuteroferrihaem were made at pH 10.5, when the polymer is ~50% helical and highly charged. Under these conditions we have found that only one lysine residue is bound to each monomeric haem complex.

(*ii*) Ferrihaem dimerization in aqueous solution profoundly influences the 'hydroperoxidase-like' activity of the complexes, monomeric ferrihaems being the predominantly active forms.[2] Binding of ferrihaem to polypeptide as a monomer may therefore enhance the catalytic activity of the complex. Deuteroferrihaem was chosen for these studies since its behaviour in aqueous solution is well characterised and, in particular, it does not possess the oxidation-sensitive vinyl groups which occur in the native protoporphyrin.

(*iii*) Binding of an iron complex to a random coil polypeptide has been shown to induce a coil to α-helix transition, to an extent dependent on the metal complex to polymer ratio, and also to bring about clustering of the polymer chains. It has been suggested that such systems may behave as a second phase in which the concentration of reactants is different from that in the bulk aqueous solution because of electrostatic factors. We have examined the possibility that a polypeptide matrix may modulate the redox reactivity of haem groups contained therein by a polyelectrolyte effect.

Experimental

Materials.—Poly-L-lysine (degree of polymerisation 80, M = *ca.* 17 000) was obtained as the hydrobromide (Sigma) and converted to the hydrochloride by dissolving the solid polymer in 1 mol dm^{-3} HCl containing 0.1 mol dm^{-3} NaCl. The solution was exhaustively dialysed against triply-distilled water and, when the conductivity of the external solution was less than 3×10^{-6} ohm^{-1} cm^{-1}, was lyophilised and the solid polymer obtained dried under vacuum to constant weight. Stock solutions of poly-L-lysine were obtained by dissolving accurately weighed samples of the dried polymer (concentrations of poly-L-lysine solutions are expressed throughout in terms of the monomeric unit).

Deuteroferrihaem was prepared as the chloro-complex from haemin chloride§ (Sigma) by the resorcinol melt method.[6] Stock solutions of 10^{-3}—10^{-4} mol dm^{-3} dfh were prepared by dissolution of weighed samples in 2 cm^3 of 0.1 mol dm^{-3} NaOH and dilution with carbonate buffer to the required volume. Triply-distilled water was used in the preparation of all reaction solutions. Stock solutions of H_2O_2 were prepared from stabilizer-free 30% H_2O_2 (Interox) and assayed cerimetrically. Unless otherwise stated all experiments were carried out at 25 °C, pH 10.5 ($NaHCO_3$–Na_2CO_3 buffer), and ionic strength 0.1 mol dm^{-3} (adjusted with NaCl).

Methods.—Binding of dfh to poly-L-lysine was studied spectrophotometrically using a Unicam 8-150 spectrophotometer. Stopped-flow studies employed a Durrum D-110 instrument. Circular dichroism (c.d.) measurements were carried out using Cary 61 and Dichrographe Mk IV instruments. The catalytic decomposition of H_2O_2 by dfh–poly-L-lysine systems was studied by mixing pre-incubated solutions of H_2O_2 and

† Chloro[3,7,12,17-tetramethylporphyrin-2,18-dipropionato-(2−)-N^{21},N^{22},N^{23},N^{24}]iron(III).

‡ Chloro[7,12-diethenyl-3,8,13,17-tetramethylporphyrin-2,18-dipropionato(2−)-N^{21},N^{22},N^{23},N^{24}]iron(III).

§ Chloro[3,7,12,17-tetramethyl-8,13-divinylporphyrin-2,18-dipropionato(2−)-N^{21},N^{22},N^{23},N^{24}]iron(III).

catalyst; reactions were quenched after appropriate times by addition of 2 mol dm^{-3} H_2SO_4 and 50 mmol dm^{-3} KF and residual H_2O_2 determined cerimetrically. Initial rates of reaction were determined in the first 60—90 s of reaction using as catalyst 20 μmol dm^{-3} dfh with 0—20 mmol dm^{-3} poly-L-lysine and 4—8 mmol dm^{-3} H_2O_2. A pseudo-first-order rate constant ($k_{obs.}$) was calculated as the ratio of the initial rate and the initial $[H_2O_2]$. Under otherwise identical conditions the values of $k_{obs.}$ were independent of $[H_2O_2]$ in the range of $[H_2O_2]$ employed.

Results and Discussion

Binding Equilibria.—Addition of poly-L-lysine to solutions of dfh at pH 10.5 results in changes in the Soret band spectrum of the haem, in which the two bands of the polymer-free dfh[7] are progressively replaced by a more intense single band ($\lambda_{max.}$ ~ 395 nm) and an isosbestic point is observed at 384 nm. The behaviour is closely similar to that observed as dfh dimer progressively dissociates into monomeric species with either decreasing pH or solubilization in detergent and reflects the formation of polymer-bound monomeric ferrihaem.[7] The dimerization constant of dfh in aqueous solution ($2M \overset{K_D}{\rightleftharpoons} D + H^+$) has been determined[7] as $K_D = 3.4 \times 10^{-2}$ at 25 °C and ionic strength 0.1 mol dm^{-3}, so that at pH 10.5 in the range of total dfh concentration, [T], employed, the free haem contributing to the optical absorption is almost entirely dimer. At constant [poly-L-lysine], solutions were found to obey Beer's law in the range studied ([T] — 1—5 μmol dm^{-3}) and the variation in the values of the observed absorption coefficient, $\varepsilon_{obs.}$, with the ratio $R = [T]/[P]$ (where [P] = [poly-L-lysine]) is shown in Figure 1. Absorption coefficients for free dfh (ε_F) and for polymer-bound dfh (ε_{PM}) were obtained at $R = \infty$ and by extrapolation to $R = 0$ respectively. If the binding equilibrium is formulated by equation (1), where F is free dfh and n the

$$F + nP \overset{K_B}{\rightleftharpoons} PM \quad (1)$$

number of lysine residues ligated to each bound haem, then K_B is given by equations (2) or (3), where $\alpha = (\varepsilon_{obs.} - \varepsilon_F)/(\varepsilon_{PM} - \varepsilon_F)$.

$$K_B = \left(\frac{\alpha}{1-\alpha}\right)\cdot\frac{1}{[P]^n} \quad (2)$$

$$\log\beta = \log(\alpha/1-\alpha) = \log K_B + n\log[P] \quad (3)$$

Figure 2 shows that our results at pH 10.5 are consistent with equation (3), with $n = 1$ and $K_B = 8.3 \times 10^3$ dm^3 mol^{-1}. In contrast, at pH 11.5, similar experiments gave $n \sim 2$.

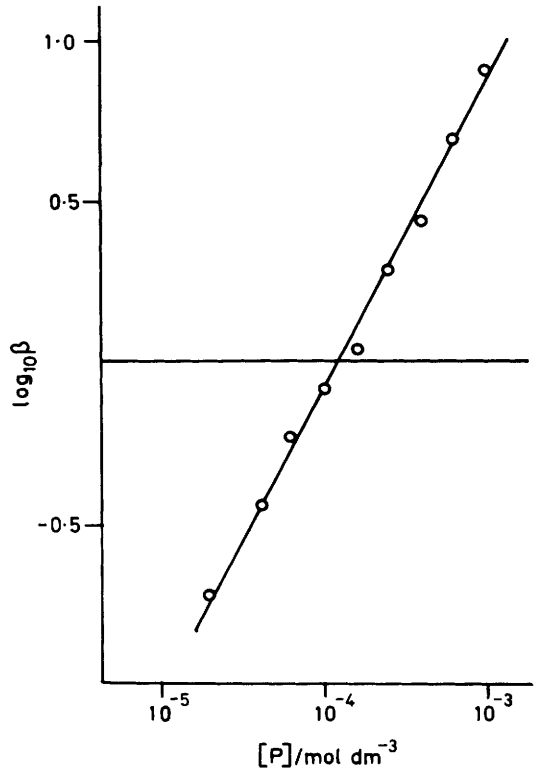

Figure 2. Variation of β with [poly-L-lysine] according to equation (3)

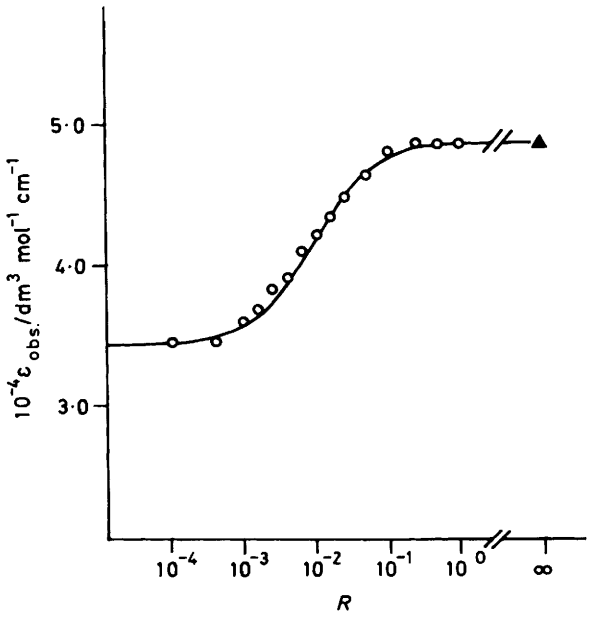

Figure 1. Variation of $\varepsilon_{obs.}$ at $\lambda = 336$ nm with the ratio (R) of [total deuteroferrihaem] to [poly-L-lysine]. ▲ Represents $\varepsilon_{obs.}$ for [P] = 0

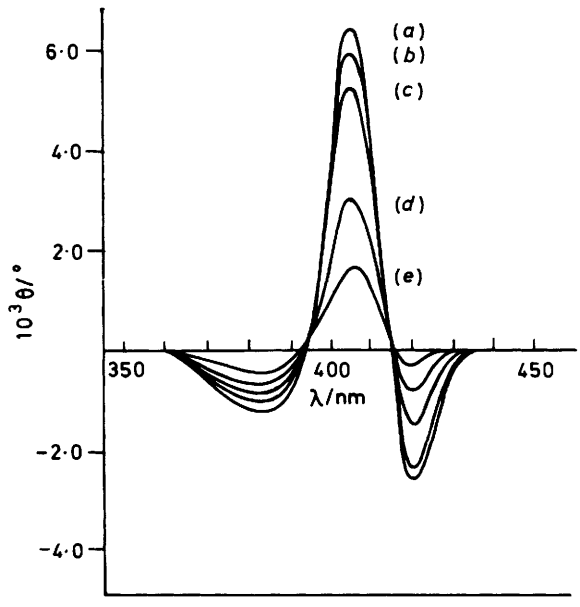

Figure 3. Circular dichroism of deuteroferrihaem–poly-L-lysine solutions at pH 10.5. {[T] = 10 μmol dm^{-3}; [P] = 1.90 (*a*), 1.32 (*b*), 1.20 (*c*), 1.00 (*d*), and 0.75 mmol dm^{-3} (*e*) respectively}

Circular Dichroism.—Measurements were made using [T] = 10 μmol dm^{-3} and various [poly-L-lysine] and the results are illustrated in Figure 3. The c.d. bands recorded at $\lambda > 230$ nm originate solely from electronic transitions of the bound ferrihaem molecules which are dissymetrically perturbed by the asymmetric polymeric substrate. The values of the induced ellipticity (θ) at the Soret band maximum are sufficiently large to suggest that the Cotton effects arise from both configurationally-induced and conformationally-induced contributions.[8] The latter would arise from the binding of monomeric ferrihaem molecules in a chiral arrangement to a helical segment of the polymer chain.

Catalytic Decomposition of H_2O_2.—The effect of varying [poly-L-lysine] on the observed rate constant ($k_{obs.}$) for the decomposition of H_2O_2 by dfh at pH 10.5 is shown in Figure 4(*a*). At low [poly-L-lysine], $k_{obs.}$ increases eventually to more than double the value observed in the absence of polymer but then falls rapidly with further increase in [poly-L-lysine]. If there are no other influences on the rate, an increase in $k_{obs.}$ with increasing [poly-L-lysine] would be expected merely as a result of the increasing fraction of dfh present as monomeric ferrihaem in the presence of the polypeptide. Detailed analysis implies that the results at low [poly-L-lysine] are quantitatively in accord with this concept. In the absence of polymer the reactivity of dfh is substantially determined by the concentration of monomeric dfh in the solution. If the reactivity of unbound dfh is unaffected by the presence of polymer then equation (4) applies, where k_M is

$$k_{obs.} = k_M[M] + k_{PM}[PM] \quad (4)$$

the catalytic constant for free monomeric dfh (M) and k_{PM} is the catalytic constant for polymer-bound monomeric dfh. k_M can be determined as 3.1×10^4 dm^3 mol^{-1} s^{-1} from experiments at [poly-L-lysine] = 0. Values of [M] and [PM] in dfh solutions containing poly-L-lysine are calculated from the previously determined values of K_D and K_B. Thus k_{PM} may be calculated from equation (5). Analysis of the results according to equation

$$k_{PM} = (k_{obs.} - k_M[M])/[PM] \quad (5)$$

(5) is shown in Figure 4(*b*). Not unexpectedly the value of k_{PM} is almost identical to k_M at low [poly-L-lysine]. However, above a critical [poly-L-lysine], k_M falls dramatically (by a factor of $\sim 10^3$) and $k_{obs.}$ falls below the level observed in the absence of poly-L-lysine, although the fraction of monomeric dfh in solution (bound and unbound) continues to increase.

Stopped-flow Experiments.—Reactions of dfh with H_2O_2 have been extensively studied using the stopped-flow spectrophotometric technique. Two processes can be distinguished: (*i*) oxidation of dfh to form the active catalytic intermediate,[9,10] which is, formally, an Fe^{IV} species[11] and which reacts further with H_2O_2 to complete the overall decomposition cycle, or, alternatively, can oxidise a wide range of other donor substrate species; (*ii*) oxidation of the porphyrin ligand of dfh to form a bile pigment, leading to catalyst inactivation.[12] The former process is conveniently studied in the Soret band region. At lower pH (<9.5) the latter process has been studied at 629 nm and the results independently confirmed by mass spectrometric analysis of product gases[12] and by computer analysis of the time course of H_2O_2 decomposition.[13] At pH 10.5 absorbancy changes at 629 nm were too small for satisfactory measurements. However, it is known from earlier work[12] that the rate constant for (*i*) increases markedly relative to that of (*ii*) with increasing pH.

We have measured the rate constants ($k'_{obs.}$) for the first-order transients observed on reaction of 5 μmol dm^{-3} dfh with 1 mmol dm^{-3} H_2O_2 at 384 nm. Figure 5 shows the variation of $k'_{obs.}$ with the fraction of dfh present as polymer-bound monomeric dfh. Although these experiments do not constitute a detailed kinetic study, the results at [P] = 0 are consistent with earlier data for the rate of formation of the catalytic inter-

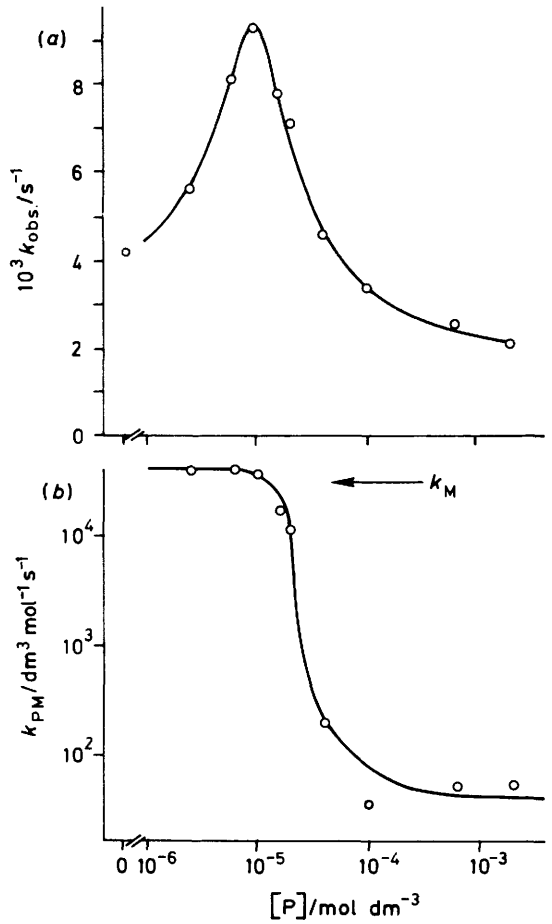

Figure 4. (*a*) Variation of $k_{obs.}$ with [P]; (*b*) variation of k_{PM} [calculated according to equation (5)] with [P]

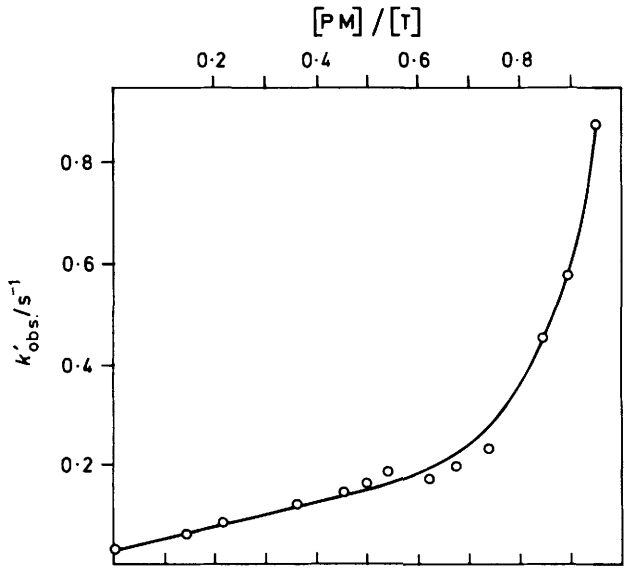

Figure 5. Variation of $k'_{obs.}$ with [PM]/[T]

mediate[10] and the marked increase of $k'_{obs.}$ with increasing [P], up to very high [P]:[T] ratios, implies that the decrease in the overall catalytic rate constant, k_{PM}, with increasing [P] [Figure 4(*b*)] does not result from a failure of the polymer-bound dfh to react efficiently with H_2O_2 in the initial phase of the reaction.

General Discussion.—At pH 10.5 dfh monomer is bound to poly-L-lysine by one axial lysine ligand. It is probable that the haem is ligated to a local helical section of the polymer chain (the polymer is ~50% helical at pH 10.5) bounded by highly-charged sections of the polymer. This charge array will inhibit binding of a second lysine ligand to the haem. This situation has parallels in the peroxidase and catalase enzymes where the haem prosthetic group is bound to a helical section of the protein and the tertiary structure of the protein creates an active site in which the sixth haem co-ordination position is either vacant (catalase)[14] or occupied by a water molecule (cytochrome c peroxidase).[15] The availability of an accessible haem co-ordination site is essential for enzyme activity.

The most striking result of our study is the collapse of the 'catalase-like' activity of polymer bound dfh with increasing polymer:haem ratios. The binding studies imply that this is not the result of additional ligation to dfh leading to haemichrome formation and this conclusion is supported by the stopped-flow studies which indicate that bound dfh continues to react rapidly with H_2O_2 at high relative polymer concentrations. Possible explanations for this result are: (*i*) the catalytic intermediate is rapidly inactivated at high polymer:haem ratios, perhaps by an internal redox process, or, (*ii*) the ability of the catalytic intermediate to oxidise hydrogen peroxide in the second phase of the catalytic cycle is markedly reduced in the polyelectrolyte environment of the haem which the polymer provides. Although at present we cannot completely exclude (*i*), the second possibility is of some interest in relation to concepts of the role of the protein environment of haem in hydroperoxidase action.

Whereas monomeric ferrihaem in aqueous solution possesses both catalase- and peroxidase-like properties, the ferrihaem-containing hydroperoxidases are sharply divided into the catalase and peroxidase classes. The only intermediate case is that of chloroperoxidase,[16] which retains some catalase-like activity. Thus the intrinsic catalase activity of ferrihaem is suppressed in the peroxidases and the peroxidase activity is largely suppressed in the catalases. In the latter case the suppression mechanism may be mainly steric in origin, since the haem prosthetic groups in the active tetrameric enzyme are deeply buried and approachable from the external milieu only *via* a narrow, intensely hydrophobic channel.[14] No satisfactory concept of the lack of catalase activity of the peroxidases has been presented. For both horse radish peroxidase and catalase the core structure of the key catalytic intermediate [compound (I)] is a $Fe^{IV}O$ 'ferryl–porphyrin' π-cation radical species.[17,18] Although the porphyrin radicals differ in ground-state symmetry in the two enzymes, this is not the origin of differences in reactivity, since a horse radish peroxidase, reconstituted with dfh in place of the native protoferrihaem, yields a porphyrin radical with the same ground-state symmetry as that in catalase compound (I), but the modified enzyme still possesses no catalase activity.[17] Modulation of the redox potential of the intermediate by differences in axial ligation of the haem prosthetic group and its protein and solvation environment have also been suggested as possible influences on reactivity.[19] However, the maximal catalytic activity of deuteroferrihaem monomer is very similar to that of catalase[20] and the peroxidatic activity of the oxidised deuterohaem intermediate, *e.g.* towards anilines[21] is closely comparable to that of horse radish peroxidase compound (I). Our results suggest that the polyelectrolyte environment provided by protein in the vicinity of the haem group may have a powerful influence on reactivity, so that not merely the immediate protein co-ordination environment of the haem but the extended protein environment in the active site overall may be important in determining the gross functional differences between catalases and peroxidases.

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