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Iodide Oxidation and Iodine Reduction Mediated by Horseradish Peroxidase in the Presence of Ethylenediaminetetraacetic Acid (EDTA): the Superoxide Effect

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ABSTRACT

Ethylenediaminetetraacetic acid (EDTA) is an inhibitor of iodide (I^-) oxidation that is catalyzed by horseradish peroxidase (HRP). HRP-mediated iodine (I_2) reduction and triiodide (I_3^-) disappearance occur in the presence of this inhibitor. It is interesting that in the presence of EDTA, HRP produces superoxide radical, a reactive oxygen species that is required for iodine reduction. Substitution of potassium superoxide (KO_2) or a biochemical superoxide generating system (xanthine/xanthine oxidase) for HRP and H_2O_2 in the reaction mixture also can reduce iodine to iodide. Thus, iodine reduction mediated by HRP occurs because HRP is able to mediate the formation of superoxide in the presence of EDTA and H_2O_2. Although superoxide is able to mediate iodine reduction directly, other competing reactions appear to be more important. For example, high concentrations (mM range) of EDTA are required for efficient iodine reduction in this system. Under such conditions, the concentration (μM range) of contaminating EDTA-Fe(III) becomes catalytically important. In the presence of superoxide, EDTA-Fe(III) is reduced to EDTA-Fe(II), which is able to reduce iodine and form triiodide rapidly. Also of importance is the fact that EDTA-Fe(II) reacts with hydrogen peroxide to form hydroxyl radical. Hydroxyl radical involvement is supported by the fact that a wide variety of hydroxyl radical (OH^-) scavengers can inhibit HRP dependent iodine reduction in the presence of EDTA and hydrogen peroxide.

Key Words: HRP, EDTA, iodide oxidation, iodine reduction, triiodide, superoxide, hydroxyl radical

I. Introduction

Peroxidases catalyze a variety of one- and two-electron oxidations (Kohler and Jenzer, 1989). Although most peroxidase substrates are organic compounds, some inorganic compounds serve as substrates. For example, myeloperoxidase, lactoperoxidase, and horseradish peroxidase (HRP) all catalyze oxidation of iodide (I^-) (Kohler and Jenzer, 1989). Two-reaction mechanisms have been proposed for peroxidase mediated iodide oxidation. The first one, proposed by Morrison and Schonbaum (1976) and by Magnusson *et al*. (1984), is summarized below:

$$E + H_2O_2 \xrightarrow{H_2O} EO \xrightarrow{I^-} [EOI^-] \longrightarrow E + IO^-, \quad \textbf{(1)}$$

$$IO^- + I^- + H^+ \longrightarrow I_2 + OH^-, \quad \textbf{(2)}$$

$$I^- + I_2 \rightleftharpoons I_3^-. \quad \textbf{(3)}$$

In reaction **(1)**, it is thought that the peroxidase [E] reacts with hydrogen peroxide to generate an activated form of the enzyme, i.e., the well characterized peroxidase intermediate known as compound 1 = [EO], which reacts with iodide to form an enzyme bound hypoiodite intermediate $[EOI]^-$. The hypoiodite ion then is thought to dissociate from the enzyme to react with free iodide to form iodine (I_2) in reaction **(2)**. Finally, triiodide (I_3^-) is formed in reaction **(3)**, the well-known triiodide equilibrium reaction. An equally plausible reaction mechanism proposed by Roman and Dunford (1972) is as follows:

$$E + H_2O_2 \xrightarrow{H_2O} EO \xrightarrow{I^-} E + I^+, \quad \textbf{(4)}$$

$$I^- + I^+ \longrightarrow I_2\,, \quad \textbf{(5)}$$

In reaction **(4)**, iodide undergoes a peroxidase mediated two-electron oxidation in the presence of hydrogen peroxide to form the iodinium ion (I^+), which is thought to react with available iodide to form iodine in reaction **(5)**. Subse-

quently, newly formed iodine and available iodide react to form triiodide, again through the triiodide equilibrium reaction (reaction **(3)**) (Roman *et al*., 1971; Roman and Dunford, 1972).

Banerjee and his associates (Banerjee *et al*., 1982, 1986; Banerjee, 1989; Bhattacharyya and Kundu, 1972; Bhattacharyya *et al*., 1993, 1994, 1996) showed that EDTA (ethylenediaminetetraacetic acid) is an inhibitor of HRP mediated iodide oxidation activity. These investigations also showed that in the presence of iodine and triiodide, HRP dependent iodine reduction and triiodide disappearance occurred in the presence of this iodide oxidase inhibitor, EDTA. This is interesting because it implies that EDTA converts HRP from an oxidase into a reductase. Banerjee (1989) concluded that iodide oxidation proceeds as in reactions **(1)** – **(3)**, and that, in the presence of EDTA, the HRP mediated reaction of hypoiodite (IO^-) with hydrogen peroxide is favored as follows:

$$IO^- + H_2O_2 \xrightarrow{\text{EDTA}} O_2 + I^- + H_2O. \qquad \textbf{(6)}$$

Banerjee (1989) proposed that EDTA prevents reaction **(2)** from occurring through an unknown mechanism. He also proposed that EDTA induces a conformational change of HRP, called "modified compound 1," that is able to reduce iodine and oxidize hydrogen peroxide to form iodide and O_2 in a pseudocatalatic reaction **(6)**.

The effects of EDTA on peroxidases have led others to investigate this phenomenon. Shah and Aust (1992) investigated EDTA mediated inhibition of iodide oxidase activity and proposed that iodide is oxidized to hypoiodite by peroxidases (lignin peroxidase H2 from *Phanerochaete chrysosporium,* HRP, lactoperoxidase and myeloperoxidase). Hypoiodite was found to be reduced by EDTA, hydrogen peroxide or iodide. In subsequent studies (Shah and Aust, 1992, 1993), the authors proposed that peroxidases are able to mediate a one-electron oxidation of iodide to the iodine atom. This reaction in turn mediates a one-electron oxidation of EDTA or other organic acids (such as oxalic acid) to form the corresponding organic acid radicals. The organic acid anion radical formed is thought to react and reduce various electron acceptors (iodine, oxygen, cytochrome c and ferric ion).

Each of the above-described reaction mechanisms is consistent with observations made by the present authors. However, unlike previous investigations (Banerjee, 1989; Shah and Aust, 1992, 1993), we show that superoxide is involved in HRP-dependent iodine reduction in the presence of EDTA. Thus, any comprehensive reaction mechanism to explain this phenomenon must include the role of superoxide. Evidence shows that superoxide is produced in the presence of EDTA, and that superoxide is required for iodine reduction. In this study, we demonstrated that in the presence of EDTA, HRP did not reduce iodine directly. Iodine reduction occurred in the system with the concomitant formation of superoxide, which promoted iodine reduction. It was also found that inhibition of HRP catalyzed iodide oxidase activity by EDTA was dependent on superoxide production and subsequent reduction of iodine, an intermediate required for iodide oxidation.

II. Materials and Methods

1. Chemicals

Horseradish peroxidase (HRP type VI, Rz = 3.0), xanthine oxidase, lactoperoxidase, sodium benzoate, isopropanol and dimethylsulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Hydrogen peroxide, ethanol, mannitol, n-butanol, t-butanol and EDTA were purchased from Fisher (Fair Lawn, NJ, U.S.A.). Potassium iodide (KI) was purchased from Mallinckrodt (St. Louis, MO, U.S.A.). Potassium superoxide (KO_2) was purchased from Aldrich (Milwaukee, WI, U.S.A.). Iodine (I_2) was purchased from Matheson, Coleman and Bell (Norwood, OH, U.S.A.). Sodium formate was purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.).

2. Enzyme Assays

All buffers were prepared using distilled deionized water prepared using a Millipore Milli-Q water purification system. All kinetic studies and spectroscopic observations were made using a CARY 3 UV-visible spectrophotometer. Iodide oxidase activity was monitored at 353 nm, the wavelength of the maximum absorbance of triiodide ion (I_3^-), which is the end product of peroxidase mediated iodide oxidation. Incubation mixtures contained HRP and variable amounts of potassium iodide and hydrogen peroxide (as noted in the figure legends) in 50 mM sodium acetate buffer, pH 5.2. The reduction of iodine and the conversion of triiodide to iodide were monitored by following the decrease in absorbance at 353 nm. This assay system contained 50 mM EDTA, 0.008 μM HRP, 1 mM H_2O_2 and triiodide. To obtain the requisite concentration of triiodide and iodine, 25 μl of a saturated aqueous solution of iodine (I_2) ($\varepsilon_{460\,nm} = 730\ M^{-1}cm^{-1}$) was added to the reaction mixture (final volume: 1 ml). Following these steps, the initial absorbance at 353 nm was approximately 0.5 – 0.7, whereas the initial absorbance at 460 nm was approximately 0.4.

3. Iodide Oxidase Assay

Reaction mixtures contained potassium iodide, HRP and H_2O_2, as indicated in the figure legends, in 50 mM sodium acetate buffer, pH 5.2. The reactions were initiated with H_2O_2, and triiodide formation was monitored at 353 nm ($\varepsilon_{353\,nm} = 2.58 \times 10^4\ M^{-1}cm^{-1}$).

4. Oxygen Evolution

Oxygen evolution from reaction mixtures was measured

using a Gilson oxygraph (Middleton, WI, U.S.A.).

5. Spin Trapping (ESR) of Superoxide-derived Hydroxyl Radicals

Reaction mixtures for enzyme assay were also analyzed for EPR following the methods described by Shah *et al.* (1992).

III. Results and Discussion

1. The Role of Hydrogen Peroxide, Iodide, and EDTA in Oxygen Evolution

Peroxidases typically mediate one electron oxidation although a number of two-electron oxidations, such as iodide oxidation (Harris *et al.*, 1993; Kohler and Jenzer, 1989; Magnusson *et al.*, 1984; Morrison and Schonbaum, 1976), do occur. A number of mechanisms exist in peroxidases that result in the evolution of molecular oxygen (Metodiewa and Dunford, 1989; Shah and Aust, 1993). In a catalatic mechanism (Arnao *et al.*, 1990) hydrogen peroxide reacts with peroxidase compound 1, undergoes a two-electron oxidation, resulting in the formation of molecular oxygen. In another pathway, hydrogen peroxide undergoes a one-electron oxidation by compound 1, resulting in the formation of compound 2 and superoxide (Arnao *et al.*, 1990; Dunford, 1982; Kohler and Jenzer, 1989). Compound 2 is then thought to react with hydrogen peroxide, undergoing a two-electron reduction to form compound 3, which dissociates to form the native enzyme and superoxide (Arnao *et al.*, 1990; Dunford, 1982; Kohler and Jenzer, 1989).

Another mechanism has shown that the reducing cosubstrate plays a critical role in promoting the evolution of molecular oxygen. Iodide, which is oxidized by lactoperoxidase and myeloperoxidase, has been studied extensively with regard to its pseudocatalatic activity (Huwiler and Kohler, 1985; Huwiler *et al.*, 1985; Lebhafsky and Wu, 1974; Lebhafsky, 1972; Magnusson *et al.*, 1984). Oxygen evolution in this system has been proposed to occur through a mechanism in which iodide binds to lactoperoxidase compound 1 to form an enzyme bound hypoiodite $[EOI]^-$ complex. The complex reacts with H_2O_2 to regenerate iodide with the concomitant production of molecular oxygen and water. In a competing reaction, iodide is thought to react with the $[EOI]^-$ complex to form iodine and, ultimately, triiodide via triiodide equilibrium (Magnusson *et al.*, 1984). This proposed mechanism for pseudocatalatic activity by lactoperoxidase is as follows (Magnusson *et al.*, 1984):

$$E + H_2O_2 \longrightarrow EO, \quad \textbf{(7)}$$

$$EO + I^- \longrightarrow EOI^-, \quad \textbf{(8)}$$

$$EOI^- + H_2O_2 \longrightarrow O_2 + H_2O + I^- + E, \quad \textbf{(9)}$$

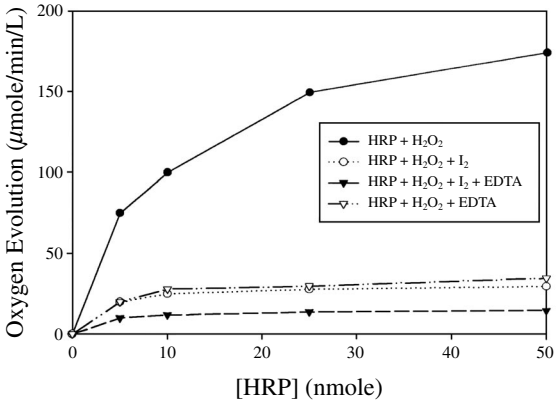

Fig. 1. Evolution of molecular oxygen was HRP-dependent and was inhibited by EDTA. The complete reaction mixtures contained iodine, 0 – 0.05 μM HRP, 50 mM EDTA, and 1 mM HRP and H_2O_2.

$$EOI^- + I^- + H_2O \longrightarrow I_2 + 2\ OH^- + E. \quad \textbf{(10)}$$

In this study, we found that iodide promoted pseudocatalatic activity in HRP-mediated reactions. The evolution of molecular oxygen during HRP mediated reactions is presented in Fig. 1.

The greatest amount of HRP mediated molecular oxygen evolution occurred in the presence of HRP, hydrogen peroxide and iodide. Inclusion of EDTA resulted in inhibition (but not elimination) of molecular oxygen evolution. However, addition of EDTA consistently inhibited oxygen evolution.

2. Formation of HRP Intermediates in the Presence of Hydrogen Peroxide and EDTA

We also found that the spectral intermediate of HRP in the presence of both 4 mM and 50 mM EDTA was HRP compound 2 (Fig. 2), not compound 1 as Banerjee (1989) reported. The spectrum of HRP was virtually identical to that of the authentic compound 2 formed in the absence of EDTA. Although these spectra are not presented here, they are indistinguishable from that shown in Fig. 2 from 500 – 700 nm. EDTA may also function as a reductant in some circumstances (Fife and Moore, 1979; Shah and Aust, 1993) and might be expected to participate in reduction of HRP compound 1 to compound 2. Under conditions where hydrogen peroxide is in great excess relative to HRP, compound 3 was found to be the major HRP intermediate formed. However, when iodide species were added to HRP compound 3, it returned immediately to the native form of the enzyme as shown in Figs. 3 and 4.

3. The Mechanism of Iodine Reduction Is Superoxide Dependent

The reduction of iodine by HRP in the presence of hydrogen peroxide and EDTA was accompanied by a transient increase in triiodide formation (Fig. 5). According to Banerjee

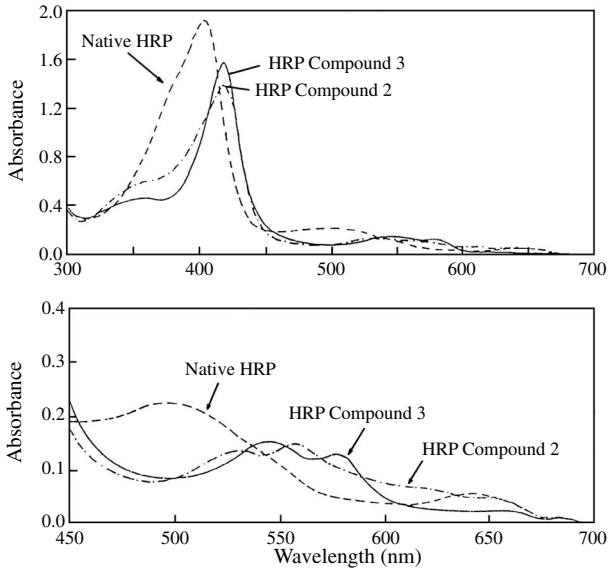

Fig. 2. Absorbance spectra of HRP native form, compound 2, and compound 3. Compound 2 was formed in the presence of one equivalent of H_2O_2 and 4 mM EDTA. Compound 3 was formed in the presence of excess H_2O_2 (1 mM). The concentration for HRP was 18.8 μM. All spectra were recorded in the presence of 4 mM EDTA in 50 mM sodium acetate buffer, pH 5.2.

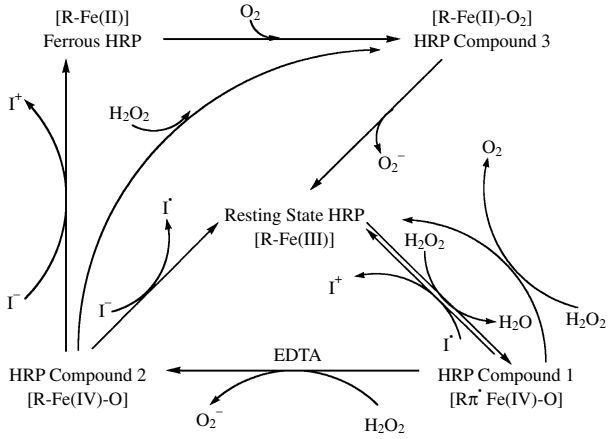

Fig. 3. Proposed interactions of iodide with horseradish peroxidase and its oxidized and reduced intermediates in the presence of EDTA.

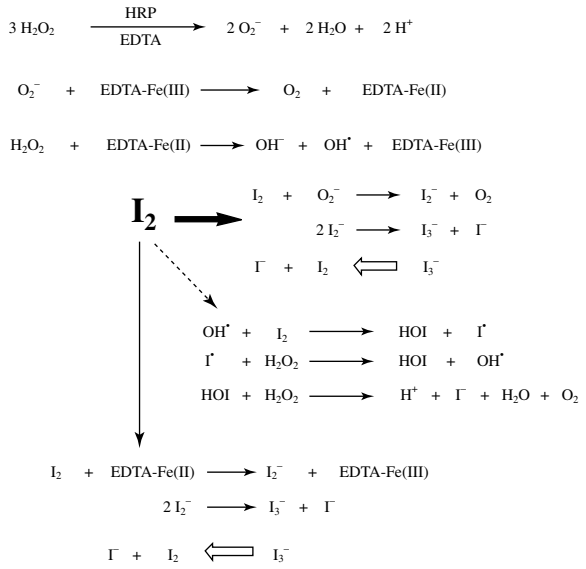

Fig. 4. Proposed mechanisms of superoxide dependent iodine reduction in the presence of HRP, EDTA and H_2O_2.

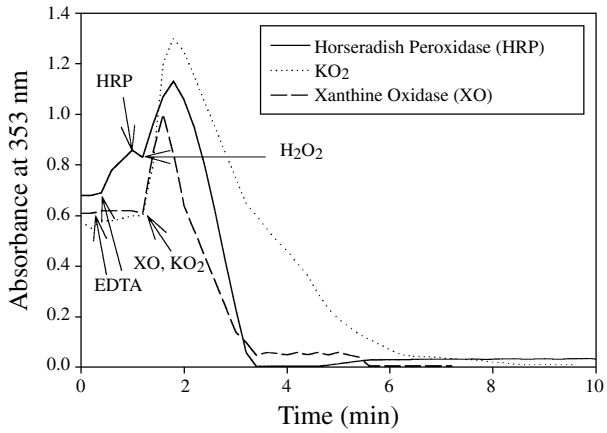

Fig. 5. Iodine reduction and triiodide appearance was superoxide-dependent. The reaction mixture contained 0.005 μM HRP, 50 mM EDTA, 1 mM H_2O_2 and iodine ($A_{353\,nm}$ = 0.5 – 0.7) in sodium acetate buffer, pH 5.2. In the KO_2 system, HRP and H_2O_2 were omitted and replaced by KO_2. In the xanthine oxidase system, HRP and H_2O_2 were omitted and replaced by 0.02 units of xanthine oxidase and 100 μM xanthine. The reactions were monitored at 353 nm.

(1989), the increased concentration of iodide, formed as a consequence of iodine reduction, reacts with existing iodine to momentarily shift the triiodide equilibrium to favor triiodide formation ($A_{353\,nm}$). However, this mechanism was also found to be true for iodine reduction, which is superoxide dependent. Indeed, when superoxide was added as potassium superoxide (KO_2), or when a superoxide generating system containing xanthine and xanthine oxidase (XO) was substituted for HRP, a transient increase in triiodide concentration accompanied by iodine reduction occurred (Fig. 5). When iodine reduction was nearly complete in the HRP dependent system, a precipitous decrease in triiodide concentration was observed (Fig. 5). EDTA may interact with HRP at a site or point in the reaction mechanism (with compound 1) where hydrogen peroxide would otherwise react as a reducing co-substrate, thus preventing the catalatic and pseudocatalatic activities of the enzyme. This has the effect of promoting the formation of superoxide (Fig. 3), which inhibits iodide oxidase activity by promoting reduction of iodine, thereby preventing formation of triiodide, the end product in iodide oxidation. The fact that superoxide (supplied chemically or biochemically) could substitute for HRP, implies the involvement of superoxide in this

series of reactions.

4. The Role of EDTA and EDTA-Fe in Iodine Reduction

EDTA inhibits iodide oxidase activity that is catalyzed by HRP, and a slight excess of Zn(II) reverses this inhibition (Fig. 6). It is also clear that EDTA promotes iodine reduction by HRP (Fig. 5). The mechanisms previously proposed (Banerjee, 1989; Shah and Aust, 1993) were detailed and, in many cases, appear to be internally consistent. However, our results demonstrate that superoxide plays an integral role in both of these processes (Figs. 3 and 4). In order to account for our observations and to provide a logical explanation for iodine reduction, it is necessary to appreciate the multiple roles that EDTA plays in the reaction mechanism by means of which iodine is reduced. It is important to note that free EDTA as well as EDTA-Fe species play important roles. It is known that EDTA preparations contain trace amounts of iron (Halliwell and Gutteridge, 1985; Rand *et al*., 1975; Wong *et al*., 1981) and high concentrations of EDTA required for complete iodide oxidase inhibition and iodine reduction. In the previous studies, the concentration of EDTA present as EDTA-Fe(III) ranged between 18 nM and 9 μM, which is in the range known to be catalytically important in mediating reactions between active oxygen species (Klebanoff, 1982; McCord and Day, 1978; Walling *et al*., 1970). For example, in the presence of EDTA-Fe(III), hydroxyl radical ($OH^{\cdot}$) is known (Bull *et al*., 1983; McCord and Day, 1978) to be formed by so-called superoxide dependent iron mediated Haber-Weiss reaction:

$$\text{EDTA-Fe(III)} + O_2^- \longrightarrow \text{EDTA-Fe(II)} + O_2, \quad \textbf{(11)}$$

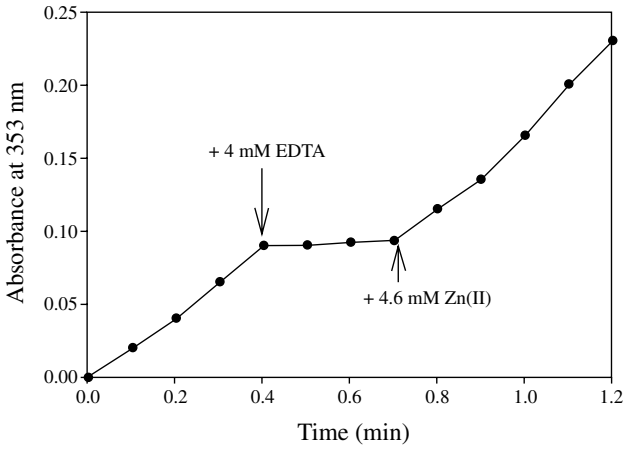

Fig. 6. Inhibition of HRP-mediated iodide oxidase activity by EDTA. Inhibition was reversed by a slight molar excess of Zn(II). Reaction mixtures contained 1.5 mM KI, 200 μM H_2O_2, and 0.001 μM HRP in sodium acetate buffer, pH 5.2.

$$\text{EDTA-Fe(II)} + H_2O_2 \longrightarrow \text{EDTA-Fe(III)} + OH^{\cdot} + OH^-. \quad \textbf{(12)}$$

It is interesting that EDTA-Fe(II) is known to reduce iodine and form triiodide. This reaction has been studied extensively by Woodruff and Margerum (1974) who showed that reduction is rapid (ca. 2×10^6 $M^{-1}s^{-1}$) at pH 5 as follows:

$$I_2 + \text{EDTA-Fe(II)} \longrightarrow I_2^- + \text{EDTA-Fe(III)}, \quad \textbf{(13)}$$

$$2\ I_2^- \longrightarrow I_3^- + I^-. \quad \textbf{(14)}$$

As iodine is depleted in this system, triiodide equilibrium, as shown in reaction **(3)**, is shifted to favor iodide formation.

5. Proposed Mechanisms of Iodine Reduction in This Study

The most likely reaction mechanism (Fig. 4) to explain observed phenomena is as follows: In the presence of hydrogen peroxide and EDTA, HRP is oxidized to form compound 1. Because EDTA inhibits catalatic and pseudocatalatic activity, compound 1 oxidizes hydrogen peroxide preferentially to form superoxide with the immediate formation of compound 2. At least three reasonable pathways (Fig. 3) are theoretically available for the return of compound 2 to the resting state.

Compound 2 may mediate the one electron oxidation of iodide to form iodine atoms and the resting state enzyme. Compound 2 may mediate the two-electron oxidation of hydrogen peroxide to form compound 3, which subsequently dissociates, forming superoxide and the resting state enzyme; compound 2 may mediate the electron oxidation of iodide, forming either HOI or I^+ (Figs. 3 and 4). In this pathway, the ferric form of the enzyme would be formed, which would be expected to immediately react with molecular oxygen to form compound 3, followed by dissociation to form superoxide and resting enzyme. This reaction pathway is supported by the fact that molecular oxygen would be required, and that iodine reduction does not occur in this system in the absence of oxygen (Fig. 1). Exceptions do occur, however, in the absence of a good reducing substrate or under conditions in which the concentration of hydrogen peroxide is considerably higher the that of the reducing co-substrate (Arnao *et al*., 1990). Although EDTA might be able to contribute to the reduction of compound 1, it is not a good reducing substrate as it is clearly unable to reduce compound 2 to resting state HRP. Iodide, however, is a good reducing substrate. If compound 1 is unavailable, it is known that compound 2 is able to catalyze slowly through the two-electron oxidation of iodide (Dunford, 1982; Roman *et al*., 1971; Roman and Dunford, 1972).

Compound 3 is formed through addition of hydrogen peroxide in excess to HRP. However, this intermediate is not

the predominant species observed during the steady state reaction. The reaction pathway probably proceeds through compound 3, followed by its immediate dissociation to form resting state HRP and superoxide. Although dissociation of compound 3 to form superoxide and resting state HRP is relatively slow (Rotilo *et al*., 1975). With a first order rate constant of 2.2×10^{-3} s^{-1} in the absence of a good reducing co-substrate, conversion to resting slate is accelerated dramatically in the presence of superoxide scavengers (*e.g*., iodine) and good reducing co-substrates (*e.g*., iodide).

We summarize the various pathways proposed for the intermediary states of HRP in its reaction cycle in the presence of EDTA and iodide (Fig. 3). According to reaction **(1)**, iodide binds to the oxyferryl moiety of compound 1. The oxyferryl region of the heme would be expected to be inaccessible to EDTA molecules. EDTA interacts on the heme moiety, possibly at the delta meso edge, which is an active site thought to be involved in catalytic activity in peroxidases (Ator and Ortiz de Montellano, 1987; Ator *et al.*, 1987). Unless HRP compound 1 has exceptional affinity for EDTA, we would not expect it to be able to completely abolish all iodide oxidase activity. The fact that EDTA does appear to abolish all iodide oxidase activity (as measured by triiodide formation) is due not to effective competition for HRP compound 1 by EDTA, but rather due to the fact that superoxide production by HRP is promoted by the presence of EDTA. Superoxide production was evidenced also by the fact that ESR data revealed the presence of hydroxyl radicals, which are the final transformed products of superoxide radicals, in the presence of EDTA (data not shown). Superoxide effectively promotes reduction of iodine (Kim *et al*., 1979; Renanathan *et al*., 1985; Schwarz and Bielski, 1986), which is also produced during reaction **(2)** back to iodide. Inhibition occurs as a consequence of iodine reduction promoted by superoxide that is generated by the presence of EDTA in the system.

In proposed mechanisms shown in Fig. 4, superoxide can directly mediate the reduction of iodine (Furrow and Noyes, 1982a, 1982b; Kim *et al*., 1979). Also, EDTA-Fe(II) rapidly reduces iodine. The direct reduction of iodine by superoxide and the reduction of EDTA-Fe(III) to form EDTA-Fe(II) produce molecular oxygen and would be expected to contribute to the evolution of molecular oxygen that is observed (Fig. 1). Hydroxyl radical formation has been proposed to occur by means of the superoxide dependent iron catalyzed Haber-Weiss reaction (McCord and Day, 1978).

6. Superoxide Dependent Iodine Reduction

In the presence of EDTA, HRP and hydrogen peroxide, we have found that a variety of hydroxyl radical scavengers inhibit iodine reduction (Table 1). Formation of hydroxyl radical is important in that this reactive oxygen species is able to oxidize iodine via the following reaction (Schwarz and Bielski, 1986):

Table 1. Inhibition of HRP (0.05 μM)-mediated Iodine Reduction in the Presence of 50 mM EDTA and 1 mM H_2O_2 by Hydroxyl Radical Scavengers

Hydroxyl radical scavenger	Concentration (mM)	Remaining of iodine reduction activity (%)
Control	—	100
Formate	20	67
Mannitol	20	52
2-Propanol	10	44
Dimethylsulfoxide	5	39
t-Butanol	25	34
Ethanol	32	30
1-Butanol	5	30
Benzoate	3	12

Notes: Reactions were monitored at 460 nm. The initial absorbance of iodine was 0.35 at A_{460}.

$$HO^{\bullet} + I_2 \longrightarrow HOI + I^{\bullet}. \qquad \textbf{(15)}$$

Both hypoiodous acid and iodine atom are reactive species. Iodine atom is thought to be reduced by hydrogen peroxide as follows (Furrow and Noyes, 1982a, 1982b):

$$I^{\bullet} + H_2O_2 \longrightarrow HOI + HO^{\bullet}. \qquad \textbf{(16)}$$

Hypoiodous acid produced by these reactions would be expected to be by hydrogen peroxide according to the reaction studied by Furrow and Noyes (1982a, 1982b) and Lebhafsky and Wu (1974):

$$HOI + H_2O_2 \longrightarrow H^+ + I^- + H_2O + O_2. \qquad \textbf{(17)}$$

Control experiments (data not shown) in a biochemical superoxide generating system (Xanthine/Xanthine oxidase) demonstrated that efficient iodine reduction occurred only in the presence of high concentrations of EDTA, suggesting that direct reduction of iodine by superoxide is not very important in this system. The reactions involve EDTA-Fe, and those initiated by hydroxyl radical are the most important reactions. These reaction mechanisms are consistent with the observation that in the presence of EDTA, superoxide is formed and subsequently inhibits HRP catalyzed iodide oxidation and promotes HRP dependent iodine reduction.

7. The Binding of EDTA and Iodide on HRP

Harris and his colleagues recently reported that several substrates that undergo two electron oxidations mediated by HRP, are oxidized at different sites on the heme moiety (Harris *et al*., 1993). This is supported by NMR evidence suggesting that iodide may interact with HRP at a site between the 1 and 8 methyl groups that is 6 – 10 Å from the heme iron atom (Sakurada *et al*.,1987; Modi *et al*., 1989). Also, Nuclear Overhauser and NMR experiments indicate that phenols and other aromatic substrates interact nearer to the 8 methyl group

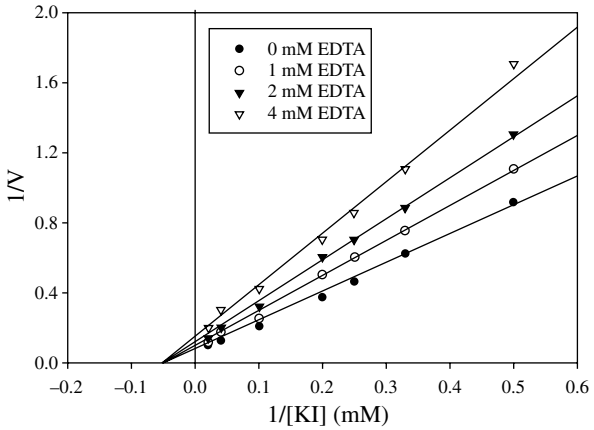

Fig. 7. Kinetic analysis (Lineweaver-Burke) of EDTA inhibition of HRP-mediated iodide oxidase activity. Reaction mixtures contained KI (2 – 50 mM), 0.005 μM HRP, 100 μM H_2O_2 and EDTA (0 – 4 mM) in 50 mM sodium acetate buffer, pH 5.2. The reaction was initiated with H_2O_2. The absorbance of triiodide was determined at 353 nm.

6 – 10 Å from the heme iron (Schejter *et al.*, 1976; Leigh *et al.*, 1975; Saxena *et al*., 1990). A third site for thioanisole oxygenation exists in close proximity to the heme iron and facilitates oxygen transfer in one of the relatively few direct oxygenation reactions mediated by peroxidase (Harris *et al*., 1993; Nakajima and Yamazaki, 1980). Interestingly, an active site other than oxyferryl heme, in close proximity to heme, was also found in lignin peroxidase (Choinowski *et al*., 1999).

The observation that different substrates may be oxidized at different sites is consistent with the fact that EDTA is a non-competitive inhibitor for iodide oxidation (Fig. 7), and with the conclusion that EDTA interacts with HRP-catalyzed iodide oxidation and with HRP-catalyzed iodine reduction. However, based on the most current evidence available, we would have to conclude that iodide interacts at the site described by Harris *et al*. (1993) rather than directly with the oxyferryl heme. However, EDTA interacts at another available but undetermined site.

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