Mechanism of inhibition of horseradish peroxidase-catalysed iodide oxidation by EDTA

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EDTA inhibits horseradish peroxidase (HRP)-catalysed iodide oxidation in a concentration and pH-dependent manner. It is more effective at pH 6 than at lower pH values. A plot of $\log K_{app}$ values as a function of pH yields a sigmoidal curve from which a pK_a value of 5.4 can be calculated for an ionizable group on the catalytically active HRP for EDTA inhibition. Among the structural analogues of EDTA, tetramethylethylenediamine (TEMED) is 80 % as effective as EDTA, whereas the EDTA–Zn²⁺ chelate and EGTA are ineffective. Kinetic studies indicate that EDTA competitively inhibits iodide oxidation. Spectral studies show that EDTA can quickly reduce compound I to compound II, but reduction of preformed compound II to the native enzyme is relatively slow, as demonstrated by the time-dependent spectral shift from 417 nm to 402 nm through an isosbestic point at

408 nm. Under steady-state conditions, in a reaction mixture containing HRP, EDTA and H₂O₂, the enzyme remains in the compound-II form, with absorption maxima at 417, 527 and 556 nm. Direct evidence for one-electron oxidation of EDTA by HRP intermediates is provided by the appearance of an e.s.r. signal of a 5,5-dimethyl-1-pyrroline *N*-oxide (spin trap)–EDTA radical adduct [a^N (hyperfine splitting constant) = 1.5 mT] in e.s.r. studies. The signal intensity, however, decreases in the presence of iodide. The K_D of the HRP–EDTA complex obtained from optical difference spectroscopy increases with an increase in iodide concentration, and the double-reciprocal plot for EDTA binding indicates that EDTA and iodide compete for the same binding site for oxidation. We suggest that EDTA inhibits iodide oxidation by acting as an electron donor.

INTRODUCTION

Peroxidase-catalysed oxidation of a variety of electron donors with H₂O₂ has been extensively studied using horseradish peroxidase (HRP), lactoperoxidase, chloroperoxidase and thyroid peroxidase [1–9]. It has been established that HRP (EC 1.11.1.7; donor–H₂O₂ oxidoreductase) catalyses these reactions through the following steps:

Compounds I and II, being two and one electron oxidation states above native ferriperoxidase respectively, oxidize the electron donor (AH₂) by two one-electron transfer reactions with the formation of substrate-free radical (AH[•]) and ferriperoxidase. In the case of halide oxidation, compound I catalyses a two-electron transfer reaction without the intermediate formation of compound II [5, 10–15]. The reaction between compound I and iodide may proceed through the formation of an enzyme–hypoiodous complex, EOI [7, 14], where EO (compound I) is an Fe(IV)-porphyrin- π -cation radical [16] and I[−] is present in an oxidation state equivalent to I⁺. EOI gives rise to the ferric form of peroxidase and HOI (pK_a of hypoiodous acid is about 11). I₃[−] is formed by the non-enzymic reaction of HOI with excess I[−]. The reactions may be represented as follows:

Aromatic donors bind HRP in a hydrophobic pocket formed by haem peripheral 8-methyl, Ty-183 and Arg-185 residues [17], whereas iodide interacts near the haem moiety [5, 12, 18, 19] at a site almost equidistant from haem peripheral 1- and 8-methyl groups [20]. Although the protonation of an ionizable group of pK_a 4–4.6 appears to be necessary for iodide oxidation [11], Sakurada et al. [20] have suggested that the distal histidine in HRP may play an important role in electron transfer from iodide to the haem ferryl group, in analogy with their finding in lactoperoxidase that iodide binding is facilitated by protonation of an ionizable group of pK_a 6–6.8 presumably provided by the distal histidine [21]. Recently we have shown by chemical-modification studies that distal histidine plays an essential role in HRP-catalysed iodide oxidation [22].

A few years back, we first reported that iodide oxidation catalysed by a partially purified gastric peroxidase is inhibited by EDTA and this is reversed by Zn²⁺ [23]. Subsequently, we observed that EDTA also inhibits iodide oxidation catalysed by HRP, and induces a new catalytic activity by which it can reduce iodine to iodide in the presence of H₂O₂ with concomitant evolution of oxygen [24]. We proposed that EDTA may bind at or near the haem causing a conformational change which is responsible for the observed effect [25]. Further kinetic, spectral and radioiodide-binding studies [26] indicated that EDTA can block HRP-catalysed iodide oxidation by interfering with iodide binding. Recently, we have shown that EDTA binds at the haem distal pocket at or near the iodide-binding site, and iodide modulates the ‘iodine reductase’ activity of the HRP by controlling EDTA binding [27]. However, the exact molecular mechanism by which EDTA inhibits iodide oxidation by HRP is not yet known. In the present paper, we present direct evidence from kinetic, spectral and e.s.r. studies that EDTA acts as an electron donor and inhibits iodide oxidation by competing with

Abbreviations used: EDDA, ethylenediaminediacetic acid, sodium salt; HRP, horseradish peroxidase; LiPH₂, lignin peroxidase H₂; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DETAPAC, diethylenetriaminepenta-acetic acid; TEMED, *NNN'*-tetramethylethylenediamine.

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iodide for oxidation by HRP as a cosubstrate. This is in contrast with the recent findings of Aust and his co-workers [28–30] that EDTA is oxidized indirectly by the primary oxidation product of peroxidase oxidation.

MATERIALS AND METHODS

HRP ($A_{403}/A_{278} = 3$), EGTA, diethylenetriaminepenta-acetic acid (DETAPAC) and ethylenediaminediacetic acid (EDDA) were purchased from Sigma. EDTA and KI (analytical grade) were obtained from Glaxo Laboratories, Bombay, India. 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was obtained from Aldrich. All other chemicals used were analytical grade. The concentrations of HRP was determined by using $\epsilon_{403} = 102 \text{ cm}^{-1} \cdot \text{mM}^{-1}$ [31].

All kinetic measurements were made in a Pye–Unicam SP8-100 u.v./visible recording spectrophotometer. HRP-catalysed iodide oxidation in the presence or absence of EDTA was monitored by following the formation of I_3^- at 353 nm [24]. The reaction mixture contained in a final volume of 3 ml: 50 mM sodium acetate buffer, pH 5.5, 1.7 mM KI, 0.5 μg of enzyme and 0.3 mM H_2O_2 added last to start the reaction. A 50 mM concentration of KI, which is ideal for I_3^- formation at pH 7 by thyroid peroxidase and lactoperoxidase [14], was not used, as this high iodide concentration causes non-enzymic oxidation of iodide by H_2O_2 at the acid pH used for HRP-catalysed iodide oxidation. To avoid this, 1.7 mM KI was used, which, although not ideal, does not cause any error in the assay of HRP activity [22,25]. HRP-catalysed reduction in the presence of EDTA was studied using ICl, cytochrome *c* or O_2 as electron acceptor. For 'iodine reductase' activity [24,25], the assay system was as described in our previous paper [27] using ICl as electron acceptor. Cytochrome *c* reduction was monitored by measuring the increase in absorbance at 550 nm in an assay system containing 50 mM sodium acetate buffer, pH 6, 2.4 μM HRP, 3 mM EDTA, 50 μM cytochrome *c* and 0.15 mM H_2O_2 in a final volume of 1 ml in the absence or presence of 50 μM KI as described by Shah and Aust [30]. O_2 -consumption was monitored in a Gilson oxygraph using the same assay system as for cytochrome *c* reduction but omitting cytochrome *c*.

Spectral studies were carried out in a Shimadzu UV-2201 spectrophotometer using quartz cells of 1 cm light path. The difference spectrum of enzyme–ligand versus enzyme was carried out as described previously [27,32]. The equilibrium dissociation constant, K_D , for complex-formation was calculated from the following expression as described by Schejter et al. [33]:

$$\frac{1}{\Delta A} = \left(\frac{K_D}{\Delta A_\infty} \right) \frac{1}{S} + \frac{1}{\Delta A_\infty} \quad (8)$$

where K_D is the dissociation constant of the enzyme–ligand complex for a given ligand, S is its concentration, ΔA is the observed absorption change at a particular wavelength and ΔA_∞ is the absorption change at saturating concentration of the ligand. The observed apparent dissociation constant, $K_{D_{\text{obs}}}$, of the EDTA–HRP complex in the presence of iodide is related to iodide concentration $[\text{I}^-]$ according to the following equation [34,35]:

$$K_{D_{\text{obs}}} = \frac{K_D[\text{I}^-]}{K_i} + K_D \quad (9)$$

where K_i is the apparent dissociation constant of the iodide–HRP complex in the absence of EDTA, and K_D is the apparent dissociation constant for the binding of EDTA to native HRP as

defined in eqn. (8). All kinetic and spectral studies were carried out at $25 \pm 2^\circ \text{C}$.

The e.s.r. spin-trapping experiments were carried out in a Varian E-112 spectrometer fitted with T-112 cavity, operating at 9.45 GHz with 100 KHz modulation frequency. The incubation mixture contained 30 mM EDTA, 100 mM DMPO and 10 μM DETAPAC in 50 mM sodium acetate buffer, pH 6.5. The reaction was started by the addition of 40 μM HRP and 0.4 mM H_2O_2 . Control incubations were run in the absence of enzyme or H_2O_2 or EDTA. A 1 ml portion was aspirated into the sample cavity within 10 s of the initiation of the reaction with H_2O_2 .

RESULTS

EDTA was found to inhibit the catalytic oxidation of iodide by HRP in a concentration- and pH-dependent manner (Figure 1). A higher concentration of EDTA is required to inhibit the activity as the pH is decreased. EDTA is more effective at pH 6 than at pH 4.5, having $K_{i_{\text{app}}}$ values of 0.22 and 10 mM respectively. A plot of $\log K_{i_{\text{app}}}$ of EDTA as a function of pH (Figure 2) produces a sigmoidal curve from which the involvement of an ionizable group of $\text{p}K_a$ 5.4 can be suggested for

Figure 1 Effect of various concentrations of EDTA on iodide oxidation as a function of pH

For I^- oxidation, 50 mM sodium acetate buffer pH 4–6.5, was used along with 1.7 mM KI, 0.5 μg of HRP and 0.3 mM H_2O_2 in the presence or absence of various concentrations of EDTA. Enzyme activity was measured as an increase in absorbance at 353 nm due to formation of I_3^- .

Figure 2 pH-dependence of the $K_{i_{\text{app}}}$ value of EDTA during iodide oxidation

The procedure was as described in the legend of Figure 1. The $K_{i_{\text{app}}}$ value of EDTA at each pH was calculated from the kinetic data, and its log value was plotted against pH.

Figure 4 Spectral evidence for the oxidation of EDTA by catalytically active HRP intermediates

(a) A is the Soret spectrum of $0.3 \mu\text{M}$ HRP; B is the spectrum of compound II (417 nm) obtained after addition of $3 \mu\text{M}$ H_2O_2 to the native HRP and this is stable for at least 10 min as shown by repeated tracings (C) at 2.5 min intervals. In (b), compound II (B) was prepared as described in (a); EDTA (1 mM) was added to it and the spectrum were recorded at intervals of 30 s until it was reduced back to the native enzyme at 402 nm. The isosbestic point was recorded at 408 nm. In (c) A represents the visible spectrum of $2.5 \mu\text{M}$ HRP; B, C, D and E represent the spectra of compound II at 7.5, 10, 13 and 30 min after the addition of a 20-fold molar excess of H_2O_2 as described by Kellin and Hartree [36]. In (d), A is the native HRP and B to E are for compound II at 1.5, 3.5, 7.5 and 10 min after H_2O_2 addition as described in (c). F–I were obtained after addition of 4 mM EDTA to compound II at 11, 13, 15 and 20 min when compound II was gradually reduced to form native enzyme.

Figure 4(c) shows that compound II formed by a molar excess of H_2O_2 [36] absorbs at 527 and 556 nm and is stable for 30 min. If EDTA is added to compound II after 10 min, it is gradually reduced to the native state within 20 min, as demonstrated by the disappearance of the visible peaks at 527 and 556 nm and reappearance of a native peak at 500 nm [36]. No visible peak at 546 or 583 nm due to formation of compound III [36] by EDTA was evident. Shah and Aust [30] have recently reported that lignin peroxidase H_2 (LiPH_2), during steady-state oxidation of EDTA in the presence of low ($50 \mu\text{M}$) iodide concentration, remains in the compound-II form absorbing at 420.1, 530 and 552 nm. However, HRP under steady-state conditions in a reaction mixture containing 0.3 mM H_2O_2 and 4 mM EDTA only remains in the compound-II state absorbing at 417, 527 and 556 nm, and, if iodide is present, it stays mainly in the native state because of immediate reduction of HRP intermediates by

iodide (results not shown). As formation of substrate free radical is usually considered as direct evidence for peroxidase-catalysed substrate oxidation by a one-electron transfer mechanism [3], the formation of EDTA radical by HRP-catalysed EDTA oxidation was studied by e.s.r. spectroscopy. Addition of HRP to a solution containing EDTA, H_2O_2 and DMPO (spin-trap) results in the appearance of a triplet signal of the DMPO–EDTA radical adduct (Figure 5a). No e.s.r. signal was observed in absence of enzyme (Figure 5b) or EDTA (Figure 5c) or H_2O_2 (Figure 5d). The signal is different from the characteristic signal of the DMPO– O_2^- radical adduct [37] likely to be generated from reduction of O_2 by the EDTA radical. The signal is also different from the characteristic signal of the DMPO– $\text{OH}^\cdot$ radical adduct [37] likely to be generated from H_2O_2 . As no other signal is observed in the absence of EDTA (Figure 5c), it is more likely that the observed signal is due to formation of the DMPO–EDTA

Figure 5 E.s.r. spectra of DMPO-trapped EDTA radical at 25 ± 2 °C

(a) E.s.r. signal of DMPO–EDTA radical adduct; the details of the incubation mixture containing HRP, EDTA and H_2O_2 are described in the Materials and methods section. The spectrometer settings were: Scan range, 80 mT; modulation amplitude, 100 KHz; time constant, 0.5 s; gain, 6.3×10^4 ; microwave power, 20 MW; scan time, 8 min. (b) Identical with (a) but HRP omitted; (c) identical with (a) but EDTA omitted; (d) identical with (a) but H_2O_2 omitted; (e) e.s.r. spectrum of DMPO–EDTA radical adduct formed in the same system in the presence of 10 mM KI. In (a) and (e), $a^N = 1.5$ mT.

radical adduct. EDTA is thus oxidized by catalytically active HRP intermediates by a one-electron transfer mechanism with the formation of EDTA free radical.

To address the question whether EDTA radical is best formed directly via the HRP– H_2O_2 system or as a secondary product in presence of iodide as reported by Shah and Aust [30], e.s.r. spectra were also compared in the presence or absence of iodide. The signal size of the EDTA radical is not increased further in the presence of iodide (Figure 5e) but rather decreased, suggesting that the EDTA radical is more efficiently formed directly by the enzyme only, and iodide reduces its formation by acting as a cosubstrate competing with EDTA oxidation.

As binding is a prerequisite for oxidation [32,33], the interaction of EDTA with the enzyme was studied by optical difference spectroscopy [27] in the absence or presence of iodide,

Figure 6 Double-reciprocal plot for EDTA binding in the absence or presence of iodide

Difference spectra of HRP–EDTA complex versus HRP were obtained in the absence (as shown in the inset) or presence of 100 mM KI as described in the Materials and methods section. The concentrations of HRP and EDTA used were 1 μM and 2–14 mM respectively. ΔA was measured at 420 nm, and $1/\Delta A$ was plotted against $1/[\text{EDTA}]$ as indicated.

and the binding constants, K_D and $K_{D_{\text{obs}}}$, were calculated from eqns. (8) and (9). The $K_{D_{\text{obs}}}$ value of EDTA increases with an increase in iodide concentration, indicating interference of EDTA binding with iodide at the active site. Figure 6 shows a double-reciprocal plot for EDTA binding in the absence (a typical difference spectrum for the HRP–EDTA complex with increasing concentration of EDTA is shown in the inset) or presence of iodide. In this experiment, the reciprocal of the difference in absorption at 420 nm (ΔA) was plotted against $1/[\text{EDTA}]$ in the absence or presence of 100 mM KI. The plot indicates that iodide competitively inhibits EDTA binding. The data suggest that EDTA and iodide compete for the same binding site on the enzyme. This plot yields a K_D for the HRP–EDTA complex of 15 mM and an apparent K_D for the HRP–iodide complex of 133 mM [(according to eqn. (9)) at this site. EDTA thus inhibits iodide oxidation by acting as a cosubstrate competing with iodide for binding and oxidation by HRP.

Recently Shah and Aust have reported that EDTA radical formed by the primary oxidation product of LiPH_2 can reduce O_2 or cytochrome *c* to O_2^- or reduced cytochrome *c* [30]. An alternative possibility was therefore considered that the EDTA radical in our system may also produce O_2^- which could be the source of the iodine-reducing activity of HRP reported earlier [24,25,27], and this may explain, at least partially, the inhibition of iodide oxidation by EDTA. The data in Table 1 show that O_2 is not reduced in our system to O_2^- , thereby excluding its role in iodine reduction. This is further substantiated by the finding that cytochrome *c* is also not reduced under the same conditions. However, only iodine is specifically reduced and both EDTA and catalytically active HRP are required for the reduction under these conditions. Formation of compound III was never observed. The data suggest that the EDTA radical formed by direct oxidation in the HRP– H_2O_2 system is capable of reducing iodine. We have further observed that, unlike LiPH_2 [30], HRP cannot

Table 1 HRP-catalysed reduction of different electron acceptors in the presence of EDTA

The assay system for measuring O_2 consumption and cytochrome *c* reduction is described in the Materials and methods section. For iodine reduction, the assay system contained in a final volume of 1 ml, 50 mM sodium acetate buffer, pH 6, 0.4 mM ICl, 4 mM EDTA, 0.5 μ g of HRP and 0.3 mM H_2O_2 , and reduction was monitored as the decrease in absorbance at 460 nm [27].

Electron acceptor	Initial rate of reduction (nmol/min)
Oxygen	0
Cytochrome <i>c</i>	0
ICl	150 $\pm$ 15
Minus EDTA	0
Minus HRP	30 $\pm$ 2
Minus H_2O_2	30 $\pm$ 2

reduce cytochrome *c* even in the presence of 50 μ M iodide under the same experimental conditions.

DISCUSSION

EDTA, a classical chelating agent [38], is generally used to chelate some bivalent cations from biological macromolecules. Several enzymes that require bivalent cations are inactivated when metal ion is chelated by EDTA and are reactivated on addition of the metal ion. Here we have presented an interesting observation that EDTA inhibits the catalytic activity of HRP not by acting as a metal ion chelator but by acting as an electron donor. HRP contains 2 mol of Ca^{2+} /mol of enzyme [39]. Only one tightly bound Ca^{2+} could be removed by very drastic treatment such as incubation with EDTA or EGTA in the presence of guanidinium chloride, as this Ca^{2+} is essential for maintaining the protein structure in the haem cavity for enzyme activity [40–42]. The observed inhibition is not due to removal of this intramolecular Ca^{2+} by EDTA as the drastic conditions required to obtain Ca^{2+} -free HRP [40–42] were not used. Insensitivity to EGTA also supports this view. Moreover, the effect of EDTA is not reversed by addition of Ca^{2+} [26], suggesting that this Ca^{2+} is not chelated by EDTA. EDTA is sometimes used as a reductant [43,44]. Thus a possibility may exist that EDTA inhibits iodide oxidation by reducing ferric HRP to the ferrous state. We have observed that the ferrous HRP produced by reduction of native HRP with dithionite absorbs at 435 nm [36], whereas EDTA causes a small hypsochromic change of HRP at 402 nm only [25,26]. This excludes the possibility of direct reduction of native HRP by EDTA. Irreversible inactivation of catalytically active HRP by EDTA or its oxidation product does not occur as it is readily reversed by dilution.

Kinetic studies indicated that EDTA competitively inhibits iodide oxidation [26,27], suggesting that it acts as a cosubstrate. EDTA also inhibits the oxidation of guaiacol competitively, and the effect is reversed by higher concentrations of the donor (D. K. Bhattacharyya, S. Adak, U. Bandyopadhyay and R. K. Bannerjee, unpublished work). However, because of the very high concentration of *o*-dianisidine used previously [26], we did not observe the inhibitory effect because of reversal of inhibition, and this led us to suggest that EDTA was not acting as an electron donor [26]. Our spectral studies further show oxidation of EDTA by HRP- H_2O_2 intermediates via a one-electron transfer mechanism. This is unlike iodide, which is oxidized by a direct two-electron transfer to compound I [5], but is similar to other organic substrates which are oxidized by one-electron

transfer mechanisms [1–3]. However, EDTA is not as good an electron donor as iodide, because it takes a few minutes to reduce compound II back to the native enzyme whereas iodide reduces compound I almost instantly [27]. We have further observed that, during steady-state oxidation, HRP remains in the compound-II state, indicating that oxidation of EDTA by compound II is slower than its oxidation by compound I. This might be the reason why previously we did not observe the reduction of compound II instantly with EDTA, and this led us to believe that some conformational change observed in the haem c.d. spectra for EDTA interaction was responsible for the observed effect [25]. Convincing evidence for one-electron oxidation of EDTA is further provided by e.s.r. spin-trap experiments in which a distinct signal for the spin-trapped EDTA radical could be detected only by enzymic oxidation of EDTA by H_2O_2 . Considering the distinctly different nature of the DMPO adduct of O_2^- and $OH^\cdot$ radicals [37], we suggest that the signals are due to formation of EDTA radical by one-electron transfer similar to other peroxidase substrates [3,45–49].

Recently Shah et al. [28] reported that EDTA is non-enzymically oxidized to the EDTA radical by the veratryl alcohol cation radical formed as a primary oxidation product in the LiPH₂ system, causing apparent inhibition of veratryl alcohol oxidation. They have further shown that EDTA is also non-enzymically oxidized to the EDTA radical by the primary oxidation product of iodide [29,30]. In contrast, HRP can directly oxidize EDTA to the EDTA radical, and its signal size is not increased further in the presence of iodide, suggesting that the EDTA radical is most efficiently formed directly by the enzyme and not as a secondary product in the presence of iodide. They have also suggested that the iodide radical ($I^\cdot$) formed as a one-electron oxidation product of low concentrations of iodide (50 μ M) may oxidize EDTA to its radical which is required for reduction of O_2 , cytochrome *c* etc., although the evidence for the formation of $I^\cdot$ is lacking [30]. The non-enzymic oxidation of EDTA in their system has been supported by a non-saturable rate of cytochrome *c* reduction with increasing EDTA concentration. Replotting their data in the conventional *x*-*y* plot with zero origin and using their statistical value for cytochrome *c* reduction rate at 2.7 μ M LiPH₂ and 3 mM EDTA, the value tends to saturate. The observation is therefore illusive unless supported by direct binding studies. We have previously shown that EDTA does bind to HRP at a specific site with a K_D value of 15 mM [27]. This site is away from the haem iron centre and aromatic donor-binding site but is close to the iodide-binding site [27]. The double-reciprocal plot of EDTA binding in the presence of iodide provides further evidence that iodide competes with EDTA for the same binding sites. This data along with the spectral and e.s.r. evidence for EDTA oxidation and kinetic evidence for competition of EDTA on iodide oxidation [26,27] suggest that EDTA interacts at or near the iodide-binding site at the haem periphery for oxidation. Shah and Aust [29,30] have also extended their views on non-enzymic EDTA oxidation by $I^\cdot$ in the LiPH₂ system to HRP and lactoperoxidase without going into details of the reaction mechanism of these peroxidases in the presence of EDTA. The catalytic activity of HRP differs in several aspects for that of LiPH₂. Shah et al. [28] have also shown that HRP does not catalyse reductive reaction using veratryl alcohol, 1,2,3-trimethoxybenzene and 1,4-dimethoxybenzene as does LiPH₂. We have observed that EDTA binds to HRP, whereas Shah and Aust [30], from their finding of a non-saturating effect of EDTA on cytochrome *c* reduction, postulate that EDTA does not bind to LiPH₂. We have shown direct oxidation of EDTA to the EDTA radical by HRP, whereas they demonstrated formation of the EDTA radical by LiPH₂ only in

the presence of iodide [30]. Shah and Aust [30] have reported that LiPH_2 in the presence of H_2O_2 , EDTA and iodide under steady-state conditions remains in the compound-II state, whereas HRP under identical conditions always remains in the native state [25]. However, HRP forms compound II only in the absence of iodide. LiPH_2 has been shown to reduce cytochrome *c* at low iodide concentration in the presence of EDTA as a result of generation of the EDTA radical by $\text{I}^\cdot$, and the same mechanism has been extended to HRP also [30]. Unfortunately, under identical conditions, we did not see cytochrome *c* reduction by HRP in the presence of 50 μM iodide. It thus appears that HRP does not behave like LiPH_2 in showing reductive activity in the presence of low iodide concentration. Our kinetic, spectral, e.s.r. and binding data clearly show that HRP can bind EDTA and directly oxidize it to EDTA radical in the absence of iodide. The EDTA radical thus formed cannot reduce cytochrome *c* or molecular O_2 to $\text{O}_2^\cdot$. However, it is capable of reducing I^+ in the $\text{HRP-H}_2\text{O}_2\text{-EDTA-I}^+$ system. Presumably, the EDTA radical formed at the active site can immediately reduce enzyme-bound I^+ complex to iodide before reducing the molecular O_2 . The fate of the EDTA radical in the presence or absence of I^+ and the mechanism of I^+ reduction is currently under investigation.

EDTA-analogue studies indicate that the nitrogen atoms of EDTA are essential for inhibition of iodide oxidation. As TEMED, which has no carboxy group, is 80% as effective as EDTA, it appears that the carboxy groups of EDTA are not essential for the inhibitory effect, but a possible role in the electrostatic interaction with some positively charged groups at the haem distal pocket cannot be excluded. The nitrogen atoms of EDTA contain a lone pair of electrons. It is tempting to speculate that EDTA is oxidized by catalytically active HRP via electron transfer from the nitrogen with the formation of EDTA free radical. This is supported by our finding that EDTA inhibition is reversed by Zn^{2+} [26], which is chelated via the lone pairs of electrons on the nitrogens, and not by Ca^{2+} or Mg^{2+} [26], which are chelated through the carboxy groups [33].

We have recently shown that EDTA binding is pH-dependent, and an ionizable group on the enzyme with a pK_a value of 5.8 appears to control this binding [27]. This group is presumably offered by the distal histidine, deprotonation of which may favour the interaction of EDTA with the enzyme [27]. The possible role of this histidine is also evident from our kinetic studies (Figure 2) in which a pH-dependent K_{app} value of EDTA shows the involvement of an ionizable group of pK_a 5.4 in catalytically active HRP for EDTA interaction. We have also shown that HRP contains an essential histidine residue (His-42) of pK_a approx. 6 for both iodide and guaiacol oxidation [22,50]. The role of this distal histidine residue in EDTA oxidation remains to be investigated.

Recently, Modi [51] reported from n.m.r. studies that EDTA binds at or near the iodide-binding site at the haem edge about 0.8 nm away from the ferric centre, and the binding is facilitated by protonation of an acid group of pK_a 4.0 in the native enzyme. This value differs from our data [27] on the native enzyme (pK_a 5.8) as well as on the catalytically active enzyme (pK_a 5.4). The latter needs a higher EDTA concentration to inhibit the enzyme activity at more acid pH (Figure 1) presumably because of decreased affinity of the active enzyme for EDTA as a result of protonation of the ionizable group of the enzyme, protonation of EDTA carboxylates (pK_1 , pK_2 , pK_3 and pK_4 = 2, 2.67, 6.16 and 10.26 respectively) and also because of decreased solubility of EDTA at acid pH. Modi [51] has not considered the influence of EDTA ionization and its decreased solubility at $\text{pH} < 5$ in this pH-dependent ^1H -n.m.r. linewidth study of EDTA in the presence of HRP. It is not clear why the interaction of EDTA with HRP

is so high at pH 3–4 [51], a pH at which solubility of EDTA is very low.

The question could be asked from our studies as to why the concentration of EDTA required to inhibit iodide oxidation at pH 6 (1 mM in Figure 1) is much lower than the K_D value [27] of EDTA (15 mM). The reason could be that EDTA may bind at a higher affinity to the catalytically active HRP than to the native enzyme, analogously with iodide binding to HRP [20]. Presumably the haem ferryl group attracts substrates more readily than the native enzyme. However, from the evidence presented, we can conclude that EDTA interacts at the iodide-binding site at the haem edge as an electron donor and inhibits iodide oxidation by competing with iodide for binding and subsequent electron flow to the haem ferryl group. Moreover, considering our observation that the EDTA radical is also capable of reducing I^+ , a partial concurrent secondary reaction between I^+ and the EDTA radical at the enzyme active site may also be partially responsible for the observed inhibition of iodide oxidation by EDTA.

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