Characterization of sheep lacrimal-gland peroxidase and its major physiological electron donor

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A soluble sheep lacrimal-gland peroxidase was purified to apparent homogeneity. It had a native molecular mass of 75 kDa with a subunit molecular mass of 82 kDa and an isoelectric point of 6.5. Western blotting showed that it shares some of the enzyme antigenic determinants in common with other soluble peroxidases. The enzyme exhibits a Soret peak at 410 nm which is shifted to 431 nm by 5 equiv. of H_2O_2 due to the formation of compound II. The latter is, however, unstable and gradually returns to the native state. The enzyme forms complexes with CN^- and N_3^- and is reduced by dithionite showing a characteristic reduced peroxidase spectrum. Although the enzyme oxidizes I^- , SCN^- and Br^- optimally at pH 5.5, 5.25 and 5.0 respectively, at physiological pH, it oxidizes I^- and SCN^- only. Since extracellular SCN^- concentration is much higher than I^- , SCN^- may

act as the major electron donor to the enzyme. The second-order rate constants for the reaction of the enzyme with H_2O_2 (k_{+1}) and of compound I with SCN^- (k_{+2}) were $4 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $8.1 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively. A plot of $\log V_{\text{max}}$ against pH yields a sigmoidal curve consistent with a single ionizable group on the enzyme with a pK_a value of 5.75, controlling thiocyanate oxidation. In a coupled system with the peroxidase, H_2O_2 , SCN^- , GSH, NADPH and glutathione reductase, peroxidase-catalysed SCN^- oxidation by H_2O_2 could be coupled to NADPH consumption. The system is proposed to operate *in vivo* for the efficient elimination of endogenous H_2O_2 .

INTRODUCTION

Both soluble and membrane-bound peroxidases (donor- H_2O_2 oxidoreductase, EC 1.11.1.7) having a wide range of biological functions are present in animal tissues [1,2]. Apart from thyroid peroxidase, they take part in either antibacterial actions or cellular defence against oxidative damage by reactive oxygen species. Of the soluble peroxidases, bovine lactoperoxidase (LPO) has been extensively studied [3,4]. A large number of other soluble peroxidases have also been purified and characterized [5–12]. In histochemical studies, peroxidases of rat salivary, lacrimal and lactating mammary glands, called LPOs [13–16], are immunologically identical with the peroxidases from bovine salivary, lacrimal and Harderian glands [3,5,17]. Peroxidases isolated from sheep and goat milk and human saliva are also immunologically similar to LPO [7–9,18–22]. Morrison et al. showed that bovine submaxillary-gland peroxidase [5] is identical with the milk peroxidase [3] from the same species. However, human salivary peroxidase was shown to have certain dissimilarities to LPO in some of its physical, catalytic and kinetic properties [7,8]. Although controversy exists about the nature and origin of human milk and colostrum peroxidases [21,22], Langbakk and Flatmark [9] have identified a classical LPO in such materials.

De [12] reported that rat lacrimal gland is the richest source of a constitutive soluble peroxidase and compared some of its physical and immunological properties with those of bovine LPO. However, neither the spectral characteristics of the catalytically active enzyme and various enzyme derivatives nor their kinetic and catalytic properties are yet known. Animal peroxidases normally utilize halides and pseudohalides as electron donors for oxidation by H_2O_2 to produce hypohalous acid:

where $\text{X}^- = \text{Cl}^-/\text{Br}^-/\text{I}^-/\text{SCN}^-$, and HOX is the corresponding hypohalous acid with bactericidal activity. Although the physiological electron donors of several peroxidases have been characterized [23–28], it is not yet clear which electron donor is used by lacrimal-gland peroxidase (LGP) to exert its antibacterial activity or for elimination of endogenous H_2O_2 . Here, we report isolation of sheep LGP and describe its physical, immunological, spectral, kinetic and catalytic properties. Evidence shows that the enzyme uses SCN^- as a major electron donor to scavenge the endogenous H_2O_2 under normal physiological conditions.

MATERIALS AND METHODS

Concanavalin A (Con A)–Sepharose, Pharmalite and isoelectric focusing (IEF) markers were obtained from Pharmacia. Methyl α -D-mannoside, molecular-mass markers, sodium dithionite, NaN_3 and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG were obtained from Sigma. CM-cellulose was obtained from SRL. Amino acid standards and other reagents for amino acid analysis were obtained from Waters, and sequence-grade 6 M HCl was from Pierce. Antiserum against rat exorbital LGP [12] was provided by Dr. P. K. De (Centre for Cellular and Molecular Biology, Hyderabad, India).

Purification of sheep LGP

Twenty pairs of fresh lacrimal glands were homogenized in 200 ml of 50 mM sodium phosphate buffer, pH 7.5. The supernatant (105000 g for 1 h in a Beckman L5-50B ultracentrifuge) was treated with $(\text{NH}_4)_2\text{SO}_4$ and the precipitate at 30–60% saturation was dissolved in 50 mM sodium phosphate buffer, pH 7.5, clarified by centrifugation, and applied to a Con A–Sepharose column (1 cm $\times$ 10 cm) equilibrated with the above buffer. After the column had been washed until the A_{280} reached zero, the bound peroxidase was eluted with the same buffer,

Abbreviations used: Con A, concanavalin A; IEF, isoelectric focusing; LGP, lacrimal-gland peroxidase; LPO, lactoperoxidase; HRP, horseradish peroxidase; TNB, 5-thio-2-nitrobenzoic acid; DTT, dithiothreitol.

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containing 1 M methyl α -D-mannoside. Active fractions were pooled, applied to a preactivated CM-cellulose column (0.8 cm $\times$ 20 cm) equilibrated with 50 mM sodium phosphate buffer, pH 6.2, and eluted with 0.25 M NaCl in the same buffer. Active fractions were used as pure enzyme. All steps were performed at 0–4 °C.

Assay of peroxidase activity

Peroxidase activity was assayed using iodide as electron donor [29–31]. SCN^- oxidation was followed by measuring the rate of oxidation of 5-thio-2-nitrobenzoic acid (TNB) by OSCN^- at 412 nm [8,24,28,32]. Protein was measured by the method of Bradford [33]. As SCN^- is oxidized by a single two-electron transfer to compound I [34], the classical mechanism may be written as:

where E is the free enzyme, EO is compound I and k_{+1} and k_{+2} are the bimolecular rate constants for the formation of compound I and OSCN^- respectively. Applying the steady-state assumption with respect to compound I, we obtain

$$v = \frac{[\text{H}_2\text{O}_2][\text{EO}][\text{SCN}^-]}{[\text{H}_2\text{O}_2]/k_{+2} + [\text{SCN}^-]/k_{+1}} \quad (4)$$

where v is the initial rate of generation of OSCN^- and $[\text{EO}]$ is initial enzyme concentration. $[\text{H}_2\text{O}_2]$ and $[\text{SCN}^-]$ are the steady-state concentrations of H_2O_2 and SCN^- respectively. The above equation is valid under the conditions in which $[\text{H}_2\text{O}_2] = 0.2$ mM, $[\text{SCN}^-] = 4$ mM and the pH is 6.0. Rearranging eqn. (4) gives

$$v/[\text{EO}] = \frac{k_{+2}[\text{SCN}^-][\text{H}_2\text{O}_2]}{k_{+2}/k_{+1}[\text{SCN}^-] + [\text{H}_2\text{O}_2]} = \frac{B_1[\text{H}_2\text{O}_2]}{B_2 + [\text{H}_2\text{O}_2]} \quad (5)$$

where B_1 and B_2 are constants for a fixed concentration of SCN^- . A plot of $v/[\text{EO}]$ against $[\text{H}_2\text{O}_2]$ yields a rectangular hyperbola, where B_1 is k_{cat} for a fixed concentration of SCN^- and B_2 is related to K_m , k_{+1} and k_{+2} could be calculated [35] from the experimentally determined B_1 and B_2 values as shown in the Results section. All kinetic constants were calculated from six sets of experiments using three different enzyme preparations.

SCN^- oxidation by LGP coupled with NADPH oxidation by glutathione reductase

SCN^- oxidation by the LGP– H_2O_2 system was measured by coupling NADPH oxidation at 340 nm in the presence of glutathione reductase and GSH [28]. The reaction mixture contained in 1 ml: 50 mM sodium phosphate buffer, pH 7.2, 1 mM SCN^- , 0.1 mM NADPH, 1 mM GSH, 0.2 mM H_2O_2 , 1 μg of LGP and 1 unit of glutathione reductase. Activity was calculated ($\epsilon_{340} = 6.3 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) from the initial rate at 1 min.

Pseudocatalase and thiol oxidase activity

The pseudocatalase activity of LGP was measured by O_2 evolution from H_2O_2 in the presence of an electron donor in a Gilson oxygraph fitted with a Clark-type electrode [28], and the thiol oxidase activity was measured by O_2 consumption in the presence of dithiothreitol (DTT) or glutathione.

Molecular-mass estimation and gel electrophoresis

The native molecular mass of LGP, dialysed extensively against 50 mM sodium phosphate buffer, pH 6.5, was estimated by linear sucrose-density-gradient (5–20%) centrifugation [36] using HRP (40 kDa) and LPO (78 kDa) as standards. The subunit molecular mass was determined by reducing SDS/PAGE (10% gels) [37] using LPO, BSA (67 kDa), HRP and carbonic anhydrase (30 kDa) as standards. LGP was also electrophoresed in 7% polyacrylamide gel under non-reducing condition without SDS and 2-mercaptoethanol [37] and the enzyme was detected by activity staining with a solution of 1.7 mM KI in 50 mM sodium acetate buffer, pH 5.2, followed by the addition of 1 mM H_2O_2 and 0.1 g/ml *o*-dianisidine. Protein was detected by silver staining. IEF of LGP and standard markers was performed in an LKB multiphore electrophoresis system for 1 h at 10 °C using a 5% polyacrylamide gel (0.5 mm thick) with 2% Pharmalite (pI 3–10) as described in the LKB operation manual. Protein was stained with Coomassie Blue R-250. For Western-blot analysis, fresh samples were subjected to SDS/PAGE (10% gels) [37] and transferred to nitrocellulose paper [38] in a Bio-Rad transblot cell at 75 mA overnight at 4 °C. After incubation with 50 mM sodium phosphate buffer, pH 7.4, in PBS containing 2% BSA for 1 h at 37 °C, the paper was extensively washed with PBS, incubated with LGP antiserum in PBS after appropriate dilution for 1.5 h at 37 °C followed by extensive washing with PBS. After incubation with HRP-conjugated goat anti-rabbit IgG for 1 h at room temperature, it was extensively washed with PBS and the protein band was detected by colour reaction with 4-chloronaphthol (10 mg in 2 ml of HPLC-grade methanol, the volume made up to 15 ml with PBS) and H_2O_2 in the dark for 5 min.

Preparation of antiserum

Antiserum to LGP was raised in a male rabbit (1.2 kg) by two successive weekly subcutaneous injections of 0.3 mg of LGP in Freund's complete adjuvant followed by three subsequent injections in incomplete adjuvant at weekly intervals.

Other physical studies

CD measurements were taken in a JASCO spectropolarimeter J-600 using a 1.2 ml cuvette of 1 mm light path, containing 100 $\mu\text{g}/\text{ml}$ enzyme with an appropriate buffer blank. The results were analysed for secondary structure by a computer program [39]. Amino acid analyses were performed in a Waters PICO-TAG system according to the PICO-TAG operation manual. The enzyme was extensively dialysed overnight against deionized water, dried and hydrolysed with 6 M HCl containing 1% distilled phenol at 150 °C for 1 h in the PICO-TAG work station. Hydrolysed sample and standard amino acids were derivatized with phenyl isothiocyanate and peaks were detected at 254 nm. Haem extraction from LGP was carried out by the method of Teale [40]. Spectral studies of the native peroxidase and its derivatives were carried out in a Shimadzu UV-2201 spectrophotometer using 1 ml cuvettes of 1 cm light path. All studies were carried out at 25 ± 1 °C.

RESULTS

Purification and physical properties

LGP was purified to apparent homogeneity with an activity yield of 44%, a protein yield of 1.5%, an RZ value (A_{410}/A_{278}) of 0.8 and a specific activity of 55×10^3 units/mg of protein (Table 1).

Table 1 Purification of a soluble peroxidase from sheep lacrimal gland

This is a typical purification chart for 20 pairs of lacrimal glands. Activity was assayed using I^- as electron donor.

	Total protein (mg)	$10^{-5} \times$ Total activity (units)	Specific activity (units/mg of protein)	Activity yield (%)	Protein yield (%)	RZ Value (A_{410}/A_{278})
Cytosol	260	5	1920	100	100	—
$(NH_4)_2SO_4$ fractionation (30–60% precipitate)	90	3.2	3555	64	34.6	—
Con A-Sepharose eluate	14	2.6	18570	52	5.3	0.3
CM-cellulose eluate	4	2.2	55000	44	1.54	0.8

Figure 1 SDS/PAGE of LGP

Lane 1, standards used; lane 2, purified LGP (4 μ g).

Figure 2 Western-blot analysis of various mammalian peroxidases

(A) Western blot of bovine LPO (lane 1), rat submaxillary gland cytosolic peroxidase (lane 2), rat cytosolic LGP (lane 3) and sheep LGP (lane 4) using sheep anti-LGP serum. (B) Western blot of bovine LPO (lane 1), rat cytosolic LGP (lane 2) and sheep LGP (lane 3) using rat anti-LGP serum.

Table 2 Amino acid composition of sheep LGP and bovine LPO

ND, not determined.

Amino acid	Composition (% by weight)		
	LGP	LPO*	LPO†
Asp	6.9	11.6	11.6
Glu	9.1	10.0	10.0
Ser	9.9	5.4	5.8
Gly	14.0	6.5	7.5
His	1.4	2.3	2.3
Arg	3.7	6.1	7.3
Thr	3.7	5.2	5.0
Ala	5.9	6.1	5.8
Pro	5.4	6.9	6.9
Tyr	1.2	2.5	2.7
Val	3.9	4.6	4.6
Met	0.41	1.8	2.0
Cys	1.2	2.6	2.4
Ile	2.0	4.4	3.2
Leu	5.4	11.1	10.3
Phe	23.0	4.0	4.0
Lys	2.9	5.6	5.6
Trp	ND	ND	ND

* Results taken from ref. [41].

† Results from present study.

In sucrose-density-gradient centrifugation, LGP sedimented (results not shown) almost identically with LPO (78 kDa), and its molecular mass was found to be 75 kDa using HRP (40 kDa) as standard. During reducing SDS/PAGE, LGP migrated as a single band (Figure 1) of subunit molecular mass 82 kDa as calculated from the plot of relative mobility versus standard molecular mass (not shown). In non-reducing PAGE, LGP showed a single diffuse protein band which corresponded in position to the activity stain (not shown). On IEF, LGP focused midway in the pH gradient (pI 3–10), the pI value calculated from the standard pI markers being 6.5 (results not shown). LGP contains more acidic (aspartic and glutamic) amino acids than basic residues (histidine, arginine and lysine) and differs significantly from the amino acid composition of LPO (Table 2). It exhibited a strong CD band in the far-UV region (190–250 nm), and computer analysis of the secondary structure [39] indicates the presence of 20% α -helix, 25% β -sheet and 55% other structures including random coil.

Immunological similarities to other mammalian peroxidases

Antiserum against sheep LGP produced a single precipitin band with the sheep enzyme described in this work during Ouchterlony immunodiffusion [42], but no visible cross-reaction was evident with rat LGP or submaxillary-gland peroxidase, bovine LPO and human salivary peroxidase (not shown). During Western-blot experiments (Figure 2A), the antiserum detected a single

Figure 3 Absorption spectra of LGP in 50 mM sodium phosphate buffer, pH 6.2

An aliquot of LGP was scanned between 200 and 700 nm to obtain UV, Soret (A) and visible absorption (B) spectra of the enzyme. (C) and (D) Formation of a complex with H_2O_2 : trace 1, native LGP; trace 2, immediately after addition of $8 \mu\text{M}$ H_2O_2 ; trace 3, after 6 min; traces 4–10, after 8, 11, 14, 18, 21, 25 and 30 min.

band at 82 kDa for the purified sheep LGP (lane 4) and 78 kDa for bovine LPO (lane 1). Both rat submaxillary-gland peroxidase and rat soluble LGP preparations showed two bands, one at 82 kDa and one at 78 kDa (lanes 2 and 3). Human salivary peroxidase [7,8], however, contains a very weakly (not shown) interacting band at 78 kDa. The antiserum raised against rat LGP [12] detected an 82 kDa immunoreactive band with the purified sheep LGP, a 78 kDa band with LPO and 82 and 78 kDa bands with rat cytosolic LGP (Figure 2B).

Spectral analysis

The purified LGP shows well-defined Soret absorbance at 410 nm and UV absorption at 278 nm with an RZ value of 0.8 (Figure 3A) and visible peaks at 500, 544 and 630 nm (Figure 3B). On addition of 5 equiv. of H_2O_2 , the Soret peak shifted to 431 nm due to the formation of compound II (Figure 3C) with visible peaks at 535 and 565 nm (Figure 3D). However, compound II was stable for 6 min and was gradually converted into the native state with a single isosbestic point at 421 nm. Cyanide interacted with LGP to give a Soret peak at 428 nm which shifted to 433 nm

Figure 4 pH-dependent oxidation of I^- , Br^- , SCN^- and Cl^- by sheep LGP

The reaction mixture contained in a final volume of 1 ml: 50 mM sodium acetate buffer, pH 4.0–5.75, or sodium phosphate buffer, pH 6.0–8.0, 0.1 mM TNB, $1 \mu\text{g}$ of LGP and electron donor. The reaction was started with 0.2 mM H_2O_2 . $\circ$, 4 mM Br^- ; $\bullet$, 4 mM SCN^- ; $\blacktriangle$, 4 mM I^- ; $\square$, 150 mM Cl^- . The inset shows the Lineweaver–Burk plot of SCN^- oxidation at pH 7.0 using $1 \mu\text{g}$ of LGP, 4 mM SCN^- and 0.2 mM H_2O_2 .

on reduction with dithionite (Table 3). The reduced form of the uncomplexed native enzyme absorbs at 433 nm. The azide complex of the enzyme absorbs at 420 nm. For comparison, the spectral characteristics of LPO and rat LGP and their derivatives are included (Table 3). The haem group from LGP could not be extracted by treatment with ethyl methyl ketone at pH 2 unlike that of HRP [40].

Kinetic and catalytic properties

Oxidation of physiological electron donor

Sheep LGP catalyses the oxidation of I^- , SCN^- and Br^- but not Cl^- optimally at pH 5.5, 5.25 and 5.0 (Figure 4) with an apparent K_m of 0.66, 0.43 and 1.8 mM respectively (Lineweaver–Burk plots not shown). Near physiological pH, the enzyme catalyses only the oxidation of I^- and SCN^- at an appreciable rate. Except in the thyroid gland, the I^- concentration in extracellular fluid

Table 3 Spectral characterization of peroxidases in the absence and presence of various ligands

CN^- and N_3^- were used at concentrations of 1 mM and 10 mM respectively for LGP. A few crystals of dithionite were used to reduce the ferric enzyme.

Enzyme	Absorption maxima (nm)				
	Fe^{3+}	$\text{Fe}^{3+}\text{--CN}^-$	$\text{Fe}^{2+}\text{--CN}^-$	Fe^{2+}	$\text{Fe}^{3+}\text{--N}_3^-$
Sheep LGP*	410, 500, 544, 630	428	433, 536, 570	433	420
Bovine LPO†	412, 501, 541, 590, 631	430, 555, 595	435, 537.5, 571.5	435, 535, 565, 596	423, 550, 587, 630
Rat LGP‡	412, 502, 542, 594, 632				

* This study.

† Results taken from ref. [63].

‡ Results taken from ref. [12].

Figure 5 pH-dependent oxidation of SCN^- by LGP

Log $V_{\max}$ of SCN^- oxidation was plotted against pH. Activity was assayed using $1 \mu\text{g}$ of LGP, 4 mM SCN^- and 0.2 mM H_2O_2 .

($0.1 \mu\text{M}$) and in other tissues [43] is too low to be the physiological electron donor *in vivo*. Moreover, both Br^- and Cl^- are not oxidized by the enzyme at physiological pH. On the other hand, the enzyme oxidizes SCN^- near physiological pH in a concentration-dependent manner showing saturation kinetics (not shown) with an apparent K_m of 0.9 mM (Figure 4, inset). However, the enzyme catalyses SCN^- oxidation at the same rate even in the presence of physiological concentrations of other halides either separately or in combination (results not shown). SCN^- oxidation was also studied as a function of H_2O_2 concentration, and a plot of $v/[\text{Eo}]$ versus $[\text{H}_2\text{O}_2]$ yields a rectangular hyperbola (not shown) with two parameters B_1 , (k_{cat}) and B_2 (K_m) as described in the Materials and methods section.

Now

$$\frac{B_1}{B_2} = \frac{k_{+2}[\text{SCN}^-]}{k_{+2}/k_{+1}[\text{SCN}^-]} = k_{+1} \quad (6)$$

and

$$\frac{B_1}{[\text{SCN}^-]} = k_{+2} \quad (7)$$

B_1 and B_2 were determined by computer best fit using experimental values of $[\text{Eo}]$, $[\text{H}_2\text{O}_2]$ and v . The second-order rate constant k_{+1} for the reaction of LGP with H_2O_2 was calculated to be $4 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ and that of k_{+2} for the reaction of compound I with SCN^- to be $8.1 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$. SCN^- oxidation is pH-dependent, and a plot of $\log V_{\max}$ against pH yields a sigmoidal curve (Figure 5) from which the involvement of an ionizable group on the enzyme having a pK_a value of 5.75 was deduced, protonation of which favours SCN^- oxidation.

Other catalytic activity

Sheep LGP has no significant catalase activity to decompose H_2O_2 to water and O_2 . However, it catalyses decomposition of H_2O_2 to evolve O_2 (pseudocatalase activity) in the presence of halide electron donors. Figure 6 shows the pseudocatalase activity as a function of pH. The activity is highest with I^- , followed by Br^- and SCN^- , having an optimum pH of 6, 4.5 and 8.5 respectively. At physiological pH, only I^- induces significant

Figure 6 pH-dependent pseudocatalase activity of LGP in the presence of different electron donors

The reaction mixture contained 50 mM sodium acetate, pH 4.0–5.75 or sodium phosphate, pH 6.0–7.5, or Tris/HCl buffer, pH 7.4–9.5, $4 \mu\text{g}$ of LGP, 0.1 mM H_2O_2 and 0.2 mM I^- ($\square$), 1.0 mM Br^- ($\bullet$) or 2.0 mM SCN^- ($\circ$) in a final volume of 2 ml . The inset (A) shows the effect of varying I^- concentration at pH 7.0, using $1 \mu\text{g}$ of LGP and 0.1 mM H_2O_2 and (B) shows the dependence on H_2O_2 concentration at the same pH using 0.2 mM I^- and $1 \mu\text{g}$ of LGP.

Figure 7 Thiol oxidase activity of LGP

The reaction mixture contained in a final volume of 2 ml : 50 mM Tris/HCl buffer, pH 8.0, $2 \mu\text{g}$ of LGP and 2 mM DTT. Inset (A) shows the variation of DTT concentration ($\circ$) and cyanide (1 mM) sensitivity ($\bullet$) and inset (B) shows the pH-dependence of DTT oxidation.

pseudocatalase activity, and SCN^- has very little effect. The activity is dependent on I^- concentration, but concentrations above 1 mM are inhibitory (inset A). The activity is also dependent on H_2O_2 concentration (inset B). The enzyme also exhibits thiol oxidase activity with DTT as shown by O_2 consumption experiments (Figure 7). The activity is dependent

on DTT concentration, showing substrate saturation and sensitivity to cyanide (inset A). The optimum activity is evident between pH 8.5 and 9.5, although significant activity is still present at physiological pH. GSH was not oxidized under identical conditions (results not shown).

Coupling of SCN^- oxidation to NADPH oxidation by LGP

At physiological pH, the major oxidation product of SCN^- by H_2O_2 is OSCN^- [44] which reacts mainly with thiol compounds [44,45]. Since GSH is the major cellular thiol, OSCN^- may oxidize it to GSSG which in turn is reduced back to SCN^- with concomitant elimination of endogenous H_2O_2 . GSH being the major cellular antioxidant should be maintained at a constant level by reduction of GSSG back to GSH by the glutathione reductase–NADPH system of the cell. Thus, for efficient elimination of endogenous H_2O_2 , peroxidase-catalysed SCN^- oxidation should be coupled with NADPH oxidation by glutathione reductase in the presence of GSH. A system containing SCN^- , H_2O_2 , LGP, GSH, NADPH and glutathione reductase can efficiently consume NADPH, is dependent on LGP concentration and sensitive to cyanide (results not shown). NADPH consumption also requires H_2O_2 , SCN^- and glutathione reductase. The activity tends to be saturated above the physiological concentration of 0.3 mM SCN^- (20 ± 2 nmol of NADPH consumed $\cdot \text{min}^{-1} \cdot \mu\text{g}^{-1}$) with the K_m value of 0.17 mM. The enzyme shows no significant NADPH oxidase activity.

DISCUSSION

The purified LGP (RZ = 0.8) is a 75 kDa protein of a single polypeptide chain of 82 kDa, similar to the rat enzyme [12] and bovine LPO [46]. However, it differs significantly from these enzymes in having a pI value of 6.5, whereas that of LPO is basic [47]. The rat enzyme(s) shows multiple molecular forms, with pI values 3.5–6.5 [12]. The difference in glycosylation may explain the microheterogeneity in LPO [41] and the rat enzyme [12]. This is not observed in sheep LGP. The sheep enzyme has a higher serine, glycine and phenylalanine content and a lower content of the rest of the amino acids except alanine than LPO. Considering the molecular masses of LPO and LGP to be 78 and 75 kDa respectively, the approximate carbohydrate content is estimated to be 9 and 30% respectively, calculated from the molecular masses obtained from amino acid analysis. Sheep LGP is 20% α -helix, 25% β -sheet and 55% other structures including random coil, the corresponding values in LPO being 23, 65 and 12% respectively [46]. However, the values are comparable with those of thyroid peroxidase which has 18% α -helix, 20% β -sheet and 62% random coil [48]. Although the primary sequence of LPO is known [49], the non-identity between the two enzymes will be proved after the determination of the primary LGP sequence. Western blotting, however, indicates the presence of some common antigenic determinants among sheep LGP, bovine LPO and rat LGP and submaxillary peroxidase. Interestingly, sheep LGP is not inactivated on incubation with its antiserum, indicating that its antigenic sites are distinct from the catalytic site.

Sheep LGP has similar Soret and visible spectra to LPO and the rat enzyme [12]. The spectra of the CN^- and N_3^- forms show strong similarity to those of LPO. No studies exist on the ligand complexes of the rat enzyme [12]. However, compound II of LGP is not very stable. The stability could not be improved by dialysing the enzyme, indicating that autoreduction of compound II to its native state was not due to a contaminating exogenous electron donor. Presumably compound II is autoreduced by one-electron transfer from an endogenous source such as sugar

Scheme 1 Plausible mechanism of elimination of endogenous H_2O_2 by LGP in the presence of SCN^- , GSH, NADPH and glutathione reductase (GR)

moieties or specific amino acid residues near the haem. Autoreduction of compound II is also evident in myeloperoxidase [50], LPO and thyroid peroxidase [51]. The possible role of O_2^- (probably generated by one-electron oxidation of H_2O_2 by compound I to compound II as in the case of myeloperoxidase [50]) in the reduction of LGP compound II is neglected, as the decay rate is quite slow [50]. Further studies are required to explore the mechanism. Like LPO [52] and mammalian cytochrome *P*-450 enzymes, the haem of LGP is tightly bound to the apoenzyme and is not liberated by acid ethyl methyl ketone.

Sheep LGP prefers SCN^- as electron donor at physiological pH. Although both I^- and SCN^- are both good substrates, consideration of the very low ($0.1 \mu\text{M}$) I^- concentration in extracellular fluid favours SCN^- as the major physiological electron donor to LGP. Moreover, the physiological concentration of SCN^- in lacrimal-gland secretion (tears) may be as high as 0.3 mM [53]. At this SCN^- concentration LGP shows appreciable activity *in vitro* (results not shown). Physiological concentrations of I^- , Br^- or Cl^- are also unable to compete with SCN^- oxidation. The kinetic parameters, k_{+1} for the reaction of LGP with H_2O_2 ($4 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$) and k_{+2} for the reaction of compound I with SCN^- ($8.1 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$), are comparable with other peroxidases [8,28,54]. From pH-dependent kinetics of SCN^- oxidation, we suggest the involvement of an ionizable group on the enzyme having a pK_a value of 5.75, protonation of which favours SCN^- oxidation. This pK_a value is close to that (6–6.5) of distal histidine, protonation of which is implicated in the binding of several electron donors in various peroxidases [55–57]. Although the enzyme exhibits significant pseudocatalase activity with I^- , Br^- and SCN^- , the activity at physiological pH is significant only with I^- . Oxidation of H_2O_2 to O_2 by oxidation products of I^- or Br^- free or in the enzyme-bound state may account for this activity [28,58–60]. Consideration of the low extracellular I^- concentration ($0.1 \mu\text{M}$), the pseudocatalase activity of LGP should be insignificant *in vivo*. LGP also shows low pseudocatalase activity with SCN^- because of the low reactivity of its oxidation product, OSCN^- with H_2O_2 [58]. Consideration of these observations leads us to propose that LGP utilizes SCN^- as a major physiological electron donor for the reduction of H_2O_2 similar to that shown for LPO [4,43,61,62]. OSCN^- should not accumulate in the cell in the presence of a high concentration of GSH. GSSG is reduced back to GSH by glutathione reductase in the presence of NADPH. In our coupled system, SCN^- oxidation by LGP is dependent on NADPH oxidation with a K_m value of 0.17 mM for the oxidation of SCN^- . This system (Scheme 1) may thus operate *in vivo* in the presence of physiological concentrations (0.3 mM) of SCN^- [53] for the efficient elimination of endogenous H_2O_2 as long as the supply of NADPH through the hexose monophosphate shunt pathway is not limiting. It could thus act as an effective lacrimal-gland antioxidant system to protect cellular components against oxidative damage. Studies are in progress to locate the SCN^- -binding site on the enzyme.

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