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pH Dependence of the Oxidation of Iodide by Compound I of Horseradish Peroxidase†

R. Roman and H. B. Dunford*

ABSTRACT: The kinetics and stoichiometry of the oxidation of iodide by horseradish peroxidase compound I have been studied as a function of pH at 25° and ionic strength 0.11. The second-order rate constant for the reaction varied from 2.1×10^6 to 7.7 M^{-1} sec^{-1} over the pH range 2.7–9.9. The pH dependence of the reaction is interpreted in terms of two ground-state ionizations on compound I; one pK_a is 4.6 and the other pK_a value lies outside the pH range of the study. It is established that the reaction of compound I proceeds without the intermediate formation of compound II in agreement with Björkstén, and involves a two-electron transfer from iodide. The possibility of formation of an iodine–peroxidase compound or complex in the time scale of the studies is excluded by the kinetic data.

Steady-state kinetic studies of the catalysis of the oxidation of iodide by hydrogen peroxide have been conducted using chloroperoxidase (Thomas *et al.*, 1970), lactoperoxidase (Morrison, 1968), thyroid peroxidase (Hosoya, 1968; Taurog, 1970), and horseradish peroxidase (Nunez and Pommier, 1968; Björkstén, 1968). The results of the present study of the reaction catalyzed by the latter enzyme (EC 1.11.1.7, donor–H_2O_2 oxidoreductase) were obtained primarily by monitoring the rate of change of concentration of the enzyme species directly.[1] Previous studies in our laboratory, concerned with other substrates, have also been conducted primarily by studying the reactions of the compounds of HRP in isolation

† From the Department of Chemistry, University of Alberta, Edmonton 7, Alberta, Canada. *Received September 30, 1971.* Supported financially by the National Research Council of Canada. R. R. is indebted to the National Research Council of Canada for a scholarship. This paper is the eighth of a series.

* To whom correspondence should be directed.

[1] Abbreviations used are: HRP, horseradish peroxidase; HRP-I and HRP-II, compounds I and II of HRP; PN, the ratio of absorbance at 403 and 280 nm.

from the enzymatic cycle (Hasinoff and Dunford, 1970; Cotton and Dunford, 1972;[2] Roman *et al.*, 1971; Critchlow and Dunford, 1972a,b). The present work on iodide oxidation, describes the second study undertaken on the pH dependence of the reactions of HRP-I, the first being the reaction with ferrocyanide (Hasinoff and Dunford, 1970).

Experimental Section

Horseradish peroxidase, obtained from Boehringer-Mannheim as an ammonium sulfate suspension, was dialyzed and passed through a Millipore filter prior to use. The PN of the resulting solution was 3.0 or greater. The concentration of HRP was determined spectrophotometrically at 403 nm using a molar absorptivity of 9.1×10^4 M^{-1} cm^{-1} (Keilin and Hartree, 1951).

All solutions were prepared from water that had been distilled from alkaline permanganate and then twice distilled from glass. Reagent grade inorganic chemicals were used without further purification. The ionic strength of all reaction mixtures was kept constant at 0.11, with 0.01 contributed by the buffer and the remainder by potassium nitrate and potassium iodide. A standard solution of Orion potassium iodide (94-53-06) was diluted for use daily. The concentrations of hydrogen peroxide solutions were determined spectrophotometrically by the HRP-catalyzed oxidation of iodide (Cotton and Dunford, 1972).[2]

An Orion Model 801 digital pH meter in conjunction with a Fisher combination electrode was used for pH measurements. A Cary 14 spectrophotometer was employed for absorption measurements and recording spectra.

The overall reaction stoichiometry for the HRP-catalyzed oxidation of iodide by H_2O_2 was determined at pH 7.24 by monitoring iodine production spectrophotometrically at 353 nm. The reaction was initiated by the addition of hydrogen peroxide from a microliter syringe to a cuvet containing a solution of 9.0×10^{-2} M potassium nitrate, 1.0×10^{-2} M potassium iodide, 4.4×10^{-6} M HRP, and phosphate buffer of ionic strength 0.01.

The stoichiometry of the reaction between HRP-I and iodide was determined by a titration of HRP-I with iodide. Solutions of HRP-I were prepared by the addition of 1 equiv concentration of hydrogen peroxide to a solution of 3.9×10^{-6} M HRP in 0.1 M potassium nitrate and pH 5.95 phosphate buffer. The titration was monitored at 411 nm using a molar absorptivity difference between HRP-II and HRP of 3.6×10^4 M^{-1} cm^{-1}, obtained from Figure 6. The stoichiometry of the reaction between HRP-II and iodide was determined by a titration of HRP-II monitored at 425 nm using a molar absorptivity difference between HRP-II and HRP of 5.5×10^4 M^{-1} cm^{-1}, interpolated from the spectrum of HRP-II measured at pH 4.2 (Critchlow and Dunford, 1972b). HRP-II was prepared by the addition of 0.6 molar equiv of *p*-cresol to a solution of 2.4×10^{-6} M HRP-I in a pH 4.03 citrate buffer of ionic strength 0.01. The spontaneous decay of both HRP-I and HRP-II was monitored until the rate of these reactions was much slower than the reactions with iodide, then solutions of potassium iodide were added from a microliter syringe and the changes in absorbance were measured.

Kinetic measurements on solutions of pH lower than 6.9 were performed on a stopped-flow apparatus. Measurements on solutions of higher pH were performed on the Cary 14 spectrophotometer. The rate of the reaction at all pH values was monitored by following the increase in absorbance at 411 nm. All measurements were made with the solution thermostatted at 25°. The method of analysis of the kinetic data has been described in an earlier publication (Roman *et al.*, 1971).

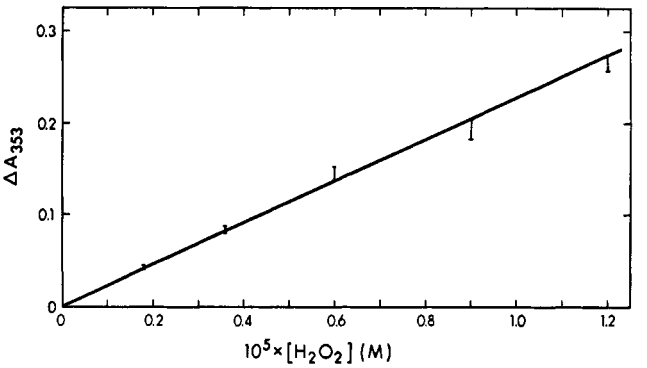

FIGURE 1: Plot of the change in absorbance at 353 nm *vs.* the concentration of hydrogen peroxide. Hydrogen peroxide was added to a solution of 4.4×10^{-6} M HRP, 1.0×10^{-2} M potassium iodide, 9.0×10^{-2} M potassium nitrate, and pH 7.24 phosphate buffer of ionic strength 0.01. The solid line was calculated assuming a 1:1 equivalence between hydrogen peroxide and iodine using the formula $\Delta A_{353} = [H_2O_2]/\epsilon_{353}(1 + 1/K[I^-])$.

The HRP-I used in kinetic studies on the stopped-flow apparatus was prepared by the addition of slightly less than 1 equiv of hydrogen peroxide to a solution of HRP (*ca.* 2×10^{-6} M). The HRP-I was stored in one syringe while a solution of iodide, buffer, and nitrate was stored in the other. The latter solution also contained *ca.* 10^{-9} M HRP to remove any oxidizing impurities. The HRP-I was sufficiently stable for its reaction to be detected up to 20 min after preparation. On the Cary 14 spectrophotometer the reactions were performed by the addition of hydrogen peroxide to a cuvet containing HRP (*ca.* 2×10^{-6} M), buffer, nitrate, and iodide. The hydrogen peroxide, from 5 to 10 μl, was added to a final concentration slightly less than that of HRP. The steady-state experiments and measurements of the spectrum of HRP-I were performed in a similar manner on the Cary 14; however, the potassium nitrate was not included in the spectral studies to minimize the spontaneous decay of HRP-I.

Results

It has been established that the overall stoichiometry of the HRP-catalyzed reaction between hydrogen peroxide and iodide is described by

$$H_2O_2 + 2I^- + 2H^+ \xrightarrow{\text{HRP}} I_2 + 2H_2O \qquad (1)$$

under conditions where the hydrogen peroxide concentration is much larger than the concentration of HRP (Björkstén, 1968). The validity of eq 1, under conditions where the concentrations of HRP and hydrogen peroxide are similar, was verified by measuring the iodine production at 353 nm on the Cary spectrophotometer and the results are illustrated in Figure 1. The linear relation in Figure 1 offers conclusive proof that iodine is not incorporated into the enzyme on the time scale of our experiments, even when the enzyme is present in large excess compared to the concentration of iodine produced.

[2] Submitted for publication.

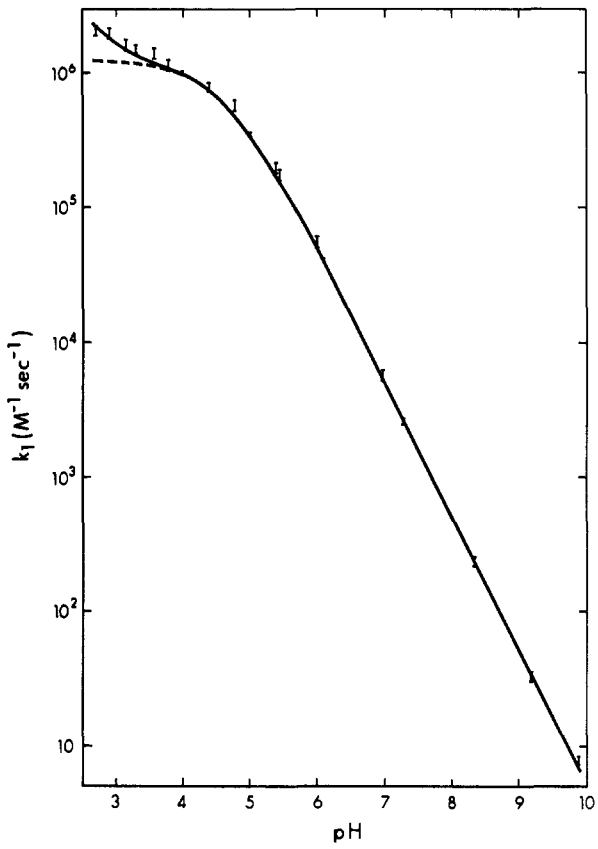

FIGURE 2: Plot of log k_1 *vs.* pH. The solid line was calculated by a weighted nonlinear least-squares analysis of eq 4. The dashed line was calculated from an analysis of eq 4 in which the $[H^+]/K_1$ term was neglected.

TABLE I: Second-Order Rate Constants for the HRP-I–Iodide Reaction.

pH	k_1 (M^{-1} sec^{-1})	$(k_{spont})/(k_{obsd})$ × 100	10^5 × Range of $[I^-]$ (M)	Buffer[a]
2.70	$(2.1 \pm 0.1) \times 10^6$	10	2–5	Pt
2.90	1.9×10^6		1–5	Pt
3.15	1.6×10^6		1–2	Pt
3.30	$(1.5 \times 0.1) \times 10^6$	3	1–4	Ci
3.58	1.4×10^6		1–2	Pt
3.79	1.1×10^6		5	A
3.99	$(9.9 \pm 0.2) \times 10^5$	<1	0.5–5	A
4.01	9.6×10^5		5	Ci
4.39	$(7.8 \pm 0.5) \times 10^5$	<1	1–4	A
4.78	5.7×10^5		2	A
4.95	4.0×10^5		5	Ci
4.99	$(3.5 \pm 0.04) \times 10^5$	<1	0.5–10	A
5.39	2.0×10^5		5	Ca
5.44	1.8×10^5		5	A
5.99	5.6×10^4		25	P
6.97	5.7×10^3		0.5–1	P
7.27	$(2.6 \pm 0.1) \times 10^3$	2	0.5–8	P
8.33	$(2.4 \pm 0.1) \times 10^2$	3	1–5 × 10	T
9.18	$(3.3 \pm 0.3) \times 10$	11	2.5–20 × 10	C
9.87	7.7 ± 0.3	9	2.5–10 × 10^2	C

[a] Buffer key: A, acetic acid–sodium acetate; C, sodium bicarbonate–sodium carbonate; Ca, cacodylic acid–sodium cacodylate; Ci, citric acid–sodium citrate, P, potassium dihydrogen phosphate–disodium hydrogen phosphate; Pt, phthalic acid–sodium phthalate; T, tris(hydroxymethyl)-aminomethane hydrochloride–tris(hydroxymethyl)aminomethane.

The titration of HRP-I with iodide, in which the ratio of the absorbance change at 411 nm to the molar absorptivity difference between HRP-I and HRP is plotted *vs.* iodide concentration, resulted in a straight line of slope 1.0 ± 0.02 as analyzed by weighted linear analysis. The titration of HRP-II with iodide resulted in a slope 1.0 ± 0.1 in a plot of the ratio of the absorbance change at 425 nm to the molar absorptivity difference between HRP-II and HRP *vs.* the concentration of iodide. These titrations indicate that the reactions of both HRP-I and HRP-II with iodide occur with a 1:1 ratio of the two reactants.

The kinetic behavior of the HRP-I-iodide system is consistent with a reaction that is first order in both HRP-I and iodide. All experiments were carried out with iodide concentrations at least ten times greater than the concentration of HRP-I; under these pseudo-first-order conditions the observed differential rate expression is

$$-\frac{d[\text{HRP-I}]}{dt} = k_{obsd}[\text{HRP-I}] \qquad (2)$$

Values of the second-order rate constant, k_1, were obtained from linear plots of k_{obsd} *vs.* iodide concentration obtained at pH values of 2.70, 3.30, 3.99, 4.39, 4.99, 7.27, 8.33, 9.18, and 9.87. The results of the linear analysis of such plots, along with their standard deviations are recorded in Table I. Errors in k_1 are equated to these standard deviations. At pH values below 3.5 and above 7 positive intercepts in the plots of k_{obsd} *vs.* $[I^-]$ were observed. These positive intercepts can be explained by the reaction of HRP-I with small concentrations of reactive impurities in the buffer, potassium nitrate or HRP solution. Thus the general relation between k_{obsd} and $[I^-]$ is

$$k_{obsd} = k_1[I^-] + k_{spont} \qquad (3)$$

where k_{spont} represents the rate of decay of HRP-I that is independent of iodide concentration. It is clear from eq 3 that even under conditions where k_{spont} is significant, that it has no effect on the value of k_1 obtained from a linear plot of k_{obsd} *vs.* $[I^-]$. The magnitudes of the k_{spont} terms expressed as percentages of the largest value of k_{obsd} obtained at any given pH are recorded in Table I. If only a single determination of k_{obsd} were made at a given pH a large concentration of iodide was used to minimize the error in calculating k_1, since under these conditions the value of k_{spont} is assumed to be zero. For these determinations of k_1 from a single value of k_{obsd} the error is estimated at ±10%. Different buffers used at the same pH yielded similar kinetic results, indicating that there were no detectable buffer effects.

The logarithm of k_1 is plotted *vs.* pH in Figure 2. As suggested by Björksten (1968), iodide ion and not hydriodic acid is the reacting species. Our results show that the reaction of hydriodic acid would require a rate constant greater by a factor of about 10^{10} than the diffusion-controlled limit (of not more than 10^{10} M^{-1} sec^{-1}). In a similar manner it was concluded that iodide ion is the reactive species with HRP-II (Roman *et al.*, 1971).

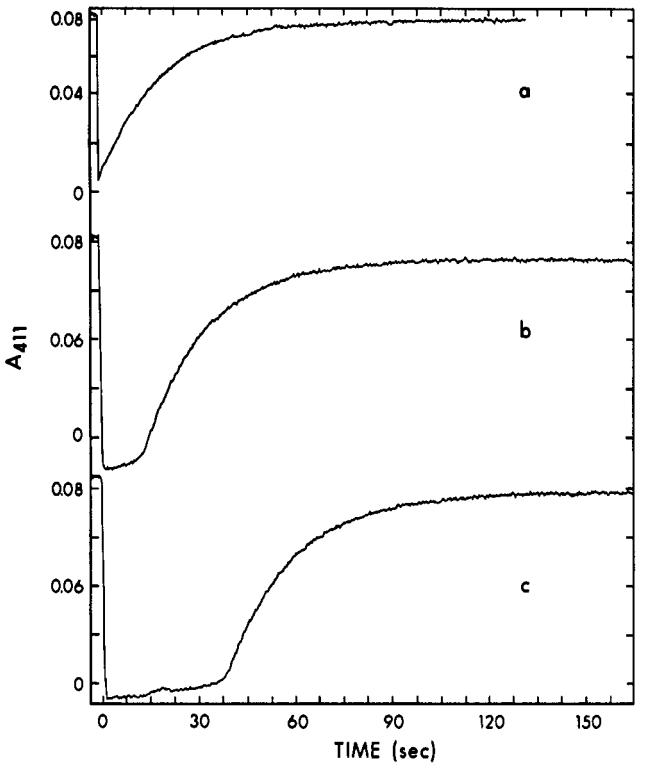

FIGURE 3: Experimental traces of absorbance at 411 nm *vs.* time. Microliter volumes of hydrogen peroxide were added to a solution of 2.7×10^{-6} M HRP, 2.5×10^{-4} M potassium iodide, 0.1 M potassium nitrate, and pH 8.4 Tris buffer of ionic strength 0.01. The concentration of hydrogen peroxide in (a) is 2.2×10^{-6} M, in (b) is 4.4×10^{-6} M and in (c) is 8.8×10^{-6} M. The observed first-order rate constants were: 5.5×10^{-2}, 5.3×10^{-2}, and 4.9×10^{-2} sec^{-1}. The absorbance values refer to an arbitrary zero.

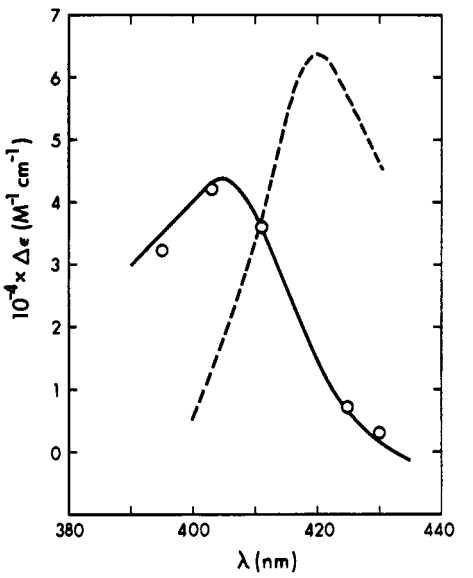

FIGURE 4: Plot of the molar absorptivity difference between HRP:HRP-I and HRP-II:HRP-I *vs.* wavelength. The solid line represents the molar absorptivity difference between HRP and HRP-I and the broken line represents the difference between HRP-II and HRP-I. The experimental points are the total change in absorbance observed for the reaction of 2.7×10^{-6} M HRP-I with 2.5×10^{-4} M potassium iodide in 0.1 M potassium nitrate and pH 8.4 Tris buffer.

The pH dependence of k_1 was analyzed using the approach of Critchlow and Dunford (1972c) where negative and positive curvatures in the plot of log k_1 *vs.* pH are represented by ground-state and transition-state dissociation constants. An interpretation involving a ground-state and transition-state pK_a, K_1, and $K_1^{\pm}$, illustrated by the solid line in Figure 2, results in satisfactory agreement with the experimental points. The line illustrates the best fit to the equation

$$k_1 = \frac{k_1'[H^+](1 + [H^+]/K_1^{\pm})}{1 + [H^+]/K_1} \qquad (4)$$

If the term $[H^+]/K_1^{\pm}$ in the numerator is neglected, the dashed line at low pH, shown in Figure 2, is obtained. The difference between this dashed line and the experimental points appears to be significant. The values of the parameters calculated from eq 4 are presented in Table II.

The commonly accepted mechanism for reactions of HRP-I involves the transfer of a single electron from an oxidizable substrate to HRP-I forming HRP-II (Brill, 1966). If this mechanism were applicable to the reaction between HRP-I and iodide, excess hydrogen peroxide in the HRP-I solution would not affect the reaction, since HRP-II reacts much more slowly than HRP-I (Roman *et al.*, 1971). It was observed, however, that if an excess of hydrogen peroxide were added to that required for complete conversion of HRP to HRP-I, the recording of absorbance *vs.* time at 411 nm exhibited an initial constant absorbance followed by an exponential decay. Increasing the concentration of hydrogen peroxide extended the duration of the linear section, but left the exponential section unchanged. Analysis of the exponential section, with and without an excess of hydrogen peroxide, resulted in the same value for the first-order rate constant. The experimental traces of absorbance *vs.* time are illustrated in Figure 3.

One explanation for the kinetic behavior of the reaction in the presence of excess hydrogen peroxide is that HRP and not HRP-II is produced directly from HRP-I. This behavior would result in the formation of a large steady-state concentration of HRP-I, which would exist until the hydrogen peroxide had been consumed. Negligible amounts of HRP and HRP-II would be present. To check this hypothesis the absorbance difference between HRP-I and the enzymatic reaction product was measured at 395, 403, 411, 427, and 430 nm. The experimental values for the total change in absorbance at the various wavelengths were converted to molar absorptivity values using the absorbance difference at 411 nm (the isosbestic point between HRP and HRP-II) as a reference. Figure 4 illustrates the molar absorptivity difference spectra calculated for the conversion of HRP-I to HRP and HRP-II. The HRP:HRP-I difference spectrum was calculated from values interpolated from Figure 6; the HRP-II:HRP-I spectrum was calculated from the spectrum of HRP-I in Figure 6

TABLE II: Ground-State and Transition-State Ionization Constants Obtained by the Nonlinear Least-Squares Analysis of Equation 4.

	Eq 4	Eq 4 Minus Term $[H^+]/K_1^{\pm}$
k_1' (M^{-2} sec^{-1})	$(5.0 \pm 0.1) \times 10^{10}$	$(4.9 \pm 0.2) \times 10^{10}$
K_1 (M)	$(2.3 \pm 0.1) \times 10^{-5}$	$(2.6 \pm 0.2) \times 10^{-5}$
$K_1^{\pm}$ (M)	$(2.1 \pm 0.35) \times 10^{-3}$	

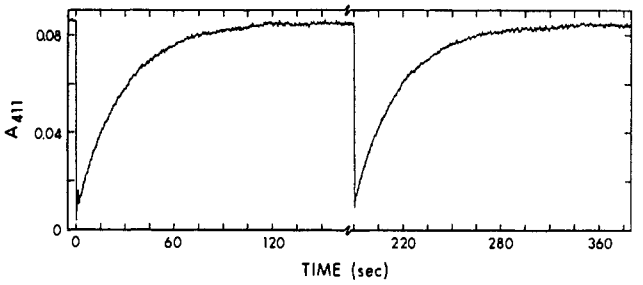

FIGURE 5: Experimental trace of absorbance at 411 nm *vs.* time resulting from the successive addition of equivalent concentrations of hydrogen peroxide to a solution of 2.4×10^{-6} M HRP in 1.5×10^{-4} M potassium iodide, 0.1 M potassium nitrate, and pH 8.4 Tris buffer. The time required for the second addition of hydrogen peroxide was 10 sec. The observed first-order rate constants were 3.6×10^{-2} and 3.8×10^{-2} sec^{-1}. The absorbance values refer to an arbitrary zero.

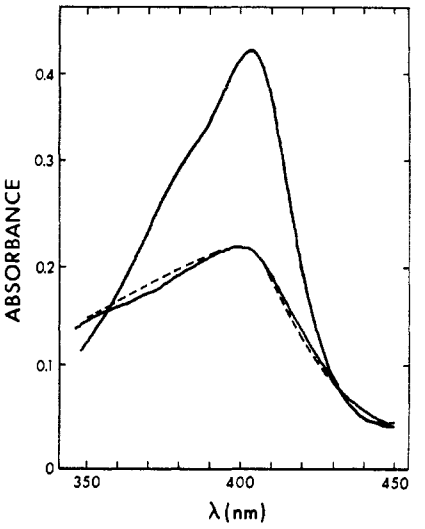

FIGURE 6: Spectrum of HRP-I compared to the spectrum of HRP. The solid line represents the spectrum scanned over a period of 40 sec following the addition of 6.0×10^{-7} mole of hydrogen peroxide to 2.0 ml of a solution containing 5.0×10^{-6} M HRP and 4.0×10^{-5} M potassium iodide in a pH 7.6 phosphate buffer. By monitoring the same reaction at 411 nm it is estimated that the HRP had completed six reaction cycles during the time taken to scan the spectrum. The broken line illustrates the corrected spectrum of HRP-I obtained by monitoring the reaction at each wavelength and extrapolating the absorbance to zero time.

and the spectrum of HRP-II published by Chance *et al.* (1967). The experimental points clearly indicate that the spectrum of the enzymatic product of the reaction between HRP-I and iodide resembles HRP.

A further test as to whether HRP-II is produced from the reaction of HRP-I was performed by making successive additions of hydrogen peroxide to solutions of HRP and iodide. As illustrated in Figure 5, the addition of hydrogen peroxide causes the rapid formation of HRP-I which then reacts more slowly with iodide. When the reaction trace at 411 nm reached its asymptotic value a second equivalent of hydrogen peroxide was added and a reaction trace identical with the first was observed. From the known rate constant for the reaction between HRP-II and iodide, the latter reaction would be less than 5% complete at the time of the second peroxide addition. The experiment was repeated at 420 nm, a wavelength that is more sensitive to the production of HRP-II, and similar results were observed.

These results indicate that for the HRP-catalyzed steady-state oxidation of iodide by hydrogen peroxide, the rate-determining step at high concentrations of hydrogen peroxide should be the reaction between HRP-I and iodide since HRP-II appears not to participate in the cycle. To test this hypothesis the steady state kinetics of the HRP-catalyzed reaction were studied over the pH range 5.4–7.0 using the technique of Björkstén (1968). The second-order rate constants for the oxidation of iodide calculated from these results were over 100 times greater than the values observed for the reaction between HRP-II and iodide (Roman *et al.*, 1971) and were in reasonable agreement with the k_1 values reported in this paper (Roman, 1972).

In iodide solutions the iodine produced is in equilibrium with triiodide ion as described by

$$I_2 + I^- \underset{}{\overset{K}{\rightleftharpoons}} I_3^- \qquad (5)$$

where K is 721 M^{-1} at 25° and the triiodide ion has a molar absorptivity (ϵ_{353}) of 2.62×10^4 M^{-1} cm^{-1} at 353 nm (Ramette and Sandford, 1965). At pH 7.0 and iodide concentration of 10^{-3} M, a single reaction was observed at 353 nm on the stopped-flow apparatus. These results indicate clearly that there is no kinetic evidence for the participation of HRP-II or other enzymatic intermediate in the reaction of HRP-I with iodide. The total change in absorbance indicated that 1 mole of iodine was produced for every mole of HRP-I which reacted. This again provides clear evidence that, within the time scale of the experiment, iodine does not react with the enzyme at a detectable rate.

The possibility of observing the spectra of enzymatic intermediates in steady-state concentrations provides a novel method for obtaining accurate spectra of these compounds. Figure 6 illustrates what we believe is a more accurate spectrum of HRP-I than those previously reported in the literature. The spectrum of HRP-I in Figure 6 was scanned over a period of time during which the HRP had completed six reaction cycles.

Since the spectrum was scanned under conditions where the term k_{spont} in eq 3 is significant, some HRP-II was formed during the time required to obtain the spectrum. The effect of this HRP-II formation was minimized in two ways: (1) by scanning the spectrum rapidly, producing the spectrum shown by the solid line in Figure 6 and (2) by observing absorbance changes with time at single wavelengths and extrapolation to zero time. The spectrum of HRP-I obtained in the latter fashion is shown by the broken line in Figure 6. The molar absorptivity values for HRP-I obtained by extrapolation of steady-state results to zero reaction time are recorded in Table III. Brill and Sandberg (1968) have published the molar absorptivity and wavelength of the Soret maximum which they use as criteria for the purity of HRP-I. The present value for the Soret maximum of 400 nm with molar absorptivity of 4.8×10^4 M^{-1} cm^{-1} indicates that our HRP-I is slightly more pure than their sample. In addition the present study shows the existence of the isosbestic points between HRP and HRP-I at 432 and 357 nm which are more sensitive to small concentrations of HRP-II than is the Soret maximum.

The molar absorptivity values in Table III were calculated assuming a molar absorptivity for HRP of 9.1×10^4 M^{-1} cm^{-1} at 403 nm (Keilin and Hartree, 1951). This value has been the subject of considerable discrepancy in the recent literature and the determination of its precise value has been

complicated by the existence of various isoenzymes of HRP. From chromatography studies on crude HRP (Roman *et al.*, 1971), we believe that our HRP is composed primarily of the B and C isozymes reported by Shannon *et al.* (1966) or isozyme III reported by Paul and Stigbrand (1970). We have chosen to use the quoted value for the molar absorptivity rather than that of Shannon *et al.* (9.5×10^4 M^{-1} cm^{-1}) or Paul and Stigbrand (1.0×10^5 M^{-1} cm^{-1}) to facilitate comparison to previously published work.

Discussion

The plot of the logarithm of the second-order rate constant for the reaction of HRP-I with iodide resembles the plot for the reaction of HRP-II with iodide (Roman *et al.*, 1971). HRP-I exhibits a rate-influencing ionization with pK_a value of 4.6 that has no observable effect on the rate of the HRP-II reaction. However, both plots have slopes of -1 in the alkaline region and neither displays a maximum rate at low pH. The similarity in the pH profiles for the two reactions leads us to speculate that the single rate-influencing ionization required to explain the reaction of HRP-II, the pK_a value of which lies outside the pH range of the study, could exert an analogous effect in the reaction of HRP-I. The similarity of the pH profiles for the reactions of ferrocyanide and iodide with HRP-I in acid solution suggests that the same ionization could also influence the rate of the ferrocyanide reaction (Hasinoff and Dunford, 1970). To determine whether the published pK_a of 5.3 for the ferrocyanide reaction could describe the same ionization observed for iodide, the HRP-I–ferrocyanide data were reanalyzed with a pK_a of 4.6 substituted as an invariant parameter. The agreement between calculated and experimental points was not substantially altered with this pK_a value, indicating that the value of the enzyme ionization constant determined from the two studies agree within experimental error. On the other hand, analysis of the HRP-I–iodide kinetic data using invariant pK_a values outside the range 4.4–4.8 resulted in significant deviations between experimental and calculated results.

Our results are in agreement with those of Björkstén (1970a) who reported that the HRP-catalyzed oxidation of iodide proceeds primarily *via* HRP-I, with HRP-II contributing at most 2% of the oxidized iodide. However we differ with the mechanism he postulates for the reaction of the main pathway

$$\text{HRP} + \text{H}_2\text{O}_2 \longrightarrow \text{HRP-I} \quad (6)$$

$$\text{HRP-I} + \text{I}^- \longrightarrow \text{ferriperoxidase–iodine} \quad (7)$$

$$\text{ferriperoxidase–iodine} + \text{H}_2\text{O}_2 \longrightarrow \text{HRP-I} + \text{I}\cdot + \text{product} \quad (8)$$

Björkstén made spectral measurements on the ferriperoxidase–iodine complex several minutes after initiation of the reactions between HRP-I and an equimolar amount of iodide. Under conditions of high iodide concentration we have never observed such a complex. Figure 1 illustrates that there is a 1:1 ratio between HRP-I reacted and iodine formed, a result that would not be expected if a portion of the oxidized iodide were incorporated into the HRP. In addition, since the ferriperoxidase–iodine complex is formed slowly compared to the reaction of HRP-I with iodide, the complex may not play an important role in the catalytic cycle. For example, at pH 6.2 under conditions where Björkstén observed the ferriperoxidase–iodine spectrum, the reaction between HRP-I and iodide is 88% complete in 10 sec (calculated using a rate constant interpolated from Figure 2) while measurements of the absorbance of the complex spectrum were made after 6 min.

TABLE III: Molar Absorptivity Values for the Spectrum of HRP-I.[a]

λ (nm)	$10^4 \times \epsilon$ (M^{-1} cm^{-1})
440	1.31
432	1.74
430	1.86
420	2.83
410	4.12
400	4.80
390	4.62
380	4.30
370	4.00
360	3.60
350	3.28

[a] Calculated using a molar absorptivity value of 9.1×10^4 M^{-1} cm^{-1} for HRP at 403 nm.

Björkstén (1970b) included atomic iodine in the reaction mechanism as a result of an attempted correlation with evidence involving the oxidation of oxalate ion. From our results the 1:1 ratio between H_2O_2 and I_2 in the stoichiometry of the overall reaction, the 1:1 ratio between HRP-I and iodide from the titration data and the direct conversion of HRP-I to HRP indicate that if the reaction between HRP-I and iodide produces atomic iodine, then the reaction must proceed *via* a mechanism

$$\text{HRP-I} + \text{I}^- \longrightarrow \text{HRP-II} + \text{I}\cdot \quad (9)$$

$$\text{HRP-II} + \text{I}\cdot \xrightarrow{\text{fast}} \text{HRP} + \text{I}^+ \quad (10)$$

The mechanism for the titration of HRP-II with iodide would then become

$$\text{HRP-II} + \text{I}^- \longrightarrow \text{HRP} + \text{I}\cdot \quad (11)$$

followed by reaction 10, which predicts that HRP-II and iodide would react in a 2:1 ratio. This ratio is excluded by our results for the titration of HRP-II with iodide. A reaction mechanism that is consistent with our results is

$$\text{HRP} + \text{H}_2\text{O}_2 \longrightarrow \text{HRP-I} \quad (12)$$

$$\text{HRP-I} + \text{I}^- \xrightarrow{k_1} \text{HRP} + \text{I}^+ \quad (13)$$

where I^+ does not necessarily exist in solution. As pointed out by Brill (1966), a distinction cannot be made between a simultaneous two-electron transfer and the formation of a transient enzyme–free-radical complex unless the decomposition of such a complex can be shown to be rate limiting under conditions of high-substrate concentration. If excess iodide is present in the solution, the production of iodine can formally be accounted for by the rapid reactions (Cotton and Wilkinson, 1966).

$$\text{I}^+ + \text{I}^- \longrightarrow \text{I}_2 \quad (14)$$

$$I^+ + OH^- \longrightarrow IOH \quad (15)$$

$$IOH + I^- \longrightarrow I_2 + OH^- \quad (16)$$

If iodide is not in excess the IOH or its degradation products could account for the slow reaction to form the ferriperoxidase–iodine complex.

The oxidation of oxalate observed by Björkstén could be explained if the iodide oxidation proceeds *via* the formation of an I^+–iron complex which is a sufficiently powerful oxidizing agent to react with oxalate. Such a complex could provide a rationale for the involvement of peroxidases in the catalysis of iodination reactions.

Acknowledgments

The authors are indebted to Dr. J. Critchlow for his helpful discussion and to Dr. M. Evett for assistance with computer programing.

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Dynamics of Fluoroescent Probe–Cholinesterase Reactions†

Richard T. Mayer and Chester M. Himel*

ABSTRACT: 1-(5-Dimethylaminonaphthalene-1-sulfonamido)-3-*N*,*N*-dimethylaminopropane and 1-(5-dimethylaminonaphthalene-1-sulfonamido)propane-3-trimethylammonium iodide are active-site-directed, equilibrium fluorescent probes. They are competitive inhibitors of horse serum cholinesterase (3.1.1.8). The basic group in each probe molecule binds at the anionic site. Subsequent binding of the fluorescent moiety is directed to an adjacent, hydrophobic site. Equilibrium dynamics of interaction of the probe–enzyme complexes were investigated with ammonium salts, organic solvents, guanidine, sodium chloride, and chlorinated hydrocarbon-type insecticides. Ammonium salts compete with the probes for the anionic site. Chlorinated hydrocarbon-type insecticides compete for the hydrophobic site. A series of organic solvents had significant effects on probe–enzyme binding. Active-site-directed, equilibrium fluorescent probes allow study of active-site dynamics.

Fluorescent probes of enzyme systems have spectral responses which reflect the environment of the probe. They can be used to monitor changes in conformation in enzymes and can be designed to be active-site-directed, equilibrium, competitive inhibitors (Himel *et al.*, 1971). The spectroscopy of intrinsic and extrinsic fluorescent probes has been reported [*cf.* Steiner and Edelhoch (1963), Chen (1967a), Chen *et al.* (1969), Edelman and McClure (1968), Stryer (1968)].

As early as 1952, Weber used 5-dimethylaminonaphthalene-1-sulfonyl chloride (dansyl chloride) to introduce a covalently bound fluorescent moiety into proteins. 8-Anilino-

† From the Department of Entomology, University of Georgia, Athens, Georgia 30601. *Received May 7, 1971.* This investigation was supported in part by a research grant, 16020 EAO, from the Environmental Protection Agency. R. T. M. was a post-doctoral research fellow, present address: Veterinary Toxicology and Entomology Research Laboratory, Agricultural Research Service, U. S. Department of Agriculture, College Station, Texas. Reprint requests and questions should be addressed to C. M. H. We are indebted to Dr. L. M. Chan for suggestions on and assistance with the manuscript.