Studies on Horseradish Peroxidase

X. THE MECHANISM OF THE OXIDATION OF P-CRESOL, FERROCYANIDE, AND IODIDE BY COMPOUND II*

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JOHN E. CRITCHLOW† AND H. BRIAN DUNFORD

From the Department of Chemistry, University of Alberta, Edmonton 7, Canada.

SUMMARY

Binding of p-cresol to native horseradish peroxidase was investigated by differential spectrophotometry, and the value $10^5 K_{\text{diss}} = 3 \text{ M}$ was obtained at neutral and acid pH; binding is not competitive with that of cyanide and hydroxide. The Soret region spectrum of Compound II of the enzyme was measured in the steady state at pH 4.26, 6.89, and 10.95, and the differences were found to be too small to be attributed to the acid dissociations of an iron-bound group. The kinetics of the reactions between Compound II and p-cresol, ferrocyanide, and iodide was investigated in 94% D$_2$O. Almost no solvent isotope effect was found on the ionization of the group of pKa 8.6. The reaction with p-cresol gave $k_D/k_H = 2.6 \pm 0.4$, which was attributed to a rate-determining proton transfer. In regions of the pH log rate profile having a slope of $-1$ ferrocyanide gave $k_D/k_H = 4.0 \pm 0.4$. The pH rate profiles, isotope effects, and over all rates of the three reactions were correlated in terms of a mechanism involving intramolecular general acid catalysis. In sufficiently acidic solutions this ultimately gives way to a specific acid-catalyzed mechanism, and the mechanistic changeover occurs at a higher pH the more difficult the over-all reaction.

This paper describes a number of experiments designed to throw additional light on the nature of the mechanism of Compound II reduction. The view that all three reactions may be regarded as essentially similar, simple bimolecular processes depends on the conclusion that the binding of p-cresol to Compound II occurs at a position away from the active site, and indirect support for this idea was therefore looked for in the interaction between p-cresol and the native enzyme. In order to investigate further the role of the group of pKa 8.6, which appears to have a marked effect on the oxidation of ferrocyanide and p-cresol but exerts no influence on that of iodide, the spectrum of Compound II was measured over a range of pH. It was felt that if this group were bound to the iron atom its protonation would cause significant changes in the Soret band. Finally the kinetics of the oxidation reactions was studied in D$_2$O in order to learn more about the mechanisms and transition states of the three reactions. It was found possible to correlate the over-all rates, pH rate profiles, and kinetic isotope effects in the three cases in terms of a model involving a transition from intramolecular general acid catalysis to specific acid catalysis.

MATERIALS AND METHODS

Binding of p-Cresol to Native Enzyme—Reagent grade potassium cyanide was obtained from Fisher Scientific, whereas horseradish peroxidase and all other materials used were obtained and purified as described previously (1). Spectrophotometric measurements were made over the range 360 to 440 nm using a Cary 14 spectrophotometer, fitted with either a 0 to 0.1 to 0.2 or 0 to 1 to 2 absorbance slide wire. Solutions were made up to an ionic strength of 0.11 by the addition of potassium nitrate, and all experiments were conducted at 25°C.

A detailed set of experiments was carried out at pH 6.91, maintained by a phosphate buffer which contributed an ionic strength of 0.04. Since preliminary studies indicated that the addition of p-cresol produced no more than a 3% change in absorbance at any wave length in the Soret region, a differential method was used, in which the reference cuvette contained enzyme at the same concentration and pH as the sample. A similar approach has been used by Theorell and Paul (4) in their study of the effect of pH on the $\alpha$- and $\beta$-bands. A typical spectrum is shown in Fig. 1. Each sample was made up directly in the cuvette by adding 1 ml of the enzyme solution to 2 ml of a buffer solution containing p-cresol, and blanks were run to check that the absorbance of the latter was negligible.

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In order to investigate the site at which p-cresol binding occurs, the above series of measurements was repeated using a solution of the enzyme 1.06 \times 10^{-4} \text{ M} in potassium cyanide in preparing both the sample and reference solutions. The dissociation constant for the enzyme-cyanide compound in the absence of p-cresol was measured by recording the change in absorbance at 403 nm upon adding successive amounts of a cyanide solution to a buffered sample of the peroxidase by means of a Hamilton microliter syringe fitted with a Chaney adaptor. This process was then repeated in the presence of 7.22 \times 10^{-3} \text{ M} p-cresol.

The above technique was used to measure the dissociation constant of the enzyme-p-cresol complex over a range of pH, and to carry out preliminary investigations for the related substances phenol, tyrosine, and aniline. The latter compounds were of reagent grade purity.

**Spectrum of Compound II as Function of pH**—The most reliable measurements of the spectrum of Compound II of horseradish peroxidase made by previous workers (5, 6) were obtained by adding an excess of peroxide and a smaller excess of a rapidly reacting reducing substrate to a sample of the enzyme. The absorption quickly reached a constant value, attributed to pure Compound II, and then showed a slow change as a result of reaction with endogenous donor or products. The method used in the present work differed from this by the use of a larger excess of a reducing substrate whose rate of reaction was slow enough to permit spectral measurements on Compound II during the finite lifetime of the steady state. The distribution of the enzyme among its three forms could then be calculated from the known rate constants and concentrations as described under "Results," and appropriate corrections made for the small amounts of Compound I or native enzyme present. In previous work the characteristics of the initial steady state reaction were not known, and with rapidly reacting substrates there was always the danger that the fate of the enzyme might depend partly on the speed of mixing.

All materials not previously mentioned were of reagent grade, and were used without further purification. Experiments at high pH were performed using a 7.3 \times 10^{-6} \text{ M} solution of the enzyme, maintained at pH 10.95 by means of a carbonate buffer. Enzyme and buffer were mixed immediately prior to reaction in order to prevent any denaturation. Ten microliters of a freshly prepared 6 \times 10^{-3} \text{ M} solution of potassium ferrocyanide were added from a Hamilton microliter syringe to 3 ml of the enzyme solution in the cuvette, and the reaction was initiated by the addition of 5 \mu l of 5 \times 10^{-6} \text{ M} hydrogen peroxide which had been previously deposited on a Teflon stirring plunger. The absorbance monitored at 420 nm quickly attained a constant value, allowing the spectrum to be scanned from 440 to 350 nm at a rate of 1 nm per s, after which the constancy of the absorption at 420 nm was checked. The latter showed a small increase over a subsequent period of about 15 min as a result of the ferrocyanide produced, at the end of which time it decreased fairly sharply, denoting the end of the steady state phase.

Ferrocyanide was also employed as reducing substrate in a phosphate buffer solution of pH 6.89. Because of the faster rate an enzyme concentration of 7.3 \times 10^{-4} \text{ M} was used and the change in absorbance on adding peroxide was measured separately at 10-nm intervals over the spectral range. Lower concentrations of ferrocyanide and peroxide were found to lead to less reproducible results, and it was therefore necessary to extrapolate the increasing absorbance due to ferrocyanide back to the time of mixing. This extrapolation amounted to no more than 0.002 absorbance units over a 6-s time interval, compared with a maximum enzyme absorbance of 0.066. The steady state ended within a minute, and the experiment was then repeated twice at each wave length with further additions of ferrocyanide and peroxide to the same enzyme solution.

A series of measurements was made in an acetate buffer of pH 4.26, using ascorbic acid as reducing substrate with the same enzyme concentration and technique employed for ferrocyanide at pH 6.89. Addition of ascorbic acid to the enzyme solution produced a slight shift in the Soret band, an effect which has previously been noted for catalase (7). On addition of hydrogen peroxide to give a solution 6 \times 10^{-3} \text{ M} in ascorbic acid and 4 \times 10^{-6} \text{ M} in peroxide a steady state was set up which lasted for only about 12 s, but since no absorbing product was formed no back extrapolation was required. A second measurement was made at the same wave length after adding fresh reducing and oxidizing reactants to give initial concentrations of 1.2 \times 10^{-4} \text{ M} and 7 \times 10^{-3} \text{ M}, respectively.

Approximate rates for the reaction of Compounds I and II with ascorbic acid were measured on the stopped flow apparatus at the same pH and ascorbic acid concentration as used above. The technique was as described in the previous paper (1), except that the reaction with Compound I was followed at 411 nm, at which point the native enzyme and Compound II are isosbestic.

**Kinetics in Deuterium Oxide**—Deuterium oxide with a stated isotopic purity of 99.8% was obtained from Columbia Chemicals and used to prepare stock solutions of p-cresol, potassium.
iodide, and potassium nitrate. The same solutions of buffer and enzyme in light water were added to both protio and deuterium oxide solution, giving the latter a final deuterium atom fraction of 94%. Hydrogen peroxide and potassium ferrocyanide were added in microliter amounts and so contributed negligibly to the final protium content.

The reactions of p-cresol and iodide ion with Compound II in light and heavy water were studied on the stopped flow apparatus using the technique described previously (1). Final reaction mixtures in the study with p-cresol contained a phosphate buffer at a pH of 6.83 in the protio solution, whereas use of the same buffer in the deuterium oxide solution gave an uncorrected pH reading of 6.93. The acetate buffer used for the reaction with iodide produced a true and apparent pH of 4.66 and 4.76, respectively. All solutions had a total ionic strength of 0.11, and the reactions were studied at 25°. Real and apparent pH meter readings were made for each solution upon completion of the reaction, using an Orion 501 digital pH meter in conjunction with a Fisher combination electrode.

The rate of the horseradish peroxidase-catalyzed oxidation of ferrocyanide by hydrogen peroxide in 94% deuterium oxide was measured in the steady state mode over a range of pH. The reaction was followed by monitoring the production of ferricyanide at 420 nm, and the initial rates were obtained as described by Hasinoff and Dunford (2). An initial ferrocyanide concentration of 1.44 x 10^-4 M was used in each run, together with an amount of enzyme ranging from 3.78 x 10^-5 to 9.45 x 10^-5 M, added by means of a 10-μl Hamilton syringe fitted with a Chaney adaptor. The same stock solution of enzyme was used throughout. The reaction was initiated by adding 10 μl of 5 x 10^-2 M hydrogen peroxide to 2 ml of solution in the cuvette with the aid of a Teflon plunger. Duplicate runs were performed in each case, and the apparent pH levels of the solutions were measured. An acetate buffer of pH 4.68 (giving an apparent pH reading of 4.78 in 94% D2O) was used to obtain data for a comparable set of runs in the two waters. The total absorbance change in all cases could be represented by an effective molar absorptivity of 1.02 x 10^3 μmol.cm^-1, in reasonable agreement with the published value for ferrocyanide of 1.05 x 10^3 μmol.cm^-1 (8).

The oxidation of ferrocyanide by Compound II in light and heavy water was also observed directly in a set of experiments carried out in the presence of a carbonate buffer of pH 10.33 (apparent pH reading in 94% D2O = 10.60). Ferrocyanide was added by means of a 10-μl syringe fitted with a Chaney adaptor to a cuvette containing 2 ml of a buffered solution 1 x 10^-4 M in enzyme. The reaction was initiated by adding sufficient hydrogen peroxide to give a concentration of 8 x 10^-3 M, and was followed at 420 nm on the Cary 14 spectrophotometer. Since the reaction of ferrocyanide with Compound I at least 4 orders of magnitude faster than that with Compound II at this pH (2), the observed absorbance change was due entirely to the conversion of Compound II to the native enzyme.

RESULTS

Binding of p-Cresol to Horseradish Peroxidase—The change of absorbance resulting from the binding of p-cresol to the native enzyme may be described by the equation,

\[ \Delta A = \Delta A_g \frac{[S]}{[S] + K_g} \]  

where \([S]\) represents the concentration of p-cresol, \(K_g\) is the dissociation constant for the complex (assuming a 1:1 stoichiometry), and \(\Delta A_g\) is the absorbance change at infinitely high \([S]\). Since the position of the base line in Fig. 1 is subject to error arising from the presence of very small amounts of extraneous matter in the solution or on the surface of the cuvettes, the algebraic difference between the absorbance changes at two wave lengths, which is simply the difference between two points on the difference spectrum, was used. This quantity obeys the analogous relation,

\[ \Delta A' = \Delta A'_{g} \frac{[S]}{[S] + K_g} \]  

The choice of 300 and 410 nm, at which wave lengths the enzyme has very similar absorbivities, was made to avoid errors resulting from small differences in either the path lengths or enzyme concentrations of the sample and reference. Fig. 2 shows the fit of the experimental data at pH 6.91 to Equation 2, as calculated by nonlinear least squares analysis, which gave \(10^6 K_g = 2.8 \pm 0.4\) M. The alternative choice of 378 and 406 nm, the peak and trough of the difference spectrum, respectively, gave \(10^6 K_g = 2.5 \pm 0.3\) M, which suggests that any unwanted differences between sample and reference were in fact no more important than the noise level of the instrument. The mean of the two results, \(10^6 K_g = 2.7\) M, was therefore taken as the best measure of the dissociation constant at this pH.

Fig. 3 shows the results for cyanide binding, plotted according to the reciprocal form of Equation 1. A small correction for dilution has been made to the measured absorbance changes. As for all ionizing substances, the quoted cyanide concentrations, [CN], refer to the total concentrations of acidic and basic forms. From the intercept and slope of the straight line a value of \(10^6 K_{CN} = 3.1 \pm 0.2\) M is obtained for the dissociation constant, in reasonable agreement with the result \(10^6 K_{CN} = 27 \pm 0.3\) M interpolated from previous kinetic data (9).

If it is assumed that p-cresol and cyanide bind at different sites on the enzyme and may therefore form a double complex S-HRP-CN, a third dissociation constant \(K_{S,CN} = [HRP-CN] [S]/[S-HRP-CN]\) is needed to define the system. The absorbance change on adding both binding species to the enzyme is then given by

\[ \Delta A = \frac{[S]}{K_s} \Delta A_s + \frac{[CN]}{K_{CN}} \Delta A_{CN} + \frac{[S][CN]}{K_{S,CN} K_{CN}} \Delta A_{S,CN} \]  

where \(\Delta A_{CN}\) and \(\Delta A_{S,CN}\) are the limiting values of \(\Delta A\) in the presence of infinite concentrations of cyanide and of both cyanide and p-cresol, respectively. In the event that the two species bind competitively at the same site, \(K_{S,CN}\) becomes infinite and

\[ \Delta A = \frac{[S]}{K_s} \Delta A_s + \frac{[CN]}{K_{CN}} \Delta A_{CN} \]  

The absorbance changes observed on adding cyanide to a solution containing a constant concentration of p-cresol are plotted against the right-hand side of Equation 4 in Fig. 4, using the values of the constants obtained above. \(\Delta A_s\) at 403 nm.
Fig. 2. Plot of algebraic difference between changes in absorbance at 390 and 410 nm versus log of p-cresol concentration; [p-cresol] in molar (M) units; [enzyme] = 7.3 \times 10^{-6} M. The smooth curve represents the best fit of the data to Equation 2.

Fig. 3. Plot of reciprocal of absorbance change at 403 nm versus reciprocal of total cyanide concentration; [enzyme] = 7.3 \times 10^{-6} M.

was calculated from Fig. 1 and used to make a correction to the measured ΔA values for the small decrease in the absorbance of the enzyme (0.0014) resulting from the presence of the p-cresol at zero [CN]. It can be seen from the dashed line of unit slope in Fig. 4 that the data are not compatible with competitive binding, and calculations showed that to make them so would require $K_{CN}$ to be reduced by a factor of 2 or $K_8$ to be increased by a factor of 7. These measurements were therefore analyzed instead by Equation 3 using the nonlinear least squares program, which yielded $10^3 K_{8, CN} = 7.1 \pm 1.2$. The value of $ΔA_{8, CN}$ was close to that of $ΔA_{CN}$, showing that the binding of p-cresol to the cyanide complex, like that to the enzyme itself, has a very small effect on the Soret band.

The data obtained on adding p-cresol to a solution containing enzyme in the presence of a constant concentration of cyanide are shown in an analogous fashion in Fig. 5. The algebraic difference between the measured decrease at 400 and 435 nm, at which wave length the cyanide complex has similar molar absorptivities, has been added to the algebraic difference between the decrease in absorbance at these two wave lengths on adding cyanide to the free enzyme (0.524) to obtain each value of $ΔA$. The results can be seen to be incompatible with the postulate of competitive binding, and nonlinear least squares analysis according to Equation 3 gave $10^3 K_{8, CN} = 5.0 \pm 1.0 M$. These two measurements of $K_{8, CN}$ under different conditions agree within the error limits of their standard deviations, and thus lend
Conversion to the hydroxide complex at pH 10.12 makes the observed at .380 and 420 nm. Although the small degree of changes to the second is given by the figures in the last column in the absence of this substrate. The ratio of the first of these
pH in the presence of infinite p-cresol with the change occurring can be judged from the results at pH 10.12 and 10.50 by com-
zyme and the cyanide complex, the degree of hydroxide binding small as those observed in its interaction with the native en-
p-cresol to the hydroxide complex produces spectral changes as incursion of a slow irreversible reaction between p-cresol and these results were unfortunately prevented by the
high pH would be expected to show whether the binding of p-cresol and cyanide at two distinct sites. It would appear
further support to the concept of simultaneous binding by p-cresol and cyanide to at two distinct sites. It would appear
that binding of p-cresol increases the dissociation constant of the cyanide complex by rather more than a factor of 2, whereas
the binding of cyanide necessarily has the same effect on the p-cresol complex.

The data obtained for the binding of p-cresol to the free enzyme over a range of pH were analyzed as described above, and the results are given in Table I. The three results at neutral pH indicate that any interference by the buffer is probably small. As in the case of the complex with Compound II, binding of p-cresol in its acid form provides the simplest explanation for the observed pH dependence. Data at very high pH would be expected to show whether the binding of p-
cresol affects the dissociation constant of the hydroxide complex, in the same way as that of the cyanide complex, but measure-
ments in this region were unfortunately prevented by the incursion of a slow irreversible reaction between p-cresol and the peroxidase. However, if it is assumed that the binding of p-cresol to the hydroxide complex produces spectral changes as small as those observed in its interaction with the native enzyme and the cyanide complex, the degree of hydroxide binding can be judged from the results at pH 10.12 and 10.50 by com-
paring the change in absorbance on going from neutral to high pH in the presence of infinite p-cresol with the change occurring in the absence of this substrate. The ratio of the first of these changes to the second is given by the figures in the last column of Table I, which have been computed from the average shifts observed at 380 and 420 nm. Although the small degree of conversion to the hydroxide complex at pH 10.12 makes the first of these figures rather imprecise, it would appear that the effect of p-cresol binding on that of hydroxide is similar in magnitude and direction to its effect on the binding of cyanide.

Data for a number of related substances are also included in Table I, and the negative results obtained with tyrosine suggest that steric factors may be important. Using a technique similar to the present one, Ricard, Santimone, and Vogt (10) showed that β-indolybutyric acid produces small spectral changes on addition to horseradish peroxidase, and obtained $K_{\text{diss}} = 3.3 \pm 0.2$ M, but no other reliable quantitative evidence appears to have been obtained for the binding of reducing substrates to the native enzyme.

**Spectrum of Compound II as Function of pH**—The generally accepted role of the enzyme in the peroxidatic reaction (11) can be represented by the scheme

\[
\text{HRP} + \text{H}_2\text{O}_2 \rightarrow k_2 \rightarrow \text{HRP-I}
\]

\[
\text{HRP-I} + R \rightarrow k_3 \rightarrow \text{HRP-II} + P
\]

where HRP, HRP I, and HRP II represent the enzyme, Compound I, and Compound II, respectively, $R$ is a reducing sub-
strate, and $P$ represents the first formed products. In the steady state the ratio of the absorbance $A$ of the enzyme system at a given wave length to the absorbance $A_0$ observed for the same concentration of the native enzyme at its maximum (403 nm) can therefore be expressed by

\[
A = \frac{A_0}{k_1[H_2O_2] + k_2[R] + k_3[R]}
\]

where $k_1$, $k_2$, and $k_3$ are the molar absorptivities of the unchanged enzyme, Compound I, and Compound II, respectively, and $A_0$ = $A_0$ at 403 nm. The experimental spectrum can be attributed entirely to Compound II only when $k_3[R] \ll k_1[H_2O_2]$. The data of Hasinoff and Dunford (2) show that the above condition was satisfied at pH 10.95, but in all other cases it was necessary to make corrections to the measured $A/A_0$ at each wave length to obtain $A/A_0$. The value of $k_2$ was taken as $1 \times 10^3$ M$^{-1}$ s$^{-1}$ at all pH levels (12), whereas for ferrocyanide at pH 6.89 $k_3 = 8.5 \times 10^4$ M$^{-1}$ s$^{-1}$ and $k_2 = 1.8 \times 10^4$ M$^{-1}$ s$^{-1}$ (2). The kinetic studies described above for ascorbic acid at pH 4.26 yielded $k_3 = 2.3 \times 10^4$ M$^{-1}$ s$^{-1}$ and $k_2 = 2.6 \times 10^4$ M$^{-1}$ s$^{-1}$, the latter value being considerably larger than that of 1 $\times 10^4$ M$^{-1}$ s$^{-1}$ reported by Chance at pH 4.7 (13). Values of $A_0$ were taken from the results of recent work in this laboratory by Roman and Dunford, although the alternative use of the spectrum reported by Chance (5) would change the calculated absorbance of Compound II by no more than 1% at any wave length. The largest corrections to $A/A_0$ made by application of Equation 5 to each set of data were at 420 nm, and amounted to 8% at pH 4.26 and 1.5% at pH 6.89. Small corrections of less than 2% were made to the results obtained with ferrocyanide at neutral and high pH for the production of ferricyanide during

<table>
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<tr>
<th>Substance</th>
<th>$10^4 \times$ concentration range</th>
<th>pH</th>
<th>$10^4 \times$ $K_{\text{diss}}$</th>
<th>Relative to values$^b$</th>
<th>Buffer and contribution to ionic strength$^a$</th>
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<tbody>
<tr>
<td>p-Cresol</td>
<td>2.4-19.6</td>
<td>5.00</td>
<td>3.2</td>
<td>Acetate, 0.01</td>
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<tr>
<td>p-Cresol</td>
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<td>6.91</td>
<td>2.7</td>
<td>Phosphate, 0.04</td>
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<tr>
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<td>7.05</td>
<td>2.5</td>
<td>Phosphate, 0.11</td>
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<td>p-Cresol</td>
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<td>2.5</td>
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<tr>
<td>p-Cresol</td>
<td>5.6-45.0</td>
<td>10.12</td>
<td>(5.6)$^e$</td>
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<td>0.7</td>
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<td>p-Cresol</td>
<td>5.6-45.0</td>
<td>10.30</td>
<td>(9.5)$^e$</td>
<td>Carbonate, 0.11</td>
<td>0.4</td>
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<td>Phenol</td>
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<td>7.05</td>
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<td>7.05</td>
<td>20</td>
<td>Phosphate, 0.11</td>
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<tr>
<td>L-Tyrosine</td>
<td>Up to 7.05</td>
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<tr>
<td>L-Tyrosine</td>
<td>Up to 7.05</td>
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<td></td>
<td>Phosphate, 0.11</td>
<td></td>
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</tbody>
</table>

$^a$ Reduced where necessary at high substrate concentrations to maintain total ionic strength of 0.11.

$^b$ See text.

$^c$ Total concentrations of acidic and basic forms.

$^d$ Figures in parentheses calculated from the average value of $K_{\text{diss}}$ at lower pH on the assumption that the increase in $K_{\text{diss}}$ at high pH coincides with the pK$_a$ of p-cresol, determined previously to be 10.12 (1).

$^e$ No effect on the spectrum could be detected at the limits of solubility.
the conversion of Compound I to Compound II in the first cycle of the steady state reaction.

The resulting values of eII at a representative number of wavelengths are recorded in Table II, using the value e0 = 9.1 X 10^4 M^-1 cm^-1 obtained by Keilin and Hartree (14). Also included are the results obtained by Chance (5) at pH 7.0, read as precisely as possible from the published spectrum, and scaled upwards by 11% to bring them into line with the above value of e0. These are in generally very good agreement with those obtained in the present work. The absorptivities at pH 6.89 lie between those at pH 4.26 and 10.95 in every case, even though the direction of the differences varies with wavelength, which suggests that the variations observed with pH are real.

Chance reported that the spectrum of Compound II in the Soret region showed a pH dependence, but his results covered a smaller range of pH and indicated no consistent trend. The effect found in the present work can be represented by a small shift of the Soret peak towards longer wavelength with increasing pH, together with a decrease in the absorbance of the shoulder in the neighborhood of 370 nm. A similar shift is noticeable for the native enzyme in the acid region, with the result that the isobestic point at 411 nm was found to be unchanged on going from pH 6.89 to 4.26. All these effects are small, being of similar magnitude to the change observed on the binding of p-cresol to horseradish peroxidase, and quite unlike the large change observed for this enzyme at pH 10.8, associated with the acid dissociation of an iron-bound water molecule (15).

**Table II**

<table>
<thead>
<tr>
<th>Molar absorptivities of Compound II over range of pH</th>
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</table>

Based on e433 = 9.1 X 10^4 M^-1 cm^-1 for the native enzyme (14).

Data of Chance (5).

At pH 10.95 λmax = 421 nm, 10^-4 e421 = 9.4 M^-1 cm^-1.

### Kinetics in Deuterium Oxide

Interpretation of the kinetic measurements in light and heavy water requires a knowledge of the relation between the pH of the solution (where L represents both hydrogen and deuterium isotopes) and the apparent pH meter reading obtained with the glass electrode. The results of Glasoe and Long (16) indicate that for a solution of 94% deuterium content, pH = pL + 0.38. This relation was confirmed for the electrode used in the present work by comparing the change of 0.10 unit observed between the pH readings for solutions containing the same acetate buffer system in light and heavy water with the value pKH - pKd = 0.48 found by Gold and Lowe (17) for acetic acid in 94% deuterium oxide. The letters H, D, and L are used here and throughout to denote quantities referring to pure H2O, pure D2O, and 94% D2O, respectively.

Under the conditions employed for the steady state peroxidation of ferrocyanide the reduction of Compound II is rate-determining, and the resulting values of the second order rate constant have been plotted in Fig. 6. Since in this paper we are not concerned with the kinetic consequences of the binding of p-cresol to Compound II and previous work has failed to reveal any detectable binding of ferrocyanide (2) or iodide (3), the second order rate constants are denoted simply by k2 instead of by a quantity of the form k2/Km. Further investigation of the kinetics of this reaction in light water has shown that the variation of log k2 with pH in the acid region is rather more simple than was at first indicated (2), showing merely a single region of positive curvature close to pH 6. By analogy with the results in light water the present data may therefore be described by Scheme 1.

the use of a single transition state acid dissociation constant $K_{zL}$ as in Scheme 2 and Equation 7

$$k_0 = k_{L} (1 + [L]/K_{zL})$$

(7)

As shown by the best fit parameters in the third column of Table III, $K_{zL}$ may be associated with the upward turn of the curve at pH 6 by a simple extension of Dixon's rules. This alternative method of analysis, which will be discussed in more detail elsewhere, is introduced here since the resulting value of $K_{zL}$ is useful in formulating a detailed description of the mechanism.

It has been found that over the whole pH range the values of $k_0$ derived from studies in the steady state mode normally lie about 20% below those obtained by direct measurement, an observation attributed to the lack of any precise knowledge of the relation between the absorbance of the enzyme and its concentration, and the values of these constants obtained previously by direct studies in light water have accordingly been listed in Table III. The quantities $K_{Z0}/K_{ZH}$ have been computed by linear extrapolation, which is unlikely to introduce significant errors over 6% of the deuterium atom fraction scale.

The value of $K_{Z0}/K_{ZH}$ lies significantly above the range found even for very strong acids (18), and is therefore of little direct use in identifying the group concerned. Since the ionization of an acid in the region of neutral pH is normally associated with an equilibrium isotope effect $K_{Z0}/K_{ZH}$ of about 0.3 (19), it follows that in the enzyme the loss of this proton must be accompanied by a significant strengthening of the vibrations of a number of other protons in the molecule. This might occur through the formation or dislocation of hydrogen bonds in a way not possible when the amino acid is studied separately in water, and so affect a number of other groups in the protein structure.

A kinetic isotope effect on an enzyme-catalyzed reaction can be interpreted most easily when measurements in both light and heavy water are conducted in regions where the rate is independent of pH or $pD$. A direct determination under these conditions is not possible for the ferrocyanide reaction, but the desired quantity can be computed from the isotope effect at high pH by making use of the measured value of $K_{Z0}/K_{ZH}$. The results obtained in the single turnover experiments at high pH are illustrated in Fig. 7. Correction to a common lyomion ion activity and 100% $D_2O$ gives the value for $K_{Z0}/K_{ZH}$ listed in Table IV. The computed pH-independent isotope effect in the neutral pH region is therefore given by

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KsD/KsH cannot be obtained, and the measured isotope effect on the quantit.y (kz+ kz/KF) in Equation 6 or

\[ \frac{k_D}{k_H} \times \frac{K_N}{K_M} = 3.4. \]

This may be regarded as the isotope effect on the quantity (k2 + k3/K2) in Equation 6 or k2 in Equation 7, and is a measure of the total change in the vibration frequencies of the protons which are present in the enzyme at neutral pH on going to the transition state.

The increase in the rate of ferrocyanide oxidation observed below pH 6 indicates that the reactive form of either the enzyme or substrate bears one more proton than does the stable form of that substance in this pH region. The reaction is now proceeding through a new transition state in which an extra proton is present at the active site, and it is therefore desirable to characterize this reaction route by a separate measurement of the kinetic isotope effect. This can be obtained from the two cases 10\( ^{-1} \) k2H = 2.6 \( \pm 0.1 \) m\( \text{s} \)\( ^{-1} \). Extrapolation of the best fit to data of Hasinoff and Dunford (2) gives 10\( ^{-1} \) k2H = 3.8 m\( \text{s} \)\( ^{-1} \). Lower plot, reaction in 94% D2O at pH 10.98, 10\( ^{-2} \) k2H = 1.6 \( \pm 0.1 \) m\( \text{s} \)\( ^{-1} \).

\[ k_D/k_H \times K_D/K_H = 3.4. \]

The results obtained in the studies of the reactions with iodide and p-cresol on the stopped flow apparatus are given in Table V. Both reactions have previously been shown to be kinetically first order in substrate at the concentrations employed (1, 3). Studies in light water showed that the second order rate constant for the reaction between Compound II and iodide increases with the first power of the hydrogen ion activity (3) so that correction of k2H to the standard pH was easily achieved. The lack of inflections in the pH rate profile for the oxidation of iodide shows that the value of k2D/H in Table IV refers to a single reaction path operative over the entire accessible pH range, but as in the case of the ferrocyanide reaction at low pH the measured quantity contains an equilibrium isotope effect for the conversion of one of the reactants into its reactive protonated form. For the reaction with p-cresol on the other hand it was possible to measure k2D/H under conditions where the pH rate profile is horizontal, so that no correction to a standard pH is necessary and the measured value can be interpreted as a simple kinetic isotope effect for a single activation process. Since there are reasons for thinking that the small inflection of the pH rate profile at pH 5.4 merely reflects the protonation of some group lying close to the active site but not taking a vital part in the catalytic process (1), the mechanism of p-cresol oxidation is essentially the same from pH 2 to 11 and may hence be characterized by this single isotope effect measurement.

An alternative and often more elegant method of analyzing the above data, which avoids the distinction between the isotope effect on the pre-equilibrium between the enzyme or substrate and its reactive protonated species and the isotope effect on the catalytic step itself, is provided by the use of fractionation factors for the ground and transition state protons (20). However, the above more familiar approach has been
used in the present case because the presence of a large number of protons in the enzyme and the lack of any prior knowledge as to the nature of the mechanism would require consideration of an undefined number of such parameters.

**DISCUSSION**

Of the three substrates whose oxidation by Compound II of horseradish peroxidase has been studied in detail, ferrocyanide (2), iodide (3), and p-cresol (1), only the latter shows any evidence of enzyme substrate binding. Since the latter reaction is also the fastest, it is tempting to attribute this to a favorable fit of enzyme and substrate in the transition state (21). However, subsequent evidence (1) that Compound II binds p-cresol at some point away from the active site has made such an interpretation far less tenable. Since this point has an important bearing on the type of detailed mechanism which may be proposed, it was considered of interest to investigate the nature of the interaction between p-cresol and the native enzyme. A demonstration that this may occur other than by direct binding to iron would provide further indirect support for the formation of an unproductive complex with Compound II.

The results of the above studies on the interaction between native horseradish peroxidase and p-cresol do in fact demonstrate that this substrate binds at a site different from that involved in the formation of the cyanide and hydroxide complexes. Since it is universally agreed that in the latter the ligand occupies one of the out-of-plane coordination positions of the heme iron (22), and since the linearity of the plot in Fig. 8 indicates that only one such position is available, it must be concluded that p-cresol binding occurs at some other site associated with the porphyrin ring or protein. The smallness of the observed spectral changes lends some additional support to this view. Moreover the influence of bound p-cresol on the stability of the cyanide and hydroxide complexes parallels its partially inhibiting effect on the rate of the oxidation reaction, although no evidence is yet available as to the mechanism by which these effects are relayed. That the complexes formed by the native enzyme and Compound II are not entirely similar is shown by the difference in the magnitude and in the behavior at low pH of their dissociation constants and by the different extents to which the Soret band is perturbed. These observations may reflect some dissimilarity in conformation between the two species.

Having established that the existence of a complex between Compound II and p-cresol does not imply any difference in reaction scheme between the oxidation of this substrate by Compound II and that of iodide and ferrocyanide, it is possible to discuss all three in terms of a common pathway. A simple and reasonable basic mechanism, which will be assumed for the purposes of the present discussion, involves a slow bimolecular displacement by the substrate of whatever group occupies the sixth coordination position in the Compound II molecule, followed by rapid electron transfer and separation of the resulting radical. All the available evidence suggests that Compound II contains low spin iron in the formal oxidation state +4, to which a protein residue or water molecule (represented below as $\text{XH}_n$) is strongly bound in its neutral or one of its basic forms (23, 24). The spectral data for Compound II obtained in the present work strongly suggest that this group does not undergo protonation between pH 4 and 11, contrary to an earlier proposal (25).

The best fit pH log $k_2$ profiles for the three reactions are collected for comparison purposes in Fig. 8, along with the $pK_a$ values associated with the kinetically important protonation equilibria in the ground state of the enzyme. Since it is important to account for all the information contained in the various pH rate profiles, the points at which these curve upwards are also indicated in Fig. 8. As previously pointed out, upward curvature reflects a change in the state of protonation of one of the reactive species and may consequently be regarded as indicating an acid dissociation equilibrium in the transition state. It can be seen from the figure that the oxidation of p-cresol is sensitive to the ionizations of functional groups in the Compound II molecule having $pK_a$ values of 8.6 and 5.4, whereas the reaction with ferrocyanide is only affected by the state of protonation of one of these groups, and the reaction with iodide is affected by none of them. The oxidation of ferrocyanide at low pH and of iodide over the entire pH range studied appears to depend on the protonation of a group of very low $pK_a$ value, but this has no influence on the kinetics of the reaction with p-cresol. Although Alberty and Bloomfield have shown that different substrates may cause an enzyme to exhibit different apparent ground state $pK_a$ values when several protonated forms can take part in a number of successive and partly rate-limiting steps (26), there is no evidence in the present case for the existence of the intermediates which this would imply. Moreover the various profiles indicate a lack of sensitivity to certain $pK_a$ values rather than an apparent shift in their values. It is therefore much more likely that the three substrates interact differently with a number of groups in the neighborhood of the active site. As pointed out previously (8), this could arise because of different steric interactions be-

![Fig. 8. Plots of log of second order rate constant for reaction of reducing substrates with Compound II versus pH.](image-url)
tween the substrate and protein or through a difference in the detailed nature of the mechanism. The very small degree of stereospecificity shown by the enzyme (27) and the fact that the isotope effects vary with the substrate both tend to favor the latter type of explanation, and as shown below this view leads to a correlation of the observed pK values, over-all rates, and kinetic isotope effects in terms of a chemically reasonable model.

Other enzyme systems normally exhibit pH rate profiles which are similar from one substrate to another and rates which can often be explained in terms of the fit between the substrate and enzyme at the active site. The hypothesis presented below may appear less familiar since it postulates instead the operation of two distinct intimate mechanisms and therefore makes use of rather different concepts. In particular it is difficult to avoid considering the configuration of the transition state, especially in discussing the magnitude of kinetic isotope effects and the pK at which one mechanism takes over from the other. An example sharing some of the features summarized by Fig. 8 is provided by the measurements of Goldsack, Eberlein, and Alberty (28) on the rates of binding of a number of small ligands to metmyoglobin, inasmuch as the pH rate profiles have different shapes from one substrate to another and a number of protonated forms of the macromolecule were found to react at different rates. In this case however, all the reactions were sensitive to the same set of pK values, and it was possible to explain the data fairly simply in terms of a model in which all interactions were considered to be electrostatic and in which proton transfer or hydrogen bonding were not required. This type of interpretation is not applicable to the present case, since the various reactions are sensitive to different combinations of ionizing groups on the enzyme, the isotope effect data show that the protons at the active site are more intimately involved in the reaction, and a comparison of a highly charged, singly charged, and neutral substrate has already excluded the possibility that electrostatic interactions are of major importance (1). The explanation given below appears to be the only one which can account for the observed behavior of Compound II.

Inspection of Fig. 8 shows that the enzyme pKα of 8.6 has a profound effect on the kinetics of the p-cresol and ferrocyanide reactions. The simplest explanation for the form of the two profiles in the alkaline region of pH is that the group in question is protonated in the transition state at some high pH, i.e., the enzyme is only active when this group is in its acid form. This conclusion receives further support in the case of p-cresol from arguments based on the diffusion-controlled limit, as discussed in the foregoing paper (1). This protonation must involve some protein residue not bound to the iron atom (which may therefore be referred to as distal), and an attractive hypothesis is that such a group acts in its acid form as a general acid catalyst, aiding the displacement of the bound group -XH, as indicated in Fig. 9, a. The hypothesis presented below is preferable. The transition state for the oxidation of p-cresol may therefore be represented by Fig. 9a. The small kinetic effect observable at pH 5.7 is presumably due to an ionizing group in the vicinity of the distal group, which affects slightly the ease of proton transfer.

The similarity between the pH rate profiles of the p-cresol and ferrocyanide reactions in the alkaline region suggests that both proceed by a similar mechanism under these conditions. Since the oxidation of ferrocyanide is considerably slower, it would be expected that the degree of proton transfer in the transition state would be greater (29), and a number of pieces of evidence support a virtually complete transfer. For example, the transition state for the oxidation of p-cresol may therefore be represented by Fig. 9a. The small kinetic effect observable at pH 5.7 is presumably due to an ionizing group in the vicinity of the distal group, which affects slightly the ease of proton transfer.

The chief of these is the kinetic isotope effect, which has the corrected value $k_2^p/k_2^n = 3.4$ in the horizontal region of the pH rate profile. This quantity reflects changes in both the proton transferred and the other protons in the molecule, and from the high value of the measured equilibrium isotope effect on the ionization of the group of pKα 8.6 it has been concluded that proton loss by this distal group is accompanied by a considerable increase in the vibrational frequencies of these secondary protons. Since ionization is almost complete in the transition state of Fig. 9a, a similar effect on the secondary protons is expected. The contribution of the transferred proton to the kinetic isotope effect must therefore be close to unity, a value which is consistent with

![Fig. 9. Proposed mechanism for reaction of Compound II.](image)

- **(a)** $\text{ArO} \longrightarrow \text{Fe} \longrightarrow \text{X} \longrightarrow \text{H} \longrightarrow \text{N}^- \text{H}_n$
- **(b)** $(\text{CN})_2\text{FeCN} \longrightarrow \text{Fe} \longrightarrow \text{X} \longrightarrow \text{H} \longrightarrow \text{N}^- \text{H}_n$
- **(c)** $\text{I} \longrightarrow \text{Fe} \longrightarrow \text{X} \longrightarrow \text{H} \longrightarrow \text{HN}^- \text{H}_n$
- **(d)** $\text{H}$

The three substrates studied, and for which a rate-determining proton transfer is strongly indicated by the isotope effect recorded in Table IV. Although the low value of $k_2^p/k_2^n$ could in principle be caused by a rate-determining removal of the hydroxyl proton from the substrate molecule, this would leave the lack of reactivity of the p-cresolate ion unexplained (1). For this reason the above explanation is preferable. The transition state for the oxidation of p-cresol may therefore be represented by Fig. 9a. The small kinetic effect observable at pH 5.7 is presumably due to an ionizing group in the vicinity of the distal group, which affects slightly the ease of proton transfer.
almost complete transfer. Another way of reaching the same conclusion without making use of the measured equilibrium isotope effect is to multiply the observed value of $k_D^P/k_H^P$ at high pH by the value of $K_D^P/K_H^P$ expected for the ionization of a simple acid group of $pK_a \approx 8$. Since this should lie between 0.25 and 0.5 (19) the corrected pH-independent kinetic isotope effect at neutral pH must be closer to unity than that for p-cresol, which is itself an upper limit because of the effect of secondary protons.

As the pH of the medium is decreased the small fraction of the enzyme in which the bound group — XIX₄ — is protonated increases, and at some point should become sufficient to allow a specific acid-catalyzed pathway to compete with the mechanism described above. Such competition should be more noticeable and hence become important at higher pH the more the transition state in the intramolecularly general acid-catalyzed mechanism resembles that for the specific acid-catalyzed route, i.e., the further the proton in the former mechanism is transferred. It is therefore entirely reasonable that the oxidation of ferrocyanide should depend on the first power of the hydrogen ion concentration at low pH whereas the reaction with p-cresol appears to proceed by the general acid-catalyzed mechanism down to the lowest pH studied. The changeover from the transition state of Fig. 9b to that of Fig. 9c representing the specific acid-catalyzed route occurs at pH 6.0, corresponding to the transition state acid dissociation constant $K_D^+$ of Scheme 2. If the proton is completely transferred in Fig. 9b, the addition of a further proton to the system to give Fig. 9c should be a little more difficult than the protonation of the free distal group on account of the energy required to break the hydrogen bond, and the value $pK_D^+$ = 6.0 is therefore consistent with this picture. The essentially identical values observed for the equilibrium isotope effects on $pK_D^+$ and $pK_I$ constitutes further evidence for the similarity of the two ionization processes, although this is a less sensitive test. The transition to a specific acid-catalyzed mechanism below pH 6 also accounts for the lack of any effect attributable to the group of $pK_a = 6.7$ on the kinetics of the ferrocyanide reaction, since the operation of the specific acid-catalyzed mechanism should not be influenced by ionizations in the neighborhood of the distal group.

For the iodide reaction it would be expected from the over-all slower rate that the specific acid-catalyzed pathway would be dominant over a larger range of pH, and inspection of the pH log $k_2$ profile shows that this is in fact the only mechanism operative over the experimental range. Protonation of the distal group at pH 8.6 accordingly has no effect on the kinetics, since it is not involved in the reaction, and no inflections are observable at pH 5.7 for the same reason. It would be expected that as the over-all reaction becomes more difficult not only should specific acid catalysis become relatively more important but the degree of Fe—X bond breaking in the transition state should also increase, a mode of behavior for which there is good evidence in other types of concerted reaction systems (30, 31). Thus the solvent-derived proton added to the —XIX₄ group in the transition state for the iodide reaction should be less acidic than that added in the reaction with ferrocyanide at low pH, and the kinetic isotope effects (although almost identical within experimental error) accordingly show a small decrease. For the same reason there is less opportunity for stabilization of the transition state of Fig. 9d by hydrogen bonding between the added proton and the free distal group at high pH, so that no inflections in the pH rate profile are observed on this account.

The terms “general” and “specific” acid catalysis have been used in the above discussion to describe mechanisms in which the proton added to the group — XIX₄ in the transition state either is or is not partly bonded to another atom. This differs from the more usual operational use of these terms to describe the dependence of the rate on the various acidic species present (32), an experimental approach which is not relevant to the present system. The mechanism for which the term general acid catalysis has been used is an example of neighboring group participation, which has often been invoked in theories of enzyme action (33). In fact a mechanistic changeover similar to that proposed for the ferrocyanide reaction has been suggested in a number of model systems exhibiting a similar pH rate profile, for example in the hydrolysis of a series of methoxymethyl ethers of substituted phenols (34) and in the hydrolysis of a number of glycosides (35).

Since it is difficult to conceive of any radically different description of the above reactions which could account for all of the observed behavior, there is good evidence for the view that the reactivity of Compound II towards various reducing substrates depends largely on purely “chemical” factors rather than on the stabilizing effect of direct substrate-protein interactions. According to the above mechanism this reactivity is more dependent on the nucleophilicity of the substrate towards iron than on its oxidation-reduction potential, which explains the rapidity of the reactions with oxygen- and nitrogen-containing unsaturated compounds, and may also have some bearing on the lack of catalytic activity shown by horseradish peroxidase.

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