

## Suicide-Peroxide inactivation of microperoxidase-11: A kinetic study

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### Abstract

The kinetics of microperoxidase-11 (MP-11) in the oxidation reaction of guaiacol (AH) by hydrogen peroxide was studied, taking into account the inactivation of enzyme during reaction by its suicide substrate, H<sub>2</sub>O<sub>2</sub>. Concentrations of substrates were so selected that: 1) the reaction was first-order in relation to benign substrate, AH and 2) high ratio of suicide substrate to the benign substrate, [H<sub>2</sub>O<sub>2</sub>] >> [AH]. Validation and reliability of the obtained kinetic equations were evaluated in various nonlinear and linear forms. Fitting of experimental data into the obtained integrated equation showed a close match between the kinetic model and the experimental results. Indeed, a similar mechanism to horseradish peroxidase was found for the suicide-peroxide inactivation of MP-11. Kinetic parameters of inactivation including the intact activity of MP-11,  $\alpha_i$ , and the apparent inactivation rate constant,  $k_i$ , were obtained as  $0.282 \pm 0.006 \text{ min}^{-1}$  and  $0.497 \pm 0.013 \text{ min}^{-1}$  at [H<sub>2</sub>O<sub>2</sub>] = 1.0 mM, 27°C, phosphate buffer 5.0 mM, pH = 7.0. Results showed that inactivation of microperoxidase as a peroxidase model enzyme can occur even at low concentrations of hydrogen peroxide (0.4 mM).

**Keywords:** Kinetic, microperoxidase, horseradish peroxidase, suicide inactivation

**Abbreviations:** MP, Microperoxidase; HRP, Horseradish Peroxidase; AH, Hydrogen Donor; A, Absorbance; C-I, Compound I; C-II, Compound II

### Introduction

Understanding the structure/function relationship of hemoproteins needs investigations on the interactions between the heme and the peptide or apoprotein matrix. For this purpose, hemoprotein models, which can simplify the complex relationships, can be used [1–3]. Hemoenzyme models such as microperoxidases are able to reproduce the coordination properties and/or the catalytic function of hemoproteins [4–7].

Microperoxidase as a heme peptide with a molecular weight of only 1900 has been found useful as an ultra-structural tracer [4]. Heme-based mini-enzymes or microperoxidases form a new generation of biocatalysts and hemoenzyme models [5,6]. Capability of these biocatalysts to act as peroxidase

[2,6–11], cytochrome C and nitric reductase [3] has made them interesting models for studying the chemistry of native peroxidases [6]. Microperoxidases have peroxidatic activity in a magnitude of about 1/4 that of HRP and 150 times that of cytochrome C [4].

Microperoxidase-11 (MP-11) is a product of the proteolytic degradation of horseheart cytochrome C [11,12] and consists of a heme prosthetic group (Iron(III) protoporphyrin IX) covalently attached to a short polypeptide chain (11 amino acid residues) through two thioether bonds. The advantage of using MP-11 over simple heme (e.g. hemin) is that MP-11 shows better water solubility, lower tendency to aggregate/dimerize, and a spatial configuration, determined by the polypeptide chain, that resembles that in true hemoproteins [13]. The microperoxidase

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family consists of four members (MP-6, MP-8, MP-9 and MP-11) [14]. The smallest member with a short chain of five residues was also known in 1999 [14]. These heme-based mini-enzymes are able to mimic peroxidase oxidation reactions [5].

Some of such studies, which include hydroperoxo-iron heme intermediate (compound I) were reported and reviewed, previously [6,9]. Also it has been reported that in the presence of a radical scavenger like ascorbate, dealkylation reactions catalyzed by microperoxidase proceeds through a non radical-type mechanism [9,10].

Another important structural feature of MP-11 is that, similar to peroxidases [13], the heme group in MP-11 is exposed directly to the solvent, allowing easy access even for bulky substrates [15–17]. Recently, MP-11 was used for oxidation of pyocyanin [13] and anthracycline [15]. Also, MP-11 can catalyze oxidation reactions in organic media [16]. Effects of the microenvironment on the function of MP-11 have been investigated [18].

Hemoproteins have been the subject of extensive mechanistic [17–23], kinetic [24–27] and thermodynamic [28–31] studies [32–35]. In the presence of high concentrations of hydrogen peroxide, all hemoproteins including peroxidases inactivate [1,36,37] through a pathway referred to as “suicide-peroxide inactivation”. Many aspects of this process have not yet been fully elucidated [1]. Microperoxidases may be also inactivated by high concentration of hydrogen peroxide, even more easily than the peroxidases because the heme group is not protected by the polypeptide chain.

The kinetic behaviour of the suicide substrates of enzymes has been analyzed [38,39] for significant [40,41] and negligible [42,43] substrate depletion. These kinetic analyses have been applied to enzymes such as tyrosinase [44], horseradish peroxidase [45], Coprinus cinereus peroxidase [46], thyroid and lactoperoxidase [47,48].

The kinetic model for suicide-peroxide inactivation of catalase (as a monosubstrate hemoenzyme) [49] and horseradish peroxidase (as a bisubstrate hemoenzyme) has been introduced previously [37]. In the latter model, the inactivation process can be easily monitored through the time-course (progress curve) of the oxidation reaction of an aromatic hydrogen donor substrate [37]. The main feature of this study was presentation of the kinetic equations and indicating the validity and reliability of the kinetic model. Since MP-11 is a peroxidase model enzyme, we examined the same kinetic model for peroxide-inactivation of microperoxidase. The present work involves a comparative study on the suicide inactivation of microperoxidase-11 and horseradish peroxidase. Validation of the kinetic model is evaluated and the comparative strength of inactivation as well as the kinetic parameters including the intact activity ( $\alpha_i$ ),

initial activity ( $\alpha_0$ ) and the apparent rate constant of inactivation of MP-11 ( $k_i$ ) were determined.

## Experimental

### Materials

MP-11 (sodium salt) and horseradish peroxidase (type II) were obtained from Sigma. Guaiacol (ortho-methoxyphenol),  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  and hydrogen peroxide 30% (V/V) were obtained from Merck. Phosphate buffer 5.0 mM, as the solvent system was prepared in  $\text{CO}_2$ -free deionized water (Barnstead NanoPure D4742 deionizer, Electrical Resistance = 18.3 M $\Omega$ ).

### Methods

Absorbance changes and electronic spectra were recorded using a Varian spectrophotometer model Cary 50 equipped with fiber optic dip probe accessory and a xenon light source. The fiber optic probe provides the data recording at the time of mixing in the reaction vessel. Using this accessory, no cuvette is required. The ionic strength and pH of the solutions were kept constant by using a 5.0 mM phosphate buffer. Temperature of the reaction solution in the jacketed laboratory reactor (4 mL) was adjusted to 25°C ( $\pm 0.1$ ) using a Lauda oil circulating thermobath equipped with an external temperature sensor. Specific activity of MP-11 was determined spectrophotometrically in a 1-min reaction time course using guaiacol as hydrogen donor [50]. The specific activity of MP-11 was equal to 52 U/mg. The same spectrophotometer in the kinetic mode was used for recording the progress curves of the inactivation process. MP-11 concentration was determined at pH = 7.0 using an extinction coefficient of 176  $\text{mM}^{-1} \cdot \text{cm}^{-1}$  at 395 nm [16].

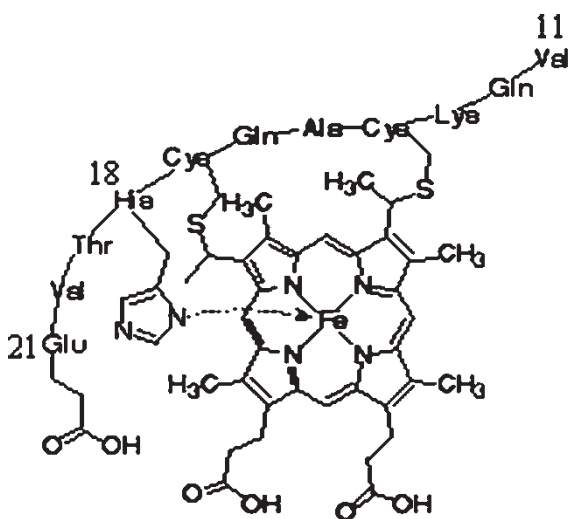
Concentration of hydrogen peroxide was estimated by measuring absorbance of the solution using  $\epsilon_{240} = 43.6 \text{ cm}^{-1} \cdot \text{M}^{-1}$  [14,51]. In all calculations, a molecular weight of 1861 was used for MP-11. Suicide-peroxide inactivation of MP-11 was monitored at 470 nm ( $\lambda_{\text{max}}$  for the product of the catalytic reaction) by difference spectrophotometry. The details of the procedure of measuring the product concentration, the rate of reaction, and determination of the progress curves have been described previously [37]. Progress curves were determined by following absorbance of the reaction mixture at 470 nm in an appropriate time course of about 30 min. After starting the reaction, 30 s was considered as the dead time for the reaction to reach the stationary state. The unreacted part of AH was obtained from the recorded absorbance data and suitable relations [37].

Each experiment was repeated three times and standard deviations were estimated for the obtained results.

“End-point procedure” experiments were carried out in order to determine the maximum elevation of absorbance ( $A_{\infty}$ ) and the value of intact activity ( $\alpha_i$ ). Thus, the reaction mixture was incubated for a prolonged time (about 2 h) according to the “end-point procedure” which is described in detail elsewhere [37]. In the end-point method by adding a fresh and excess quantity of MP-11 solution to the incubated mixture, values of  $A_{\infty}$ , the unreacted parts of AH ( $[AH]_{\infty}$ ) and also the intact activity of the enzyme ( $\alpha_i$ ), could be estimated. Intact activity ( $\alpha_i$ ) is defined as the enzyme activity before it is exposed or reacted with the substrate.

## Results and discussion

An important structural feature of MP-11 is that, similar to most peroxidases, the fifth coordination position of iron in the heme is occupied by the imidazole group of histidine present in the polypeptide chain (His.18). The sixth position is occupied by a water molecule [6] and this weak ligand can be easily replaced by the peroxide substrate [52]. The chemical structure of MP-11 is shown below:



In the catalytic cycle, displacement of the water molecule by hydrogen peroxide and the subsequent formation of compound I can easily occur. In comparison to hemoproteins, the heme periphery of MP-11 is exposed directly to the solvent, allowing easy access even for bulky substrates. From the kinetic point of view, formation of compound I is the rate-limiting step [10]. Indeed, in the presence of  $H_2O_2$ , microperoxidases form a reactive intermediate (an analog of peroxidase compound I which is responsible for the catalytic activity [13,52]). Thus, in accordance with the spectroscopic data it is believed that microperoxidase and HRP have a similar kinetic mechanism [6].

There are two major catalytic pathways for the kinetics of peroxidase: 1) a two-electron transfer as the catalase pathway and 2) a one-electron transfer as the compound III pathway, which is a minor pathway compared with that of the catalase reaction [1,53].

It has been shown that in the presence of high concentrations of peroxide, in addition to the two above described major catalytic pathways, peroxidase can also enter into an inactivation pathway namely “suicide-substrate inactivation” [1,37,54–57]. The inactivation process starts by the reaction of excess  $H_2O_2$  with compound-I which has a central role in the suicide-substrate inactivation pathway. The product of the inactivation process (**P-670**) is a catalytically inactive form of peroxidase ( $E_i$  in Scheme I) [37,58–60]. This inactive form indicates a  $\lambda_{max}$  in its electronic spectra at 670 nm (see Figure 5). P-670 has two characteristics: 1) It has a low molar absorptivity that would involve the use of high enzyme concentrations and 2) P-670 is unstable and may convert into the other inactive forms. Hence, following the inactivation of peroxidase and peroxidase models is not recommended through the quantitative monitoring of P-670.

Among the MP active forms (including MP, C-I and C-II), Compound I as a high valent intermediate and strong oxidant shows the maximum capacity and potential to oxidize aromatic substrates.

According to Scheme I, two major reactions could be attributed to the reaction system:

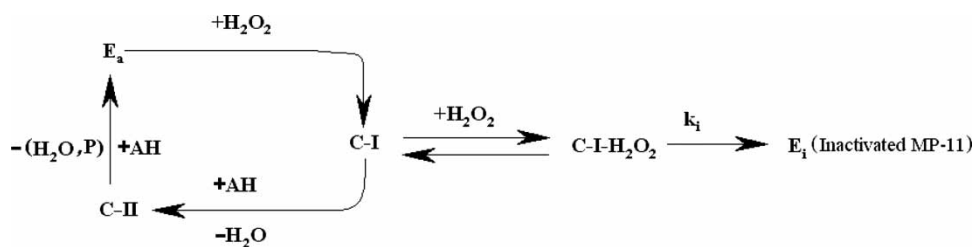


Reaction **a** is the inactivation process and **b** (oxidation of guaiacol by peroxide) is the catalyzed reaction which is also used to monitor the inactivation process.

$E_a$ ,  $E_i$ ,  $AH$ , and  $P$  denote the whole forms of active enzyme, inactivated enzyme, hydrogen donor (reductant-substrate) and the catalytic reaction product (tetraguaiacol), respectively. Parameters of  $k_i$  and  $\alpha$  denote the apparent rate constant of inactivation (reaction a) and the apparent catalytic rate constant of the oxidation reaction (reaction b), respectively. In the catalytic reaction cycle, the active enzyme species are free active enzyme ( $E_a$ ), C-I and C-II. The rate constants of formation of C-I, C-II and  $E_a$  are extremely large compared with the  $k_i$  value. Accordingly, the whole active enzymes are supposed to be in form of C-I, which may undergo inactivation by the peroxide (see Scheme I). The following conditions are recommended for the simultaneous advancement of the two reactions (**a** and **b**):

**a)** Higher concentration of hydrogen peroxide with respect to the concentration of hydrogen donor (AH)

**b)** Low concentration of hydrogen donor (“benign substrate”, a substrate that does not damage the



Scheme I. The catalytic reaction cycle and the inactivation pathway for MP-11.

enzyme); so the donor substrate does not saturate the enzyme (according to the initial linear part of the Michaelis-Menten plot).

The reaction **b** is essentially first-order in relation to the hydrogen donor and an expected linear dependence on the initial velocity of reaction **b** and lower range of AH concentration was observed (data not shown) [37]. As a result, the rate of reaction **a** seems to be proportional to the active enzyme concentration. Using a steady-state approximation, it is possible to derive the differential and integrated kinetic equations. In principle, by monitoring AH concentration in the catalytic reaction cycle, one can estimate the extent of decrease in the level of active forms of the enzyme (due to the suicide inactivation process).

Combination of the rate equations of the two above reactions i.e. consumption of substrate and inactivation of enzyme, can be written as:

$$\alpha = \alpha_0 \times e^{-k_i \cdot t} \quad (1)$$

where  $\alpha_0$  can be defined as the value of  $\alpha$  at the start time of measurements ( $t = 0$ ). The remaining amount of guaiacol (ortho-methoxy phenol,  $[\text{AH}]_t$ ) was determined using the following relationships:

$$A_\infty \propto [\text{AH}]_0; [\text{AH}]_{\text{consumed}} = (A_t/A_\infty) \times [\text{AH}]_0 \quad (2)$$

$$[\text{AH}]_t = [\text{AH}]_0 - [\text{AH}]_{\text{consumed}} \quad (3)$$

where  $A_\infty$  and  $A_t$  are the absorbance of the colored product (at 470 nm) and concentration of guaiacol at time  $t$  and  $t = \infty$  (end of the catalytic reaction), respectively.  $[\text{AH}]_t$  is concentration of guaiacol (AH) at time  $t$ .

The present model involves the advantage of monitoring of the inactivation reaction with no need to measure the concentrations of enzyme species ( $E_a$ , C-I, C-II,  $E_i$ ). In fact, the overall concentration of active enzyme species,  $\alpha$ , (in the form of C-I) defines the first order rate law at each time ( $\alpha = \alpha_0 e^{-k_i \cdot t}$ ). Instead of activity measurements,  $\alpha_0$  can be obtained by fitting the experimental data into the overall integrated kinetic equation (Equation (4)) of the process. Indeed, the rate of the consumption of reductant substrate (AH) and the overall rate of

conversion of active enzyme ( $E_a$ ) to the inactivated form ( $E_i$ ) enables us to obtain the differential kinetic equation as well as the integrated kinetic relation (according to our previous model) [37,49,61]:

$$[\text{AH}]_t = [\text{AH}]_0 \times e^{\alpha_0/k_i(e^{-k_i \cdot t}-1)} \quad (4)$$

where  $[\text{AH}]_0$  is the molar concentration of AH at  $t = 0$ . Equation (4) is the relationship between the remaining concentration of the aromatic substrate and time, whilst the decrease in enzyme activity during the simultaneous inactivation reaction has been taken into account. Equation (4) can be used for the determination of  $\alpha_0$  and  $k_i$  in a non-linear regression manner by fitting the experimental data into the equation. Common computer software such as **Excel Solver** was used for this purpose. Table I indicates the obtained values of the kinetic parameters for suicide inactivation of MP-11.

On the other hand, at adequately low concentrations of  $\text{H}_2\text{O}_2$  (about 0.1–0.2 mM) the normal catalytic cycle proceeds without the suicidal inactivation effect of hydrogen peroxide. Figure 1 shows the various types of inactivation progress curves at different concentrations of hydrogen peroxide. Progress curves were obtained as described in the *Methods*. Details of the experiments are described in the legend of Figure 1. The excellent coincidence between experimental data (points) and the fitted curve (calculated) based on Equation (4) was observed as shown in Figure 1. On the other hand, Figure 1 illustrates the accuracy of the kinetic model and the Equation (4) for the suicide-peroxide inactivation of microperoxidase-11.

Table I.  $k_i$  and  $\alpha_i$  values for HRP and MP-11 were obtained for a time course of 10 min of the progress curves data at 27°C, pH = 7.0, phosphate buffer 5.0 mM.

Biocatalyst	$[\text{H}_2\text{O}_2]$ (mM)	$k_i$ ( $\text{min}^{-1}$ )	$\alpha_0$ ( $\text{min}^{-1}$ )	$\alpha_i$ ( $\text{min}^{-1}$ )
[HRP] = 20.0 nM	3.0	0.046	0.18	0.20
[MP-11] = 1.0 $\mu\text{M}$	1.0	0.491	0.26	0.28
[MP-11] = 1.0 $\mu\text{M}$	3.0	1.30	0.19	0.28



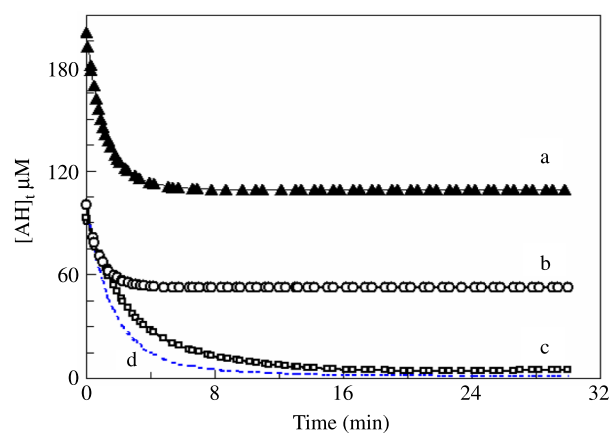


Figure 1. Progress curves for suicide inactivation of MP-11 by hydrogen peroxide, as the variation of guaiacol concentration versus reaction time, according to Equation (4). Inactivation rate constant,  $k_i$  and the initial activity of MP-11,  $\alpha_o$ , were estimated by successive approximation and non-linear fitting of the experimental data into Equation (4). Solid lines indicate the calculated curve data using the obtained  $k_i$  and  $\alpha_o$  parameters in Equation (4). Values of the kinetic parameters are shown in Table I. Each parameter was systematically adjusted to produce best fit curves that gave a minimum value of the sum-of-squares of residuals (SSR, as the differences between observed and calculated values of progress curves, i.e.  $SSR = \sum (y_{obs.} - y_{calc.})^2$ ). An Excel Solver program was used for this purpose. A detailed description of the suicide-peroxide inactivation model is given in references [33] and [37]. Reactions were started by adding hydrogen peroxide to the mixture of AH and MP-11 (1.0  $\mu\text{M}$ ) having an initial activity of 0.3  $\text{min}^{-1}$ , time course of about 30 min at 27°C, phosphate buffer 5.0 mM, pH = 7.0. **a)**  $[\text{AH}] = 200 \mu\text{M}$ ,  $[\text{H}_2\text{O}_2] = 1.0 \text{ mM}$ ,  $k_i = 0.487 \text{ min}^{-1}$  **b)**  $[\text{AH}] = 100 \mu\text{M}$ ,  $[\text{H}_2\text{O}_2] = 1.0 \text{ mM}$ ,  $k_i = 0.491 \text{ min}^{-1}$ , **c)**  $[\text{AH}] = 100 \mu\text{M}$ ,  $[\text{H}_2\text{O}_2] = 0.40 \text{ mM}$ ,  $k_i = 0.120 \text{ min}^{-1}$  **d)** The hypothetical curve with  $k_i = 0$  (using value of  $k_i = 10^{-8}$  for solving Equation (4)), i.e. without suicidal effects of peroxide. Here  $[\text{AH}]_o$  and  $\alpha_o$  were considered to be the same as state (c). AH concentration was followed from the absorbance of the reaction mixture at 470 nm using Equations (2) and (3). In order to reach the stationary state of the reaction, a dead time of 30 seconds was considered for processing the progress curves data.

Generally, there is a delay from the start of the reaction to the start of measurements ( $t = 0$ ) for homogenizing the mixture and allowing the reaction to reach the steady state condition. Certainly, some of the enzyme activity may be lost in such a delay period.  $\alpha_o$  is the activity of enzyme at the end of this delay time. Therefore, a correction is necessary for calculation of  $\alpha_i$  as the intact value of activity just at the start of reaction. The intact activity defines by a modified form of Equation (1)  $\{\alpha_i = \alpha_o \times e^{-k_i \cdot \tau}\}$  in which  $\tau$  is the delay time of measurement. The inactivation rate constant,  $k_i$ , must be independent of changes in the initial value of either enzyme or AH concentration. Figures 2a and 2b shows such independency of  $k_i$  from both of the above variables. Figure 2c depicts the independency of  $\alpha_o$  versus changes in the concentration of reductant substrate ( $[\text{AH}]$ ).

Determination of  $k_i$  and  $\alpha_i$  is also possible from the “end-point” approach, using linear plots. Based on

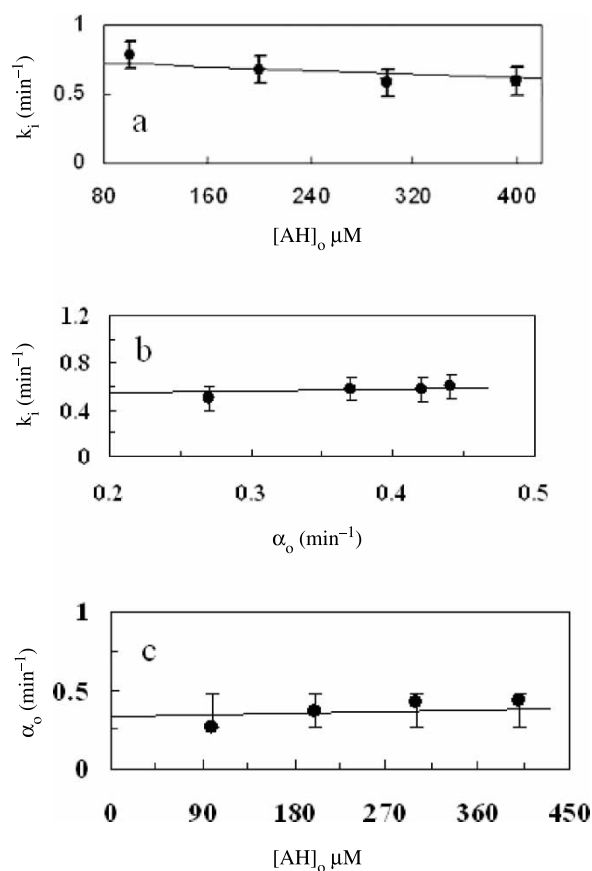


Figure 2. **a)** Variation of  $k_i$  with initial concentration of guaiacol, AH.  $k_i$  values were obtained (27°C, phosphate buffer 5.0 mM, pH = 7.0) from the related progress curves and Equation (4) as described in legend of Figure 1.  $[\text{MP} - 11] = 1.0 \mu\text{M}$ ;  $[\text{H}_2\text{O}_2] = 1.0 \text{ mM}$ ; **b)**  $k_i$  as a function of activity (concentration) of MP-11,  $\alpha_o$ . Values of  $k_i$  and  $\alpha_o$  were obtained (27°C, phosphate buffer 5.0 mM, pH = 7.0,  $[\text{H}_2\text{O}_2] = 1.0 \text{ mM}$ ) from the related progress curves and Equation (4) as described in the text and in the legend of Figure 1. **c)**  $\alpha_o$  as a function of concentration of hydrogen donor substrate,  $[\text{AH}]$ . Values of  $\alpha_o$  were obtained (27°C, phosphate buffer 5.0 mM, pH = 7.0,  $[\text{H}_2\text{O}_2] = 1.0 \text{ mM}$ ) from the related progress curves and Equation (4) as described in the text and in the legend of Figure 1.

the end-point procedure, the relation between the remaining concentration of substrate at the end of reaction ( $t_\infty$ ) assigned by  $[\text{AH}]_\infty$  and the other variables can be predicted from Equation (4) by setting  $t = \infty$ , as below:

$$[\text{AH}]_\infty = [\text{AH}]_i \times e^{-\alpha_i/k_i} \quad (5)$$

$$\ln[\text{AH}]_\infty = \ln[\text{AH}]_i - \alpha_i/k_i \quad (6)$$

$$\ln([\text{AH}]_i/[\text{AH}]_\infty) = 1/k_i \times \alpha_i \quad (7)$$

where  $[\text{AH}]_i$  is the initial concentration of AH. According to Equation (5) a linear plot of  $[\text{AH}]_\infty$  versus  $[\text{AH}]_i$  represents a slope of  $e^{(-\alpha_i/k_i)}$ . Figure 3a shows a linear relation between  $[\text{AH}]_\infty$  versus  $[\text{AH}]_i$ . Also, Figure 3b shows the linear plot of  $\ln[\text{AH}]_\infty$  versus  $\ln[\text{AH}]_i$  with a calculated slope of unity and Y-intercept of  $-\alpha_i/k_i$ , according to Equation (6).

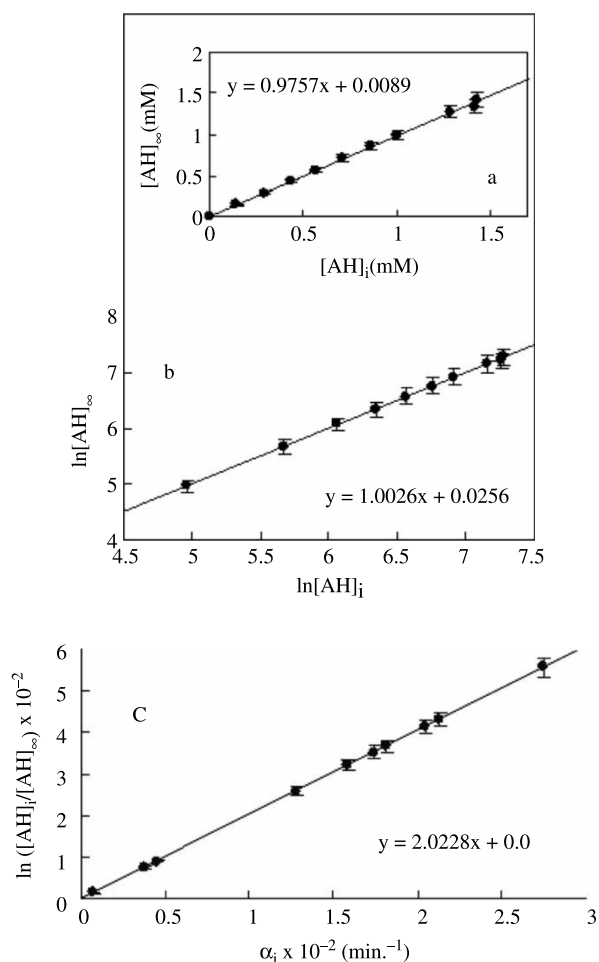


Figure 3. Linear plots based on the end-point procedure (Equations 5 to 7) illustrating dependence of initial concentration of AH ( $[AH]_i$ ) on the remaining (unreacted) concentration of AH ( $[AH]_\infty$ ). Reactions were carried out at constant activity of MP-11. **a)** Linear plot based on Equation (5). A mixture of MP-11 (1.0  $\mu\text{M}$ ) and various initial concentrations of guaiacol ( $[AH]_i$ ) were incubated in the presence of  $[H_2O_2] = 1.0 \text{ mM}$  at  $27^\circ\text{C}$  for 2 h ( $t = \infty$ ). Reactions were carried out at constant activity of MP-11 ( $0.3 \text{ min}^{-1}$ ). Slope =  $\text{Exp}(-\alpha_i/k_i) = 0.976$  and  $\alpha_i/k_i = 0.0243$ , Y-intercept = 0.009. **b)** Logarithmic plot based on Equation (6). A mixture of MP-11 (1.0  $\mu\text{M}$ ) and various initial concentrations of guaiacol ( $[AH]_i$ ) were incubated in the presence of  $[H_2O_2] = 1.0 \text{ mM}$  at  $27^\circ\text{C}$  for 2 h ( $t = \infty$ ). Reactions were carried out at constant activity of MP-11 ( $0.3 \text{ min}^{-1}$ ). Slope = 1.003, Y-intercept =  $-\alpha_i/k_i = -0.0256$  and  $\alpha_i/k_i = 0.0256$ . **c)** Linear plot according to Equation (7) for the variation of  $\ln([AH]_i/[AH]_\infty)$  with the initial activity of MP-11,  $\alpha_i$ . The mixture of AH and MP-11 (1.0  $\mu\text{M}$ ) were incubated in the presence of 1.0 mM  $H_2O_2$ , phosphate buffer 5.0 mM at  $27^\circ\text{C}$  for 2 h ( $t = \infty$ ). Slope =  $1/k_i = 2.023$  and  $k_i = 0.494 \text{ min}^{-1}$ , Y-intercept = 0.0.

Furthermore, on the basis of Equation (7) a plot of  $\ln([AH]_i/[AH]_\infty)$  versus initial activity of MP-11 would be a line with the slope of  $1/k_i$ . This linear plot is shown in Figure 3c. Each of the above plots i.e. Figures 3a,b,c were obtained from independent experiments. Good agreements between the predicted and the experimental values for the slopes and

intercepts of the lines (according to the related equation) indicate the validity and reliability of the kinetic model. The related slopes and intercepts are shown in the legend of the Figures. Equations (5–7) demonstrate that 1) variation of initial concentration of aromatic substrate ( $[AH]_i$ ) must result in a proportional change in its final concentration ( $[AH]_\infty$ ), when the other parameters remain unchanged, 2) variation of the intact activity parameter ( $\alpha_i$ ) must lead to the expected changes in the ratio of ( $[AH]_i/[AH]_\infty$ ) according to Equation (7).

On the other side, effect of variation of  $H_2O_2$  concentration on  $k_i$  is shown in Figure 4a which indicates that the rate of inactivation is proportional to the concentration of suicide substrate, a factor which drives the free MP-11 to a substantially reactive intermediate, compound I and then crashes the latter as a catalytically inactive derivative, P-670. According to Figure 4a, a linear relation in the form of  $k_i = 0.445 \times [H_2O_2]$  can be easily used to predict and estimate the apparent rate constant of inactivation of MP-11 by hydrogen peroxide. Figure 4b shows that the intact activity ( $\alpha_i$ ) of MP-11 is not influenced by the suicide substrate,  $H_2O_2$ . This means that the rate limiting step of the catalytic reaction cycle is not the  $H_2O_2$  consuming step.

Figure 5 shows the electronic spectra for the native and inactivated MP-11 (in the presence of 3.0 mM  $H_2O_2$ ) after 1 minute of incubation. The inactivated form shows a maximum absorption at 670 nm which is the same as for inactivated HRP. Also during the inactivation, due to the conversion of the active heme into the inactive verdoheme, the Soret band absorbance is expected to be decreased as shown in Figure 5.

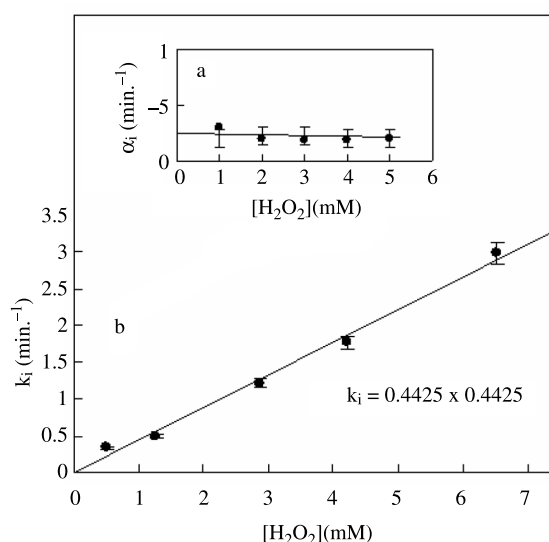


Figure 4. **a)** Dependence of  $k_i$  on concentration of suicide-substrate,  $[H_2O_2]$ .  $[AH] = 0.4 \text{ mM}$ ,  $[MP-11] = 1.0 \mu\text{M}$ ,  $\text{pH} = 7.0$ , phosphate buffer 5.0 mM and temperature of  $27^\circ\text{C}$ . **b)** Independency of  $\alpha_i$  on  $[H_2O_2]$ .  $[AH] = 0.4 \text{ mM}$ ,  $\text{pH} = 7.0$ , phosphate buffer 5.0 mM and temperature of  $27^\circ\text{C}$ .

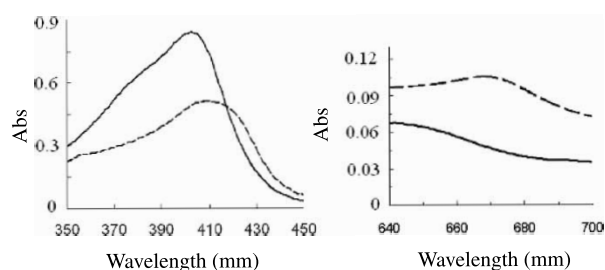


Figure 5. Electronic spectra of native and inactivated microperoxidase 11 (150  $\mu\text{M}$ ) at pH = 7.0, phosphate buffer 5.0 mM. **Solid line**, Native MP-11 (in the absence of hydrogen peroxide). **Dashed line**, produced inactivated MP-11 (or P-670) after 5 min incubation with  $[\text{H}_2\text{O}_2] = 3.0$  mM.

All of the above mentioned findings show that MP-11 and HRP have similar a suicide inactivation mechanism regarding both the inactivated product (see Figure 5) and kinetics of suicide-peroxide inactivation. On the other hand, similar to HRP, MP-11 also has two substrates: one of them is AH, the "benign substrate" and the other is  $\text{H}_2\text{O}_2$ , the damaging substrate. The benign substrate which does not damage the enzyme, is only used for monitoring the progress of the reaction.

Comparison of the inactivation parameters for HRP and MP-11, in addition to providing an estimation of kinetic parameters for the microperoxidase family also gives the extent of inactivation for each biocatalyst. Suicide-peroxide inactivation parameters for MP-11 and horseradish peroxidase are shown in Table I. Comparing the  $k_i$  values (see Table I) shows that the rate of inactivation of MP-11 is about 10 times faster than HRP. It must be noted that the minimum concentration for detection of inactivation of HRP was about 2 mM while the same value for MP-11 is about 0.4 mM of  $\text{H}_2\text{O}_2$ . This result was expected because of the availability of the heme group to the suicide substrate ( $\text{H}_2\text{O}_2$ ). Furthermore, in the presence of 3.0 mM of hydrogen peroxide, MP-11 inactivates much more rapidly with a rate constant of  $k_i = 1.30 \text{ min}^{-1}$  which shows a 2.65 times (1.30/0.491) increase in the strength of inactivation in relation to  $[\text{H}_2\text{O}_2] = 1.0$  mM (see Table I). In the peroxidase, the heme group is sandwiched and protected between two helices and only the edge of the pyrrole (IV) of heme is accessible to the solvent or substrate.

For HRP the extent of inactivation is negligible below  $[\text{H}_2\text{O}_2] < 0.5$  mM and/or  $[\text{H}_2\text{O}_2]/[\text{AH}] \sim 1.3$  ( $[\text{AH}] = 0.4$  mM,  $k_i = 0.009 \text{ min}^{-1}$ ), while for the ratio of  $[\text{H}_2\text{O}_2]/[\text{AH}] \sim 25$  peroxidase inactivates completely ( $[\text{H}_2\text{O}_2] = 10$  mM,  $[\text{AH}] = 0.4$  mM,  $k_i = 0.18 \text{ min}^{-1}$ ) within a 10 minute reaction cycle [37].

According to Table I, the inactivation extent for MP-11 at  $[\text{H}_2\text{O}_2] = 3.0$  mM becomes about 2.65 times greater in relation to 1.0 mM  $\text{H}_2\text{O}_2$ . On the other side, in comparison with HRP the extent of inactivation of MP-11 at  $[\text{H}_2\text{O}_2] = 3.0$  mM is

about 28.3 times (1.30/0.046) faster (see Table I). This problem needs to be solved for the application of MP-11 in systems containing hydrogen peroxide. This is the subject of our future research on protecting the active site of MP-11 against the destroying effect of hydrogen peroxide, by using efficient water soluble polymers and osmolytes.

## Conclusion

Suicide-peroxide inactivation of microperoxidase takes place in a manner and mechanism similar to horseradish peroxidase, but at much more rapid rates. Inactivation of MP-11 as a peroxidase model enzyme can occur even at low concentrations of hydrogen peroxide (0.4 mM). A complete and fast inactivation of MP-11 takes place within about 20 minutes at  $[\text{H}_2\text{O}_2] \sim 3$  mM. This clearly indicates a significant need to proceed with when such enzyme models are used for clinical, biochemical or chemical purposes. Furthermore, introducing the means and solutions for protection and preventing such severe inactivation effects is of prior and great importance, and is currently in progress in our laboratory.

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