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Mechanism-Based Inhibition of Lactoperoxidase by Thiocarbamide Goitrogens†

Daniel R. Doerge
Department of Agricultural Biochemistry, University of Hawaii, Honolulu, Hawaii 96822
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ABSTRACT: The irreversible inactivation of bovine lactoperoxidase by thiocarbamide goitrogens was measured, and the kinetics were consistent with a mechanism-based (suicide) mode. Sulfide ion inactivated, 2-mercaptobenzimidazole-inactivated, and 1-methyl-2-mercaptoimidazole-inactivated lactoperoxidases have different visible spectra, suggesting different products were formed. The results support a mechanism in which reactive intermediates are formed by S-oxygenation reactions catalyzed by lactoperoxidase compound II. It is proposed that the reaction of electron-deficient intermediates with the heme prosthetic group is responsible for the observed spectral changes and inactivation by thiocarbamides.

The inhibition of TPX-[1] and LPX-catalyzed reactions by thiocarbamides has been described (Ohtaki et al., 1985) and a relationship established with the physiological state of goiter (Gilman & Murad, 1975). A substantial body of experimental evidence suggests that modification of the iron porphyrin cofactor of these mammalian peroxidases occurs concomitant to the loss of enzymatic activity (Engler et al., 1982; Ohtaki et al., 1982; Nakamura et al., 1984). LPX was shown to be similar to TPX by several criteria, including inactivation by thiocarbamides (Ohtaki et al., 1982). The production of green, inactive derivatives of LPX by treatment of hydrogen peroxide oxidized enzyme intermediates with sodium sulfide and MMI was described (Nakamura et al., 1984); however, a mechanistic description of LPX inactivation supported by experimental results has not been presented.

A recent report from this laboratory described the catalysis of S-oxygenation reactions by LPX (Doerge, 1986). These observations provided a clue to the mechanism of inactivation of LPX by thiocarbamides and suggested the involvement of enzymatic sulfoxidation. This report describes the reactions of thiocarbamide goitrogens with LPX and provides data in support of a mechanism-based (suicide) mode of inhibition (Waley, 1985).

Materials and Methods

Bovine LPX was purchased from Sigma Chemical Co. and the activity measured by guaiacol oxidation [3×10^4 mol min^{-1} (mol of LPX)$^{-1}$] and iodide ion oxidation [3.3×10^4 mol min^{-1} (mol of LPX)$^{-1}$] at 22 °C in the presence of 0.2 mM hydrogen peroxide, 33 mM guaiacol, or 5 mM potassium iodide in 0.1 M phosphate buffer, pH 7.0, as previously described (Morrison, 1970). LPX concentration was determined spectrophotometrically (Morrison, 1970). MBI was obtained from Eastman Chemical Co. and NMBI from Aldrich Chemical Co., and both were recrystallized from aqueous ethanol. MMI was obtained from Sigma Chemical Co. Purity was checked by thin-layer or high-pressure liquid chromatography. Hydrogen proxide, obtained as a 30% solution from Sigma Chemical Co., was standardized periodically (Cotton & Dunford, 1973), and dilute solutions were prepared daily. Kinetic measurements and spectra were made with a Hewlett-Packard 8541A recording spectrophotometer.

For determination of enzymatic activity, the iodide ion oxidation method was employed because of greater reproducibility of initial rates. Assays were initiated by the addition of aliquots containing LPX at a final concentration of ca. 1 nM into a cuvette containing the reagents listed above, and initial rates were determined in the first 30 s of reaction.

The time-dependent inactivation of LPX by thiocarbamides was determined by incubation of LPX (0.25–2.0 nM) in the presence of hydrogen peroxide (0.05 mM) and inhibitor such that the ratio [LPX]/[inhibitor] was constant (Waley, 1985). The inhibitors were added as ethanolic solutions, and it was determined that addition of small amounts of ethanol had no effect on the inactivation reaction. Hydrogen peroxide was added to a mixture of LPX plus inhibitor to initiate the inactivation at 0 °C, and at various times an aliquot was removed and analyzed for enzymatic activity. The plots of enzyme inactivation vs. time were approximately first order for 50–90% inactivation, and the inactivation half-times ($t_{1/2}$) were determined from this portion of the curve where hydrogen peroxide dependent inactivation was negligible.

Stoichiometric measurements for the inactivation of LPX by thiocarbamides were made by adding excess hydrogen peroxide (0.2 mM) to a mixture of LPX (0.005 mM) plus

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[1] Abbreviations: EDTA, ethylenediaminetetraacetate; HRP, horseradish peroxidase; LPX, lactoperoxidase; MBI, 2-mercaptobenzimidazole; MMI, 1-methyl-2-mercaptoimidazole; NMBI, 5-nitro-2-mercaptobenzimidazole; TPX, thyroid peroxidase.

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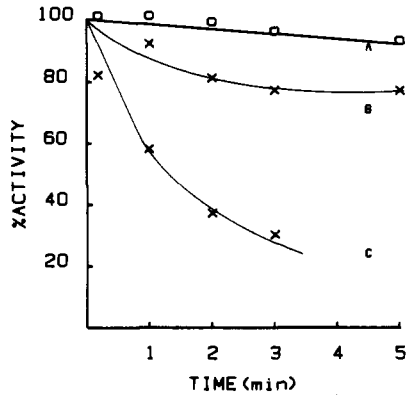


FIGURE 1: LPX activity at various times of preincubation with NMBI. LPX was incubated with NMBI as described under Materials and Methods, and aliquots were removed and analyzed for activity. (A) Control incubation; (B) 0.04 mM NMBI plus 5 mM NaSCN; (C) 0.04 mM NMBI.

inhibitor (0–0.30 mM) in 0.1 M phosphate buffer, pH 7.0. After a 1-min incubation at 0 °C, the mixture was diluted and the enzymatic activity of an aliquot determined. Stoichiometric measurements for hydrogen peroxide were made in the presence of excess MBI or MMI (0.2 mM) as described above with 0–0.05 mM hydrogen peroxide added to initiate the reaction.

Sephadex G-25 chromatography of LPX samples was carried out with a 1 × 10 cm column equilibrated and eluted with 0.1 M phosphate buffer, pH 7.0, at a flow rate of 0.2 mL/min.

Thiocarbamide-inactivated LPX for spectrophotometric measurements was prepared by addition of 0.05 mM hydrogen peroxide to 0.005 mM LPX plus inhibitor at a concentration greater than $4K_i$ ([MBI] = [MMI] = 0.1 mM) and the spectrum recorded. The enzymatic activity of $^1/_{4000}$-fold diluted samples was determined.

Reduction of ferric MMI–LPX was carried out by addition of a 10-fold excess of ferrous EDTA to the cuvette (Andersson et al., 1984). Oxidation of ferrous MMI–LPX was carried out by addition of a 10-fold molar excess of potassium ferricyanide.

RESULTS

The inactivation of LPX by the thiocarbamide goitrogens met the criteria associated with mechanism-based inhibition (Waley, 1985). Figure 1 shows the time-dependent inhibition of LPX by NMBI in the presence of hydrogen peroxide. Figure 1 also shows the slow loss of activity in the presence of hydrogen peroxide alone and the ability of the pseudo-halide LPX substrate thiocyanate ion to reduce the rate of NMBI-induced inactivation.

The steady-state kinetic treatment of Waley was used to support a suicide inactivation mechanism of LPX by thiocarbamides (Waley, 1985). Varying [I] while fixing the ratio [LPX]/[I] yields plots of $[I]t_{1/2}$ vs. [I], which are linear. These plots showed good linear correlation ($r^2 = 0.99$) for all thiocarbamides tested, and the calculated kinetic constants (k_i and K_i, the first-order inactivation rate constant and the enzyme-inhibitor equilibrium binding constant, respectively) are listed in Table I. MBI and MMI showed similar kinetic behavior, and the substitution of MBI with an electron-withdrawing nitro substituent increased K_i 100-fold while k_i was less sensitive to the change.

The nature of the inhibition was investigated for MMI and MBI and was found to be irreversible. Inactivated enzyme was prepared as described under Materials and Methods, and the enzyme was separated from small molecules by chromatography on Sephadex G-25. Elution from the column was monitored by heme absorbance (410 nm) and enzymatic activity (iodide ion oxidation). It was determined that all protein fractions remained more than 98% inhibited following removal of loosely bound inhibitors. Control experiments showed that at least 90% of the enzymatic activity was recovered from incubates of LPX and hydrogen peroxide in the absence of inhibitor under identical incubation and chromatographic conditions. After chromatography on Sephadex G-25 and standing at 0 °C overnight, MBI-inactivated LPX showed no recovery of enzymatic activity.

Table I: Kinetic Constants for the Inactivation of LPX by Thiocarbamides

inhibitor	k_i (min^{-1})	K_i ($M^{-1} \times 10^6$)
MBI	4.4	0.2
MMI	3.2	0.2
NMBI	1.4	24

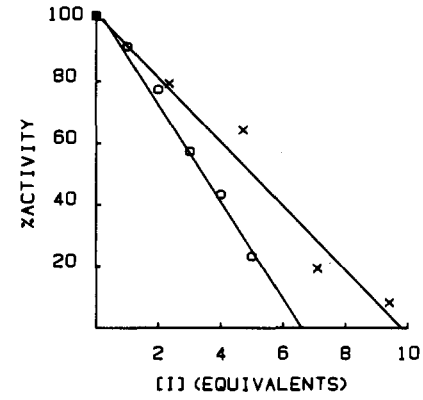


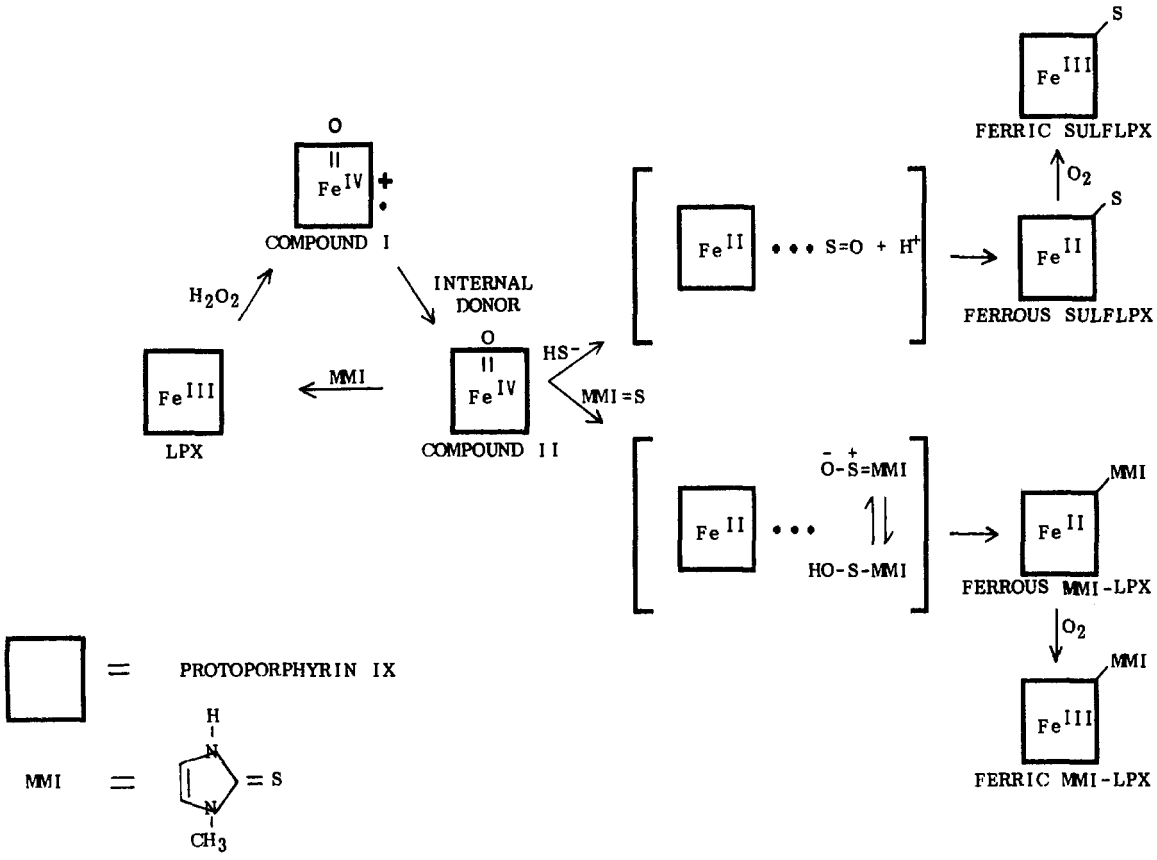
FIGURE 2: Determination of partition ratio for MBI and MMI in the presence of excess hydrogen peroxide. LPX was incubated with inhbitor and hydrogen peroxide added as described under Materials and Methods. MBI, O; MMI, ×.

The partition ratios for the thiocarbamide inactivators were determined, and the plots for MBI and MMI are shown in Figure 2. Approximately 7, 10, and 90 equiv of MBI, MMI, and NMBI, respectively, were required for the complete inactivation of LPX in the presence of excess hydrogen peroxide.

It was observed that the absorption spectrum from MMI-inactivated LPX was not identical with sulf-LPX in contrast to the previous description (Nakamura et al., 1984). Addition of excess ferrous EDTA to the final product from the reaction of LPX with excess MMI and excess hydrogen peroxide caused the production of a compound with a visible spectrum identical with the initial spectral form observed during the MMI inactivation of LPX (λ_{max} = 612 nm; cf. Figure 4). Alternatively, treatment of the final product with excess ferricyanide ion followed by chromatography on Sephadex G-25 gave a compound with an unchanged visible spectrum, consistent with a modified ferric LPX.

When LPX was inactivated in the presence of excess MBI and excess hydrogen peroxide, the spectral properties of the inhibited product were different from those observed for native or MMI-inactivated LPX (cf. Figure 3). No intermediates were observed, and the final spectrum was not that of sulf-LPX or MMI–LPX. The MBI-modified LPX spectrum is characterized by a 25% decrease in the Soret band (410 nm) and smaller shifts in absorbance wavelength maxima as well as extinction coefficients at longer wavelengths. Unlike ferric sulf-LPX or MMI–LPX, MBI-inactivated LPX was unchanged by the addition of excess ferrous EDTA as the visible spectrum after this treatment was essentially identical.

Scheme I: Proposed Reactions of LPX Compound II with Sulfide Ion and MMI

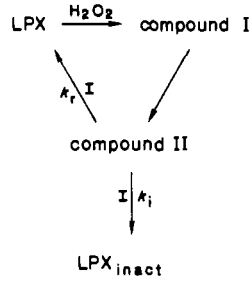


Discussion

The results of this study provide experimental support for a mechanism-based mode of inhibition of LPX by thiocarbamide goitrogens. When hydrogen peroxide was added to a mixture of LPX and inhibitor, a rapid loss of enzymatic activity was observed. The inactivation rate was greatly retarded when the incubations were carried out in the presence of an alternate substrate, thiocyanate ion (cf. Figure 1). Thiocyanate ion was previously shown to be a substrate for LPX, which was competitive with iodide ion (Tenovuo, 1978), a putative biological substrate (Morrison & Schonbaum, 1976). Iodide ion was not used to retard the inhibitory effects of NMBI due to the ability of enzymatically produced oxidized iodine species (e.g., HOI) to oxidize thiocarbamides nonenzymatically (Edelhoch et al., 1979). The inhibition was presumed to be covalent as previously shown for MMI-modified TPX (Engler et al., 1982) since (a) separation of excess unbound inhibitor from the enzyme by chromatography on Sephadex G-25 did not restore enzymatic activity and (b) the modified visible spectra from MMI- and MBI-inactivated LPX were unchanged after chromatography on Sephadex G-25. The inhibition was determined to be irreversible since no recovery of activity was observed from MBI-inactivated LPX following Sephadex G-25 chromatography and a 24-h incubation at 0 °C.

The reaction of LPX with hydrogen peroxide and the production of oxidized enzymic intermediates (compounds I and II) were previously studied by stopped-flow visible spectrophotometry (Ohtaki et al., 1982). The initial product from the reaction of hydrogen peroxide with LPX had spectral properties and reactivity characteristic of a two-electron-oxidized intermediate similar to compound I from other hemoproteins like HRP (Hewson & Hager, 1979). This intermediate was rapidly transformed ($t_{1/2}$ = 0.36 s) by unidentified

Scheme II: Kinetics of Conversions of LPX in the Presence of Inhibitor and Hydrogen Peroxide



donors in the protein to another intermediate with spectral similarities to compound II of HRP (Hewson & Hager, 1979). This intermediate was called LPX compound II although the unpaired electron spin has probably been transferred from the porphyrin cation radical of compound I to form another unidentified radical center on the protein. A similar phenomenon was described for the reaction of ferrylmyoglobin with styrene (Ortiz de Montellano & Catalano, 1985). This contrasts HRP compound II, which is formed from the reaction of HRP compound I with reducing substrates in solution. Ohtaki et al. concluded that MMI increased the rate of transformation of compound I to compound II and that MMI reacted with compound II to give inactivated LPX (Ohtaki et al., 1982). Their conclusion that compound II reacted with MMI and sulfide ion to yield modified LPX products is supported by the results of this study.

An explanation of these observations is that sulfur-containing compounds, including sulfide ion, MBI, MMI, and NMBI, react with LPX compound II, the oxoferrylporphyrin form of LPX, to yield a reactive intermediate that is capable of irreversibly inactivating critical active-site residues, including the heme prosthetic group (cf. Scheme I). Scheme II provides

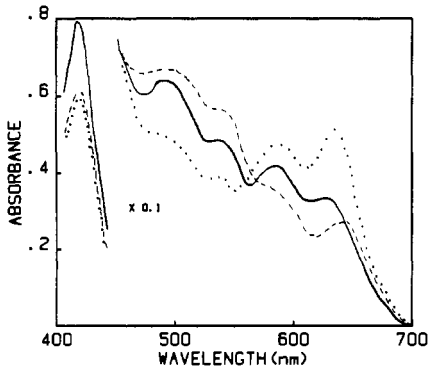


FIGURE 3: Visible spectra for LPX and inactivated LPX. LPX samples were prepared and spectra recorded as described under Materials and Methods. Native LPX, —; MBI-inactivated LPX, - - -; MMI-inactivated LPX, ····.

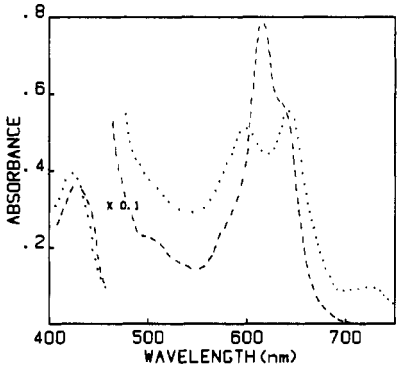


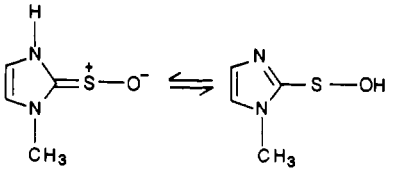
FIGURE 4: Visible spectra for ferrous and ferric MMI–LPX. LPX samples were prepared and spectra recorded as described under Materials and Methods. Ferrous MMI–LPX, - - -; ferric MMI–LPX, ····.

a framework for these observations and shows the branched pathway characteristic of mechanism-based inhibitors (Waley, 1985). Depending on the relative values of k_r and k_i (the rates of reduction and inactivation in the presence of inhibitor, respectively), partitioning of LPX compound II will occur between reductive (turnover) and inactivation pathways.

The abscissa intercepts of Figure 2 for MBI and MMI were determined to be approximately 7 and 10, respectively, in the presence of excess hydrogen peroxide. The kinetic treatment of Waley permits the calculation of k_r from the values of k_i and the partition ratio for inactivation vs. turnover pathways (Waley, 1985). The partition ratio "r" is calculated from the intercepts of Figure 2 by the relation intercept = $1 + r$. From the relation $r = k_r/k_i$, k_r values for MBI, MMI, and NMBI are 26, 28, and 129 min^{-1}, respectively.

The addition of excess hydrogen peroxide to mixtures of LPX plus MMI or MBI produced rapid, irreversible changes in the visible spectra of the heme prosthetic group of LPX and products with different visible spectra (cf. Figure 3). The products from sulfide ion and MMI inactivation of LPX were observed to be different on the basis of dissimilar visible spectra of the ferrous and ferric forms of the products. This is in contrast to the previous observation that the products from reaction of sulfide ion and MMI with LPX compound II were identical (Nakamura et al., 1984). When hydrogen peroxide was added to a mixture of LPX and MMI, a transient green intermediate (λ_{max} = 612 nm; cf. Figure 4) was observed, which was converted ($t_{1/2}$ ~5 min) to a stable spectral form. Treatment of this final product with ferrous EDTA caused the rapid conversion to a product with the same visible spectrum as the initial intermediate. This is in contrast to the spectrum of ferrous sulf-LPX produced by the reaction of a

Scheme III: MMI Sulfine and Formamidinesulfenic Acid Tautomers



stoichiometric amount of sodium sulfide with LPX compound II (λ_{max} = 638 nm) (Nakamura et al., 1984). This study provides experimental support for the assignment of the intermediate with a 612-nm absorption maximum as the ferrous MMI adduct of LPX and establishes a common mechanism for the reaction of sulfide ion and MMI with LPX compound II although different products are observed. Scheme I proposes reactions of LPX compound II with MMI and sulfide ion, and Figure 4 shows the visible spectra for ferrous and ferric MMI–LPX.

Treatment of the stable MMI–LPX product with ferricyanide ion caused only small changes in the visible spectrum, and the observed product had a visible spectrum consistent with the assignment of the ferric MMI–LPX adduct (λ_{max} = 725, 638, and 595 nm). This also contrasted the spectrum of ferric sulf-LPX (λ_{max} = 727 and 605 nm) (Nakamura et al., 1984).

Sulf-LPX is similar to sulfmyoglobin in (a) the method of production from hydrogen peroxide and sulfide ion and (b) visible spectral properties (Berzofsky et al., 1971). The chemical identity of sulfheme proteins has not been unambiguously determined, and several thiochlorin structures have been proposed for the heme prosthetic group (Andersson et al., 1984).

MBI-inactivated LPX has a spectrum that is distinct from native LPX but also different from MMI-inactivated LPX and sulf-LPX. Although heme modification is also deemed likely in this case, modification of active-site amino acid residues or other reactions that affect iron ligation cannot be excluded.

A recent report from this laboratory described S-oxygenation reactions catalyzed by LPX, including the first reported peroxidase-mediated S-oxygenation of the thioamide functional group (Doerge, 1986). These observations provided a rationale for the suicide inactivation of LPX by sulfur-containing compounds in the presence of hydrogen peroxide. A mechanism involving heme modification by active-site-produced electrophilic formamidinesulfenic acids (cf. Scheme III) from the S-oxygenation of the thiocarbamides by LPX compound II is consistent with the chemistry of these compounds. The related alkyl- and arylsulfenic acids (RSOH) are electron-deficient compounds (Hogg, 1979; Kice, 1980) with reactivity sufficient for potential addition to electron-rich sites of a metalloporphyrin (Fuhrhop, 1975). The analogous production of sulfur monoxide from the reaction of sulfide ion with LPX compound II would also yield a reactive electrophilic intermediate at the enzyme active site (Dodson & Sauers, 1967).

Scheme I is consistent with the final oxidation state of iron observed for the ferrous sulf-LPX produced in the reaction of stoichiometric amounts of sulfide ion with LPX compound II at pH 7.0 (Nakamura et al., 1984) and the production of ferrous MMI–LPX in the reaction of MMI with LPX compound II. In analogous model reactions, oxoferrylhemes converted triphenylphosphine to the corresponding phosphine oxide with concomitant formation of ferrous hemes (Chin et al., 1980). The ferrous forms of sulf-LPX and MMI–LPX are unstable under the aerobic experimental conditions as oxidation to the ferric state is facile.

These studies expand the scope of LPX-catalyzed S-oxygenation reactions and are of significance in the general mechanisms of enzymatic activation of goitrogenic drugs and carcinogens through S-oxygenation reactions mediated by mammalian peroxidases.

Registry No. LPX, 9003-99-0; MBI, 583-39-1; MMI, 60-56-0; NMBI, 6325-91-3.

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