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THE HORSERADISH PEROXIDASE-CATALYZED OXIDATION OF IODIDE. OUTLINE OF THE MECHANISM

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SUMMARY

1. Horseradish peroxidase (donor: H_2O_2 oxidoreductase, EC 1.11.1.7), Compound I, is shown to react with I^- to yield a ferriperoxidase–iodine complex. The complex has a spectrum similar to that of ferriperoxidase (the unmodified enzyme), which suggests that the oxidation state of the heme moieties of the two species is the same.

2. Ferriperoxidase–iodine reacts very rapidly with H_2O_2 to give Compound I and an iodine-containing product. It reacts with reducing agents to give ferriperoxidase and I^-.

3. The main pathway for the horseradish peroxidase-catalyzed oxidation of I^- can probably be characterized as:

Compound I + I^- → ferriperoxidase–iodine
ferriperoxidase–iodine + H_2O_2 → Compound I + product(s)

A side pathway identical with the mechanism described by CHANCE[1,2] and by GEORGE[3-5] contains the reaction steps

ferriperoxidase + H_2O_2 → Compound I
Compound I + I^- → Compound II + product(s)
Compound II + I^- → ferriperoxidase + product(s)

4. When the H_2O_2 concentration is optimal for I^- oxidation, an estimated 50% of the enzyme will be in the form of Compound II in the steady state. Since turnover along the side pathway is slow, only about 2% of the I^- oxidation can occur by this route.

INTRODUCTION

The mechanism for the horseradish peroxidase-catalyzed (donor: H_2O_2 oxidoreductase, EC 1.11.1.7) oxidation of $K^4Fe(CN)_6$ and ascorbic acid was clarified by

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CHANCE[1,2] and by GEORGE[3–5]. Many of their results have recently been confirmed by YONETANI[6]. The mechanism arrived at is usually summarized with the equations

$$\text{ferriperoxidase} + H_2O_2 \rightarrow \text{Compound I} \tag{1}$$

$$\text{Compound I} + \text{AH} \rightarrow \text{Compound II} + \text{A} \tag{2}$$

$$\text{Compound II} + \text{AH} \rightarrow \text{ferriperoxidase} + \text{A} \tag{3}$$

where ferriperoxidase is the unmodified enzyme and AH symbolizes a substrate undergoing one-equivalent oxidation to A.

Most, if not all, iron-porphyrin peroxidases oxidize I^- to I_2 in a reaction which can be described by the overall equation

$$H_2O_2 + 2\,I^- + 2\,H^+ \xrightarrow{\text{peroxidase}} I_2 + 2\,H_2O \tag{4}$$

Enzymes known to catalyze the reaction include chloroperoxidase[7], horseradish peroxidase[8–12], lactoperoxidase[10], myeloperoxidase[12] and thyroid peroxidase[13,14].

The mechanism of the peroxidase-catalyzed oxidation of I^- has been the subject of only a few studies. NUNEZ AND POMMIER[15] have described the formation of a horseradish peroxidase–iodine complex. BJÖRKSTÉN[11] investigated reaction steps in which I^- and H^+ are added to the horseradish enzyme. Although these studies are relevant, they do not permit any deeper insight into the mechanism.

Our preliminary studies showed that the main pathway for the horseradish peroxidase-catalyzed I^- oxidation could not be identical with the Chance–George mechanism of Eqns. 1–3 (ref. 16). The present investigation is an effort to elucidate the nature of the main pathway.

MATERIALS AND METHODS

Reagents and equipment

The horseradish peroxidase used was a commercial (Worthington) electrophoretically purified lyophilized preparation. It had an absorbance ratio $A_{403\,\text{nm}}/A_{275\text{nm}}$ of 2.8–3.0, which indicates high purity[17]. Usually a stock solution containing approx. 15 μM peroxidase in 30 mM potassium phosphate (pH 6.2) was prepared. The solution was left to stand at 4° for at least 10 days before use. It was observed that the absorbance at 403 nm increased during this time and that aged peroxidase solutions gave more reproducible results than fresh ones. The peroxidase concentration was determined spectrophotometrically using $\varepsilon_M = 9.0 \cdot 10^4\ \text{cm}^{-1} \cdot \text{M}^{-1}$ at 403 nm (ref. 18).

H_2O_2 solutions were prepared by diluting 30% H_2O_2 (Merck). Their concentration was assayed through titration with $KMnO_4$ in acidic solution.

Rates of absorbance change and spectra were measured with a Beckman DK-1 or a Perkin–Elmer 137 spectrophotometer. A Beckman DU or a Hitachi–Perkin–Elmer 137 instrument was used for other spectrophotometric measurements. Cells with a 1-cm light path and containing 3 ml of solution were always used. The reference cell always contained water. Reactions and measurements were carried out at 25°, except where otherwise indicated.

Determination of the rate of reaction between peroxidase species and reducing agents

In all cases ferriperoxidase was first converted to Compound I by adding an equimolar or somewhat lower amount of H_2O_2 (see Eqn. 1). When Compound II was needed, Compound I was converted to Compound II by adding an equimolar amount of $K_4Fe(CN)_6$ (see Eqn. 2). When the reactivity of Compound I or the ferriperoxidase–iodine complex was under study, Compound I was converted to ferriperoxidase–iodine by an excess of I^- according to the equation

$$\text{Compound I} + I^- \xrightarrow{k_a} \text{ferriperoxidase–iodine} \tag{5}$$

The bimolecular rate constants k_a, k_b and k_c, which were measured, are defined by Eqn. 5 and the equations

$$\text{Compound II} + I^- \xrightarrow{k_b} \text{ferriperoxidase} + \text{product(s)} \tag{6}$$

$$\text{ferriperoxidase–iodine} + \text{reducing agent} \xrightarrow{k_c} \text{ferriperoxidase} + I^- + \text{product(s)} \tag{7}$$

When k_a or k_c was measured, the reaction was followed by recording the absorbance as a function of time at 403 nm, where ferriperoxidase and ferriperoxidase–iodine have an absorption maximum. When k_b was measured, the wavelength used was 418 nm, where Compound II has a maximum. The rate constants were calculated using the equations

$$k_a = \frac{1}{[\text{Compound I}]\,[I^-]} \cdot \frac{dA}{dt} \cdot \frac{1}{\varepsilon_{M_6} - \varepsilon_{M_3}} \tag{8}$$

$$k_b = \frac{1}{[\text{Compound II}]\,[\text{reducing agent}]} \cdot \frac{dA}{dt} \cdot \frac{1}{\varepsilon_{M_2} - \varepsilon_{M_5}} \tag{9}$$

$$k_c = \frac{1}{[\text{ferriperoxidase–iodine}]\,[\text{reducing agent}]} \cdot \frac{dA}{dt} \cdot \frac{[\text{ferriperoxidase–iodine}]}{\Delta A} =$$

$$= \frac{1}{[\text{reducing agent}]} \cdot \frac{dA}{dt} \cdot \frac{1}{\Delta A} \tag{10}$$

TABLE I

MOLAR ABSORBANCE COEFFICIENTS FOR HORSERADISH PEROXIDASE SPECIES

Peroxidase species	*λ (nm)*	*Symbol*	*Numerical value ($cm^{-1} \cdot mM^{-1}$)*	*Source*
Ferriperoxidase	403	ε_{M_1}	90	Ref. 18
	418	ε_{M_2}	53	Fig. 4
Compound I	403	ε_{M_3}	49	Fig. 1
Compound II	403	ε_{M_4}	61	Ref. 1*
	418	ε_{M_5}	87	Ref. 19
Ferriperoxidase–iodine	403	ε_{M_6}	107	Fig. 4

* The value given in the table is 12% higher than in the reference to compensate for the discrepancy in spectral data reported for ferriperoxidase by CHANCE[1] and by KEILIN AND HARTREE[18].

In Eqns. 8–10 dA/dt is the initial rate of absorbance change, $\varepsilon_{M_1}, \varepsilon_{M_2} \ldots \varepsilon_{M_6}$ are molar absorbance coefficients defined in Table I, and ΔA is the absorbance change observed when all ferriperoxidase–iodine present was instantaneously reduced (*e.g.* by $Na_2S_2O_3$) to ferriperoxidase.

When k_a was measured, the sample cell originally contained 30 mM potassium phosphate (pH 6.19) and 5.8 μM ferriperoxidase. H_2O_2 to a final concentration of 5 μM and 20 sec later KI to a concentration of 1.5 μM were added. The dA/dt obtained after the KI addition was used for calculations.

When k_b was measured, the sample cell originally contained 30 mM potassium phosphate (pH 6.19) and 5 μM ferriperoxidase. Other reagents were added at 10-sec intervals to the following final concentrations: 5 μM H_2O_2, 5 μM $K_4Fe(CN)_6$ and 50 μM KI. In a "blank" experiment KI was omitted. The dA/dt was measured after the last reagent addition. The dA/dt from the blank experiment was subtracted from the dA/dt obtained in the experiment with KI, and the difference between the dA/dt's was used in the calculation of k_b by Eqn. 9.

Measurement of the spectrum of the ferriperoxidase–iodine complex

In the experiment the spectrum of 10 μM ferriperoxidase was first recorded. The enzyme was then converted to ferriperoxidase–iodine by adding amounts of KI and H_2O_2 equimolar to the enzyme. The spectrum was recorded again 6 min after the additions. To obtain the concentration of Compound II formed through side reactions, the spectrophotometer was immediately readjusted to record the absorbance at 403 nm as a function of time. Then $Na_2S_2O_3$ to a final concentration of 100 μM was added to convert the ferriperoxidase–iodine to ferriperoxidase. From the absorbance (A) observed immediately after the $Na_2S_2O_3$ addition, the concentration of Compound II was calculated using the equation

$$[\text{Compound II}]\,\varepsilon_{M_4} + ([\text{peroxidase}] - [\text{Compound II}])\,\varepsilon_{M_1} = A \qquad (12)$$

$$[\text{Compound II}] = \frac{A - [\text{peroxidase}]\,\varepsilon_{M_1}}{\varepsilon_{M_4} - \varepsilon_{M_1}} \qquad (13)$$

In Eqns. 12 and 13 [peroxidase] is the total peroxidase concentration.

A calculated "true" spectrum for ferriperoxidase–iodine was obtained from the recorded spectrum by compensating for the presence of the observed Compound II concentration (1.5 μM). The spectral data for Compound II used in the calculations were those given by CHANCE[1] but increased by 12% to compensate for the discrepancy in spectral data reported for ferriperoxidase by CHANCE[1] and by KEILIN AND HARTREE[18].

RESULTS

Formation of Compound II as an intermediate

An experiment was performed in which the spectrum of a peroxidase solution was followed, while the peroxidase was gradually converted from Compound I to ferriperoxidase or a related species through the stepwise addition of an oxidizable substrate. The procedure resembles a spectrophotometric titration, but the results should be interpreted in a qualitative rather than quantitative manner. The reason

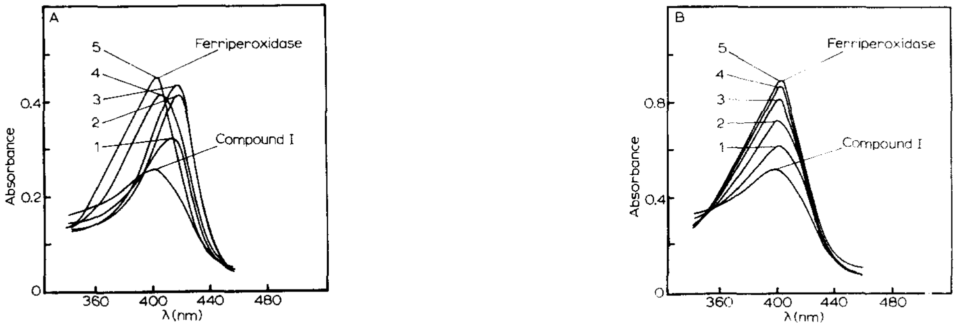


Fig. 1. Spectrophotometric detection of peroxidase Compound II formed as an intermediate. The peroxidase was initially in the form of ferriperoxidase and was converted to Compound I by the addition of an equimolar quantity of H_2O_2 in 25 μl of water. Both the spectrum of the original ferriperoxidase and that of Compound I were recorded. Beginning 1 min after the H_2O_2 addition the portionwise addition of an oxidizable substrate was started. The volume of each addition was 5 μl, and the additions were made at 1-min intervals. Spectrum 1 was recorded beginning 30 sec after the first addition, Spectrum 2, 30 sec after the second, *etc.* In A the sample cell contained 30 mM potassium phosphate (pH 6.2) and 5 μM peroxidase; $K_4Fe(CN)_6$ was added in amounts raising the concentration by 1.67 μM. In B there was 30 mM potassium phosphate (pH 6.2) and 10 μM peroxidase; KI was added in 1.67 μM portions.

is that the process is rather slow, which permits reactive enzymic intermediates and products formed from substrates to undergo side reactions to some extent.

When K_4FeCy_6 was used as the oxidizable substrate, Compound I was clearly first converted to Compound II (maimum at 418 nm) and only then to ferriperoxidase (Fig. 1A). When I^- was used as substrate, a gradual conversion of the spectrum of Compound I to a spectrum resembling that of ferriperoxidase was seen (Fig. 1B). No extensive formation of Compound II or of any other enzymic intermediate could be detected.

When I is oxidized by horseradish peroxidase, concentrations can be chosen in such a manner that the rate of the overall reaction is first order with respect to peroxidase and I^-, and zero order with respect to H_2O_2 (ref. 11). From observed data a bimolecular rate constant k_d corresponding to the hypothetical reaction step[14] can

$$\text{peroxidase} + I^- \xrightarrow{k_d} \text{products} \qquad (14)$$

be calculated. The value obtained (from Fig. 4, ref. 11) is $k_d = 5.9 \cdot 10^3\ M^{-1} \cdot sec^{-1}$ at pH 6.19. The constant k_d must be equal to or smaller than the bimolecular rate constant corresponding to the slowest step in which I^- truly participates along the main pathway in the mechanism for I^- oxidation.

Iodide was found to reduce Compound II as described by Eqn. 6. The rate constant for this reaction was found to be $k_b = 0.18 \cdot 10^3\ M^{-1} \cdot sec^{-1}$ at pH 6.19. This value is only about 1/30 of the minimum required ($= k_d$) to make Reaction 6 a possible step along the main pathway. Thus it appears that Compound II can only be formed along a side pathway, if at all, when I^- is oxidized.

Rate of reaction between Compound I and I^-

Compound I will react with I^- to yield a peroxidase species with a spectrum similar to but not identical with that of ferriperoxidase. For the rate constant k_a

(defined by Eqn. 5) a value $k_a = 13 \cdot 10^3\ M^{-1} \cdot sec^{-1}$ at pH 6.19 was found. This value can be compared with the value $k_d = 5.9 \cdot 10^3\ M^{-1} \cdot sec^{-1}$ for the rate constant corresponding to the hypothetical rate-limiting step (Eqn. 14). Since $k_a \approx 2\ k_d$, it can be assumed that Reaction 5 affects the overall rate, but that it cannot be the only rate-limiting step. These arguments are true when concentrations are chosen in such a manner that the overall reaction is first order with respect to peroxidase and I^- and zero order with respect to H_2O_2.

Titration of Compound I with I^-

The spectrophotometric titration of Compound I with I^- was complicated by the slowness of the main reaction and the occurrence of side reactions. The side reactions caused a slow linear rise in the absorbance extending beyond the time when the main reaction should have gone to completion (see Fig. 2). Presumably the side reactions involved conversion of Compound I first to Compound II and then to ferriperoxidase. To limit the effects of side reactions, only one I^- addition (corresponding to one measurement) to each peroxidase sample was performed. In addition low temperature and an extrapolation procedure were used (see Fig. 2).

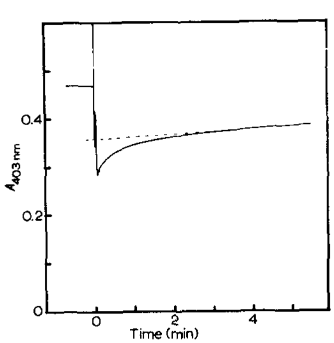
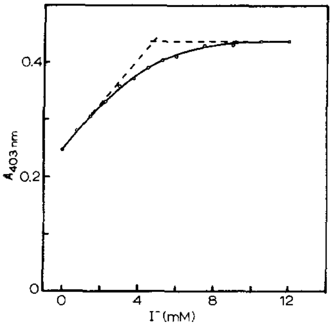


Fig. 2. Typical experiment performed to titrate Compound I spectrophotometrically with I^-. The absorbance at 403 nm was followed as a function of time at a temperature of 4°. At the start the sample cell contained 30 mM potassium phosphate (pH 6.0), 5.25 μM ferriperoxidase and 3 μM KI. At zero time 5 μM H_2O_2 was added and caused the immediate formation of 5 μM Compound I (sharp drop in absorbance), which began to react with I^- (rise in absorbance). The slow linear increase in the absorbance beyond 3 min is largely due to side reactions. To obtain an absorbance value unaffected by side reactions and corresponding to a time when the main reaction was nearly complete, the following extrapolation procedure was used: a tangent to the straight portion of the absorbance curve extending beyond 3 min was drawn, and the absorbance corresponding to zero time was read off it.

To clarify phenomena observed in the titration experiment, the rate constant k_a was determined under the conditions of the experiment, that is at 4° and pH 6.0 The value found was $k_a = 7 \cdot 10^3\ M^{-1} \cdot sec^{-1}$.

In the titration experiment 5 μM Compound I was originally present. Using this concentration, the above value for k_a and the assumption that Compound I and I^- react with each other in a 1 : 1 molar ratio, it can be calculated that the reaction will be 99% complete at 2 min, when the original I^- concentration is close to zero, and 83% complete at 2 min, when the original I^- concentration is 5 μM. At higher I^- concentrations the reaction will again go more rapidly to completion.



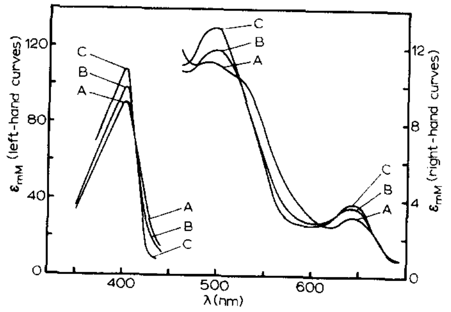


Fig. 3. Spectrophotometric titration of 5 μM peroxidase Compound I with I . The absorbance values were obtained from experiments resembling the one in Fig. 3. Due to the slow completion of the reaction, the absorbances corresponding to I^- concentrations around 5 μM are too low. To obtain a "true" titration curve tangents have been drawn to the hyperbola-like experimental curve. The tangents cross at 4.4 μM I^-, which is taken as the end point of the titration.

Fig. 4. Spectrum A is that of ferriperoxidase, B is the spectrum of the ferriperoxidase-iodine complex contaminated by 15% Compound II, and C is the calculated spectrum for the ferriperoxidase-iodine complex obtained when Spectrum B was corrected for the presence of Compound II.

Extrapolation was used to eliminate anomalies caused by the slow completion of the reaction. The end point of the titration was then found to be 4.4 μM I^- (Fig. 3). This corresponds to a stoichiometric ratio Compound I : I^- of 1 : 0.88.

Properties of a ferriperoxidase-iodine complex

Efforts to study the species which is formed when Compound I reacts with I^- were complicated by our inability to prepare it in a state free from other peroxidase forms. Our best samples were contaminated by some 15% Compound II and possibly by a smaller amount of ferriperoxidase. In spite of this, it should be readily apparent from Fig. 4 that the complex in question (ferriperoxidase-iodine) has a spectrum similar to but not identical with that of ferriperoxidase. The spectra differ most at 530 nm, where ferriperoxidase has a shoulder, while no feature is seen in the spectrum of the new complex.

Due to the spectral similarities we designate the complex a ferriperoxidase derivative and describe its formation as indicated by Eqn. 5. In a subsequent paper[20] it will be shown that spontaneous liberation of I_2 from ferriperoxidase-iodine occurs only slowly if at all.

It was found that when H_2O_2 was added to ferriperoxidase-iodine, the spectrum immediately shifted to that of Compound I. This suggests that ferriperoxidase-iodine will react very rapidly with H_2O_2:

$$\text{ferriperoxidase-iodine} + H_2O_2 \rightarrow \text{Compound I} + \text{product(s)} \quad (15)$$

If $S_2O_3^{2-}$ was added to ferriperoxidase-iodine, the spectrum of ferriperoxidase immediately appeared. This suggests the reduction

$$2\ \text{ferriperoxidase-iodine} + 2\ S_2O_3^{2-} \rightarrow 2\ \text{ferriperoxidase} + 2\ I^- + S_4O_6^{2-} \quad (16)$$

The occurrence of these reactions is illustrated by the experiment of Fig. 5A where the absorbance at 403 nm was recorded while reactions were carried out. It

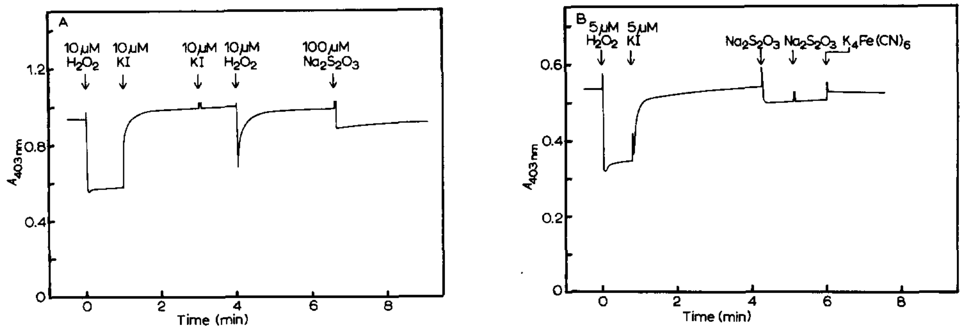


Fig. 5. The interconversion of peroxidase species was followed by recording the absorbance at 403 nm as a function of time. The peroxidase concentration was in A 10.4 µM and in B 5.3 µM. In both cases the peroxidase was originally in the form of ferriperoxidase. The medium concontained 30 mM potassium phosphate (pH 6.2). Reagents were added in the concentrations and at the times apparent from the figure.

can be seen how ferriperoxidase reacted rapidly with an equimolar amount of H_2O_2 and then more slowly with an equimolar amount of I^-, but not with subsequent additions of I^-. A second equimolar amount of H_2O_2 caused a sharp drop in the absorbance to a level approaching that of Compound I (Reaction 15). Compound I immediately started to react with the I^- present and was again transformed into ferriperoxidase–iodine. A final addition of $S_2O_3^{2-}$ caused the absorbance to drop to a level somewhat below that of the original ferriperoxidase (Reaction 16). At the end a slow rise of the absorbance to a level close to that of the original ferriperoxidase was seen. This should be due to the slow reduction of Compound II (Reaction 6) formed in side reactions.

The formation of Compound II *via* side reactions is also evident from the experiment of Fig. 5B. Here ferriperoxidase–iodine is shown to be completely reduced to ferriperoxidase by an approximately equimolar amount of $S_2O_3^{2-}$ (Reaction 16). The reduction is accompanied by a drop in the absorbance to a level slightly below that of pure ferriperoxidase. The addition of $K_4(Fe)CN_6$ immediately brings the absorbance up to a steady level close to that of pure ferriperoxidase. The $K_4(Fe)CN_6$ apparently reduced the Compound II present to ferriperoxidase (Eqn. 3).

In order to study whether the iodine in ferriperoxidase–iodine might be present

TABLE II

RATE CONSTANTS (k_c) FOR THE REACTION BETWEEN FERRIPEROXIDASE-IODINE AND REDUCING AGENTS

To a spectrophotometer cell reagents were added in the following order to the following final concentrations: 30 mM potassium phosphate (pH 6.2), 5 µM ferriperoxidase, 50 µM KI and 5 µM H_2O_2. 1 min after the H_2O_2 addition 10 µM of one of the reducing agents mentioned in the table was added, and the initial dA/dt was measured.

Reducing agent	k_c *($M^{-1} \cdot sec^{-1}$)*
Cysteine	$>2 \cdot 10^4$
1-Methyl-2-mercaptoimidazole	$9 \cdot 10^2$
$Na_2S_2O_3$	$>2 \cdot 10^4$

in the form of a sulfenyl iodide (-SI) group, the rate of reaction between ferriperoxidase–iodine and some reducing agents was examined. Reaction with $S_2O_3^{2-}$ and cysteine was very rapid and reaction with 1-methyl-2-mercaptoimidazole somewhat slower (Table II). CUNNINGHAM[21] found that protein sulfenyl iodide groups react fast with 1-methyl-2-mercaptoimidazole and quite slowly with cysteine, *i.e.* the opposite of what was now observed. This speaks against the presence of a sulfenyl iodide group.

DISCUSSION

In this study horseradish peroxidase Compound I has been found to react with I^- in an equimolar ratio to yield a complex characterized as ferriperoxidase–iodine (Eqn. 5). The complex is probably identical with a horseradish peroxidase–iodine complex described by NUNEZ AND POMMIER[15]. Ferriperoxidase–iodine has a visible spectrum similar to that of ferriperoxidase, which suggests identical oxidation states for the heme moieties in the two enzyme species. Consequently the complex has been named as a ferriperoxidase derivative. That iodine indeed remains bound to ferriperoxidase–iodine is perhaps best shown by the fact that iodinating activity can be liberated from it by treatment with H_2O_2 (ref. 20) (see also Eqn. 15).

Reducing agents readily reduce ferriperoxidase–iodine to ferriperoxidase + I^-. Rates of reaction with different reducing agents are not similar to those observed for the reduction of protein sulfenyl iodide groups. Therefore the iodine in ferriperoxidase–iodine is probably not present as a sulfenyl iodide group. It has been suggested that such groups are formed in the thyroid as a result of peroxidase activity[22,23]. Protein iodotyrosyl or iodohistidyl groups would not be affected by the reducing agents used, and the iodine therefore cannot be located in such a group.

There is some circumstantial, but inconclusive, evidence, which indicates that the iodine in ferriperoxidase–iodine might be bound to the prosthetic heme group. Thus attachment of I^- to the enzyme affects the visible spectrum. The iodine is liberated by H_2O_2, which is known to react with the heme.

We found that the present lyophilized peroxidase preparation underwent a transformation after being dissolved in potassium phosphate buffer at pH 6.2. The absorbance at 403 nm increased slowly and so did the stability of the ferriperoxidase–iodine complex obtainable from the preparation. Ferriperoxidase–iodine prepared from a fresh peroxidase solution appeared to undergo spontaneous breakdown to yield ferriperoxidase; in fact the half-life of the ferriperoxidase– iodine was so short that direct spectrophotometric observations were difficult to make. On the other hand, ferriperoxidase–iodine prepared from a peroxidase solution aged for 10 days or more had a half-life on the order of tens of minutes. Only aged peroxidase solutions were used for experiments reported here.

Present kinetic results show that the main pathway for the peroxidase-catalyzed oxidation of I^- is not the Chance–George mechanism of Eqns. 1–3. The most significant evidence of this is that Compound II is reduced at only 1/30 the rate needed to permit the rates observed for the overall reaction (Eqn. 4) to be reached over a mechanism in which Compound II is an obligatory intermediate.

In spite of this, there is good evidence that some Compound II is formed. Thus, when efforts were made to transform ferriperoxidase quantitatively into the ferriperoxidase–iodine complex *via* Reactions 1 and 5, the preparations obtained were always contaminated by Compound II. It must then be assumed that the Chance–George mechanism is operative as a side pathway. In the present case it can be described with the equations

$$\text{Ferriperoxidase} + H_2O_2 \rightarrow \text{Compound I} \tag{1}$$

$$\text{Compound I} + I^- \rightarrow \text{Compound II} + \text{product(s)} \tag{17}$$

$$\text{Compound II} + I^- \rightarrow \text{ferriperoxidase} + \text{product(s)} \tag{6}$$

In a previous study the properties of reaction steps in which ferriperoxidase and Compound II participate were studied by observing the steady-state kinetics of I^- oxidation[11]. The results support the presence of the side pathway. The main pathway could not be detected, since ferriperoxidase and Compound II are not on it.

A second pathway is characterized by the equations

$$\text{Compound I} + I^- \xrightarrow{k_a} \text{ferriperoxidase–iodine} \tag{5}$$

$$\text{Ferriperoxidase–iodine} + H_2O_2 \rightarrow \text{Compound I} + \text{product(s)} \tag{15}$$

The reaction steps of this mechanism are fast enough to account for the rate of the overall reaction. The rate constant k_a (Eqn. 5) was found to be twice as large as would be expected from the rate (k_d) of the overall reaction when the I^- concentration is the rate-limiting factor. This is close enough to make it probable that Eqns. 5 and 15 characterize the main pathway. The discrepancy between k_a and k_d is explained if it is assumed that half the enzyme is diverted to the side pathway in the steady state and exists as Compound II. Turnover *via* the side pathway is slow, and under the mentioned conditions only about 2% of the I^- oxidation would proceed by this route.

It should be stressed that Eqn. 15 undoubtedly summarizes several still unresolved reaction steps. The nature of the products formed from I^- and the fate of the oxidizing equivalents will be discussed in the subsequent paper[20].

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