Irreversible inactivation of lactoperoxidase by mercaptomethylimidazole through generation of a thiyl radical: its use as a probe to study the active site

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The mechanism of suicidal inactivation of lactoperoxidase (LPO) by mercaptomethylimidazole (MMI) has been studied. Analogue studies indicate a specific requirement for the thiol group of MMI for inactivation of LPO in the presence of H_2O_2 . MMI is oxidized via one-electron transfer by LPO compound II as demonstrated by a spectral shift from 430 to 412 nm through an isosbestic point at 421 nm. A decrease in Soret absorbance at 412 nm and the appearance of visible peaks at 592 and 636 nm are the characteristics of the inactivated enzyme. The one-electron oxidation product of MMI was identified by e.s.r. spectroscopy as the 5,5'-dimethyl-1-pyrroline *N*-oxide (DMPO) adduct of the sulphur-centred thiyl radical. Both inactivation and spectral change are prevented by the radical trap DMPO, suggesting

involvement of the thiyl radical in inactivation. pH-dependent inactivation kinetics indicate the involvement of an ionizable group on LPO (pK_a 6.1), deprotonation of which favours inactivation. The enzyme is protected by iodide and not by guaiacol, suggesting that MMI interacts at or near the iodide-binding site which is away from the aromatic-donor-binding site. The inactive enzyme can form compound II and bind aromatic donor, indicating that the MMI oxidation product does not attack haem iron or aromatic-donor-binding site. We suggest that MMI interacts at the iodide-binding site for oxidation and the reactive product, probably the thiyl radical, is incorporated into the adjacent electron-rich site of haem porphyrin to cause inactivation.

INTRODUCTION

The mechanism of inactivation of thyroid peroxidase (TPO) and lactoperoxidase (LPO) by mercaptomethylimidazole (MMI), a potent antithyroid drug of the thionamide group, has been a subject of continuous research for the past three decades (Moris and Hager, 1966; Taurog, 1976; Davidson et al., 1978; Michot et al., 1979; Edelhofer et al., 1979; Engler et al., 1982; Ohtaki et al., 1982; Nakamura et al., 1984; Doerge, 1986a,b; Mason and Ramakrishna Rao, 1990). Inhibition of TPO by MMI is either reversible or irreversible depending on the ratio of iodide to drug (Taurog, 1976). Kinetic, spectral and radiolabelled drug-binding studies indicate that MMI irreversibly inactivates the enzyme by interacting with the haem of the oxidized TPO, and low concentrations of iodide protect it by keeping the enzyme (TPO- I_{ox}) in the reduced state, conditions under which MMI does not bind (Engler et al., 1982). That a reactive oxidation product of MMI interacts with the haem to cause inactivation (Engler et al., 1982) was also previously postulated (Davidson et al., 1978). As LPO resembles TPO (Carlström, 1969; Taurog et al., 1974; Ohtaki et al., 1982), the mechanism of inactivation was further studied with this enzyme. MMI is directly oxidized by the LPO- H_2O_2 system at a lower rate, and iodide increases the rate of drug oxidation and decreases the inactivation of LPO without changing the binding constant of MMI (Edelhofer et al., 1979). MMI was also shown to accelerate the reduction of both TPO and LPO compound I to compound II via one-electron transfer, and inactivation was found to be due to reaction of MMI with compound II to form an adduct (Ohtaki et al., 1982). This

adduct was later characterized as a 'sulphactoperoxidase' with stable visible peaks at 592 and 636 nm (Nakamura et al., 1984). However, the yield of the adduct was only 20 % whereas 80 % of LPO compound II was reduced to the ferric state by one-electron transfer (Nakamura et al., 1984) forming the free radical of MMI. Doerge (1986a) presented kinetic evidence for suicidal (mechanism-based) inactivation of LPO by MMI and suggested that the sulphonylation reaction between LPO compound II and MMI produces a reactive MMI intermediate which interacts with the haem to cause inactivation.

The plausible role of the reactive free radical of MMI (Ohtaki et al. 1982; Nakamura et al., 1984) in the inactivation of TPO and LPO has been ignored, as catalytic oxidation may not proceed because of concurrent inactivation of the enzyme. If 12 % of MMI is oxidized by the LPO- H_2O_2 system and about 100 mol of MMI is turned over per mol of LPO (Edelhofer et al., 1979), the amount of free radical generated should be sufficient to kill the enzyme. The role of this free radical therefore remains to be investigated, especially as a view is emerging that peroxidase-catalysed oxidation of some substrates generates highly reactive free radicals which cause suicidal inactivation of the enzyme by incorporation into haem (Sinha, 1982; Ortiz de Montellano, 1987; Ator et al., 1987; Ortiz de Montellano et al., 1988). MMI may also undergo a partial sulphonylation reaction with LPO compound II (Nakamura et al., 1984), and Doerge et al. (1993) attempted to identify the product as imidazole-2-sulphonic acid by chemical oxidation of some MMI analogues with 3-chloroperbenzoic acid, although significant differences may exist in the chemical and enzymic oxidation products. Doerge also suggested

Abbreviations used: MMI, 1-methyl-2-mercaptoimidazole; LPO, lactoperoxidase; TPO, thyroid peroxidase; DETAPAC, diethylenetriaminepenta-acetic acid; DMPO, 5,5'-dimethyl-1-pyrroline *N*-oxide.

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the possible role of this sulfoxidation product (Doerge, 1986a,b, 1988; Doerge et al., 1993) but direct evidence for imidazole-2-sulphenic acid as the reactive MMI intermediate is still lacking.

Here we present direct evidence for the formation of a sulphur-centred MMI thiyl radical by univalent oxidation of MMI by LPO compound II and its role in enzyme inactivation via interaction with the haem. Specific protection by halide and not by aromatic donor has provided further insight into the spatial relationship of the binding sites of these electron donors at the haem periphery of LPO.

EXPERIMENTAL

LPO ($A_{412}/A_{280} = 0.8$), MMI, 2-mercaptoimidazole, imidazole, 1-methylimidazole, resorcinol, guaiacol and diethylenetriaminepenta-acetic acid (DETAPAC) were obtained from Sigma. 5,5'-Dimethyl-1-pyrroline *N*-oxide (DMPO) was from Aldrich. LPO concentration was determined by using $\epsilon_{412} = 114 \text{ cm}^{-1} \cdot \text{mM}^{-1}$ (Morrison and Bayse, 1970).

Kinetic and spectrophotometric measurements were performed at $25 \pm 1^\circ \text{C}$ in a Shimadzu UV-2201 spectrophotometer. Peroxidase activity was measured using iodide or guaiacol as electron donor (Alexander 1962; Banerjee et al., 1986; De and Banerjee, 1986; Bandyopadhyay et al., 1992; Bhattacharyya et al., 1993). Enzyme activity was expressed as nmol of I_3^- or nmol of tetraguaiacol formed/min, calculated from molar and millimolar absorption coefficients of I_3^- and tetraguaiacol of 26340 (Huwiler and Kohler, 1984) and 6.48 (Hosoya, 1960) respectively.

Inactivation of LPO

Inactivation of LPO by MMI or its analogues was carried out in a $30 \mu\text{l}$ preincubation mixture containing LPO (10 nM), H_2O_2 (100 μM), MMI (3 μM) and sodium phosphate buffer (50 mM, pH 7.2) for 3 min at $25 \pm 1^\circ \text{C}$. Peroxidase activity was finally assayed by measuring iodide oxidation or guaiacol oxidation. For iodide oxidation, whole preincubation mixture was transferred to a 1 ml iodide assay solution containing sodium acetate buffer (50 mM, pH 5.2), KI (1.7 mM) and H_2O_2 (0.27 mM). For guaiacol oxidation, preincubation mixture was transferred to a 1 ml guaiacol assay solution containing sodium phosphate buffer (50 mM, pH 7.2), guaiacol (20 mM) and H_2O_2 (0.27 mM). To measure substrate protection against inactivation, electron donors were added to the preincubation mixture before the addition of MMI and H_2O_2 . The effect of pH on the inactivation by MMI in the presence of H_2O_2 was determined by incubating the enzyme for 3 min in sodium acetate, phosphate or Tris/HCl buffer (50 mM) over the pH range 5–8.

E.s.r. studies

E.s.r. measurements were carried out at $20 \pm 1^\circ \text{C}$ in a Varian E-104 spectrometer equipped with a TM_{110} microwave cavity and an aqueous flat cell. The incubation mixture contained LPO (13 μM), MMI (10 mM), Tris/HCl buffer (50 mM, pH 8), DETAPAC (1 mM) and DMPO (90 mM) in a final volume of 1 ml. Reaction was initiated by the addition of H_2O_2 (0.5 mM). It was vortexed and transferred to the flat cell. E.s.r. spectra were recorded 30 min after the addition of H_2O_2 .

Binding of donor to native and MMI-modified LPO measured by difference spectroscopy

For modification of LPO with MMI, 100 μM H_2O_2 was added four times at 5 min intervals to a microfuge tube containing a solution of excess MMI (5 mM), LPO (20 μM) and phosphate buffer (0.1 M, pH 7.5) in a total volume of 200 μl at $25 \pm 1^\circ \text{C}$.

Under these conditions, the enzyme showed complete loss of activity with 70% reduction of its Soret peak at 412 nm. The reaction mixture was then passed through a Sephadex G-25 spin column to remove small molecules. Spectral studies using control and modified enzyme were carried out in a Shimadzu UV-2201 spectrophotometer in a 1 ml cuvette of 1 cm light path. The difference spectra of the MMI-modified LPO–resorcinol complex versus the modified LPO and the native LPO–resorcinol complex versus native LPO were recorded as described previously (Hosoya et al., 1989; Bhattacharyya et al., 1993). Initially, both the sample and reference cuvettes were filled with 1 ml of enzyme solution, and a baseline trace were recorded. A small volume (usually 5–20 μl) of the donor solution of appropriate concentration was then successively added to the sample cuvette with concomitant addition of the same volume of buffer to the reference cuvette. The contents of each cuvette were stirred well with a plastic rod before the difference spectrum was recorded.

RESULTS

Involvement of the SH group of MMI in inactivation

We have observed that MMI acts as a suicidal substrate and inactivates LPO following mechanism-based kinetics (results not shown) similar to those of gastric peroxidase (Bandyopadhyay et al., 1993). In order to ascertain which group in MMI is essential for inactivation, the effect of various MMI analogues was investigated in the presence or absence of H_2O_2 . The results (Table 1) indicate that LPO loses its ability to oxidize iodide and guaiacol when preincubated with MMI and H_2O_2 together. No inactivation occurs in absence of H_2O_2 , indicating that either catalytically active enzyme is prone to inactivation by MMI or MMI is oxidized by catalytically active LPO to an inactivating species which concurrently kills the enzyme. 2-Mercaptoimidazole which is devoid of the methyl group is equally effective. On the other hand, 1-methylimidazole which is devoid of the SH group is not effective at all. Imidazole has no effect. Thus the SH group of MMI appears to be essential for inactivation of catalytically active LPO.

Table 1 Effect of preincubation of LPO with MMI or its analogues in the presence or absence of H_2O_2 on iodide and guaiacol oxidation

LPO was incubated with MMI (3 μM) or its analogues (3 μM) in the presence or absence of H_2O_2 (100 μM) in a final volume of 1 ml containing 50 mM sodium phosphate buffer. After 3 min, remaining peroxidase activity was monitored by following both iodide and guaiacol oxidation as described in the Experimental section.

	Peroxidase activity	
	Iodide oxidation (nmol of I_3^- /min)	Guaiacol oxidation (nmol of tetra- guaiacol/min)
LPO	31.13	67.18
LPO + H_2O_2	26.20	62.50
LPO + MMI	24.67	60.94
LPO + MMI + H_2O_2	4.93	3.12
LPO + 2-mercaptoimidazole	25.05	62.50
LPO + 2-mercaptoimidazole + H_2O_2	4.55	4.68
LPO + 1-methylimidazole	26.57	64.06
LPO + 1-methylimidazole + H_2O_2	25.81	62.50
LPO + imidazole	25.43	65.62
LPO + imidazole + H_2O_2	26.20	65.06

Figure 1 Spectral changes in LPO compound II on the addition of MMI

The concentrations were 5.6 μM LPO, 30–80 μM H_2O_2 and 25 μM MMI in 0.1 M sodium phosphate buffer, pH 7.2. A, Native LPO; B, LPO compound II formed after the addition of 5 equiv. of H_2O_2 to LPO; C, as for B + MMI; D, as for C + 50 μM H_2O_2 . Each scan was recorded 1 min after the last addition. The scale on the right indicates absorbance in the visible region. Inset: correlation between decrease in Soret absorbance with percentage of inactivation at various concentrations of H_2O_2 . It was a separate experiment in which activity (Δ) and A_{412} ($\circ$) were monitored by successive addition of H_2O_2 , as indicated, to an LPO (6 μM) plus MMI (100 μM) mixture in a final volume of 1 ml containing 50 mM sodium phosphate buffer, pH 7.2.

Figure 2 E.s.r. spectra of DMPO-MMI thiyl radical adduct

A, E.s.r. spectrum obtained with 13 μM LPO, 10 mM MMI, 400 μM H_2O_2 and 1 mM DETAPAC in 50 mM Tris/HCl buffer, pH 8.0; B, as for A but in the absence of H_2O_2 ; C, as for A but in the absence of LPO. Instrument conditions: microwave power 20 mW; modulation amplitude 1×10 G; time constant 1 s; scan rate 12.5 G/min; gain 5×10^4 .

Evidence for one-electron oxidation of MMI

As MMI acts as a suicidal substrate to LPO, it must be oxidized by the catalytically active LPO before inactivation. Figure 1 shows the spectral change in LPO compound II caused by MMI. MMI has no significant effect on the Soret spectra of native LPO at 412 nm. It causes an immediate reduction in compound II (trace B) to the ferric state (trace C) as indicated by the spectral shift from 430 to 412 nm through an isosbestic point at 421 nm, suggesting one-electron oxidation by MMI by compound II. However, unlike conventional electron donors, MMI causes two significant spectral changes in the Soret and visible region. First, the peak height of the Soret spectrum (trace C) is significantly lower than that of the native enzyme (trace A). Secondly, there is an initial appearance of a visible peak at 612 nm which is

Figure 3 Protection of haem from MMI free radical by the spin trap DMPO

The concentrations were 1 μM LPO, 10 μM MMI, 100 μM DMPO and 5 μM H_2O_2 in a final volume of 1 ml containing 50 mM sodium phosphate buffer, pH 7.2. A, Native LPO; B, as for A + H_2O_2 ; C, as for B + DMPO; D, as for C + MMI.

finally stabilized to form new peaks at 592 and 636 nm (results not shown). Assay of peroxidase activity at this stage indicated only 30 % loss of activity. The loss of activity and the associated spectral change correlate well with the concentration of H_2O_2 added to this system. Inactivation gradually increases along with the loss of absorbance at 412 nm and increase in absorbance at 592 and 636 nm (trace D). The decrease in Soret spectrum with concurrent increase in inactivation is a function of H_2O_2 concentration as shown in Figure 1 inset. Thus turnover of the enzyme in a few catalytic cycles to generate a sufficient amount of the oxidation product of MMI is required for complete inactivation, and the observed spectral change is due to concurrent interaction of the MMI oxidation product with the haem of the ferric enzyme concomitantly formed from compound II.

Generation of MMI radical

One-electron oxidation of MMI by LPO compound II is expected to generate a free radical of MMI, which is confirmed by e.s.r. spectroscopy using DMPO as a spin trap. The e.s.r. spectrum (Figure 2, spectrum A) was due to formation of spin-trapped MMI thiyl radical with hyperfine splitting constants of $a^N = 15$ G, $a^{\text{H}} = 3.2$ G which are consistent with the splitting constants of the known DMPO-thiyl radical adduct (Mottley et al., 1987). In the absence of H_2O_2 (Figure 2, spectrum B) or LPO (Figure 2, spectrum C), no significant e.s.r. signal was detected. The signal characteristic of O_2^- or $\text{OH}^\bullet$ (Finkelstein et al., 1980) was not detected.

Demonstration that MMI oxidation product is the interacting and inactivating species

In order to find out whether MMI or its oxidation species or secondary product interacts with the active enzyme, spectral studies were carried out in the presence of the spin trap, DMPO. When MMI was added to the reaction mixture containing LPO, H_2O_2 and DMPO, it caused immediate reduction of compound II to the native enzyme absorbing at 412 nm without any decrease in the Soret peak at 412 nm (Figure 3). The unstable visible band at 612 nm and subsequent stable bands at 592 and 636 nm characteristic of inactivated enzyme did not appear (not shown). This indicates that MMI thiyl radical is involved in the inactivation, and, when it is scavenged by DMPO, the enzyme is protected against inactivation. The results in Figure 4 also indicate that, whereas LPO is inactivated on preincubation with MMI and H_2O_2 , it is almost completely protected in the presence of DMPO. The inset of Figure 4 further shows DMPO protection

Figure 4 Protection of LPO activity against MMI inactivation by DMPO

Activity was monitored 3 min after preincubation of LPO (0.01 μM) with MMI (3 μM) and H_2O_2 (100 μM) by following I_3^- formation as described in the Experimental section. MMI was added last during the preincubation. ●, Activity of the control LPO; ○, activity of (3 μM) MMI-treated LPO; ▲, activity in the presence of DMPO (100 μM) and MMI (3 μM). Inset: protection by DMPO against various concentrations of MMI. Percentage remaining activity was measured by incubating LPO and H_2O_2 with various concentrations of MMI, as indicated, in the presence (△) or absence (○) of 100 μM DMPO.

against LPO inactivation by various concentrations of MMI. Thus MMI thiyl radical inactivates LPO either directly or through generation of a secondary product.

Figure 5 (a) pH-dependence of the inactivation rate of LPO with MMI and (b) experimentally obtained pseudo-first-order rate constants plotted according to eqn (1)

(a) LPO (0.01 μM) was incubated with 2.5 μM MMI and 100 μM H_2O_2 in 50 mM sodium acetate, phosphate or Tris/HCl buffer (pH 5–8) in a final volume of 30 μl . The pseudo-first-order rate constants obtained were plotted against pH (○). —, Theoretical curve. (b) The $\text{p}K_a$ value obtained from the slope was 6.1.

pH-dependence of LPO inactivation by MMI

In order to investigate the possible role of an ionizable group on LPO in the control of MMI-induced inactivation, kinetic analysis of the inactivation was carried out at different pH values. No significant inactivation occurred at pH values below 5.25 whereas it increased above this pH, attaining a maximum at pH 7.5–8.0. The pH-dependence of inactivation indicates that an ionizable group on the enzyme controls the inactivation process. The $\text{p}K_a$ value of this ionizable group can be calculated from the kinetic data by use of the following expression:

$$K_{\text{obs.}} = \frac{(K_{\text{obs.}})_{\text{max.}}}{1 + \frac{[\text{H}^+]}{K_a}} \quad (1)$$

which in linear form may be expressed as:

$$K_{\text{obs.}} [\text{H}^+] = K_a (K_{\text{obs.}})_{\text{max.}} - K_a K_{\text{obs.}} \quad (2)$$

where K_a is the dissociation constant of the reacting group and $(K_{\text{obs.}})_{\text{max.}}$ is the pseudo-first-order rate constant of the unprotonated reacting group (Takeuchi et al., 1986). The pseudo-first-order rate constant was determined and plotted against pH (Figure 5a). The open circles indicate experimental data and the solid line indicates the theoretical curve. From a plot of $K_{\text{obs.}} [\text{H}^+]$ against $K_{\text{obs.}}$ (values taken from Figure 5a), a straight line is obtained, the slope of which gives a $\text{p}K_a$ value of 6.1 (Figure 5b). It thus appears that ionization of a group on the enzyme with $\text{p}K_a$ 6.1 greatly enhances the rate of inactivation. As the $\text{p}K_a$ of thiolimidazole is 10.8 (Stowell, 1961), the role of MMI ionization at the pH range of inactivation is too small to be excluded.

Specific protection of LPO against MMI inactivation by halides

The irreversible inactivation of LPO by MMI could be almost completely prevented by preincubation with halides such as iodide or bromide but not with aromatic donors such as guaiacol or resorcinol (Figure 6). Only 20 μM iodide could protect 90% of the activity, bromide, under identical conditions, being slightly less effective. Neither guaiacol nor resorcinol at concentrations as high as 10 mM could protect the enzyme at all. The inset further shows that protection is a function of iodide concentration

Figure 6 Role of electron donors in MMI inactivation

Electron donors were added before the addition of MMI (3 μM) and H₂O₂ (100 μM) in the preincubation system containing LPO (0.01 μM) in 50 mM sodium phosphate buffer, pH 7.2, in a final volume of 50 μl. In each case, remaining peroxidase activity was assayed 3 min after the addition of H₂O₂ by following iodide oxidation as described in the Experimental section. ▲, Control; ●, +20 μM I⁻; ○, +20 μM Br⁻; ■, +10 μM I⁻; □, +5 μM I⁻; ×, +2.5 μM I⁻; ⊙, +10 mM guaiacol; △, +10 mM resorcinol; ▽, control + MMI.

Table 2 Role of two different substrates in the protection of LPO against inactivation as measured by guaiacol and iodide oxidation at two different pH values

The preincubation system is the same as that described in the legend of Figure 6 except that 20 μM iodide or 100 μM guaiacol was used. For guaiacol oxidation at pH 5.2 and iodide oxidation at pH 7.2, 50 μl of the preincubation mixture was assayed whereas for iodide oxidation at pH 5.2 and guaiacol oxidation at pH 7.2, 5 μl was used. Iodide oxidation at pH 7.2 was monitored using 8 mM instead of 1.7 mM KI for standard assay.

	Peroxidase activity			
	Guaiacol oxidation (nmol of tetraguaiacol/min)		Iodide oxidation (nmol of I ₃ ⁻ /min)	
	pH 5.2	pH 7.2	pH 5.2	pH 7.2
LPO + H ₂ O ₂	30.8	61.72	24.99	18.46
LPO + H ₂ O ₂ + MMI	0.30	1.54	1.53	0.84
LPO + H ₂ O ₂ + I ⁻ + MMI	27.00	55.66	24.07	18.07
LPO + H ₂ O ₂ + guaiacol + MMI	0.30	1.23	1.15	0.46

and is a saturable process. The difference between the two types of substrate in protecting the enzyme against inactivation under identical preincubation conditions was further confirmed by assaying the activity at both pH 7.2 and 5.2 using guaiacol and iodide as electron donor. The results in Table 2 indicate that, whereas iodide protects LPO by 90–100%, guaiacol offers no

Figure 7 Effect of electron donors on haem interaction with MMI free radical

The concentration were 10 μM MMI, 15 μM H₂O₂, 10 μM KI or 15 μM guaiacol in 50 mM sodium phosphate buffer, pH 7.2, in a volume of 1 ml. (a) A, Native LPO (2.5 μM); B, as for A + H₂O₂ + KI + MMI. (b) A, Native LPO (0.7 μM); B, as for A + H₂O₂ + guaiacol + MMI (immediate); B', scan of B after 5 min.

protection at all, as demonstrated by assay of both guaiacol and iodide oxidation at two different pH values. Spectral studies further indicate that, in the presence of iodide, MMI is unable to interact with the enzyme, as shown by the absence of any decrease in Soret spectrum at 412 nm (Figure 7a) and the lack of appearance of visible bands at 592 and 636 nm (not shown), whereas, in the presence of guaiacol, the enzyme shows typical MMI-induced spectral change at 412 nm characteristic of inactivation (Figure 7b). Specific protection by iodide suggests that MMI interacts at or near the iodide-binding site but not at the binding site of the aromatic donor for oxidation.

Absence of interaction of MMI oxidation product with the haem iron or aromatic-donor-binding site

In order to discover whether the MMI oxidation product binds the haem iron, LPO was inactivated with H₂O₂ and MMI, and spectral studies were carried out to detect compound-II formation after removal of the small molecules through a spin column of Sephadex G-25. Figure 8(a) shows that MMI-inactivated enzyme can readily form compound II absorbing at 430 nm with H₂O₂. This indicates that haem iron remains free in the ferric state, and the interaction of the MMI oxidation product elsewhere does not interfere with the reactivity of the enzyme with H₂O₂ which is required for donor oxidation.

It is possible that the enzyme is inactivated because of interaction of the product at the substrate-binding site. To investigate this, aromatic-donor binding was studied by difference spectroscopy using resorcinol as substrate. The difference spectrum of native LPO–resorcinol complex versus LPO (Figure 8b) shows a peak at 427 nm and a trough at 407 nm indicating binding of resorcinol to LPO (Hosoya et al., 1989). The difference spectrum of MMI-modified LPO–resorcinol complex versus modified LPO shows the same absorption characteristics as those of the native LPO–resorcinol complex. This difference spectrum thus indicates that resorcinol does bind at the aromatic-donor site of the inactivated LPO. The slight perturbation of the spectrum may be due to steric hindrance caused by incorporation of MMI oxidation product close to this site. Thus the MMI

Figure 8 (a) Absorption spectra of MMI-modified LPO and its interaction with H_2O_2 and (b) binding of resorcinol by native LPO and MMI-modified LPO

In (a), A, Soret spectrum (LPO 0.25 μM); B, as for A + 5 μM H_2O_2 . (b) After sample and reference cuvettes were filled with 1 ml of native LPO (4 μM) or modified LPO (4 μM) in 0.1 M phosphate buffer, pH 7.2, resorcinol was added (20 mM final concentration) to the sample cuvette. An equal volume of buffer was added to the reference cuvette. Difference spectra were recorded within 1 min. A, Native LPO versus native LPO–resorcinol; B, modified LPO versus modified LPO–resorcinol. Details of the modification of LPO with MMI and difference spectroscopy of modified LPO with resorcinol are given in the Experimental section.

oxidation product must interact at the haem site away from the iron centre but close to the iodide- and aromatic-donor-binding sites to cause inactivation of LPO.

DISCUSSION

Evidence has been presented to show that MMI is oxidized to a highly reactive free radical before irreversibly inactivating LPO. Spectral data suggest that MMI is oxidized by LPO compound II which is reduced to the ferric state by a one-electron-transfer mechanism (Ohtaki et al., 1982; Nakamura et al., 1984). Reduction of compound II to the ferric state is also demonstrated by the finding that the inactivated enzyme can again form compound II with H_2O_2 . The decrease in the Soret peak at 412 nm and the appearance of new visible peaks at 592 and 636 nm can be explained by concurrent incorporation of the highly reactive MMI oxidation product into the electron-rich site of the haem moiety. The shift of the unstable 612 nm visible peak to stable peaks at 592 and 636 nm might be due to structural adjustment of the haem moiety after incorporation of the MMI oxidation product. The primary oxidation product has been identified by e.s.r. studies as MMI free radical. From the similarity of the hyperfine splitting constants to those of the known thiyl radicals formed by LPO (Mottley et al., 1987), we suggest that the oxidation product is a sulphur-centred thiyl radical (Dunford, 1982; Harman et al., 1984; 1986; Mottley et al., 1987; Mason and Ramakrishna Rao, 1990). It is evident that MMI radical and not MMI is involved in the inactivation because DMPO, a free-radical trap, prevents both inactivation

and associated spectral change. If MMI were the species that reacted with compound II (Doerge, 1986a,b), both inactivation and spectral change would have occurred in the presence of DMPO. It is possible that MMI disulphide or another secondary product generated from the thiyl radical (Taurog et al., 1989) might be the active species but, as a radical is more reactive than the stable product, inactivation by the thiyl radical is the more plausible mechanism. Moreover, MMI acts as a suicidal substrate for LPO (Doerge, 1986a,b), and suicidal substrates of peroxidases are now known to inactivate via generation of a more reactive radical which interacts with the specific haem site to block electron transfer (Ortiz de Montellano, 1987; Ator et al. 1987; Ortiz de Montellano et al., 1988; Hosoya et al., 1989). The pH-dependence of inactivation of LPO indicates the requirement for an ionizable group of pK_a 6.1 on the enzyme, deprotonation of which favours inactivation. As distal histidine in the haem pocket of LPO exhibits a pK_a value around 6 and is involved in the binding of halide (Sakurada et al., 1987) and pseudohalide (Modi et al., 1989), we suggest that this residue may control inactivation by influencing the binding and oxidation of MMI.

MMI reversibly inhibits the catalytic activity of chloroperoxidase (Moris and Hager, 1966), horseradish peroxidase and prostaglandin synthetase–peroxidase (Petry and Eling, 1987) but irreversibly inactivates TPO (Engler et al., 1982), LPO (Doerge, 1986a,b) and gastric peroxidase (Bandyopadhyay et al., 1993). In the latter three, MMI presumably binds at the active site as a substrate whereas in the former it may react with the substrate free-radical intermediate or the product (Moris and Hager, 1966; Petry and Eling, 1989) without binding at the active site. Substrate protection against inactivator usually sheds more light on the nature, location, orientation and mode of interaction of both substrate and inhibitor at the active site. Inactivation of LPO is prevented specifically by iodide (halide) and not by guaiacol (aromatic donor). Although both are electron donors, the specific protection by iodide indicates that MMI interacts at or near the iodide-binding site and not at the guaiacol-binding site. In the presence of iodide, MMI neither binds nor is oxidized to inactivate the enzyme whereas, in the presence of guaiacol, it binds to the iodide-binding site, competes with guaiacol for oxidation and the reactive product formed concurrently inactivates the enzyme. It is also possible that the LPO-catalysed primary oxidation product of iodide either in the free state (I^+) or in the enzyme-bound state (EOI) may oxidize MMI so that I^+ is reduced to I^- (Taurog, 1976). This cyclic process may maintain a large enough I^- concentration at the active site of LPO to prevent MMI binding for oxidation. Although MMI-inactivated enzyme can form compound II and bind aromatic donor, it cannot oxidize it because the MMI oxidation product attacks a specific haem site which must be very close to both iodide- and aromatic-donor-binding sites and through which electrons from the donors are transferred to the haem ferryl group. The spatial alignment of iodide-binding and aromatic-donor-binding sites in LPO is still obscure. Proton and ^{127}I n.m.r. studies indicate that iodide binds to LPO in the vicinity of the haem methyl protons but close enough to interact with the distal histidine residue which possibly mediates electron transport in iodide oxidation (Sakurada et al., 1987). Iodide significantly increases the K_D value of resorcinol binding, suggesting a close proximity of iodide-binding and aromatic-donor-binding sites (Sakurada et al., 1987; Hosoya et al., 1989; Modi et al., 1989). Our studies on the specific protection of LPO by iodide and not by guaiacol indicate that the iodide- and MMI-binding sites are the same or very close to each other but away from the aromatic-donor-binding site. As resorcinol binding in MMI-inactivated enzyme is slightly perturbed compared with that of the native enzyme, the

reactive oxidation product appears to interact at a specific haem site close to the aromatic-donor-binding site. We therefore conclude that MMI interacts at or near the iodide-binding site of LPO compound II and is oxidized by one-electron transfer to form the thiyl radical which either directly or indirectly (through generation of a secondary product) inactivates the ferric enzyme by incorporation into a specific site of the haem prosthetic group.

U.B. gratefully acknowledges the award of a fellowship from the University Grants Commission (UGC) during this work.

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