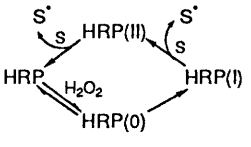
Identification of Catalytic Pathways in the Peroxidatic Reactions of the Haem Octapeptide Microperoxidase-8

Ian D. Cunningham* and Giles R. Snare
Department of Chemistry, University of Surrey, Guildford GU2 5XH, UK

Reaction of microperoxidase-8 (MP-8) with H_2O_2 leads to the formation of an oxidized intermediate whose subsequent reduction by 2,4-dimethoxyphenol occurs in two stages and results in regeneration of MP-8. Analysis of the kinetics of these reactions in comparison with those of the normal peroxidatic reaction shows that at least four oxidized forms of MP-8 are capable of oxidizing the phenol. Possible structures and modes of action for these intermediates are discussed.

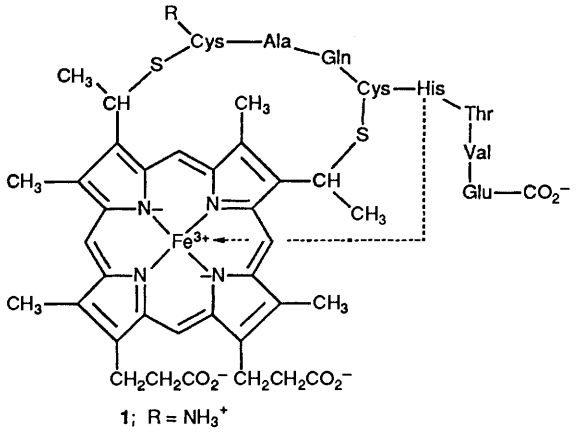
The peroxidases are a class of iron porphyrin-containing enzymes important in the oxidation of a range of organic and inorganic substrates.[1] In general the reaction mechanism involves the oxidation by H_2O_2 or an organic hydroperoxide of the enzyme to a species, commonly called compound I, which is two oxidizing equivalents above the resting enzyme. Subsequent one electron reduction [1d] by a substrate (such as a phenol) yields a species one equivalent above the resting enzyme (compound II), which in turn can undergo one electron reduction to regenerate the enzyme. The horseradish peroxidase enzyme (HRP) is typical and its mechanism can be outlined in Scheme 1 where HRP(I) and HRP(II) represent compounds I and II, respectively. Recently, Van Wart [2] has proposed a pre-complex between HRP and the peroxide [HRP(0)] and this is also included in Scheme 1.



Scheme 1

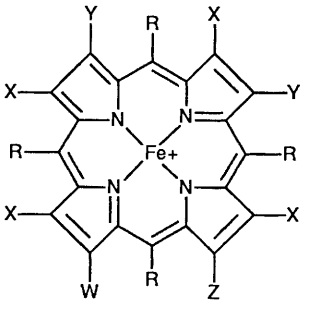
The nature of compound I appears to vary for the different enzymes, with the first oxidizing equivalent being in the form of an oxyferryl (Fe^{IV}), but the second being in the form of a porphyrin radical cation (HRP, catalase) [1e] or a protein amino acid residue radical cation (cytochrome c peroxidase); [1f] compound II is assumed to be an Fe^{IV} porphyrin.

A popular approach to the study of these enzymes is the use of model compounds, ranging from iron porphyrins bearing oligopeptide substituents [3–7] such as microperoxidase-8 (MP-8) **1** [3a,3b,4–7] or MP-11 [3c] **2**, through those with a proximal



1; R = NH_3^+
2; R = Lys—Gln—Val—NH_3^+

imidazole (*e.g.* **3**),[8] to relatively simple haem compounds such as coproferrihaem **4**.[9] The simple haem models tend to



3; R = H, X = CH_3, Y = CH═CH_2, W = $CH_2CH_2CO_2CH_3$
Z = $CH_2CH_2CONH(CH_2)_3$—N⟨N⟩
4; R = H, X = CH_3, W = Y = Z = $CH_2CH_2CO_2^-$
5; R = Aryl, W = X = Y = Z = H

aggregate in aqueous solution and are oxidized to unstable intermediates. These problems can be overcome to an extent by the use of iron porphyrins bearing substituents (often *ortho*-substituted aryls) (*e.g.* **5**);[10] a recent series of papers by Lindsay Smith and co-workers exemplifies this approach.[11]

The peroxidase model MP-8 is an attractive one because of the oligopeptide substituent to the porphyrin ring and because it can be studied in aqueous solution with minimal aggregation.[3a] The outline mechanism is based on Scheme 1,[3b] but recent studies by one of us,[6] and by Adams [5] have shown that the mechanism is complex, involving additional decomposition steps and catalysis by an MP-8–substrate complex under certain conditions. In the case of MP-8 the evidence for the intermediacy of compound I and II analogues is mainly kinetic. However, it has been tentatively suggested that at least one oxidized intermediate can be detected spectroscopically;[4] similarly in a recent study of acetyl MP-8 (Ac-MP-8)[12] evidence is presented for the spectroscopic identification of compound 0, I and II analogues.

Of particular interest to the organic chemist is the nature of the oxidized intermediates in the above reactions, and their mode of interaction with organic substrates. In this paper we present the results of a study of the interaction of oxidized forms of MP-8 with a phenolic substrate (2,4-dimethoxyphenol) and speculate on their nature and mode of interaction with the substrate.

Experimental

Materials and Spectroscopic Methods.—All materials were as

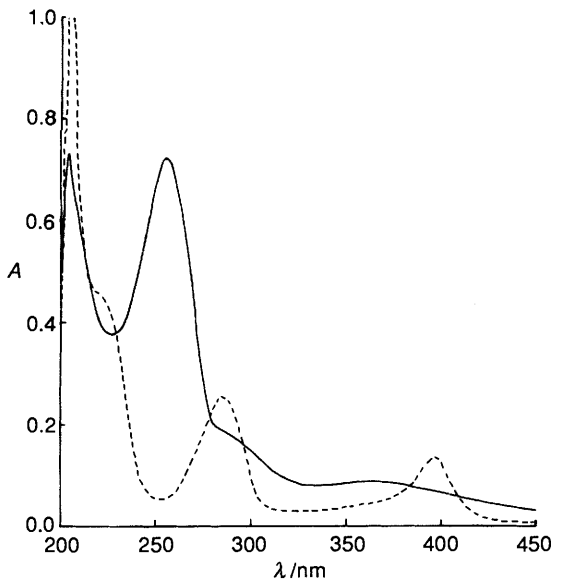


Fig. 1 Spectrum (- - - -) of 2,4-dimethoxyphenol (7.0×10^{-5} mol dm^{-3}) with added MP-8 (9.3×10^{-7} mol dm^{-3}) and of (——) its oxidation product in the presence of excess H_2O_2 (66×10^{-5} mol dm^{-3}). (NOTE: the MP-8 is rapidly bleached by excess H_2O_2 at the end of the reaction).

Table 1 UV–VIS spectra of 2,4-dimethoxyphenol and its product on reaction with H_2O_2 in aqueous phosphate buffer (0.1 mol dm^{-3}, pH 7)

	2,4-Dimethoxyphenol	Oxidation product
λ_{max}/nm	286	256
ε_{256}	781[a]	9222[b]
ε_{286}	3309[a]	—
ε_{397}	0[a]	644[b]

[a] In units of mol^{-1} dm^3 cm^{-1}. [b] In units of mol^{-1} of 2,4-dimethoxyphenol consumed dm^3 cm^{-1} ($\varepsilon'\rho$).

used previously.[6] 2,4-Dimethoxyphenol was prepared from 2,4-dimethoxybenzaldehyde (Aldrich) according to a published procedure;[13] purification was by preparative TLC and recrystallization.

A Philips PU 8720 spectrophotometer was used throughout and reactions were carried out using 1 cm stoppered silica cuvettes thermostatted at 25 °C. Reactions were generally initiated by injection of the appropriate reagent and manual mixing, but where rapid (<2 s) mixing was required a preloaded Teflon plunger inside the cuvette was used.

Oxidation of 2,4-Dimethoxyphenol.—UV–VIS changes on HRP-catalysed H_2O_2 oxidation of 2,4-dimethoxyphenol were determined by adding 5 mm^3, and then a further 60 mm^3, of stock 0.0233 mol dm^{-3} aqueous H_2O_2 solution to a solution of aqueous phosphate buffer (3 cm^3; 0.1 mol dm^{-3}, pH 7), 19.4×10^{-5} mol dm^{-3} in 2,4-dimethoxyphenol, and containing HRP (0.67 μg cm^{-3}). No change in the product spectrum was noted on standing in the presence of excess H_2O_2 subsequent to completion of the reaction.

Reaction Stoichiometry.—Aliquots (1 mm^3) of stock 0.0233 mol dm^{-3} aqueous H_2O_2 solution were added to aqueous buffer (2 cm^3; 0.1 mol dm^{-3}, pH 7), 13.1×10^{-5} mol dm^{-3} in 2,4-dimethoxyphenol, and containing HRP (1.0 μg cm^{-3}). After each addition several minutes was allowed to elapse for any reaction to subside, and addition was continued until no further increase in absorbance at 256 nm resulted.

Formation of Oxidized MP-8 and its Reaction with 2,4-Dimethoxyphenol.—A typical run was as follows. To aqueous buffer (2.5 cm^3; 0.1 mol dm^{-3}, pH 7), 1.01×10^{-6} mol dm^{-3} in MP-8, was added 0.0023 mol dm^{-3} stock aqueous H_2O_2 solution (5.5 mm^3) (to yield a solution 5.06×10^{-6} mol dm^{-3} in H_2O_2). The resultant absorbance (A_t) decrease at 397 nm was monitored every 5 s. After 130 s (or after the appropriate time interval τ in other runs), 0.097 mol dm^{-3} stock aqueous 2,4-dimethoxyphenol solution (1.7 mm^3) was added (to give a solution 6.6×10^{-5} mol dm^{-3} in the phenol) and the subsequent exponential increase in absorbance was monitored every 5 s; an infinity reading was taken 715 s after addition of the phenol.

A first-order rate constant for the H_2O_2-induced decrease in absorbance k'_{obs} was determined from a plot of $\ln(A_t - A_1)$ against t where A_1 is the absorbance just prior to addition of the phenol. The constant for the phenol-induced exponential increase k_{obs} was determined similarly using A_3, the absorbance value after about 10 half-lives. Extrapolation, using k_{obs} back to τ allowed A_2, the absorbance just after addition of the phenol, to be calculated.

Steady State Oxidation of 2,4-Dimethoxyphenol.—The reaction was initiated by the addition of 0.0233 mol dm^{-3} stock aqueous H_2O_2 solution (57 mm^3) to aqueous buffer (2.0 cm^3; 0.1 mol dm^{-3}, pH 7), 6.98×10^{-7} mol dm^{-3} in MP-8 and 5.9×10^{-5} mol dm^{-3} in 2,4-dimethoxyphenol, in a thermostatted cuvette at 25 °C. The resulting absorbance increase at 256 nm was monitored until reaction ceased. dA_t/dt was determined from the linear portion of the change (no deviation from linearity could be detected over >90% of the absorbance increase). The run was duplicated. A similar experiment was carried out using a solution in which the MP-8 ($[MP\text{-}8]_0 = 1.33 \times 10^{-6}$ mol dm^{-3}) had been 'regenerated' by addition, after 200 s, of 2,4-dimethoxyphenol to the H_2O_2-oxidized catalyst, and to which extra 2,4-dimethoxyphenol and H_2O_2 were added to give their concentrations as 6.6×10^{-5} mol dm^{-3} and 63×10^{-5} mol dm^{-3}, respectively.

Results

Oxidation of 2,4-Dimethoxyphenol.—Treatment of 2,4-dimethoxyphenol (6.8–19.4 × 10^{-5} mol dm^3) in aqueous buffer (pH 7.0) with 2–11-fold molar excess of H_2O_2 in the presence of HRP or MP-8 gave a product showing a UV spectrum (Fig. 1) the details of which are summarized in Table 1; the spectra obtained using either catalyst are identical. Although the product has not been characterized, 2,4-dimethoxyphenol has been used as substrate (a) because of the stability of its product spectrum, (b) because of the product's low absorbance at 397 nm (λ_{max} of MP-8), and (c) because of its high reactivity (see below).

The stoichiometry of the reaction was determined to be 1:1 in terms of 2,4-dimethoxyphenol and H_2O_2.

Oxidation of MP-8 by H_2O_2.—On treatment of MP-8 (0.58–1.40×10^{-6} mol dm^{-3}) with H_2O_2 (5.06×10^{-6} mol dm^{-3}) the absorbance due to the MP-8 at 397 nm decreased over several minutes as shown in Fig. 2(*a*). The initial decrease appeared exponential, but an invariant 'infinity absorbance' was not obtained; a slower decay followed until the peak at 397 nm had disappeared ('bleaching'). Although a precise calculation of k'_{obs} for the initial decrease is not possible, an estimation of the range within which it lies was determined by use, in a series of experiments, of A_1, the absorbance at 70, 130 or 300 s, as a 'pseudo-A_{inf}'; in this way k'_{obs} was found to lie in the range 24–38×10^{-3} s^{-1}. When the H_2O_2 concentration was doubled the rate was too fast to measure precisely, but k'_{obs} was approximately doubled. Assuming, therefore, a linear dependence of

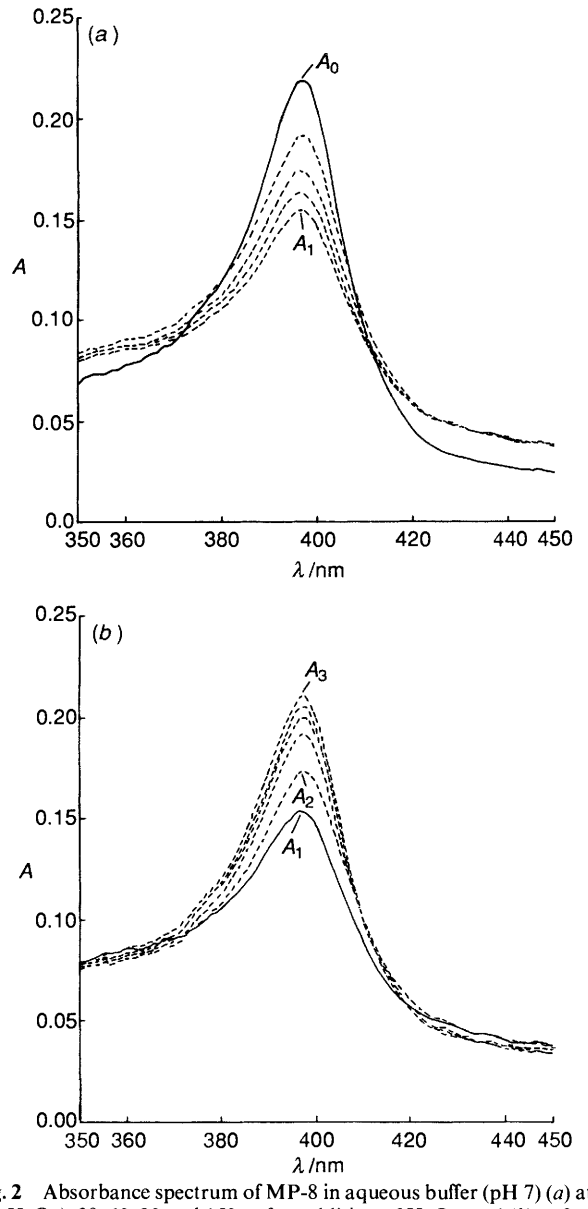


Fig. 2 Absorbance spectrum of MP-8 in aqueous buffer (pH 7) (*a*) at 0 (no H_2O_2), 30, 60, 90 and 150 s after addition of H_2O_2, and (*b*) at 0 (no phenol), 10, 70, 130, 190, 720 s after subsequent addition of 2,4-dimethoxyphenol. $[MP\text{-}8]_0$ = 1.4 μmol dm^{-3}, $[H_2O_2]_0$ = 5.06 μmol dm^{-3} and [2,4-dimethoxyphenol] = 68 μmol dm^{-3}.

Table 2 Changes in absorbance (*A*) at 397 nm upon addition of H_2O_2 (5.06×10^{-6} mol dm^{-3}) followed after an interval (τ) by 2,4-dimethoxyphenol (S) along with observed rate constants for absorbance changes

A_1/A_0 (%)	τ/s	10^5 [S]/ mol dm^{-3}	$\Delta A_{2-3}/\Delta A_{1-2}$	A_3/A_0 (%)	$10^3 k_{obs}/s^{-1}$
34	300	6.6	1.6	79	10.8 ± 0.3
47	130	6.6	2.9	87	10.4 ± 0.9
37	130	24.3	1.9	86	29.6 ± 4.4
54	130	48.6	2.2	92	55.1 ± 2.1
55	70	6.6	1.6	93	9.8 ± 0.5

k'_{obs} on H_2O_2 concentration the second-order rate constant $k' = k'_{obs}/[H_2O_2]_0$ has a value in the range 4.7–7.5 × 10^3 dm^3 mol^{-1} s^{-1}.

Reaction of Oxidized MP-8 with 2,4-Dimethoxyphenol.—Following addition of H_2O_2 to the MP-8 solution, addition of a solution of 2,4-dimethoxyphenol (to yield solutions 6.6–48.6 × 10^{-5} mol dm^{-3} in the phenol) after 70, 130 and 300 s (τ) caused the peak at 397 nm to be regenerated; a typical reaction is shown in Fig. 2(*b*). This behaviour is summarized quantitatively in Table 2 where A_0 is the initial absorbance at 397 nm and A_1, A_2 and A_3 are defined in the Experimental section and shown on the spectra of Fig. 2. On addition of the phenol there was an 'instantaneous' (on the scale of our measurements) increase in absorbance (ΔA_{1-2}) followed by a slower increase in absorbance (ΔA_{2-3}). The initial increase in absorbance occurred within the mixing time which was typically 5–10 s, but in experiments where mixing was rapid it was seen that this increase occurred within the first 2 s. Irrespective of τ or the concentration of added phenol the ratio $\Delta A_{2-3}/\Delta A_{1-2}$ is seen to be 1.6–2.9:1. The slower regeneration of the 397 nm peak (ΔA_{2-3}) followed first-order kinetics and the observed rate constant (k_{obs}) for this process is given in Table 2. The final absorbance (A_3) was found to be between 79 and 93% of the original value (A_0) depending on τ and the amount of phenol added. For addition of 2,4-dimethoxyphenol at 130 s, k_{obs} was found to vary linearly with phenol concentration and from the slope of a plot of k_{obs} against [2,4-dimethoxyphenol] (including the zero point) a value for the second-order rate constant k of 110 dm^3 mol^{-1} s^{-1} was calculated.

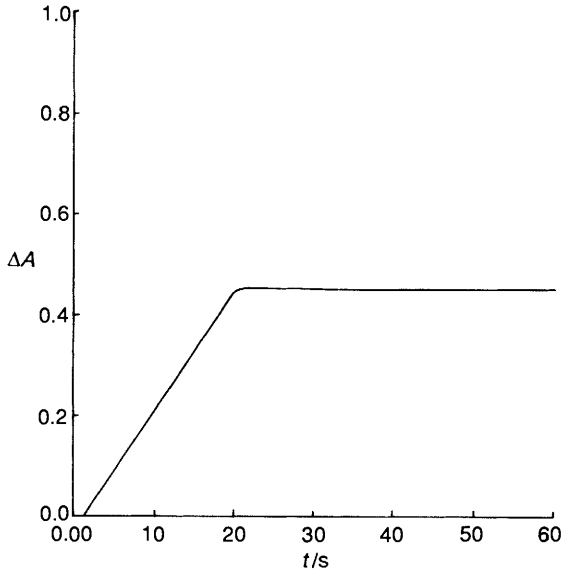
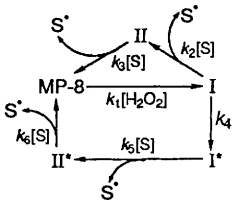


Fig. 3 Absorbance change at 256 nm on addition of H_2O_2 to aqueous buffer (pH 7) containing MP-8 and 2,4-dimethoxyphenol. $[MP\text{-}8]_0$ = 0.70 μmol dm^{-3}, $[H_2O_2]_0$ = 660 μmol dm^{-3} and [2,4-dimethoxyphenol] = 59 μmol dm^{-3}.

Uncertainties in absorbances and in values derived from them were $< \pm 15\%$; rate constants were obtained from duplicated runs and quoted uncertainties in the tables are deviations from the mean value.

Steady-state H_2O_2-Oxidation of 2,4-Dimethoxyphenol Catalysed by MP-8.—Oxidation of 2,4-dimethoxyphenol (5.9×10^{-5} mol dm^{-3}) by an excess of H_2O_2 (66.4×10^{-5} mol dm^{-3}) in the presence of MP-8 (6.98×10^{-7} mol dm^{-3}) resulted in an increase in absorbance at 256 nm which followed zero-order kinetics (Fig. 3). The zero-order kinetics show that the stoichiometrically deficient component (2,4-dimethoxyphenol) does not appear in the rate equation and additionally that the MP-8 catalyst is not significantly deactivated during the reaction (*c.f.* the oxidation of other substrates by this catalyst, where some relatively unreactive substrates allow competing deactivation of the MP-8).[6] The MP-8-catalysed H_2O_2-oxidation of a range of phenol, aniline and other substrates has been investigated by us[6] and others;[3b,5] in all cases the rate equation at low substrate concentration has been found to be of the form



Scheme 2

$-d[H_2O_2]/dt = k''\cdot[H_2O_2]\cdot[MP\text{-}8]$ and it seems reasonable in view of the observed substrate-independence of the rate in this case to assume a similar rate equation. It can be shown therefore, that dA_t/dt should equal $k''\cdot\{\varepsilon'_p - \varepsilon_s\}\cdot[H_2O_2]_0\cdot[MP\text{-}8]_0$; ε'_p is defined in Table 1, ε_s is the extinction coefficient of 2,4-dimethoxyphenol at 256 nm and k'' is the second-order rate constant for loss of H_2O_2. Based on duplicate experiments to determine dA_t/dt (as in Fig. 3), and using values from Table 1 allows a value for k'' of 6.19 ± 0.3 × 10^3 dm^3 mol^{-1} s^{-1} to be calculated.

A steady-state reaction under the similar conditions, but using a solution where MP-8 had been regenerated also gave a zero-order plot with $dAbs/dt$ within 5% of the expected value based on the constants above.

Discussion

Clearly, on treatment of MP-8 with H_2O_2, an oxidized species, MP-8(ox), is generated; it shows a UV maximum at about 397 nm (similar to MP-8), but has a lower extinction coefficient. Although approximate, the value of the second-order rate constant k' (between 4.7 and 7.5 × 10^3 dm^3 mol^{-1} s^{-1}) shows that MP-8(ox) is, or is formed *via*, the same intermediate that is involved in the steady-state peroxidatic cycle for MP-8 (see refs. 3*b*, 4, 5, 6 and the value of k'' above). In the absence of added reducing agent a slow loss of MP-8(ox) subsequently occurs as shown by the drop in A_1/A_0 as τ is increased.

Addition of the phenolic reducing agent causes a regeneration of MP-8 as shown by the reformation of the peak at 397 nm with its associated peroxidatic activity characteristic of MP-8. It is clear however, that two types of regenerative behaviour are occurring, a fast (ΔA_{1-2}) and a slow (ΔA_{2-3}). There are several possible explanations for this, each of which is considered below. The first possibility is that MP-8(ox) consists of two species, both of which show different reactivity towards the phenol and one of which is being slowly converted into the other. However, this is unlikely as the relative amounts of 'fast' and 'slow' regeneration (about 1:2) do not change significantly between interception at 70 and 300 s. This objection is avoided if the species are not interconvertible. However, it is a fundamental postulate of our argument that the regeneration of MP-8 from MP-8(ox) by reaction with the phenolic reducing agent requires that the various MP-8 species involved in this reaction differ only in oxidation state, electronic distribution and/or nature of group coordinated to iron. Given this, it is difficult to see why, particularly with excess of H_2O_2 present, conversion of one into the other would not be possible. A fast equilibrium between two species can be discounted since reaction with the phenol would be *via* the more reactive species and a separate slow reaction of the less reactive species would not be seen, while a slow equilibrium would be similar to the first possibility above.

The second explanation is that reaction of the phenol with MP-8(ox) involves initial formation of a complex, MP-8(ox)–phenol (ΔA_{1-2}) followed by its conversion (ΔA_{2-3}) into MP-8. However, the results in Table 2 are not compatible with this; if the ratio [complex]/[MP-8(ox)] is low, increasing the concentration of 2,4-dimethoxyphenol would be expected to cause an increase in ΔA_{1-2}. On the other hand a high ratio of complex to MP-8(ox) would result in a subsequent reaction independent of phenol concentration.

The third possibility is that MP-8(ox) is reduced in a rapid one electron-transfer reaction with the phenol to give a second, less highly oxidized form MP-8(ox)′, which is subsequently and more slowly reduced by phenol to MP-8. This seems the most likely explanation for the observed behaviour, and furthermore the assignation to MP-8(ox) and MP-8(ox)′ of oxidation states 2 and 1 units, respectively, above MP-8 seems reasonable [the two-stage reduction observed is difficult to reconcile with MP-8(ox) being formally in a (IV)-oxidation state, or with regeneration of MP-8 from MP-8(ox) *via* a two electron pathway].

However, we will argue below that these oxidized forms of MP-8 are not the intermediates on the normal (substrate initially present) MP-8-peroxidatic pathway, and that MP-8(ox)′, at least, is unlikely to be an HRP(II) analogue. It can be seen from Table 2 that the pseudo-first-order rate constant for conversion of MP-8(ox)′ into MP-8 at the phenol concentration used in the steady state reaction (the 'normal' catalytic cycle) has a value of about 10^{-2} s^{-1} (and is dependent on [phenol]), yet the steady state kinetics show that the rate-limiting step of the cycle is the initial interaction of MP-8 with H_2O_2 (independent of [phenol]) with a pseudo-first-order rate constant (equal to $k''\cdot[H_2O_2]_0$) of 6.19 × 10^3 × 66 × 10^{-5} s^{-1}, that is about 408 × 10^{-2} s^{-1}. It is self-evident that a reaction slower than the rate-limiting step in a cyclic process cannot be part of that cycle. Furthermore, it is hard to see how MP-8(ox) can be on the cycle if MP-8(ox)′ is not, and therefore, we propose an additional catalytic peroxidatic cycle as shown in Scheme 2.

The novel feature in this scheme is the rapid (k_4 faster than $k_1\cdot[H_2O_2]$) 'rearrangement' of the highly oxidizing initially-formed intermediate I to a more stable intermediate MP-8(ox) (I* in Scheme 2), unless the more favourable reductive interaction with the substrate 'S' ($k_2\cdot[S]$ faster than k_4 and followed by fast $k_3\cdot[S]$) is possible. Subsequent one electron reduction of I* ($k_5\cdot[S]$ fast and corresponding to ΔA_{1-2}) yields MP-8(ox)′ (II* in Scheme 2). Compound II* undergoes a slow reduction (corresponding to ΔA_{2-3}) back to MP-8 with a second-order rate constant $k_6 = k$ (110 dm^3 mol^{-1} s^{-1}). It should be noted that there is no evidence that additional steps involving an MP-8–substrate complex [5] or substrate-involved decompositon [6] are significant in this work.

We conclude by speculating on the nature of the intermediates involved in this reaction and their mode of interaction with the phenol. There is strong evidence that the initial reaction of H_2O_2 with MP-8,[3b,4–7] and MP-11 [3c] involves heterolytic cleavage of the peroxide to yield an oxy-iron species two equivalents above the resting catalyst. Apart from ourselves in this study, only Clore, in a paper [3c] dealing with the H_2O_2-induced oxidation and decomposition of MP-11 appears to have suggested that the initially-formed oxidized species can undergo internal oxidation reduction reactions. Based on Clore's scheme, the initially-formed Fe^{IV}=O-por$^{\bullet+}$ would transfer (in the absence of phenol) an electron from oxygen to the Fe. We propose an alternative, although perhaps more controversial, mechanism where the initially-formed intermediate (I) is a species, perhaps an Fe^{V}, which in the absence of reducing agent converts into Fe^{IV}=O-por$^{\bullet+}$ (I*). We cite a recent report by Van Wart [12] which details spectroscopic evidence for the formation of an oxoferryl-porphyrin cation radical (Fe^{IV}=O-por$^{\bullet+}$) from Ac-MP-8; however, the conditions under which it is generated appear similar to ours and we question whether this species might be an analogue of our I* rather than I. HRP-analogous one electron reduction of the porphyrin cation radical I* would lead to a compound II analogue; yet we believe that II* is not a HRP(II) analogue,

since its maximum absorbance is very close to that of MP-8 and compound II analogues tend to absorb at significantly longer wavelengths than their unoxidized forms.[12,14] We propose that I* (Fe^{IV}=O-por$^{\bullet+}$) is reduced, not by transfer of an electron to the porphyrin ring, but by transfer of an electron to the iron to give an Fe^{III} porphyrin cation radical (this may or may not be accompanied by transfer of a proton to the oxygen). This is in contrast to the accepted mechanism of interaction of HRP(I), although a recent study of cytochrome c peroxidase has shown that the intermediate oxy-ferryl amino acid radical cation (compound I) is reduced by electron transfer to the iron prior to transfer to the amino acid radical cation.[1f] A different mode of substrate interaction with HRP and with MP-8 is not unreasonable. With HRP(I) it seems likely that oxidation of the substrate occurs at the edge of the porphyrin ring, close to the outer surface of the protein shell; the porphyrin cation radical nature of the HRP(I) is most appropriate in this respect (ref. 8*b* and refs. cited therein). With MP-8 intermediates the haem portion is more open to attack from other directions.

Concluding, we believe that studies of peroxidase models have over-emphasized the analogy with enzyme systems, particularly HRP, in postulating intermediate structure, and that the existence of other oxidizing intermediates and modes of interaction with organic substrate cannot be ignored.

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