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Transient-State Kinetics of the Reactions of 1-Methoxy-4-(methylthio)benzene with Horseradish Peroxidase Compounds I and II[†]

Ubaldo Pērez[‡] and H. Brian Dunford*

Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2G2

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ABSTRACT: Transient-state reactions of horseradish peroxidase compounds I and II with 1-methoxy-4-(methylthio)benzene (a para-substituted thioanisole) were studied over the pH range from 3.4 to 10.5. The pH-jump technique was applied to the compound II reactions at pH values below 8.6. The reactions of both compound I and compound II with the para-substituted thioanisole consisted predominantly of an initial burst. The burst was followed by a steady-state phase that became more obvious at lower concentrations of the thioanisoles. The burst phase for both compounds I and II can be explained in terms of two independent transient-state reactions with 1-methoxy-4-(methylthio)benzene as follows: (i) a single reaction of compound I (or compound II) with the substrate and (ii) the formation of a complex between compound I or II and the substrate followed by reaction of the productive complex with another molecule of sulfide. The overall rate of reaction path ii is faster than that of path i. The preference for path i or ii is highly dependent upon the concentration of sulfide with step ii favored at higher sulfide concentrations. The experimental results obtained on the overall reaction under both pseudo-first-order and single-turnover conditions indicate that compound II reacts competitively with both the organic sulfide substrate and the sulfur cation radical produced from compound I oxidation of sulfide.

Despite the importance of the enzymatic oxidation of organic sulfides in mammalian detoxification processes, little information is available. Watanabe et al. (1980, 1981a,b) reported that oxidation of alkyl sulfides by mammalian liver microsomes and by purified cytochrome P-450 occurs via a sulfur cation radical intermediate. Both sulfur cation radical formation and sulfoxide formation were also noted with cytochrome P-450 by Guengerich and MacDonald (1984).

Kobayashi et al. (1986, 1987) reported evidence for oxygen atom transfer from H_2O_2 and from H_2O to organic sulfides (thioanisoles) catalyzed by HRP.[1] Their results were in accord with the formation of a cation radical intermediate in the rate-limiting step and with an overall mechanism always involving single-electron transfers.

More recently, Blēe and Schuber (1989) demonstrated that the hemoprotein soybean sulfoxidase catalyzes oxygen transfer from 13(*S*)-hydroperoxylinoleic acid to para-substituted thioanisoles. On the basis of their results they suggested a

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[*] Correspondence should be addressed to this author.

[‡] Present address: Department of Biochemistry, Faculty of Biology, University of Havana, Havana, Cuba.

[1] Abbreviations: HRP, horseradish peroxidase; HRP-I, horseradish peroxidase compound I; HRP-II, horseradish peroxidase compound II.

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mechanism similar to that proposed for S-oxygenation by mammalian NADPH-dependent cytochrome P-450 (Watanabe et al., 1981a,b, 1982) involving single-electron transfer with the formation of a sulfenium radical cation. This intermediate could be ultimately transformed into the corresponding sulfoxide.

All the previous suggestions about the mechanism of oxidation of organic sulfides by HRP have been made on the basis of spectral data (Pérez & Dunford, 1990) or steady-state kinetics combined with isotopic labeling (Kobayashi et al., 1987). In this paper we provide more evidence on the reaction mechanism obtained from transient-state kinetic experiments on the oxidation of 1-methoxy-4-(methylthio)benzene by HRP-I and HRP-II.

MATERIALS AND METHODS

Materials. HRP (grade I) purchased from Boehringer-Mannheim as an ammonium sulfate suspension was extensively dialyzed against deionized water and passed through a Millipore filter prior to use. The RZ number of the resulting solution was 3.3. The concentration of HRP was determined spectrophotometrically at 403 nm with a molar absorptivity of $1.02 \times 10^5\ M^{-1}\ cm^{-1}$ (Ohlsson & Paul, 1976).

All solutions were prepared from water that had been twice distilled and deionized by a Milli-Q water purification system.

1-Methoxy-4-(methylthio)benzene, a para-substituted thioanisole was purchased from Aldrich Chemical Co. and *p*-cresol from BDH. All chemicals were of the highest grade and were used without further purification. The concentration of H_2O_2 solutions was determined spectrophotometrically by the HRP-catalyzed oxidation of iodide (Cotton & Dunford, 1973). Solutions of *p*-cresol were prepared by weighing, and the concentration was checked spectrophotometrically at 277 nm (Critchlow & Dunford, 1972). Stock solutions of 0.1 M 1-methoxy-4-(methylthio)benzene in 98% ethanol were prepared daily. The ethanol is necessary to solubilize the sulfide in the aqueous reaction mixture.

HRP-I was prepared with 1.0 molar equiv of H_2O_2 (Hewson & Dunford, 1976). Buffer contributed an ionic strength of 0.01 M, and potassium sulfate was added to a final total ionic strength of 0.11 M. HRP-II was formed with 1.0 molar equiv of buffered H_2O_2 and 0.45 molar equiv of *p*-cresol at pH 9.0 in 2.5 mM Tris-HCl buffer (Hasinoff & Dunford, 1970).

The spectra of HRP-I and HRP-II solutions were monitored repeatedly to confirm the stability of the compounds. A 1 μM solution of either compound was stable for at least 7 min. At higher enzyme concentrations the stability of both compounds decreases (Bohne et al., 1987).

A Fisher Accumet Model 420 digital pH meter was used for pH measurements.

Stopped-Flow Kinetic Experiments. Kinetic measurements were made on a Union Giken rapid-reaction analyzer model RA-601 equipped with a 1-cm observation cell. Rate constants performed on the Union Giken apparatus were determined by nonlinear least-squares curve fitting analysis, carried out by computer.

All reactions were studied at 25 °C. HRP, HRP-I, and HRP-II final concentrations were typically 1.0 μM. Concentrations of 1-methoxy-4-(methylthio)benzene varied from 10 to 300 μM in order to ensure pseudo-first-order conditions.

The concentration of ethanol in the final solutions was always negligible. In a blank experiment no change in the stopped-flow reaction signal was observed upon the addition of a volume of ethanol, equivalent to the highest sulfide concentration (12 μL), to a total reaction volume of 2 mL. Also, no change in the pH reading was noted upon ethanol addition.

The reaction of HRP-I was monitored at 411 nm, the isosbestic point between HRP and HRP-II with a band-pass of 3.5 nm. In a typical experiment 2 mL of freshly prepared HRP-I solution was placed in one storage compartment of the stopped-flow apparatus and a buffered sulfide solution in the other. The different buffer solutions employed were citrate, acetate, phosphate, Tris-HCl, and bicarbonate in a pH range from 3.4 to 10.5 and ionic strength of 0.01.

The reaction of HRP-II was monitored at 427 nm, the isosbestic point between HRP and HRP-I with a band-pass of 3.5 nm. Since a high yield of HRP-II cannot be obtained at pH values lower than 8.0, the reaction of HRP-II was performed by means of a pH jump (Bohne et al., 1987). Thus, 2 mL of freshly prepared HRP-II solution weakly buffered at pH 9 was placed in one compartment of the stopped-flow apparatus and in the other strongly buffered sulfide solutions at the desired pH. The buffers employed for the pH-jump experiments were sodium acetate (pH 4.0–5.4), sodium phosphate (pH 6.05–7.95), and sodium carbonate–bicarbonate (pH 9.1) to a final ionic strength in reaction mixture of 0.10.

Repeated determinations (between six and eight) of the reaction rate constants (k_{obs}) were always achieved in a period of time that never exceeded 5 min and the data were stored directly in an on-line computer memory. The rate of autodecomposition of HRP-I or HRP-II was negligible compared to the rate of reaction. An alternative way of measurement of HRP-II formation and reaction at 427 nm was achieved in the rapid-reaction analyzer by starting with native HRP. In a typical experiment 2 mL of HRP solution was placed in one compartment of the stopped-flow apparatus and in the other buffered sulfide solution in the presence of 1 molar equiv of H_2O_2 to a final ionic strength of 0.11.

Single-turnover kinetic measurements were conducted spectrophotometrically on a Cary 219 recording spectrophotometer for the HRP reaction with sulfide (1 and 2 molar equiv of sulfide compared to the concentration of HRP) in the presence of 1 molar equiv of H_2O_2. The reaction was followed by measuring the increase followed by the decrease in absorbance at 427 nm due to formation and reaction of HRP-II. HRP (2 μM), sulfide solution, buffered at pH 5.0, and K_2SO_4 to a final ionic strength of 0.11 were placed in a cuvette of the Cary spectrophotometer. The final volume was 2 mL. The reaction was started by the addition of 1.0 molar equiv of H_2O_2. The reaction occurred over a range up to 10–15 s.

RESULTS

Oxidation of 1-Methoxy-4-(methylthio)benzene by HRP-I. Under conditions of excess of sulfide (at least 10 times) the pseudo-first-order constant (k_{obs}) was obtained as a parameter from a weighted nonlinear least-squares analysis of the experimental curves. The first-order kinetics should be described by

$$-\frac{d[\text{HRP-I}]}{dt} = k_{1,obs}[\text{HRP-I}] \qquad (1)$$

For the larger concentrations of sulfide (0.25–0.3 mM) simple exponential traces were observed (although at sufficiently long times a linear portion of finite slope could be detected). Below 0.2 mM sulfide, as shown in Figure 1, simple exponential traces occurred only during the first third of the total sampling time followed by a linear portion of positive slope. A poor fit for an exponential decay to the entire trace was obtained. Similar behavior was observed in the transient-state kinetics of prostaglandin H synthase (MacDonald et al., 1989).

Thus, a rapid disappearance of HRP-I was observed prior to its slower reaction. It appears that a burst occurs followed

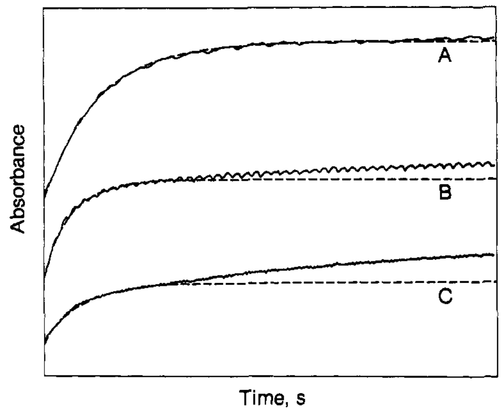


FIGURE 1: Stopped-flow traces of the relative absorbance at 411 nm for the reaction of HRP-I with 1-methoxy-4-(methylthio)benzene in acetate buffer, pH 5.0, ionic strength 0.11; [HRP-I], 1.0 μM; temperature, 25 °C; [sulfide] (mM) and sampling reaction time (s): (A) 0.3, 0.3; (B) 0.1, 0.8; (C) 0.05, 2. Broken line denotes curve fitting.

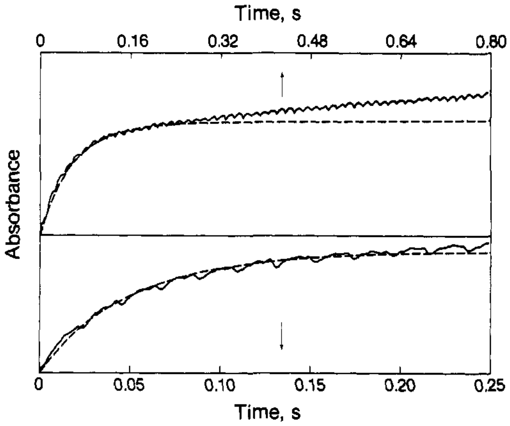


FIGURE 2: Stopped-flow traces of the relative absorbance at 411 nm for the reaction of HRP-I with 1-methoxy-4-(methylthio)benzene in acetate buffer, pH 5.0, ionic strength 0.11; [HRP-I], 1.0 μM; temperature, 25 °C; [sulfide], 0.1 mM. The arrows indicate the sampling reaction time employed in the experiments. Broken line denotes curve fitting.

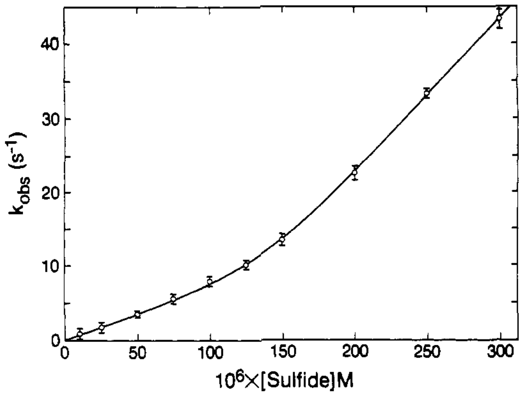


FIGURE 3: Plot of k_{obs} vs [sulfide] under the same conditions as described in Figure 1. The nonlinear response illustrates the deviation from first-order behavior in 1-methoxy-4-(methylthio)benzene. The line was obtained by fitting eq 3 to the experimental data through a nonlinear least-squares analysis. The error bars show the standard deviation calculated from several measurements of k_{obs} for each [sulfide] value.

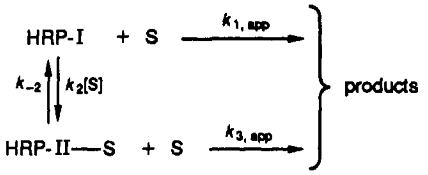
by a steady-state reaction. For each determination in the rapid-reaction analyzer, the spontaneous decomposition of HRP-I was tested and found to be negligible compared to the HRP-I reaction with sulfide.

In order to separate the apparent two steps in the reaction of HRP-I with 1-methoxy-4-(methylthio)benzene, kinetic determinations were performed in longer reaction times which encompassed both parts of the reaction (Figure 2, top) and in shorter sampling times which encompassed the first portion only, and where a good exponential fit was obtained to all of the data (Figure 2, bottom). At low substrate concentrations where the reaction is obviously biphasic, the restricted sampling time was required to obtain accurate k_{obs} values for the exponential portion. At sulfide concentrations below 25 molar equiv of the HRP-I concentration, the value of k_{obs} for the initial portion becomes too small to determine accurately, although the biphasic nature of the trace can still be detected.

When the pseudo-first-order rate constants, obtained from eq 1 for the reaction of HRP-I with 1-methoxy-4-(methylthio)benzene, are plotted against the concentration of the excess substrate, nonlinear behavior is observed at all pH values. A typical result is shown in Figure 3, where an approximately linear dependence upon substrate concentration is obtained at lower substrate concentrations; at higher substrate concentrations another approximately linear region is observed of greater slope. Thus the plot may be described as convex with respect to the abscissa.

Nonlinear behavior has been described for the reactions of HRP-II and HRP-I with *p*-cresol (Critchlow & Dunford, 1972; Hewson & Dunford, 1976) and for HRP-I and HRP-II with *p*-aminobenzoic acid (Dunford & Cotton, 1975). In these latter cases the nonlinear responses were concave toward the abscissa, and Michaelis–Menten kinetics were inadequate. A scheme in which both uncomplexed enzyme compound (I or II) and a nonproductive or less productive complex can react bimolecularly with a substrate molecule (Critchlow & Dunford, 1972; Hewson & Dunford, 1976) was required to fit the experimental data. Similarly, the dimerization of dimethylaniline has been proposed to involve enzyme–substrate complexes (Slaughter & O'Brien, 1986).

As a first approximation our experimental results (convex curves) could be considered as opposite to those described above for compounds like *p*-cresol (concave curves). A possible reaction scheme is



(2)

where $k_{1,\text{app}}$ and $k_{3,\text{app}}$ are the apparent second-order rate constants. The rate constants k_2 and k_{-2} are for the reversible formation of an HRP-II complex from HRP-I and sulfide. This scheme leads to the equation for $k_{1,\text{obs}}$ (see Appendix):

$$k_{1,\text{obs}} = \frac{k_{1,\text{app}}[\mathrm{S}] + \dfrac{k_{3,\text{app}}k_2[\mathrm{S}]^2}{k_{3,\text{app}}[\mathrm{S}] + k_{-2}}}{1 + \dfrac{k_2[\mathrm{S}]}{k_{3,\text{app}} + k_{-2}}} \qquad (3)$$

It was found that eq 3 would fit the $k_{1,\text{obs}}$ data obtained as a function of substrate concentration at each pH. The kinetic parameters $k_{1,\text{app}}$, $k_{3,\text{app}}$, k_2, and k_{-2} obtained with a nonlinear least-squares analysis are listed in Table I.

Figure 4 shows the log of the kinetic parameters versus pH for the oxidation of 1-methoxy-4-(methylthio)benzene by HRP-I.

Oxidation of 1-Methoxy-4-(methylthio)benzene by HRP-II. There are some similarities between the reaction of HRP-II with sulfide and the corresponding HRP-I reaction. In the

Table I: Parameters for Nonlinear Least-Squares Analysis of Dependence of Pseudo-First-Order Rate Constants (k_{obs}) on 1-Methoxy-4-(methylthio)benzene Concentration[a] for the HRP-I Reaction

pH[b]	$k_{1,app}$ (M^{-1} s^{-1})[c]	$k_{3,app}$ (M^{-1} s^{-1})[c]	k_2 (M^{-1} s^{-1})[c]	k_{-2} (s^{-1})[c]
3.4	$(3.7 \pm 0.2) \times 10^4$	$(1.02 \pm 0.06) \times 10^5$	$(7.2 \pm 0.1) \times 10^5$	$(1.8 \pm 0.1) \times 10^2$
3.8	$(3.5 \pm 0.2) \times 10^4$	$(1.47 \pm 0.02) \times 10^5$	$(7.7 \pm 0.2) \times 10^5$	$(2.6 \pm 0.1) \times 10^2$
4.4	$(2.8 \pm 0.2) \times 10^4$	$(2.03 \pm 0.07) \times 10^5$	$(5.9 \pm 0.1) \times 10^6$	$(1.3 \pm 0.2) \times 10^3$
5.0	$(2.5 \pm 0.3) \times 10^4$	$(5.6 \pm 0.05) \times 10^5$	$(1.17 \pm 0.05) \times 10^7$	$(1.0 \pm 0.1) \times 10^4$
5.5	$(2.6 \pm 0.3) \times 10^4$	$(5.9 \pm 0.1) \times 10^5$	$(1.4 \pm 0.2) \times 10^7$	$(1.2 \pm 0.1) \times 10^4$
6.0	$(3.2 \pm 0.2) \times 10^4$	$(4.9 \pm 0.2) \times 10^5$	$(8.1 \pm 0.6) \times 10^6$	$(6.8 \pm 0.6) \times 10^3$
6.5	$(3.2 \pm 0.2) \times 10^4$	$(4.2 \pm 0.3) \times 10^5$	$(4.5 \pm 0.4) \times 10^6$	$(5.0 \pm 0.3) \times 10^3$
7.0	$(3.9 \pm 0.2) \times 10^4$	$(3.5 \pm 0.2) \times 10^5$	$(3.9 \pm 0.2) \times 10^6$	$(4.4 \pm 0.2) \times 10^3$
7.5	$(4.5 \pm 0.2) \times 10^4$	$(3.6 \pm 0.2) \times 10^5$	$(4.1 \pm 0.2) \times 10^6$	$(4.3 \pm 0.3) \times 10^3$
8.0	$(6.2 \pm 0.4) \times 10^4$	$(2.9 \pm 0.2) \times 10^5$	$(4.0 \pm 0.1) \times 10^6$	$(3.4 \pm 0.2) \times 10^3$
8.6	$(6.8 \pm 0.3) \times 10^4$	$(2.7 \pm 0.2) \times 10^5$	$(3.9 \pm 0.2) \times 10^6$	$(2.8 \pm 0.2) \times 10^3$
9.1	$(5.5 \pm 0.2) \times 10^4$	$(2.4 \pm 0.3) \times 10^5$	$(3.2 \pm 0.2) \times 10^6$	$(9.0 \pm 0.5) \times 10^2$
10.0	$(1.3 \pm 0.1) \times 10^4$	$(1.5 \pm 0.1) \times 10^5$	$(1.5 \pm 0.1) \times 10^6$	$(1.6 \pm 0.1) \times 10^2$
10.5	$(1.2 \pm 0.2) \times 10^4$	$(1.4 \pm 0.1) \times 10^5$	$(6.9 \pm 0.2) \times 10^5$	$(1.1 \pm 0.1) \times 10^2$

[a] Concentration of 1-methoxy-4-(methylthio)benzene from 0.1×10^{-4} to 3.0×10^{-4} M. [b] Errors in pH values estimated at ±0.03. [c] Errors of rate constants represent the standard deviation calculated from data at a given pH.

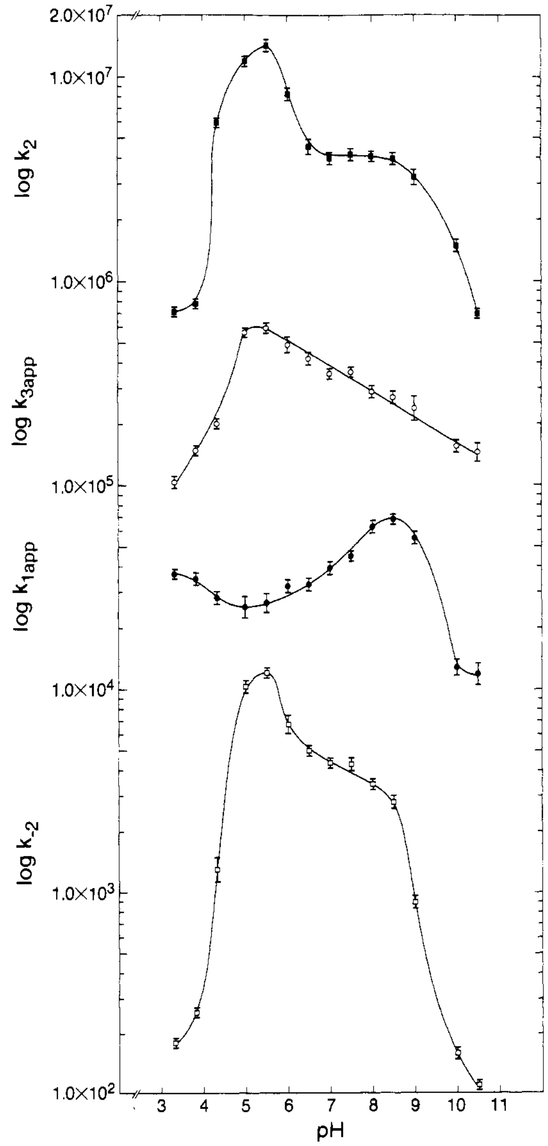


FIGURE 4: Plots of the values of the log of the kinetic parameters calculated from eq 3 versus pH for the reaction of HRP-I with 1-methoxy-4-(methylthio)benzene.

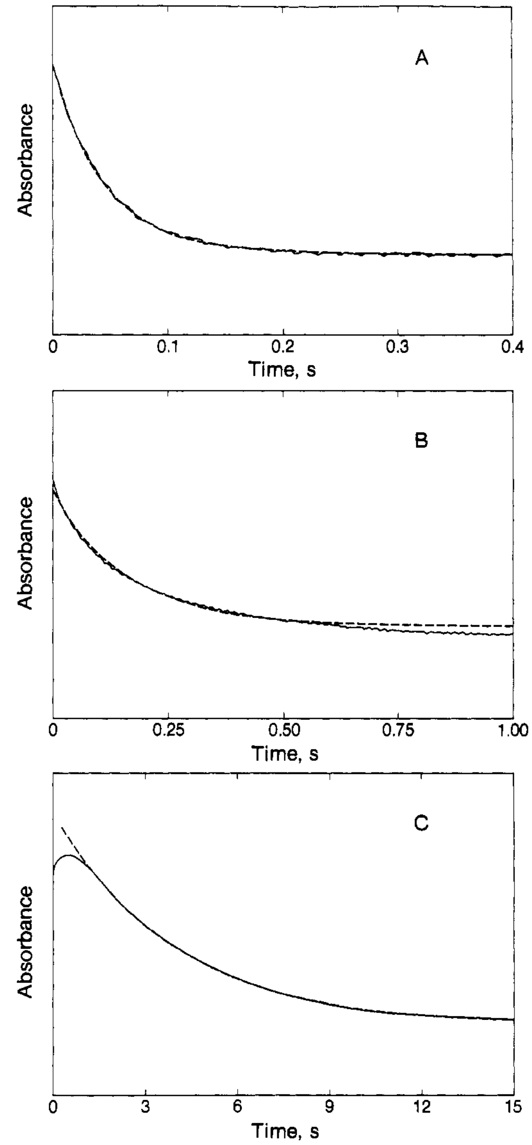


FIGURE 5: Stopped-flow traces of the relative absorbance at 427 nm for the reaction of HRP-II with 1-methoxy-4-(methylthio)benzene obtained by means of a pH jump from 9.0 to 5.0. (A and B) Ionic strength, 0.1; temperature, 25 °C. (A) [HRP-II], 1 μM; [sulfide], 0.3 mM. (B) [HRP-II], 1 μM; [sulfide], 0.1 mM. (C) [HRP], 1 μM; [H_2O_2], 1 μM; [sulfide], 0.1 mM; acetate buffer, pH 5.0; ionic strength, 0.11. Broken line denotes curve fitting.

HRP-II reaction only simple exponential traces were found for the larger concentrations of sulfide (0.2–0.3 mM). For sulfide concentrations below 0.15 mM exponential fits could be obtained only for the first part of the curves. An example is shown in Figure 5.

The plot of the pseudo-first-order rate constants (k_{obs}) versus the concentrations of the excess of sulfide also showed a

Table II: Parameters for Nonlinear Least-Squares Analysis of Dependence of Pseudo-First-Order Rate Constants (k_{obs}) on 1-Methoxy-4-(methylthio)benzene Concentration[a] for the HRP-II Reaction

pH[b]	$k_{4,app}$ ($M^{-1}\ s^{-1}$)[c]	$k_{6,app}$ ($M^{-1}\ s^{-1}$)[c]	k_5 ($M^{-1}\ s^{-1}$)[c]	k_{-5} (s^{-1})[c]
4.1	$(9.1 \pm 0.1) \times 10^2$	$(3.0 \pm 0.4) \times 10^4$	$(3.6 \pm 0.1) \times 10^5$	$(1.2 \pm 0.2) \times 10^2$
5.0	$(6.9 \pm 0.2) \times 10^3$	$(7.5 \pm 0.4) \times 10^4$	$(1.2 \pm 0.1) \times 10^6$	$(3.0 \pm 0.5) \times 10^2$
6.05	$(4.3 \pm 0.4) \times 10^3$	$(7.1 \pm 0.7) \times 10^4$	$(1.1 \pm 0.2) \times 10^6$	$(1.3 \pm 0.1) \times 10^2$
7.0	$(3.0 \pm 0.2) \times 10^3$	$(7.4 \pm 0.4) \times 10^4$	$(9.6 \pm 0.6) \times 10^6$	$(1.2 \pm 0.2) \times 10^2$
8.1	$(1.2 \pm 0.2) \times 10^3$	$(5.5 \pm 0.6) \times 10^4$	$(6.3 \pm 0.3) \times 10^5$	$(1.1 \pm 0.05) \times 10^2$
9.1	$(4.4 \pm 0.2) \times 10^2$	$(1.1 \pm 0.3) \times 10^4$	$(8.0 \pm 0.5) \times 10^4$	$(9.5 \pm 0.5) \times 10^1$

[a] Concentration of 1-methoxy-4-(methylthio)benzene from 0.1×10^{-4} to 3.0×10^{-4} M. [b] Errors in pH values estimated at ±0.03. [c] Errors of rate constants represent the standard deviation calculated from data at a given pH.

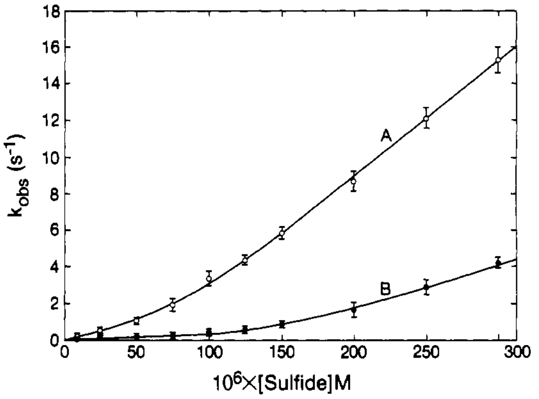
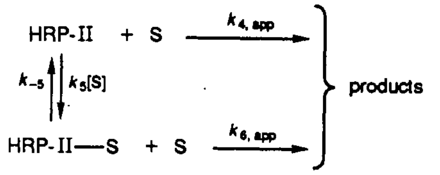


FIGURE 6: Plot of k_{obs} vs [sulfide]. (A) Starting from pure HRP-II at the same conditions as described in Figures 5 (A and B). (B) Starting from native HRP at the same conditions as described in Figure 5 (C). The nonlinear response illustrates the deviation from first-order behavior in 1-methoxy-4-(methylthio)benzene. The error bars show the standard deviations calculated from several measurements of k_{obs} for each [sulfide] value.

nonlinear behavior (Figure 6A).

Thus a similar kinetic response for the HRP-II reaction suggests a scheme mathematically similar to that for HRP-I:



(4)

where $k_{4,app}$ and $k_{6,app}$ are the apparent second-order rate constants for product formation and k_5 and k_{-5} the rate constants for the reversible formation of the HRP-II–sulfide complex. By analogy with eq 3, the pseudo-first-order rate constant for the HRP-II reaction, $k_{2,obs}$, may be related to the four kinetic parameters by

$$k_{2,obs} = \frac{k_{4,app}[S] + \dfrac{k_{6,app}k_5[S]^2}{k_{6,app}[S] + k_{-5}}}{1 + \dfrac{k_5[S]}{k_{6,app} + k_{-5}}} \qquad (5)$$

The kinetic parameters are summarized in Table II. It can be seen that the k_{app} values for the HRP-II reaction are approximately 5 and 10 times lower than those for HRP-I (comparison of Tables I and II). Figure 7 shows the log of the kinetic parameters versus pH for the oxidation of 1-methoxy-4-(methylthio)benzene by HRP-II.

An alternative approach also was used to study the reaction of HRP-II with the sulfide. Starting with HRP and H_2O_2 and allowing the formation of HRP-I, its conversion into HRP-II with excess sulfide and the subsequent reaction of HRP-II were monitored. Under conditions of large values of delay time and long reaction time in the stopped-flow apparatus, the signal recorded at 427 nm followed only the formation of HRP-II and its further reaction (Figure 5C).

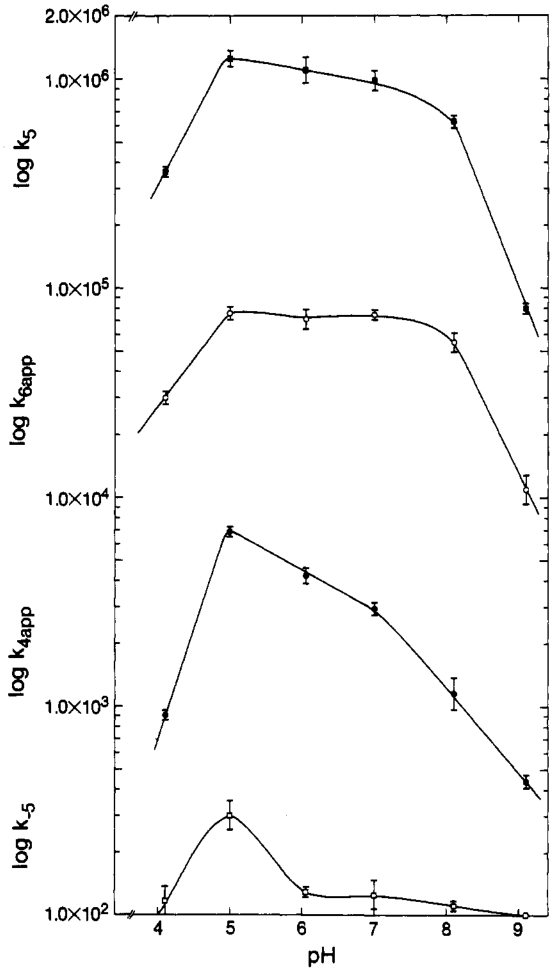


FIGURE 7: Plots of the values of the log of the kinetic parameters calculated from eq 5 versus pH for the reaction of HRP-II with 1-methoxy-4-(methylthio)benzene. $k_{4,app}$ (●); $k_{6,app}$ (○); k_5 (■); k_{-5} (□).

The plot of the pseudo-first-order rate constant for the reaction of HRP-II against the concentrations of sulfide (Figure 6B) showed also a nonlinear concave behavior. However, the values of the pseudo-first-order rate constants were around 5 times smaller than those obtained in the pure HRP-II reaction. Because of the different behavior observed for HRP-II for these conditions, we label the pseudo-first-order rate constant $k_{3,obs}$. The $k_{3,obs}$ data for the decay of HRP-II starting the reaction from HRP-I cannot be fit to eq 5.

In order to get more knowledge about the HRP-II reaction, the complete enzymatic cycle was studied under conditions of single turnover with only 1 or 2 molar equiv of sulfide and 1 molar equiv of H_2O_2 to form HRP-I. Under second-order conditions the kinetics of the reaction followed at 427 nm (isosbestic point between HRP-I and native HRP) showed that

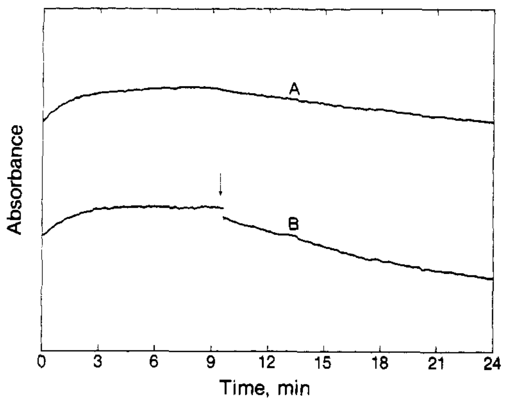


FIGURE 8: Reaction traces (relative absorbance versus time) at 427 nm under second-order conditions of [HRP-I] = [sulfide] for the reaction of HRP-I with 1-methoxy-4-(methylthio)benzene at pH 5.0, ionic strength 0.11; [HRP], 1 μM; [H_2O_2], 1 μM; temperature, 25 °C. (A) In the presence of 1 μM sulfide. (B) In the presence of 1 μM sulfide and a second addition of 1 μM sulfide indicated by the arrow.

the reaction between HRP-I and 1-methoxy-4-(methylthio)-benzene (Figure 8) proceeds in two steps; both of them are slow reactions. The first step, conversion of HRP-I to HRP-II, is faster (half-life ≈ 105–110 s) than the reaction of HRP-II, causing an increase in the absorbance at 427 nm. The second step is slower (half-life ≈ 700 s), and the decrease in absorbance corresponds to reaction of HRP-II (Figure 8A). When another molar equivalent of sulfide was added at a time corresponding to optimum formation of HRP-II, the second step is accelerated (half-life ≈ 330 s; Figure 8B).

DISCUSSION

Our transient-state kinetic results on the HRP-I and HRP-II reactions with 1-methoxy-4-(methylthio)benzene (a para-substituted thioanisole) point out the complexities of these reactions. At all concentrations of sulfide, the reaction exhibited an initial exponential burst followed by a linear phase. The linear phase became more pronounced at lower sulfide concentrations.

The exponential burst is the transient-phase reaction of HRP-I with sulfide. When the sulfide concentration is large, the reaction pathway is largely via the reaction with rate constant $k_{3,\mathrm{app}}$ (eq 2), and when it is small, the pathway is via the reaction with rate constant $k_{1,\mathrm{app}}$. The linear portion indicates zero-order kinetics and appears to be a steady-state phase. The simplest explanation is that some autoxidation of sulfide has occurred to produce a peroxide or peracid, which causes recycling of the enzyme. Thus the conversion of HRP-I to HRP-II is followed by the reaction of HRP-II to generate native enzyme which reacts with the autoxidation product to start the normal peroxidase cycle. In the steady-state cycle the rate-limiting step would be the formation of HRP-I. However, since the concentration of autoxidation product is not known, we cannot extract the rate constant for HRP-I formation from the linear phase.

The typical nonlinear curve of the pseudo-first-order rate constant k_{obs} for the HRP-I reaction versus the concentration of sulfide could be fit accurately with eq 2. At high sulfide concentrations the rate for formation of the complex is large (the ratio $k_2[\mathrm{S}]/k_{-2}$ is also large), and $k_{3,\mathrm{app}}$ is larger than $k_{1,\mathrm{app}}$. This explains the second part of the curve with large slope in Figure 3. On the contrary at low concentrations of sulfide the ratio $k_2[\mathrm{S}]/k_{-2}$ is small, and there is less formation of the productive complex. Under these conditions the main step must be the single reaction of HRP-I with sulfide with a very low rate constant (first part of the curve in Figure 3).

No rapid-scan spectral evidence for complex formation was reported in a previous paper on the HRP-I and HRP-II reactions with sulfide (Pérez & Dunford, 1990). Neither was there any appreciable change in the native HRP visible or Soret spectra upon addition of a high concentration of sulfide. However, this does not rule out the formation of a highly reactive complex at high sulfide concentrations. Such a complex would have a very short half-life and hence would exist at a very low concentration.

A mathematical analysis, identical with that for the HRP-I reaction, fits the reaction of HRP-II with sulfide, which displayed similar features. The main difference is in the values of the rate constants $k_{1,\mathrm{app}}$ and $k_{3,\mathrm{app}}$ for the HRP-I reaction and $k_{4,\mathrm{app}}$ and $k_{6,\mathrm{app}}$ for the HRP-II reaction. The ones for the HRP-II reaction were 8–10 times smaller than the values of the rate constants for HRP-I reaction (Tables I and II) for pH values between 4 and 8.

The computer analysis that yielded the rate constants $k_{1,\mathrm{app}}$, $k_{3,\mathrm{app}}$, k_2, and k_{-2} for the HRP-I reaction as a function of pH (Figure 4) reproduces the experimental values of k_{obs}. The formation of a reactive complex is favored below pH 7 since k_2 and $k_{3,\mathrm{app}}$ increase to a maximum at pH 5.5 and 5.0, respectively.

The lack of a hydrogen atom on the sulfur center means the thioanisoles are different from the common reducing substrates of HRP-I such as phenols (Job & Dunford, 1976), which can be considered hydrogen atom donors. Thus for the reaction of HRP-I with thioanisoles the requisite proton must be obtained elsewhere. A likely source is hydronium ion or water (Pérez & Dunford, 1990). Thus sulfur cation radical formation occurs during oxidation of sulfide by HRP-I, as has been suggested by Watanabe et al. (1981a,b) and Kobayashi et al. (1987).

The values of the rate constants $k_{4,\mathrm{app}}$, $k_{6,\mathrm{app}}$, k_5, and k_{-5} for the HRP-II reaction, obtained as a function of pH, also reproduce the experimental values of the pseudo-first-order rate constants. The profiles for these four kinetic parameters in Figure 5 show the role of the ionization of an acid group with a pK_a value of 8.6 in the catalytic activity of HRP-II (Dunford & Stillman, 1976; Dunford, 1982). Below pH 8 the values of the kinetic parameters related with the formation of a more active complex (k_5 and $k_{6,\mathrm{app}}$) increase and maintain their highest values at pH 7, 6, and 5. The same occurs to $k_{4,\mathrm{app}}$, showing an inverse behavior to the corresponding rate constant $k_{1,\mathrm{app}}$ in the unimolecular reaction of HRP-I.

All four kinetic parameters decrease at pH 4. Perhaps for the anisoles the acid group with pK_a ~ 0 in HRP-II has an inverse role to that for substrates such as ferrocyanide or *p*-aminobenzoic acid in which the effect of the group of pK_a = 8.6 is superceded at low pH by the effect of a very acid group (Critchlow & Dunford, 1972; Cotton & Dunford, 1973; Hubbard et al., 1975).

One important point to be explained is the difference in the values of the pseudo-first-order rate constants for the HRP-II reaction obtained starting from pure HRP-II and deduced from the complete enzymatic cycle starting with native HRP. A plausible explanation is a dual pathway in which HRP-II formed during reduction of HRP-I with sulfide could react either with the original sulfide substrate or with the sulfur cation radical produced from the sulfide (Pérez & Dunford, 1990).

The results of the single-turnover kinetic experiments obtained on the Cary spectrophotometer appear to support this hypothesis. The decrease in absorbance at 427 nm after HRP-II was formed from reaction of 1 molar equiv of sulfide with HRP-I suggests that HRP-II can react with the sulfur cation radical, which is the only reactant available under these conditions. Moreover, the addition of another molar equivalent of sulfide at the point of optimum HRP-II formation more than doubles the rate of the HRP-II reaction. Thus the difference in the values of k_{obs} for HRP-II obtained under different experimental conditions can be explained on the basis of two different available substrates, thioanisole and the sulfur cation radical.

Direct transfer of oxygen from hydroperoxide to the sulfur has been observed during oxidation of thioanisoles catalyzed by HRP (Kobayashi et al., 1986) and by soybean sulfoxidase (Blée & Durst, 1987). Since the one-electron reduction of HRP-I has been demonstrated as the only type of reaction with para-substituted thioanisoles (Pérez & Dunford, 1990), the direct transfer of the ferry oxygen atom is not possible. The latter oxene mechanism has been suggested for the reactions catalyzed by chloroperoxidase and cytochrome P-450 (Ortiz de Montellano, 1987). In the HRP–phenylhydrazine reaction it appears that the activated ferryl oxygen and the substrate are physically separated by a protein-imposed barrier (Ator & Ortiz de Montellano, 1987).

The following sequence of reactions for HRP oxidation of organic sulfides is consistent with our experimental results:

$$\mathrm{Fe^{3+} + H_2O_2 \rightarrow (Fe{=}O)^{3+} + H_2O} \tag{6}$$

$$\mathrm{(Fe{=}O)^{3+} + -\ddot{S}- \rightarrow (Fe{=}O)^{2+} + S^{\bullet+}} \tag{7}$$

$$\mathrm{(Fe{=}O)^{2+} + S^{\bullet+} \rightarrow Fe^{3+} + S{=}O} \tag{8}$$

$$\mathrm{(Fe{=}O)^{2+} + -\ddot{S}- \rightarrow Fe^{3+} + S^{\bullet+}} \tag{9}$$

$$\mathrm{2S^{\bullet+} \rightarrow S^{2+} + -\ddot{S}-} \tag{10}$$

$$\mathrm{S^{2+} + H_2O \rightarrow S{=}O + H^+} \tag{11}$$

where $\mathrm{Fe^{3+}}$, $\mathrm{(Fe{=}O)^{3+}}$, and $\mathrm{(Fe{=}O)^{2+}}$ represent native HRP, HRP-I, and HRP-II, respectively, and $\mathrm{\ddot{S}}$, $\mathrm{S^{\bullet+}}$, $\mathrm{S^{2+}}$, and $\mathrm{S{=}O}$ represent the sulfide, sulfur cation radical, dication, and sulfoxide.

The lack of stereospecificity in sulfoxidation catalyzed by HRP (Kobayashi et al., 1987) might be considered as a result of a reaction occurring in the heme edge. Then the close proximity of reactants in the active site leads to a tight coupling during the step of oxygenation (eq 8) through a hydroxyl radical released from the ferryl oxygen of HRP-II. This hydroxyl radical could attack easily the electron-deficient sulfur cation, forming the corresponding sulfoxide. This hypothesis does not rule out the reaction of HRP-II with sulfide that has been demonstrated during the transient-state kinetics experiments.

APPENDIX

Refer to eq 2:

$$v = k_{1,\mathrm{app}}[\mathrm{E}][\mathrm{S}] + k_{3,\mathrm{app}}[\mathrm{ES}] \tag{12}$$

$$[\mathrm{E}] = [\mathrm{E}]_0 - [\mathrm{ES}] \tag{13}$$

where [E] and [ES] represent [HRP-I] and [HRP-I-S], respectively.

$$\frac{d[\mathrm{ES}]}{dt} = k_2[\mathrm{E}][\mathrm{S}] - (k_{-2} + k_{3,\mathrm{app}}[\mathrm{S}])[\mathrm{ES}] \tag{14}$$

Substitution of eq 13 in eq 14 yields

$$[\mathrm{ES}] = \frac{k_2[\mathrm{E}]_0[\mathrm{S}]}{k_2[\mathrm{S}] + k_{3,\mathrm{app}}[\mathrm{S}] + k_{-2}} \tag{15}$$

Substitution of eqs 13 and 15 in eq 12 yields

$$\frac{v}{[\mathrm{E}]_0} = \frac{k_{1,\mathrm{app}}[\mathrm{S}] + \dfrac{k_{3,\mathrm{app}}k_2[\mathrm{S}]^2}{k_{3,\mathrm{app}}[\mathrm{S}] + k_{-2}}}{1 + \dfrac{k_2[\mathrm{S}]}{k_{3,\mathrm{app}} + k_{-2}}} \tag{16}$$

Since $v = k_{obs}[\mathrm{E}]_0$, then

$$k_{obs} = \frac{k_{1,\mathrm{app}} + \dfrac{k_{3,\mathrm{app}}k_2[\mathrm{S}]^2}{k_{3,\mathrm{app}}[\mathrm{S}] + k_{-2}}}{1 + \dfrac{k_2[\mathrm{S}]}{k_{3,\mathrm{app}} + k_{-2}}} \tag{17}$$

Registry No. HRP, 9003-99-0; 1-methoxy-4-(methylthio)benzene, 1879-16-9.

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