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UNIVERSITY OF ALBERTA

OXIDATION OF PHENOLS AND ASCORBATE BY PEROXIDASES

BY 

HONGCHANG ZHANG

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE.

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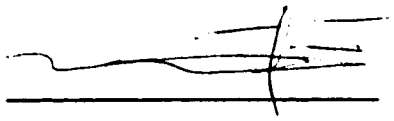
Signature: 

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and research for acceptance, a thesis entitled OXIDATION OF PHENOLS AND ASCORBATE BY PEROXIDASES submitted by HONGCHANG ZHANG in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE.

H. B. Dunford, Supervisor

J. E. Bertie, Committee Chairman

B. Leskiw, External Examiner

DATE: June 28, 1993

This thesis is dedicated to my wife, Yuwei

and my daughter, Fengyi

ABSTRACT

A stopped-flow kinetic study shows that the reaction rates of lactoperoxidase compound II with substituted phenols are greatly dependent upon the substituent on the benzene ring. Of the 15 phenols we studied, it has been possible to relate the reaction rate constants with the ionization constants of 11 phenols by a linear Hammett free-energy relationship: ($\log (k_X/k_H) = \rho\sigma$) at pH 7.0 and 25.0°C. The 11 *m*- and *p*-substituted phenols yield a ρ value of -2.7 ± 0.2. The data do not correlate with σ^+ values using the Brown-Okamoto equation. These results can be explained by a mechanism in which the substrate simultaneously gives an electron and proton to lactoperoxidase compound II, and they indicate that the active site is accessible to substrate. The correlation for these 11 phenols is worsened by introducing an additional Hansch term π for hydrophobicity of the substituent. On the other hand, for the four phenols which do not fit the simple Hammett relation, introduction of π values leads to a much better correlation. The rates of reduction of lactoperoxidase compound II by *p*-iodophenol have been studied from pH 6.5 to 11.2. The kinetics are influenced by an acid group of pK_E 9.5 in the active site of lactoperoxidase compound II and by the ionization of the substrate (pK_S 9.0). The acidic form of lactoperoxidase compound II and the electrically neutral, unionized form of phenol are the reactive species. A re-analysis of data on reactions of horseradish peroxidase compounds I and II with substituted phenols using more recent σ values yielded Hammett ρ values of -5.5 ± 0.3 and -4.5 ± 0.2.

The kinetics of ascorbic acid oxidation catalyzed by lactoperoxidase compound II and horseradish peroxidase compound I and II were investigated intensively as a function of pH at 25 ^{0}C in aqueous solutions of ionic strength 0.11. For reactions of lactoperoxidase compound II, deviations from first order behavior with respect to the enzyme were observed in the pH range of 4.5-6.7; no deviation was observed below pH 4.5. For horseradish peroxidase compound II, in the range from pH 2.8 to 6.4

deviations were observed in the pH range of 4.1-5.3 which may be attributed to the deprotonation of ascorbic acid. No deviation was observed in reactions of horseradish peroxidase compound I with ascorbic acid over the entire pH range (pH 2.7-7.1) of our study. The deviations behavior from first order behavior were explained by a mechanism involving formation of an enzyme-substrate complex which reacts with an additional molecule of substrate. The reduction of lactoperoxidase compound II by ascorbic acid appeared to be influenced only by the protonation/deprotonation of ascorbic acid. The rate-pH profile indicates that the acidic form of ascorbic acid is more reactive, also we observed that the Michaelis constant $K_{1,M}$ is pH independent. For horseradish peroxidase compound I and II, it seems that both the protonation/deprotonation of ascorbic acid and enzyme affect the reaction rate constant. In contrast to the reduction of lactoperoxidase compound II, the deprotonated form of ascorbic acid and protonated form of horseradish peroxidase compound I and II are more reactive. Also we found that a slope of +1 can be obtained from the plot of $1/K_{2,M}$ versus pH.

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CHAPTER ONE

INTRODUCTION

1.1 CLASSIFICATION OF PEROXIDASES

Peroxidases are enzymes which catalyze the oxidations of molecules by hydrogen peroxide or hydroperoxides of the type ROOH. The hemin (ferriprotoporphyrin) enzymes have been classified together with non-heme peroxidases as oxidoreductases with EC 1.11.1X, where X depends on the reductant of the biological system.

Among the hemin-containing peroxidases, three superfamilies can be classified (1):

1) plant peroxidase superfamily;

2) animal peroxidase superfamily;

3) catalase superfamily;

The detailed information about these three superfamilies are listed in Table 1.1.

The sequence of lactoperoxidase (LPO) has not been published but available biochemical data suggest it also belongs to the animal peroxidase superfamily. It appears that lactoperoxidase and myeloperoxidase have protohemin covalently bound to the apoprotein (2,3).

The three superfamilies listed in Table 1.1 are all thought to catalyze the herterolytic cleavage of the peroxide O-O bond (4).

1.2 STRUCTURE AND PROPERTIES OF PEROXIDASES

The loss in enzymatic activity during the catalytic cycle of peroxidase has been known since 1949 in the reaction of peroxidase with excess peroxide (5, 6). By using kinetic analysis, Acosta et al. reported the inactivation process of horseradish peroxidase by different hydroperoxides (7).

Horseradish peroxidase (HRP) is the most intensively studied peroxidase. HRP consists of 308 amino acid residues with a molecular weight of about 42,000 M_r, 18%

Table 1.1 Characteristics of hemin-containing peroxidase superfamilies

Superfamily	Origin	Families (examples)	EC number	Min. size Amino-acids	Heme	Axial Ligand	Catalytic residues
Plant peroxidase				300	Protohemin	His	RXXXH
	Plant	Acidic-suberin	1.11.1.7			(hydrogen	RLHFHD
	Plant	Acidic-lignin	1.11.1.7			bonded to	RLHFHD
	Plant	Neutral-basic	1.11.1.7			Asp)	RLHFHD
	Plant	Highly basic	1.11.1.7				RLHFHD
	Yeast	Cytochrome c peroxidase	1.11.1.5				RLAWHT
	Fungus	Coprinus	1.11.1.				
	Fungus	Ligninase	1.11.1				RLVFHD
	Fungus	MnII-dependent	1.11.1				RLTFHD
	Bacterium	Peroxidase-catalase	1.11.1.6				RMAWH
Animal peroxidase				600	Protohemin	?	REHNR
	Animal	Thyroid peroxidase	1.11.1.8				REHNR
	Animal	Eosinophil peroxidase	1.11.1.7				REHNR
	Animal	Myeloperoxidase	1.11.1.7		Protohemin-SH		REHNR
	Animal	Lactoperoxidase	1.11.1.7		Protohemin-SH		
	Animal	Prostaglandin endoperoxide Sythetase res 290-510	1.11.1				REHNR
Catalase	Animal,	plant, fungus, yeast	1.11.1.6	500	Protohemin	Tyr	His, Asn

of which is carbohydrate (8). HRP contains a single iron(III) protoporphyrin IX prosthetic group in which the iron center is coordinated to a proximal histidine residue in the fifth position (9). It had been assumed that the sixth coordination position of the iron(III) of native HRP was occupied by a water molecule. Over the past 16 years, evidence has accumulated that this distal-side coordination position is unoccupied. Longitudinal relaxation and ^{17}O ESR measurements indicated absence of water (10, 11). Photolysis of the iron(III)-nitric oxide complexes of metmyoglobin and HRP at liquid helium temperature led to a metmyoglobin species different from native metmyoglobin which is known from its crystal structure to contain water in the sixth coordinate position (12); the iron(III) HRP was the same before NO addition and after the NO was removed by photolysis (13). Optical spectra of complexes of metmyoglobin and HRP, such as fluoride, are similar, but spectra of the native species are different. All of this evidence points to HRP having a five-coordinate heme iron.

Generally, peroxidases consist of a heme and glycoprotein. The heme prosthetic group of most peroxidases is ferriprotoporphyrin IX (Fig. 1.1).

The protein portion of LPO consists of a single polypeptide chain of about 78,000 M_r (14-17). LPO is a glycoprotein with a carbohydrate content of about 10%. It has been proposed that LPO has two poles; one is hydrophilic and the other is hydrophobic (17). Based on microcalorimetry studies, the protein consists of two domains; a thermally labile domain containing the heme prosthetic group and a more stable domain (18). The overall protein structure of LPO consists of 65% β-sheets, 23% α-helix and 12% random coil (17). The primary structure of LPO is not available yet, but amino acid compositions have been reported by three labs (14-17, 19). LPO is a polycation due to the high content of basic lysine, arginine, and histidine residues.

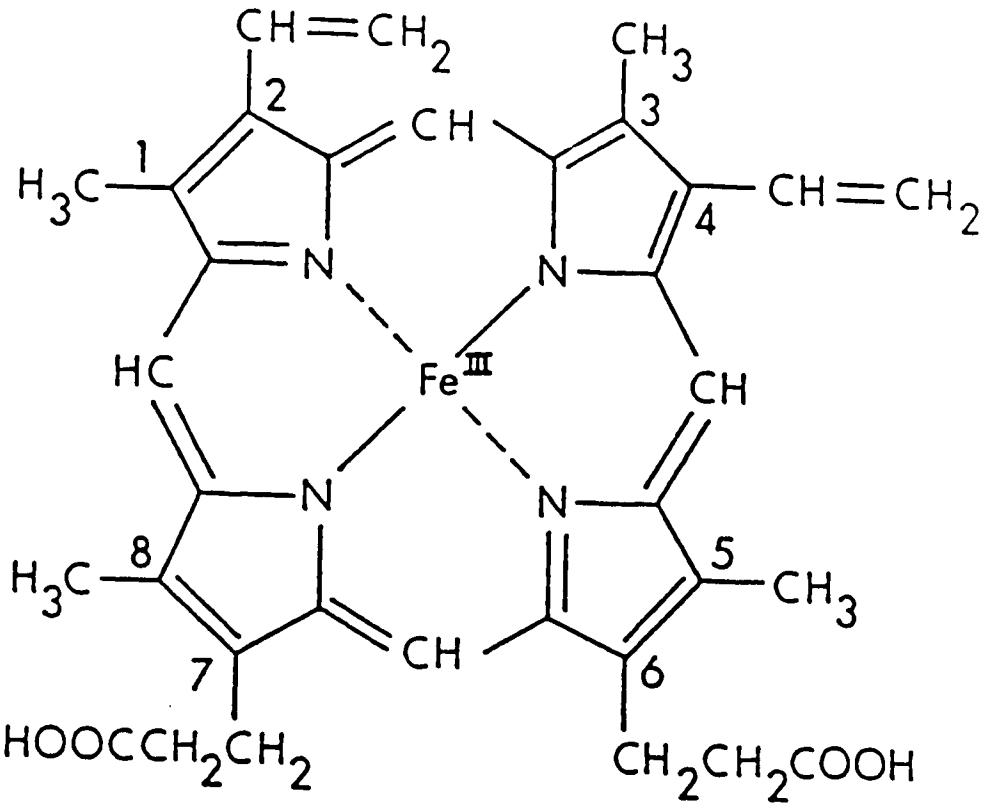


Figure 1.1. Structure of ferriprotoporphyrin IX: the prosthetic group for most peroxidases.

Also it was proposed that the cysteine residues are organized into as many as eight intramolecular disulfide bonds (17).

The structure of the heme of LPO and the nature of its association with the apoprotein have been controversial. Recently, it has been proposed that the heme of LPO is covalently linked to protein through a disulfide bond to a cysteine residue (3). The heme appeared identical to protoporphyrin IX except for the substitution of a $-CH_3SH$ group for the $-CH_3$ group at the C-8 position in Fig. 1.1.

There are some disputes about the sixth coordination position of LPO. For example, resonance Raman spectroscopy (20) and magnetic circular dichroism spectroscopy (21) suggest a ligand may occupy the sixth coordinate position. In contrast, proton-NMR spectroscopy (22) suggested that the sixth position is vacant as in HRP. Now, it is well accepted that the ferric iron in the heme of LPO is five coordinate, four of them are from the porphyrin ring, the fifth one is from the imidazole nitrogen of the proximal histidine residue (21, 23-25).

1.3. ENZYME INTERMEDIATES AND CATALYTIC MECHANISM

In the normal catalytic cycle, the native enzyme first reacts with a hydrogen peroxide to form enzyme compound I, which is two oxidation states higher than the native enzyme. The metal center, Fe(III), which activates the heterolytic cleavage of the hydroperoxide, stores one oxidizing equivalent and the porphyrin ring stores another oxidizing equivalent (26). In most cases, enzyme compound I undergoes a one-electron reduction by a reducing substrate (AH_2) to form compound II and a free radical, followed by a further one-electron reduction to give the native enzyme and a free radical (27, 28). In simplified terms the normal peroxidase cycle may be represented by the following:

[1.1] Peroxidase + H_2O_2 ⟶ Compound I + H_2O

[1.2] Compound I + AH_2 ⟶ Compound II + $\cdot AH$

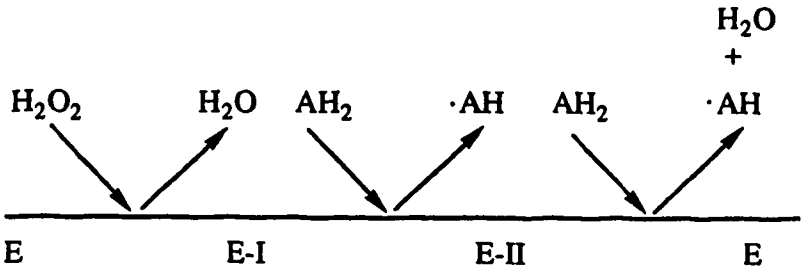
[1.3] Compound II + AH_2 ⟶ Peroxidase + $\cdot AH$ + H_2O

The sum of the above reactions is:

[1.4] $H_2O_2 + 2AH_2 = 2\cdot AH + 2H_2O$

There are several possible fates for the free radical product, depending upon its chemistry. It may dimerize, react with another substrate molecule, or attack another species causing cooxidation. It may reduce molecular oxygen to superoxide or it may be scavenged by molecular oxygen to form a peroxyl radical. The above scheme can also be represented as a modified type of ping-pong kinetics:

[1.5]



The stability of the above two intermediates are different for different enzymes. For HRP, its compound I is stable around neutral pH; and its compound II is stable at pH 9. For LPO, its compound I can only exist for about 25 ms and its compound II can be stable for a few minutes around neutral pH.

The three enzyme species of native enzyme, enzyme compound I and compound II can be easily distinguished by their Soret and visible spectra (29, 30).

1.4 pH EFFECTS ON THE ENZYMATIC ACTIVITIES OF PEROXIDASES

In general, enzymes are only active over a limited range of pH and in most cases a definite optimal pH is observed. This optimal pH may be due to (a) ionizations in the active site; (b) an effect of pH on the affinity of substrate to enzyme, the fall on either side of the optimum being due to a decreased saturation of the enzyme with substrate, due to a decreased affinity, or (c) an effect of pH on the stability of the enzyme, which may become irreversibly destroyed on one or both sides of the optimum. These effects may occur in combination.

The activities of many enzymes vary with pH in the same way that simple acids and bases ionize. The active sites of enzymes generally contain important acidic or basic groups. It is to be expected that if only one protonic form of the acid or base is catalytically active, the catalysis will depend on the concentration of the active form.

Although enzymes contain a multitude of ionizing groups, it is usually found that plots of rate against pH take the form of simple single or double ionization curves. This is because the only ionizations that are of paramount importance are those of groups that are directly involved in catalysis at the active site, or those of groups elsewhere that are responsible for mantaining the active conformation of the enzyme.

The reduction of enzyme compound I to compound II, and of compound II to native enzyme can be represented as:

[1.6] $\quad E\text{-}I + H^+ + e^- \rightarrow E\text{-}II$

[1.7] $\quad E\text{-}II + H^+ + e^- \rightarrow E$

It has been shown from NMR experiments that the proton does not add to the ferryl oxygen, but rather to the apoenzyme (31); it has also been established that an acid group with a pK_a of 8.6 is essential for most reactions of HRP-II (32-35). An

acid group of pK_a 5.1 to 5.4 has been shown to be important in most one-electron reductions of compound I of HRP (29, 32); and the distal His42 has been implicated.

Powers and Chang (36, 37) investigated the active sites of HRP-II, LPO, Lactoperoxidase compound III and reduced form of LPO at different pH values by using X-ray absorption spectroscopy. They concluded that with increasing pH, the bond length of the sixth coordinated ligand of the active site decreases from 2.04 to 1.91 Å for HRP-II; The five coordinated ferrous form of LPO was stable at pH 9, but at pH 6 it was rapidly converted to the six-coordinated form with a distal ligand at 2.18 Å.

1.5 BIOLOGICAL FUNCTIONS OF PEROXIDASES

The most studied enzymes in connection with the body's defense against invading micro-organisms, parasites and tumor cells are myeloperoxidase and lactoperoxidase (38, 39). LPO is detected in human saliva, milk and tears (39-41). Antimicrobial activity of peroxidases is due to their ability to catalyze H_2O_2-dependent oxidation of halide ions or thiocyanate (SCN^-) to produce powerful oxidizing agents (42). The produced oxidizing agent attacks microbial components, resulting in chemical modification of essential enzymes, transport systems and other functional components. Even though H_2O_2 is a powerful oxidizing agent, it reacts slowly with biological materials; and most cells have enzymes that could decompose H_2O_2 rapidly. Thus peroxidase-catalyzed oxidation of halides and thiocyanate conserves the oxidizing power of H_2O_2 in forms that react more rapidly, and for which the target cell may have no defense (38).

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CHAPTER TWO

HAMMETT ρσ CORRELATION FOR REACTIONS OF LACTOPEROXIDASE COMPOUND II WITH PHENOLS

2.1 INTRODUCTION

The reactions of phenols (and other organic substrates) with lactoperoxidase may be summarized as follows (1-3):

[2.1] $LPO + H_2O_2 \rightarrow LPO\text{-}I + H_2O$

[2.2] $LPO\text{-}I + AH_2 \rightarrow LPO\text{-}II + \cdot AH$

[2.3] $LPO\text{-}II + AH_2 \rightarrow LPO + \cdot AH$

[2.4] $2\cdot AH \rightarrow A_2H_2$ or $A + AH_2$

where lactoperoxidase and its two oxidized intermediates, compound I and II, are represented by LPO, LPO-I, and LPO-II, the phenols by AH_2, and their free radical product by ·AH. Because of their ability either to scavenge free radicals or to react directly with oxidant (4), phenols are widely used as antioxidants. An example of physiological significance is the fat-soluble α–tocopherol (Vitamin E) (5). *p*-Methoxyphenol exhibits action against maligant melanomas (6). It has been suggested that peroxidases could be used commercially to remove phenol contaminants from water supplies (7-9).

In this study, 15 phenols are reacted with LPO-II in order to gain information on the mechanism of their reactions and to determine whether there is any linear free energy correlation in their relative reactivities. In one case the pH-rate profile is obtained.

A version of this chapter has been submitted to Can. J. Chem. for publication

2.2 MATERIALS AND METHODS

Lactoperoxidase (EC 1.11.1.7, donor-H_2O_2 oxidoreductase) was purchased from Sigma. The RZ (A_{412}/A_{280}) of the sample used in experiments was 0.70-0.85 and its concentration was determined by using a molar absorptivity of 1.12×10^5 $M^{-1}cm^{-1}$ at 412 nm (10). LPO-II (λ_{max} 430 nm) (11) was obtained after mixing 2 μM LPO with 4 μM H_2O_2. It did not exhibit significant decay over a period of 6 min. Hydrogen peroxide was obtained as a 30% solution from Fisher; the concentrations of diluted stock solutions were determined by the peroxidase assay (12).

Solutions of all substrates were prepared each day by dissolving a weighed amount of the compound in an appropriate volume of water (deionized in a Milli-Q water purification system) to make fresh stock solutions of about 10 mM. The solutions were stored in bottles wrapped in aluminum foil and placed in ice to prevent photochemical reactions. The substrate solutions for each experiment were then made by diluting portions of the stock solution to give a desired range of concentrations, using phosphate buffer of pH 7.0 (0.025 M, ionic strength $\mu = 0.11$). In the case of *p*-iodophenol, buffers in the range of pH 6.5 to 11.2 were used in the determination of the pH-rate profile. At least a 10-fold excess of substrate over the enzyme was always used so that the reaction occurred by a pseudo-first-order process. Stock buffer solutions used for both enzyme and substrate were made up to ionic strength $\mu = 0.11$ M by adding sufficient KNO_3 to 0.025 M buffer. For solutions of pH 6.0 to 7.0, citrate-phosphate buffers were used. Phosphate buffers were used between pH 7.0 and 9.0, and carbonate buffers were used above pH 9.0.

Kinetic measurements were made on a Photal (formerly Union Giken) stopped-flow spectrophotometer model RA-601. The 1-cm cells were thermostated at 25.0°C for all the experiments. One of the drive syringes of the stopped-flow spectrophotometer was filled with LPO-II solution, freshly prepared prior to the

experiment, and the other syringe was filled with substrate. The reaction was then followed by monitoring the appearance of the native LPO at 412 nm. Each result quoted in this paper is an average value of 10-20 experimental values.

2.3 RESULTS

Pseudo-first-order traces were observed when the substrate concentration was 10 times larger or more compared to that of LPO-II. An example is shown in the inset to Fig. 2.1. The apparent second-order rate constant, k_{app}, was found to be related to the observed pseudo-first-order rate constant, k_{obs}, by the expression:

[2.5] $$k_{obs} = k_{app}[S] + k_0$$

where [S] is the concentration of substrate. Thus k_{app} was determined from the slope of the plot of k_{obs} against substrate concentration. Fig. 2.1 is an example, where k_{app} is 1.13×10^6 $M^{-1}s^{-1}$. The intercept k_0 is close to but not equal to zero in Fig. 2.1, which can be explained by the small autodecay of LPO-II (15). Second-order rate constants determined in this study for 15 *meta-* and/or *para*-substituted phenols reacting with LPO-II at pH 7.0 are listed in Table 2.1 with corresponding literature values for Hammett σ (13), Brown-Okamoto σ^+ (14) and Hantsch π (13) parameters.

Hammett plot

A Hammett plot (16) according to equation [2.6]

[2.6] $$\log (k_X/k_H) = \rho\sigma$$

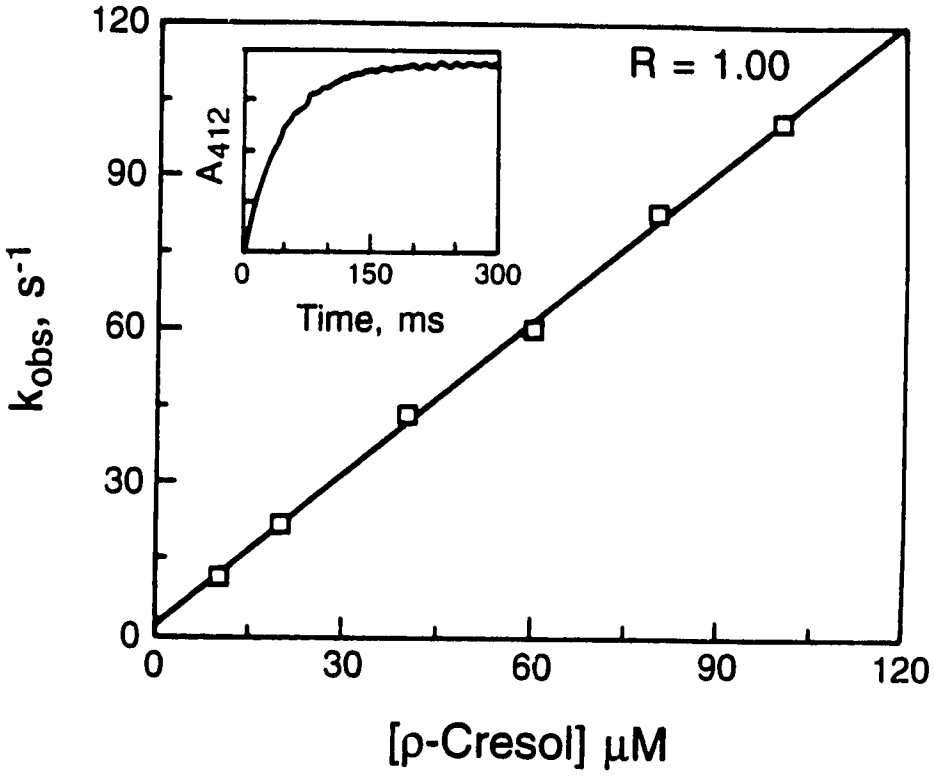


Fig. 2.1. Linear plot of pseudo-first order constant versus [*p*-cresol] for the reaction between LPO-II and *p*-cresol. pH 7.0; ionic strength 0.11 M; temperature 25.0 °C; [LPO-II] 1.0 μM. The inset shows an example of a first order exponential trace (20 μM p-cresol) from which k_{obs} was obtained.

Table 2.1: Rate constants for the reactions of LPO-II with *m*- and *p* -substituted phenols at 25°C, pH=7.0 and ionic strengh 0.11 M; Hammett σ values[a], Brown-Okamoto σ^+ values[b] and Hansch π values[a].

Substituent	k_{app} ($M^{-1}s^{-1}$)	σ	π	σ^+
p -COO⁻*	$(9.70 \pm 0.10)\cdot 10^3$	0.00	-4.36*	0.421
p -CHO	$(3.20 \pm 0.04)\cdot 10^4$	0.42	-0.65	
m -CHO	$(1.45 \pm 0.05)\cdot 10^5$	0.35	-0.65	
p-Cl*	$(1.12 \pm 0.03)\cdot 10^6$	0.23	0.71*	0.114
m-coumaric acid	$(1.72 \pm 0.08)\cdot 10^5$	0.19	-0.22	
p-I*	$(1.66 \pm 0.01)\cdot 10^6$	0.18	1.12*	0.135
m-OC_2H_5*	$(3.80 \pm 0.20)\cdot 10^6$	0.10	0.38*	
p-coumaric acid	$(2.44 \pm 0.04)\cdot 10^5$	0.03	-0.22	
H	$(1.40 \pm 0.02)\cdot 10^6$	0.00	0.0	0.00
m-CH_3	$(1.09 \pm 0.05)\cdot 10^6$	-0.07	0.56	-0.066
m, p-$(CH_3)_2$	$(1.91 \pm 0.05)\cdot 10^6$	-0.083	1.12	
p-CH_3	$(1.10 \pm 0.10)\cdot 10^6$	-0.17	0.56	-0.311
p-OC_2H_5	$(1.90 \pm 0.10)\cdot 10^6$	-0.24	0.38	
p-OCH_3	$(2.40 \pm 0.06)\cdot 10^6$	-0.13[c]	-0.02	-0.778
p-OH	$(1.16 \pm 0.07)\cdot 10^7$	-0.37	-0.67	-0.92

[a]Reference (13) , [b]Reference (14), [c]Reference (30).

*Phenols for which π values led to an improved correlation.

is shown in Fig. 2.2. A rate constant, k_H, of $1.40 \times 10^6\ M^{-1}s^{-1}$ for unsubstituted phenol reacting with LPO-II is used as reference; k_X is the rate constant for the substituted phenol. From the slope of the linear plot in Fig. 2.2 the value of ρ, the Hammett reaction constant or susceptibility factor, is -2.7 ± 0.2. The electronic substituent constant σ depends on the nature and position of the substituent group. It can be seen that four of the phenols, labelled with asterisks in Table 2.1 and as open squares in Fig. 2.2, do not fit the plot. The correlation among the 11 phenols which fit the Hammett correlation was worsened by attempting to introduce a hydrophobicity term in addition to the electronic substituent effect.

Attempted Brown-Okamoto Plot

An attempted plot according to the Brown-Okamoto equation (14):

[2.7] $$\log (k_X/k_H) = Q\sigma^+$$

is shown in Fig. 2.3 for the eight phenols for which σ^+ values are available. Q is the reaction constant, which depends on the reaction and the conditions under at which it occurs. It is obvious that there is not a linear correlation.

Possible Role of a Hydrophobic Factor

Since four phenols do not fit the Hammett correlation, we used an extended equation [2.8] which covers both electronic and hydrophobic effects:

[2.8] $$\log (k_X/k_H) = \rho\sigma + r\pi + c$$

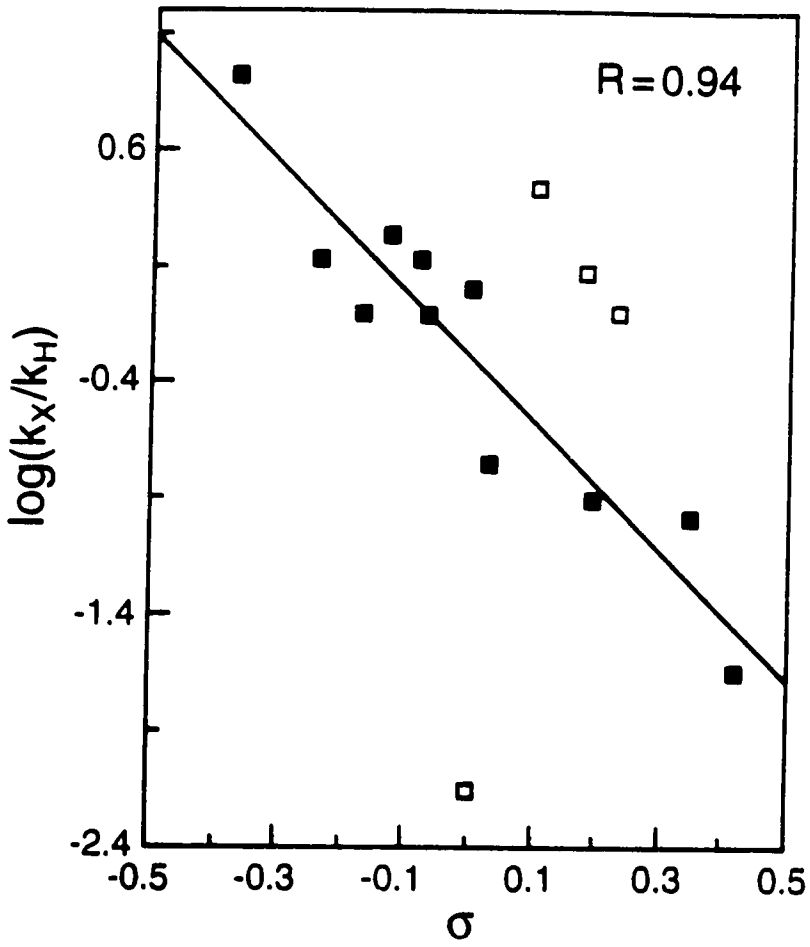


Fig. 2.2. Hammett plot for reactions of LPO-II with 15 phenols at 25°C, pH 7.0 and ionic strength 0.11 M. The correlation coefficient, R, of 0.94 is for the 11 phenols denoted by solid squares.

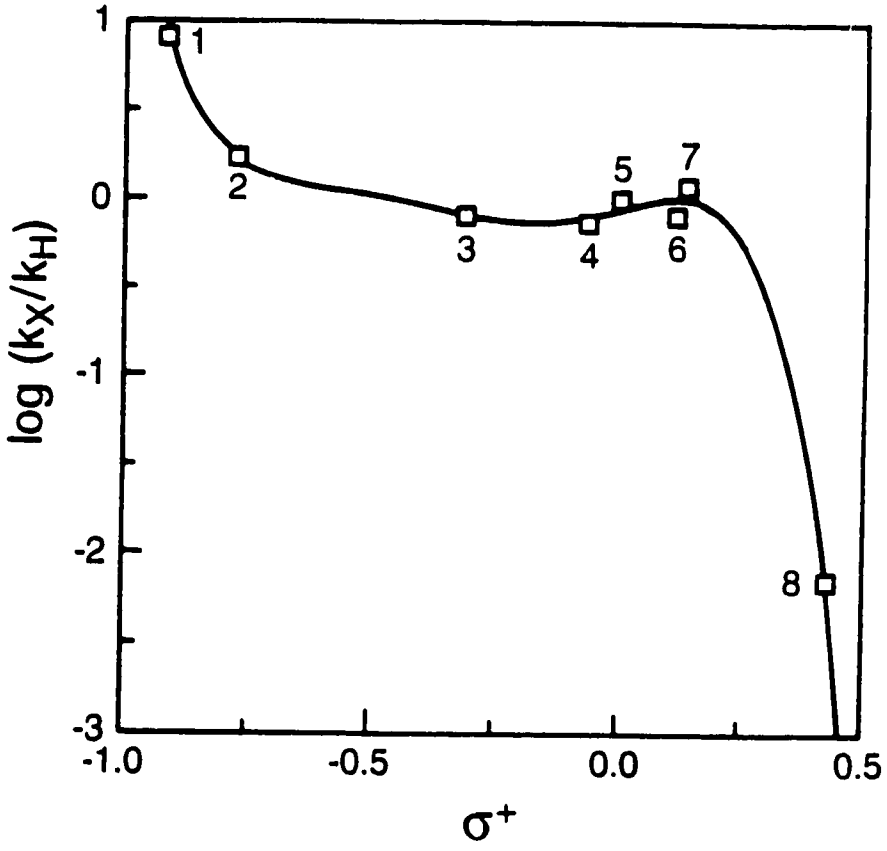


Fig. 2.3. Brown-Okamoto plot for the rate constants of LPO-II reduction by various substituted phenols at pH 7.0, ionic strength 0.11 M and temperature 25°C. Substituent: (1) *p*-OH, (2) *p*-OCH_3, (3) *p*-CH_3, (4) *m*-CH_3, (5) H, (6) *p*-Cl, (7) *p*-I, (8) *p*-COO^-.

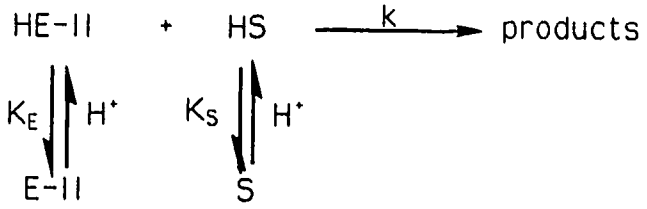
In equation [2.8], π is the Hansch hydrophobic parameter, r is its coefficient and c is a constant. The term $\rho\sigma$ has the same meaning as in the Hammett relation. Among the 15 phenols we studied, 11 of them fit the Hammett plot very well, which means that for these 11 phenols, the electronic effect plays the key role for enhancing or decreasing the reaction rate constant. The hydrophobic effect of these 11 phenols is not significant. Therefore their π values were set equal to zero. Using all σ values and four π values (for those four phenols marked with an asterisk in Table 2.1), the results of a multiple linear regression analysis are $\rho = -2.5 \pm 0.2$, $r = 0.5 \pm 0.1$ and $c = -0.11 \pm 0.04$. By plotting the measured values of log (k_X/k_H) against values calculated in the above manner from equation [2.8] for all 15 phenols, we obtained a correlation coefficient $R = 0.93$. If all the π values for 15 phenols are used in the multiple linear regression analysis, the results are $\rho = -2.0 \pm 0.2$ (which is far removed from $\rho = -2.7$ obtained from the simple Hammett correlation), $r = 0.42 \pm 0.1$ and $c = -0.14 \pm 0.05$. By plotting the experimental values of log (k_X/k_H) against values calculated in the above manner from equation [2.8] for all 15 phenols, we obtained a correlation coefficient of $R = 0.88$ which is worse than the value from the former analysis in which hydrophobicity factors are selectively applied.

Our conclusion is that although hydrophobic factors π appear to account for the deviation of four phenols from the Hammett correlation, there is no obvious reason why π values should be applied selectively. However there appears to be no other obvious explanation for the deviations.

pH dependence

A log k_{app} *versus* pH plot is shown in Fig. 2.4 for the reaction of *p*-iodophenol with LPO-II. The reaction mechanism which is used to fit the experimental data is:

[2.9]



where HE is the protonated form of LPO-II and HS is unionized *p*-iodophenol. Equation [2.9] leads to:

[2.10]

$$k_{app} = \frac{k}{\left[1 + \frac{K_E}{[H^+]}\right]\left[1 + \frac{K_S}{[H^+]}\right]}$$

where k_{app} is the experimentally determined pH-dependent second order rate constant and k is defined in equation [2.9]. K_E and K_S are acid dissociation constants of LPO-II and substrate respectively. A best fit to the experimental data was obtained using a non-linear least squares program in which k, K_E and K_S are adjustable parameters. From the fit, $k = (1.9 \pm 0.2) \times 10^6\ M^{-1}s^{-1}$, $pK_E = 9.5 \pm 0.4$ and $pK_S = 9.0 \pm 0.3$), which are in agreement with reported values for pK_E of 9.7 (17) and pK_S of 9.3 (18). The best fit line is shown in Fig. 2.4. If one simplifies equation [10] by setting either equilibrium constant K equal to zero, and allows the non-linear least squares program to adjust the remaining single K to its best fit value, a value of $pK_a = 8.9$ is obtained. The best-fit curve in this case is shown as a dashed line in Fig. 2.4, which is not a satisfactory fit.

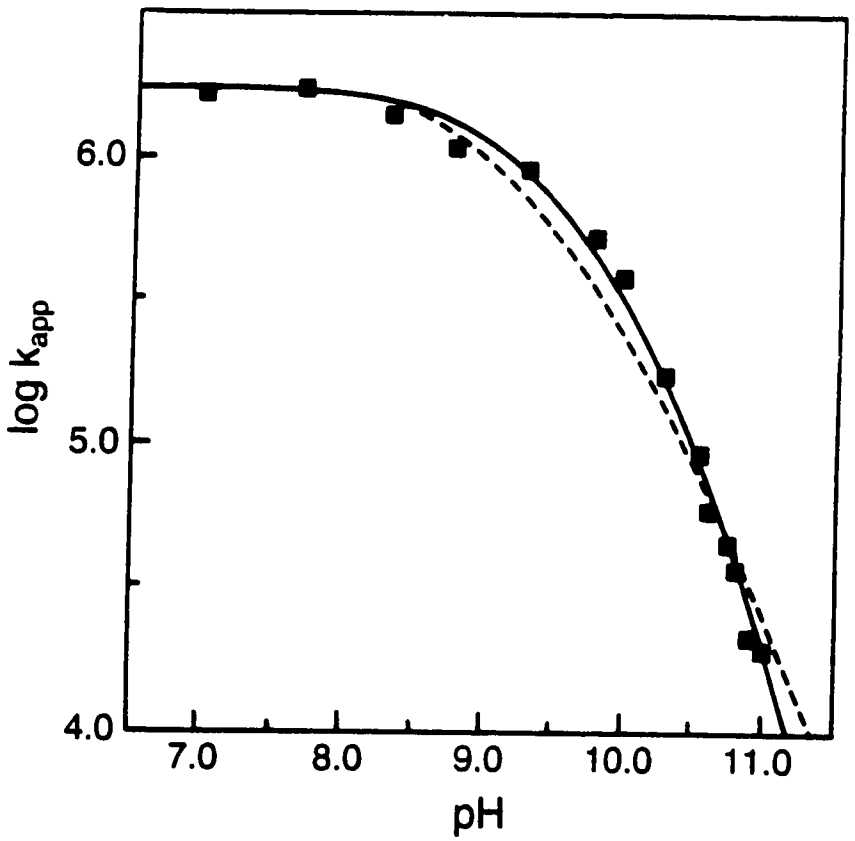


Fig. 2.4. pH-rate profile for the reaction of LPO-II with *p*-iodophenol at 25°C and ionic strength 0.11M. Experimental (solid squares), best-fit curve with two K's (solid line), best-fit curve with single K (dashed line). The fit is obviously better with the inclusion of two ionization constants.

2.4 DISCUSSION

The Hammett plot in Fig. 2.2 shows a reasonable correlation of log (k_X/k_H) *versus* σ values of 11 phenols. Thus, for those 11 phenols, the electron-donating or withdrawing effect of *m*- and *p*-substituents explains the relative reactivities; no hydrophobicity factor is required. Electron donating groups (negative σ values) enhance the reaction rate and the converse is also true. From Fig. 2.2, it can be seen that four points do not fit the Hammett plot. The observed values of k_X for *p*-Cl, *p*-I , *m*-OC_2H_5 substituted phenols are 10 times greater and for *p*-COO^- is 100 times less than that predicted by the linear correlation for the other substituted phenols. The improved correlation obtained by introduction of Hansch π parameters is evidence that the hydrophobic (or hydrophilic) effect of the substituent on the benzene ring, for those four phenols deviating from the Hammett plot for LPO-II, accounts for their anomalously high (or low) reactivities. *p*-COO^- is a strongly hydrophilic group; this will interfere with the accessibility of *p*-COO^- substituted phenol to the active site of LPO-II, so the reaction rate is slowed. However, *p*-Cl, *p*-I and *m*-OC_2H_5 are hydrophobic groups that will promote binding to a hydrophobic region in the active site of LPO-II. Therefore their reaction rates are increased.

We have re-analyzed the data of Job and Dunford (19) for the reactions of HRP-I with phenols, using the σ values of Hansch and Leo (13), which were published three years after the original HRP-I publication. The result is a value of ρ of -5.5 ± 0.3 compared to the original value of -6.94. We regard the new value as more accurate. Because of their enhancement of luminescence of the HRP-luminol reaction (20), the reactions of three more phenols with HRP-I have been studied (21) and they are more reactive than predicted by the Hammett correlation. These are phenols with the substituents *p*-phenyl, *p*-I and *p*-Cl which all have hydrophobic π values.

We have also re-analyzed the data for the Hammett correlation of HRP-II-phenol reactivities. The originally published ρ value was -4.6 (22). Since then another study of HRP-II reactions with phenols has been completed (23), which is generally in agreement with the earlier data (22). An exception is the rate of reaction of *p*-Cl phenol and we have reason to believe the newer value is more accurate. Using only σ values of Hansch and Leo where possible, we obtain an identical ρ value of -4.5 ± 0.2 by inclusion of data for 20 of 23 phenols from refs. (22) and (23). Of the remaining three phenols, two are exceptionally unreactive phenols (*p*-NH_2 and *p*-COO^- substituents) and we note that they are hydrophilic. The other is more reactive (*m*-Cl substituent). The phenolic luminescence enhancers, with *p*-phenyl, *p*-I and *p*-Cl substituents, are also more reactive with HRP-II than predicted by the Hammett correlation (23). All four of the latter phenols are hydrophobic. Therefore the trend for both HRP-I and HRP-II reactions is the same as for LPO-II: most phenols fit well to a Hammett correlation; and for the few exceptions strongly hydrophilic substituents retard the reaction and hydrophobic groups enhance it.

A recent study showed that the rate constants for phenols reacting with prostaglandin H synthase (PGHS) compound II also fit a Hammett plot, with ρ = -2.0 at 4°C (24). There were dual effects of phenols on the cyclooxygenase activity of PGHS, with small concentrations of phenols enhancing cyclooxygenase activity and large concentrations causing inhibition. Both electronic and hydrophobic effects are evident. Multiple linear regression analysis according to equation [2.8] led to ρ values of -2.2 (stimulation) and -2.6 (inhibition) at 25°C (24).

The pH-rate profile for the reaction of LPO-II with *p*-iodophenol (Fig. 2.4) is not the same as that reported by Hodgson and Jones (20) for the reaction between the same substrate and horseradish peroxidase compound II (HRP-II), where the pH dependence of log k_{app} above pH 8.0 is linear with a slope of -1. In our case, with increasing pH, the plot of log k_{app} *versus* pH has a slope which changes from zero to

increasingly negative values, as LPO-II and phenol each lose a proton. By inclusion of ionization constants for both LPO-II and phenol, the excellent fit shown by the solid line in Fig. 2.4 is obtained. If one postulates that only a single ionization is affecting the kinetics the fit to the experimental data is not as good, as shown by the dashed line in Fig. 2.4. The data are entirely consistent with a protonated form of LPO-II reacting with the unionized *p*-iodophenol. The phenoxide ion appears to be unreactive (22).

Brewster *et al.* (25) used semiempirical molecular orbital methods to calculate several properties of substrate molecules and compared them with rates of reaction of HRP-II. They did not make a choice between a hydrogen atom *versus* an electron transfer mechanism. Sakurada *et al.* (23) showed that the HRP-II reaction rates with phenols correlate well HOMO and LUMO energy levels.

Our major conclusion, applicable to substrates such as phenols and aromatic amines (23, 26) which react via a normal peroxidatic cycle, native peroxidase --> compound I --> compound II --> native peroxidase, is the following. The Hammett $\rho\sigma$ correlations, in themselves providing a rationale for the rates of most phenols and aromatic amines, indicate that these substrates react by hydrogen atom transfer: an electron and a proton are transferred simultaneously from the reducing substrate to the enzyme. Hydrophobicity (or hydrophilicity) can account, at least qualitatively, for most deviations from the Hammett correlations. The hydrogen atom transfer mechanism is in accord with the proton balance for conversions of compound I to II and compound II to native enzyme (27). The same mechanism explains the results obtained with enols. Schematic representations of the mechanisms for enols and phenols (28, 29) and aromatic amines (26) have been published, which show that the active sites of compounds I and II are accessible to substrate.

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CHAPTER THREE

KINETICS AND MECHANISM OF THE OXIDATION OF ASCORBIC ACID CATALYZED BY PEROXIDASES

3.1 INTRODUCTION

Peroxidases (donor: H_2O_2 oxidoreductase with EC number of 1.11.1.) are enzymes which catalyze the oxidation of a wide variety of molecules by hydroperoxides of the type ROOH. The normal peroxidatic cycle involves the intermediate compounds I and II (1-4). Myeloperoxidase plays an important role in the anti-bacterial function of mammalian white blood cell (5). Lactoperoxidase (LPO) is a constituent of mammalian milk, saliva and tears. It is often used as a model for a drug oxidizing system. Horseradish peroxidase (HRP) is an example of plant peroxidases. It has been proposed that HRP is the key enzyme in the rapid adaption process to changes in environment for whole plants or for some of their organs (6).

Ascorbic acid (AA) is an antioxidant (7). AA oxidation leads to formation of the ascorbate free radical in a one electron transfer reaction (8). The ascorbate free radical scavenges other more destructive radicals (7, 9). AA has also been implicated in various physiological functions. It was found to stimulate the chlorinating and antimicrobial activity of human myeloperoxidase (10).

Herein we reported on the kinetics and mechanisms of the reactions between lactoperoxidase compound II (LPO-II), horseradish peroxidase compound I (HRP-I), horseradish peroxidase compound II (HRP-II) and ascorbic acid as a function of pH.

3.2 MATERIALS AND METHODS

Materials. Lactoperoxidase (EC 1.11.1.7, donor-H_2O_2 oxidoreductase) was purchased from Sigma. The RZ (A_{412nm}/A_{280nm}) of the sample used in experiments was 0.70-0.85 and its concentration was determined by using a molar absorptivity of 1.12×10^5 $M^{-1}cm^{-1}$ at 412 nm (11).

A version of this chapter will be submitted for publication

Horseradish peroxidase, grade I, was purchased from Boehringer-Mannheim as an ammonium sulfate suspension. Stock solutions were prepared by dialyzing the suspension against deionized water and passing it through a Millipore filter prior to use. The purity number RZ (A_{403nm}/A_{280nm}) of the HRP stock solutions was greater than 3.3. The concentration of HRP was determined spectrophotometrically at 403 nm using a molar absorptivity of 1.02×10^5 $M^{-1}cm^{-1}$ (12).

L-ascorbic acid was purchased from Sigma. Aqueous solutions were freshly prepared by using an appropriate volume of deionized water from a Milli-Q water purification system each day before experiments were performed. The solutions were stored in bottles wrapped in aluminum foil and placed in ice to prevent photochemical reactions. The stability of a 0.2 mM solution of ascorbic acid was tested by measuring the absorbance at 265 nm as a function of time (13). At 4.0 °C, it is stable for 9 h. All chemicals were reagent grade and used without further purification.

H_2O_2 (30%) and $K_4Fe(CN)_6$ were obtained from Fisher; the concentrations of $K_4Fe(CN)_6$ stock solutions were determined by weight while those of H_2O_2 were determined by peroxidase assay (14).

All experiments were carried out at 25 ± 0.5 °C. Stock buffer solutions used for both enzyme and substrate were made up to ionic strength μ = 0.11 M by adding sufficient Na_2SO_4 to buffer. For solutions of pH 3.0 to 5.0, citrate buffers were used. Phosphate buffers were used between pH 5.0 and 7.5.

Preparation of LPO-II. Lactoperoxidase compound II (λ_{max} 430 nm) stable for 6 minutes without significant autodecay was obtained after mixing 2 μM LPO with 4 μM H_2O_2 (15).

Preparation of HRP-I and HRP-II. HRP-I was prepared by the addition of one equivalent of H_2O_2 to the native HRP at the desired pH (16). Because of the difficulty of preparing pure compound II at neutral and acidic pH's, stable HRP-II was obtained in 2.5 mM Tris-HCl buffer of pH 9.0 by adding one equivalent of H_2O_2

and one equivalent of $K_4Fe(CN)_6$ to the native HRP solution (1). Then a pH jump method was used to obtain HRP-II at the desired pH.

Stopped-flow kinetic experiments. At least a 10-fold excess of substrate over the enzyme was always used so that the reaction occurred by a pseudo-first-order process. Kinetic measurements were made on a Photal (formerly Union Giken) stopped-flow spectrophotometer model RA-601. The 1-cm cells were thermostated at 25 ± 0.5 °C. One of the drive syringes of the stopped-flow spectrophotometer was filled with enzyme solution, freshly prepared prior to the experiment, and the other syringe was filled with substrate.

Rapid scan spectral analyses. Spectral analyses were made on a Photal (formerly Union Giken) model RA-601 rapid reaction analyzer which is equipped with a solid-state photodiode array. Spectral changes during reactions of HRP-I and HRP-II with ascorbic acid were monitored in the Soret region.

The rate of reaction of LPO-II was followed by monitoring the disappearance of LPO-II at 430 nm which is the isosbestic point between LPO and LPO-I; HRP-I was monitored at 411 nm, the isosbestic point between HRP and HRP-II; and the reaction of HRP-II was monitored at 427 nm, the isosbestic point between HRP and HRP-I. Each result is an average value of 10-20 experimental values.

3.3 RESULTS

The first-order kinetics observed in the presence of excess AA are described by:

$$[3.1] \qquad -\frac{d[E]}{dt} = k_{obs}[E]$$

where E represents any of the peroxidase intermediate compounds.

Oxidation of AA by LPO-II

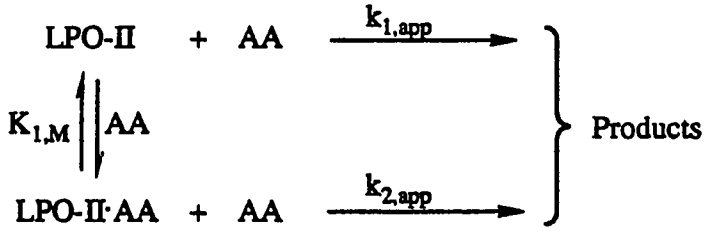
At pH values less than 4.5 exponential traces were obtained (Fig. 3.1a). Above pH 4.5, biphasic stopped-flow traces were observed consisting of a rapid disappearance followed by a slower disappearance of LPO-II (Fig. 3.1b). Good fits can only be obtained for the latter part of the biphasic stopped-flow traces. Similar cases were observed in the transient-state kinetics for the reactions of HRP-I and HRP-II with 1-methyoxy-4-(methylthio)-benzene and with *p*-aminobenzoic acid (17, 18).

Below pH 4.5, the plots of k_{obs} versus concentrations of AA are linear (Fig. 3.2a). In this case, the apparent second-order rate constant $k_{1,app}$ can be obtained from the slope.

[3.2] $$k_{1,obs} = k_{1,app}\,[AA]$$

Above pH 4.5, the plots of $k_{1,obs}$ versus concentrations of AA are convex with respect to the abscissa (Fig. 3.2b). In order to fit the observed kinetics of LPO-II with AA, a possible reaction scheme is proposed:

[3.3]



in which both uncomplexed LPO-II and complexed LPO-II can react with AA. From Eq. 3.3, Eq. 3.4 can be derived:

[3.4] $$k_{1,obs} = \frac{k_{1,app}\,[AA] + k_{2,app}\,[AA]^2/K_{1,M}}{1 + [AA]/K_{1,M}}$$

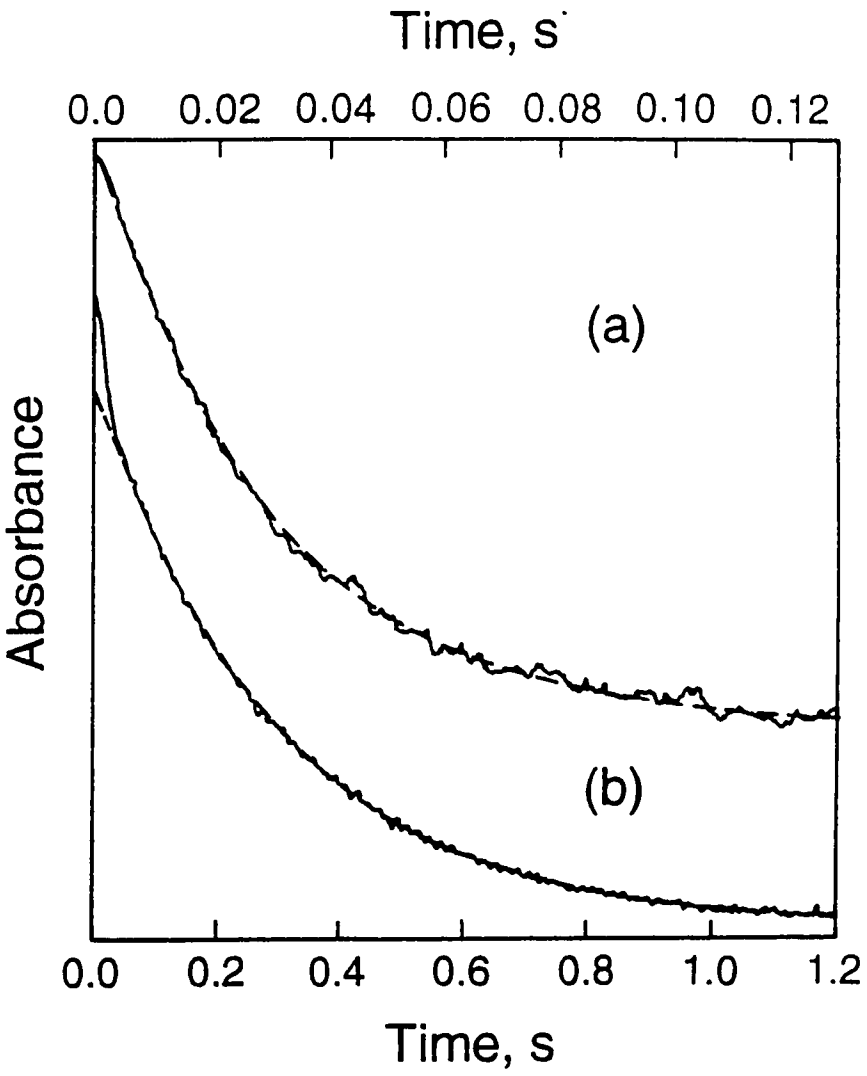


Fig. 3.1 Stopped-flow time traces of the reaction between LPO-II and AA at different pH. μ 0.11 M; temp. 25 ^{0}C; final conc. of [LPO-II] 1.0 μM, [AA] 0.1 mM; (a) pH 3.9 (b) pH 5.4. The solid lines are the experimental traces and the dashed lines are the best-fit curves.

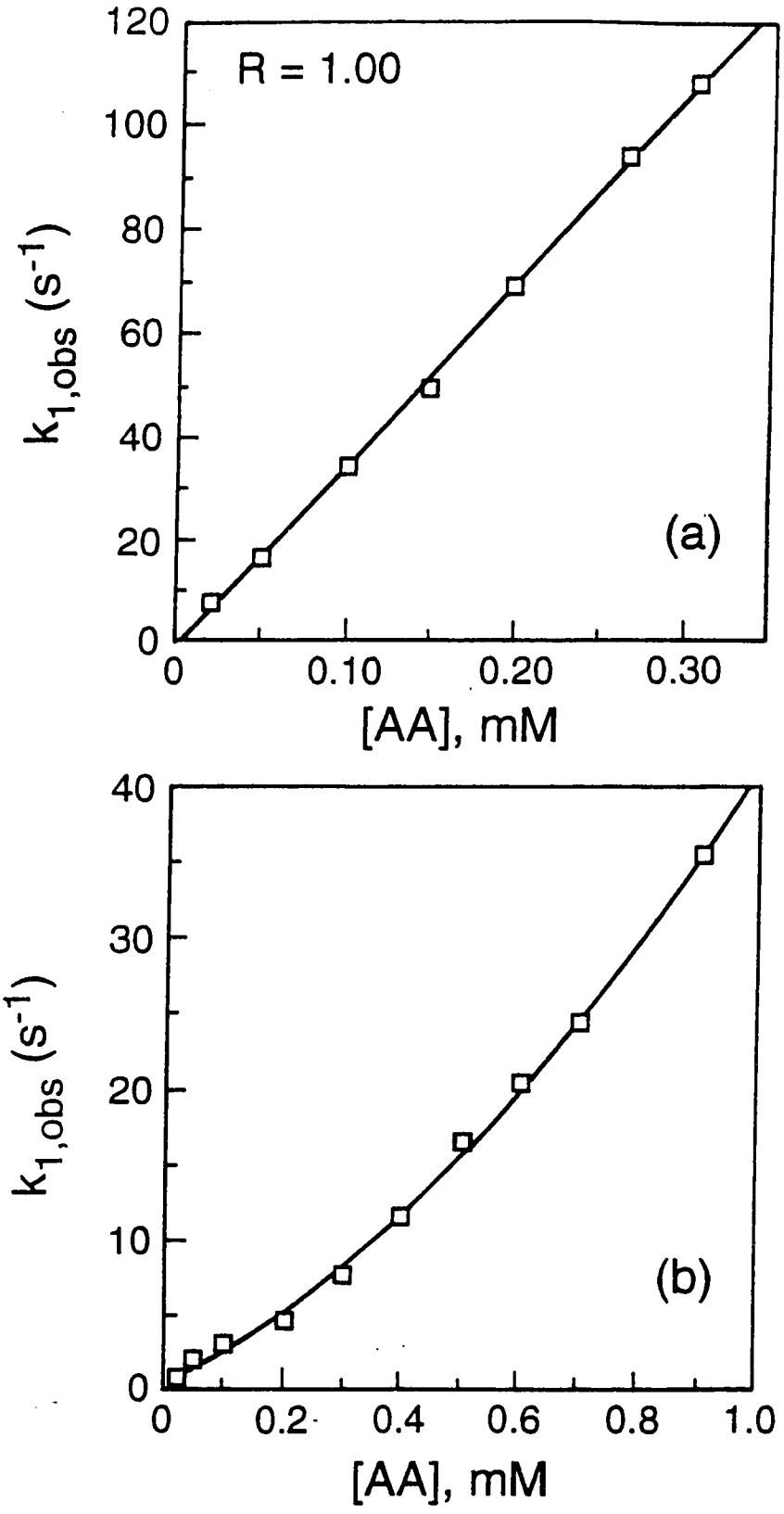


Fig. 3.2 Plot of k_{obs} vs. AA for the reaction of LPO-II with AA under the same conditions as described in Fig. 3.1. (a) pH 3.9 (b) pH 5.4

A nonlinear least squares analysis was used to determine the best fit curve (Fig. 3.2b) and the parameters $k_{1,app}$, $k_{2,app}$ and $K_{1,M}$ for pH values >4.5, where $k_{1,app}$ and $k_{2,app}$ are the apparent second-order rate constants and $K_{1,M}$ is the Michaelis constant. The results are listed in Table 3.1.

The plots of $k_{1,app}$, $k_{2,app}$ and $1/K_{1,M}$ versus pH are shown in Fig. 3.3. The acidity of the reaction medium affects the kinetics of the oxidation of AA by LPO-II, apparently by protonation of AA. As can be seen from Fig. 3.3, as the pH increases, $k_{1,app}$ and $k_{2,app}$ decrease until they approach a constant small value. The constant $K_{1,M}$ is pH independent.

Oxidation of AA by HRP-I

The spectral changes observed during the reaction indicate that HRP-I is first reduced to HRP-II, then converted to the native state (Fig. 3.4) showing that there are two one-electron reactions occurring.

The stopped-flow traces for the reactions between HRP-I and AA are pseudo-first order yielding rate constants $k_{2,obs}$. The plots of $k_{2,obs}$ versus concentrations of AA are linear over the whole pH range of our studies yielding second-order rate constants $k_{3,app}$ which are listed in Table 3.2.

The approximately bell-shaped plot of $k_{3,app}$ versus pH is shown in Fig. 3.5. At least to a first approximation both the increase and decrease in rate with increasing pH can be accounted for by deprotonations.

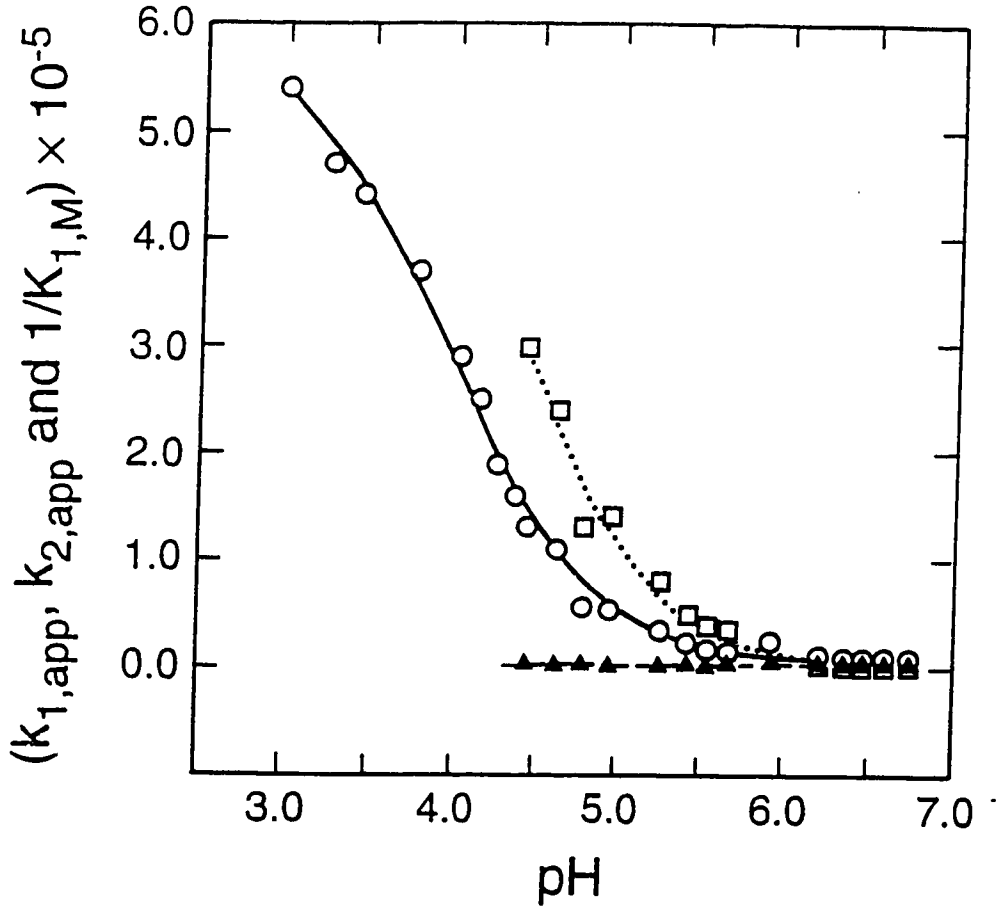


Fig. 3.3 pH rate profiles of $k_{1,app}$, $k_{2,app}$ and $1/K_{1,M}$ for the reaction of LPO-II with AA at 25 ^{0}C, μ 0.11 M; (open circles) $k_{1,app}$, (open squares) $k_{2,app}$, (solid triangles) $1/K_{1,M}$

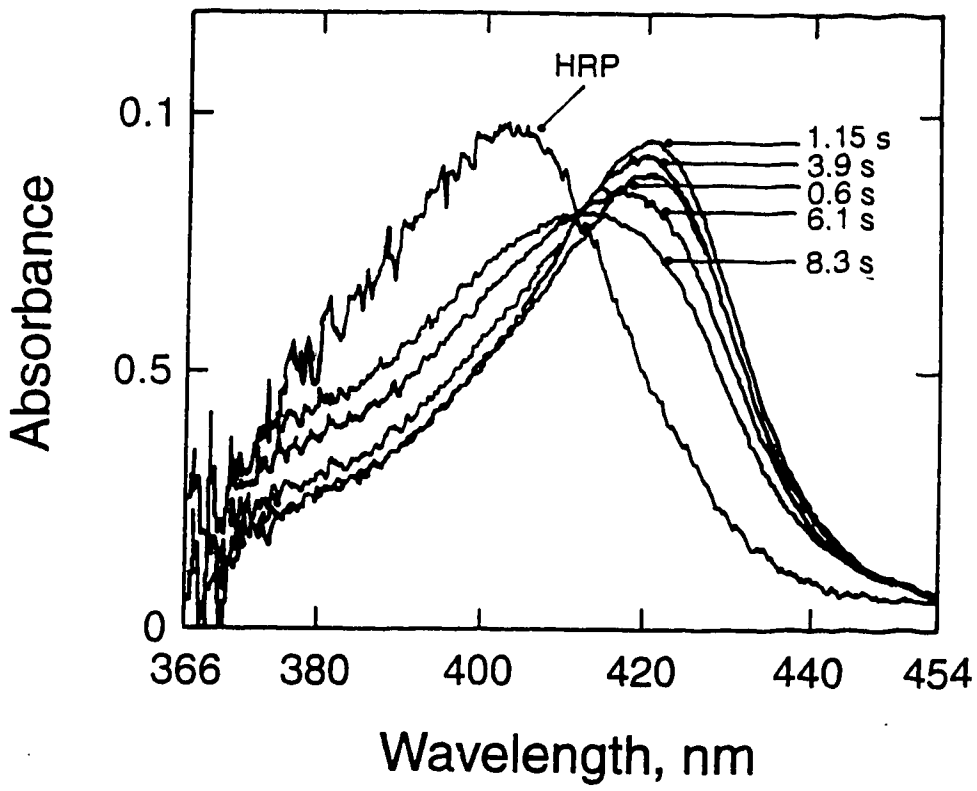


Fig. 3.4 Rapid scan spectra for reaction between HRP-I (1.0 μM) and AA (0.1 mM) at pH 6.5. μ 0.11 M; temp. 25 ^{0}C

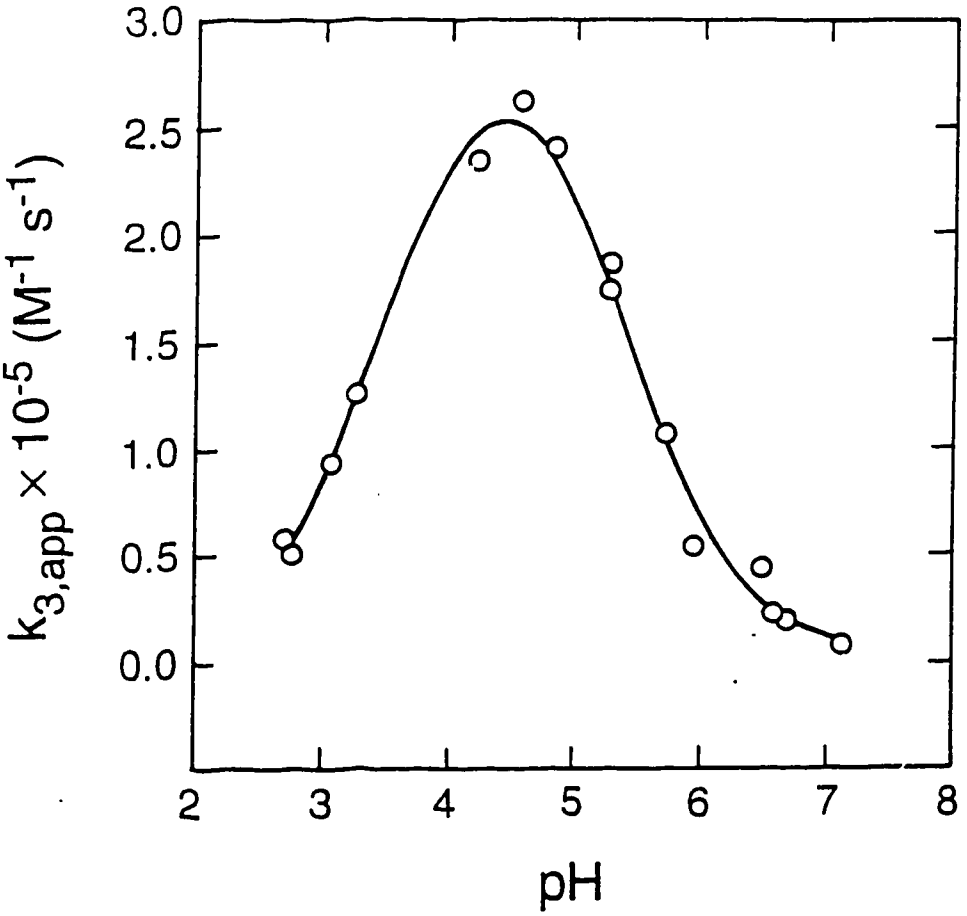


Fig. 3.5 pH rate profiles of $k_{3,app}$ for the reaction of HRP-I with AA at 25 ^{0}C, μ 0.11 M. Open circles are experimental results. Smooth curve is the best-fit curve from Eq. 3.14.

Table 3.1 Reaction of LPO-II with AA. Values of $k_{1,app}$, $k_{2,app}$, $1/K_{1,M}$ and $1/K_{1,M}^{cal}$ as a function of pH

pH	$k_{1,app}$ $M^{-1}s^{-1}$	$k_{2,app}$ $M^{-1}s^{-1}$	$1/K_{1,M}$	$1/K_{1,M}^{cal}$
3.00	$(5.4 \pm 0.5)\times10^{5}$			
3.26	$(4.7 \pm 0.2)\times10^{5}$			
3.45	$(4.4 \pm 0.3)\times10^{5}$			
3.78	$(3.7 \pm 0.3)\times10^{5}$			
4.04	$(2.9 \pm 0.2)\times10^{5}$			
4.16	$(2.5 \pm 0.3)\times10^{5}$			
4.27	$(1.9 \pm 0.1)\times10^{5}$			
4.37	$(1.6 \pm 0.2)\times10^{5}$			
4.45	$(1.3 \pm 0.9)\times10^{5}$	$(3.0 \pm 1.3)\times10^{5}$	$(3.0 \pm 0.6)\times10^{3}$	$(3.6 \pm 0.92)\times10^{3}$
4.64	$(1.1 \pm 0.5)\times10^{5}$	$(2.4 \pm 1.0)\times10^{5}$	$(1.9 \pm 0.2)\times10^{3}$	$(2.6 \pm 1.3)\times10^{3}$
4.79	$(5.7 \pm 2.5)\times10^{4}$	$(1.3 \pm 0.5)\times10^{5}$	$(1.2 \pm 0.7)\times10^{3}$	$(1.7 \pm 0.4)\times10^{3}$
4.96	$(5.4 \pm 0.7)\times10^{4}$	$(1.4 \pm 0.7)\times10^{5}$	$(9.1 \pm 1.1)\times10^{2}$	$(6.0 \pm 3.0)\times10^{2}$
5.26	$(3.3 \pm 0.5)\times10^{4}$	$(8.0 \pm 2.5)\times10^{4}$	$(9.1 \pm 0.6)\times10^{2}$	$(1.0 \pm 0.6)\times10^{3}$
5.44	$(2.2 \pm 1.0)\times10^{4}$	$(4.9 \pm 0.3)\times10^{4}$	$(1.4 \pm 0.6)\times10^{3}$	$(2.0 \pm 0.7)\times10^{3}$
5.55	$(1.7 \pm 0.9)\times10^{4}$	$(4.0 \pm 5.9)\times10^{4}$	$(9.1 \pm 1.6)\times10^{2}$	$(5 \pm 10)\times10^{2}$
5.68	$(1.5 \pm 0.4)\times10^{4}$	$(3.4 \pm 5.0)\times10^{4}$	$(1.2 \pm 0.1)\times10^{3}$	$(1.0 \pm 0.3)\times10^{3}$
5.93	$(2.3 \pm 0.5)\times10^{4}$	$(8.0 \pm 3.5)\times10^{3}$	$(4.3 \pm 7.0)\times10^{3}$	$(4 \pm 10)\times10^{3}$
6.21	$(1.0 \pm 0.2)\times10^{4}$	$(3.5 \pm 1.6)\times10^{3}$	$(2.0 \pm 2.2)\times10^{3}$	$(2.0 \pm 0.3)\times10^{3}$
6.36	$(7.9 \pm 0.3)\times10^{3}$	$(1.6 \pm 0.2)\times10^{3}$	$(8.3 \pm 5.0)\times10^{2}$	$(8.3 \pm 4.0)\times10^{2}$
6.48	$(7.2 \pm 0.4)\times10^{3}$	$(2.0 \pm 1.0)\times10^{3}$	$(1.3 \pm 2.5)\times10^{3}$	$(1.4 \pm 1.9)\times10^{3}$
6.6	$(6.7 \pm 0.7)\times10^{3}$	$(1.6 \pm 1.4)\times10^{3}$	$(1.2 \pm 0.3)\times10^{3}$	$(1.5 \pm 1.4)\times10^{3}$
6.74	$(4.7 \pm 0.5)\times10^{3}$	$(1.3 \pm 1.0)\times10^{3}$	$(2.4 \pm 1.3)\times10^{3}$	$(2.5 \pm 0.2)\times10^{3}$

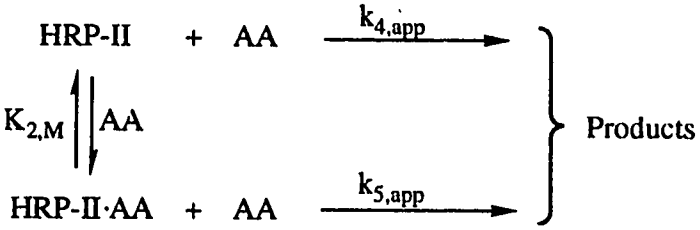
Table 3.2 Reaction of HRP-I with AA. Values of $k_{3,app}$ as a function of pH

pH	$k_{3,app}$ $M^{-1}s^{-1}$
2.68	$(5.8 \pm 0.3)x10^4$
2.74	$(5.0 \pm 0.1)x10^4$
3.07	$(9.3 \pm 0.3)x10^4$
3.26	$(1.3 \pm 0.2)x10^5$
3.78	$(2.2 \pm 0.2)x10^5$
4.21	$(2.3 \pm 0.5)x10^5$
4.52	$(2.6 \pm 0.4)x10^4$
4.81	$(2.4 \pm 0.1)x10^5$
5.26	$(1.7 \pm 0.2)x10^5$
5.27	$(1.9 \pm 0.5)x10^5$
5.71	$(1.1 \pm 0.5)x10^5$
5.95	$(5.4 \pm 0.2)x10^4$
6.48	$(4.4 \pm 0.4)x10^4$
6.57	$(2.2 \pm 0.5)x10^4$
6.65	$(1.9 \pm 0.6)x10^4$
7.11	$(7.6 \pm 0.3)x10^3$

Oxidation of AA by HRP-II

As shown in Fig. 3.4, HRP-II is reduced directly to native HRP by AA; thus a one-electron transfer reaction is occurring. Pseudo-first-order rate constants $k_{3,obs}$ are obtained. The plots of $k_{3,obs}$ versus AA concentrations are shown on Fig. 3.6. From pH 4.1 to 5.3, the plots are concave with respect to the abscissa (Fig. 3.6a). At all other pH values, the plots are linear (Fig. 3.6b). From the linear plots, the second-order rate constants $k_{4,app}$ can be obtained from the slope. A possible reaction mechanism is:

[3.5]



which is identical to that for LPO-II. HRP-II·AA is the complex formed between HRP-II and AA. The values of $k_{3,obs}$ are related to the three kinetic parameters as follows:

[3.6] $$k_{3,obs} = \frac{k_{4,app}[AA] + k_{5,app}[AA]^2/K_{2,M}}{1 + [AA]/K_{2,M}}$$

The best-fit values of $k_{4,app}$, $k_{5,app}$ and $1/K_{2,M}$ are listed in Table 3.3.

The plots of $k_{4,app}$, $k_{5,app}$ and $1/K_{2,M}$ versus pH are shown in Fig. 3.7. The effect of the acidity of the reaction medium on the kinetics of the oxidation of AA by HRP-II is strikingly different from the LPO-II reaction with AA.

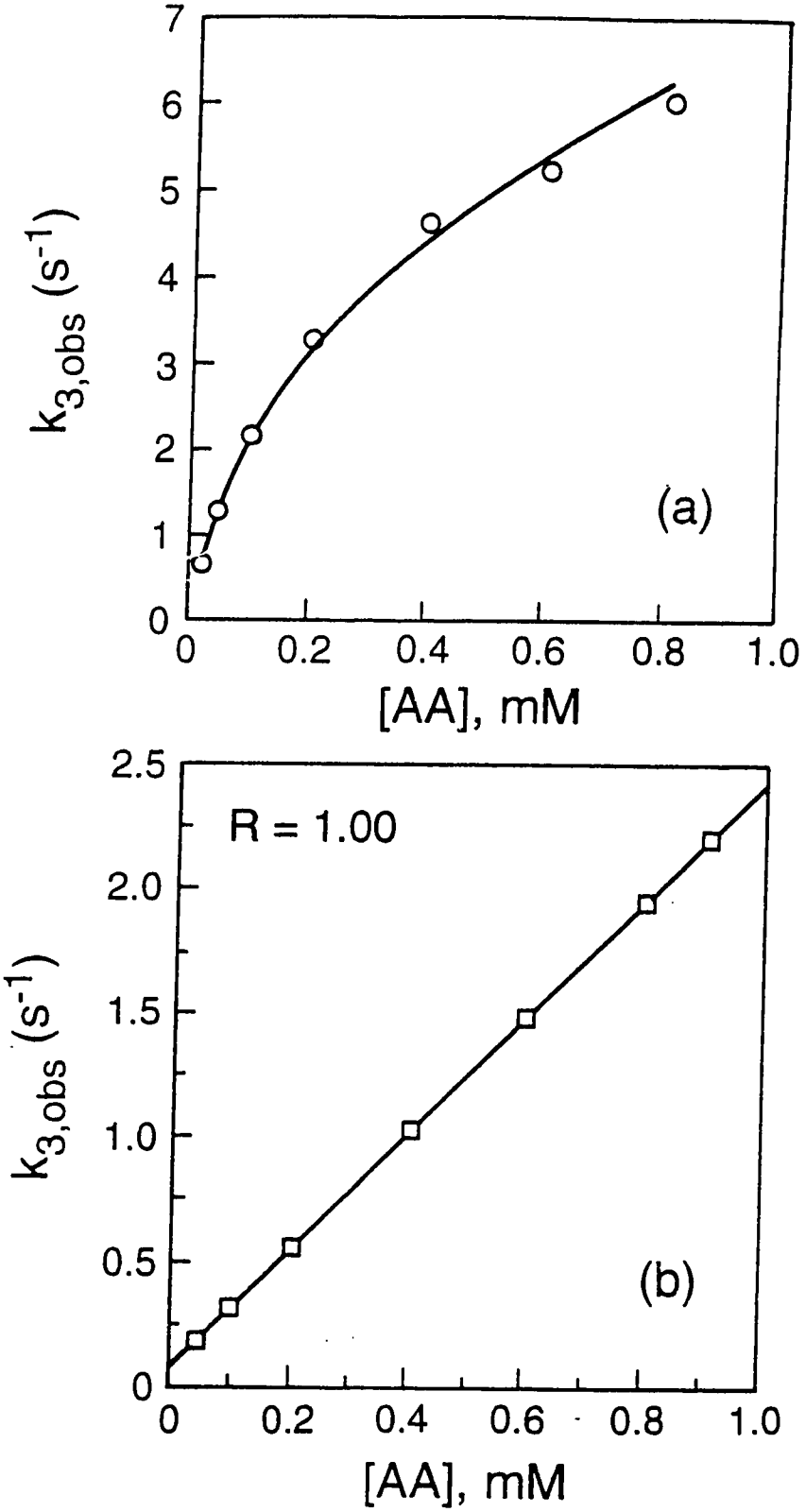


Fig. 3.6 Plot of k_{obs} vs. AA for the reaction of HRP-II with AA. μ 0.11 M; temp. 25 ^{0}C; (a) pH 4.1 (b) pH 5.7

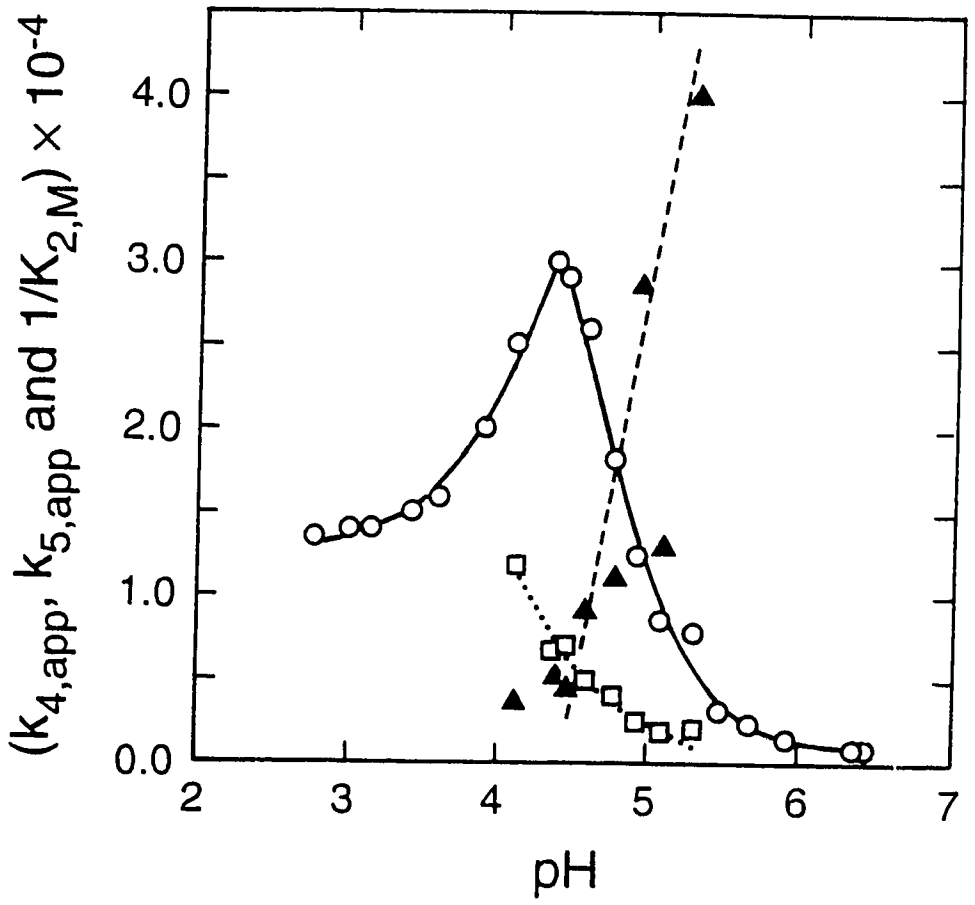


Fig. 3.7 pH rate profiles of $k_{4,app}$, $k_{5,app}$ and $1/K_{2,M}$ for the reaction of HRP-II with AA at 25 ^{0}C, μ 0.11 M; (open circles) $k_{4,app}$, (open squares) $k_{5,app}$, (solid triangles) $1/K_{2,M}$

Table 3.3 Reaction of HRP-II with AA. Values of $k_{4,app}$, $k_{5,app}$, $K_{2,M}$ as a function of pH

pH	$k_{4,app}$ $M^{-1}s^{-1}$	$k_{5,app}$ $M^{-1}s^{-1}$	$1/K_{2,M}$
2.77	$(1.3 \pm 0.6)x10^4$		
3.00	$(1.4 \pm 0.3)x10^4$		
3.15	$(1.4 \pm 0.4)x10^4$		
3.41	$(1.5 \pm 0.5)x10^4$		
3.58	$(1.6 \pm 0.2)x10^4$		
3.89	$(2.0 \pm 0.8)x10^4$		
4.11	$(2.5 \pm 0.7)x10^4$	$(1.2 \pm 0.3)x10^4$	$(4.0 \pm 5.0)x10^4$
4.38	$(3.0 \pm 1.0)x10^4$	$(6.9 \pm 0.6)x10^3$	$(1.3 \pm 0.6)x10^4$
4.46	$(2.9 \pm 0.9)x10^4$	$(7.1 \pm 0.5)x10^3$	$(2.9 \pm 4.5)x10^4$
4.58	$(2.6 \pm 0.5)x10^4$	$(4.9 \pm 0.4)x10^3$	$(1.1 \pm 1.4)x10^4$
4.77	$(1.8 \pm 0.6)x10^4$	$(4.0 \pm 0.6)x10^3$	$(9.3 \pm 3.6)x10^3$
4.93	$(1.2 \pm 0.7)x10^4$	$(2.5 \pm 0.3)x10^3$	$(4.4 \pm 3.3)x10^3$
5.09	$(8.6 \pm 0.5)x10^3$	$(2.9 \pm 0.5)x10^3$	$(5.3 \pm 4.8)x10^3$
5.30	$(7.8 \pm 1.0)x10^3$	$(2.0 \pm 0.4)x10^3$	$(6.0 \pm 1.6)x10^3$
5.47	$(3.1 \pm 0.2)x10^3$		
5.68	$(2.3 \pm 0.1)x10^3$		
5.93	$(1.4 \pm 0.2)x10^3$		
6.36	$(8.1 \pm 0.2)x10^2$		
6.43	$(7.9 \pm 0.3)x10^2$		

It should be noted that obedience to Eq. 3.2 does not disprove the existence of an enzyme-substrate complex, but it does impose limits on the pertinent constants. For instance, if productive binding were occurring, linear plots would still be obtained if the unimolecular reaction of the complex going to products was much faster than the rate of formation of the complex. Furthermore, if the Michaelis constant is large, the terms, $k_{2,app}$ $[AA]^2/K_{1,M}$, $[AA]/K_{1,M}$, $k_{5,app}$ $[AA]^2/K_{2,M}$, $[AA]/K_{2,M}$, in Eq 3. 4 and Eq. 3.6 become vanishingly small, whereupon Eqs. 3.4 and 3.6 become equal to Eq. 3.2.

3.4 DISCUSSION

AA is a dibasic acid (pK_{a1} = 4.2; pK_{a2} = 11.3) with a bifunctional ene-diol group built into a heterocyclic lactone ring. Peroxidases can oxidize ascorbate to the free radical, semidehydroascorbate (19). It was reported that the ascorbate dependent peroxidase action starts with the formation of Fe^{3+}-ascorbate charge transfer complex intermediate (20). Ascorbic acid is a reducing agent and radical scavenger that is found in normal human plasma at concentrations of 50-150 μM and in the cytosol of human neutrophils at concentrations of 1.0-1.4 mM (21). Its function has been suggested to be the protection of neutrophils or host tissues by the scavenging of oxygen radicals (22-24).

pH profile for the reaction of LPO-II with AA

Referring to Fig. 3.3, there is only one inflection for both $k_{1,app}$ and $k_{2,app}$ in their plots as a function of pH, which can be most readily assigned as K_S for the acid dissociation constant of AA.

To explain the pH profile of $k_{1,app}$, we propose the following mechanism:

[3.7] $$\text{LPO-II} + \left\{ \begin{array}{l} \text{HAA} \xrightarrow{k_1} \\ \text{H}^+ \rightleftharpoons K_{1,S} \\ \text{AA} \xrightarrow{k_2} \end{array} \right\} \text{Products}$$

where HAA is the protonated form and AA is the deprotonated form of ascorbic acid. From Eq. 3.7, we obtain:

[3.8] $$k_{1,app} = \frac{\frac{k_1}{K_{1,S}}[H^+] + k_2}{1 + \frac{[H^+]}{K_{1,S}}}$$

The best-fit values for the kinetic parameters in Eq. 3.8 were obtained by a nonlinear least squares analysis and are compiled in Table 3.4. The corresponding best-fit line is shown in Fig. 3.3.

For the pH profile of $k_{2,app}$, we propose a mechanism similiar to that for $k_{1,app}$:

[3.9] $$\text{LPO-II}\cdot\text{AA} + \begin{array}{c} \text{HAA} \\ \text{H}^+ \rightleftharpoons K_{2,S} \\ \text{AA} \end{array} \xrightarrow{k_3} \text{Products}$$

from Eq. 3.9, we get:

[3.10] $$k_{2,app} = \frac{k_3}{1 + \frac{K_{2,S}}{[H^+]}}$$

Values of the parameters in Eq. 3.10 are listed in Table 3.4 and the best-fit line is marked by short dashes in Fig. 3.3.

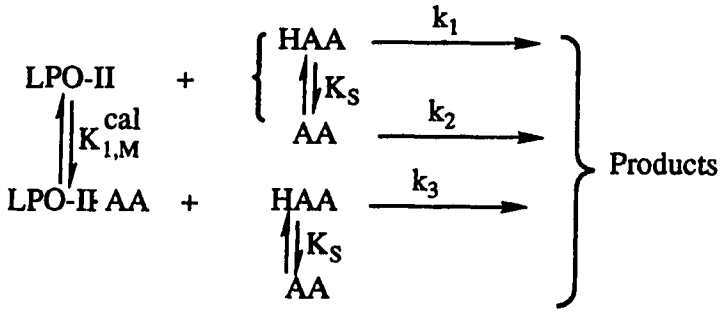
Table 3.4 Parameters for k_{app} versus pH profile

Reactions	Parameters	Calculated from Eq. 3.8	Calculated from Eq. 3.10
LPO-II + AA	k_1 ($M^{-1} s^{-1}$)	$(5.8 \pm 0.2)x10^5$	
	k_2 ($M^{-1} s^{-1}$)	$(3.4 \pm 3.9)x10^3$	
	k_3 ($M^{-1} s^{-1}$)		$(8 \pm 2)x10^5$
	$K_{1,S}$ (M)	$(1.0 \pm 0.1)x10^{-4}$	
	$K_{2,S}$ (M)		$(6 \pm 3)x10^{-5}$
	Parameters	Calculated from Eq. 3.14	
HRP-I + AA	k_4 ($M^{-1} s^{-1}$)	$(3.1 \pm 0.1)x10^4$	
	k_5 ($M^{-1} s^{-1}$)	$(4.4 \pm 4.9)x10^3$	
	$K_{3,S}$ (M)	$(3.0 \pm 0.5)x10^{-4}$	
	$K_{1,E}$ (M)	$(4.5 \pm 0.7)x10^{-6}$	
	Parameters	Calculated from Eq. 3.16	Calculated from Eq. 3.18
HRP-II + AA	k_6 ($M^{-1} s^{-1}$)	$(1.3 \pm 2.9)x10^4$	
	k_7 ($M^{-1} s^{-1}$)	$(1.0 \pm 0.9)x10^4$	
	k_8 ($M^{-1} s^{-1}$)	$(9.1 \pm 0.6)x10^4$	
	k_9 ($M^{-1} s^{-1}$)	$(2.4 \pm 0.5)x10^3$	
	$K_{4,S}$ (M)	$(1.8 \pm 1.5)x10^{-4}$	
	$K_{2,E}$ (M)	$(9.5 \pm 0.7)x10^{-6}$	
	k_{10} ($M^{-1} s^{-1}$)		$(2.9 \pm 0.8)x10^4$
	$K_{5,S}$ (M)		$(1.1 \pm 0.4)x10^{-4}$

The trends of the pH dependence of $k_{1,app}$ and $k_{2,app}$ are similar and the fitted K_S values from both Eq. 3.8 and Eq. 3.10 are also very close. This implies that the proton transfer mechanisms for the uncomplexed and complexed forms of AA reacting with LPO-II are the same.

By analyzing $1/K_{1,M}$ values in Table 3.1, we conclude that $K_{1,M}$ is independent of pH. The implication is that ionizations of AA and LPO-II do not affect the Michaelis constant $K_{1,M}$. Therefore Eqs. 3.7 and 3.9 can be combined as follows:

[3.11]



from Eq. 3.11, we get:

[3.12]

$$k_{1,obs} = \frac{\left(k_{1,app} + k_{2,app} \frac{[AA]_{tot}}{K_{1,M}^{cal} \left(1 + \frac{[H^+]}{K_S} \right)} \right) [AA]_{tot}}{1 + \frac{[AA]_{tot}}{K_{1,M}^{cal} \left(1 + \frac{[H^+]}{K_S} \right)}}$$

Therefore, at a given pH value, by using values of $k_{1,app}$, $k_{2,app}$ and K_S (25) and fitting $k_{1,obs}$ to $[AA]_{tot}$, we can get a calculated Michaelis constant $K_{1,M}^{cal}$ which is listed in Table 3.1 as $1/K_{1,M}^{cal}$. Comparison of $1/K_{1,M}$ and $1/K_{1,M}^{cal}$ in Table 3.1 , shows that they are consistent , which provides evidence that:

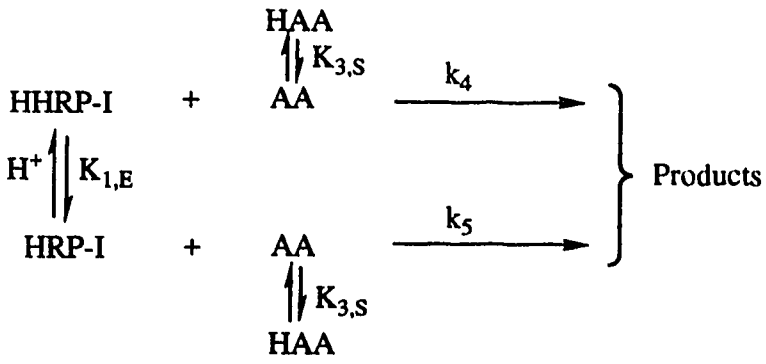
1) $K_{1,M}$ is independent of pH.

2) the mechanisms [7] and [9] which we have proposed are correct.

Reaction of HRP-I

A different mechanism is required for the reaction between HRP-I and AA:

[3.13]



where HHRP-I is the protonated form and HRP-I is the deprotonated form of horseradish peroxidase compound I, $K_{1,E}$ is its dissociation constant. $K_{3,S}$ is the dissociation constant of ascorbic acid.

From the above mechanism, Eq. 3.14 can be obtained:

[3.14]

$$k_{3,app} = \frac{k_4 + \frac{k_5 K_{1,E}}{[H^+]}}{\left[1 + \frac{K_{1,E}}{[H^+]}\right]\left[1 + \frac{[H^+]}{K_{3,S}}\right]}$$

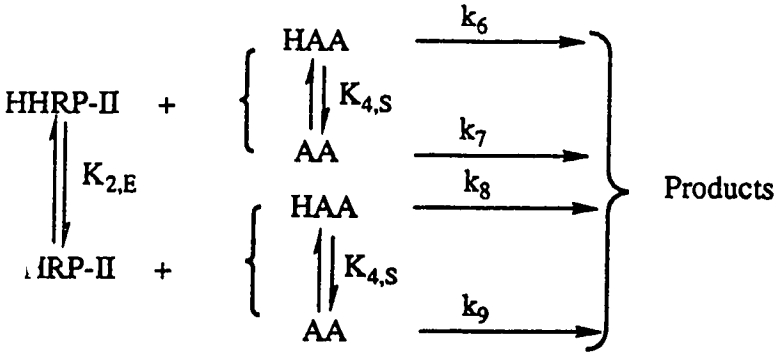
A non-linear least-square analysis program was used to obtain the best-fit parameters which are listed in Table 3.4. The best-fit line is shown in Fig. 3.5.

Reaction of HRP-II

The pH rate profile of HRP-II with AA is the most complicated. With increasing pH, $k_{4,app}$ gradually increases. After reaching a maximum value at pH 4.4 ($pK_S = 4.2$ of ascorbic acid), it starts decreasing rapidly, then after pH 5, decreases more slowly. Several mechanisms were tried to fit the pH rate profile, but we were unable to fit the whole pH range by using a single mechanism (a similar case could be found for the

pH rate profile of reaction between myeloperoxidase compound II with AA) (26). The best mechanism we were able to obtain is shown as follows:

[3.15]



where HHRP-II is the protonated form and HRP-II is the deprotonated form of horseradish peroxidase compound II, $K_{2,E}$ is the dissociation constant. $K_{4,S}$ is the dissociation constant of ascorbic acid.

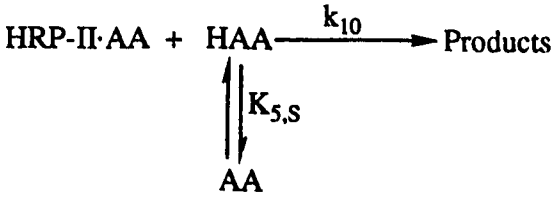
Eq. 3.16 can be obtained from the above mechanism:

$$k_{4,app} = \frac{\frac{k_6[H^+]^2}{K_{2,E}K_{4,S}} + \frac{k_7[H^+]}{K_{2,E}} + \frac{k_8[H^+]}{K_{4,S}} + k_9}{\left[1 + \frac{[H^+]}{K_{4,S}}\right]\left[1 + \frac{[H^+]}{K_{2,E}}\right]} \qquad [3.16]$$

Since a satisfactory fit could not be obtained if we use Eq. 3.16 to fit the whole pH range, a separate fitting procedure was used for different pH ranges. For the pH range of 2.8-4.4, the value of $K_{2,E}$ was fixed after which best-fit values of $K_{4,S}$ and other four parameters were determined; For the pH range of 4.4-6.4, the value of $K_{4,S}$ was fixed and values of $K_{2,E}$ and other four parameters were determined. All the average values of the parameters in Eq. 3.16 are tabulated in Table 3.4.

The pH rate profile of $k_{5,app}$ is simple. The dissociation of ascorbic acid is the only factor affecting the reaction rate constant. The mechanism can be described as:

[3.17]



where $K_{5,S}$ is the dissociation constant of ascorbic acid. From the above mechanism, Eq. 3.18 can be obtained:

[3.18]

$$k_{5,app} = \frac{k_{10}}{1 + \frac{K_{5,S}}{[H^+]}}$$

The non-linear least square curve fitting gives the values of k_{10} and $K_{5,S}$ which are listed in Table 3.4.

The effect of pH on Michaelis constants had been discussed in detail by Dixon and Webb (27). For the reaction between LPO-II and AA, the Michaelis constant $K_{1,M}$ is pH independent. A similar case was reported for the reaction between HRP-I and *p* -cresol (16). For the reaction between HRP-II and AA, a slope of +1 can be obtained from the plot of $1/K_{2,M}$ versus pH.

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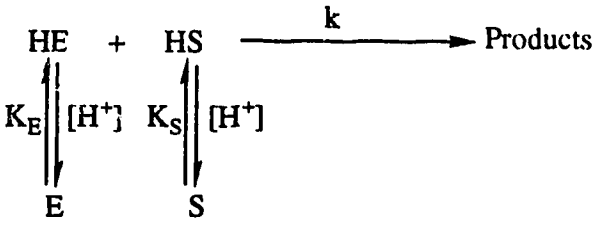
APPENDICES

Appendix I

(Chapter Two)

For the following reaction mechanism:

[I.1]



The reaction rate can be expressed like:

[I.2] $$v = k\,[HE][HS]$$

By using the following mass conservation relations and dissociation constants,

$$[E]_0 = [E] + [HE] \quad \text{and} \quad [HS]_0 = [S] + [HS]$$

$$K_E = [H^+]\,[E] / [HE] \quad \text{and} \quad K_S = [H^+]\,[S] / [HS]$$

We can get:

[I.3] $$[HE] = \frac{[E]_0}{1 + K_E / [H^+]} \quad \text{and} \quad [HS] = \frac{[HS]_0}{1 + K_S / [H^+]}$$

By plugging Eq. [I.3] in [I.2] and using $k_{app} = v / ([E]_0\,[HS]_0)$ relation, we get:

[I.4] $$k_{app} = \frac{k}{\left[1 + \frac{K_E}{[H^+]}\right]\left[1 + \frac{K_S}{[H^+]}\right]}$$

Appendix II

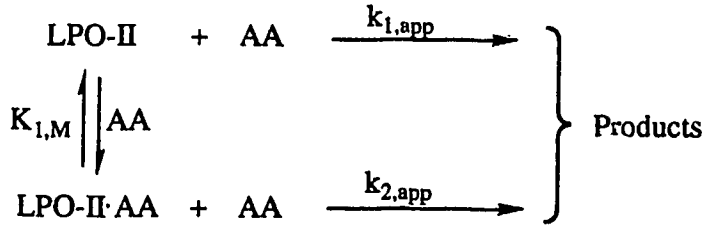
(Chapter Three)

Derivation of the equations in chapter three

I: The correlation between pseudo-first-order rate constant and second-order -rate constants

For the kinetic reaction scheme as following:

[II.1]



The reaction rate can be expressed like:

[II.2] $$v = k_{1,app}[\text{LPO-II}][AA] + k_{2,app}[\text{LPO-II}\cdot AA][AA]$$

Since $$[\text{LPO-II}]_0 = [\text{LPO-II}] + [\text{LPO-II}\cdot AA]$$

and $$K_{1,M} = [\text{LPO-II}][AA] / [\text{LPO-II}\cdot AA]$$

Thus

[II.3] $$[\text{LPO-II}] = [\text{LPO-II}]_0 / (1 + [AA]/K_{1,M})$$

and $$[\text{LPO-II}\cdot AA] = [\text{LPO-II}]_0 / (1 + K_{1,M}/[AA])$$

Putting Eq.[II.3] in [II.2] and also using correlation of $k_{1,obs} = v / [\text{LPO-II}]_0$, we obtain:

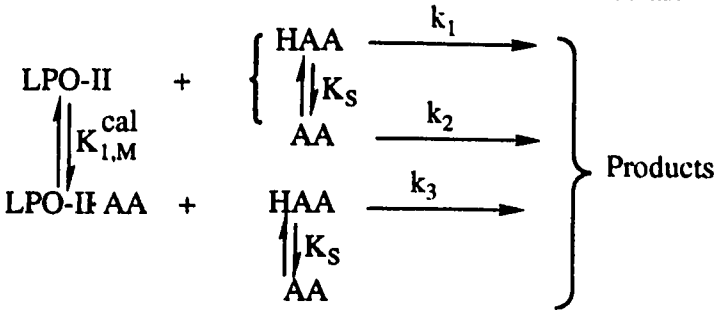
[II.4] $$k_{1,obs} = \frac{k_{1,app}[AA] + k_{2,app}[AA]^2/K_{1,M}}{1 + [AA]/K_{1,M}}$$

II: The correlation between second-order -rate constants and $[H^+]$

1) Reaction of LPO-II

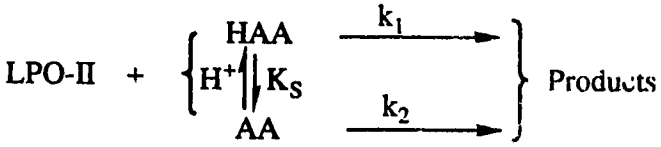
The reaction mechanism of LPO-II with ascorbic acid can be described as:

[II.5]



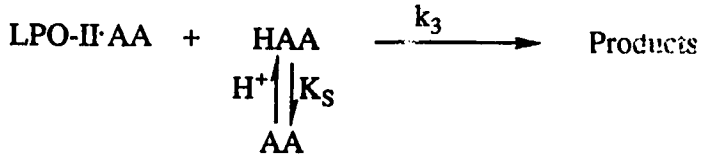
Since $K_{1,M}$ is pH independent, Eq. [II.5] can be divided into two parts:

[II.6]



and

[II.7]



By using the similar method as indicated in appendix I, we obtain:

[II.8]

$$k_{1,app} = \frac{\frac{k_1}{K_S}[H^+] + k_2}{1 + \frac{[H^+]}{K_S}}$$

and

[II.9]

$$k_{2,app} = \frac{k_3}{1 + \frac{K_S}{[H^+]}}$$

The reaction rate of Eq. [II.5] can be represented as following:

[II.10]

$$v = k_1 [\text{LPO-II}][\text{HAA}] + k_2 [\text{LPO-II}][\text{AA}] + k_3 [\text{LPO-II·AA}][\text{HAA}]$$

Applying the following correlations of mass conservation and dissociation constants:

[II.11] $[\text{LPO-II}]_0 = [\text{LPO-II}] + [\text{LPO-II}\cdot\text{AA}]$ and

$K = [\text{LPO-II}]\,[\text{AA}] / [\text{LPO-II}\cdot\text{AA}]$

[II.12] $[\text{AA}]_{tot} = [\text{LPO-II}\cdot\text{AA}] + [\text{HAA}] + [\text{AA}]$ since

$[\text{LPO-II}\cdot\text{AA}] << [\text{AA}]_{tot}$

$[\text{AA}]_{tot} = [\text{HAA}] + [\text{AA}]$ and $K_S = [H^+]\,[\text{AA}] / [\text{HAA}]$

We obtain:

[II.13] $$[\text{LPO-II}] = \frac{[\text{LPO-II}]_0}{(1 + [\text{AA}] / K)} \quad \text{and} \quad [\text{LPO-II AA}] = \frac{[\text{AA}]}{K} \frac{[\text{LPO-II}]_0}{(1 + [\text{AA}] / K)}$$

and

[II.14] $$[\text{AA}] = \frac{[\text{AA}]_{tot}}{(1 + [H^+] / K_S)} \quad \text{and} \quad [\text{HAA}] = \frac{[H^+]}{K_S} \frac{[\text{AA}]_{tot}}{(1 + [H^+] / K_S)}$$

Plugging in Eqs. [II.13] and [II.14] in Eq. [II.10], and rearranging it, we get:

[II.15] $$v = \frac{[\text{LPO-II}]_0\,[\text{AA}]_{tot}}{1 + [\text{AA}] / K} \left[\frac{k_1 [H^+]}{K_S} \frac{1}{1 + [H^+] / K_S} + \frac{k_2}{1 + [H^+] / K_S} \right]$$

$$+ \frac{[\text{AA}]}{K} \frac{[\text{LPO-II}]_0\,[\text{AA}]_{tot}}{1 + [\text{AA}] / K} \left[\frac{k_3 [H^+]}{K_S} \frac{1}{1 + [H^+] / K_S} \right]$$

Introducing $k_{1,obs} = v / [LPO\text{-}II]_0$; $K_{1,M}^{cal} = K$; substitute $k_{1,app}$ and $k_{2,app}$ into Eq. [II.15] and rearrange it, we obtain:

[II.16]
$$k_{1,obs} = \frac{\left(k_{1,app} + k_{2,app} \frac{[AA]_{tot}}{K_{1,M}^{cal} \left(1 + \frac{[H^+]}{K_S} \right)} \right) [AA]_{tot}}{1 + \frac{[AA]_{tot}}{K_{1,M}^{cal} \left(1 + \frac{[H^+]}{K_S} \right)}}$$

2) Reaction of HRP-I

The derivation method is similar to the method in appendix I.

3) Reaction of HRP-II

The derivation method is similar to the method in above (Reaction of LPO-II).