

行政院國家科委員會專題研究計劃成果報告

題目：微過氧化酵素之動力學及光譜研究

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中文摘要：

利用停止流光譜法偵測微過氧化酵素在每一催化步驟中之中間體(I, II)動力學參數。 $k_{1,obs}$ 隨 pH 之變化為鐘型曲線，其 pK_a 為 8.6 及 9.6。 $k_{2,obs}$ 在 pH 高於 9 時，急遽增加。在 pH 7 易形成 I，pH 10.7 易形成 II。鐵-微過氧化酵素催化不同基質其速率可相差至四個數量級；隨甲醇 (v/v)% 增加 $\log k_1$ 呈線性下降；GdnHCl 和 Tris 在 pH 7 使速率明顯加快，而在 pH 10.7 則分別有些微的減小與增加，由熱力學參數顯示在 pH 7 時，活化能(E_a) 與亂度 (S^\ddagger) 同時增加，在 pH 10.7 則同時減小，由於活化能與亂度互相補償的作用下，於不同條件時，因穩定反應物與過渡態的程度不同，造成不同速率之結果。綜合各結果提出一催化機制。

關鍵詞：微過氧化酵素、動力學

GdnHCl: Guanidine hydrochloride

Abstract

The stopped-flow technique has been used to determine the rate constant of each individual step in the catalytic pathway of MP-catalyzed reaction. We have characterized two intermediates (I and II) spectroscopically. The pH-dependence of $k_{1,obs}$ is bell-shaped with

pK_a values of 8.6 and 9.6, while the value of $k_{2,obs}$ increases dramatically at pH above 9. This is consistent with the fact that I dominates at pH 7 and II dominates at pH 10.7. The reactivity of II depends on the substrates used. The rate constants differ by four order of magnitudes for different substrates. The presence of methanol causes a linear decrease in $\log k_1$ as %CH₃OH increases. GdnHCl and Tris significantly increase the reaction rate at pH 7. At pH 10.7, GdnHCl slightly decreases the rate, while Tris slightly increases the rate. Both E_a and S^\ddagger decrease at pH 7 but increase at pH 10.7. Because the opposite effect of E_a and S^\ddagger , the rate depends on the relative magnitudes of these two parameters. We also proposed a mechanism of MP-catalyzed reaction.

Keywords: Microperoxidase, Kinetics

計劃緣由與目的：

Hemoproteins play important roles in biological functions: such as transport of O₂ (hemoglobin, myoglobin) or electron (cytochromes), reduction of O₂ (cytochrome c oxidase), oxidation of substrate (peroxidase), decomposition of

H₂O₂ (catalase), etc. Microperoxidase (MP), obtained from the proteolytic digestion of cytochrome c by pepsin and trypsin, is an excellent model for these heme enzymes because its heme c moiety still contains a covalently-linked oligopeptide with a histidine serving as the proximal ligand. Kinetic studies of MP are usually carried out at high concentration of H₂O₂, which may cause partial degradation of MP and the formation of a mixture of products. These will complicate the interpretation of the kinetic results and affect the reliability of the obtained rate constants. In this report, we have carried out the kinetic study of MP at low concentration of H₂O₂ under the single turn-over condition using the stopped-flow (time-resolved and fixed wavelength) technique. We have characterized the intermediate, **II**, of MP at pH 10.7 and measured the rate of its formation and reactivity against various substrates. We also investigate the effects of pH, methanol, GdnHCl, and temperature on the rate constants. These results are important in understanding the mechanism of MP-catalyzed reaction.

結果與討論：

Characterization of Intermediates

The absorption and difference spectra of the reaction of MP8 and H₂O₂ in carbonate buffer, pH 10.7 at 25°C clearly show the disappearance of the original peak of MP8 at 398 nm and the appearance of new bands at 415, 522,

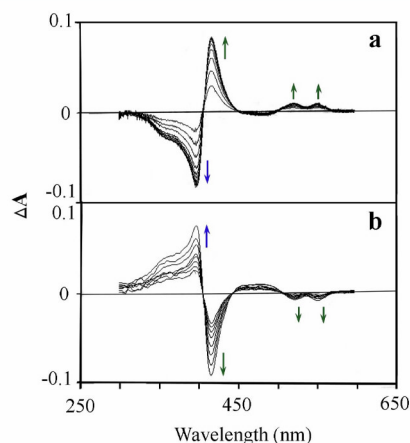
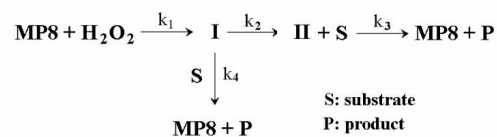


Fig. 1 Time-resolved difference spectra for (a) the reaction of native MP and H₂O₂ (0.24 s/trace). (b) the reaction of **II** and OMP (0.03s/trace). In carbonate buffer, pH10.7.

and 549 nm (Fig. 1). These bands are characteristics of **II** of heme enzymes or model compounds. The sharp isosbestic point at 405 nm indicates the direct transformation of MP8 to **II**. **I** is not observed at pH 10.7 probably because of its fast decay such that no significant accumulation occurred. Addition of the substrate, OMP (*o*-methoxyphenol), to **II** results in a gradual decrease in absorbance at 415, 522, and 549 nm and a concomitant increase in absorbance at 398 nm. These data clearly indicate the conversion of **II** to native MP8 by the presence of substrate. Based on the above results and the results from the following sections, we proposed the following mechanism for MP-catalyzed reactions.



The extinction coefficients of **II** at pH 10.7 are $\Delta\epsilon_{398 \text{ nm}} = 3.76 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ and $\Delta\epsilon_{415 \text{ nm}} = 3.86 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$. Under the condition of $k_2 \gg k_1$, the first step (k_1) is

rate determining, the initial rate of formation of **II** is given by $k_1[\text{MP8}][\text{H}_2\text{O}_2]$ and the initial rate for the reaction of **II** and OMP is given by $k_3[\text{II}][\text{OMP}]$. The rate constants, determined from the absorbance changed at 398 and 415 nm, are $k_1=1.7\times 10^5 \text{ M}^{-1}\text{s}^{-1}$ and $k_3=4.4\times 10^6 \text{ M}^{-1}\text{s}^{-1}$, respectively.

At pH 7, a significant decrease in absorbance at 398 nm was observed due to the formation of **I**. There is no change in absorbance at 415 nm indicating that the conversion of **I** to **II** is too slow to be detected. The values of $\Delta\epsilon_{398 \text{ nm}}$ and k_1 are $1.31\times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ and $6.3\times 10^3 \text{ M}^{-1}\text{s}^{-1}$, respectively.

Effect of Substrate on rate constants

The reactivity of **II** depends on the substrate used. (Fig. 2). The rate constants (k_3) for various substrates differ by four orders of magnitudes. This result is quite different from the data by Pratt et. al,¹ which shows similar rate constants for various substrates.

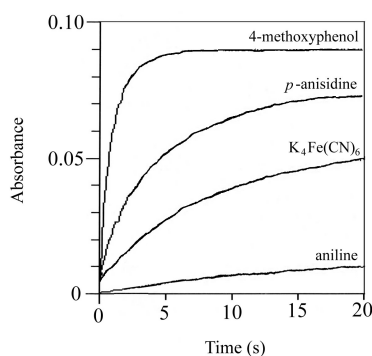


Fig. 2 Time courses of the change in absorbance at 415 nm of compound **II** reacted with different substrates at pH 10.7.

pH Dependence of rate constants

MP8 exhibits maximum activity at

pH9. The pH-dependence of $k_{1,\text{obs}}$ is bell-shaped with pK_a values of 8.6 and 9.6, which are assigned to the ionizations of MP-bound H_2O_2 and MP-bound water, respectively. The value of $k_{2,\text{obs}}$ increases dramatically at pH above 9. This result is consistent with the fact that **I** dominates at pH 7 and **II** dominates at pH 10.7.

Effect of Solvent on k_1 and k_3

The rate constant (k_1) of MP8 and H_2O_2 , at pH 10.7, is decreased by the presence of an increasing amount of methanol. The plot of $\log k_1$ v.s. $\% \text{CH}_3\text{OH}$ is linear. Heterolytic cleavage to form **I** requires a significant charge separation of peroxide in the transition state. The decrease in the dielectric constant at high percentage of methanol is unfavorable for the charge separation process, causing an increase in the activation energy and hence a reduction in rate. Both k_1 and k_3 decrease five times by the presence of 50% methanol. The rate is not affected by the presence of methanol at pH 7, in consistent with the results of P. Jones al.²

Effect of GdnHCl

Addition of GdnHCl causes an acceleration of the rate of formation of **I** at pH 7 as illustrated in Fig. 3. Hydrogen bonding between GdnH^+ and charge-separated HO_2^- results in a stabilization of the transition state (lowering of the activation energy) and hence an enhancement of the rate.^{3,4}

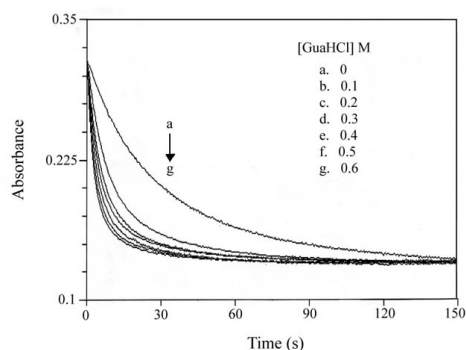
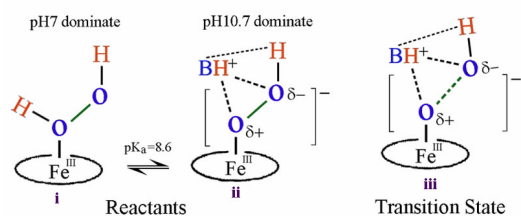


Fig. 3. Time courses of the change in absorbance at 398 nm of GdnHCl effect on the reaction of MP8 with hydrogen peroxide at pH 7.

T-dependence of rate constants

The thermodynamic constants from the temperature dependence experiments are given below.

kcal/mol	E _a	G [‡]	H [‡]	S [‡] (eu)
MP + H ₂ O ₂				
pH7	12.08	12.63	11.48	-3.86
pH10.7	3.74	10.75	3.15	-25.49
Containing [GdnH ⁺]=0.5M				
pH7	9.41	11.33	8.82	-8.42
pH10.7	9.59	11.15	9.00	-7.23
Containing [Tris]=0.5M				
pH7	5.81	11.39	5.21	-20.7
pH10.7	5.63	10.38	5.04	-17.91



Addition of GdnHCl or Tris causes a significant increase in k_1 at pH 7.0. This is due to the stabilization of the transition state (iii), relative to the reactant (i), through electrostatic interaction and hydrogen bonding with GdnHCl or Tris, as confirmed by the

reduction in E_a and S^{\ddagger} . The effect of E_a is greater than that of S^{\ddagger} , causing an increase in rate. At pH 10.7, a slight decrease in k_1 was observed for the addition of GdnHCl, whereas a slight increase in k_1 was observed upon addition of Tris. The presence of GdnHCl and Tris causes a greater stabilization of the reactant (ii) as compared to the transition state (iii), in consistent with the increase in E_a and S^{\ddagger} . Because of the compensation effect, the rate depends on the relative magnitudes of these two parameters.

計劃成果自評：

本計劃利用停止流光譜儀鑑定出微過氧化酶催化反應之中間體，並決定其各步驟之速率常數。同時也完成不同基質、離子強度、溶劑、酸鹼值及溫度對催化速率的影響。根據本計劃的結果，我們提出一機制有助於對此酶之催化反應有進一步的瞭解。至於不同中心金屬 MP 之研究尚在進行中，詳細結果有待後續之研究。

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