Mechanism-based inactivation of lacrimal-gland peroxidase by phenylhydrazine: a suicidal substrate to probe the active site

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Humans are exposed to various hydrazine derivatives for therapeutic control of several diseases, and mammalian peroxidases are implicated in the oxidative metabolism of many drugs. The results presented here indicate that lacrimal-gland peroxidase is irreversibly inactivated in a mechanism-based way by phenylhydrazine, which acts as a suicidal substrate in the presence of H_2O_2 . The pseudo-first-order kinetic constants for inactivation at pH 5.5 are $K_1 = 18 \mu\text{M}$, $k_{\text{inact}} = 0.25 \text{ min}^{-1}$ and $\tau_{50} = 2.75 \text{ min}$, with a second-order rate constant of $0.75 \times 10^4 \text{ M}^{-1} \cdot \text{min}^{-1}$. Approx. 27 mol of phenylhydrazine and 54 mol of H_2O_2 are required per mol of enzyme for complete inactivation. The pH-dependent inactivation kinetics indicate the involvement of an ionizable group on the enzyme with a pK_a value of 5.4, protonation of which favours inactivation. SCN^- , the plausible physiological electron donor of the enzyme, protects it from inactivation. Binding studies by optical difference spectroscopy indicate that phenylhydrazine interacts with the enzyme with a K_D value of $60 \mu\text{M}$, and its binding is prevented by the presence of SCN^- . The enzyme is also protected by 5,5-dimethyl-1-pyrroline *N*-oxide, a free-radical trap, suggesting the involvement of a radical species in the inactivation. ESR studies indicate the formation of a spin-trapped phenyl radical ($a^N = 15.9 \text{ G}$ and

$a^{\beta\text{H}} = 24.8 \text{ G}$) generated on incubation of phenylhydrazine with the enzyme and H_2O_2 . A 75% loss of the Soret spectrum is observed when the enzyme is completely inactivated. However, in the presence of the spin trap, spectral loss is prevented and the enzyme compound II is readily reduced to the native state by phenylhydrazine. The phenylhydrazine-inactivated enzyme reacts with H_2O_2 or CN^- to form compound II or the cyanide complex with a characteristic spectrum, indicating that haem iron is protected from attack by the radical species. The inactivated enzyme binds SCN^- with a K_D value similar to that of the native enzyme ($15 \pm 3 \text{ mM}$), suggesting that the donor-binding site remains unaffected. CD studies of the inactive enzyme show complete disappearance of the Soret band at 409 nm with the appearance of a new band at 275 nm. This indicates that the haem environment of the enzyme is perturbed in the inactive form. As benzene, the end product of phenylhydrazine oxidation, has no effect on the enzyme, we suggest that the phenyl radical formed by one-electron oxidation by catalytically active enzyme inactivates it by incorporation in the vicinity of its haem moiety. The data support the use of phenylhydrazine as a probe for structural and mechanistic analysis of the active site of the lacrimal-gland peroxidase.

INTRODUCTION

Peroxidase (EC 1.11.1.7; donor- H_2O_2 oxidoreductase) catalyses the oxidation of various substrates including halides, pseudohalides, aromatic electron donors and xenobiotics. Halides are oxidized by a single two-electron transfer to compound I whereas aromatic donors are oxidized by two one-electron transfers through the intermediate formation of compound I and compound II [1]. In animal systems, soluble peroxidases similar to lactoperoxidase (LPO) are present in saliva, milk and uterine fluid [2]. Tears, the secretion of the lacrimal gland, contain a soluble peroxidase that is immunologically similar to LPO [3]. Peroxidase activity has also been demonstrated in rat lacrimal gland [4], from which the enzyme has been purified and characterized [5]. We have also purified a soluble peroxidase from sheep lacrimal gland (LGP) and characterized its physical, catalytic and kinetic properties [6]. The enzyme is a single-subunit (82 kDa) glycoprotein of native molecular mass 75 kDa having similarity to LPO. The haem of LGP is tightly bound to the apoenzyme and cannot be dissociated by standard methods [7,8]. The enzyme catalyses the oxidation of SCN^- , I^- and Br^- but not Cl^- [6]. Considering the physiological concentration of SCN^- and its oxidation at physiological pH, SCN^- is the plausible physiological electron donor of the enzyme

[6]. The enzyme can scavenge endogenous H_2O_2 effectively through SCN^- oxidation in the presence of glutathione, NADPH and glutathione reductase [6]. H_2O_2 may be generated intracellularly from oxidative metabolism or extracellularly from microbial cell growth. H_2O_2 and its metabolites such as hydroxyl radical ($\text{OH}^\cdot$) are involved in the oxidative damage of the lens proteins during cataract formation [9]. Although human tears possess antibacterial activity, it cannot be ascribed solely to the action of lysozyme [10]. The peroxidase/ H_2O_2 / SCN^- system has also been implicated in the control of bacterial flora in the ocular area [3,4,11] together with lysozyme. Thus LGP and its secretion plays an important role in host defence against oxidative damage and microbial infection.

It is now known that various drugs and xenobiotics are oxidized by peroxidases [12] in addition to the cytochrome *P*-450 system [13]. Hydrazine derivatives such as isocarboxazid (monoamine oxidase inhibitor), hydralazine (anti-hypertensive), isoniazid (anti-tubercular) and iproniazid (tranquilizer) have therapeutic importance but exert a number of toxic side effects. Phenylhydrazine (PH) (used to control polycythemia vera), acetylphenylhydrazine and isoniazid are well-known haemolytic agents [14–16]. Iproniazid causes severe liver damage [17], and hydrazine causes lupus erythematosus [18] and base pair mutation in bacterial and mammalian cells [19,20]. Hydrazine is already

Abbreviations used: PH, phenylhydrazine; DETAPAC, diethylenetriaminepenta-acetic acid; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; LGP, lacrimal-gland peroxidase; LPO, lactoperoxidase; HRP, horseradish peroxidase.

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known to inactivate haemoglobin [21], catalase [22], horseradish peroxidase (HRP) [23], lactoperoxidase (LPO) [24,25], thyroid peroxidase [26] and monoamine oxidase [27]. Although the mechanism of inactivation of haemoglobin [21], catalase [22] and HRP [28] are available, very little is known about the detailed mechanism of inactivation of mammalian peroxidases. As LGP prevents oxidative damage and bacterial infection [3,4,6,11], it is of interest to investigate whether this enzyme is also inactivated by PH. We present evidence here to show that PH is a suicidal substrate of LGP which is inactivated via a mechanism-based mode of action. The enzyme oxidizes it to a carbon-centred phenyl radical which is incorporated at the haem vicinity and prevents electron flow from bound donors to the haem ferryl group. The PH-modified enzyme provides interesting information on the haem environment of the enzyme.

MATERIALS AND METHODS

Phenylhydrazine hydrochloride (PH), resorcinol, guaiacol, KCN, sodium dithionite and diethylenetriaminepenta-acetic acid (DETAPAC) were obtained from Sigma. 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was purchased from Aldrich. All other chemicals used were of analytical grade.

LGP was purified as described previously [6]. Briefly, the soluble supernatant was treated with $(\text{NH}_4)_2\text{SO}_4$, and the precipitate at 30–60% saturation was applied to a concanavalin A–Sephacrose column. The bound peroxidase was eluted with 1 M methyl α -D-mannoside. Active fractions were pooled and applied to a preactivated CM-cellulose column and eluted with 0.25 M NaCl. The active fractions (absorption ratio of LEP at 410/280 nm = 0.8–0.85) were pooled and used as pure enzyme.

Enzyme assay

Kinetic and spectrophotometric measurements were performed at $25 \pm 1^\circ\text{C}$ in a Shimadzu UV-2201 computerized spectrophotometer. Peroxidase activity was measured using iodide as electron donor [29–31].

Inactivation by PH

Inactivation of LGP by PH was carried out by preincubating 0.66 μM LCP with 100 μM H_2O_2 and 5–30 μM PH in 50 mM acetate buffer, pH 5.5, in a final volume of 50 μl . At 2, 5, 8 and 12 min after the addition of H_2O_2 , an aliquot was transferred to a quartz cuvette containing 1.0 ml of assay mixture containing 50 mM sodium acetate buffer, pH 5.2, and 2 mM KI. The reaction was started by the addition of 300 μM H_2O_2 to assay the peroxidase activity. The residual activity at various concentrations of PH was plotted against time in order to calculate the kinetic constants. The effect of pH on enzyme inactivation by PH and H_2O_2 was determined by incubating the enzyme at various pHs before assay of an aliquot for peroxidase activity. To assess substrate protection against inactivation, various concentrations of SCN^- were added to the preincubation mixture before the addition of PH (30 μM) and H_2O_2 (100 μM). The rate constants of inactivation were determined in the absence (K_o) and presence (K) of various SCN^- concentrations. The protection constant (K_p) was then determined by the method of Chuang and Bell [32].

Partition ratio

LGP (0.66 μM) was incubated with 30 μM PH at various concentrations of H_2O_2 (0–100 μM) in 50 mM sodium acetate buffer, pH 5.0, for 10 min before assay of the enzyme activity.

Partition ratio with respect to PH was determined by varying the concentration of PH (0–30 μM) with a fixed concentration of H_2O_2 (100 μM).

ESR spectroscopy

ESR spectra were recorded at room temperature ($20 \pm 1^\circ\text{C}$) in a Varian E-112 spectrometer equipped with a TM_{110} microwave cavity. The incubation mixture contained 15 μM LGP, 10 mM PH, 50 mM sodium phosphate buffer, pH 7.0, 1 mM DETAPAC and 100 mM DMPO in a final volume of 0.5 ml. The reaction was started with 0.4 mM H_2O_2 ; the mixture was stirred well and immediately transferred to a flat cell. Spectra were recorded 5 min after the addition of H_2O_2 .

Donor binding to native and PH-modified LGP

The difference spectra of the enzyme–donor complex versus enzyme [25,33] were recorded in a Shimadzu UV-2201 computerized spectrophotometer at $25 \pm 1^\circ\text{C}$. Initially both the cuvettes were filled with 1.0 ml of the enzyme solution and the baseline was recorded. A small volume (5–20 μl) of donor solution of appropriate concentration was successively added to the sample cuvette along with the addition of the same amount of buffer to the reference cuvette. The contents were stirred well before the difference spectrum was recorded. The apparent equilibrium dissociation constant (K_D) for complex-formation was calculated from the following expression [34]:

$$1/\Delta A = [(K_D/\Delta A_x)/S] + (1/\Delta A_x) \quad (1)$$

where ΔA is the absorbance of peak minus trough of the spectrum or peak minus baseline, S is the substrate concentration and ΔA_x is the change in absorbance at saturating concentration of the substrate. In some cases where the donor (e.g. resorcinol) itself absorbs in the working region of the spectrum and where the absorption of the donor cannot be disregarded, it was subtracted from ΔA . For donor binding to the PH-modified enzyme, 2 ml of 16 μM LGP was incubated with 0.3 mM PH in the presence of 0.3 mM H_2O_2 for 30 min in 50 mM sodium acetate buffer, pH 5.2. The mixture was dialysed extensively against distilled water to remove all small molecules. Under these conditions, the enzyme showed complete loss of activity. Donor binding to this enzyme was studied by optical difference spectroscopy as described.

CD spectroscopy

CD spectra were recorded in an automated spectropolarimeter, JASCO J-720 using a rectangular cell in a thermostatically controlled cell holder at $20 \pm 1^\circ\text{C}$. Both native and PH-modified enzyme were dialysed extensively against deionized water and spectra were recorded from 200 to 450 nm with a scan speed of 50 nm/min.

RESULTS

Kinetics of inactivation

Incubation of LGP with various concentrations of PH in the presence of H_2O_2 resulted in a time-dependent loss of enzyme activity (Figure 1a). Inactivation was irreversible, as the activity could not be restored by dilution or dialysis. The inactivation followed pseudo-first-order kinetics with a second-order rate constant of $0.75 \times 10^4 \text{ M}^{-1} \cdot \text{min}^{-1}$ obtained from the plot shown in Figure 1(b). The half-life of inactivation ($t_{1/2}$) at each PH concentration when plotted against the reciprocal of PH con-

Figure 1 Time-dependent inactivation of LGP by PH at pH 5.5

(a) LGP ($0.66 \mu\text{M}$) was incubated with various concentrations of PH [0 (■), 5 (□), 10 (●), 15 (▲) and 30 μM (○)] in the presence of 100 μM H_2O_2 . (b) Determination of second-order rate constant of inactivation of LGP by PH and H_2O_2 . The slopes of the straight lines obtained from (a) were plotted against the concentrations of PH. The slope of this plot indicates the second-order rate constant of inactivation, which is $0.75 \times 10^4 \text{ M}^{-1} \cdot \text{min}^{-1}$. (c) Kinetic constants of mechanism-based inactivation of LGP by PH. The time required for half-inactivation at each concentration of PH obtained from the straight lines of (a) was plotted against the corresponding reciprocal PH concentrations. τ_{50} was calculated from the y-axis intercept, K_i from the x-axis intercept and k_{inact} by dividing the first-order rate constant by the τ_{50} value [35].

Figure 2 Partition ratios for the inactivation of LGP by H_2O_2 and by PH

The loss of activity is plotted against the $[\text{PH}]/[\text{Enz}]$ ratio in (a) and $[\text{H}_2\text{O}_2]/[\text{Enz}]$ ratio in (b) to calculate the partition ratio. The experimental details are described in the Materials and methods section.

centration yielded a straight line (Figure 1c) from which τ_{50} (minimum time for half-inactivation in the presence of saturating concentration of the inhibitor), K_i (apparent dissociation constant for the inhibitor) and k_{inact} were calculated to be 2.7 min, 18 μM and 0.25 min^{-1} respectively.

Partition ratio

The loss of enzyme activity was measured as a function of PH and H_2O_2 concentration to determine the turnover of enzyme inactivation. Titration of LGP with PH in the presence of excess H_2O_2 (100 μM) indicated that 27 equivalents of PH are required for complete inactivation (Figure 2a). Similarly, when the enzyme was titrated with H_2O_2 in the presence of excess PH (100 μM), it required 54 equivalents of H_2O_2 for complete inactivation (Figure

Figure 3 pH-dependence of the inactivation rate of LGP with PH and H_2O_2

LGP ($0.66 \mu\text{M}$) was incubated with 30 μM PH and 100 μM H_2O_2 in 50 mM acetate or phosphate buffer, pH 4.5–7.5. The pseudo-first-order rate constants obtained were plotted against pH. The curve obtained was a theoretical curve from which a $\text{p}K_a$ value of 5.4 was ascertained.

2b). Thus 2 mol of H_2O_2 are consumed for oxidation of each mol of PH for complete loss of activity.

pH-dependence of inactivation

The inactivation of LGP by PH was found to be dependent on the pH of the incubation medium. The rate of inactivation was higher at acidic pH and lower above pH 6.0. The pH-dependence of inactivation may reflect the requirement for an ionizable group on the enzyme which controls the rate of inactivation. The $\text{p}K_a$ value of each ionizable group was therefore calculated from the kinetic data according to the following equation [36]:

$$K_{\text{obs}} = \frac{[K_{\text{obs}}]_{\text{max}}}{1 + \frac{[\text{H}^+]}{K_a}} \quad (2)$$

From the plot of the pseudo-first-order rate constant (K_{obs}) of inactivation against pH (Figure 3), a theoretical curve was obtained from which a $\text{p}K_a$ value of 5.4 could be ascertained for the control of inactivation.

Substrate protection

Inactivation of LGP could be prevented by preincubation with SCN^- in a concentration-dependent manner, and 75 μM SCN^- was found to completely protect the enzyme. To determine the dissociation constant (K_p) of the enzyme–protector complex as described by Chuang and Bell [32], $(K_o - K)$ when plotted against SCN^- concentration yielded a hyperbolic plot as shown in Figure 4. The plot of $1/(K_o - K)$ against $1/[\text{SCN}^-]$ was linear and yielded a value of 24 μM for the dissociation constant of the enzyme– SCN^- complex. Iodide was also found to protect the enzyme at a relatively higher concentration (200 μM) with a K_p value of 66 μM (not shown).

PH binding

The binding of PH to LGP was studied by optical difference spectroscopy. The difference spectrum for the enzyme–PH complex had an absorption maximum at 439 nm and a minimum at

Figure 4 Protection of LGP activity from PH inactivation by SCN^-

LGP ($0.66 \mu\text{M}$) was incubated with various concentrations of SCN^- in the presence of $30 \mu\text{M}$ PH and $100 \mu\text{M}$ H_2O_2 added last to start the reaction. The protection constant (K_p) was determined from the plot shown in the inset after determining the rate constants of inactivation in the absence (K_0) and presence (K) of SCN^- .

Figure 5 Binding of PH to LGP

Difference spectra of LGP–PH complex versus PH were obtained by using $3 \mu\text{M}$ LGP and various concentrations of PH at pH 6.5. The inset is the plot for the determination of the K_D value of PH binding.

412 nm (Figure 5). The apparent K_D value when calculated from the plot was found to be $60 \mu\text{M}$. No difference spectrum was observed when PH binding was studied in the presence of SCN^- , indicating that SCN^- prevents PH binding to LGP.

Protection by the free-radical trap, DMPO

The rate of inactivation of LGP was sensitive to the spin trap, DMPO (Figure 6), which protects it in a concentration-dependent manner; $250 \mu\text{M}$ DMPO completely prevented inactivation. The result indicates the involvement of free-radical species generated by enzymic oxidation of PH; when scavenged by DMPO, the enzyme is protected. Spectral studies indicate that the addition of PH to preformed compound II causes time-dependent loss of the haem spectra (Figure 7a), which correlates with the loss of enzyme activity. Extensively dialysed inactivated enzyme showed 75% loss of Soret absorption at 410 nm (Figure 7b). However, if PH was added to compound II in the presence of DMPO, no loss of compound II spectrum was evident. On the other hand, compound II was reduced to the native enzyme as shown by the shift of the spectrum from 430 nm to 410 nm (Figure 7c). Only

Figure 6 Protection of LGP activity against PH inactivation by DMPO

Activity was monitored after preincubation of LGP ($0.66 \mu\text{M}$) with PH ($30 \mu\text{M}$) and H_2O_2 ($100 \mu\text{M}$) in the absence or presence of various concentrations of DMPO. The initial rate of enzyme activity was plotted against DMPO concentration. The native enzyme has a rate of $1.4 \Delta A_{353}/\text{min}$.

Figure 7 Spectral studies on the interaction of PH with LGP in the presence of H_2O_2

(a) Spectrum 1, LGP ($1.8 \mu\text{M}$); spectrum 2, LGP + H_2O_2 ($8 \mu\text{M}$); the other spectra were obtained at an interval of 2, 5, 10, 15, 20, 25 and 30 min after the addition of $10 \mu\text{M}$ PH. (b) Absorption spectrum of PH-modified LGP ($16 \mu\text{M}$) with $100 \mu\text{M}$ PH and $200 \mu\text{M}$ H_2O_2 for 30 min. The reaction mixture was dialysed overnight against deionized water with a few changes before the spectrum was recorded. (c) Protection of LGP by DMPO. Spectrum 1, LGP ($1.6 \mu\text{M}$); spectrum 2, LGP + H_2O_2 ($8 \mu\text{M}$); spectrum 3, LGP + H_2O_2 + DMPO ($250 \mu\text{M}$); spectrum 4, LGP + H_2O_2 + DMPO + $10 \mu\text{M}$ PH. The spectrum was recorded 1 min after each addition.

Figure 8 ESR spectrum of the DMPO-trapped radicals obtained by oxidation of PH with LGP and H_2O_2

The ESR spectrum was obtained with 15 μM LGP, 10 mM PH, 100 mM DMPO, 1 mM DETAPAC and 400 μM H_2O_2 added last to start the reaction. The ESR settings were: field set 3360 G, modulation frequency 100 kHz, modulation amplitude 10 G, receiver gain 6.3×10^3 , time constant 0.25 s, and microwave gain 15 mW.

Figure 9 Optical spectra of native and PH-modified enzyme in the presence of H_2O_2 and CN^-

Sample and reference cuvettes were filled with 1 ml of 1 μM native or PH-modified LGP. H_2O_2 (30 μM) was added to the sample cuvette for the formation of stable compound II. Difference spectrum was recorded within 1 min of the addition of H_2O_2 : spectrum 1, native enzyme; spectrum 2, PH-modified LGP. (b) Cyanide complex of PH-modified enzyme. Spectrum 1, PH-modified enzyme; spectrum 2, after addition of freshly prepared KCN solution (final concentration 2 mM); spectrum 3, after reduction of the cyanide complex with a few crystals of sodium dithionite.

DMPO, however, could not reduce compound II to the ferric enzyme.

Generation of phenyl radical

Incubation of PH with LGP and H_2O_2 in the presence of DMPO resulted in the formation of a DMPO-adduct of carbon-centred radical with the splitting constants $a^{\text{N}} = 15.9$ G and $a^{\text{H}} = 24.8$ G (Figure 8), which are identical with the values reported previously for carbon-centred phenyl radical [37,38]. No signal was obtained in the absence of PH or LGP (not shown). Signal characteristic of O_2^- or $\text{OH}^\cdot$ [39] was also absent.

Table 1 Difference spectral characterization and apparent dissociation constant of LGP-donor complexes

The donor binding in native and PH-modified LGP was studied by optical difference spectroscopy. The data were obtained from three different experiments. The K_{D} results are means $\pm$ S.E.M.

Enzyme	Donor	Spectrum complex (nm)		K_{D} (mM)
		max	min	
Native LGP	SCN^-	426	405	15 ± 3
	Resorcinol	430	409	167 ± 30
PH-modified LGP	SCN^-	426	403	21 ± 2
	Resorcinol	428	409	200 ± 20

Complex-formation of PH-modified enzyme with H_2O_2 and cyanide

The inactivation of PH-modified LGP is not due to its inability to form catalytically active enzyme intermediates with H_2O_2 . The difference spectra for native (1) and PH-modified (2) LGP in the presence of H_2O_2 are shown in Figure 9(a). Both showed a peak at 433 nm for compound II formation and a trough at 411 nm, although the optical difference spectrum between the peak and trough of spectrum 2 was about 25% of that of spectrum 1. This reduced difference spectrum is not due to the existence of unmodified LGP in the preparation, since the activity loss was more than 95% under these conditions. Rather it suggests that PH-inactivated LGP is more or less capable of forming H_2O_2 complex to a similar extent to the native enzyme. However, significant loss of absorption intensity was due to the lower absorption coefficient of the compound II form of the modified enzyme. A similar observation was also made in the case of modification of LPO with PH [25]. Although, because of the difficulty of splitting haem from LGP, it cannot be demonstrated at present that haem has been modified similarly to HRP [28], the comparable change in intensity without the shift of the Soret band indicates modification of the haem environment. Moreover PH-inactivated LGP also forms a complex with ferric cyanide that absorbs at exactly the same position (428 nm) as the native enzyme (Figure 9b). After the addition of dithionite, the ferric cyanide complex is reduced to the ferrous form like the native enzyme. These data suggest that the haem iron of the inactivated LGP remains free in the ferric state and the inactivation is not due to the formation of ferrous state.

Donor binding in the PH-modified enzyme

Binding of electron donors such as SCN^- and resorcinol was studied in both native and PH-modified enzyme. Table 1 shows the K_{D} values and absorption maxima and minima of the LGP-donor complexes. It is noteworthy that the K_{D} values of the LGP-donor complexes before and after modification were more or less the same at least in the order of magnitude. This indicates that donor-binding ability of LGP was not altered significantly by PH modification.

CD spectra

Native LGP had a negative haem CD band at 409 nm and a near-UV band at 285 nm (Figure 10). On modification with PH, the negative ellipticity at the Soret region reduced to zero with

Figure 10 CD spectra of native and PH-modified LGP

Both native and PH-modified enzymes were dialysed extensively to remove all small molecules and CD spectra were recorded as described in the Materials and methods section. -----, Native LGP; —, PH-modified LGP.

the appearance of a new negative ellipticity at 275 nm. These data indicate a significant change in the haem environment of LGP after modification with PH.

DISCUSSION

The observation of pseudo-first-order inactivation kinetics, irreversibility of inactivation and an absolute requirement for H_2O_2 identify PH as a suicidal substrate [40,41] for LGP. In the presence of excess H_2O_2 , about 27 mol of PH are required per mol of enzyme for complete inactivation. Conversely, in the presence of excess PH, approx. 54 mol of H_2O_2 are required per mol of enzyme to achieve the same effect. Thus inactivation of each mol of LGP requires oxidation of 27 mol of PH with the consumption of 54 mol of H_2O_2 . The stoichiometry of 2:1 of H_2O_2 /PH can only be explained if one equivalent of H_2O_2 is consumed to generate phenyldiazene from PH and another is required for enzymic oxidation of phenyldiazene to form the oxidation products by the active enzyme intermediates.

LGP inactivation is completely prevented by SCN^- , the major physiological electron donor of the enzyme [6]. Since PH cannot bind to LGP in the presence of SCN^- , inactivation is prevented. Alternatively, SCN^- can protect the enzyme by acting as a better substrate with a high turnover, thereby limiting the oxidation of PH and the generation of the inactivating species. The K_p value of SCN^- ($24 \mu\text{M}$) when compared with the K_d value of the enzyme- SCN^- complex (15 mM) was found to be significantly lower. The reason for this might be that SCN^- binds to the catalytically active enzyme intermediates (ferryl enzyme) with much higher affinity than to the native enzyme, similar to the binding of iodide to HRP [42].

The pH-dependence of inactivation indicates a requirement for an ionizable group on the enzyme with a pK_a value of 5.4, protonation of which favours inactivation. A distal histidine residue in the haem pocket with a pK_a value around 6.0 is implicated in the oxidation of various electron donors in peroxidases [43–49]. Since the observed pK_a is a kinetically derived value, its assignment to the histidine group of LGP remains to be established by further experiments.

ESR studies indicate that PH is oxidized to a carbon-centred phenyl radical with hyperfine splitting constants ($a^N = 15.9 \text{ G}$, $a^{\text{H}} = 24.8 \text{ G}$) similar to those reported previously [21,37,38]. When the radicals are scavenged by DMPO, the enzyme is protected. Since phenyl radical is more reactive than its end product, benzene [50], the enzyme is probably inactivated by the phenyl radical. The loss of Soret spectrum indicates that either haem or its vicinity is the target of this radical. Although a stable iron-phenyl complex is formed in the interaction of PH with the haem iron of myoglobin [51,52], haemoglobin [21,53], catalase [22] and cytochrome *P*-450 [54], no such complex was observed in HRP, but a δ -meso phenyl- and 8-hydroxymethyl-haem derivative as well as a protein adduct are formed, indicating that haem is buried in the protein structure [28]. Inactivated LGP forms a ferric cyanide complex which can be reduced by dithionite to the ferrous cyanide form, indicating that the haem iron remains free in the ferric state where phenyl radical does not interact. Formation of compound II by H_2O_2 is further evidence for the existence of the ferric state after inactivation. Moreover, as no Soret shift was observed, direct interaction with the haem iron is also excluded. These studies suggest that the phenyl radical cannot diffuse to the haem iron of LGP as it is probably buried in the protein structure, as in HRP [46,55]. As the haem of LGP cannot be dissociated by standard methods [7,8], we are unable to provide direct evidence on the exact site of interaction of the phenyl radical. However, the loss of Soret absorbance without any detectable shift indicates that the haem environment is modified by the radical. The loss of haem CD band at 409 nm and the appearance of a negative ellipticity at 275 nm indicate that the haem environment is significantly altered by incorporation of the radical. In myoglobin, haemoglobin and other haemoproteins, a coupled oscillatory interaction between the π - π^* transition of the group introduced and the nearby aromatic amino acid residue is responsible for most of the induced rotational strength of haem transition [56]. The intense positive cotton effect in the Soret region and strong negative ellipticity at the near-UV region are also unique to modified haem peroxidase [57]. Thus the change in Soret-associated ellipticity in the PH-modified LGP may be due to coupled oscillatory interaction between the π - π^* transition of the incorporated phenyl group near the haem and nearby aromatic amino acid residues. Since the secondary structure of the modified LGP in the far-UV region remains the same as that of the native enzyme (not shown), inactivation is not due to any gross structural change caused by random incorporation of radicals into the amino acid residues. Binding studies with the PH-modified enzyme indicate that SCN^- and resorcinol interact with K_D values similar to but slightly higher than that of the native enzyme, presumably because of steric hindrance created by the incorporated phenyl group. The radical may thus bind at a site some distance away from these donor-binding sites. As SCN^- binding to native LGP is not affected in the presence of the aromatic donor (results not shown), the binding sites for these donors appear to be different.

From the results presented, we suggest that PH acts as a suicidal substrate of LGP, which oxidizes it through one-electron transfer to phenyl radicals which interact in the vicinity of the haem to prevent electron transfer from the bound donor to the haem ferryl group causing irreversible inactivation. Identification of the radical-binding site will shed more light on the electron-transfer pathway of this enzyme. Irreversible inactivation of LGP by PH may lead to oxidative damage of the eye and susceptibility to microbial infection.

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