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ORIGINAL ARTICLE

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Stopped-flow kinetic study of the H_2O_2 oxidation of substrates catalyzed by microperoxidase-8

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Abstract We have studied the oxidation of microperoxidase-8 (MP-8) by H_2O_2 and the subsequent reaction of the intermediates with substrate by stopped-flow experiments. Oxidation of MP-8 by H_2O_2 gives two intermediates, **I** and **II**. The observed rate constant for the formation of **I** is linearly dependent on $[H_2O_2]$ and exhibits a bell-shaped dependence on pH with pK_a values of 8.90 and 10.60, which are attributed to the deprotonation of MP-bound H_2O_2 and H_2O, respectively. The observed rate constant for the conversion of **I** to **II** is independent of $[H_2O_2]$, but increases sharply at pH > 9.0. The predominant forms of the intermediate at pH 7.0 and 10.7 are **I** and **II**, respectively. Addition of substrate to the intermediates at pH 9.0 gives rise to three distinct stages, corresponding to the three steps (in decreasing order of rate): **I**→**II***, **II**→MP, and **II***→MP. The rates of these steps are all linearly dependent on the substrate concentration and each individual rate constant has been determined. Substrate reactivity at pH 10.7 covers over two orders of magnitude, ranging from 1.36×10^7 M^{-1} s^{-1} for 1-naphthol to 4.03×10^4 M^{-1} s^{-1} for ferrocyanide. The substrate reactivity is linearly correlated with its reduction potential, indicating that an electron transfer process is involved in the rate-limiting step.

Keywords Microperoxidase-8 · Kinetic study · Substrate reactivity

Abbreviations *HRP*: horseradish peroxidase · *MP-8*: microperoxidase-8 · *OMP*: *o*-methoxyphenol

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Introduction

Microperoxidase-8 (MP-8) is a heme-containing octapeptide obtained from proteolytic digestion of horse heart cytochrome *c* with pepsin and trypsin [1]. MP-8 retains residues 14–21 (^{14}Cys-Ala-Gln-Cys-His-Thr-Val-^{21}Glu) of the cytochrome *c* protein. The heme *c* remains linked to the octapeptide through two thioether bonds, with ^{18}His serving as a fixed proximal ligand. With the presence of the proximal ligand and the heme *c* moiety, MP-8 and other related MPs serve as excellent model compounds for hemoproteins. MPs with oligopeptides of 5, 6, 8, 9, 11, 17, and 50 amino acids have been described [2, 3,!4, 5, 6]. Lack of a specific substrate binding site enables these minienzymes to catalyze a wide variety of reactions, including peroxidase-like reactions [7, 8, 9, 10], cytochrome *P*-450-like reactions [4, 5], and the alkoxylating dehalogenation of halophenol [11, 12].

The general mechanism of peroxidases, including MP, involves the oxidation of the enzyme by hydrogen peroxide to form intermediate **I**, which is two oxidizing equivalents above the resting enzyme. Subsequent one-electron reduction by a substrate yields another intermediate **II** (with one equivalent above the resting enzyme), which in turn can undergo one-electron reduction by the substrate to regenerate the native enzyme [13, 14]. The evidence for the presence of **I** and **II** for MP is mainly kinetic. Most kinetic studies have been carried out at high concentrations of H_2O_2, which will lead to a significant degradation of MP, the occurrence of multiple turnovers, and the formation of a mixture of products. These will complicate the interpretation of the kinetic data. Moreover, under the multiple turnover conditions, the differences in substrate reactivities will not be differentiated if the rate for the formation of the intermediates is much slower than that of the subsequent reaction of the intermediates with substrate. In addition, the reactivity of each intermediate of MP-8 against various substrates has never been reported. In this

paper, we have used the stopped-flow technique to study the kinetics of MP-catalyzed reactions. We have investigated the formation of the intermediates and the subsequent regeneration of MP-8 separately. Oxidation of MP-8 by H_2O_2 results in the formation of either **I** or **II** or both, depending on the pH used. Upon addition of substrate to the intermediates under single turnover conditions, three distinct phases were observed during the regeneration of MP-8. We have proposed a mechanism to describe the observed kinetic results and have determined the rate constants for each individual step. We have also measured the substrate reactivity of various species against the intermediates. The substrate reactivity varies over two orders of magnitude and is correlated with the reduction potential.

Materials and methods

Materials

Horse heart cyctochrome *c*, tris(hydroxymethyl)aminomethane (Tris), pepsin, and trypsin were purchased from Sigma (USA). 1-Naphthol, nitrobenzene, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), aniline, and *o*-methoxyphenol (OMP) were obtained from Aldrich (USA). *p*-Anisidine, *p*-methoxyphenol, sodium phosphate, sodium carbonate, and homovanillic acid were from Acros (Belgium). Sodium chloride and phenol were from Merck (Germany) and potassium ferrocyanide from Kanto (Japan). All the reagents were of the highest grade of purity. MP-8 was prepared from the proteolytic digestion of cytochrome *c* with pepsin and trypsin according to the procedure in the literature [1].

Stopped-flow measurements

The stopped-flow experiments were performed on a Hitech SF-61 stopped-flow instrument (Hitech Scientific, UK) equipped with a sample handling unit (SHU-61), a dual detection accessory (OPT-680), a photodiode array assembly (MG-6010), a 75-W xenon lamp, and a circulating water bath for temperature control. It can be operated in either the absorption (time resolved or fixed wavelength) or the fluorescence mode. For measuring the rates of formation of the intermediates, changes in absorbance at 398 or 415 nm were monitored continuously after the mixing of MP-8 and H_2O_2, which were stored in two separate syringes in the stopped-flow equipment. For measuring the rate of the MP regeneration process, MP-8 and H_2O_2 were premixed in one syringe with the substrate stored in the other syringe. After an appropriate delay time (normally 10–100 s, depending on the nature and concentration of substrate used), the two solutions were mixed and the changes in absorbance (or fluorescence) were recorded continuously. The built-in software of the stopped-flow instrument allows one- or two-exponential fitting of the traces. All the stopped-flow experiments were carried out at 25 °C. In different pH ranges, the following buffers (10 mM each) were used and adjusted with HCl or NaOH to the desired pH: sodium phosphate (pH 7–8.3), Tris (pH 8.3–9.5), sodium carbonate (pH 9.5–12). For each kinetic analysis, at least 10 replicate measurements were performed and the relative standard deviation for the obtained rate constants was 3–4%.

Measurement of oxidation potential

The oxidation potential of the substrate was determined by a Bioanalytic System (Ind., USA) potentiostat, model CV-50V. Square wave voltametry was conducted with the use of a three-electrode cell in which a BAS glassy carbon electrode was used as the working electrode. The auxiliary compartment contained a platinum wire separated by a medium-size glass frit. All cell potentials were taken with the use of a Ag[braceexAgCl[braceexKCl (sat.) reference electrode.

Results

Formation of intermediates

Figure 1 shows the stopped-flow time-resolved difference absorption spectra for the oxidation of MP-8 by H_2O_2 at pH 7.0, 9.0, and 10.7. At pH 7.0, the

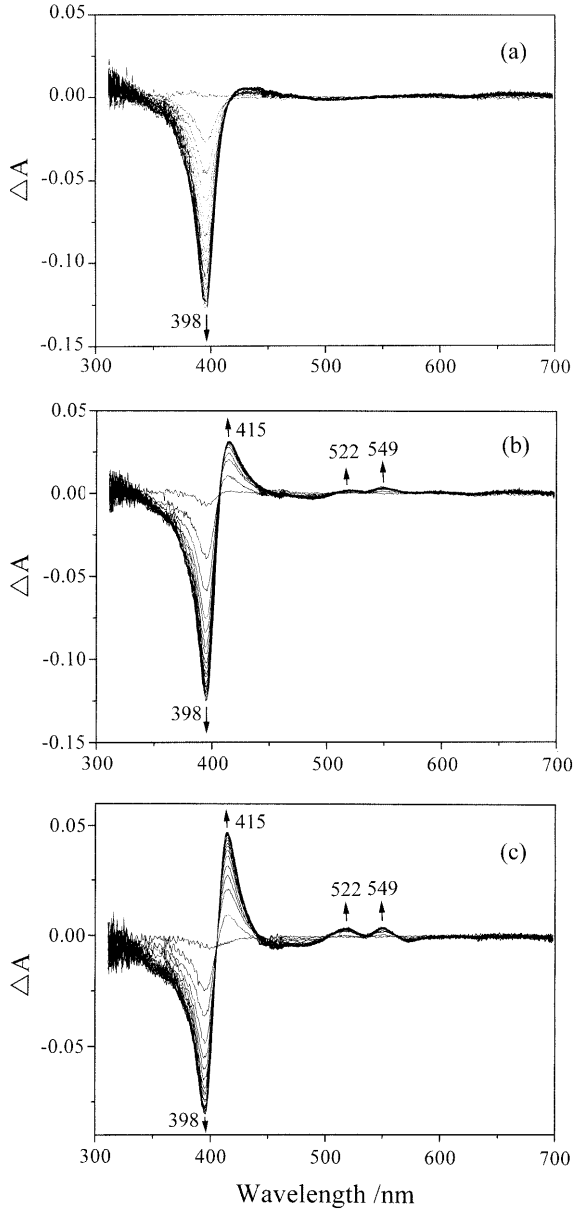


Fig. 1 Stopped-flow time-resolved difference absorption spectra for the oxidation of MP-8 (1.4 μM) by H_2O_2 (14.7 μM) at pH **a** 7.0, **b** 9.0, and **c** 10.7. The time interval per trace is 5 s for **a** and 0.25 s for **b** and **c**

absorbance of the Soret band decreases (with essentially no shift in peak position) as the reaction proceeds (Fig. 1a), indicating the formation of **I**. Similar changes in absorbance of the Soret band have been reported for horseradish peroxidase (HRP) at low pH [15]. At pH 10.7, the difference spectra (Fig. 1c) clearly show the disappearance of the peak at 398 nm and the appearance of a new peak at 415 nm and a doublet at 522 and 549 nm with sharp isosbestic points at 404, 448, and 499 nm. These spectral features of **II** are similar to the characteristics of **II** of HRP [15, 16]. The conversion of **I** to **II** is accomplished by the abstraction of an electron from some exogenous reducing agent in the solution. At pH 9.0, the decrease in absorbance at 398 nm is much greater than that at 415 nm (Fig. 1b), suggesting the simultaneous presence of both **I** (major component) and **II**. Quantitative formation of **I** at pH 7.0 and **II** at pH 10.7 was obtained when several folds of H_2O_2 were added to MP-8, and their absorption spectra are shown in Fig. 2. The changes in molar absorptivities are: $\Delta\epsilon_{398}(\text{MP}\rightarrow\textbf{I}) = 1.29\times10^5$, $\Delta\epsilon_{398}(\text{MP}\rightarrow\textbf{II}) = 3.76\times10^4$, and $\Delta\epsilon_{415}(\text{MP}\rightarrow\textbf{II}) = 3.86\times10^4$ M^{-1} cm^{-1}. These values will be used to estimate the concentrations of **I** and **II** during the reaction.

Figure 3 shows the time courses for the changes in absorbance for the reaction of MP-8 and H_2O_2 at pH 7.0, 9.0, and 10.7. The stopped-flow trace at pH 9.0 (Fig. 3b) is biphasic, which can be described by the following process:

$$\text{MP-8} \xrightarrow{k_1[H_2O_2]} I \xrightarrow{k_2} II \qquad (1)$$

The time variation of the absorbance (A) can be deduced by introducing standard expressions for the time dependence of [MP-8], [**I**], and [**II**] for a two-step series reaction and is given by:

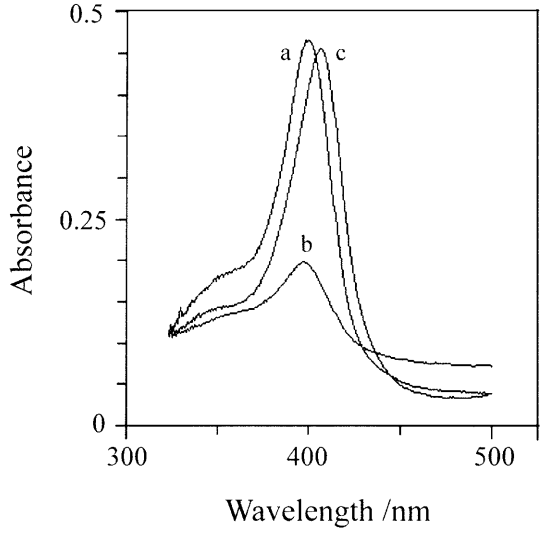


Fig. 2 Absorption spectra of *a* MP-8, *b* **I**, and *c* **II**. The spectrum of MP-8 (4 μM) was recorded at pH 7.0. The spectra of **I** and **II** were obtained by treating MP-8 (4 μM) with a 10 molar excess of H_2O_2 at pH 7.0 and 10.7, respectively

$$A = a + b\exp(-k_1[H_2O_2]t) + c\exp(-k_2t) \qquad (2)$$

where a, b, and c are constants. If the concentration of H_2O_2 is much greater than that of MP-8, $[H_2O_2]$ can be assumed to be nearly unchanged during the reaction. Fitting of the stopped-flow traces to a two-exponential equation yields two observed rate constants ($k_{1,obs}$ and $k_{2,obs}$). The dependence of $k_{1,obs}$ and $k_{2,obs}$ on the concentration of H_2O_2 is shown in Fig. 4. The results show that $k_{1,obs}$ is linearly dependent on $[H_2O_2]$, while $k_{2,obs}$ is independent of $[H_2O_2]$, in accord with Eq. 2. The values of k_1 and k_2 are determined to be 7.2×10^4 M^{-1} s^{-1} and 0.043 s^{-1}, respectively.

At pH 7.0, only **I** was observed (Fig. 1a), indicating that the conversion of **I** to **II** is extremely slow. Under this circumstance, the time variation of the absorbance of the system is given by:

$$A = a + b\exp(-k_1[H_2O_2]t) \qquad (3)$$

The trace in Fig. 3a is single exponential, in accord with Eq. 3. The obtained value of k_1 at pH 7.0 is 2.9×10^3 M^{-1} s^{-1}. At pH 10.7, the time course for the change in absorbance at 415 nm (Fig. 3c) is also single expo-

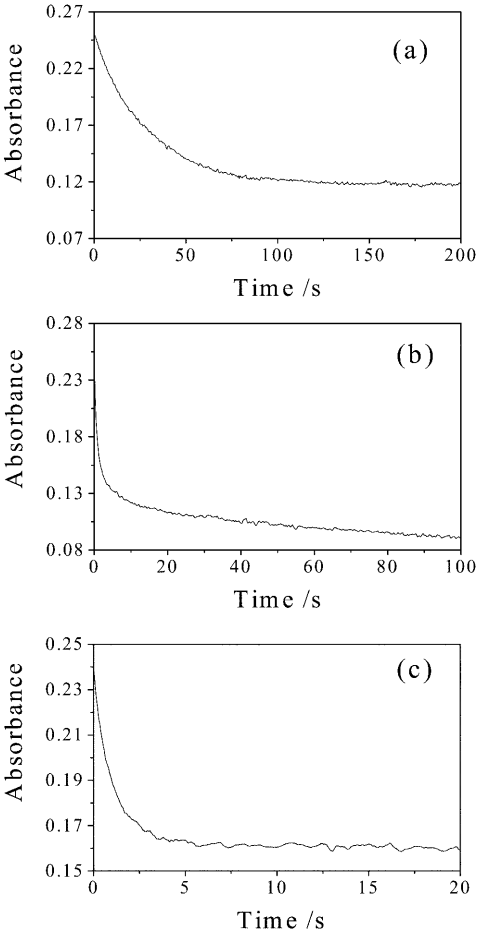


Fig. 3 Time courses for the changes in absorbance at 398 nm for the oxidation of MP-8 (1.4 μM) by H_2O_2 (14.7 μM) at pH **a** 7.0, **b** 9.0, and **c** 10.7

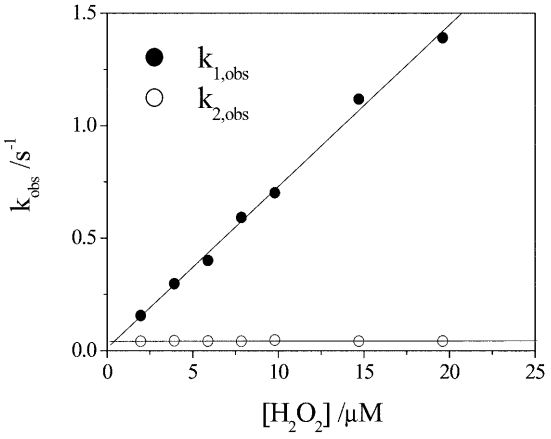


Fig. 4 Dependence of (●) $k_{1,obs}$ and (○) $k_{2,obs}$ on the concentration of H_2O_2 for the oxidation of MP-8 (1.4 μM) by H_2O_2 at pH 9.0

nential. This requires that either the k_1 step in Eq. 1 is much faster than the k_2 step, or the reverse. Since no accumulation of **I** was observed, the latter possibility is favored. Under the condition that the k_1 step is rate determining, the time variation of the absorbance of the system is also given by Eq. 3. The rate constants obtained from the single-exponential fitting of the stopped-flow traces are linearly dependent on $[H_2O_2]$ (data not shown). The obtained value of k_1 is 7.8×10^4 M^{-1} s^{-1} at pH 10.7.

pH dependence of k_1 and k_2

The stopped-flow experiments for the oxidation of MP-8 by H_2O_2 have also been carried out at various pH values and the pH dependence of k_1 and k_2 are plotted in Fig. 5. The curve for k_1 is bell shaped, which gives two pK_a values of 8.90 and 10.60 by curve fitting. The curve for k_2 contains two linear portions with a rapid increase in k_2 occurring at above pH≈9.0.

Regeneration of MP-8 by the donor substrate

Addition of the substrate (OMP) to **II** at pH 10.7 leads to the regeneration of MP-8, as revealed by the time-resolved difference spectra in Fig. 6. The difference spectra clearly show the disappearance of the peaks at 415, 522, and 549 nm for **II** and the appearance of the peak at 398 nm for MP-8. To measure the substrate reactivity, the concentration of H_2O_2 used is always less than that of MP-8 to minimize the chance of multiple turnovers. The recovery of MP-8 is 90–95%, indicating that degradation of MP-8 is not significant. This process can be described by:

$$II \xrightarrow{k_3[S]} \text{MP-8} \qquad (4)$$

where [S] is the concentration of substrate, which is usually much greater than that of **II** and is assumed to be

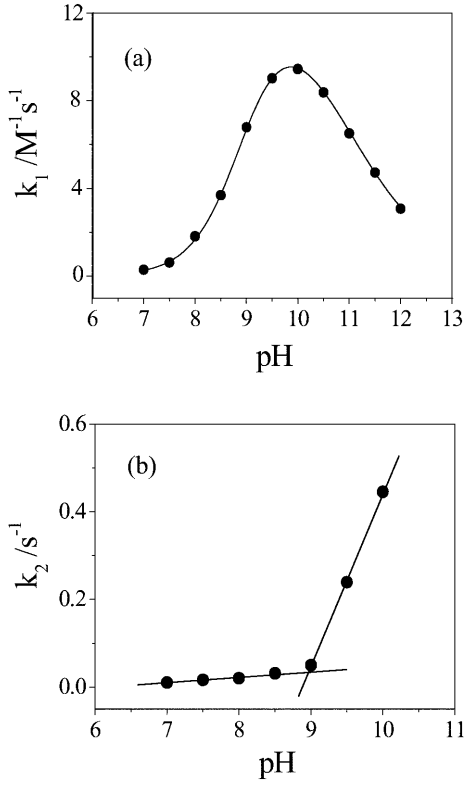


Fig. 5 pH dependence of **a** k_1 and **b** k_2 for the oxidation of MP-8 (1.4 μM) by H_2O_2 (14.7 μM). The values for the pK_a obtained from the pH dependence of k_1 are 8.90 and 10.60

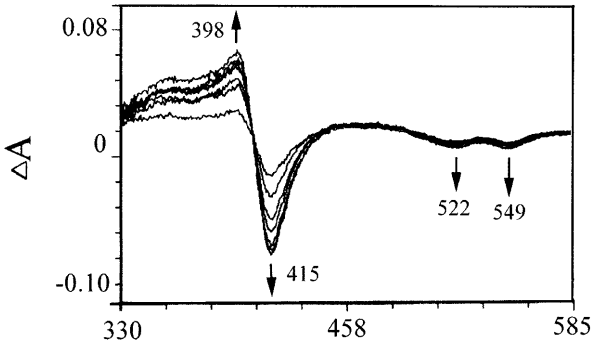


Fig. 6 Time-resolved difference absorption spectra (0.10 s/trace) for the reaction of **II** and OMP (10 μM) at pH 10.7. **II** was formed by premixing MP-8 (4.0 μM) and H_2O_2 (4.0 μM) in one syringe for 10 s and then mixed with OMP in the other syringe

unchanged during the reaction. The time variation of the absorbance is given by:

$$A = a + b\exp(-k_3[S]t) \qquad (5)$$

The time course for the change in absorbance at 415 nm is single exponential with the observed rate constant linearly dependent on [S] (data not shown). The obtained value of k_3 is 6.88×10^5 M^{-1} s^{-1}.

The time course for the regeneration of MP-8 by OMP at pH 7.0 is shown in Fig. 7a. The stopped-flow

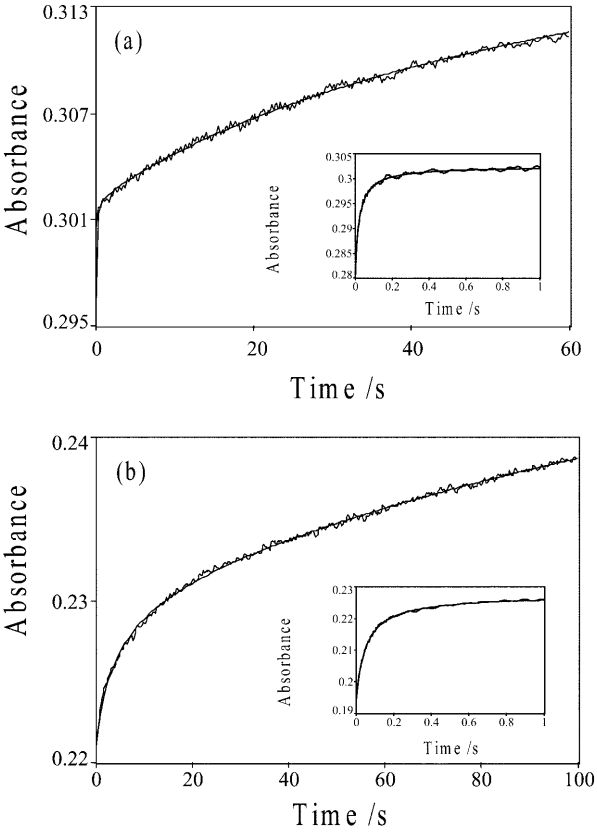
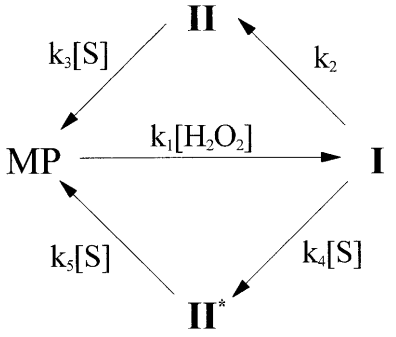


Fig. 7 Time courses for the changes in absorbance at 398 nm for the reaction of intermediates and OMP at pH **a** 7.0 and **b** 9.0. The intermediates were formed by mixing MP-8 (4.4 μM) and H_2O_2 (3.92 μM) for 90 s. [OMP] = 60 μM in **a**, 89 μM in **b**, and 0.89 μM in the *inset* to **b**. Two steps were observed in **a** and three steps in **b**

traces contain two stages: a very fast process, which is completed within 0.4 s (Fig. 7a, inset), and a slow process. The time-resolved absorption spectra reveal a slight red-shift (~2 nm) for the fast step followed by a small blue-shift for the slow step to regenerate the spectrum of MP-8 (data not shown). Good recovery of MP-8 (85–92%) was observed. The fast process thus corresponds to the conversion of **I** to an intermediate **II***, whose Soret band (~400 nm) is quite different from that of **II** (~408 nm) formed in the absence of the substrate. An intermediate analogous to **II*** has also been reported by Cunningham and Snare [17]. Combining all the kinetic results, the overall mechanism of the MP-catalyzed reaction can be described by Scheme 1. Thus the reaction between **I** and the substrate at pH 7.0 contains the k_4 and k_5 steps. Under the condition that $[S]_0 >> [\text{intermediate}]_0$, the time variation of the absorbance for the regeneration of MP-8 is given by:

$$A = a + b\exp(-k_4[S]_0 t) + c\exp(-k_5[S]_0 t) \quad (6)$$

Fitting of the traces in Fig. 7a to Eq. 6 allows the determination of the rate constants of the individual steps. The observed rate constants are all linearly dependent on the substrate concentration (data not



Scheme 1

shown). The values of k_4 and k_5 are given in Table 1. The regeneration of MP-8 by 1-naphthol also contains two steps (Fig. 9a). Similar treatment of the kinetic data yields the rate constants k_4 and k_5, which are also given in Table 1.

At pH 9.0, three distinct phases were observed for the regeneration of MP-8 upon addition of OMP, as shown in Fig. 7b. The fastest phase (Fig. 7b, inset), which is observable only at very low concentrations of substrate (< 8 μM), corresponds to the k_4 step in Scheme 1. The second fastest step is attributed to the reaction of **II**, which is already present in the solution, with OMP, because this step has occurred, though slowly, in the early stage of the regeneration process (at an appropriate low concentration of substrate) when **II*** has not been generated appreciably. Moreover, the amplitude of the fast step in Fig. 7b is only 35% of the slow step, which is consistent with the fact that the amount of **II** is smaller than that of **II*** (or **I**) at pH 9.0 (Fig. 1b). Thus the slowest step is assigned to the k_5 step in Scheme 1. The time variation of the absorbance for the regeneration of MP-8 is expressed as:

$$A = a + b\exp(-k_3[S]_0 t) + c\exp(-k_4[S]_0 t) + d\exp(-k_5[S]_0 t) \quad (7)$$

The concentration of donor substrate employed is usually much greater than that of intermediate to satisfy Eq. 7. For the fastest step, [MP-8] = 1.0 μM, $[H_2O_2]$ = 0.96 μM, and [OMP] = 0.45–6.7 μM. Since the conversion of MP-8 to **I**/**II** is only ~50% when $[H_2O_2]$/[MP-8] ≈ 1, the concentration of OMP is much greater than that of the intermediate when [OMP] > 5 μM. Biexponential fitting of the traces in Fig. 7b, using two appropriate exponential terms (i.e., keep the k_3 and k_4 terms at low substrate concentrations and the k_3 and k_5 terms at high substrate concentrations) in Eq. 7, allows the determination of three rate constants. The three rate constants are all linearly dependent on the substrate concentration, as shown in Fig. 8. The slopes give rise to k_3, k_4, and k_5, which are all given in Table 1. Similar stopped-flow experiments and kinetic treatments were also carried out for 1-naphthol and the obtained rate constants are given in Table 1.

Table 1 The rate constants (in M^{-1} s^{-1}) for the regeneration of MP-8 by OMP and 1-naphthol at pH 7.0, 9.0, and 10.7

	OMP			1-naphthol		
	k_3	k_4	k_5	k_3	k_4	k_5
pH 7.0	–	4.42×10^5	2.10×10^2	–	1.25×10^7	2.67×10^3
pH 9.0	1.80×10^3	2.12×10^7	1.10×10^2	5.50×10^3	1.21×10^8	1.56×10^2
pH 10.7	6.88×10^5	–	–	1.36×10^7	–	–

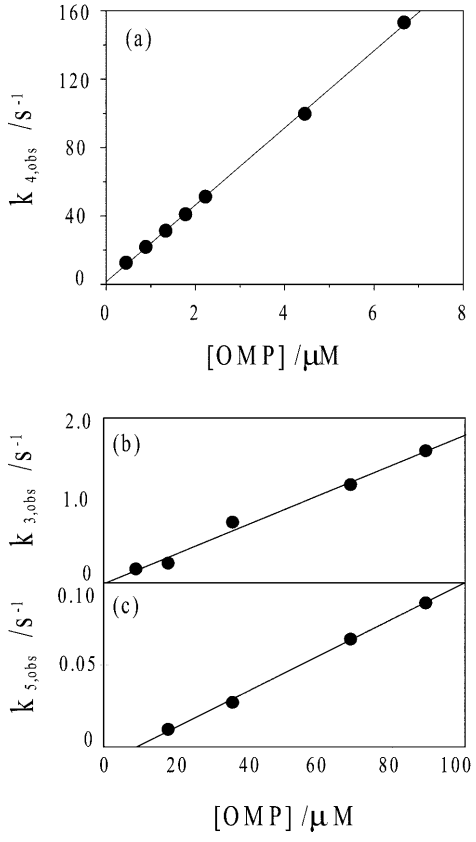


Fig. 8 Plots of **a** $k_{4,obs}$, **b** $k_{3,obs}$, and **c** $k_{5,obs}$ as a function of the concentration of OMP at pH 9.0. The concentrations (after final mixing) of MP-8 and H_2O_2 employed were 1.0 and 0.96 μM, respectively

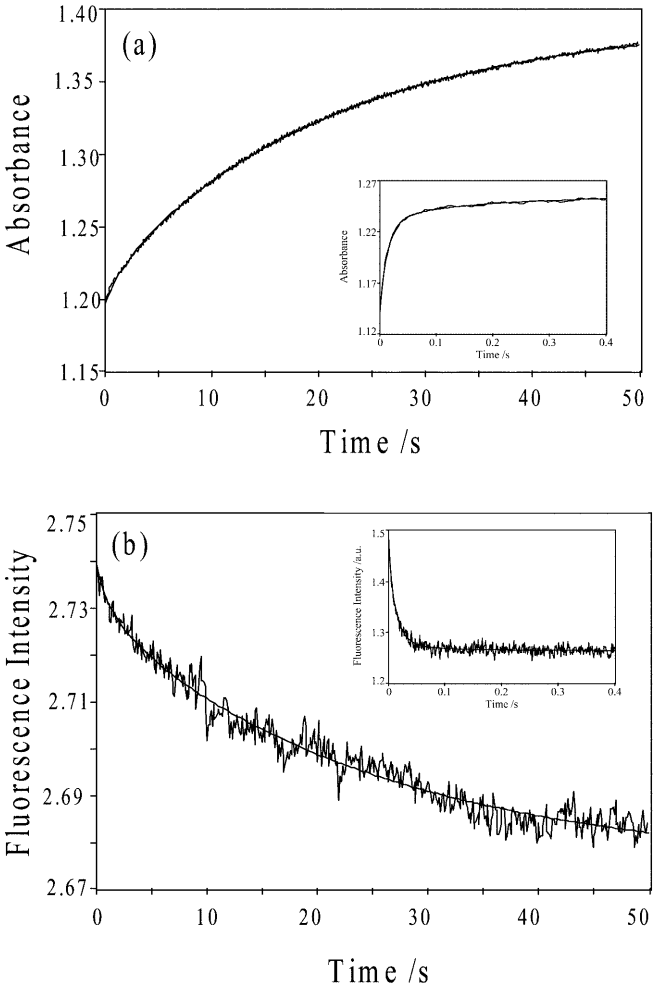


Fig. 9 Time courses for the changes in **a** absorbance at 398 nm and **b** fluorescence (λ_{ex} = 330 nm) for the reaction of **I** and 1-naphthol (10 μM) at pH 7.0. **I** was formed by mixing MP-8 (10 μM) and H_2O_2 (9.8 μM) for 90 s. Two steps were observed for each mode and the rate constants for each corresponding step are in good agreement with each other

Regeneration of MP-8 and product formation

We used the substrate 1-naphthol to deduce whether the rates for the regeneration of MP-8 (k_3–k_5 steps) reflect the substrate reactivity. 1-Naphthol exhibits a fluorescence maximum at 465 nm when excited at 330 nm. In the presence of MP-8, 1-naphthol is oxidized by H_2O_2 to form a non-fluorescent product. The time course for the change in fluorescence during the regeneration of MP-8 upon addition of 1-naphthol at pH 7.0 is shown in Fig. 9b. The disappearance of substrate occurs in two stages. The observed rate constants for these two steps are in good agreement with those obtained from the changes in absorbance for the regeneration of MP-8 (Fig. 9a). Good agreement in the rate constants for the corresponding steps obtained from the two modes of spectral measurements was also observed at pH 9.0 and 10.7. Therefore the rate constant obtained from the regeneration of MP-8 can serve as a good measure of the substrate reactivity against the intermediate.

Substrate reactivity

We have also carried out similar stopped-flow experiments for the regeneration of MP-8 upon addition of various donor substrates at pH 10.7. Different

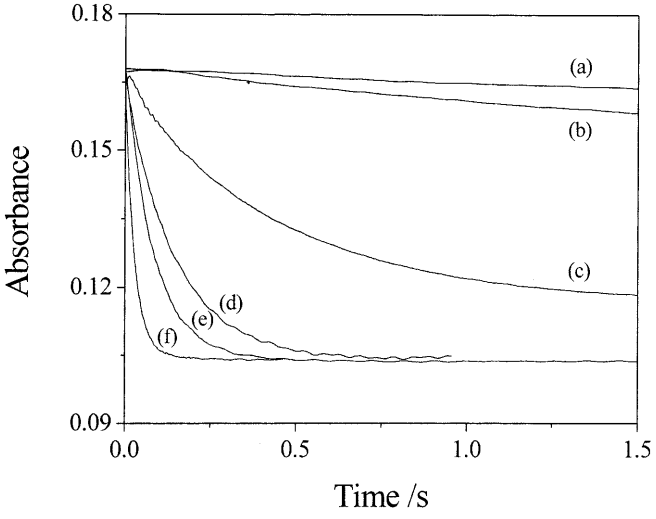


Fig. 10 Time courses for the change in absorbance at 415 nm for the reaction of **II** with various substrates (10 μM each) at pH 10.7. **II** was formed by mixing MP-8 (4.4 μM) and H_2O_2 (1.96 μM) for 20 s. The substrates are: *a* ferrocyanide, *b* aniline, *c* *p*-anisidine, *d* OMP, *e* *p*-methoxyphenol, and *f* ascorbic acid

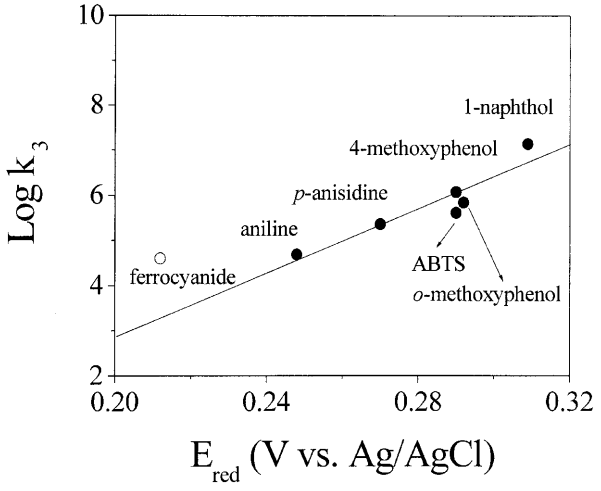


Fig. 11 The plot of log k_3 versus E_{red} at pH 10.7. A linear correlation was observed for log k_3 and E_{red}

Table 2 The rate constants k_3 and reduction potentials for various substrates at pH 10.7

Substrate	k_3 (M^{-1} s^{-1})	E_{red} (V)
1-Naphthol	1.36×10^7	0.309
Ascorbic acid	3.68×10^6	–
p-Methoxyphenol	1.18×10^6	0.290
Homovanillic acid	7.18×10^5	–
o-Methoxyphenol	6.88×10^5	0.292
ABTS	4.08×10^5	0.290
p-Anisidine	2.30×10^5	0.270
Aniline	4.86×10^4	0.248
Ferrocyanide	4.03×10^4	0.212

substrates exhibit very different rates for the regeneration of MP-8, as shown in Fig. 10. Similar kinetic analysis using Eq. 5 gives the values of k_3 and the results are listed in Table 2. The values of k_3 differ by more than two orders of magnitude, ranging from 1.36×10^7 M^{-1} s^{-1} for 1-naphthol to 4.03×10^4 M^{-1} s^{-1} for ferrocyanide. The reduction potentials of some of the substrates have been determined by scanning square-wave voltammetry and the results are listed in Table 2. A good correlation between the substrate reactivity (log k_3) and the reduction potential was observed, as illustrated in Fig. 11. The results indicate that the easier the oxidation of the substrate, the greater the substrate reactivity.

Discussion

The intermediates obtained from the oxidation of MP-8 by H_2O_2 depend on the pH used. It produced mostly **I** at pH 7.0 and mostly **II** at pH 10.7, while both **I** and **II** were clearly observed at pH 9.0 (Fig. 1). Whether **I** or **II** is favored depends on the relative magnitudes of k_1 and k_2, which in turn are pH dependent. The pH dependence of k_1 is bell shaped (Fig. 5a) with pK_a values of 8.90 and 10.60, which are assigned to the deprotonation of MP-bound H_2O_2 and MP-bound H_2O, respectively [18]. It is proposed that MP-bound HO_2^- is the active form for the formation of **I** by the heterolytic cleavage of the O-O bond [7], which accounts for the increase in k_1 between pH 7.0 and 9.0. At high pH, the MP-8-bound OH^- will inhibit the binding of HO_2^- to MP, causing a decrease in k_1. The value of k_2 increases rapidly above pH≈9.0, suggesting that the exogeneous reducing agent involved in the k_2 step is probably related to some ionized species (e.g., HO_2^-) whose concentration is strongly pH dependent. Since $k_1[H_2O_2] >> k_2$ at pH 7.0 and the reverse is true at pH 10.7 (Fig. 1), the dominant intermediate at pH 7.0 and 10.7 are **I** and **II**, respectively.

Neither MP-8 nor H_2O_2 alone can oxidize the substrates employed in this study. The formation of product is due to the reaction of the intermediates with the substrate. The values of k_3 cover over two orders of magnitude (Table 2). The diversity in reactivity depends on the nature, especially the reducing power, of the substrate. Of course, the identity of the porphyrin, including the central metal ion and the substituents, also has a profound effect on the reactivity. The substrate reactivity correlates with its oxidation potential (Fig. 11), with the easier oxidized substrate showing higher reactivity. The linear correlation suggests that an electron transfer process is involved in the rate-limiting step. A large positive deviation from the straight line was observed for ferrocyanide, which may result from the contribution to the reactivity by some other factors. Recently, Rietjens and co-workers [19] have reported similar linear correlations for the catalytic constants (k_{cat}) and the half-wave potentials ($E_{1/2}$) of substrates for HRP-catalyzed reactions. They obtained two separate straight lines, deviating by 1–2 orders of magnitude, for the phenol and aniline derivatives, respectively.

Substrate reactivity differing by 2–3 orders of magnitude has also been observed for HRP [20].

If the reaction is carried out at a high concentration ratio of H_2O_2 to MP-8, multiple turnovers will take place and the difference in substrate reactivity may not be differentiated. Cunningham et al. [10] measured the rate of product formation for the MP-8-catalyzed oxidation of a range of phenols, naphthols, and anilines by H_2O_2. They found that the rate of reaction is independent of the nature of the substrate. It is understandable that if the rate of the regeneration of MP-8 is much faster than that for the formation of intermediates such that the later process becomes rate limiting, the rate of reaction will be dictated by the oxidation of MP-8, regardless of the substrate used. Consequently, the kinetic treatment under single turnover condition described in this study is useful for the differentiation of substrate reactivity.

The regeneration of MP-8 upon addition of substrate is more complex at pH 9.0 than at pH 7.0 and 10.7. Three distinct stages (corresponding to the k_3–k_5 steps in Scheme 1) have been observed. The reactivities of the intermediates depend strongly on the pH and substrate used (Table 1). Intermediate **I** is three orders of magnitude more reactive than **II** towards the substrate OMP or 1-naphthol. This is typical of the peroxidase reaction [20, 21]. The rate constant for each individual step is greater for 1-naphthol than for OMP at a given pH (Table 1). This may indicate that similar differences in substrate reactivities, such as those given in Table 1, will also be observed at other pH values as well. The results also show that the reactivity of **II*** towards the substrate is about an order of magnitude smaller than that of **II**. Since the rates for the k_3–k_5 steps are all linearly dependent on [S] and the rate constants (k_3–k_5) are very dependent on the substrate used, particular attention should be given to the choice of the appropriate range of substrate concentration in order to resolve the three distinct steps for each substrate.

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