Iodide modulation of the EDTA-induced iodine reductase activity of horseradish peroxidase by interaction at or near the EDTA-binding site

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Horseradish peroxidase (HRP) catalyses the reduction of iodine ion (I^+) to iodide by H_2O_2 in the presence of EDTA. I^+ reduction occurs optimally at pH 6 whereas the enzyme catalyses iodide oxidation optimally at pH 3.5. Thus the two activities reside on the same enzyme with two characteristic pH optima. Iodide modulates the expression of the reductase activity by EDTA. Higher concentrations of iodide inhibit the reductase activity by EDTA. Nitrite, an electron donor, acts similarly to iodide. Both EDTA and nitrite competitively inhibit iodide oxidation, indicating that they compete with iodide for the same binding site for electron flow to the haem iron group. However, unlike iodide, EDTA converts compound I, not into

the native enzyme, but into a compound absorbing at 416 nm which reduces I^+ and then returns to the native form. The apparent equilibrium dissociation constant, K_D , for the formation of the EDTA–HRP complex (15 mM) is doubled in the presence of iodide, indicating interference with EDTA binding by iodide. EDTA binds away from the haem iron centre and not through intramolecular Ca^{2+} . The pH-dependence of EDTA binding indicates that an ionizable group of the enzyme with pK_a 5.8, presumably a distal histidine, controls the binding. The data suggest that iodide competes with EDTA for compound I and modulates the iodine reductase activity by limiting the formation of the 416 nm-absorbing active compound.

INTRODUCTION

Horseradish peroxidase (HRP; EC 1.11.1.7; donor- H_2O_2 oxidoreductase) catalyses the oxidation of a wide variety of electron donors by H_2O_2 through intermediate formation of compound I and compound II [1–5]. The mechanism of iodide oxidation by compound I through a two-electron transfer reaction has been extensively studied [5–8]. Iodide binds near the haem moiety [7,9–11] at an almost equal distance from the haem peripheral 1- and 8-methyl groups and moves closer to the distal histidine by protonation of an ionizable group with a pK_a value of 4 [12]. In lactoperoxidase, the binding of iodide is facilitated by protonation of an ionizable group of pK_a value 6–6.8 which is presumably distal histidine [13]. From these studies, Sakurada et al. [13] have suggested that the approach of iodide to the distal histidine is a prerequisite for iodide oxidation, and distal histidine may bind to both iodide and $Fe(IV) = O$ of compound I to form a route for electron transport during oxidation. Aromatic donors have also been proposed to bind to HRP in the vicinity of the haem peripheral 8-methyl group [14], and a route connecting the donor molecule to the iron may be formed by distal His-42 and other amino acids such as Arg-38 and Arg-183 for electron flow [14]. Ortiz de Montellano and his co-workers [15–19] have proposed the active-site model for HRP and suggested that substrates interact at the haem edge and are oxidized by the flow of electrons through the δ -meso carbon to the haem iron group. However, the distal histidine appears to play an important role in the binding of substrates and electron flow during catalysis [12,13]. It is highly conserved in several peroxidases [20] and may be involved in the formation of compound I [21]. Subsequently, by the use of chemical modification studies, the role of the distal histidine in chloroperoxidase-catalysed chlorination [22] and HRP-catalysed iodide oxidation [23] has been established.

A few years ago we reported that HRP can exhibit a 'new'

catalytic activity, namely reduction of oxidized iodine species (I_2 or I_3^-) by H_2O_2 to form iodide and O_2 in the presence of EDTA [24]. Further studies indicated that EDTA causes a conformational change at the haem site and alters the haem reactivity with azide and H_2O_2 [25]. It also causes a hypsochromic effect on HRP at 402 nm and a bathochromic red shift of compound I (412 nm) to 416 nm [25]. The EDTA effect can be reproduced by its structural analogue, tetramethylethylenediamine, but not by EGTA or EDTA–Zn chelate [25]. Further kinetic and radioiodide-binding studies indicated that EDTA can block HRP-catalysed iodide oxidation by interfering with iodide binding [26]. However, it is not clear yet where EDTA binds, how this binding is controlled and how it induces the iodine reductase activity of the HRP. In this paper, we present evidence showing that iodide oxidation and iodine reduction are two independent catalytic activities of HRP. Iodide modulates the reductase activity by competing with EDTA for compound I which is converted by EDTA into a 416 nm-absorbing compound, an active species that reduces iodine. Evidence is also presented indicating that iodide interferes with EDTA binding at the haem periphery and an ionizable group of pK_a 5.8 controls EDTA binding.

MATERIALS AND METHODS

HRP ($A_{403}/A_{278} = 3$) and EGTA were purchased from Sigma. EDTA and KI (analytical grade) were from Glaxo Laboratories, Bombay, India. Iodine monochloride was purchased from Aldrich. All other chemicals used were analytical grade. The concentration of HRP used was determined from the ϵ_{403} of $102 \text{ cm}^{-1} \cdot \text{mM}^{-1}$ [27].

All kinetic studies were carried out at room temperature (28 °C) in a Pye-Unicam SP8-100 u.v./visible recording spectrophotometer. Peroxidase-catalysed iodide oxidation was

Abbreviation used: HRP, horseradish peroxidase.

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monitored at 353 nm by following the formation of I_3^- with iodide as electron donor as described previously [24–26]. The assay system contained, in a final volume of 3 ml: 50 mM sodium acetate buffer, pH 5.5, 1.7 mM KI, 0.5–1 μ g of HRP and 0.27 mM H_2O_2 added last to start the reaction. For assay of iodine reductase activity [24,25], the assay system contained in a final volume of 3 ml: 50 mM sodium acetate buffer, pH 6, 0.44 mM iodine monochloride or a portion of fresh saturated solution of iodine giving a final A_{460} of 0.4, 4 mM EDTA, 1 μ g of HRP and 0.27 mM H_2O_2 added last to start the reaction. The decrease in A_{460} was monitored at intervals of 15 s to measure the activity. The peroxidase and reductase activities were expressed as $\Delta A_{353}/\text{min}$ and $\Delta A_{460}/\text{min}$ respectively after correction for the non-enzymic rate, if any. All spectral studies were carried out in a Hitachi U-2000 computerized spectrophotometer at 28 °C with quartz cells of 1 cm light-path. For measurement of difference spectra of enzyme–ligand versus the enzyme, both the reference and sample cuvettes were filled with 1 ml of enzyme solution (1 μ M) to provide the baseline trace. This was followed by addition of a small volume of the ligand to the sample cuvette with concomitant addition of the same volume of buffer to the reference cuvette. The contents were stirred with a plastic rod before the spectrum was recorded. The equilibrium dissociation constant (K_D) for complex-formation was calculated from the following expression as described by Schejter et al. [28]:

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

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 thermodynamic parameter, free energy change ($\Delta G'$), for binding of ligand to HRP, was calculated from the K_D value by the following equation [29]:

$$\Delta G' = RT \ln K_D \quad (2)$$

where R is the universal gas constant and T is the temperature in degrees Kelvin.

RESULTS

We have previously shown that HRP can catalyse the reduction of oxidized iodine species, I_3^- and I_2 , to iodide by H_2O_2 in the presence of EDTA [24,25]. Since I_3^- itself contains iodide, which might affect the reaction rate, it was decided to investigate whether it can be replaced by iodine monochloride and to compare the rate of reduction with that of I_2 or I_3^- as substrate. Figure 1 shows that HRP can effectively reduce I^+ in the presence of EDTA, and the activity is entirely dependent on EDTA. The reaction product was found to be I^- as demonstrated by the formation of I_3^- on further addition of HRP, H_2O_2 and chelation of EDTA with equimolar Zn^{2+} at the time indicated by the arrow. The turnover number of the iodine reductase activity, as calculated from the absorption coefficient of I^+ , was $300 \pm 10 \mu\text{mol}$ of I^+ reduced/min per mg of HRP. When I^+ was replaced by I_2 or I_3^- , the turnover number was found to be 270 ± 8.5 and $65 \pm 2.8 \mu\text{mol}$ of I_2 or I_3^- reduced/min per mg of HRP respectively. Thus I^+ or I_2 appears to be a better substrate than I_3^- .

HRP is known to catalyse the oxidation of a number of inorganic substrates such as iodide, nitrite and thiocyanate [3–5].

Figure 2 shows the pH optima of HRP-catalysed iodide oxidation and HRP-catalysed I^+ reduction. Iodide oxidation takes place optimally at pH 3.5, whereas I^+ reduction occurs most effectively at pH 6 where iodide oxidation is only 5% of the optimum activity. However, in the presence of EDTA, which induces iodine reductase activity, iodide oxidation is further reduced to an insignificant level at this pH. No reduction of I^+ occurs at pH 3.5 where iodide oxidation is optimal. Thus two well-defined separate pH optima are the characteristics of the two different activities of the same enzyme, being expressed in the absence or presence of EDTA.

Iodide appears to modulate the iodine reductase activity of HRP. As shown in Table 1, iodine reductase activity of HRP decreases with an increase in iodide concentration, showing complete loss of activity at 3.4 mM. While studying the mechanism of the inhibitory effect of iodide on EDTA-induced iodine reductase activity, we considered two possibilities: (1) iodide, being the product of the reaction, shifts the equilibrium of the reaction, causing apparent inhibition; (2) iodide competes with EDTA for the same binding site and blocks iodine reduction. To clarify the first point, nitrite was used as the inorganic substrate as it can also, like iodide, be oxidized by HRP [3,4]. The result (Table 1) indicates that nitrite, similarly to iodide, can completely block EDTA-induced iodine reductase activity. Thiocyanate could not be used as it reduces iodine non-enzymically. Chloride, which is not oxidized by HRP, has no effect. The effect of nitrite

Figure 1 Kinetics of iodine reduction (●) by HRP in the presence of EDTA

The assay system is described in the text. When most of the I^+ had been reduced after 5 min, 4 mM $ZnSO_4$, 5 μ g of HRP and 0.27 mM H_2O_2 were added, as indicated by the arrow, to demonstrate the formation of I_3^- at 353 nm from iodide formed by the reduction of I^+ (○).

Figure 2 pH optima of I^- oxidation (○) and I^+ reduction (●)

Assay conditions were as described in the text, except that 50 mM sodium acetate buffer of varying pH was used. The amount of HRP used was 0.5 μ g for I^- oxidation and 1 μ g for I^+ reduction.

Table 1 Effect of I^- , NO_2^- , NO_3^- and Cl^- on EDTA-induced I_2 reduction I_2 reduction was assayed as described in the text.

System	I_2 reduction ($\Delta A_{460}/\text{min}$)
Control	0.065
+0.8 mM I^-	0.050
+1.7 mM I^-	0.040
+3.4 mM I^-	0.00
+0.85 mM NO_2^-	0.043
+1.7 mM NO_2^-	0.030
+3.4 mM NO_2^-	0.00
+3.4 mM Cl^-	0.068
+3.4 mM NO_3^-	0.060

Figure 3 Lineweaver-Burk plot of the effect of EDTA and nitrite on iodide oxidation

Iodide oxidation was measured at various concentrations of iodide as described in the text in presence or absence of 0.85 mM nitrite or 4 mM EDTA.

is not non-specific, as nitrate at the same concentration has no significant effect. It is thus feasible that both iodide and nitrite, being electron donors of the enzyme, displace EDTA from its binding site and thereby block the reductase activity. Figure 3

shows Lineweaver-Burk plots for oxidation of iodide in the presence or absence of EDTA or nitrite. The kinetic studies indicate that both EDTA and nitrite competitively inhibit iodide oxidation, as the K_m value for iodide is changed without alteration of the V_{max} value. The data indicate that EDTA competes with iodide similarly to nitrite, and they probably bind at the same site and compete with one another for the flow of electrons to the haem iron group. This would explain the reversal of the EDTA-induced block of iodide oxidation by higher concentrations of iodide and loss of iodine reductase activity. Figure 4 shows the effect of iodide, EDTA and I^+ on catalytic intermediates of HRP. Whereas iodide (Figure 4a) immediately reduces compound I (412 nm, trace 2) back to the native enzyme at 402 nm (traces 3 and 4), EDTA converts (Figure 4b) compound I into a more stable compound absorbing at 416 nm (traces 1 and 2). This compound is the active species for iodine reduction, as demonstrated by its time-dependent conversion into the native enzyme on addition of I^+ (traces 3–9). It is thus clear that both iodide and EDTA compete for compound I. Whereas iodide can reduce it directly by a two-electron mechanism to the native enzyme, EDTA converts it into a 416 nm-absorbing active compound for iodine reduction.

The binding of EDTA to HRP was studied by optical difference spectroscopy. Figure 5(a) shows the optical difference spectra of the HRP-EDTA complex versus HRP at various EDTA concentrations. The apparent K_D value for the binding of EDTA to HRP, as calculated from the plot of $1/\Delta A_{420}$ against $1/S$ as shown in the inset, was 15 mM. EDTA causes a hypsochromic change in the optical spectra of the HRP-CN complex at 421 nm (not shown) where CN^- is known to bind irreversibly to the iron centre. Figure 5(b) shows the difference spectra of the HRP-CN-EDTA complex versus HRP-CN. The K_D value for the binding of EDTA to the HRP-CN complex, as calculated from the plot of $1/\Delta A_{424}$ against $1/S$ as shown in the inset, was 19 mM, close to the value obtained for the binding of EDTA to the native enzyme. Thus EDTA appears to bind away from the haem iron centre. The K_D values for the binding of EDTA to the native HRP and HRP-CN complex and their corresponding $\Delta G'$ values are shown in Table 2. The result indicates that EDTA binds spontaneously to HRP both in its native form and when complexed to cyanide as demonstrated by

Figure 4 Effect of iodide, EDTA and I^+ on the catalytic intermediates of HRP

(a) HRP (3 μM ; trace 1), +10 μM H_2O_2 (trace 2), +7.5 μM KI at the end of 0.5 min (trace 3) and at the end of 1.5 min (trace 4) in 1 ml of 50 mM sodium acetate buffer, pH 6.0. (b) HRP (3 μM) +10 μM H_2O_2 +0.4 mM EDTA (trace 1), +0.4 mM EDTA (trace 2), +0.24 mM ICl with traces recorded after 2 min (trace 3), 5 min (trace 4), 7 min (trace 5), 9 min (trace 6), 12 min (trace 7), 14 min (trace 8) and 16 min (trace 9) in 1 ml of 50 mM sodium acetate buffer, pH 6.0.

Figure 5 Optical-difference spectra for the binding of EDTA to HRP and its cyanide complex

(a) Difference spectra of HRP-EDTA versus HRP at pH 6 at various EDTA concentrations. The initial (i) and final (f) concentrations of HRP were $1\ \mu\text{M}$ and $0.95\ \mu\text{M}$ respectively and final EDTA concentration was $14.5\ \text{mM}$. (b) Difference spectra of HRP-CN-EDTA versus HRP-CN under identical conditions. CN⁻ used was $200\ \mu\text{M}$. The final concentration of EDTA was $10\ \text{mM}$. The inset in each case represents the plot of $1/\Delta A$ against $1/S$ to calculate the K_D value. ΔA was measured at $420\ \text{nm}$ in the case of (a) and $424\ \text{nm}$ in the case of (b).

the identical $\Delta G'$ values. However, the binding is weaker than that of cyanide, which binds irreversibly to haem iron with a $\Delta G'$ value of $-29.8\ \text{kJ/mol}$. In order to investigate whether iodide

Figure 6 pH-dependence of the binding of EDTA to HRP

K_D values for the formation of the HRP-EDTA complex were determined at various pH values as indicated in Figure 5. The data presented are means $\pm$ S.E.M. for three to four experiments.

can affect EDTA binding, the latter was studied in the presence of $100\ \text{mM}$ iodide. The results (not shown) indicated that the binding of EDTA to the native HRP is altered only in the presence of iodide. The K_D value for the HRP-EDTA complex ($15\ \text{mM}$) is doubled ($31.2\ \text{mM}$) in the presence of iodide, suggesting that iodide competes with EDTA for the same binding site. This is not a non-specific effect of high salt concentration as nitrate has no such effect under identical conditions. The K_D for EDTA binding remains unaltered after pretreatment of the enzyme with $10\ \text{mM}$ EGTA (results not shown), suggesting that EDTA does not bind to the intramolecular Ca^{2+} of HRP [30]. The binding of EDTA to HRP was studied as a function of pH to investigate whether an ionizable group on the enzyme controls the binding. A plot of $\log K_D$ against pH (Figure 6) produced a sigmoidal curve showing near-saturation of binding between pH 6 and 7. The binding constant, K_D , is in the range $15\text{--}5\ \text{mM}$ at pH 6–7. The data indicate that an ionizable group on the enzyme with a $\text{p}K_a$ of 5.8 is responsible for controlling the binding of EDTA to the enzyme and this is presumably the distal histidine.

DISCUSSION

HRP is known to have a number of catalytic properties besides its usual peroxidase activity. These include its NADPH oxidase activity [31] and its thiol oxidase activity [32]. We have demonstrated that HRP also exhibits reductase activity in the presence of EDTA [24,25]. This activity has primarily been monitored by using oxidized iodine species such as I_3^- and I_2 [24,25], but this study indicates that I^+ can also be used as substrate. When the turnover number was calculated, the enzyme was found to reduce I^+ and I_2 more effectively than I_3^- , suggesting that the positively charged or non-polar oxidized species are better substrates than the negatively charged association complex of iodine (I_3^-). Presumably the iodide in the association complex inhibits the reductase activity.

Table 2 K_D and $\Delta G'$ values for the binding of EDTA and CN^- to HRP and the HRP-ligand complex

K_D values were determined as described in the text and $\Delta G'$ values were calculated from the K_D values by using eqn. (2).

Ligand	K_D			$\Delta G'$ (kJ/mol)		
	HRP	HRP-EDTA	HRP-CN	HRP	HRP-EDTA	HRP-CN
EDTA	$15\ \mu\text{M}$	—	$19\ \text{mM}$	-10.5	—	-10.05
CN ⁻	$5.8\ \mu\text{M}$	$7.7\ \mu\text{M}$	—	-29.8	-29.6	—

While studying how EDTA converts the iodide-oxidizing enzyme to an iodine-reducing enzyme, we asked three important questions. First, how is the reductase activity modulated and what is the role of iodide in the conversion process? Second, where is the binding site of EDTA in relation to iodide? Third, what is the active species that catalyses iodine reduction? The present study indicates that iodide plays an important role in modulating the catalytic activities of this enzyme. The peroxidase and reductase activities of the same enzyme have two well-separated pH optima, indicating that two different specific groups may be involved in the expression of each activity. Previous workers have shown that iodide oxidation by HRP takes place optimally at pH 4, and a group of pK_a 4, presumably contributed by haem propionic acid, is involved in the process [10,12]. At pH 6, at which iodine reduction takes place optimally in the presence of EDTA, iodide oxidation occurs at a very low rate. Without EDTA, iodine cannot be reduced at this pH. However, addition of EDTA blocks iodide oxidation at this pH almost completely and induces reductase activity at the same time. It is interesting to note that iodide actually controls the two activities. Higher iodide concentrations reverse the EDTA-induced block of iodide oxidation and at the same time inhibit the iodine reductase activity, presumably because EDTA is unable to bind to its binding site in the presence of excess iodide.

As nitrite is an oxidizable substrate of HRP [33], it also blocks iodine reduction similarly to iodide, possibly by displacing EDTA from the same site. We have reported previously, with the use of radioiodide-binding studies, that EDTA blocks iodide oxidation by limiting the binding of iodide to HRP [26]. The kinetic studies presented in this paper also show that EDTA inhibits iodide oxidation by competing with iodide for oxidation. This has been further substantiated by spectral studies in which iodide has been shown to reduce compound I back to the native enzyme by a fast two-electron reduction, whereas EDTA converts it into a stable 416 nm-absorbing compound. Optical-difference spectroscopic studies indicate that the binding constant, K_D , of EDTA to the native HRP is significantly increased in the presence of iodide, suggesting that iodide specifically interferes with the binding of EDTA. This strengthens the possibility that the iodide- and EDTA-binding sites are the same or very close to each other. As iodide appears to bind to the enzyme near the haem peripheral 1- and 8-methyl groups [12], EDTA may be presumed to bind at or near this site and compete with iodide for electron flow. EDTA does not bind to the haem iron, as it also binds to the HRP-CN complex in which CN^- is known to bind irreversibly to the haem iron with a K_D of $2.9 \mu M$ [33]. The K_D of EDTA binding was also found to be increased, similarly to that of iodide, in the presence of SCN^- (results not shown) which appears to bind at the iodide site at the haem peripheral 1- and 8-methyl groups [34]. The pH-dependence of EDTA binding shows that the binding is controlled by an ionizable group of pK_a 5.8 which is presumably distal histidine. The role of the distal histidine in chloroperoxidase-catalysed chlorination [22] and HRP-catalysed iodide oxidation [23] has been established. Evidence has also accumulated showing that distal histidine (pK_a = 6–6.8) controls the binding of iodide, thiocyanate and aromatic donors in lactoperoxidase [13,35,36] and salicyl-hydroxamic acid to myeloperoxidase [37]. It is therefore even more likely that EDTA binding at the haem distal side is also regulated by the distal histidine.

How EDTA converts the iodide-oxidizing enzyme into the iodine-reducing enzyme is not yet known. Kinetic studies have shown that EDTA does not bind and inactivate HRP irreversibly during catalytic turnover of the compound, as in the case of alkylhydrazine and azide which cause suicidal inactivation of

HRP [15–19]. On the other hand, it binds reversibly, competes with iodide and its K_D and $\Delta G'$ values are comparable with the values reported for the binding of aromatic donors to HRP [29,38]. Spectral studies indicate that EDTA converts compound I into a stable 416 nm-absorbing active species which is responsible for the reduction of iodine. As the spectrum of this compound was found to be similar to that of compound II [39], one can speculate that EDTA may reduce compound I by a one-electron mechanism to compound II with concomitant formation of EDTA free radical. If EDTA is rapidly oxidized by compound I and the conversion of compound II into native enzyme is slow, it can explain the formation of the stable compound that absorbs at 416 nm. Further studies are required to investigate the mechanism of iodine reduction by these compounds. However, from the evidence presented so far, we can suggest that iodide modulates the iodine reductase activity of HRP by competing with EDTA for binding near the same site at the haem periphery, competing for compound I and thereby limiting the formation of the 416 nm-absorbing active compound required for iodine reduction.

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