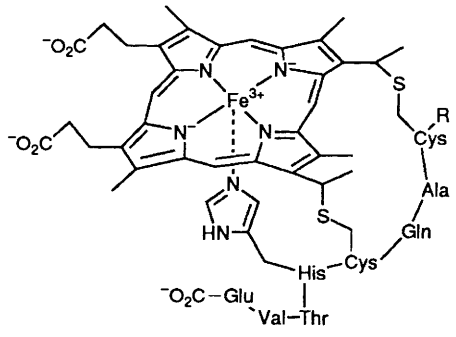
Peroxidatic Activity of Haem Octapeptide Complexes with Anilines, Naphthols and Phenols

Ian D. Cunningham,* John L. Bachelor and John M. Pratt
Department of Chemistry, University of Surrey, Guildford, UK GU2 5XH

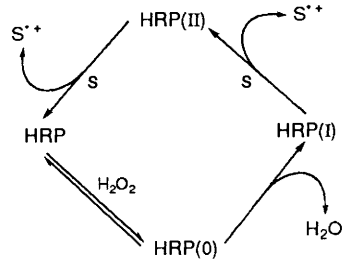
Complexation of the title substrates to microperoxidase-8 (MP-8) results in inhibition of the normal MP-8 catalytic pathway, but introduces an alternative pathway, probably leading to the same oxidised intermediate. This pathway must involve a different type of H_2O_2–MP-8 interaction to that proposed for HRP, and a preliminary interaction of peroxide with the porphyrin ring, rather than the Fe, of the MP-8:substrate complex is suggested for this pathway.

The haem octa- and undeca-peptides (microperoxidases), MP-8[1] (**1**) and MP-11[2] (**2**), respectively, along with derivatives such as *N*-acetyl-MP-8 (AcMP-8)[3] (**3**) have been shown to be effective oxidation[1-3] and oxygenation[4] catalysts. They have been used as models for P-450[4b,5] and, more commonly, the



1 R = NH_3^+
2 R = Lys-Gln-Val-NH_3^+
3 R = NH—Ac

peroxidatic enzymes typified by horseradish peroxidase (HRP) and their relevance to these enzymes has been dealt with at some length.[1c,6,7] They are generally assumed to involve a catalytic cycle analogous to that of HRP shown in its simplest form in Scheme 1, where I and II are species two and one oxidising



Scheme 1

equivalents, respectively, above the resting catalyst. In addition, Van Wart has proposed a compound 0, equivalent to or formed from an initial complex between catalyst and H_2O_2 or HO_2^-, which is formed prior to I in reactions involving HRP[8] and AcMP-8.[3] In recent publications, however, we have shown that the catalytic cycle for MP-8 is much more complex than that for HRP, and have proposed the existence of a pathway by which interaction of an oxidised intermediate of the catalyst with organic substrate leads to deactivation and ultimately destruction of the MP-8.[1b] Furthermore, we have shown that there exists an alternative catalytic cycle, and have questioned the assumption that the microperoxidase catalytic cycle involves exact analogues of the HRP compound I and II.[1a] Haem-based enzymes can be grouped into three types according to their biological function; catalysis of oxygen transfer (cytochrome P-450), redox reactions (peroxidases) or electron transport (cytochrome b_5).[9] An important structural factor in the last type is the di-axial coordination to the haem iron, which appears to suppress redox catalysis and/or enhance electron transport. Although one of us has recently published the results of a comprehensive investigation of the coordination of amines to MP-8,[10] little is known about the effect of such complexation on peroxidatic behaviour. Adams has examined the effect of one compound, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS)[7] and has proposed an MP-8:ABTS complex which is capable of showing peroxidatic activity. During our earlier work[1b] we noted a similar effect when using a range of phenol, aniline and naphthol substrates; we subsequently examined this effect in more detail and we report our findings in this paper.

Experimental

Materials.—All materials were as used previously.[1] AcMP-8 was prepared according to the method of Van Wart.[3]

Kinetics.—All reactions were carried out in 0.1 mol dm^{-3} phosphate buffer, pH 7.0, at 25 °C, in thermostatically controlled cuvettes. In general, reaction solutions were prepared by injection of a suitable volume of stock substrate solution(s), followed by stock MP-8 (or AcMP-8) solution, into a measured volume of buffer. Reactions were initiated by injection of a suitable volume of stock aqueous H_2O_2 solution, and were monitored by following the change in absorbance (A) due to product formation at a suitable wavelength. Initial rates were calculated from the absorbance change during the first 5% of the reaction and standard deviations over several determinations of $dA/dt_{t=0}$ were < ±8%. The term $\Delta A/[H_2O_2]_{consumed}$ which is used to derive $d[H_2O_2]/dt$ from dA/dt for each substrate was determined in earlier work.[1b]

Results

Initial Rate vs. *Substrate Concentration.*—The variation of the initial rate of the MP-8-catalysed H_2O_2-oxidation of substrate (S) with substrate concentration ([S]) at fixed concentrations of H_2O_2 and MP-8 was determined for 2-methoxyphenol, 4-methoxyphenol, ferrocyanide, 4-methoxynaphth-1-ol, 4-methoxyaniline and 2,4-dimethoxyaniline. The results are given in Tables 1–4; included are results for 2-methoxyphenol using AcMP-8. The initial rate of loss of H_2O_2, $-d[H_2O_2]/dt$, is derived from dA/dt by dividing by the

Table 1 Variation of the initial rate of the MP-8-catalysed H_2O_2 oxidation of 2-methoxyphenol, 4-methoxyphenol and ferrocyanide[a]

Substrate	[Substrate]/ 10^{-4} mol dm^{-3}	$dA/dt_{t=0}$/ 10^{-4} s^{-1}	λ/nm	$-d[H_2O_2]/dt_{t=0}$/ 10^{-7} mol dm^{-3} s^{-1}
2-Methoxyphenol	0.10–110[b]	16.7 ± 1.3	470	3.21 ± 0.25
2-Methoxyphenol[c]	0.10–100[b]	10.7 ± 0.9	470	2.06 ± 0.17
4-Methoxyphenol	0.10–1000	3.79–10.84	315	1.40–4.01
Ferrocyanide	0.54–107	6.17–7.13	420	2.80–3.24

[a] $[H_2O_2]_0 = 1.02 \times 10^{-4}$, $[MP\text{-}8]_0 = 4.0 \times 10^{-7}$ mol dm^{-3}. [b] See text for reaction at higher concentrations. [c] Reaction catalysed by *N*-acetyl-MP-8 (6.0×10^{-7} mol dm^{-3}) in 20% methanol–0.1 mol dm^{-3} phosphate buffer pH 7.0.

Table 2 Variation of the initial rate of the MP-8-catalysed H_2O_2 oxidation of 4-methoxynaphth-1-ol[a]

[4-Methoxynaphth-1-ol]/ 10^{-4} mol dm^{-3}	$dA/dt_{t=0}$ at 620 nm/10^{-4} s^{-1}	$-d[H_2O_2]/dt_{t=0}$/ 10^{-7} mol dm^{-3} s^{-1}
0.09	12.07	2.84
0.51	8.87	2.09
1.02	8.30	1.95
2.01	6.78	1.60
4.93	5.17	1.22

[a] $[H_2O_2]_0 = 1.02 \times 10^{-4}$, $[MP\text{-}8]_0 = 4.0 \times 10^{-7}$ mol dm^{-3}.

Table 3 Variation of the initial rate of the MP-8-catalysed H_2O_2 oxidation of 4-methoxyaniline[a]

[4-Methoxyaniline]/ 10^{-4} mol dm^{-3}	$dA/dt_{t=0}$ at 494 nm/ 10^{-4} s^{-1}	$-d[H_2O_2]/dt_{t=0}$/ 10^{-7} mol dm^{-3} s^{-1}
0.14	10.35	2.03
0.49	9.51	1.86
0.95	7.92	1.55
2.05	7.10	1.39
5.05	4.67	0.92
10.10	3.44	0.67

[a] $[H_2O_2]_0 = 1.13 \times 10^{-4}$, $[MP\text{-}8]_0 = 3.0 \times 10^{-7}$ mol dm^{-3}.

Table 4 Variation of the initial rate of the MP-8-catalysed H_2O_2 oxidation of 2,4-dimethoxyaniline[a]

[2,4-Dimethoxyaniline]/ 10^{-4} mol dm^{-3}	$dA/dt_{t=0}$ at 500 nm/10^{-4} s^{-1}	$-d[H_2O_2]/dt_{t=0}$/ 10^{-7} mol dm^{-3} s^{-1}
0.09	10.49	2.10
0.52	10.04	2.01
1.03	8.00	1.60
2.03	6.49	1.30
5.15	3.34	0.67
10.31	2.58	0.52

[a] $[H_2O_2]_0 = 1.02 \times 10^{-4}$, $[MP\text{-}8]_0 = 3.0 \times 10^{-7}$ mol dm^{-3}.

Table 5 Variation of the initial rate of the MP-8-catalysed H_2O_2 oxidation of 4-methoxynaphth-1-ol along with the extent of reaction[a]

[4-Methoxynaphth-1-ol]/ 10^{-4} mol dm^{-3}	$dA/dt_{t=0}$ at 620 nm/10^{-4} s^{-1}	A_{inf} at 620 nm
0.50	3.88	0.13
1.03	2.93	0.14
1.53	2.88	0.16
2.02	2.44	0.15

[a] $[H_2O_2]_0 = 0.49 \times 10^{-4}$, $[MP\text{-}8]_0 = 3.4 \times 10^{-7}$ mol dm^{-3}.

$\Delta A/[H_2O_2]_{consumed}$ term appropriate to the substrate being oxidised and the wavelength at which the reaction was monitored; this term, its method of determination and its meaning have been dealt with in a previous paper.[1b] For each substrate the lower concentration limit was dictated by the requirement that ΔA be sufficient to allow determination of the initial rate, while the upper limit was dictated by solubility of the substrate or the intensity of substrate absorbance in the UV spectrum.

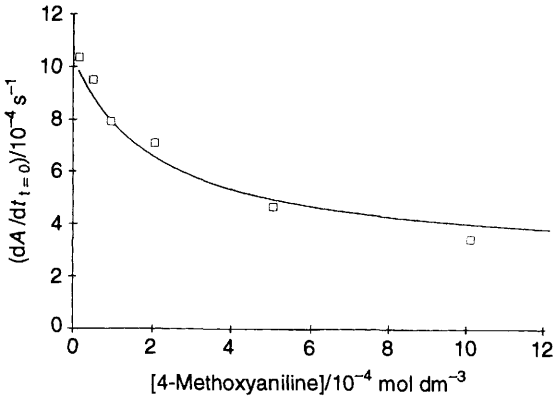


Fig. 1 Variation of the initial rate of the MP-8-catalysed H_2O_2 oxidation of 4-methoxyaniline with 4-methoxyaniline concentration: λ = 494 nm, $[H_2O_2]_0 = 1.13 \times 10^{-4}$, $[MP\text{-}8]_0 = 3.0 \times 10^{-7}$ mol dm^{-3}

In earlier work we examined the variation of rate with substrate concentration over a limited range at the lower limit and found that the rate was more or less invariant for many of the above substrates.[1b] It is clear that over a wider concentration range significant variation occurs for some. In particular, a clear decrease in rate with increasing substrate concentration is observed for 4-methoxynaphthol, 4-methoxyaniline and 2,4-dimethoxyaniline; this is illustrated for 4-methoxyaniline in Fig. 1. In contrast the rate for 2-methoxyphenol (using MP-8 or AcMP-8) is almost constant, a slight increase in rate is observed for ferrocyanide, while 4-methoxyphenol shows a slightly greater increase which levels off slightly. In all cases (except for 2-methoxyphenol at very high concentration, *vide infra*) the decrease affects only the rate, while the amount of product eventually formed (as determined by monitoring ΔA at a suitable wavelength) does not vary significantly; results for 4-methoxynaphth-1-ol are shown in Table 5.

Effect of Substrate Concentration on MP-8 Spectrum.—Amines are known to complex MP-8 with resultant shifts of 6–9 nm in the position of the Soret band.[10] Treatment of 0.6×10^{-6} mol dm^{-3} MP-8 with 4-methoxyaniline leads to a shift in the MP-8 Soret band from 398 nm at [4-methoxyaniline] = 0.1×10^{-3} mol dm^{-3} to 408 nm at 1.0×10^{-3} mol dm^{-3} with no further shift above this concentration; this trend roughly parallels that of the inhibition curve (Fig. 1). Over a similar concentration range no shift in the MP-8 Soret band was observed for 4-methoxynaphth-1-ol, despite the observed inhibition.

Reactions with Two Substrates.—The reaction was carried out using ferrocyanide (a substrate showing little variation in rate) along with 4-methoxyaniline (at a concentration where this substrate shows some inhibition) and was monitored at 420

Table 6 MP-8 catalysed H_2O_2 oxidations of substrate mixtures[a]

Substrate/ 10^{-4} mol dm^{-3}	Substrate/ 10^{-4} mol dm^{-3}	$dA/dt_{t=0}/10^{-4}$ s^{-1} (λ/nm)	$-d[H_2O_2]/dt_{t=0}$/ 10^{-7} mol dm^{-3} s^{-1}
Ferrocyanide	4-Methoxyaniline		
1.01	0.00	6.05 (420)	2.75
0.00	10.20	4.17 (494)	0.82
1.01	10.20	1.37 (420)	0.62–0.44
2,4-Dimethoxyaniline	4-Methoxyaniline		
0.92	0.00	9.40 (500)	1.88
0.00	9.98	3.14 (494)	0.62
0.92	9.98	3.01 (500)	0.60
4-Methoxynaphthol	2-Methoxyphenol		
1.02	0.00	10.2 (620)[b]	2.40[b]
0.00	>0.100	25.1 (470)[c]	4.82[c]
1.02	509.00	20.8 (620)[b]	4.89[b]

[a] $[H_2O_2]_0 = 1.03 \times 10^{-4}$, $[MP\text{-}8]_0 = 3.0 \times 10^{-7}$ mol dm^{-3}. [b] $[MP\text{-}8]_0 = 6.0 \times 10^{-7}$ mol dm^{-3}. [c] = Calculated from values obtained at $[MP\text{-}8]_0 = 4.0 \times 10^{-7}$ mol dm^{-3}.

nm. The dA/dt measured at this wavelength is the sum of the absorbance changes due to oxidation of both substrates (although it appeared that it was mostly due to ferricyanide), and Table 6 shows the two extreme values of $d[H_2O_2]/dt_{t=0}$ calculated using $\Delta A/[H_2O_2]_{consumed}$ appropriate to ferrocyanide oxidation only, or the value for 4-methoxyaniline oxidation only [determined as per ref. 1(*b*)]. It is clear that the high level of 4-methoxyaniline has reduced the overall rate from the higher 'ferrocyanide' level to that of 4-methoxyaniline.

A similar result was obtained with a mixture of 2,4-dimethoxyaniline and 4-methoxyaniline (Table 6). For these the 'oxidation product' λ_{max} values (500 and 494 nm), and the $\Delta A/[H_2O_2]_{consumed}$ values (5000 and 5100)[1b] are similar and monitoring at 494–500 nm allows a reliable value of $d[H_2O_2]/dt_{t=0}$ to be derived; again the high concentration of one substrate has inhibited the overall reaction.

The oxidation products of 4-methoxynaphth-1-ol and 2-methoxyphenol show very different maxima, at 620 nm (and a minimum around 470 nm) and 470 nm, respectively. When subjected to MP-8-catalysed oxidation, a mixture of these two substrates gave UV–VIS spectral changes due only to 4-methoxynaphth-1-ol oxidation product until, on consumption of this substrate, the spectrum of 2-methoxyphenol oxidation product began to appear; this was seen even for a 500-fold excess of 2-methoxyphenol. Unlike the two reactions dealt with above, in this case the rate of reaction was at the higher level appropriate to the 2-methoxyphenol (non-inhibiting) substrate (Table 6).

Reaction at Higher Levels of 2-Methoxyphenol.—The initial rate for 2-methoxyphenol ('guaiacol') was found to be more or less independent over a wide range of substrate concentration (*vide supra*). However, at very high concentrations, above 0.01 mol dm^{-3}, with deficient H_2O_2, the absorbance at 470 nm was found to rise to a maximum before dropping. This is typical of two consecutive reactions where 'tetraguaiacol' (the complex and poorly characterised 2-methoxyphenol reaction product) formed in the first reaction is destroyed in the second. It has been proposed that 'tetraguaiacol' can undergo as yet unspecified reactions with both light and unreacted 2-methoxyphenol.[11] We found evidence for the reaction with excess 2-methoxyphenol; when an amount of 'tetraguaiacol' equivalent to 1.02×10^{-4} mol dm^{-3} of H_2O_2 consumed was formed, subsequent addition of further 2-methoxyphenol to 0.091 mol dm^{-3} caused a drop in the 'tetraguaiacol' absorbance with an observed pseudo-first order rate constant of 0.017 s^{-1}. That this behaviour is not catalyst related is shown by the fact that a similar effect was observed using AcMP-8 or the HRP enzyme.

Discussion

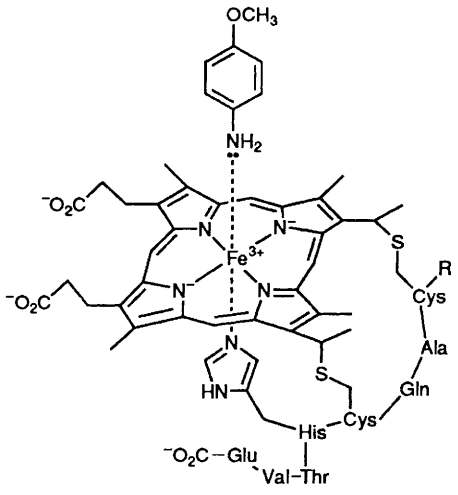
All studies of the peroxidatic reactivity of MP-8 have shown that the rate-limiting step is the initial interaction of the catalyst with H_2O_2 to form the highly reactive oxidised intermediate. Therefore, the substrate-induced decrease in the initial rate for 4-methoxyaniline, 2,4-dimethoxyaniline and 4-methoxynaphthol indicates either a reversible competitive inhibition of the unoxidised catalyst MP-8, or an irreversible inhibition or deactivation. Considering the A_{inf} values in Table 5 and the value for $\Delta A/[H_2O_2]_{consumed}$ (4250),[1b] it can be seen that the consumption of H_2O_2 is incomplete for all entries; this is due to destruction of the catalyst subsequent to the rate limiting interaction with H_2O_2 as explained in ref. 1(*b*). Any additional irreversible deactivation of the catalyst would further reduce consumption of H_2O_2 and be reflected in decreasing product yield. It is clear that no enhancement of this destruction occurs for 4-methoxynaphthol, showing that the inhibition is reversible. Indeed, no significant changes in 'product yield' (as determined by UV–VIS absorbance changes) were noted for any of the substrates investigated (with exceptions mentioned below) showing the generality of this effect. In recent work[10] it has been shown that coordination of aniline occurs in 20% aqueous methanol with an equilibrium constant (for substitution of coordinated H_2O by aniline) given by $\log(K/dm^3\ mol^{-1}) = 2.67$, and a shift in the MP-8 Soret band from 397 to 406 nm. The fact that similar behaviour is observed for 4-methoxyaniline in this work supports inhibition due to coordination of the inhibitor to the MP-8 as in **4**.

Despite the above, peroxidatic activity does not fall to zero for the three inhibiting substrates, even at high concentration. This suggests that complexes such as **4** also act as catalysts with an activity within an order of magnitude of the 'free' catalyst. The proposed mechanism is outlined in Scheme 2, and a kinetic analysis relates the initial rate of reaction $v = -d[H_2O_2]/dt_{t=0}$ to eqn. (1) where S is the organic substrate and the other terms

$$v = \frac{(k + k'K[S])[H_2O_2]_0[MP\text{-}8]_0}{1 + K[S]} \quad (1)$$

are given in Scheme 2. At very low [S] v will approach $v_0 = k[H_2O_2]_0[MP\text{-}8]_0$ and become independent of substrate concentration, as is observed,[1b] while at high [S] v will approach $v_S = k'[H_2O_2]_0[MP\text{-}8]_0$ and reflect catalysis (k') by the MP:S complex only. The inhibition constant K is obtained by rearranging eqn. (1) to give eqn. (2). If the rates at the lowest

$$K = \frac{(v_0 - v)}{(v - v_S)[S]} \quad (2)$$



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[S] and at highest [S] are taken as approximations to v_0 and v_S, respectively, values of log (K/dm^3 mol^{-1}) for 4-methoxynaphth-1-ol, 4-methoxyaniline and 2,4-dimethoxyaniline are calculated to be 4.16 ± 0.08, 3.68 ± 0.21 and 3.7 ± 0.5, respectively; values consistent with binding constants found for complexing of similar compounds to MP-8.[10]

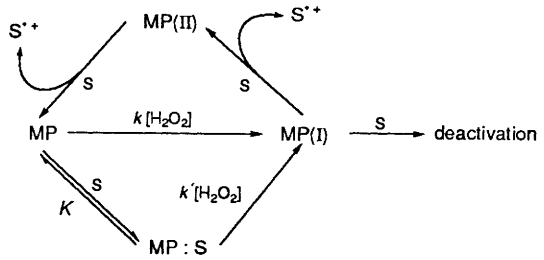
In the mixed 4-methoxyaniline/2,4-dimethoxyaniline experiment the fact that the reaction proceeds *via* the complex which, based on the above values of K and the conditions given in Table 6, would be dominant, is consistent with Scheme 2.

In the case of ferrocyanide no inhibition is observed unless 4-methoxyaniline is present (Table 6). This suggests that ferrocyanide (and possibly the aniline also) reacts with an MP-8 intermediate formed *via* the MP-8 : aniline complex, with MP-8 to ferrocyanide complexation being unimportant under these conditions.

No inhibition is observed for the two phenol substrates; either these substrates do not complex to the MP-8, or the catalytic activity of the MP : S complex is comparable to that of MP-8. In the mixed 4-methoxynaphth-1-ol/2-methoxyphenol experiment (although results from such experiments must be treated with some caution) the results of Table 6 show that the reaction is *not* proceeding *via* the less reactive MP-8 : naphthol complex which would be formed by 'free' MP-8 in the presence of 4-methoxynaphth-1-ol alone, but *via* a catalyst formed in preference to this and which is of similar reactivity to the 'free' MP-8 with the most likely candidate being an MP-8 : 2-methoxyphenol complex. The results also seem to show that the intermediate oxidised catalyst oxidises 4-methoxynaphth-1-ol in preference to 2-methoxyphenol, although we cannot be sure than an initially-formed product derived from the phenol does not oxidise the naphthol.

As an aside we point out the relative instability of the so-called 'tetraguaiacol' oxidation product of 2-methoxyphenol, commonly used in peroxidase enzyme assays, at high concentrations of the unoxidised substrate.

Are the intermediates formed by oxidation of the MP : S complexes the same as MP(I)? Considering the RCE (defined earlier[1b] as A_{inf}/4250[MP-8]$_{deactivated}$) as reflecting the balance between the oxidised MP being deactivated or continuing the cycle of product production (reflected in A_{inf}) the constancy of A_{inf} (Table 5) over the region of 'free' MP-8 catalyst (substrate-independent kinetics) and the MP : S catalyst region suggests that the intermediate is the same in both regions; a similar argument applies to 4-methoxyaniline [Table 3 and ref. 1(*b*)].



Scheme 2

The apparent preferential oxidation of the non-complexing substrate in the mixed ferrocyanide/4-methoxyaniline and 2-methoxyphenol/4-methoxynaphthol experiments is difficult to reconcile with a highly oxidised intermediate still bearing the complexed substrate.

The fact that hexacoordinated iron, MP : S, can be oxidised directly to MP(I) without vacating a ligand binding site has implications for the initial H_2O_2/MP-8 interaction. An initial HO_2^- complex (compound 0) as proposed for the HRP enzyme and AcMP-8 by Van Wart, while consistent in part for MP-8 (the partial inhibition), is not compatible with oxidation of the MP : S complex to MP(I).* One possibility is reaction of H_2O_2 or HO_2^- to give an isoporphyrin,[8a] while substrate remains bound to iron, with further reaction resulting in loss of the coordinating substrate and formation of MP(I); the relatively[9] open porphyrin ring of the microperoxidases **1**–**3** (even with a complexed substrate) may facilitate this.

* Although this intermediate is two oxidizing equivalents above the resting catalyst, we are reluctant to call it compound I in the case of MP-8, since evidence has been presented[1a] that questions whether it is an exact analogue of the HRP compound I.

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