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Kinetics and thermodynamics of the reaction of peroxides with manganese-reconstituted horseradish peroxidase: a stopped-flow transient kinetic investigation

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The reaction of manganese-reconstituted horseradish peroxidase (MnHRP) with peroxides of different nature has been investigated by stopped-flow kinetics in order to understand the nature of the active site of the enzyme, the differences in the mechanism of the formation of MnHRP–I with different peroxides and possible resolution of the intermediate(s) involved. The reaction of *m*-$ClC_6H_4CO_3H$ with MnHRP shows that only the unionised species of the peracid reacts. *tert*-Butyl hydroperoxide reacted with MnHRP, although it does not with native HRP. The reaction shows saturation kinetics at room temperature for the formation of MnHRP–I at high concentrations of Bu^tO_2H. This observation provides direct evidence for a reversible binding step of the enzyme and peroxide before the product formation. The thermodynamic parameters for the formation of MnHRP–I are similar to those for HRP–I formation, indicating that the mode of formation of both peroxide compounds may be similar.

Hemoproteins contain iron protoporphyrin (3,7,12,17-tetramethyl-8,13-divinylporphyrin-2,18-dipropanoic acid) as prosthetic group and exhibit diverse physiological functions in nature.[1,2] The substitution of iron in such proteins by other metal ions has been carried out to understand the effect of the metal ion on the function and related properties of metalloproteins.[3–16]. Manganese porphyrin-reconstituted hemoprotein is one of the most commonly studied metal-substituted hemoproteins because of the similarity between manganese and iron.[4–11]. The overall conformation of the proteins is found to be restored in manganese-reconstituted hemoproteins, though their biological properties are different.[5] Manganese-reconstituted cytochrome b_5, hemoglobin and myoglobin do not show any activity,[6] while manganese-reconstituted horseradish peroxidase and cytochrome c peroxidase (CcP) have been found to be catalytically active though their activity is lower.[8] Manganese-reconstituted horseradish peroxidase (MnHRP) has been shown to form only one peroxide compound on reaction with H_2O_2 as compared to two in the case of the native horseradish peroxidase (HRP).[3,8,17] The peroxide compound formed reacts very slowly with oxidisable substrates such as ferrocytochrome c, $[Fe(CN)_6]^{4-}$ and ascorbate.[8] Further, the peroxide compounds I of MnHRP (MnHRP–I) and HRP (HRP–I) differ in the radical which is formed due to the reaction of H_2O_2. The radical resides on the protein in the former, while in the latter it resides on the porphyrin ring.[3,9] Recent NMR study on the interaction of aromatic donor molecules with MnHRP has shown that the binding site of several organic and inorganic substrates is however the same, both in the native HRP and MnHRP.[11]

Recently we reported a detailed transient kinetic investigation of the reaction of H_2O_2 with MnHRP.[17] The pH dependence of the reaction revealed the involvement of two ionisable groups, in contrast to the reaction of H_2O_2 with native HRP which is independent of pH above 5.0,[18] suggesting some change in the active site of the protein on its reconstitution. Peroxides of different nature and size have been used in the past to understand the nature of the peroxide intermediate and the mechanism of the reaction of peroxides with HRP and CcP.[19,20] In continuation of this work, we report here a transient kinetic study of the reaction of MnHRP with two different oxidising agents. It reveals a significant difference in the mechanism of the reaction of different peroxides with the reconstituted enzyme. The reaction of *tert*-butyl hydroperoxide with MnHRP shows saturation kinetics for the formation of the peroxide compound, which is unique for any natural or synthetic peroxidase under physiological conditions, and provides evidence for a reversible binding of the enzyme with peroxide before the product formation.

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Experimental

m-Chloroperbenzoic acid [*m*-$ClC_6H_4C(O)O_2H$] and *tert*-butyl hydroperoxide Bu^tO_2H were obtained from E. Merck. All other reagents were of analytical grade. Crude HRP with R_z (A_{403}/A_{280}) = 2.0, essentially as a salt-free lyophilised powder, was obtained from Sigma and purified to a R_z value of 3.2.[21] The preparation of horseradish apoperoxidase, its reconstitution with manganese(III) protoporphyrin IX and the purification were carried out as reported earlier.[17] The concentration of *m*-chloroperbenzoic acid was determined by monitoring the formation of iodide at 354 nm. The buffers used were acetate (below pH 5.9), phosphate (pH 6.0–8.0), Tris (pH 8.0–9.0) and carbonate (pH > 9.3). The concentration of the buffer was kept constant at 50 mM and ionic strength was adjusted with potassium nitrate to 0.2 M.

Absorption spectra and stopped-flow kinetics

The UV/VIS absorption spectra were recorded with a Shimadzu UV-2100 spectrophotometer equipped with a TCS-200 temperature controller using a cell with 1 cm path length.

The kinetic measurements were performed on a microprocessor-controlled stop-flow machine (HITECH-500), and the temperature of the system was maintained using a water-bath thermostat. The formation of MnHRP–I was monitored by observing the increase in absorbance at 412.5 nm. The concentration of $Mn^{III}HRP$ was kept constant at 1.0 μM. The concentration of peroxides was at least ten times greater than that of the enzyme. Under these conditions the reaction followed a pseudo-first-order rate law.

In a study of the MnHRP-catalysed oxidation of ferrocytochrome c by H_2O_2, Yonetani and Asakura[8] tentatively

Table 1 Apparent rate constants for MnHRP and HRP

Substrate	k_{app}(MnHRP)/M^{-1} s^{-1}	Ref.	k_{app}(HRP)/M^{-1} s^{-1}	Ref.
H_2O_2	1.70×10^3	17	1.15×10^7	18
m-$ClC_6H_4CO_3H$	1.62×10^5	Present work	1.18×10^8	22
Bu^tO_2H	9.64 ± 2.2	Present work	0.0	23

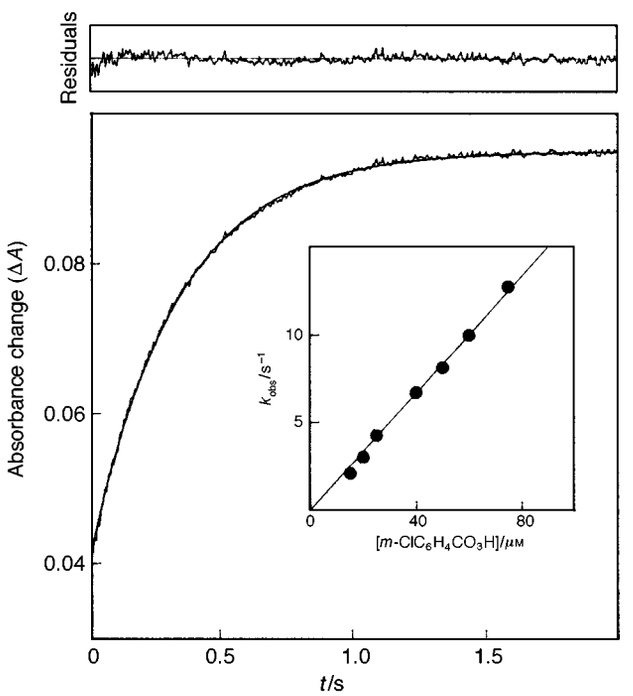

Fig. 1 Typical stopped-flow trace for the change in absorbance due to reaction of MnHRP (1.0 μM) with *m*-chloroperbenzoic acid (30 μM) at pH 7.0 showing the increase in absorbance at 412.5 nm. The line drawn through the experimental trace is the computer fit to a single exponential function, $A_t = A_0 \exp(-k_{obs}t) + B$, where A_t is the absorbance change signal with time, A_0 is the amplitude of the absorbance change, k_{obs} is the observed rate constant, t the time in seconds and B is the equilibrium absorbance signal (offset). Upper curve: the residual $A_{obs} - A_{calc}$ showing the accuracy of the computer-generated fit. Inset: the observed pseudo-first-order rate constant (k_{obs}) as a function of the *m*-chloroperbenzoic acid concentration at 25 °C; the solid line through the points is fit using equation (3)

suggested the existence of a H_2O_2-bound MnHRP complex which finally forms the product. A general mechanism for the transient reaction of MnHRP with peroxides can be given as in equation (1)[8,17] where S is a peroxide. This gives expression (2)

$$\text{MnHRP} + \text{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [\text{MnHRP–S}] \overset{k_2}{\rightleftharpoons} \text{MnHRP–I} \quad (1)$$

$$k_{obs} = \frac{k_2[\text{S}]}{K_D + [\text{S}]} \quad (2)$$

for k_{obs} where K_D ($= k_{-1}/k_1$) is the dissociation constant of the enzyme–peroxide complex and k_2 the rate of formation of the product from this complex or the turnover number of the reaction.

Results

Transient kinetics of the formation of MnHRP–I

(i) *m*-Chloroperbenzoic acid. Manganese-reconstituted horseradish peroxidase MnHRP was observed to form a peroxide compound on quantitative addition of *m*-chloroperbenzoic acid. The optical spectrum was identical to that reported for MnHRP–I[9,17] and showed a decrease in the intensity of the peaks at 374 and 482 nm and an increase in the intensity of that at 412.5 nm. The stopped-flow trace monitored at 412.5 nm shows a monophasic increase in the absorbance due to the formation of MnHRP–I (Fig. 1). The pseudo-first-order rate constant (k_{obs}) for the formation of MnHRP–I was calculated from the single-exponential fit of the experimental data (see Fig. 1); k_{obs} was found to be 4.16 s^{-1} at pH 7.0 at 30 μM *m*-$ClC_6H_4CO_3H$.

A plot of k_{obs} *vs.* [*m*-$ClC_6H_4CO_3H$] is linear (see Fig. 1, inset). For $K_D \gg [S]$, equation (2) reduces to (3) where $k_{app}(= k_2/K_D)$ is

$$k_{obs} = k_{app}[\text{S}] \quad (3)$$

the apparent rate constant for the formation of MnHRP–I. The data in Fig. 1 (inset) are consistent with equation (3). The value of k_{app} determined from the slope was found to be 1.62×10^5 M^{-1} s^{-1} at 25 °C (pH 7.0). This is two orders of magnitude higher than that for the reaction of MnHRP with H_2O_2 (see Table 1). It has been observed that k_{app} for the reaction of HRP with *m*-$ClC_6H_4CO_3H$ is also higher than the corresponding rate of reaction with H_2O_2, though to a lesser extent; it was explained by assuming preferential binding of the aromatic end group to the active site of the enzyme.[22,24] The active site of MnHRP may perhaps be more hydrophobic in nature than the native enzyme, giving *m*-$ClC_6H_4CO_3H$ easier access to the active site of MnHRP. Since k_{obs} varies linearly with the concentration of *m*-$ClC_6H_4CO_3H$, individual value of K_D and k_2 could not be calculated.

(ii) *tert*-Butyl hydroperoxide. On reaction with *tert*-butyl hydroperoxide MnHRP gives MnHRP–I (optical spectra not shown). This formation of MnHRP–I is interesting since Bu^tO_2H does not react with the native HRP[23] and manganese-reconstituted myoglobin MnMb.[25] The transient kinetic stopped-flow trace for the reaction of MnHRP with Bu^tO_2H is shown in Fig. 2, and k_{obs} was determined by a single-exponential fit to the data. The plot of k_{obs} against [Bu^tO_2H] shows saturation behaviour at high concentrations (see Fig. 2, inset). However, the complete asymptotic behaviour could not be approached due to a limitation on the use of higher concentrations of Bu^tO_2H during its reaction with MnHRP. A double reciprocal plot ($1/k_{obs}$ *vs.* $1/[H_2O_2]$) was also linear with a finite intercept (not shown), and thus provides further support for the saturation kinetics. The involvement of the latter requires the existence of equilibrium formation of the precursor complex prior to the formation of the compound I species. The formation of such a complex is now well documented for peroxidases.[26,27] With special reference to the manganese-reconstituted protein, our earlier results provided evidence of the formation of the precursor complex in the reaction of manganese-reconstituted myoglobin with H_2O_2.[25] The observation of saturation kinetics has not been reported earlier for the formation of compound I for any natural or synthetic peroxidase at ambient temperature, though it has been reported for HRP at subzero temperature.[26,27] The present observation provides direct evidence for the reversible binding step of MnHRP and the peroxide compound in the catalytic activity of MnHRP as in equation (1). The saturation behaviour of the formation of MnHRP–I can be expressed by equation (2) if $K_D \approx [S]$. The values of K_D and k_2 were determined from the double-reciprocal plot, as 140 ± 12 mM and 0.59 ± 0.1 s^{-1} at 20 °C respectively.

Table 2 Thermodynamic parameters for the formation of MnHRP and HRP

Enzyme	Substrate	$E^{\ddagger}_{app}$/kJ mol^{-1}	$\Delta G^{\ddagger}_{app}$/kJ mol^{-1}	$\Delta S^{\ddagger}_{app}$/kJ K^{-1} mol^{-1}	$\Delta H^{\ddagger}_{app}$/kJ mol^{-1}
HRP	H_2O_2	14.6 ± 4.2	31.3 ± 8.9	−48.5 ± 9.7	17.1 ± 3.6
MnHRP	H_2O_2	20.4 ± 0.7	54.4 ± 1.9	−105.9 ± 3.6	22.9 ± 0.8
	m-$ClC_6H_4CO_3H$	23.4 ± 1.2	43.2 ± 2.2	−58.2 ± 2.9	25.8 ± 1.3

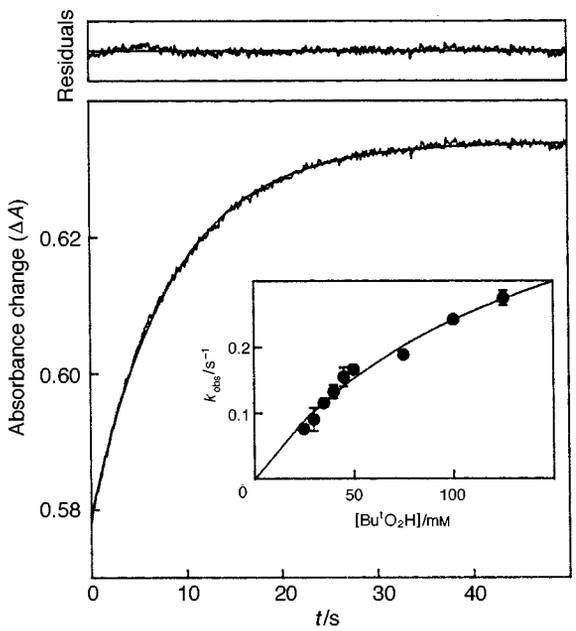

Fig. 2 Typical stopped-flow trace for the change in absorbance due to reaction of MnHRP (1.0 μM) with Bu^{t}O$_2$H (15 mM) at pH 7.0 showing the increase in absorbance at 412.5 nm. The line drawn through the experimental trace is the computer fit to a single-exponential function. Upper curve: residual as in Fig. 1. Inset: the observed pseudo-first-order rate constant (k_{obs}) as a function of Bu^{t}O$_2$H concentration at 20 °C showing saturation kinetics; the solid line shows a theoretical fit to the experimental data. The apparent values of K_D and k_2 were obtained from the slope and intercept of a double-reciprocal plot

A plot of k_{obs} *vs.* [S] for the reaction of MnHRP with H_2O_2 and *m*-$ClC_6H_4CO_3H$ is linear (see ref. 17 and Fig. 1, inset). This is expected since in both cases $K_D \gg [H_2O_2]$ or [*m*-$ClC_6H_4CO_3H$]. However, for the reaction of Bu^{t}O$_2$H with MnHRP saturation is observed when [Bu^{t}O$_2$H] ≈ K_D (Fig. 2). Physically, the observation of saturation kinetics implies that MnHRP forms a more stable enzyme–substrate complex with Bu^{t}O$_2$H than that with H_2O_2 or *m*-$ClC_6H_4CO_3H$. Since the electronic effect is important in the formation of the complex, and Bu^{t}O$_2$H has a very rich electron-donating substituting group, the MnHRP–Bu^{t}O$_2$H complex may be less reactive towards the product (compound I) formation.

pH Dependence

The pH dependence of k_{app} for the reaction of MnHRP with *m*-$ClC_6H_4CO_3H$ was studied in the pH range 5.0–10.3 (see Fig. 3). The rate of the reaction of MnHRP with H_2O_2 shows a bell-shaped variation with pH, which has been interpreted in terms of the involvement of two proton-ionisable groups having pK_a 4.8 and 10.6.[17] The result in Fig. 3 cannot, however, be explained on this basis. The marked fall in the rate of reaction as the pH passes through the pK_a of *m*-$ClC_6H_4CO_3H$ (= 7.5)[22] suggests that the ionisation of *m*-$ClC_6H_4CO_3H$ is an important factor in determining the rate of reaction. Since the pK_a of *m*-$ClC_6H_4CO_3H$ lies in the pH range of this study, the peracid can react either in unionised or ionised form. Considering two possible mechanisms where (i) only the unionised form reacts with the enzyme and (ii) both unionised and ionised forms, an attempt was made to fit the experimental data. An acceptable fit

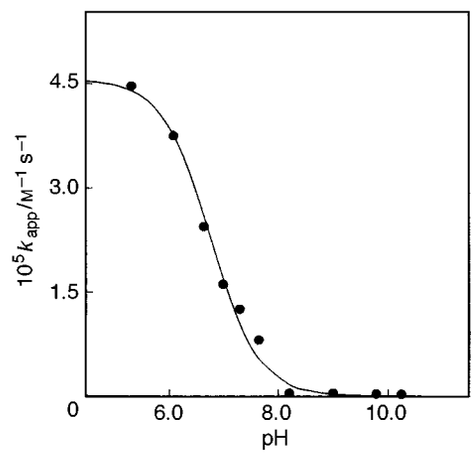

Fig. 3 Plot of k_{app} as a function of pH for the formation of MnHRP–I from the reaction of MnHRP and *m*-$ClC_6H_4CO_3H$ at 25 °C. The solid line through the experimental points is the fit using the equation $k_{app} = k^{max}_{app}/(1 + [H^+]K_s^{-1})$ considering that only the unionised form of *m*-$ClC_6H_4CO_3H$ reacts with the enzyme. Here, k^{max}_{app} is the second-order rate constant independent of pH and K_S is the equilibrium ionisation constant of *m*-$ClC_6H_4CO_3H$

was obtained only using the first model, *i.e.* when unionised *m*-$ClC_6H_4CO_3H$ reacts with MnHRP. It may be noted that the native HRP also reacts only with unionised *m*-$ClC_6H_4CO_3H$.[22] The fitted values of k^{max}_{app} and pK_a for the reaction of MnHRP with *m*-$ClC_6H_4CO_3H$ were found to be (4.56 ± 1.4) × 10^5 M^{-1} s^{-1} and 6.8 ± 0.1.

Temperature dependence

The value of k_{app} for the reaction of MnHRP with *m*-chloroperbenzoic acid was measured over the temperature range 20 to 35 °C. An Arrhenius plot constructed from the data was linear (not shown) and the apparent activation energy ($\Delta E^{\ddagger}_{app}$) for the formation of MnHRP–I was determined from the slope. The apparent activation enthalpy ($\Delta H^{\ddagger}$), free energy ($\Delta G^{\ddagger}$) and entropy ($\Delta S^{\ddagger}$) of the reaction were also calculated as described earlier.[17] The values are listed in Table 2.

Discussion

The spectra of MnHRP–I obtained from the reaction of MnHRP with different peroxides were found to be the same irrespective of the nature and size of the peroxides used. Similar observations have been made for HRP and cytochrome c peroxidase.[19,20] A comparison between the rates of reaction of different peroxides with HRP and MnHRP shows that rate constants for the formation of the peroxide compound for MnHRP are several orders of magnitude lower than the corresponding rate constants for HRP (see Table 1). The substitution of iron in cytochrome c peroxidase and myoglobin by manganese is also known to lower the peroxidase activity of these proteins.[8,28] Thus, manganese (a weak Lewis acid) seems to be a poor substitute for iron as the central metal in the peroxidase activity of these proteins.

The reactivity of different oxidants towards peroxidases would depend on the electrophilic characters of the oxidants, and also on the active site architecture of the enzyme molecules. In the case of peroxidase enzymes, the active site structure of

the distal (site for electrophiles such as peroxides) and proximal sites are known to play crucial roles in the function of these enzymes. Horseradish peroxidase contains two important amino acid residues (histidine and arginine) at the distal site. These residues are involved in specific interactions with the catalytically relevant −O–O linkage of the peroxide molecules and greatly enhance the binding affinity of the peroxides at the distal cavity of the enzyme. It has earlier been observed that the weak binding affinity of peroxides makes peroxide enzymes unreactive at low concentration of the peroxide substrate.[25,29] The present results indicate that the apparent second-order rate constants (M^{-1} s^{-1}) for the formation of MnHRP–I are 1.62×10^5 (*m*-$ClC_6H_4CO_3H$) > 1.7×10^3 (H_2O_2) > 9.64 (Bu^tO_2H).

tert-Butyl hydroperoxide does not react with the native HRP,[23] while it was found to react with MnHRP. This suggests some structural change at the active site of the enzyme due to reconstitution. The preferential access of *m*-$ClC_6H_4CO_3H$ to the active site of MnHRP as compared to the native enzyme (see Table 1) and the bell-shaped pH dependence of the rate of reaction of H_2O_2 with HRP which is pH independent[17,18] are also consistent with the above suggestion.

The apparent activation energy for the formation of compound I of native HRP has been reported to be 14.7 ± 4.2 kJ mol^{-1}.[30] The corresponding energy for the formation of MnHRP–I is slightly higher for all the peroxides (see Table 2). The minimum theoretical energy of activation for a reaction in water (E_{act}^{water}) has been found to be 16.3 kJ mol^{-1},[31] which is lower than the energy of activation reported for the formation of HRP–I. This has been explained on the basis that the enthalpy of association of HRP with H_2O_2 may be negative.[31,32] The higher activation energy for the formation of MnHRP–I compared to E_{act}^{water} indicates that the change in enthalpy associated with the binding of MnHRP to the peroxides may be positive.

The apparent thermodynamic activation parameters for the formation MnHRP–I and HRP–I are similar (Table 2), which suggests that the modes of formation of peroxide compound I for HRP and MnHRP may be similar. However, a higher positive apparent free-energy change for the reaction of peroxides with MnHRP as compared to HRP suggests a stronger thermodynamic opposition to the formation of MnHRP–I.

In conclusion, the present study brings out the differences in the mechanisms of reaction of different peroxides with MnHRP, and the mechanistic resolution of any possible intermediate(s) during the reaction. It highlights the subtle differences in the reaction of the different peroxides with MnHRP, especially Bu^tO_2H, which appears to suggest structural changes in the active site of the enzyme upon metal reconstitution. The reaction of Bu^tO_2H with MnHRP shows saturation kinetics and provides evidence for a reversible binding step of the enzyme and peroxide before product formation. The stronger thermodynamic opposition, a possible change in the active site structure on reconstitution, and weak Lewis-acid character of manganese could be possible reasons for the lower activity of the reconstituted enzyme.

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