The Catalase Activity of Ferrihaems

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1. The variation of the specific stoichiometric catalytic activity of proto- and deuterioferrihaem with total ferrihaem concentration has been studied at 25°C over a wide range of pH. For deuterioferrihaem the results imply that only monomeric ferrihaem species contribute significantly to the catalytic activity. Protoferrihaem is more highly dimerized in solution and, in this system, contributions to the catalytic activity from both monomeric and dimeric ferrihaem species were observed. The ratio of the specific activity of protoferrihaem monomer to that of dimer varied from ~20 at pH 7 to 5×10^4 at pH 12.2. 2. The specific activity of protoferrihaem monomer closely resembles that of deuterioferrihaem monomer, both in magnitude and pH-dependence. In both cases the activity is inversely proportional to $[H^+]$. In contrast, the activity of catalase is independent of pH in the range 5–10. At pH 13 the activity of ferrihaem monomer becomes equal to the maximal activity of catalase. The results are in good agreement with those reported by Brown *et al.* (1970b) and provide support for the assumptions upon which this previous analysis relied. 3. Information from the literature concerning the catalytic activity and dimerization of the iron(III) complex of 4,4',4'',4'''-tetrasulphophthalocyanine (Waldmeier & Sigel, 1971; Sigel *et al.*, 1971) have been re-analysed. The results imply that both the monomeric and dimeric complexes contribute to catalytic activity and these activities closely resemble those of the corresponding protoferrihaem species.

Iron-porphyrin complexes (haems) play a central role as prosthetic groups in the actions of diverse biological catalysts and elucidation of the properties of these species is thus of importance in a variety of connexions. The complex protoferrihaem [the iron(III) complex of 1,3,5,8-tetramethyl-2,4-divinylporphyrin-6,7-propionic acid] is the prosthetic group of the enzyme catalase (EC 1.11.1.6) and it has long been known (von Euler & Josephson, 1927) that ferrihaems possess, in common with the enzyme, the ability to catalyse the dismutation of hydrogen peroxide. This paper continues the examination by the authors of the behaviour of both the catalase enzyme (Jones & Suggett, 1968*a,b,c*; Jones & Middlemiss, 1972) and the ferrihaem complexes (Brown & Jones, 1968*a,b*; Brown *et al.*, 1968, 1969, 1970*a,b,c*). The objective of these detailed comparative studies is an understanding of the manner in which catalase protein effects the transformation of a prosthetic group, of modest catalytic power at physiological pH, into a catalytic species which is superlative under these conditions.

Kinetic studies of the catalytic behaviour of ferrihaems yield results which give an appearance of considerable complexity, particularly in the effects of

pH. Brown *et al.* (1970b) argued, on the basis of a comparative study of proto- and deuterioferrihaem, that the occurrence of ferrihaem dimerization equilibria in solution is a major complicating factor in the catalytic behaviour of these species. This argument was supported by spectrophotometric measurements of the dimerization constants of the complexes (Brown *et al.*, 1970*a*).

In the analysis presented the observed catalytic constant (a_o) was resolved into contributions from ferrihaem monomer (catalytic constant a_M) and dimer species (a_D) with the following results: (a) $a_M \gg a_D$ under all conditions; (b) $a_M \propto 1/[H^+]$; a_D has a much smaller pH variation; (c) a_M becomes equal to the maximal activity of catalase at $pH > pK(H_2O_2)$.

These results depended upon the validity of a number of assumptions, which, although plausible, were not susceptible to direct test with the data then available. It was noted that the thesis advanced could be tested directly by studying, over an adequate range, the effect on a_o of changing total ferrihaem concentration. The present paper reports such experiments and the results provide substantial justification for the previous analysis. A preliminary report of some of the work described has been presented (Jones,

1971). The paper also includes a re-analysis of some recent data for the catalytic properties and dimerization equilibria of the iron(III) complex of 4,4',4'',4'''-tetrasulphophthalocyanine (Sigel *et al.*, 1971; Waldmeier & Sigel, 1971). The results are shown to be in good agreement with the behaviour of ferrihaems.

Experimental

The specification of materials and the experimental methods used have been described previously (Brown *et al.*, 1970*a,b*), with the addition that, for measurements in carbonate buffers, the buffer solutions were prepared from AnalaR Na_2CO_3 and NaHCO_3 . Measurements in unbuffered solutions were performed in a pH-stat (Radiometer, Copenhagen, Denmark) to measure and control the pH of the reaction solutions. The additions of NaOH (10 mM) that were required to maintain the pH during the reactions did not significantly alter the volumes or ionic strengths of the reaction solutions. Ferrihaem solutions were prepared by method A (Brown *et al.*, 1970*c*). Kinetic studies of catalytic activity of deuterioferrihaem were made at 25°C, $I = 0.1$ (added electrolyte NaCl), by measuring the initial rate of decomposition (V_i) of solutions which were 4 mM with respect to

H_2O_2 (after the first 15% of the reaction). Measurements with protoferrihaem were made under similar conditions, except that the initial concentration of H_2O_2 in the reaction solutions was in the range 1–4 mM.

Results and Discussion

We employ the notation of Brown *et al.* (1970*b*); a'_0 is the apparent second-order catalytic constant (the specific stoichiometric catalytic activity) for protoferrihaem at a particular ferrihaem concentration and pH; a''_0 is the corresponding quantity for deuterioferrihaem. a'_M and a''_M are the catalytic constants (at a particular pH) for proto- and deuterio-ferrihaem monomers respectively. a'_D and a''_D are the catalytic constants for proto- and deuterio-ferrihaem dimers respectively.

In the previous analysis (Brown *et al.*, 1970*b*) it was assumed that $a'_M = a''_M = a_M$ and $a'_D = a''_D = a_D$ and the result $a_M \gg a_D$ was obtained. On the basis of the values of a_M and a_D previously reported it is unlikely that a dimer contribution to the total reaction rate could be detected directly for deuterioferrihaem in the range of ferrihaem concentration accessible for activity measurements. The experiments reported below yield no evidence for a dimer contribution in the

Fig. 1. Examples of the dependence of the rate of catalytic decomposition of hydrogen peroxide, V_i (at $[\text{H}_2\text{O}_2] = 4 \text{ mM}$), upon total concentration of deuterioferrihaem, $[T]$, at 25°C, 10.1 M

$\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$ buffers (67 mM): $\circ$, pH 6.75; $\blacktriangle$, pH 7.02; $\triangle$, pH 7.40; $\bullet$, pH 7.55; $\square$, pH 7.75; $\blacksquare$, pH 8.10.

activity of deuteroferrahaem. With protoferrahaem, the extent of dimerization is so large that a dimer contribution should be detectable, certainly at lower pH. Our measurements support the thesis that both monomeric and dimeric protoferrahaem species contribute significantly to the total catalytic activity of these solutions.

Deuteroferrahaem

Fig. 1 illustrates the results of measurements of the variation of V_1 with total deuteroferrahaem concentration, $[T]$, under a variety of conditions. The variation has a complex form and it is clear that, if we define a_0'' by (Brown *et al.*, 1970b)

$$V_1 = -\frac{1}{2}d[H_2O_2]/dt = a_0''[T][H_2O_2] \quad (1)$$

then a_0'' is not a true second-order constant. Introducing the concept that monomer and dimer species separately contribute to V_1 we obtain

$$a_0'' = \alpha'' a_M'' + \frac{1}{2}(1 - \alpha'')a_D'' \quad (2)$$

where α'' is the fraction of deuteroferrahaem present in monomeric form (Brown *et al.*, 1970a). In the present work the values of α'' were computed by using the most recent value for the dimerization constant of deuteroferrahaem; $K = K_{obs.}[H^+] = 3.4 \times 10^{-2}$ (Prudhoe, 1971). Rearranging eqn. (2):

$$a_0'' = (a_M'' - \frac{1}{2}a_D'')\alpha'' + \frac{1}{2}a_D'' \quad (3)$$

Plots of the data according to eqn. (3) yielded straight lines through the origin at all pH values, implying that no dimer contribution to the total activity was detectable and that values of a_M'' are good second-order rate constants. In this situation the result may be represented simply by eqn. (4), where

$$V_1 = a_M''[M][H_2O_2] \quad (4)$$

$[M]$ is the concentration of deuteroferrahaem monomer. This form (Fig. 2) gives a direct comparison with the plots according to eqn. (1) shown in Fig. 1.

Fig. 3 shows the variation of a_M'' with pH. The full line in the figure has unit positive slope and accurately describes the variation of a_M'' with pH ($a_M'' \propto 1/[H^+]$) in carbonate buffers (pH 8.80–10.05), in Na_2HPO_4 –NaOH buffers (pH 9.75–10.60) and in unbuffered (pH-stat) NaOH solutions (pH 7.55–11.70). The values of a_M'' in Na_2HPO_4 – KH_2PO_4 buffers (pH 6.75–8.10) show positive deviations from the line. Additional experiments in these phosphate buffers, in which the total buffer concentration was varied at constant pH, showed that a_M'' decreased with decreasing total buffer concentration and fell to the value obtained in unbuffered solutions in the limit of vanishing buffer concentration. This behaviour implies a specific effect of the Na_2HPO_4 – KH_2PO_4 buffer

Fig. 2. Examples of the dependence of V_1 on the concentration of deuteroferrahaem monomer ($[M]$)

The data are those illustrated in Fig. 1.

Fig. 3. pH-dependence of the catalytic activity of deuteroferrahaem monomer (a_M'') at 25°C, $I = 0.1$ M

○, Na_2HPO_4 – KH_2PO_4 buffers (concentration 67 mM); ▽, Na_2CO_3 – $NaHCO_3$ buffers; □, Na_2HPO_4 –NaOH buffers; △, unbuffered (pH-stat) solutions. The continuous line has unit positive slope.

system, although the origin of this effect remains unclear.

Protoferrahaem

Fig. 4 shows results obtained in studies of the variation of the pseudo-first-order constant $V_1/[H_2O_2]$ with $[T]$ in protoferrahaem solutions. The data represent the extremes of the pH range studied. Similar curved

Fig. 4. Examples of the dependence of $V_i/[H_2O_2]$ upon the total concentration of protoferrihaem, $[T]$, at $25^\circ C$, $I = 0.1 M$

(a) Δ , Unbuffered NaOH solution, pH 12.23. (b) $\circ$, pH 6.51 (Na_2HPO_4 - KH_2PO_4 buffer).

Fig. 5. Dependence of $a'_0[T]^{\frac{1}{2}}$ against $[T]^{\frac{1}{2}}$ for protoferrihaem (according to eqn. 8)

The data are those of Fig. 4.

plots were obtained under all conditions. Thus a'_0 is not a good second-order rate constant.

Protoferrihaem is so highly dimerized under the conditions of these experiments that it is a good approximation to write ($[D] \gg [M]$):

$$[D] = \frac{1}{2}[T] \quad (5)$$

and

$$[M] = (2K_{obs.})^{-1/2} [T]^{1/2} \quad (6)$$

where $K_{obs.}$ is the dimerization constant at a particular pH (Brown *et al.*, 1970a). Then, if both monomer and dimer contribute to the observed catalytic activity at a particular pH (a'_0):

$$V_i = a'_M(2K_{obs.})^{-1/2} [T]^{-1/2} [H_2O_2] + \frac{1}{2}a'_D[T][H_2O_2] \quad (7)$$

whence:

$$a'_0[T]^{1/2} = a'_M(2K_{obs.})^{-1/2} + \frac{1}{2}a'_D[T]^{1/2} \quad (8)$$

Typical plots of the data according to eqn. (8) are shown in Fig. 5. If monomeric protoferrihaem alone contributes to the catalytic activity the points should fall on a straight line parallel with the abscissa. The positive slopes observed indicate (according to eqn. 8) the contributions of dimeric ferrihaem species to the total rate. Plots of this type are very sensitive to errors in the primary kinetic data, but the results nevertheless

conformed well with the form predicted by eqn. (8). Least-squares analysis, using the value $K = K_{obs.}$, $[H^+] = 4.5$ (Brown *et al.*, 1970a), yielded the values of a'_M and a'_D shown in Fig. 6. At pH 12.86 the slope obtained was zero within the experimental error, i.e. a dimer contribution could not be detected under these conditions (see Table 1).

The full line in Fig. 6 is taken from Fig. 3 (i.e. it describes the variation of a'_M with pH).

It is clear that a'_M resembles a'_M closely both in magnitude and in pH-dependence. These results provide substantial justification for the assumption, $a'_M = a'_M$, employed in the previous analysis (Brown *et al.*, 1970b). There is again evidence for a specific effect of the Na_2HPO_4 - KH_2PO_4 buffer system in the lower pH range.

The values of a'_D show a much smaller pH-dependence (again in agreement with the previous work) and $a'_M \gg a'_D$. The ratio a'_M/a'_D varies from ~ 20 at pH 7 to 5×10^4 at pH 12.2. The effect of this result upon the relative contributions made by monomer and dimer to the total activity is shown in Table 1.

Values of R_D (deuteroferrihaem) were calculated on the basis $a'_D = a'_D$ and are also shown in Table 1. Contributions from values of a'_D of this order would not be experimentally detectable, although on the borderline of detectability at low pH. From our results we may only state that $0 \leq a'_D \leq a'_D$.

Fig. 6. pH-dependence of the catalytic activity (second-order catalytic constants) for various species at 25°C

Protoferrrihaem monomer (a'_M): $\circ$, Na_2HPO_4 – KH_2PO_4 buffers; ∇ , Na_2CO_3 – NaHCO_3 buffers; $\square$, Na_2HPO_4 – NaOH buffers; $\triangle$, unbuffered NaOH solutions. Protoferrrihaem dimer (a'_D): $\bullet$, Na_2HPO_4 – KH_2PO_4 buffers; $\blacktriangledown$, Na_2CO_3 – NaHCO_3 buffers; $\blacksquare$, Na_2HPO_4 – NaOH buffers; $\blacktriangle$, unbuffered NaOH solutions. Fe(III)TPS (Phosphate buffers): $\diamond$, monomer activity; $\blacklozenge$, dimer activity. The data were taken from Waldmeier & Sigel (1971) and re-analysed according to Fig. 7. —, Values from Fig. 3 (for deuteroferrrihaem); ----, activity of bacterial catalase at 20°C (Jones & Suggett, 1968a).

Table 1. Percentage dimer contribution (R_D) to V_i under various conditions

The values of R_D are experimental for protoferrrihaem. Those for deuteroferrrihaem were calculated on the assumption that $a'_D = a'_M$.

pH	[T](μM)	...	R_D (proto-)		R_D (deutero-)	
			20	2	20	2
7			56	28	9	2.4
9			35	15	4.5	1.4
12			13	4.6	1.3	0.4

Iron(III)–4,4',4'',4'''-tetrasulphophthalocyanine complex [Fe(III)TPS]

The aggregation in solution of Fe(III)TPS has been studied by Sigel *et al.* (1971). Waldmeier & Sigel (1971) reported measurements of the catalytic activity of the complex and discussed the relationship between dimerization and catalytic behaviour. They suggested

Fig. 7. Catalytic activity of Fe(III)TPS in phosphate buffers, at 25°C

V_0 is the initial rate of decomposition of H_2O_2 , obtained by re-measurement of Fig. 6 of the paper of Waldmeier & Sigel (1971). The data have been plotted according to eqn. 8. $\blacktriangle$, pH 7.25, $[\text{H}_2\text{O}_2] = 1.25 \text{ mM}$; $\triangle$, pH 7.25, $[\text{H}_2\text{O}_2] = 2.5 \text{ mM}$. $\circ$, $\bullet$, pH 5.5, $[\text{H}_2\text{O}_2] = 1.25 \text{ mM}$ in both cases.

that, in this system, the catalytic activity was carried by the Fe(III)TPS monomer species although their experimental results showed substantial deviations from this model. Fe(III)TPS solutions are highly dimerized [the dimerization constant $K_D = [\text{dimer}]/[\text{monomer}]^2 = 8.6 \times 10^7 \text{ M}^{-1}$ ($I = 0.1$, 25°C), independent of pH] and, in view of the results reported in the previous section for protoferrrihaem, it was desirable to re-examine the results of Waldmeier & Sigel (1971). Values of the initial rate of H_2O_2 decomposition at various concentrations of Fe(III)TPS were obtained by re-measurement of Fig. 6 of Waldmeier & Sigel (1971). The experiments were performed under conditions where the rate was first-order with respect to H_2O_2 . These results were plotted according to eqn. (8), as shown in Fig. 7.

The catalytic constants for monomeric and dimeric Fe(III)TPS , calculated from Fig. 7 by using eqn. (8), are included in Fig. 6. The resemblance to the results for the ferrrihaems is striking. Waldmeier & Sigel (1971) noted that the dimerization constant of Sigel *et al.* (1971) must be used with circumspection because their spectrophotometric measurements at

[Fe(III)TPS] > 10 μ M yielded effects that they suggested arose from polymerization. Thus conclusions based upon the above analysis must remain tentative at present.

Conclusions

The broken horizontal line in Fig. 6 represents the observed activity of bacterial catalase from *Micrococcus lysodeikticus* (Jones & Suggett, 1968a). It is clear that, at pH 13, the catalytic activity of ferrihaem monomer becomes identical with that of the enzyme (catalytic constant $3.8 \times 10^9 \text{ M}^{-1} \cdot \text{min}^{-1}$). At pH 7 the enzyme is 10^6 -fold more active than ferrihaem monomer. Thus, in a comparison of the enzyme activity with that of the prosthetic group model system, the conditions under which the comparison is made require careful specification. The high activity of catalase is no longer remarkable if comparison with the ferrihaem monomer model system is made under conditions of maximal activity for each system.

There are undoubtedly several factors that contribute to the efficiency of the catalase enzyme, but a more intimate comparison of catalase action with that of the ferrihaem model systems requires precise mechanistic formulations for each system. That persuasive evidence exists to support a generalized 'peroxidatic' mechanism for these Fe(III)-centred catalysts [including the aqua-Fe(III) ion] has recently been argued by Jones (1971). The evidence derives not only from similarities of kinetic form, but also from examination of the formation and roles of reaction intermediates. Nevertheless, it is not, as yet, possible to pursue a quantitative comparative analysis of rate constants and reaction energetics in these systems. The second-order catalytic constants that we have reported above are composite quantities in systems that obey Michaelis-Menten kinetics and further studies over a wide range of substrate concentrations are required for resolution.

At the phenomenological level it is clear that the different form of pH-dependence (the absence of a pH effect in catalase action) enables the enzyme to exert the maximal catalytic effect of its ferrihaem prosthetic group at physiological pH. There is little doubt that it is the quaternary structure of the catalase protein which provides this uniquely effective environment for the catalase protein (Jones & Suggett, 1968a; Jones *et al.*, 1970). The sparse information available concerning the catalytic properties of horseradish peroxidase and metmyoglobin (Keilin & Hartree, 1955; Brown *et al.*, 1970d) indicate activities (at pH 5.9, 20°C) comparable with that of ferrihaem monomer for these species.

A variety of Fe(III) complexes have been studied as 'catalase models'. These investigations have not commonly involved detailed studies of the nature of

the species present in the solutions and catalytically active in the system, and comparisons have been based on stoichiometric catalytic constants or turnover numbers. A general problem in these systems is that catalytic activity appears to require the availability of a substrate co-ordinate site, which at the same time is a potential site for olation reactions leading to dimerization. Change in ligand environment from the aqua-Fe(III) ion to ferrihaem does not, according to our interpretation, substantially alter the catalytic activity of the Fe(III) centre but merely permits the reaction to proceed at higher pH. We may tentatively include Fe(III)TPS in this generalization.

The ammine complexes studied by Wang (1955a,b); Jarnagin & Wang (1958) and Beck & Görög (1959) yield activities similar to that of ferrihaem monomer at pH 7 but substantially lower at pH 10. This result may appear to weaken the argument that these species derive additional catalytic prowess from their presumed facility for bidentate co-ordination of HO_2^- but it would clearly be desirable to examine the possible role of catalyst dimerization in these systems. Fleischer *et al.* (1971) have studied the dimerization of tetra-(*p*-sulphophenyl)porphyrin-iron(III) but the catalytic properties of this complex have not been examined. Melnyk & Busch (1972) reported the complex (I) to be 'a very effective catalase model'. The kinetic studies were made in the presence of large excesses of H_2O_2 , when the reaction was found to be second order with respect to H_2O_2 . It is not possible to make a direct comparison of the third-order rate constant deriving from this work with the second-order catalytic constants discussed above. It is noteworthy that aqua-Fe(III) ion also shows catalysis via a third-order pathway at high substrate concentration (Jones *et al.*, 1959; Lewis *et al.*, 1963).

The use of borate buffers in the study of these systems appears to introduce severe additional complications. Gatt & Kremer (1968) found that the activity of protoferrihaem decreased with increasing pH in borate buffers, in contrast with the increase observed in carbonate buffers and in unbuffered systems. Waldmeier & Sigel (1971) found that the kinetic

form of the Fe(III)-TPS reaction changed to an approximate $[H_2O_2]^2$ dependence in borate buffers and the activity became almost independent of pH. It is known that H_2O_2 substrate interacts with the components of borate buffers, leading to formation of peroxoborate (Edwards, 1953; Kemp, 1956). It would seem to be desirable in future work to avoid the additional complexity that borate buffers may introduce in these reactions.

The pathway involving ferrihaem dimers as catalytic species is a novel reaction in systems of this type. Preliminary temperature-jump studies of the rate of equilibration in the deuterioferrihaem monomer-dimer reaction (Prudhoe, 1971) show that this is a very mobile equilibrium. It is therefore possible that the reaction involves the formation of μ -peroxo-bridged species, which are not uncommon in peroxide-transition-metal ion chemistry (Sykes & Weil, 1970). Some evidence exists for a pathway of this type in aqua-Fe(III) ion catalysis at very high substrate concentrations (Jones *et al.*, 1959). The data available do not, at present, permit other than very tentative discussion of the mechanism of this reaction.

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