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Catalase Model Systems

DECOMPOSITION OF HYDROGEN PEROXIDE CATALYSED BY MESOFERRIHAEM, DEUTEROFERRIHAEM, COPROFERRIHAEM AND HAEMATOFERRIHAEM

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The catalytic decomposition of H_2O_2 by deuteroferrihaem, mesoferrihaem, coproferrihaem and haematoferrihaem was studied as a model for the mechanism of action of catalase. For haematoferrihaem, anomalous but reproducible results were obtained, which could not be adequately explained. For each of the other ferrihaems studied, both monomeric and dimeric species catalysed decomposition, although the activity of monomer (a_M) was much greater than that of dimer (a_D). The pH variation of a_D in the range 6.5–11 was consistent with an inverse dependence on $[H^+]^{\frac{1}{2}}$. The molecular mechanism whereby such a dependence could be achieved is not apparent. A study of the pH-dependence of a_M in the range 6.5–11 revealed a linear inverse relationship with $[H^+]$. This is interpreted in terms of attack by HO_2^- on ferrihaem monomer. The specific pH-independent rate constants for this reaction were in the order coproferrihaem > protoferrihaem ≥ mesoferrihaem ≃ deuteroferrihaem. The order of magnitude of these rate constants is the same as that for catalysis by $Fe(H_2O)_6^{3+}$ and the second-order rate constant for decomposition of H_2O_2 by catalase. The implications on the mechanism of action of catalase are discussed.

Although the decomposition of H_2O_2 to O_2 and water is superficially one of the simplest of reactions catalysed by haemoproteins, the mechanism of action of catalase (EC 1.11.1.6) is incompletely understood. It is one of the few enzymes whose prosthetic group alone (protoferrihaem, Scheme 1) catalyses the same reaction as the enzyme itself and, indeed, the precise catalytic site is the iron atom in both cases. However, at physiological pH protoferrihaem is less efficient than the enzyme by many orders of magnitude. Previous studies (Kremer, 1965; Brown *et al.*, 1970*a*,*c*; Jones *et al.*, 1973) have suggested that the mechanisms of decomposition in the enzyme and model systems may be qualitatively similar, and that the difference in activity reflects, among other factors, differences in protonation of the substrate in the micro-environment of the catalytic centre (Brown *et al.*, 1970*c*).

The study of ferrihaems as catalase models is therefore potentially of value in determining the mechanism of action of the enzyme itself. In addition considerable interest has been shown in the possible technological use of ferrihaem–H_2O_2 systems in bleaching. In the case of protoferrihaem, however, several complicating factors arise (Brown *et al.*, 1970*a*). The most serious of these is the aggregation of protoferrihaem in aqueous solution to form dimers or higher aggregates (Falk, 1964, p. 46; Brown *et al.*, 1970*b*; Blauer & Zvilichovsky, 1970). Below pH 11, aggregation appears to be limited to dimerization (Brown *et al.*, 1970*c*) according to the equation:

$$2M \overset{K}{\rightleftharpoons} D + H^+$$

from which:

$$K = [D][H^+]/[M]^2 \qquad (1)$$

The value of K (which is dimensionless) was 4.5 (I 0.1).

The haem moieties in such a dimer are linked via their iron atoms with the formation of an oxo (Fe–O–Fe) bridge (Brown *et al.*, 1969; Fleischer & Srivastava, 1969). Since the iron atom is the centre of catalytic activity, it might be expected that dimerization would change the catalytic properties of protoferrihaem. In a spectrophotometric study, Brown *et al.* (1970*b*) showed that protoferrihaem was much more highly dimerized than deuteroferrihaem (Scheme 1) under similar conditions and, correspondingly, was considerably less active in the catalase reaction. These results were interpreted by suggesting that monomeric ferrihaems were much more active than the corresponding dimers. The activities of monomeric protoferrihaem and deuteroferrihaem were inversely proportional to $[H^+]$.

The usefulness of ferrihaems as catalase models can be extended by a study of additional substituted ferrihaems, particularly those that exhibit less tendency to dimerization. Brown & Hatzikonstantinou have investigated the dimerization of deuteroferrihaem, mesoferrihaem, haematoferrihaem and coproferrihaem (Scheme 1) which, as with protoferrihaem, occurs according to eqn. (1). For mesoferrihaem and deuteroferrihaem in phosphate buffer

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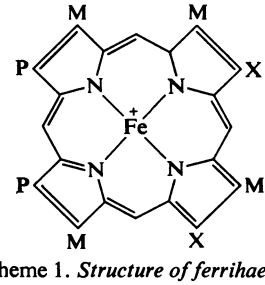
(I 0.05), $K = 5.47\times10^{-2}$ and 2.62×10^{-2} respectively (Brown & Hatzikonstantinou, 1978; Brown *et al.*, 1978), whereas for haematoferrihaem and coproferrihaem, $K = 1.0\times10^{-2}$ (I0.1) and 1.76×10^{-3} (I0.05) respectively (Hatzikonstantinou, 1977). In the present work the behaviour of these compounds as catalase models has been investigated.

Experimental

Mesoferrihaem chloride was readily obtained from protoferrihaem chloride by reduction with H_2 using 10% palladium in charcoal by the method of Davies (1940). The detailed preparation is described by Brown & Hatzikonstantinou (1978). Coproferrihaem hydroxide was prepared by iron insertion (Falk, 1964, p. 133) into coproporphyrin III (Hatzikonstantinou, 1977). Haematoferrihaem was similarly obtained by iron insertion into haematoporphyrin and was precipitated as the hydroxide (Found: C, 61.2; H, 5.6; Cl, nil; Fe, 8.3; N, 8.7; $C_{34}H_{36}N_4O_6FeOH$ requires C, 61.0; H, 5.5; Cl, nil; Fe, 8.4; N, 8.4%). The electronic spectrum of the pyridine haemochrome was in good agreement with data reported by Falk (1964, p. 240). Other materials and solutions were as previously described (Brown *et al.*, 1970*c*).

Special care was taken to make measurements on haematoferrihaem solutions within 15 min of solution preparation. This procedure was adopted in an attempt to avoid problems caused by specific adsorption of haematoferrihaem to the glass walls of reaction vessels, a process that occurs to a significant extent over longer periods (S. B. Brown & H. Hatzikonstantinou, unpublished work). All rate measurements were made at 25°C as previously described, H_2O_2 being estimated by titration (Brown *et al.*, 1970*c*). However, in view of the finding that high concentrations of phosphate buffer components significantly increase dimerization (Brown & Hatzikonstantinou, 1978) and enhance catalytic activity (Kelly *et al.*, 1977), the measurements reported here have been carried out at lower buffer concentrations (0.017M, I 0.05) than those reported for protoferrihaem and deuteroferrihaem.



Scheme 1. *Structure of ferrihaems*
M, $-CH_3$; P, $-CH_2CH_2CO_2H$. For protoferrihaem, X, $-CH{=}CH_2$; deuteroferrihaem, X, $-H$; mesoferrihaem, X, $-CH_2CH_3$; coproferrihaem, X, $-CH_2CH_2CO_2H$.

Results and Discussion

Fig. 1 shows a selection of plots of the variation in H_2O_2 concentration with time for the mesoferrihaem reaction at several mesoferrihaem concentrations. Similar plots were obtained for all the ferrihaems studied, except for haematoferrihaem (see below). It is clear that catalytic decomposition ceases before the H_2O_2 concentration reaches zero, owing to oxidation and destruction of the catalyst by H_2O_2 (Brown & Jones, 1968). For this reason, it is essential to measure the initial rate of decomposition, V_i defined by:

$$V_i = -\tfrac{1}{2}\frac{d[H_2O_2]}{dt} = k_0[T][H_2O_2] \qquad (2)$$

where [T] is the stoicheiometric ferrihaem concentration and k_0 is a second-order rate constant. Measurements of V_i were made by construction of tangents to curves such as those in Fig. 1 at zero time.

Mesoferrihaem

Fig. 2 shows results of measurements of the variation of V_i with total mesoferrihaem concentration, [T], at several pH values and constant initial H_2O_2 concentration (4mM). It is clear that the variation is not linear and that k_0, as defined by eqn. (2), is not a true second-order rate constant, independent of [T]. This result is expected if the monomeric and dimeric species contribute differently to the catalytic activity, since the proportions of monomer and dimer vary

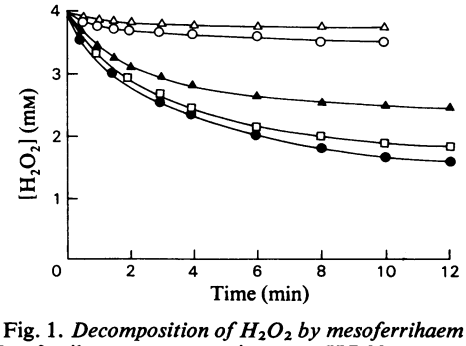


Fig. 1. *Decomposition of H_2O_2 by mesoferrihaem*
Mesoferrihaem concentrations at pH 7.02 were: △, 11.9 μM; ○, 19.8 μM; ▲, 59.4 μM; □, 79.2 μM; ●, 98.3 μM. For each experiment, reaction ceases before complete loss of H_2O_2 owing to oxidation of the catalyst.

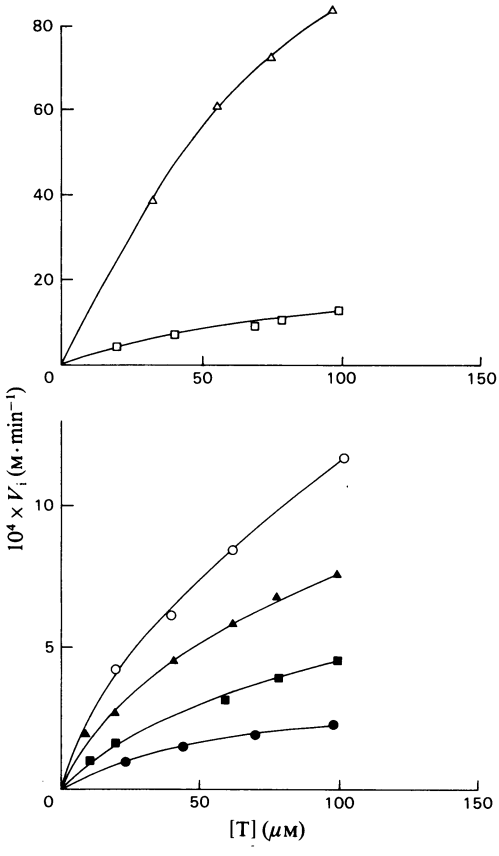


Fig. 2. *Dependence of V_i on [T] for mesoferrihaem* △, pH10.92; □ pH8.11; ○, pH7.73; ▲, pH7.41; ■, pH7.02; ●, pH6.62.

with [T]. Assuming a contribution from both species, the initial rate may be expressed as:

$$V_i = a_M[M][H_2O_2] + a_D[D][H_2O_2] \quad (3)$$

where a_M, a_D represent second-order rate constants for reaction catalysed by monomer and dimer respectively. Substitution in eqn. (3) from eqn. (1) yields:

$$V_i = [a_M M][H_2O_2] + a_D \frac{K}{[H^+]}[M]^2[H_2O_2] \quad (4)$$

Rearranging eqn. (4) gives:

$$\frac{V_i}{[M]} = a_M[H_2O_2] + a_D \frac{K}{[H^+]}[H_2O_2][M] \quad (5)$$

In previous work for deuteroferrihaem, plots of V_i versus [M] were approximately linear, from which it was deduced that no dimer contribution to the catalytic activity was detectable (Jones *et al.*, 1973). However, plots of V_i/[M] versus [M] are more sensitive since the intercepts (equal to $a_M[H_2O_2]$) reflect only the monomer activity, whereas the slopes (equal to a_D $K[H_2O_2]/[H^+]$) reflect only the dimer activity. Series of values of [M] at each pH studied were computed by using eqn. (1), the known dimerization constant (K) for mesoferrihaem and the relationship [T] = [M]+2[D].

Fig. 3 shows several plots of V_i/[M] against [M]. The linear relationships obtained show that the experimental data are consistent with eqn. (5). It is clear that all of the lines have positive slopes indicating that a dimer contribution is detectable. Values of a_M and a_D for each pH value were computed from the intercepts and slopes in Fig. 3. It is evident that both a_M and a_D show a pH variation. Assuming that a_M can be related to [H$^+$] by the general relationship:

$$a_M = a_M^0[H^+]^n \quad (6)$$

where a_M^0 is a pH-independent rate constant and n may be positive or negative, it can be shown that:

$$\log a_M = \log a_M^0 - n \cdot \mathrm{pH} \quad (7)$$

Fig. 4 shows the data for mesoferrihaem plotted according to eqn. (7) from which the best straight line through the points yields a value of $n = -0.93$. The true value of n is therefore probably -1 and the data are best represented by the line in Fig. 4 that has been drawn with a slope of unity.

The values of a_D, although dependent on pH, clearly do not follow the same pattern observed for a_M values. Assuming that the [H$^+$] variation can be represented by the relationship $a_D = a_D^0[H^+]^m$, where a_D^0 is the pH-independent rate constant in a similar fashion to the treatment for the monomeric species, then a plot of $\log a_D$ versus pH should be linear, slope $-m$. The straight line in Fig. 4 relating to the values of a_D has been drawn with a slope of 0.5, from which:

$$a_D = a_D^0/[H^+]^{\frac{1}{2}} \quad (8)$$

Although the data are consistent with this interpretation, the precision with which values of a_D may be determined is not sufficient to exclude other possibilities.

Deuteroferrihaem

Although deuteroferrihaem has been investigated at higher buffer concentrations (Jones *et al.*, 1973) a few additional measurements have been made at the concentrations of the present work to provide comparative data. In addition the possible role of the dimeric species has been reinvestigated in the light of the above results for mesoferrihaem. Fig. 5(*a*) shows plots of V_i versus [T] at three pH values. It is clear that a non-linear relationship exists, confirming that monomeric and dimeric species exhibit different activities. Values of [M] were calculated for each [T]

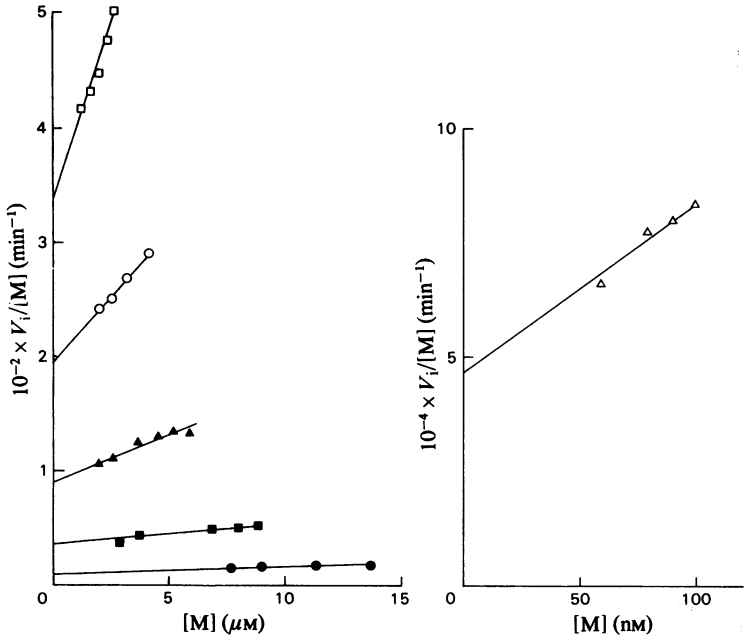


Fig. 3. *Plots of $V_i/[M]$ against [M] for mesoferrihaem*
Symbols are as in Fig. 2.

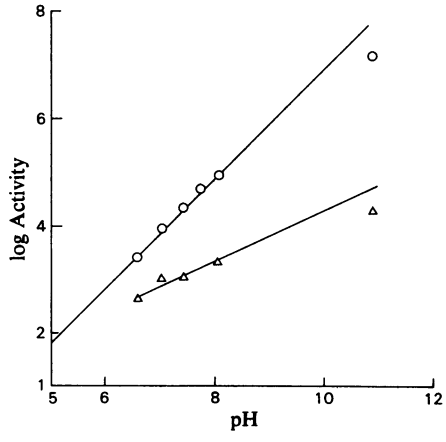


Fig. 4. *pH-dependence of catalytic activity of monomeric and dimeric mesoferrihaem*
○, Monomeric mesoferrihaem; the solid line has been drawn with unit slope. △, Dimeric mesoferrihaem; the solid line has been drawn with a slope of 0.5.

value and Fig. 5(*b*) shows plots of $V_i/[M]$ versus [M], from which it is evident that by using this more sensitive plot than that used previously, a dimer contribution is detectable at each pH value. Values of a_M and a_D were determined as described above and their pH variation is shown in Fig. 5(*c*). The lines for monomer and dimer have been drawn with slopes equal to 1.0 and 0.5 respectively, showing that these results are consistent with the interpretation for mesoferrihaem given above and with results for deuteroferrihaem monomer (Jones *et al.*, 1973).

Coproferrihaem

For the coproferrihaem-catalysed reaction it was apparent that reaction rates were considerably faster than those for mesoferrihaem or deuteroferrihaem. At the higher pH values studied, more than 50% decomposition occurred in less than 10s. Since it was impossible with the techniques available to make measurements in times less than about 8s, accurate values of V_i could not be determined under these conditions. Measurements of V_i for coproferrihaem were therefore limited to pH values below 7.74. Fig. 6(*a*) shows the variation of V_i with total coproferrihaem concentration [T] at each of four pH values. As was found for mesoferrihaem and deuteroferrihaem, the relationship between V_i and [T] is clearly not linear. Fig. 6(*b*) shows the same data plotted in the form $V_i/[M]$ versus [M], values for which were determined as described above. The experimental scatter in these plots for coproferrihaem is higher than that observed previously. This is partly due to the error in measurement of the large V_i values at higher pH. Also, because the proportion of dimer

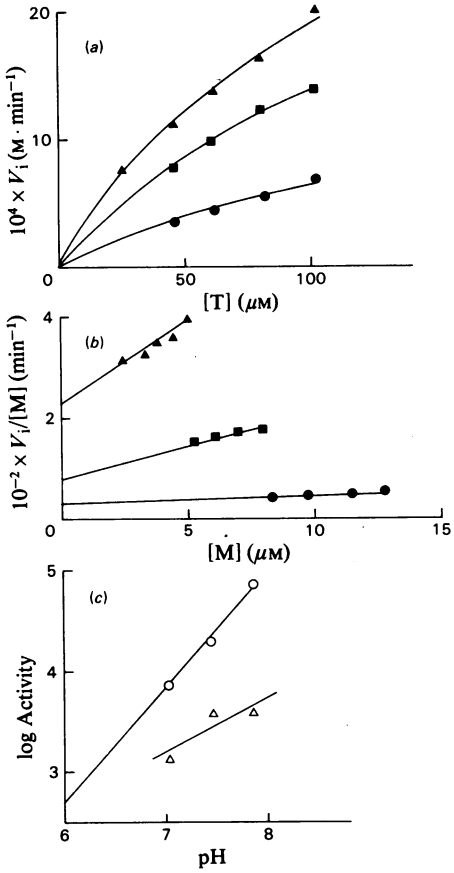


Fig. 5. *Data for catalytic decomposition of H_2O_2 by deuteroferrihaem*

(*a*) Dependence of V_i on [T]; ▲, pH7.86; ■, pH7.45; ●, pH7.02. (*b*) Plots of V_i/[M] against [M], symbols as in (*a*) above. (*c*) pH-dependence of catalytic activity of monomeric (○) and dimeric (△) deuteroferrihaem. The lines for monomeric and dimeric species have been drawn with slopes of unity and 0.5 respectively.

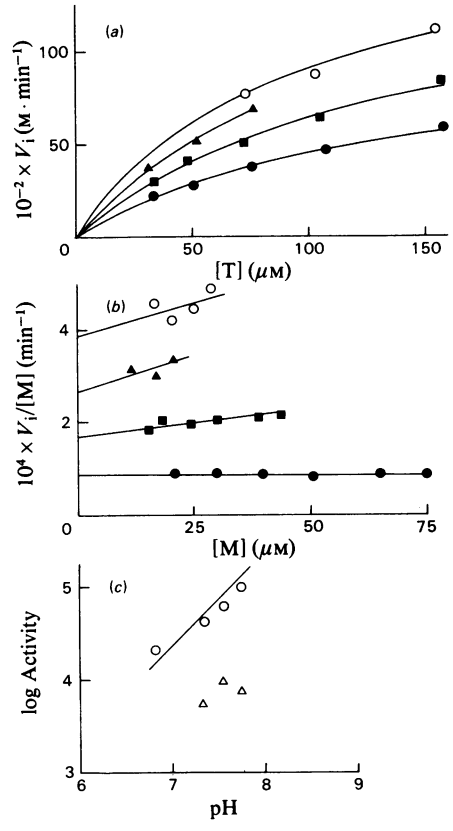


Fig. 6. *Data for catalytic decomposition of H_2O_2 by coproferrihaem*

(*a*) Dependence of V_i on [T]; ○, pH7.74; ▲, pH7.56; ■, pH7.35; ●, pH6.81. (*b*) Plots of V_i/[M] against [M], symbols as in (*a*) above. (*c*) pH-dependence of catalytic activity of monomeric (○) and dimeric (△) coproferrihaem. The line for monomeric species has been drawn with unit slope.

present is much lower than in the other ferrihaems studied, the slopes of the lines in Fig. 6(*b*) are relatively small and the precision in the measurement of a_D is correspondingly low. For pH6.81, the dimer contribution was too small to permit measurement. The pH-dependence of a_M and a_D is shown in Fig. 6(*c*). The solid line through the data for a_M represents the best straight line with a slope of unity. Within experimental error, it appears therefore that the results for coproferrihaem monomer conform to the pH-dependence observed for the other ferrihaems studied. The results for a_D are also shown in Fig. 6(*c*), but the large scatter precludes analysis of the pH-dependence.

Haematoferrihaem

Decomposition rate data for the haematoferrihaem-catalysed reaction were obtained by using the precautions discussed in the Experimental section in an attempt to avoid problems caused by adsorption of the catalyst to glass vessels. However, it was immediately apparent that haematoferrihaem behaved differently from other ferrihaems. In every case, the H_2O_2 concentration (as measured iodometrically) appeared to decrease in the expected manner, but then surprisingly began to increase again before reaching a constant value. Several curves illustrating this phenomenon are shown in Fig. 7(*a*). These effects were accurately reproducible. Further, the values of the H_2O_2 concentration at zero

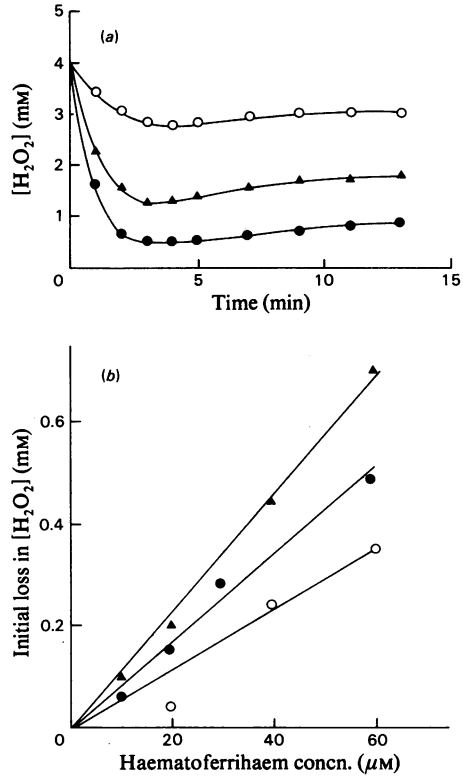


Fig. 7. *Anomalous catalytic behaviour of haematoferrihaem*

(*a*) Curves showing apparent increase in $[H_2O_2]$ during decomposition at pH6.60. Haematoferrihaem concentrations were: ○, 20μM, ▲, 40μM; ●, 60μM. (*b*) Initial apparent loss in $[H_2O_2]$ as a function of haematoferrihaem concentration: ▲, pH7.69; ●, pH 7.44; ○, pH6.60.

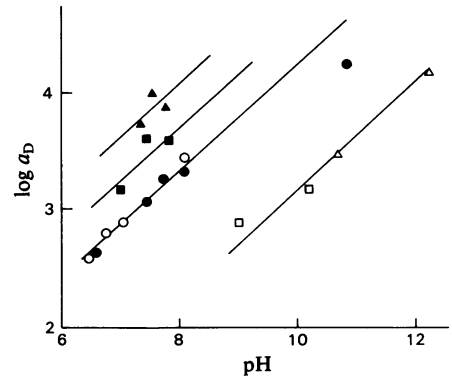


Fig. 8. *pH-dependence of catalytic activities of various dimeric ferrihaem species*

▲, Coproferrihaem (present work); ■, deuteroferrihaem (present work); ●, mesoferrihaem (present work); ○, protoferrihaem in KH_2PO_4/Na_2HPO_4 buffer, *I* 0.1 (Jones *et al.*, 1973); □, protoferrihaem in $Na_2CO_3/NaHCO_3$ buffer, *I* 0.1 (Jones *et al.*, 1973); △, protoferrihaem in $Na_2HPO_4/NaOH$ buffer, *I* 0.1 (Jones *et al.*, 1973). As was found for the monomeric species, $H_2PO_4^-$ buffer ions appear to enhance dimer catalytic activities (see data for protoferrihaem). The lines have been drawn with slopes of 0.5. The data for mesoferrihaem and protoferrihaem in KH_2PO_4/Na_2HPO_4 buffers are so close that they are best represented by the same line.

time (determined in the presence of haematoferrihaem), were significantly less than the values calculated from the addition of known quantities of H_2O_2 to reaction mixtures. (For other ferrihaems studied, good agreement was obtained between measured and calculated values.) It was also clear that these 'losses' in H_2O_2 were directly dependent on the total ferrihaem concentration in an approximately linear fashion as shown in Fig. 7(*b*). Although it was possible to obtain reproducible V_i values for haematoferrihaem, the data were not consistent with the model established above and did not appear to follow any coherent pattern. At present, no satisfactory molecular explanation is available for the unusual behaviour of haematoferrihaem.

General discussion

The results obtained in this work show that monomeric mesoferrihaem and coproferrihaem species are considerably more active than their dimeric counterparts, in agreement with previous data for deuteroferrihaem and protoferrihaem. The data also demonstrate that coproferrihaem is the most active ferrihaem model for catalase yet studied, consistent with its comparatively low dimerization constant.

Although the values of a_D are considerably less than corresponding a_M values, they are nevertheless significant, since dimeric species often predominate in ferrihaem solutions. Fig. 8 shows values of log a_D plotted against pH for all ferrihaem species so far studied under various conditions. The lines have been drawn through the data with slopes of 0.5. For protoferrihaem, the experimental scatter is relatively small, probably because the dimeric species makes the major contribution to the catalytic activity. It is clear that these data are in good agreement with an inverse dependence of a_D on $[H^+]^{\frac{1}{2}}$. Although the results for mesoferrihaem, coproferrihaem and deuteroferrihaem show considerable scatter, they are also consistent with this interpretation. However, the molecular mechanism by which such a rate dependence could be produced is not apparent. It is evident from Fig. 8 that although the values of a_D for the various ferrihaems at fixed pH show small differences, they are of the same order of magnitude.

There is, however, a pronounced effect of phosphate buffer ($H_2PO_4^-$), as seen in the two lines for protoferrihaem.

The values of a_M show an inverse dependence on $[H^+]$ up to pH10.5 approx. [This agrees with results for deuteroferrihaem and protoferrihaem (Jones *et al.*, 1973).] An explanation of this pH-dependence must therefore be sought in a protonation reaction with $pK \geqslant 11$. Since proteolytic equilibria involving the ferrihaem catalyst are unlikely to contribute to such a high pK value, it seems probable that the pH-dependence arises from the substrate owing to the equilibrium $H_2O_2 \rightleftharpoons HO_2^- + H^+$ (for which $pK_a = 11.6$; Kelly *et al.*, 1977) and that HO_2^- is the true reacting species. Assuming that this is the case, the reaction rate, V_i, is given by:

$$V_i = a_M^0[M][HO_2^-]$$

where a_M^0 represents the second-order rate constant for reaction of ferrihaem monomer with HO_2^- and is independent of pH. Values of a_M^0 are readily calculated from graphs of $\log a_M$ versus pH (Figs. 4, 5 and 6) and the known value of K_a and are shown in Table 1 for each monomeric species (I 0.05). Also shown is the value of a_M^0 for protoferrihaem calculated from previous data at I0.1 (Jones *et al.*, 1973). Under certain conditions, the decomposition of H_2O_2 by $Fe(H_2O)_6^{3+}$ also follows the same rate law (Brown *et al.*, 1970*c*) and the value of a_M^0 for this reaction is also shown in Table 1. For each catalytic species, the nominal net charge is shown, assuming that the carboxy groups are fully ionized and that no other acid–base equilibria occur. For protoferrihaem, because of phosphate catalysis, the value of a_M^0 at I0.05 is likely to be somewhat lower than that shown in Table 1. The activities of the ferrihaems therefore follow the sequence coproferrihaem > protoferrihaem ≃ mesoferrihaem ≃ deuteroferrihaem. The high activity of coproferrihaem may be related to its increased negative charge. However, the values of a_M^0 are remarkably similar in view of the different ligands for Fe(III) and the widely differing net nominal charges on the molecules.

The activity of bacterial catalase (a_c) defined by the equation:

$$-\tfrac{1}{2}\frac{d[H_2O_2]}{dt} = a_c[\text{catalase}][H_2O_2]$$

is pH-independent over the range where the protein exists in its native conformation and is shown in Table 1. It is clear that the order of magnitude of the values of a_M^0 obtained in the present work is the same as that of a_c for catalase, confirming earlier suggestions (Jones *et al.*, 1973) that the rate of reaction of ferrihaems with HO_2^- is very similar to that of bacterial catalase with H_2O_2.

The results presented here, along with earlier work, suggest several ways in which catalase apoprotein is able to confer high activity on its haem group. (*a*) By enveloping its captive haem group, each catalase polypeptide chain prevents dimerization and the consequent loss in activity; (*b*) catalase apoprotein is able to minimize haem oxidation and the consequent catalyst destruction; (*c*) the data in Table 1 suggest that catalase apoprotein may be able to exert maximum activity at physiological pH by accepting H_2O_2 and 'delivering' HO_2^- to the iron atom, i.e. effectively reducing the pK of H_2O_2 in the micro-environment of the catalytic site. The present results and previous work suggest, therefore, that an explanation of the superactivity of catalase should be sought in terms of the effect of the apoprotein on acid–base equilibria, rather than the effect on redox properties of the Fe(III) centre. These results also give added confidence in the relevance of studies on ferrihaems as catalase and peroxidase models, for example, in the determination of the structure and role of catalase–H_2O_2 complexes.

Table 1. *Activities of Fe(III)-centred catalysts*
The results for deuteroferrihaem, coproferrihaem and mesoferrihaem are from the present work. Those for protoferrihaem (I0.1), $Fe(H_2O)_6^{3+}$ and catalase were derived from data quoted by Brown *et al.* (1970*c*). The values of a_M^0 refer to activity towards HO_2^-. The activity for catalase, a_c, refers to reaction with H_2O_2 (see the text).

Fe(III) catalyst	$10^{-9} \times a_M^0$ ($M^{-1} \cdot min^{-1}$)	Nominal net charge
Protoferrihaem	0.64	−1
Deuteroferrihaem	0.28	−1
Coproferrihaem	1.0	−3
Mesoferrihaem	0.28	−1
$Fe(H_2O)_6^{3+}$	0.8	+3
	$10^{-9} \times a_c$ ($M^{-1} \cdot min^{-1}$)	
Catalase	4.0	−1

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