-

I　II　I

(k$_1$)　　pH

I　II

(k$_2$)　　pH>9

pH 7　　　**I**

pH 10.7　　　**II**

pH 9　　　**I　II**

I　II*　II　MP　II*

MP　　　(k$_3$, k$_4$　k$_5$)

H$_2$O$_2$

<u>1</u>　　　　pH

<u>1</u>　　　　　Mn(III)-

MP8

2-3

2-25

Abstract

Oxidation of Fe(III)-MP8 by H$_2$O$_2$ gives two intermediates, **I** and **II**. The rate constant for the formation of **I** is linearly dependent on [H$_2$O$_2$] and exhibits a bell-shaped dependence on pH. However, the observed rate constant for the conversion of **I** to **II** is independent of [H$_2$O$_2$], but increases sharply at pH>9.0. Both intermediates were observed at pH 9.0. Addition of substrate to the intermediates at pH 9.0 gives rise to three distinct stages: **I　II*** (k$_3$) **II　MP** (k$_4$) **II*　MP** (k$_5$). The rates of these steps are all linearly dependent on the substrate concentration. Oxidation of Mn(III)-MP8 by H$_2$O$_2$ yields only one intermediate, <u>1</u>, and the rate for the formation of <u>1</u> increases sharply as the pH increases. Addition of substrate to <u>1</u> regenerates Mn(III)-MP8. The substrate reactivity of both systems covers two to three orders of magnitudes, and is 2-25 times smaller for Mn(III)-MP8 than for Fe(III)-MP8 at pH 10.7. The substrate reactivity is linearly correlated with the reduction potential for most of the substrates studied, with the easier oxidized species showing greater reactivity.

The peroxidase-type reaction of Fe(III)-MP8 has been extensively studied. Most kinetic studies have been carried out at high concentration of H$_2$O$_2$, 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. Many Mn(III)-porphyrins, which are less reactive (and perhaps undergo less degradation) than their iron counterparts, have been frequently employed to help elucidate the catalytic mechanism. However, only very few studies on Mn(III)-MP8 have been reported. We have investigated the formation of the intermediates and the subsequent regeneration of Fe(III)- and Mn(III)-MP8 separately and proposed mechanisms to describe the observed kinetic results of the both systems and have determined the rate constants of each individual step. We have also measured the substrate reactivity of various species.

Formation of intermediates of Fe-MP8

The spectral characteristics of **I** and **II** have been described [1]. The oxidation of Fe-MP8 with H_2O_2 yields only **I** at pH 7.0 (Fig. 1a) and only **II** at pH 10.7(Fig. 1c). At pH 9.0, both **I** and **II** were produced. The stopped-flow trace at pH 9.0 (Fig. 2b) is biphasic, which can be described by the following process:

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

The standard expression of a two-step series reaction is given by:

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

Fitting of the stopped-flow traces to a two-exponential equation yields two observed rate constants ($k_{1,obs}$ and $k_{2,obs}$).
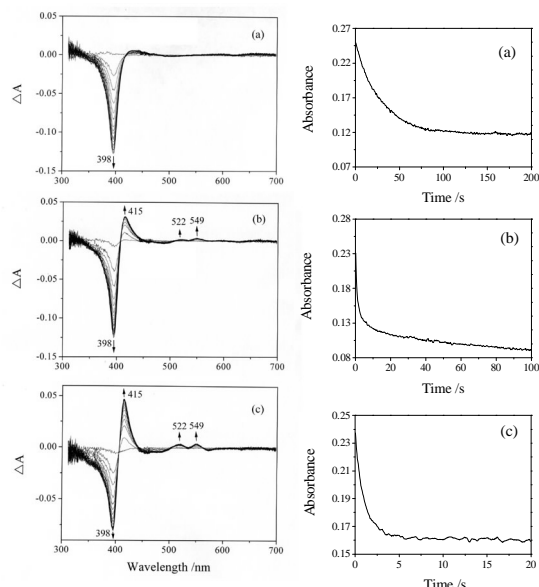

Fig. 1. The stopped-flow time-resolved difference absorption spectra for the oxidation of MP-8 by H_2O_2 at (a) pH 7.0, (b) 9.0 and (c) 10.7.

Fig 2. 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.

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 equation (2) (Fig. 3). The values of k_1 and k_2 are 7.2×10^4 $M^{-1}s^{-1}$ and 0.043 s^{-1}, respectively. At pH 7.0, **I** converts to **II** extremely slowly. The trace in Fig. 2a is single exponential, in accord with equation (3).
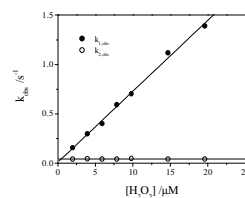

Fig 3. Dependence of (a) $k_{1,obs}$ and (b) $k_{2,obs}$ on the concentration of H_2O_2 for the oxidation of MP-8 (1.4 μM) by H_2O_2 at pH 9.0.

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

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. 2c) is also single exponential according to equation (3). The rate

constants obtained are linearly dependent on [H_2O_2] (data not shown). Similar kinetic analysis yields k_1 of 7.8 × 10^4 $M^{-1}s^{-1}$ at pH 10.7.

Regeneration of MP-8 by the substrate

Addition of the substrate (OMP) to **II** at pH 10.7 leads to the regeneration of MP-8 (data not shown). This process can be described by:

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

The time variation of the absorbance is given by:

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

The observed rate constant was linearly dependent on [S] (data not shown). The obtained value of k_3 is 6.88 × 10^5 $M^{-1}s^{-1}$.
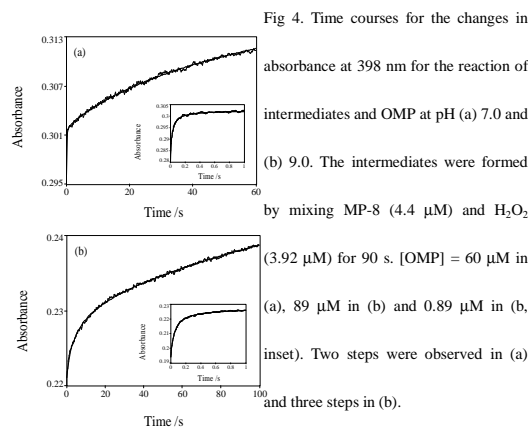

Fig 4. Time courses for the changes in absorbance at 398 nm for the reaction of intermediates and OMP at pH (a) 7.0 and (b) 9.0. The intermediates were formed by mixing MP-8 (4.4 μM) and H_2O_2 (3.92 μM) for 90 s. [OMP] = 60 μM in (a), 89 μM in (b) and 0.89 μM in (b, inset). Two steps were observed in (a) and three steps in (b).

The time course for the regeneration of MP-8 by OMP at pH 7.0 contains two stages: a very fast process, which is completed within 0.4 s (Fig. 4a, inset), and a slow process (Fig. 4a). In the first step, an intermediate with a different Soret band (400 nm) from that of **II** (408 nm) was generated and was designated as **II***. Combined all the kinetic results, the overall mechanism of the MP-catalyzed reaction can be described by scheme 1.
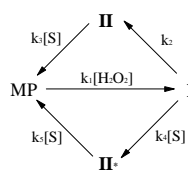
Scheme 1

At pH 7.0, under the condition that [S]$_o$ >> [intermediate]$_o$, the time variation of the absorbance for the regeneration of MP-8 is given by:

$$A = a + b \exp(-k_4[S]_o\, t) + c \exp(-k_5[S]_o\, t) \qquad (6)$$

The rate constants obtained from fitting of the traces in Fig. 4a to equation (6) are all linearly dependent on the substrate concentration (data not shown). At pH 9.0, three distinct phases were observed as shown in Fig. 4b. The fastest phase is k_4-step. The second fastest step is attributed to the reaction of **II**, which is already present in the solution, with OMP. The slowest step is assigned to the k_5-step. The time variation of the absorbance for the regeneration of MP-8 is expressed as:

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

Biexponential fitting of the traces in Fig. 4b, using two appropriate exponential terms (i.e., keep k_3 and k_4 terms at low substrate concentrations and k_3 and k_5 terms at high substrate concentrations) in equation (7), allows the determination of three rate constants. The three rate constants are all linearly dependent on the substrate concentration (data not shown).

Formation of Intermwdiates of Mn-MP8

Oxidation of Mn(III)-MP8 with H_2O_2 results in a gradual disappearance of the absorption bands at 368 and 464

nm and the concomitant appearance of a new peak at 404 nm (Fig. 5) [2]. Sharp isosbestic points at 389 and 418 nm were also observed indicating the direct conversion of Mn(III)-MP8 to an intermediate (called $\underline{1}$). The process and the formation rate can be described as:
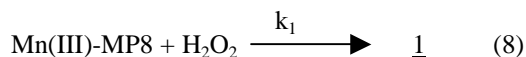
$$Mn(III)\text{-}MP8 + H_2O_2 \xrightarrow{k_1} \underline{1} \qquad (8)$$

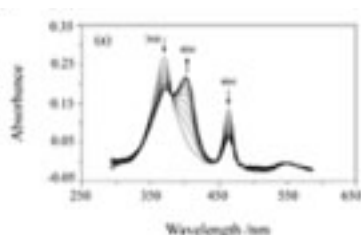$$Rate = k_1[Mn(III)\text{-}MP8][H_2O_2] \qquad (9)$$

Figure 5. Stopped-flow time-resolved absorption spectra (2.5 s per trace) for the oxidation of Mn(III)-MP8 (4 µM) with H_2O_2 (4 µM) at pH 10.7.

The stopped-flow time courses for the changes in absorbance at 368 and 404 nm (data not shown) were analyzed by the method of initial rate. The average value of k_1 is $2.9 \times 10^4 \ M^{-1}s^{-1}$.

Reaction of $\underline{1}$ with substrate

The regeneration of Mn(III)-MP8 by adding OMP to $\underline{1}$ as revealed by a gradual disappearance of the band at 404 nm for $\underline{1}$ and a concomitant appearance of the bands at 368 and 464 nm for Mn(III)-MP8 (data not shown). This process and the time variation of the absorbance are expressed as:

$$\underline{1} \xrightarrow{k_2[S]} Mn(III)\text{-}MP8 \qquad (10)$$
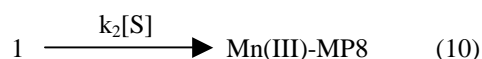
$$A = a + b \exp(-k_2[S]t) \qquad (11)$$

The observed rate constants ($k_{2,obs}$) are linearly dependent on [OMP] and the slope yields $k_2 = 2.95 \times 10^4 \ M^{-1}s^{-1}$.

Substrate reactivity

(a) ferrocyanide (b) aniline

(c) *p*-anisidine (d) OMP

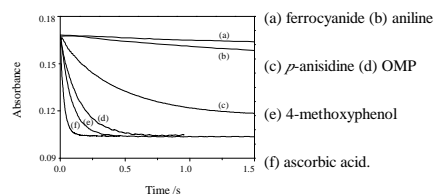(e) 4-methoxyphenol

(f) ascorbic acid.

Figure 6. 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 90 s.

Different substrates exhibit very different rates for the regeneration of MP-8 as shown in Fig. 6. The regeneration rate constants in both catalytic systems differ by more than two orders of magnitude. The substrate reactivity is 2-25 times smaller for Mn-MP8 than for Fe-MP8. The reduction potentials of some of the substrates have a good correlation with the substrate reactivity (data not shown). The results indicate that the easier the oxidation of the substrate, the greater the substrate reactivity.

luminol

References:

1. H. –C. Yeh, J. –S. Wang, Y. O. Su, and W. –Y. Lin *JBIC* (in press)
2. H. –C. Yeh, C. –H. Yu, J. –S. Wang, S. –T. Chen, Y. O. Su, and W. –Y. Lin *JBIC* (in press)