Kinetics of the Oxidation of Iodide Ion by Lactoperoxidase Compound II†

R. J. Maguire and H. B. Dunford*

ABSTRACT: The kinetics of the oxidation of iodide ion by lactoperoxidase II have been studied as a function of pH at 25° and an ionic strength of 0.05. Both a first-order and a second-order dependence on the concentration of iodide ion were detected. The second-order rate constant for the reaction of lactoperoxidase II with iodide ion decreases from 4.2×10^6 to 2.6×10^{-1} M^{-1} sec^{-1} and the third-order rate constant decreases from 2.7×10^{10} to 1.4×10^8 M^{-2} sec^{-1} with increasing pH over the pH range 2.9–10.1. The pH dependence of the reaction is explained in terms of an acid dissociation outside the pH range of this study.

The horseradish peroxidase catalyzed oxidation of iodide ion was first observed by Bach (1904, 1907) at the turn of the century. In the thyroid gland a peroxidase with physical properties similar to those of the horseradish enzyme oxidizes iodide ion as a step in thyroid hormone biosynthesis (Hosoya and Morrison, 1967a). Hosoya and Morrison (1967b) have demonstrated that lactoperoxidase is more active in catalyzing the oxidation of iodide ion than is thyroid peroxidase, myeloperoxidase, or horseradish peroxidase. The chloroperoxidase of *Caldariomyces fumago* plays a similar role in the synthesis of caldariomycin, which contains chlorine (Hager *et al.*, 1966). Hence the peroxidase-catalyzed oxidation of halide ions is of particular interest. Previous studies on lactoperoxidase from this group have dealt with the binding of small ions including fluoride (Dolman *et al.*, 1968; Segal *et al.*, 1968; Maguire and Dunford, 1971). A recent study dealt with the reaction of lactoperoxidase with hydrogen peroxide (Maguire *et al.*, 1971).

Experimental Section

Lactoperoxidase was obtained as a lyophilized powder from Calbiochem. In this form, the samples exhibited a PN[1] of about 0.6. The enzyme was purified by gel filtration on Sephadex G-200 at 4° using a phosphate buffer of pH 7 and ionic strength 0.05 as eluent. Enzyme fractions obtained in this way which exhibited PN's greater than 0.8 were used in this study. The concentration of lactoperoxidase was determined spectrophotometrically at 412 nm using a molar absorptivity of 1.14×10^5 M^{-1} cm^{-1} (Morrison *et al.*, 1957).

The buffers used in this study, prepared from reagent grade chemicals and of an ionic strength of 0.05, are listed in Table I. An Orion Model 801 digital pH meter in conjunction with a Fisher combination electrode was used for all pH measurements.

Potassium iodide was obtained from Alfa Inorganics and the McArthur Chemical Co., and sodium iodide from Orion Research, Inc. The three iodide samples were found to exhibit identical chemical behavior within experimental error.

Solutions of hydrogen peroxide were prepared by diluting a 30% solution of hydrogen peroxide obtained from the Fisher Scientific Co. Concentrations of hydrogen peroxide were determined according to the method of Ovenston and Rees (1950). The water used in all solutions was distilled once from alkaline permanganate and once from glass.

The rate of reaction of LP-II with iodide ion was followed spectrophotometrically at 412 nm, the wavelength of maximum absorbance by native lactoperoxidase. The concentration of iodide ion was at least ten times that of LP, maintaining pseudo-first-order conditions. LP-II was prepared in most cases by the addition of 1 equiv of hydrogen peroxide to a LP solution, and had a half-life of over 20 min at most pH's. In Tris-HCl buffer, about 1.2–1.5 equiv of hydrogen peroxide was required, which may be due to the presence of some reducing agent in this buffer. LP and LP-II were found to exhibit an isosbestic point at 422 nm, and LP-II an absorption maximum at 432 nm, in agreement with the spectra obtained by Chance (1950).

The reactions between LP-II and iodide ion at 25° were studied using a Cary 14 recording spectrophotometer (equipped with 0–0.2 and 0–2 absorbance slide-wires), and a stopped-flow apparatus constructed in this laboratory which has a dead time of 6 m sec. The stopped-flow data were obtained as an amplified photomultiplier voltage print-out digitalized at 30 equally spaced intervals of time. The optical detection system has been described elsewhere (Ellis and Dunford, 1968).

In stopped-flow experiments, one driving syringe contained the LP-II in unbuffered aqueous solution, and the other syringe contained iodide ion in the appropriate buffer. This procedure minimized the possibility of enzyme denaturation at the low pH's at which some experiments were performed. In addition the total absorbance change was kept small ($\Delta A < 0.04$) so that the relative voltage changes observed, ΔV, were proportional to ΔA.

† From the Department of Chemistry, University of Alberta, Edmonton 7, Alberta, Canada. *Received October 4, 1971.* R. J. M. is the recipient of a 1967 Science Scholarship from the National Research Council of Canada, which also supported this work financially. This paper is the fifth of a series. References to preceding papers are included in the text.

*To whom to address correspondence.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: LP, lactoperoxidase; LP-I, the primary lactoperoxidase–hydrogen peroxide compound; LP-II, the secondary lactoperoxidase–hydrogen peroxide compound; HRP-II, the secondary horseradish peroxidase–hydrogen peroxide compound; PN, purity number, the ratio of the absorbance of a solution of lactoperoxidase at 412 nm to that at 280 nm; μ, ionic strength; k_{obsd}, the pseudo-first-order rate constant for the reaction of LP-II with iodide; k_1, k_2: second and third order rate constants, respectively, for the reaction of LP-II with iodide ion; ΔV, ΔA, changes in voltage and absorbance, respectively.

TABLE 1: Rate Constants with Standard Deviations for the LP-II–Iodide Reaction at 25.0° and Ionic Strength 0.05.

pH	$[I^-]$ (M)	k_1 (M^{-1} sec^{-1})	k_2 (M^{-2} sec^{-1})	Buffer[a]
2.86	7×10^{-6} to 2×10^{-5}	$4.20 \pm 0.55 \times 10^{6}$	$2.72 \pm 0.24 \times 10^{10}$	C
3.76	1×10^{-5} to 5×10^{-5}	$5.36 \pm 0.48 \times 10^{5}$	$4.15 \pm 0.37 \times 10^{9}$	C
4.17	1×10^{-5} to 5×10^{-5}	$1.24 \pm 0.07 \times 10^{5}$	$1.91 \pm 0.09 \times 10^{9}$	C
4.81	2×10^{-5} to 2×10^{-4}	$7.02 \pm 1.19 \times 10^{4}$	$3.42 \pm 0.51 \times 10^{8}$	C
5.01	1×10^{-5} to 8×10^{-5}	$1.19 \pm 0.12 \times 10^{4}$	$1.70 \pm 0.17 \times 10^{8}$	A
6.08	7×10^{-5} to 8×10^{-4}	$2.03 \pm 0.14 \times 10^{3}$	$1.50 \pm 0.24 \times 10^{7}$	P
6.35	5×10^{-5} to 5×10^{-4}	$6.20 \pm 0.56 \times 10^{2}$	$1.03 \pm 0.13 \times 10^{7}$	P
6.95	4×10^{-5} to 1×10^{-4}	$3.19 \pm 0.29 \times 10^{2}$	$1.48 \pm 0.18 \times 10^{6}$	P
7.04	2×10^{-4} to 5×10^{-3}	$2.01 \pm 0.24 \times 10^{2}$	$1.19 \pm 0.07 \times 10^{6}$	P
7.41	5×10^{-5} to 2×10^{-4}	$5.99 \pm 1.44 \times 10^{1}$	$6.21 \pm 0.62 \times 10^{5}$	T
7.93	3×10^{-4} to 7×10^{-3}	$6.62 \pm 0.73 \times 10^{1}$	$1.81 \pm 0.05 \times 10^{5}$	T
7.98	3×10^{-4} to 1×10^{-3}	$1.42 \pm 0.21 \times 10^{1}$	$1.69 \pm 0.13 \times 10^{5}$	T
8.81	3×10^{-4} to 8×10^{-4}	1.82 ± 0.38	$2.39 \pm 0.05 \times 10^{4}$	T
10.07	4×10^{-4} to 4×10^{-3}	0.26 ± 0.04	$1.41 \pm 0.17 \times 10^{3}$	Car

[a] Buffer key: C, citric acid–sodium citrate; A, acetic acid–sodium acetate; P, potassium dihydrogen phosphate–disodium hydrogen phosphate; T, tris(hydroxymethyl)aminomethane hydrochloride–tris(hydroxymethyl)aminomethane; Car, sodium bicarbonate–sodium carbonate.

The kinetic data were analyzed as described by Roman *et al.* (1971). At each pH and for a given concentration of iodide ion, three to five experiments were performed on the Cary spectrophotometer, or about ten experiments were performed if the stopped-flow apparatus was used. When it was possible, concentrations of iodide ion were used which would yield reaction rates which overlapped the time scales pertaining to the Cary spectrophotometer and the stopped-flow apparatus; using both instruments, experiments at the same concentration of iodide ion (and the same pH) yielded values for a pseudo-first-order rate constant, k_{obsd}, which were equal within experimental error.

Blank reaction rate experiments were conducted at various pH's in the absence of LP, and in no case was an appreciable rate measured (by observing absorbance changes at 353 nm, an absorbance maximum of triiodide ion), for the uncatalyzed reaction between iodide ion and hydrogen peroxide.

The products produced in the reaction between LP-II and iodide ions (iodine atoms or molecular iodine) may react further with lactoperoxidase, perhaps with aromatic amino acid residues. It was noticed in experiments performed using the stopped-flow apparatus that there exists a second, much slower, reaction. At pH 5, this reaction is 50 times slower than the reaction between LP-II and iodide ion, and so caused no detectable interference in measurements of the rate of the reaction of LP-II with iodide. Calculation of rate constants using the initial time course of the LP-II–iodide reaction led to values which were slightly higher (*ca.* 3%) than those rate constants obtained by computer analysis of the whole first-order curve. Because this value is within experimental error, it was not necessary to apply the correction, and it was concluded that this second, unidentified, reaction did not interfere with observation of the reaction between LP-II and iodide ion.

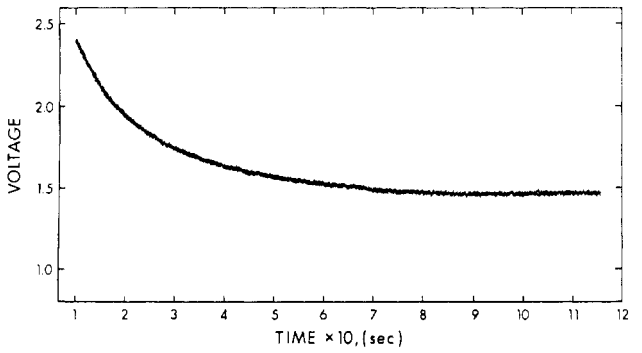


FIGURE 1: An oscilloscope trace of voltage *vs.* time for the reaction of LP-II with iodide ion at pH 4.17 citrate, $\mu = 0.05$. The reaction was observed at a monochromator setting of 412 nm. The initial concentrations of iodide ion and LP-II were 1.0×10^{-5} and 9.1×10^{-7} M, respectively. Data points from this trace are plotted in Figure 2.

Results

The kinetics of the reaction between LP-II and iodide under conditions of high iodide ion concentration in relation to the concentration of LP-II are consistent with the differential rate expression

$$-\frac{d[\text{LP-II}]}{dt} = k_{obsd}[\text{LP-II}] \quad (1)$$

Linear semilogarithmic plots (over 4 or more half-lives) of ΔV (or ΔA) *vs.* time proved the validity of eq 1. An example of an experimental curve is shown in Figure 1; Figure 2 shows a semilogarithmic plot of the curve in Figure 1, and also plots of curves obtained at two other pH values.

Figure 3 is a plot of k_{obsd} *vs.* the concentration of iodide ion, using data obtained at pH 6.95. The nonlinearity is evidence that a higher order in concentration of iodide ion is involved. Values of k_{obsd} as a function of the concentration of iodide ion were found to fit the relation

$$k_{obsd} = k_1[I^-] + k_2[I^-]^2 \quad (2)$$

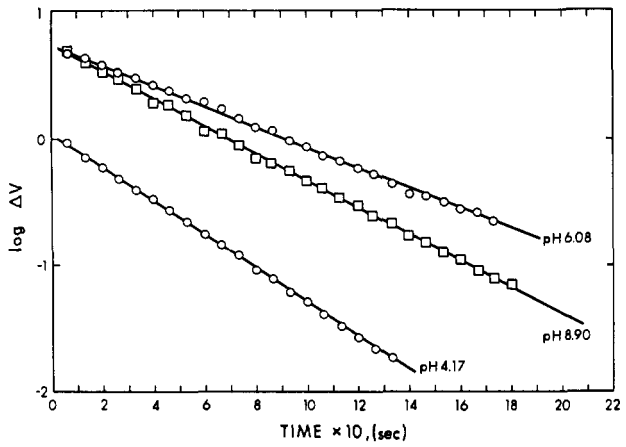


FIGURE 2: Semilogarithmic plots of ΔV *vs.* time for the reaction of LP-II with iodide ion at three pH's. Experimental conditions: pH 6.08, $[I^-] = 2.0 \times 10^{-4}$ M; pH 8.90, $[I^-] = 4.5 \times 10^{-2}$ M; pH 4.17, $[I^-] = 1.0 \times 10^{-5}$ M. In all cases the concentration of LP-II was about 9×10^{-7} M. Linear semilogarithmic plots of ΔV (or ΔA) *vs.* time (over more than four half-lives) over the pH range 2.8–10.1 proved the validity of eq 1.

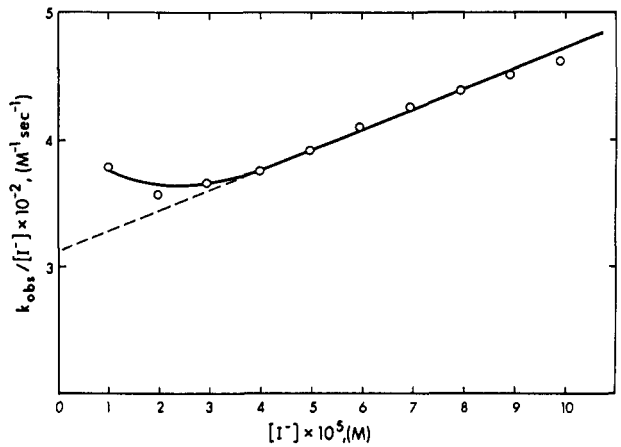


FIGURE 4: A plot of $k_{obsd}/[I^-]$ *vs.* $[I^-]$ at pH 6.95 for the LP-II–iodide reaction. The straight line was obtained by least-squares analysis. The slope, k_2, with its standard deviation is $1.48 \pm 0.18 \times 10^6$ M^{-2} sec^{-1}. The intercept, k_1, is $3.19 \pm 0.29 \times 10^2$ M^{-1} sec^{-1}.

where k_1 and k_2 are second and third-order rate constants, respectively. A plot of $k_{obsd}/[I^-]$ *vs.* $[I^-]$ yields k_2 from the slope and k_1 from the intercept. A plot of $k_{obsd}/[I^-]$ *vs.* $[I^-]$ using data obtained at pH 6.95 is shown in Figure 4. The plot curves up at low concentrations of iodide ion because of the spontaneous decay of LP-II. The values of the rate constants k_1 and k_2 were determined by least-squares analysis. The rate constant k_1 varies from $2.6 \pm 0.4 \times 10^{-1}$ to $4.20 \pm$

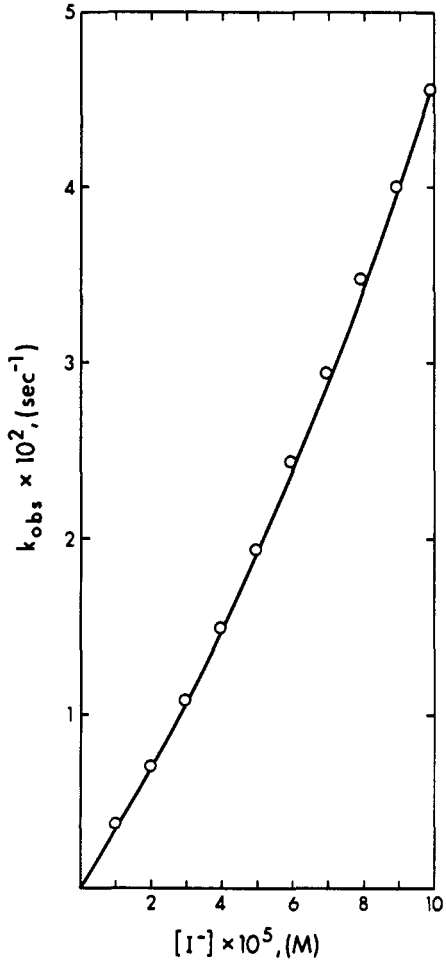


FIGURE 3: A plot of k_{obsd} *vs.* $[I^-]$ at pH 6.95 phosphate, $\mu = 0.05$. The curvature shows that the reaction is not simply first order in the concentration of iodide ion.

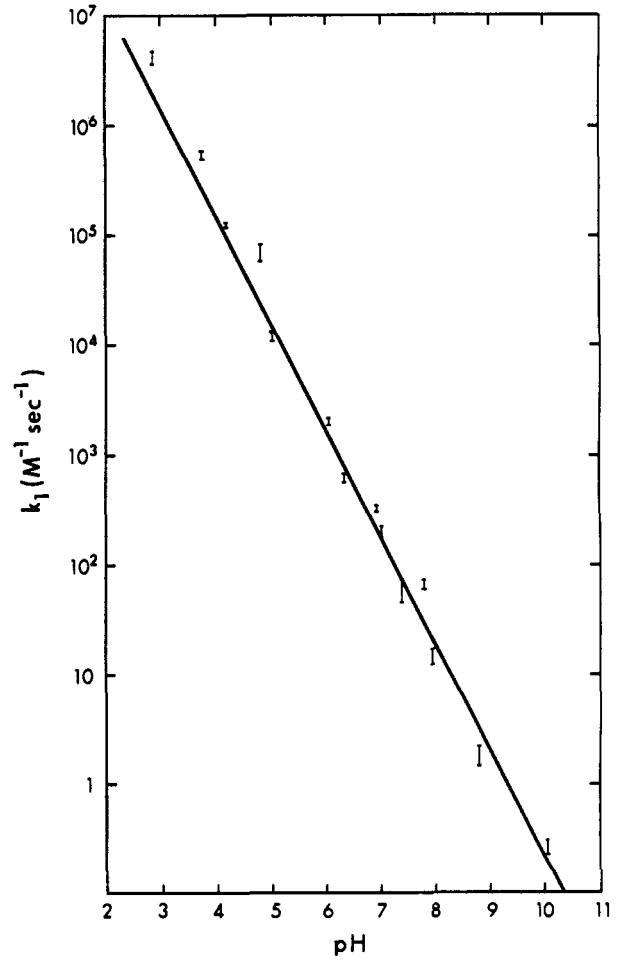


FIGURE 5: A semilogarithmic plot of k_1 *vs.* pH for the reaction of LP-II with iodide ion. The error bars on the points are standard deviations obtained by analysis of plots such as Figure 4 at each pH. The solid line is a least-squares fit, with a slope of -0.98 ± 0.04.

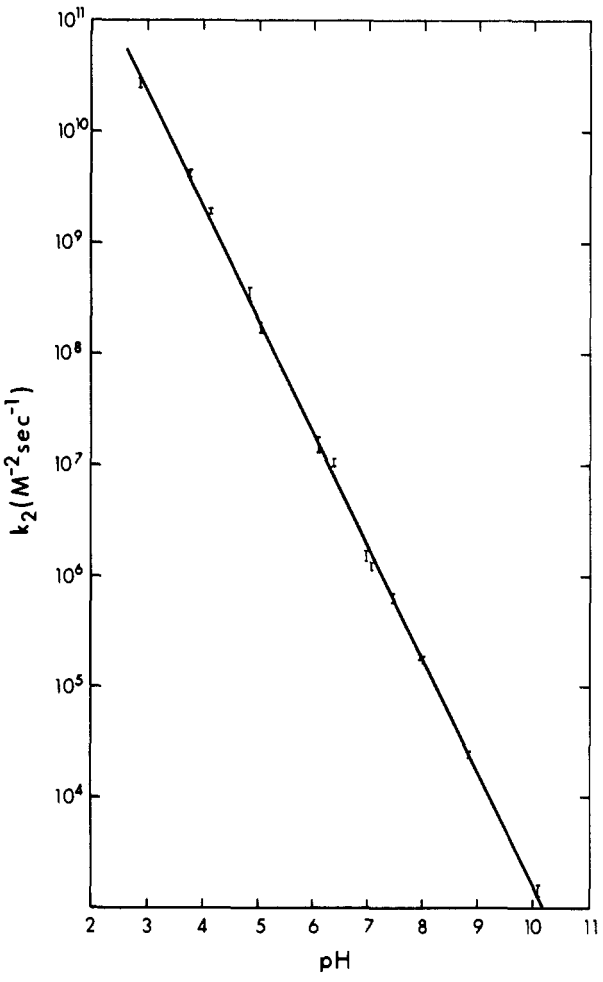


FIGURE 6: A semilogarithmic plot of k_2 *vs.* pH for the reaction of LP-II with iodide ion. The error bars on the points are standard deviations obtained by analysis of plots such as Figure 4 at each pH. The solid line is a least-squares fit, with a slope of -1.03 ± 0.10.

0.44×10^6 M^{-1} sec^{-1} over the pH range 10.07–2.86, while the rate constant k_2 varies from $1.41 \pm 0.17 \times 10^3$ to $2.72 \pm 0.24 \times 10^{10}$ M^{-2} sec^{-1} over the same pH interval.

The rate constants k_1 and k_2 are plotted logarithmically *vs.* pH in Figures 5 and 6, and listed in Table I. The slope (obtained by least-squares analysis) of the log k_2 *vs.* pH plot is -1.03 ± 0.10; the slope of the log k_1 *vs.* pH plot is -0.98 ± 0.04. The error in k_1 is greater than the error in k_2 because of the interference of the spontaneous rate of decay of LP-II at the low concentrations of iodide ion in plots such as Figure 4.

A slight perturbation of the Soret spectrum of lactoperoxidase was observed when the concentration of iodide ion was in very large excess over that of lactoperoxidase. Figure 7 shows a difference spectrum at pH 7 of LP + I^- *vs.* LP. The same difference spectrum is obtained at pH 4.6. There is a maximum in the difference spectrum at 431 nm, and a minimum at 410 nm, an observation made previously by Morrison *et al.* (1970). It is not clear if there is binding of iodide ion to the iron of lactoperoxidase. Perhaps the perturbation of the Soret spectrum is due to a structural alteration induced by the high concentration of salt in the enzyme solution; we observed that nitrate ion at the same concentration produced the same sort of difference spectrum. In addition, we were unable to remove cyanide ion bound to LP (by observing a shift of the Soret maximum of LP-CN at 432 nm back to

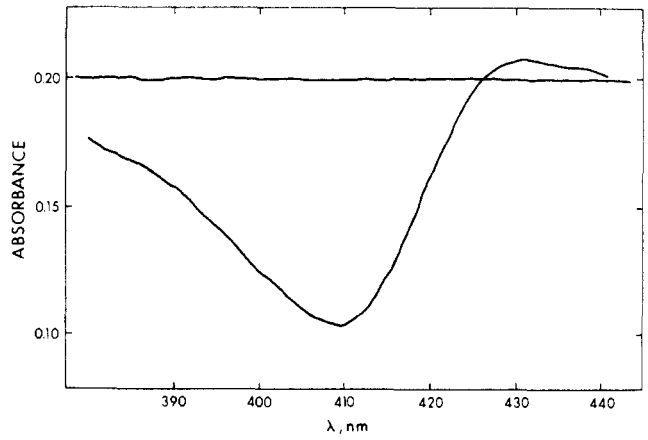


FIGURE 7: A difference spectrum of LP plus iodide ion *vs.* LP at pH 7. There is a maximum at 431 nm and a minimum at 410 nm. The baseline is LP *vs.* LP balanced to 0.2 absorbance unit. The concentrations of LP and iodide ion are 1.02×10^{-5} and 1.06 M, respectively.

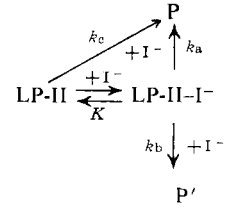
412–413 nm) by adding iodide ion up to very high concentrations (*ca.* 0.25 M). In general hemoprotein halide complexes form more readily at low pH; however, our spectral results give no indication of such a trend for iodide and indeed provide little evidence for any binding of iodide. It can at least be said that if iodide ion binds to the iron of lactoperoxidase, the extent of binding is much less than that of fluoride (Segal *et al.*, 1968) and cyanide (Dolman *et al.*, 1968).

Discussion

The conversion of LP-II to LP is clearly indicated in our kinetic results, and so the oxidation of iodide by LP-II occurs by the expected one-electron transfer. We were unable to perform successfully the difficult task of titrating LP-II with iodide however. Comparable experiments in the better behaved HRP-II system confirmed the stoichiometry: HRP-II + $I^- \rightarrow$ HRP + $0.5I_2$ (R. Roman and H. B. Dunford, submitted for publication).

The plots of log k_1 *vs.* pH and log k_2 *vs.* pH are linear with a slope of -1 within experimental error. This behavior can be explained by a kinetically important ionization on either the enzyme or substrate. The pK_a of hydriodic acid has been estimated to be about -9 (Bell, 1959). Using this value, it can be shown that the rate constant k_1 for the reaction of LP-II with HI would exceed the maximum value of the diffusion-controlled limit (10^{10} M^{-1} sec^{-1}) by about eight orders of magnitude. Therefore iodide ion is the reacting species, and the kinetically important ionization is that of an acid group on the enzyme.

A simple mechanism which incorporates the effects of first- and second-order concentrations of iodide ion (but not the ionization in the enzyme) is



where P and P′ are the products.

LP-II–I^- is a complex formed between LP-II and iodide ion, and K is its dissociation constant. One may be able to make some choice as to which steps are more important in the mechanism. From the work of Roman *et al.* (1971) it is known that HRP-II does not form a long-lived complex with iodide ion. However, the second-order rate constant k_1 for the reaction of LP-II with iodide is larger by a factor of 12 over the comparable constant for HRP-II. This indicates greater efficiency in the LP-II oxidation and therefore favors the formation of the LP-II–iodide complex. The direct reaction, with rate constant k_c, cannot be excluded on the basis of a purely kinetic argument however. The reaction which is second order in iodide points clearly to the importance of a complex between LP-II and iodide ion. The probability of termolecular collisions would appear too small to account for a significant proportion of the total rate; this possibility has not been shown in the mechanism. The alternative involves the reaction between iodide ion and the LP-II–iodide complex, an example of substrate activation.

It is possible to place limits on the value of the dissociation constant, K_a, of the kinetically important ionizing group on the enzyme. From the lack of curvature at low pH of the log k_1 *vs.* pH plot, an upper limit can be assigned of $pK_a \leq 2.8$. A lower limit of pK_a is obtained by extrapolation of the log k_1 *vs.* pH plot to the diffusion-controlled limit. Depending upon whether one uses 10^8 or 10^{10} M^{-1} sec^{-1} for this diffusion-controlled rate (Alberty and Hammes, 1958) one obtains $pK_a \geq 1.2$ or -0.8. This pK_a value lies outside the range of ionization constants for the common amino acids found in proteins, and may be due to the influence of the heme group on the ionization of an acid, or the ionizing group may be a component of the heme group. An acid group bound in the fifth or sixth coordination position of the heme iron appears an attractive possibility, but an acid group obtained from protonation of the porphyrin ring cannot be excluded. The catalytic influence of the acid group cannot occur through a rate-controlling proton transfer, since this leads to a prediction of general acid catalysis, a result clearly excluded by our experimental results.

The reaction of LP-II with iodide is similar to the HRP-II iodide reaction in that the catalytic importance of a single acid group on the enzyme is clearly demonstrated in both reactions (Roman *et al.*, 1971). However, the faster rate and the importance of a term second order in iodide in the LP-II reaction, point clearly to the pitfalls if one attempts to extrapolate results from one enzymatic reaction to the analogous reaction for a related enzyme. Recently Björksten (1970) concluded that HRP-I oxidizes iodide by a two-electron transfer and this finding has been confirmed in our laboratory (R. Roman and H. B. Dunford, submitted for publication). This implies that HRP-II is absent from the steady-state cycle in the HRP-catalyzed oxidation of iodide by H_2O_2. In practice it is found that HRP-II does accumulate slowly in the steady-state process, but this can be accounted for by the spontaneous conversion of a small fraction of HRP-I to HRP-II and by the fact that HRP-I reacts much more rapidly than HRP-II with iodide (R. Roman and H. B. Dunford, submitted for publication). Thus, establishment of the rate of the LP-II-iodide reaction will help to determine whether the LP-catalyzed steady-state oxidation of iodide by H_2O_2 proceeds by a similar mechanism to that of the HRP-catalyzed reaction.

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