Concurrent Reduction of Iodine and Oxidation of EDTA at the Active Site of Horseradish Peroxidase: Probing the Iodine Binding Site by Optical Difference Spectroscopy and Steady State Kinetic Analysis for the Formation of Active Enzyme−I^+−EDTA Ternary Complex for Iodine Reductase Activity[†]

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ABSTRACT: Horseradish peroxidase (HRP) catalyzes the reduction of iodine to iodide by EDTA with pseudocatalatic degradation of H_2O_2 to O_2 (Banerjee et al., (1986) *J. Biol. Chem. 261*, 10592−10597; and Banerjee (1989) *J. Biol. Chem. 264*, 9188−9194). The reduction of iodine (I^+) is dependent on EDTA concentration and is blocked by spin trap, DMPO, indicating the involvement of free radical species in the reduction process. Incubation of EDTA with both HRP and H_2O_2 results in the appearance of triplet ESR signal of spin-trapped EDTA radical (a^N = 15 G), indicating its one-electron oxidation to a nitrogen-centered monocation radical (N-N^+). The latter oxidizes H_2O_2 to evolve O_2 and regenerate EDTA. In the presence of I^+, a ternary complex of compound I−I^+−EDTA is formed, which generates compound II−$I^•$ complex and both nitrogen-centered dication radical (N^+-N^+) through intermolecular electron transfer from EDTA nitrogens. Compound II−$I^•$ complex is further reduced similarly by another molecule of EDTA to form ferric enzyme, I^-, and (N^+−N^+).(N^+-N^+) the oxidation product of EDTA, which may be released from the active site and, being more reactive, oxidizes H_2O_2 to O_2 at a faster rate to regenerate EDTA. The existence of (N^+-N^+) is suggested from the similarity of its ESR signal with that of single nitrogen-centered monocation radical (N-N^+). EDTA degradation by oxidative decarboxylation due to two-electron oxidation from the same or both nitrogen atoms is not evident, and EDTA concentration remains the same throughout the reactions. While EDTA binds (K_D = 15 mM) at or near the iodide binding site (Bhattacharyya et al. (1993) *Biochem. J. 289*, 575−580), I^+ binds to HRP with a K_D value of 20 ± 7 μM. I^+ binding in the HRP−CN complex (K_D = 20 ± 8 μM) indicates that its site is away from the heme iron center. I^+ binding remains unaltered by guaiacol or vice versa, suggesting that I^+ binds away from the aromatic donor binding site. As I^+ reduction occurs at a saturating concentration of EDTA, I^+ binding at the EDTA site could be excluded. A plot of log K_D of I^+ binding against various pHs shows the involvement of an ionizable group on the enzyme having pK_a = 4.8, contributed by an acidic group, deprotonation of which favors I^+ binding. Systematic variation of the concentrations of H_2O_2, EDTA, and I^+ under steady state condition yields sets of kinetic parameters containing both kinetic and mechanistic information. Four distinct enzyme species are involved in I^+ reduction: native enzyme, compound I, compound I−I^+, and compound II−$I^•$ complex; and rate constants for individual steps are calculated. The kinetic experiments support the view that an active enzyme−I^+−EDTA ternary complex is formed during I^+ reduction by EDTA at the enzyme active site.

Horseradish peroxidase (HRP;[1] EC 1.11.1.7; donor−H_2O_2 oxidoreductase) catalyzes the oxidation of various electron donors with H_2O_2 through intermediate formation of compound I and compound II (Chance, 1949; George, 1952; Yamazaki et al., 1960; Saunders et al., 1964; Dunford & Stillman, 1976). Compound I and compound II, having two- and one-electron oxidation state higher over ferriperoxidase, respectively, oxidize electron donors (AH_2) by two one-electron transfers with the formation of substrate free radicals ($AH^•$) and ferriperoxidase. However, the reaction between compound I and iodide (halide) is a direct two-electron transfer (Björkstein, 1970; Roman et al., 1971; Roman & Dunford, 1972; Pommier et al., 1973) through the formation of enzyme−hypoiodous complex, EOI (Morrison & Schonbaum, 1976; Magnusson et al., 1984), where EO, the compound I, is an oxo−Fe(IV) porphyrin−π-cation radical (Dolphin et al., 1971) and I^- is present in an oxidation state equivalent to I^+. EOI gives rise to ferriperoxidase and HOI, which in the presence of excess I^- forms I_2 and I_3^- nonenzymatically. Aromatic donors bind to HRP in a hydrophobic pocket formed by the heme peripheral 8-methyl group, tyrosine-183, and arginine-185 (Sakurada et al., 1986), while iodide interacts at a site almost equidistant from the heme peripheral 1- and 8-methyl groups (Sakurada et al., 1987). Recently, Ortiz de Montellano (1987) and Harris et al (1993) have proposed a model for the active site topology

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[1] Abbreviations: HRP, horseradish peroxidase; EDTA (N-N), ethylenediaminetetraacetate sodium salt; N-N^+, single nitrogen-centered EDTA monocation radical; N^+-N^+, both nitrogen-centered EDTA dication radical; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DETAPAC, diethylenetriaminepentaacetic acid.

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of HRP and suggested that electron donors interact at the heme edge and are oxidized by the flow of electrons through the δ-meso carbon to the heme ferryl group.

We first reported a novel reaction of HRP-catalyzed reduction of iodine by EDTA with pseudocatalatic decomposition of H_2O_2 to molecular O_2 (Banerjee et al., 1986). This is associated with concurrent loss of iodide oxidation by HRP (Banerjee et al., 1986). We proposed that EDTA might bind at or near the heme moiety, causing a conformational change responsible for the observed effect (Banerjee, 1989a). Subsequently, we observed that EDTA binds (K_D = 15 mM) at or near the iodide binding site and competitively inhibits iodide oxidation as a cosubstrate (Banerjee, 1989b; Bhattacharyya et al., 1993a, 1994). Iodide modulates iodide oxidase and iodine reductase activities of HRP by competing with EDTA for oxidation by compound I, and we proposed that EDTA oxidation product, presumably its free radical, is involved in the iodine reductase activity (Bhattacharyya et al., 1993a). However, the exact mechanism of iodine reductase and pseudocatalase activities of HRP by EDTA is not clear yet. It is not known whether I^+ binds at the enzyme active site and is reduced through concurrent oxidation of EDTA. Here, we present evidence to show that EDTA is oxidized to generate nitrogen-centered EDTA radicals in the absence and presence of I^+. I^+ binds to HRP at a specific site away from the heme iron center or aromatic donor binding site. A steady state kinetic analysis provides further evidence for the formation of an active HRP$-I^+-$EDTA ternary complex during enzymatic reduction of I^+ by EDTA. A plausible mechanism of HRP-catalyzed I^+ reduction through enzymatic generation of EDTA radical at the enzyme active site along with its pseudocatalatic activity has been proposed. This study represents a novel observation on the peroxidase-catalyzed oxidation of an electron donor and reduction of an electron acceptor occurring concurrently at the enzyme active site.

MATERIALS AND METHODS

Horseradish peroxidase (HRP Type VI, RZ = 3.0), guaiacol, DETAPAC, and sodium azide were purchased from Sigma, USA. ICI and DMPO were obtained from Aldrich, USA. EDTA was a product of Glaxo laboratories, India. Eriochrome Black T was procured from Loba Chemie, India. All other chemicals used were analytical grade.

Assay of Enzyme Activity. The concentration of HRP was determined from $\epsilon_{403} = 102\ cm^{-1}\ mM^{-1}$ (Aibara et al., 1982). A stock of 0.2 M EDTA solution was prepared in warm distilled water, and its pH was adjusted to 6−6.5 with dilute NaOH. All reactions were carried out in the dark at 25 ± 1 °C. For the assay of iodine reductase activity (Banerjee et al., 1986; Banerjee, 1989a), the assay system contained the following in a final volume of 1 mL: 50 mM sodium acetate buffer, pH 6.0, 0.5 mM freshly prepared ICI, 4 mM EDTA, 0.3 mM H_2O_2, and 2.5 nM HRP (added last to start the reaction). The decrease in absorbance at 460 nm was recorded in a Shimadzu UV-2201 computerized spectrophotometer at an interval of 30 s to measure the activity (Bhattacharyya et al., 1993a). The pseudocatalase activity of HRP was measured by O_2 evolution in a Gilson oxygraph. The assay system contained the following in a final volume of 2 mL: 50 mM sodium acetate buffer, pH 6.0, 4 mM EDTA, 1 μM HRP, and 0.3 mM H_2O_2 in the absence or presence of 200 μM ICI. The initial rate of O_2 evolution was determined from the linear part of the curve after correction for the nonenzymatic rate, if any. H_2O_2 consumption was assayed by measuring the concentration of H_2O_2 (Hildebrandt & Roots, 1975). The assay system contained in the following in a final volume of 1.2 mL: 50 mM sodium acetate buffer, pH 6.0, 4.0 mM EDTA, 1 μM HRP, and 0.3 mM H_2O_2 in the absence or presence of 200 μM ICI. An aliquot of 0.1 mL was removed at different time intervals, and the reaction was stopped by adding the aliquot to 3 mL of 80 mM HCl. The rest of the procedure was the same as described (Hildebrandt & Roots, 1975). EDTA concentration was measured by the standard titration procedure (Bhattacharyya, 1984), and the assay system was the same as described for the H_2O_2 consumption assay. An aliquot of 0.2 mL was removed at different time intervals, and the reaction was stopped by 5 nM catalase. It was titrated against standard zinc acetate solution using Eriochrome Black T as indicator. Oxidative decarboxylation of EDTA was measured by $^{14}CO_2$ evolved from EDTA-*carboxy*-^{14}C (Sigma). The reaction was carried out for 30 min in a Warburg flask containing 2 mL of reaction mixture as described for the H_2O_2 consumption assay, except that 6.5×10^6 cpm of radiolabeled EDTA was included. The reaction was started by the addition of H_2O_2 (final concentration 0.3 mM) from the side arm. $^{14}CO_2$ absorbed in KOH-soaked filter paper in the central well was measured by scintillation counting.

Steady State Kinetic Experiments. For steady state kinetic analysis (Lambeir & Dunford, 1983) of the reaction of HRP with H_2O_2, I^+, and EDTA, the reaction was initiated with addition of different concentrations of H_2O_2 to a reaction mixture (final volume 1 mL) containing 50 mM sodium acetate buffer, pH 6.0, ICI (at a fixed concentration between 0.09 and 0.56 mM), 4 mM EDTA, and 2 nM HRP. I^+ reduction was recorded by the loss of absorbance at 460 nm. The initial rate was calculated from the linear part of the assay after correction for the nonenzymatic part, if any, and the mean value was determined from three experiments. The ionic strength was maintained at 60 mM with potassium sulfate as inert salt, if necessary. The plots of initial rate (V) versus substrate concentration [S] were fitted as rectangular hyperbolae with the help of a weighted nonlinear least-squares program in the equation:

$$\frac{V}{E_{tot}} = \frac{B_1[S]}{B_2 + [S]}$$

where B_1 and B_2 are constants at a particular set of concentrations of two other substrates. B_1 and B_2 were determined by computer best fit using the experimental values of E_{tot}, [S], and V, where E_{tot} is the total HRP concentration and [S] is the third substrate concentration. The equations for various experimental conditions are derived in the Appendix.

Binding Studies. Enzyme−ligand complex formation was demonstrated by optical difference spectroscopy (Critchow & Dunford, 1972; Hosoya et al., 1989) as described earlier (Bhattacharyya et al., 1993a,b), and the equilibrium dissociation constant, K_D, for complex formation was calculated from the following expression (Schejter et al., 1976):

$$\frac{I}{\Delta A} = (K_D/\Delta A_\infty)\frac{I}{S} + \frac{I}{\Delta A_\infty}$$

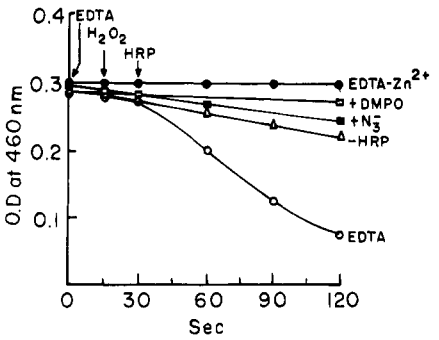

FIGURE 1: HRP-catalyzed I^+ reduction by EDTA: The assay system has been described under Materials and Methods. After addition of ICI, 1 min was allowed for stabilization of absorbance at 460 nm before the addition of EDTA. The arrows indicate the time of addition of each reactant. The concentrations of $ZnSO_4$, DMPO, and NaN_3 used were 4, 10, and 1 mM, respectively, and addition was done after EDTA.

where ΔA is the change in absorbance, S is the substrate concentration, and ΔA_∞ is the change in absorbance at saturating concentration of the substrate.

Detection of EDTA Radicals by ESR Spectroscopy. EDTA free radicals were detected as spin adduct with DMPO by ESR spectroscopy. The reaction mixture contained 50 mM sodium acetate buffer, pH 6.0, 30 mM EDTA, 100 mM DMPO, 10 μM DETAPAC, 40 μM HRP, and 0.5 mM H_2O_2 added last to start the reaction. One milliliter of the reaction mixture was aspirated into the sample cavity within 10 s after initiation of the reaction with H_2O_2 at 25 °C. ESR spectra were recorded in a Varian E-112 spectrometer fitted with T-112 cavity operating at 9.45 GHz with 100 KHz modulation frequency.

Statistical Analysis. Data were presented as mean ± SEM of at least three experiments.

RESULTS

HRP-Catalyzed I^+ Reduction by EDTA. I^+ reduction by EDTA is catalyzed by HRP and is sensitive to N_3^- (Figure 1). No significant rate was evident in the absence of HRP or in the presence of EDTA−Zn^{2+} chelate. The reaction is blocked in the presence of DMPO, a free radical trap, indicating involvement of free radical in the reduction process.

ESR Spectroscopy of EDTA Radicals. Since EDTA competitively inhibits HRP-catalyzed iodide oxidation (Banerjee, 1989a,b; Bhattacharyya et al., 1993a, 1994) and is often used as reductant (Fife & Moore, 1979; Heelis, 1982), we looked for the generation of EDTA free radical as intermediate in HRP-catalyzed EDTA oxidation. The triplet ESR signal for nitrogen-centered EDTA cation radical (N−N^+) (a^N = 15 G) was observed by ESR spectroscopy (Figure 2A) using DMPO as spin trap. The concentration of the radical is dependent on EDTA concentration (not shown), and no signal appears in the absence of HRP (B), EDTA (C), or H_2O_2 (D). No signal for DMPO−O_2^- or DMPO−OH• adduct (Finkelstein et al., 1980) was detected. EDTA is thus directly oxidized by HRP and H_2O_2 through one-electron transfer to form a nitrogen-centered monocation radical (N−N^+). A similar ESR signal was also detected in the presence of I^+ (Figure 2E) in the system where I^+ is reduced to I^-. From the similar nature, we speculate that the latter is due to both nitrogen-centered EDTA dication

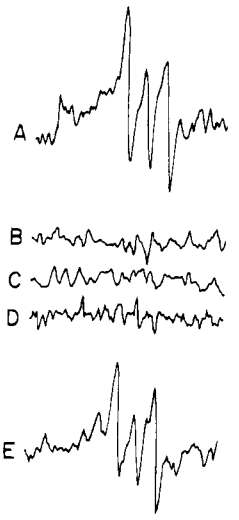

FIGURE 2: ESR spectra of DMPO−EDTA radical adduct in the HRP−H_2O_2 system in the absence or presence of I^+: The incubation system for the detection of the ESR spectra for DMPO−EDTA radical adduct (A) generated from oxidation of EDTA by HRP and H_2O_2 has been described under Materials and Methods. The spectrometer settings were as follows: scan range, 800 G; modulation amplitude, 100 kHz; time constant, 0.5 s; gain, 6.3 × 10^4; microwave power, 20 mW; and scan time, 8 min. (B) −HRP; (C) −EDTA; (D) $-H_2O_2$. (E) indicates the ESR signals obtained with the same system as (A) but in the presence of 30 mM KI to generate sufficient I^+ in the system. I^+ generated from I^- equimolar to EDTA is reduced by the HRP−H_2O_2 system (Banerjee et al., 1986).

Table 1: Oxidative Decarboxylation of EDTA by HRP in the Absence or Presence of ICI[a]

system	nmol of CO_2 evolved
−HRP + H_2O_2 + EDTA ± ICI	0.22 ± 0.01
+HRP + H_2O_2 + EDTA	0.25 ± 0.02
+HRP + H_2O_2 + EDTA + ICI	0.28 ± 0.02

[a] Experimental conditions have been described under Materials and Methods.

radical (N−N^+) formed by the transfer of one electron from second nitrogen to I^+. The alternate possibility that electron is being donated from the same nitrogen-centered radical (N−N^+) to I^+ should result in immediate degradation of EDTA by oxidative decarboxylation evolving CO_2. Using EDTA-*carboxy*-^{14}C, no significant CO_2 was found to be evolved in the absence or presence of ICI (Table 1), suggesting that second electron transfer does not take place from the same nitrogen-centered monocation radical.

Fate of EDTA Radicals. The fate of EDTA monocation radical was studied using electron acceptors such as ICI, cytochrome *c*, or molecular oxygen. Only ICI was specifically reduced, whereas no reduction was observed with either cytochrome *c* or oxygen (Bhattacharyya et al., 1994). The rate of reduction of iodine is already shown in Figure 1, and its sensitivity with DMPO suggests that I^+ is reduced by the monocation radicals formed by peroxidation of EDTA. Surprisingly, in the absence of I^+, instead of O_2 consumption, O_2 was evolved. A typical HRP-dependent O_2 evolution in a system containing EDTA and H_2O_2 is shown in the inset of Figure 3. The result also shows that O_2 evolution is dependent on the concentration of H_2O_2 (Figure 3). Thus, HRP behaves as a pseudocatalase in the presence of EDTA. The result further shows that its pseudocatalatic activity is increased in the presence of iodine which is concurrently

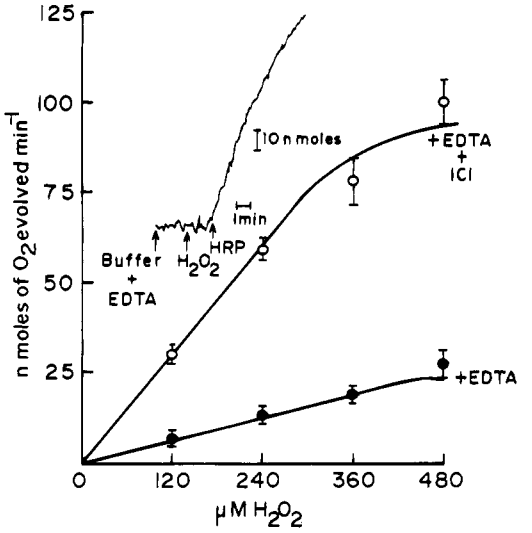

FIGURE 3: Effect of varying concentration of H_2O_2 on O_2 evolution by the HRP–H_2O_2–EDTA system in the absence or presence of ICI: The procedure was the same as described under Materials and Methods except that O_2 evolution was measured as a function of various H_2O_2 concentrations. The inset shows a typical O_2 evolution in HRP-catalyzed EDTA oxidation. For stoichiometry, O_2 evolution was calculated under the assay conditions at 0.3 mM H_2O_2 concentration.

Table 2: Effect of DMPO on the Pseudocatalase Activity of HRP by EDTA in the Absence or Presence of ICI[a]

	pseudocatalase activity: initial rate of O_2 evolution (nmol/min)
complete system	30 ± 5
+DMPO	0
+ICI	93 ± 10
+DMPO + ICI	0

[a] Pseudocatalase activity was measured by O_2 evolution in a complete system in the absence or presence of ICI as described under Materials and Methods, except that the concentrations of DMPO and H_2O_2 were 10 mM and 480 μM, respectively.

reduced to iodide. This pseudocatalase activity in the absence or presence of ICI is completely blocked by DMPO (Table 2), indicating involvement of EDTA free radicals in O_2 evolution from H_2O_2. In order to get further evidence for O_2 being evolved from H_2O_2 through oxidation by EDTA free radicals, H_2O_2 consumption was measured in the absence or presence of ICI (Figure 4). The data indicate that the rate of H_2O_2 consumption is significantly increased in the presence of ICI. No significant H_2O_2 consumption was observed in the absence of HRP except the small amount (1 μM) required for compound I formation. At 300 μM H_2O_2 concentration (Figures 3 and 4), 15 nmol of O_2 was evolved, with consumption of 30 nmol of H_2O_2/min, in the absence of ICI. This corresponds to 1 mol of O_2 produced per 2 mol of H_2O_2 consumption. In the presence of ICI, 75 nmol of O_2 was evolved with the consumption of 105 nmol of H_2O_2/min. This leads to 2 mol of O_2 evolved per 3 mol of H_2O_2 consumed in this reaction. If H_2O_2 is oxidized to O_2 by EDTA radicals, the latter should be reduced back to EDTA. Figure 4 shows that EDTA concentration under identical conditions remains more or less the same throughout the reaction in the absence or presence of ICI, indicating regeneration of EDTA. However, if EDTA is degraded to a nanomole level within the variability of the assay, it could not be detected. The data in Table 1 show that EDTA

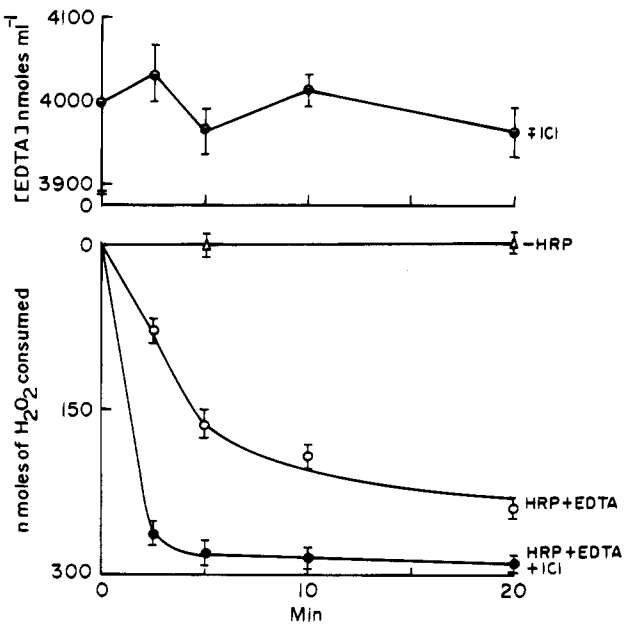

FIGURE 4: HRP-catalyzed consumption of H_2O_2 in the absence or presence of ICI and the concurrent EDTA concentration: The assay systems for the consumption of H_2O_2 and measurement of EDTA concentration (upper panel) in HRP-catalyzed oxidation of EDTA in the absence or presence of ICI have been described under Materials and Methods.

degradation by oxidative decarboxylation is insignificant. This strengthens the observation that EDTA radicals are reduced by H_2O_2, with the regeneration of EDTA and evolution of O_2.

I^+ Binding Site. The specificity of I^+ reduction led us to investigate whether the reduction process takes place at or near the enzyme active site where EDTA radical may be formed to reduce the enzyme-bound iodine. We have shown earlier that EDTA binds (K_D = 15 mM) at or near the iodide binding site of the heme distal pocket for oxidation (Bhattacharyya et al., 1993a). Now, we present spectroscopic evidence for the binding of iodine to the enzyme active site. The binding of I^+ gives a characteristic difference spectra of HRP–I^+ complex versus HRP, having a minimum at 423 nm and a broad peak at about 400 nm (Figure 5A). The equilibrium dissociation constant, K_D, for HRP–I^+ complex as calculated from the plot of [I]/ΔA versus [I]/[I^+] (inset of Figure 5A) was 20 μM. I^+ also binds to the HRP–CN complex to show characteristic difference spectra (Figure 5B) with a K_D value of 20 μM (inset of Figure 5B). This indicates that I^+ interacts at a site away from the heme iron center. This is further substantiated by the finding (Table 3) that the K_D of the HRP–CN complex (3.3 μM) is not altered in the presence of I^+ (3 μM). I^+ binding in the HRP–CN complex was also studied in the presence of guaiacol, which does not alter its K_D value (25 μM as shown in Table 3). The K_D of the HRP–guaiacol complex (9 mM) also remains unaltered by I^+. These data suggest that I^+ binds away from the aromatic donor binding site. Since I^+ reduction takes place only in the presence of EDTA and both show saturation kinetics in the presence of saturable concentrations of the other, the possibility of I^+ binding at the EDTA binding site (Bhattacharyya et al., 1993a) could be excluded. The difference spectrum for the formation of HRP–I^+ complex disappears on addition of iodide, with the appearance of a hump near 460 nm and a peak at 350 nm

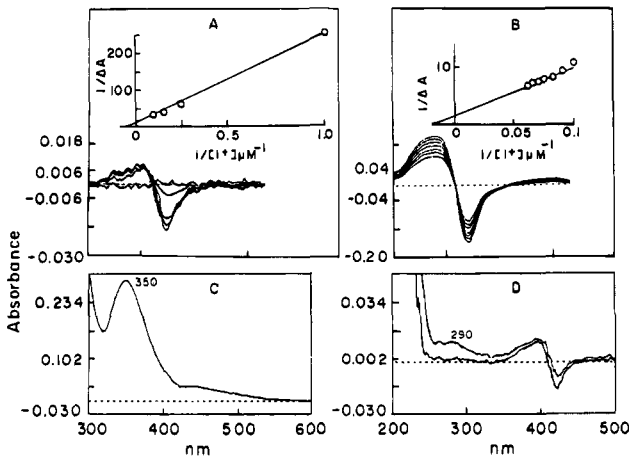

FIGURE 5: I^+ binding on HRP by optical difference spectroscopy. (A) Difference spectra of HRP−I^+ versus HRP at pH 5.0. The concentration of HRP used was 10 μM, and the final ICI concentration was 10 μM. The plot of [I]/ΔA versus [I]/[I^+] (inset) was used for calculating the K_D of I^+. (B) Difference spectra of HRP−CN−I^+ versus HRP−CN. [HRP] = 10 μM, and final [CN^-] = 200 μM; the inset is the similar plot for K_D calculation. (C) Evidence for reversible binding of I^+ in the HRP−I^+ complex. Disappearance of difference spectra of HRP−I^+ versus HRP by I^-. After getting the difference spectrum for HRP−I^+ versus HRP as described in (A), 1 mM KI was added, and the scan was taken after 1 min. Note the disappearance of the difference spectrum with the appearance of the products, I_2 and I_3^-. (D) Disappearance of difference spectra of HRP−I^+ versus HRP by tyrosine. The procedure was the same as described in (C) except that 1 mM tyrosine was used instead of iodide. Note the partial loss of the difference spectrum of the HRP−I^+ complex with the appearance of the peak at 290 nm for the formation of monoiodotyrosine by iodination.

Table 3: Difference Spectral Characterization and Apparent Dissociation Constants (K_D) of HRP−Ligand Complexes[a]

enzyme	ligand	spectrum of complex (nm) min	spectrum of complex (nm) max	K_D	$\Delta\epsilon_{peak\text{-}trough}$ (mM^{-1} cm^{-1})
HRP	I^+	423	400	20 ± 7.0 μM	7.0 ± 2.0
HRP−CN	I^+	423	400	20 ± 8.0 μM	62.5 ± 10.0
HRP	CN^-	393	425	3.3 ± 1.0 μM	95.6 ± 3.0
HRP−I^+	CN^-	393	425	3.0 ± 1.0 μM	95.6 ± 5.0
HRP−CN−guaiacol	I^+	423	400	25 ± 8.0 μM	35.0 ± 5.0
HRP	guaiacol		409	9 ± 4.0 mM	4.0 ± 1.0
HRP−I^+	guaiacol		409	8 ± 2.0 mM	3.6 ± 1.0

[a] The data were obtained from three experiments.

(Figure 5C) due to transfer of bound I^+ to I^- to form I_2 and I_3^-, respectively. This indicates that I^+ is not covalently bound with the HRP and is readily transferable. Addition of tyrosine instead of I^- causes, however, a partial transfer of I^+ to form monoiodotyrosine absorbing at 290 nm (Sun & Dunford, 1993) with partial loss of the difference spectrum (Figure 5D). Thus, although the bound I^+ is transferable, the HRP−I^+ complex acts as a poor iodinating species. The binding of I^+ is also dependent on the pH. A plot of log K_D of I^+ binding at various pHs (Figure 6) shows a sigmoidal curve, from which the involvement of an ionizable group on the enzyme having pK_a = 4.8 can be implicated to control the I^+ binding.

Steady State Kinetic Analysis for Active HRP−I^+−EDTA Ternary Complex. The initial rate steady state enzyme kinetics support our hypothesis that EDTA radical formed at or near the active site reduces enzyme-bound I^+ through

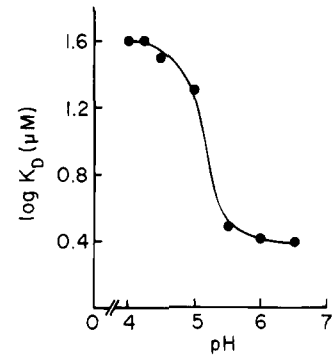

FIGURE 6: pH dependence of I^+ binding to HRP. K_D values at different pH were calculated as described in the legend of Figure 5A.

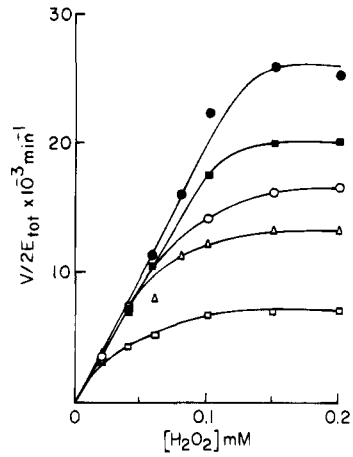

FIGURE 7: Effect of increasing H_2O_2 concentration on the initial rate of ICI reduction at 50 mM acetate buffer, pH 6.0. ICI concentrations used were as follows: 0.09 ± 0.01 mM (□); 0.18 ± 0.02 mM (△); 0.27 ± 0.03 (○); 0.36 ± 0.04 (■); and 0.55 ± 0.05 mM (●). The EDTA concentration was 4.0 mM for every plot. The data were fitted using eq 10 (see supporting information).

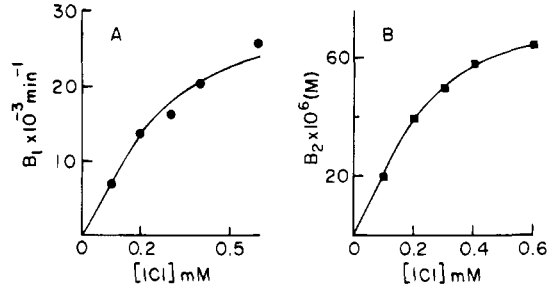

FIGURE 8: (A) Dependence of B_1 on ICI concentration at 50 mM acetate buffer, pH 6.0. The data were fitted using eq 11 (see supporting information). (B) Dependence of B_2 on ICI concentration. The data were fitted according to eq 13 (see supporting information). Experimental conditions were the same as shown in Figure 6.

the formation of active enzyme−I^+−EDTA ternary complex. When increasing concentrations of H_2O_2 were added to a system containing fixed concentrations of HRP, EDTA, and I^+, the resultant rectangular hyperbolae (Figure 7) obtained by plotting $V/2E_{tot}$ versus [S] are characterized by two parameters: B_1 and B_2. It is evident from Figure 8 that both B_1 (Figure 8A) and B_2 (Figure 8B) are increased with increasing concentration of I^+ and that the plots are rectangular hyperbolae at constant EDTA concentration. The simplest mechanism to explain these results is presented in Figure 9. The derivatives of appropriate equations are shown

Table 4: Kinetic Parameters Obtained from Steady State Analysis of I^+ Reduction by HRP, H_2O_2, and EDTA[a]

pH	k_1 (M^{-1} s^{-1} × 10^{-6})	k_2 (M^{-1} s^{-1} × 10^{-6})	k_3 (M^{-1} s^{-1} × 10^{-6})	k_4 (M^{-1} s^{-1} × 10^{-5})
6.0	6.66 ± 0.4	2.0 ± 0.1	(10−100)k_4	1.0 ± 0.06

[a] $k_1 = C_1/D_1$; $k_2 = (C_1/C_2 + C_1/D_2)/2$; $k_4 = C_1/[\text{EDTA}]$ and $k_3 \approx (10-100)k_4$.

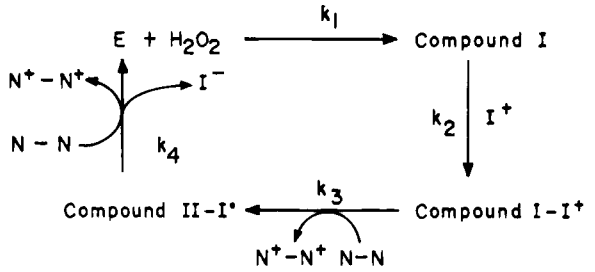

FIGURE 9: Proposed mechanism for I^+ reduction by HRP, H_2O_2, and EDTA. E represents HRP.

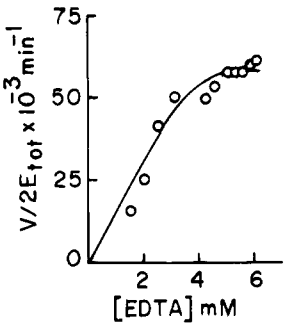

FIGURE 10: Effect of varying concentrations of EDTA on the initial rate of ICI reduction at pH 6.0. [HRP] = 8 nM, [ICI] = 0.5 mM, and $[H_2O_2] = 0.3$ mM. The enzymatic reduction step $k_4 = 3.33 \times 10^5$ M^{-1} s^{-1}. Data were fitted using eq 15 (see supporting information).

in the Appendix. The kinetic parameters obtained from a steady state analysis of the reaction are shown in Table 4. In the presence of 0.6 mM I^+ at pH 6, B_1 and B_2 values do not appreciably change when the EDTA concentration is varied between 4 and 6 mM. It is therefore possible to calculate the lower limit of k_4 under these conditions. The following inequality should be satisfied for eqs 11 and 13 (Appendix) to be independent of EDTA concentration:

$$k_4[\text{EDTA}]/k_2 \gg [I^+] \quad \text{or} \quad k_4 \gg 2 \times 10^5\ M^{-1}\ s^{-1}$$

Using this lower limit for k_4, it was possible to estimate conditions in which the EDTA dependence of initial rate could be expected. The rate of step 2 (Figure 9) could be increased by a higher concentration of either I^+ or H_2O_2. This creates two problems: (i) higher I^+ inactivates the enzyme, and (ii) higher H_2O_2 nonenzymatically reduces I^+ in the presence of EDTA. To avoid these problems, the rate was increased by higher HRP concentrations. From the data plotted in Figure 10, the rate constant k_4 was found to be $(3.33 \pm 0.02) \times 10^5$ M^{-1} s^{-1}.

DISCUSSION

The salient features of the present study are as follows: (1) EDTA is oxidized by HRP catalytic intermediates to its free radical by one-electron oxidation; (2) I^+ has a specific binding site on HRP; (3) I^+ reduction takes place concurrently by EDTA radical formed at or near the active site through the formation of an active enzyme−I^+−EDTA ternary complex; and (4) EDTA radical oxidizes H_2O_2 to O_2 to show the pseudocatalase activity. That EDTA is oxidized by active HRP intermediates to EDTA monocation radical ($N\text{-}N^+$) is evident from ESR studies showing characteristic a triplet signal (a^N = 15 G) for nitrogen-centered cation radical. This is supported by our previous kinetic studies that EDTA acts as an electron donor and competitively inhibits iodide oxidation by acting as a cosubstrate (Bhattacharyya et al., 1993a). Further studies indicated that EDTA also competes with iodide for binding at the same site for oxidation (Bhattacharyya et al., 1993a, 1994). Spectral studies also indicate that EDTA is readily oxidized by compound I but slowly oxidized by compound II, and under steady state oxidation, compound II is the predominating species (Bhattacharyya et al., 1994). The slow oxidation of EDTA by compound II (Bhattacharyya et al., 1994) is presumably due to slow release of the EDTA monocation radical from the active site of the compound I, creating hindrance to the binding of the second EDTA molecule to compound II. However, in the presence of I^+, the rate of oxidation of EDTA is increased significantly because of further oxidation of EDTA monocation radical ($N\text{-}N^+$) by I^+ to form both nitrogen-centered dication radical ($N^+\text{-}N^+$). The latter, being the oxidation product, is likely to be released from the active site, allowing concurrent entry of another EDTA molecule to the compound II state. Compound II and $I^\bullet$ are again reduced concurrently to ferriperoxidase and I^-, forming another ($N^+\text{-}N^+$) radical. This is how the peroxidative cycle continues, with the reduction of enzyme-bound I^+ to I^- and concomitant oxidation of EDTA to ($N^+\text{-}N^+$). As the ESR spectrum of ($N^+\text{-}N^+$) was found to be similar to that of ($N\text{-}N^+$), the former is speculated to be a nitrogen-centered radical also. We still do not know whether there is a precedent elsewhere for this type of both nitrogen-centered EDTA radical. The postulation of the formation of the radical in our system is based on the finding that CO_2 is not evolved by oxidative decarboxylation if EDTA undergoes two-electron oxidation from the same nitrogen atom. Oxidative decarboxylation was not evident even if EDTA undergoes two-electron oxidation from two different nitrogen atoms, generating ($N^+\text{-}N^+$). Moreover, instead of CO_2, oxygen is evolved, which is due to oxidation of H_2O_2 in the reaction medium by the cation radical (Barr & Aust, 1993). Furthermore, tetramethylethylenediamine (TEMED), an analogue of EDTA having no carboxyl group, is 80% as effective as EDTA in iodine reduction (Banerjee, 1989a), suggesting that one-electron transfer from each of the two nitrogen atoms appears to be the more likely mechanism. However, direct demonstration of this radical by some other technique or isolation of the di-DMPO−EDTA derivative will support the existence of this radical, and further studies will be made in this direction. The active site of HRP should be constructed in a way that facilitates intermolecular electron transfer from bound EDTA to the heme ferryl group and to bound iodine simultaneously from two nitrogens of the EDTA molecule. I^+ should therefore

be bound at a site different from but close to the EDTA binding site. EDTA binds at the iodide binding site at an equal distance from the heme peripheral 1- and 8-methyl groups (Sakurada et al., 1987), about 8 Å away from the heme iron center (Modi, 1993). The I^+ binding site should be closer to the EDTA site for efficient electron transfer between bound I^+ and reactive EDTA molecules. I^+ binding to the HRP−CN complex indicates that I^+ binds away from the heme iron center. Guaiacol does not interfere with I^+ binding, nor does the latter interfere with guaiacol binding. This suggests that I^+ binds away from the aromatic donor binding site, a hydrophobic pocket constituted by heme peripheral 8-methyl and tyrosine-183 and arginine-185 residues (Sakurada et al., 1986). As I^+ reduction takes place on the catalytically active enzyme only in the presence of EDTA through the formation of a ternary complex, I^+ binding at the EDTA binding site (vis-a-vis iodide binding site) of the enzyme (Bhattacharyya et al., 1993a, 1994; Modi, 1993; Sakurada et al., 1987) could be excluded. However, the pH-dependent I^+ binding studies suggest that I^+ binding is facilitated by deprotonation of an ionizable residue of pK_a value 4.8. This is close to the pK_a value of 4−4.3 reported earlier for an acidic group (Ugarova et al., 1981; Sakurada et al., 1987) and is presumably contributed by the heme peripheral propionate (Morishima & Ogawa, 1979), although further studies are required to prove this. I^+ binds noncovalently with the active site residue in the heme pocket. The difference spectrum for the HRP−I^+ complex is abolished on addition of iodide due to formation of I_2 and I_3^- through iodine equilibria (Huwiler & Kohler, 1984). This suggests that I^+ is not covalently bound with the residue by iodination. Partial loss of difference spectral intensity by tyrosine further supports this view and is due to partial transfer of bound I^+ to nucleophilic tyrosine to form monoiodotyrosine. However, our data indicate that the HRP−I^+ complex is a poor iodinating species and does not account for the mechanism of iodination of tyrosine by H_2O_2 and iodide catalyzed by HRP (Dunford & Ralston, 1983). By extensive kinetic analysis, it is now established that HRP-catalyzed iodination of tyrosine occurs nonenzymatically by HOI or I_2 in the medium whereas LPO-bound hypoiodite complex (LPO−O−I^-) is the efficient iodinating species (Sun & Dunford, 1993) instead of free HOI, as claimed by Magnusson et al. (1984).

From mechanistic point of view, if I^+ is to be reduced at the active site with concomitant oxidation of EDTA by catalytically active HRP, a ternary complex of active enzyme−I^+−EDTA should be formed under steady state reaction. A steady state kinetic analysis of the reaction of HRP with H_2O_2, I^+, and EDTA was used to investigate the mechanism of these enzymatic reactions. Systematic variation of the concentration of these three substrates under steady state conditions yields sets of kinetic parameters containing both kinetic and mechanistic information. From the rate of reaction against substrate concentration, two important parameters, B_1 and B_2, were computed. B_1 means the k_{cat}, the maximum turnover number for fixed concentration of I^+ and EDTA. B_2 is related to the Michaelis constant, K_m, the H_2O_2 concentration for half-maximum velocity. However, B_2 also includes the effect of I^+ binding to the native enzyme (see Appendix for the equation). From the mechanism (Figure 8) proposed, four important enzymatic species are involved in the reaction cycle: native enzyme, compound I, compound I−I^+, and compound II−$I^\bullet$ intermediates. This mechanism predicts the hyperbolic dependence of B_1 and B_2 on I^+ concentration and also the dependence of initial rate on EDTA concentration, which have been demonstrated experimentally (Figures 6, 7, and 10). From the kinetic analysis of the data, we can suggest that the enzymatic intermediates of compound I−I^+ and compound II−$I^\bullet$ exist during steady state oxidation of EDTA with simultaneous reduction of I^+ to I^-. On the basis of this mechanism, we can tentatively propose the reaction sequence as follows:

$$E + H_2O_2 \rightarrow \text{compd I} + H_2O \quad (1)$$

$$\text{compd I} + I^+ + (\text{N-N}) \rightarrow \text{compd I}{-}I^+{-}(\text{N-N}) \quad (2)$$

$$\text{compd I}{-}I^+{-}(\text{N-N}) \rightarrow \text{compd II}{-}I^\bullet + (N^+\text{-}N^+) \quad (3)$$

$$\text{compd II}{-}I^\bullet + (\text{N-N}) \rightarrow \text{compd II}{-}I^\bullet{-}(\text{N-N}) \quad (4)$$

$$\text{compd II}{-}I^\bullet{-}(\text{N-N}) \xrightarrow{2H^+} E + I^- + (N^+\text{-}N^+) + H_2O \quad (5)$$

$$\text{sum:} \quad H_2O_2 + I^+ + 2(\text{N-N}) \xrightarrow[2H^+]{E} I^- + 2H_2O + 2(N^+\text{-}N^+) \quad (6)$$

$(N^+\text{-}N^+)$, being extremely reactive, oxidizes H_2O_2 to molecular O_2 and is reduced back to EDTA. This explains the pseudocatalase activity of the enzyme in the presence of I^+, and regeneration of EDTA accounts for the unchanged EDTA concentration observed during I^+ reduction. The following reaction sequence may occur:

$$(N^+\text{-}N^+) + H_2O_2 \rightarrow (\text{N-}N^+) + O_2^- + 2H^+ \quad (7)$$

$$(\text{N-}N^+) + H_2O_2 \rightarrow (\text{N-N}) + O_2^- + 2H^+ \quad (8)$$

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (9)$$

$$\text{sum:} \quad (N^+\text{-}N^+) + H_2O_2 \rightarrow (\text{N-N}) + O_2 + 2H^+ \quad (10)$$

$$\text{or:} \quad 2(N^+\text{-}N^+) + 2H_2O_2 \rightarrow 2(\text{N-N}) + 2O_2 + 4H^+ \quad (11)$$

In the absence of I^+, HRP oxidizes EDTA through one-electron transfer to form EDTA monocation radical (N-N^+), which oxidizes H_2O_2 to O_2 at a lower rate than in the presence of I^+ and is reduced back to EDTA.

$$(\text{N-}N^+) + H_2O_2 \rightarrow (\text{N-N}) + O_2^- + 2H^+ \quad (12)$$

$$\text{sum (2 × eq 12):} \quad 2(\text{N-}N^+) + H_2O_2 \rightarrow O_2 + 2(\text{N-N}) + 2H^+ \quad (13)$$

O_2^- thus produced will disproportionate according to reaction 9 and should be detected by the reduction of ferricytochrome *c*. However, no significant reduction of cytochrome *c* in our system was observed, probably due to nonoptimal conditions of the assay system. Alternatively, O_2^- may immediately reduce N-N^+ back to EDTA so that reduction of ferricytochrome *c* becomes limited. That cation radical oxidizes H_2O_2 to O_2 through generation of O_2^- has also been reported recently (Barr & Aust, 1993). The reaction

sequence, 2 to 5, further shows that two EDTA are required to reduce one I^+ bound at the active site. One EDTA binds at the compound I state, donating one electron to compound I and one electron to bound I^+ simultaneously from the two nitrogen atoms, and the resulting (N^+-N^+) may then be released as oxidation product from the active site. The second EDTA binds to the same enzyme at the compound II state, reducing it to ferric state and bound $I^\bullet$ to I^- concurrently before releasing into the medium as (N^+-N^+). Reactions 1 and 13 further indicate that, for EDTA oxidation, 1 mol of O_2 should evolve per 2 mol of H_2O_2 consumption, whereas in the presence of ICI (reactions 1 and 11). 2 mol of O_2 should be produced from 3 mol of H_2O_2 consumed. This is farily satisfied by the experimental data derived from Figures 3 and 4. Our results also suggest that the mechanism of reduction of I^+ to I^- at the active site of HRP by EDTA could be mediated through one-electron reduction. Similar one-electron reduction (nonenzymatic) of $I^\bullet$ to I^- by EDTA has recently been suggested (Shah et al., 1993a). Thus, our proposed mechanism for HRP-catalyzed I^+ reduction by EDTA appears reasonable although the plausible formation of (N^+-N^+) in this system remains to be established conclusively by further experiments. We, therefore, suggest that the catalytically active HRP has the unique property to oxidize EDTA and reduce iodine simultaneously at the active site through intermolecular electron transfer to account for the iodine reductase activity in the presence of EDTA. Its pseudocatalase activity is manifested due to oxidation of H_2O_2 by EDTA cation radical similar to other systems reported earlier (Magnusson et al., 1984a; Huwiler & Kohler, 1984; Shah & Aust, 1993b; Barr & Aust, 1993).

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APPENDIX[2]

Under steady state conditions:

$$-\frac{d[E]}{dt} = \frac{d[Cpd^I]}{dt} = k_1[E][H_2O_2] - k_2[Cpd^I][I^+] = 0 \quad (A1)$$

$$\frac{d[Cpd^I{-}I^+]}{dt} = k_2[Cpd^I][I^+] - k_3[Cpd^I{-}I^+][EDTA] = 0 \quad (A2)$$

$$\frac{d[Cpd^{II}{-}I^\bullet]}{dt} = k_3[Cpd^I{-}I^+][EDTA] - k_4[Cpd^{II}{-}I^\bullet][EDTA] = 0 \quad (A3)$$

$$\frac{d[E]}{dt} = k_4[Cpd^{II}{-}I^\bullet][EDTA] - k_1[E][H_2O_2] = 0 \quad (A4)$$

Initial rate expression:

$$V = -\frac{d[I^+]}{dt} = \frac{d[EDTA]}{dt} = k_3[Cpd^I{-}I^+][EDTA] + k_4[Cpd^{II}{-}I^\bullet][EDTA] \quad (A5)$$

Mass balance:

$$[E_{tot}] = [E] + [Cpd^I] + [Cpd^I{-}I^+] + [Cpd^{II}{-}I^\bullet] = \frac{k_3[Cpd^I{-}I^+][EDTA]}{k_1[H_2O_2]} + \frac{k_3[Cpd^I{-}I^+][EDTA]}{k_2[I^+]} + [Cpd^I{-}I^+] + \frac{k_3[Cpd^I{-}I^+][EDTA]}{k_4[EDTA]} \quad (A6)$$

$$\therefore [Cpd^I{-}I^+] = ([E_{tot}]k_1k_2k_4[H_2O_2][I^+][EDTA])/(k_3k_2k_4[I^+][EDTA]^2 + k_3k_1k_4[H_2O_2][EDTA]^2 + (k_4 + k_3)[H_2O_2][I^+][EDTA]k_1k_2) \quad (A7)$$

Again,

$$[E_{tot}] = \frac{k_4[Cpd^{II}{-}I^\bullet][EDTA]}{k_1[H_2O_2]} + \frac{k_4[Cpd^{II}{-}I^\bullet][EDTA]}{k_2[I^+]} + \frac{k_4[Cpd^{II}{-}I^\bullet][EDTA]}{k_3[EDTA]} + [Cpd^{II}{-}I^\bullet]$$

$$\therefore [Cpd^{II}{-}I^\bullet] = ([E_0]k_1k_2k_3[H_2O_2][I^+][EDTA])/(k_2k_3k_4[I^+][EDTA]^2 + k_1k_3k_4[H_2O_2][EDTA]^2 + (k_4 + k_3)k_1k_2[H_2O_2][I^+][EDTA]) \quad (A8)$$

Putting the values of $[Cpd^I{-}I^+]$ and $[Cpd^{II}{-}I^\bullet]$ into eq A5,

$$V = (k_1k_2k_3k_4(2)[E_{tot}][EDTA]^2[H_2O_2][I^+])/(k_2k_3k_4[I^+][EDTA]^2 + k_1k_3k_4[H_2O_2][EDTA]^2 + k_1k_2(k_4 + k_3)[H_2O_2][I^+][EDTA]) \quad (A9)$$

Rearranging eq A9,

$$\frac{V}{2[E_0]} = \frac{\frac{1}{(1/k_2[I^+]) + (k_4 + k_3)/(k_3k_4[EDTA])}[H_2O_2]}{\frac{1/k_1}{(1/k_2[I^+]) + (k_4 + k_3)/(k_3k_4[EDTA])} + [H_2O_2]} = \frac{B_1[H_2O_2]}{B_2 + [H_2O_2]} \quad (A10)$$

For most substrates of HRP, k_3 is about 10 times greater than k_4.

$$\therefore \frac{k_3k_4}{k_3 + k_4} \approx \frac{1}{k_4}$$

where

[2] $[E_0]$ indicates total enzyme concentration $[E_{tot}]$ in the Appendix. Compound I and compound II are written as Cpd^I and Cpd^{II} to avoid confusion with I^+ and $I^\bullet$.

$$B_1 = \frac{1}{(1/k_2[I^+]) + (1/k_4[EDTA])} = \frac{k_4[EDTA][I^+]}{(k_4/k_2)[EDTA] + [I^+]}$$

$$= \frac{C_1[I^+]}{C_2 + [I^+]} \quad \text{(A11)}$$

$$\therefore C_1 = k_4[EDTA]; \quad C_2 = (k_4/k_2)[EDTA] \quad \text{(A12)}$$

$$B_2 = \frac{1/k_1}{(1/k_2[I^+]) + (1/k_4[EDTA])} =$$

$$\frac{(k_4/k_1)[EDTA][I^+]}{(k_4/k_2)[EDTA] + [I^+]} = \frac{D_1[I^+]}{D_2 + [I^+]} \quad \text{(A13)}$$

$$\therefore D_1 = (k_4/k_1)[EDTA]; D_2 = (k_4/k_2)[EDTA] \quad \text{(A14)}$$

Again rearranging eq A9 as a function of EDTA concentration:

$$\frac{V}{2[E_0]} = \frac{\frac{1}{(1/k_1[H_2O_2]) + (1/k_2[I^+])}[EDTA]}{\frac{(k_3 + k_4)/(k_3k_4)}{(1/k_1[H_2O_2]) + (1/k_2[I^+])} + [EDTA]}$$

$$= \frac{E_1[EDTA]}{E_2 + [EDTA]} \quad \text{(A15)}$$

where

$$E_1 = \frac{k_1k_2[H_2O_2][I^+]}{k_2[I^+] + k_1[H_2O_2]} \quad \text{(A16)}$$

$$E_2 = \frac{1}{k_4}\frac{k_1k_2[I^+][H_2O_2]}{(k_2[I^+] + k_1[H_2O_2])} \quad \text{(A17)}$$

$$E_1 = k_4E_2$$

The rate constant for the individual step can be calculated from the coefficient obtained in the equation:

$$k_1 = \frac{C_1}{D_1} \quad k_2 = \frac{C_1/C_2 + C_1/D_2}{2} \quad k_4 = \frac{C_1}{[EDTA]}$$

$$k_3 \approx 100k_4$$

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