Mechanism-Based Inhibition of Lactoperoxidase by Thiocarbamide Goitrogens. Identification of Turnover and Inactivation Pathways†

Daniel R. Doerge
Department of Agricultural Biochemistry, University of Hawaii, Honolulu, Hawaii 96822
Received October 14, 1987; Revised Manuscript Received January 19, 1988

ABSTRACT: Direct evidence is presented in support of mechanism-based (suicide) inactivation of lactoperoxidase by thiocarbamide thyroid inhibitors. The turnover of 1-methylbenzimidazolidine-2-thione was demonstrated by identifying the inhibitor-derived products 1-methylbenzimidazole and bisulfite ion that are formed concurrent to enzyme inactivation. The turnover of a hydroperoxide cosubstrate, 5-phenyl-4-pentenyl hydroperoxide, was quantitated from formation of the corresponding alcohol during enzyme inactivation. A specific inactivation pathway is suggested by the covalent binding of 1 mol of ^{14}C- and ^{35}S-labeled benzimidazolidine-2-thione and 1-methylbenzimidazolidine-2-thione per mole of inactivated lactoperoxidase. These results are explained by partitioning of inhibitor-derived S-oxygenated intermediates between turnover and inactivation pathways. The properties of the inactivation process are unique among thiono-sulfur compounds and suggest that benzimidazolinesulfenic acids are the reactive intermediates.

Derivatives of thiourea depress thyroid function by inhibiting the TPX[1]-catalyzed iodination and coupling reactions that are required in the synthesis of thyroid hormones (Gilman & Murad, 1976). A previous study, using the closely related LPX, provided kinetic and spectral evidence for mechanism-based (suicide) inhibition by the therapeutic antithyroid agent MMI and MBI derivatives (Doerge, 1986a). Organosulfur substrates and inhibitors of LPX showed the same linear relation between electrochemical oxidation potential and the logarithm of the apparent LPX binding constant, K_m or K_i, respectively (Doerge et al., 1987). This finding provided further indirect support for the proposal that LPX-catalyzed S-oxygenation of thiocarbamides produces reactive intermediates that bind covalently to the active-site heme.

A suicide inactivation mechanism is characterized by branched pathways leading concurrently to enzyme inactivation and turnover of the suicide substrate (Waley, 1985). This branching results from the partitioning of inhibitor-derived reactive intermediates between addition to critical groups at the enzyme active site and other reactions leading to turnover, e.g., solvolysis or addition to noncritical protein groups (Ables & Maycock, 1976). The present paper provides direct evidence for a turnover pathway that occurs concomitant to LPX inactivation by identifying products derived from the thiocarbamide inhibitor and the reduction of a hydroperoxide cosubstrate. In addition, the stoichiometry of covalent binding by ^{14}C- and ^{35}S-radiolabeled thiocarbamides to inactivated LPX provides strong evidence for a specific inactivation pathway.

Materials and Methods

Bovine LPX was purchased from Sigma Chemical Co., and the purity was checked by polyacrylamide gel electrophoresis as previously described (Doerge et al., 1987). Hydrogen peroxide, obtained as a 30% solution from Sigma, was periodically standardized by iodometric titration (Kolthoff et al., 1969). MMBI (Harrison & Ralph, 1965), benzimidazole (Wagner & Millet, 1966), MeBI (Kikugawa, 1981), synthesized by published procedures, and MBI (Eastman Kodak Co.) were purified by recrystallization from aqueous ethanol. PPA and PPHP were synthesized and analytically determined as previously described (Weller et al., 1985). The concentration of bisulfite ion was measured spectrophotometrically (Doerge & Ingraham, 1981). Reference solutions of bisulfite ion were standardized iodometrically (Kolthoff et al., 1969). ^{14}C- and ^{35}S-labeled MBI and MMBI were synthesized with radiopurity of at least 98% and with specific activities of 2.1–5.3 mCi/mmol (Doerge, 1988).

Enzymatic oxidation of iodide ion was determined at 22.0 ± 0.1 °C by stopped-flow spectrophotometry using a Hi-Tech SFA-11 rapid kinetics accessory and a Hewlett-Packard 8451A UV–vis spectrophotometer (total system dead time <0.2 s). Initial rates were determined from the linear phase of the reaction (<2 s) with the final concentrations of hydrogen peroxide, 0.2 mM; LPX, 25 nM; potassium iodide, 5 mM; and 0.1 M phosphate, pH 7.0.

LPX was inactivated by addition of limiting amounts of hydroperoxide (0–0.3 mM) to mixtures of LPX (0.005 mM) and excess inhibitor (MBI, 0.1 mM; MMBI, 0.5 mM). After 30 s at 22 °C, an aliquot of 0.05 mL was quenched by 100-fold dilution into 10 mM potassium iodide in 0.1 M phosphate, pH 7.0, and the remaining activity was determined spectrophotometrically. Except where specifically noted, 500 units of bovine catalase (Sigma) was then added to terminate uncatalyzed hydroperoxide-dependent reactions. Under these conditions, irreversible inactivation of LPX was complete in less than 30 s (Doerge, 1986a). Concentrations of bisulfite ion were determined immediately after catalase quenching of the reactions.

The complex of radiolabeled inhibitor bound covalently to LPX was separated from unbound inhibitor and products by

† This work was supported in part by a Research Starter Grant from the Pharmaceutical Manufacturers Association Foundation and by BSRG S07 RR 07026 awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health, and is contributed as Journal Series No. 3191 from the Hawaii Institute of Tropical Agriculture and Human Resources.

[1] Abbreviations: BI, benzimidazole; LPX, lactoperoxidase; MBI, benzimidazoline-2-thione; MeBI, 1-methylbenzimidazole; MMBI, 1-methylbenzimidazolidine-2-thione; MMI, 1-methylimidazoline-2-thione; NBI, 5-nitrobenzimidazole; NMBI, 5-nitrobenzimidazoline-2-thione; PPA, 5-phenyl-4-pentenyl alcohol; PPHP, 5-phenyl-4-pentenyl hydroperoxide; TPX, thyroid peroxidase.

0006-2960/88/0427-3697$01.50/0

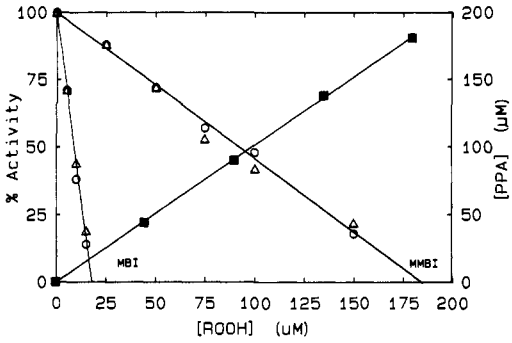


FIGURE 1: Hydroperoxide-dependent turnover and inactivation of LPX by benzimidazoline-2-thiones. LPX was inactivated with various concentrations of hydroperoxide (hydrogen peroxide, O; PPHP, Δ) in the presence of excess MBI or MMBI. The activity remaining was determined spectrophotometrically, and the concentration of PPA (■) was determined from MMBI-inactivated samples by HPLC.

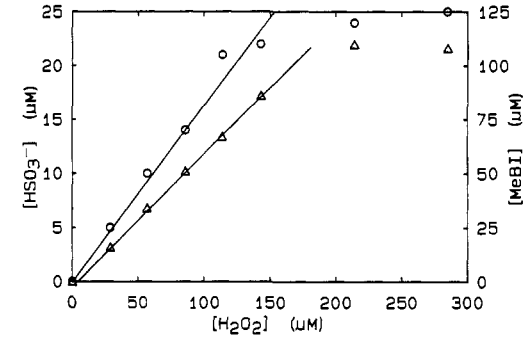


FIGURE 2: Formation of bisulfite ion and MeBI during MMBI inactivation of LPX. LPX was inactivated by different concentrations of hydrogen peroxide in the presence of excess MMBI. The concentration of bisulfite ion (O) was determined spectrophotometrically, and the concentration of MeBI (Δ) was determined by HPLC.

dialysis or gel permeation chromatography. Changes in protein concentration during chromatography and dialysis were monitored spectrophotometrically at 280 and 410 nm. Radioactive product fractions were quantitated by liquid scintillation counting (Beckman DPM 100). In radioactivity release studies, LPX was completely inactivated with either ^{14}C- or ^{35}S-labeled MBI and MMBI, the reaction terminated and treated with either 100 mM potassium cyanide or 100 mM 2-mercaptoethanol for 1 h at 22 °C, and the radioactivity of the dialysate determined.

The production of MeBI was measured by HPLC (Nova-PAK C_{18}, Waters Associates, 25% acetonitrile/water containing 0.01% triethylamine, 1.5 mL/min flow rate, and 260-nm absorbance detection). For PPA and PPHP determinations, reactions were terminated after 30 s by aspiration through 3-mL C_{18} SPE columns (J. T. Baker Chemical Co.), eluted with methanol, and analyzed by HPLC (Nova-PAK C_{18}, 50% acetonitrile/water, 2.0 mL/min flow rate, and 257-nm absorbance detection). Quantitative HPLC measurements were made by comparing sample peak heights with those generated by known amounts of synthetic standards.

Results

Incubation of limiting amounts of PPHP with LPX and excess MBI or MMBI resulted in quantitative production of PPA concomitant to LPX inactivation (Figure 1). When excess PPHP was added to LPX alone, incomplete conversion to PPA was observed (40%). The formation of other unidentified products is likely since under these conditions 90% of the PPHP was consumed. Figure 1 also shows the hydrogen peroxide dependent loss of LPX activity in the presence of inhibitor. The coincident lines demonstrate the stoichiometric equivalence of PPHP and hydrogen peroxide for LPX inactivation. The partition ratios for MBI and MMBI, correlated with actual hydroperoxide turnover as shown in Figure 1, are 2.5 and 35.5, respectively (Waley, 1985).

Incubation of MMBI with LPX and either PPHP or hydrogen peroxide yields MeBI and bisulfite ion (Figure 2). The production of bisulfite ion and MeBI were linearly dependent on the amount of hydroperoxide added up to the maximal conversion at approximately 0.185 mM hydroperoxide. Approximately 5 and 22 equiv of bisulfite and MeBI, respectively, were produced per equivalent of LPX inactivated. Since peroxidative reagents convert MMBI to these same products, catalase was used to terminate nonenzymatic reactions. The termination procedure was judged effective since addition of hydroperoxides to MMBI in the absence of LPX produced MeBI and bisulfite ion in concentrations less than 2% of the enzyme-catalyzed levels during analogous 30-s reaction intervals.

Incubation of MBI or MMI with LPX under the same conditions did not produce measurable amounts of bisulfite ion or the corresponding imidazole. This could arise from the approximately 15-fold lower partition ratio for MBI vs MMBI. If this same ratio holds for product formation, 0.4 and 1.5 equiv of bisulfite and BI, respectively, are predicted. The estimated limit of detection for both bisulfite ion and BI is 1 equiv.

Sulfate ion was not detected in reactions of LPX and hydroperoxides with MBI or MMBI. No decrease in supernatant radioactivity was observed following the addition of barium chloride to and centrifugation of mixtures of LPX previously inactivated with [^{35}S]MBI or [^{35}S]MMBI (Vogel, 1978). Positive control experiments showed that approximately 90% of the radioactivity could be precipitated by the same procedure after oxidation with excess bromine.

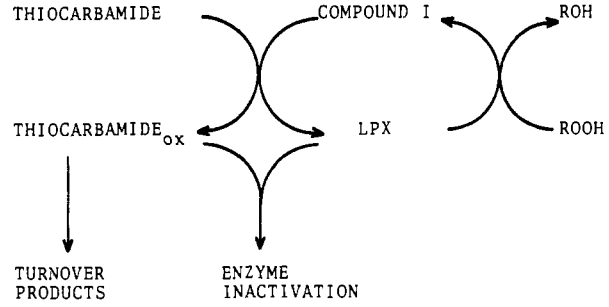
The covalent binding of ^{14}C- and ^{35}S-labeled MBI and MMBI to LPX was measured following chromatography on Sephadex G-25 or dialysis. One mole of [2-^{14}C]MBI (0.9 ± 0.1), [^{35}S]MBI (1.0 ± 0.1), [1′-^{14}C]MMBI (1.0 ± 0.2), and [^{35}S]MMBI (1.0 ± 0.1) binds per mole of LPX concomitant to total enzyme inactivation. The bound radioactivity was determined after complete inactivation of LPX with hydroperoxide (0.018 or 0.185 mM, respectively). In another experiment, LPX activity was titrated with limiting amounts of hydroperoxide (0.005–0.02 mM) in the presence of excess [^{14}C]- and [^{35}S]MBI and the bound radioactivity determined as above. Equivalent amounts of ^{14}C- and ^{35}S-labeled inhibitors were bound to LPX, and the number of moles of radiolabeled MBI that bound covalently to LPX was directly related to the number of moles of LPX inactivated (slope = 0.93 MBI bound/LPX inactivated).

The release of LPX-bound inhibitors by thiophilic reagents was investigated with 2-mercaptoethanol and cyanide ion since these compounds are effective nucleophiles in substitution reactions at divalent sulfur (Parker & Kharasch, 1960). These two reagents released similar amounts of either ^{14}C or ^{35}S from MBI-inactivated LPX. Equivalent release of the radiolabels was also observed with MMBI-inactivated LPX. Cyanide ion caused the release of 49 ± 5% of bound MBI but no release of bound MMBI. In contrast, no MBI and small amounts of MMBI (13 ± 6%) were released by 2-mercaptoethanol.

Discussion

The results of this study provide the first direct evidence for mechanism-based inhibition of LPX by identifying both turnover and inactivation pathways for benzimidazolidine-2-

Scheme I: Suicide Inactivation of LPX by Thiocarbamides



thiones. Bisulfite ion and MeBI are products derived from MMBI, and the quantitative conversion of PPHP to PPA demonstrates turnover of the peroxidase cycle during inactivation of LPX by MBI and MMBI. The covalent binding of 1 mol of ^{14}C- and ^{35}S-labeled MBI and MMBI concomitant to LPX inactivation and heme modification results from the enzyme inactivation pathway. These observations, in conjunction with the indirect evidence of previous studies, provide strong support for the proposed mechanism of suicide inactivation shown in Scheme I.

Previous studies on the stoichiometry of LPX inactivation suffered from the limitation that actual consumption of hydroperoxide was not determined (Doerge, 1986a). In the present study, a direct measure of the quantitative turnover of hydroperoxide is accomplished by the use of PPHP (Weller et al., 1985). The UV absorbance of the reduction product, PPA, allows its sensitive detection and quantitation by HPLC from peroxidase-catalyzed reactions. The cosubstrate activity of PPHP with several peroxidases, including LPX, was previously demonstrated (Marnett & Weller, 1986). Substoichiometric conversion of PPHP to PPA was observed only when inhibitor concentration was limiting. Therefore, it is concluded that a more accurate determination of partition ratios is obtained by using limiting amounts of hydroperoxide and excess inhibitor. Partition ratios determined in this way accurately reflect enzymatic turnover stoichiometry since it is known that each peroxidase cycle requires 1 mol of hydroperoxide (Hewson & Hager, 1979).

MBI and MMBI were chosen as model compounds for the antihyperthyroid drug, MMI. Inactivation of LPX by MMI gives the same final spectral form as MMBI (data not shown), but MBI gives a different spectral form (Doerge, 1986a). MMBI was selected because of its 15-fold higher partition ratio relative to MBI and MMI, which permits the formation of detectable amounts of turnover products during the inactivation reaction.

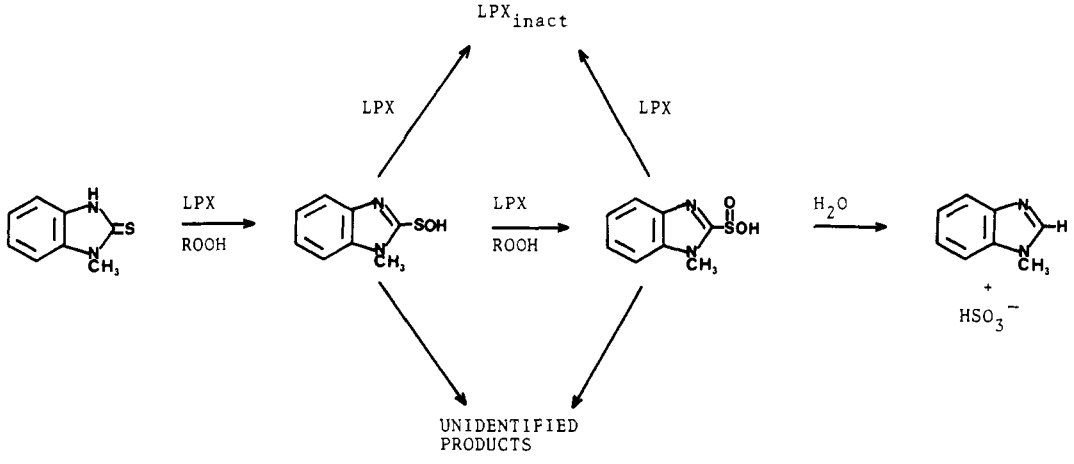
Oxygenation of MBI and MMBI with *m*-chloroperbenzoic acid or hydrogen peroxide results in the formation of bisulfite ion and the corresponding benzimidazole (data not shown). The ability of LPX to catalyze S-oxygenation reactions (Doerge, 1986b) suggested that these products would be formed by the action of LPX on imidazolidine-2-thiones. The identification of MeBI and bisulfite ion as products from MMBI provides indirect evidence for the formation of LPX-derived reactive S-oxygenated intermediates. The partitioning of the putative reactive intermediate between addition to the heme prosthetic group and further reactions, including solvolysis, leads to inactivation and product formation, respectively (Scheme II).

Bisulfite ion and the corresponding imidazole product are not detected during inactivation of LPX by MMI and MBI. It is likely that this is due to the much lower partition ratios for MBI and MMI since inactivation with NMBI, another high partition ratio inhibitor (r = 49), also results in the formation of detectable amounts of bisulfite ion and NBI. This suggests that the reactive intermediates derived from MMI and MBI preferentially react by pathways that do not lead to the formation of the turnover products as seen with MMBI (Scheme II). However, until all products derived from MBI, MMI, and MMBI are identified, definitive conclusions about the reactivity of the intermediates cannot be made.

The covalent binding of both ^{14}C- and ^{35}S-labeled inhibitors to inactivated LPX in stoichiometric amounts suggests that the entire molecule binds rather than the separate binding of sulfur-containing and carbon-containing fragments. This pattern of binding is consistent with the addition of a sulfenic acid intermediate to the prosthetic heme as previously proposed (Doerge, 1986a). This contrasts the action of cytochrome P450 on thiono-sulfur xenobiotics (carbon disulfide, MMI, and parathion) that leads to unequal binding of sulfur and carbon portions of the compounds (Neal, 1980). Neal and co-workers concluded that singlet atomic sulfur was a major reactive species from cytochrome P450 catalyzed oxygenation of MMI, parathion, and carbon disulfide and was responsible for the observed covalent binding to hepatic microsomal protein. In these studies, it was found that the thiophilic reagents cyanide ion and dithiothreitol caused the release of protein-bound ^{35}S label (Neal, 1980; Halpert et al., 1980). This reactivity was consistent with that of a hydrodisulfide derivative of cysteine.

Treatment of MBI- or MMBI-inactivated LPX with the thiophilic reagents cyanide ion and 2-mercaptoethanol caused the release of similar amounts of ^{14}C or ^{35}S radiolabel for each inhibitor. This equivalent release of label also supports the conclusion that the sulfur- and carbon-containing portions of

Scheme II: Partitioning Reactions of Proposed Intermediates Derived from MMBI



both inhibitors are bound to LPX by a common covalent linkage. The difference in the release by cyanide ion of covalently bound radiolabel from MBI- and MMBI-inactivated LPX (49% vs 0%, respectively) suggests that different adducts are formed by the two inhibitors. This conclusion is also supported by the observation of different visible spectra of MBI- and MMBI-inactivated LPX (Doerge, 1986a). Treatment of MBI-inactivated LPX with cyanide ion does not cause the release of the modified heme since the total absorbance of the Soret band in the visible spectrum is unchanged following gel permeation chromatography. However, changes in the long-wavelength bands of the modified LPX spectrum are consistent with an altered adduct structure. The molecular basis for the different MBI and MMBI adducts and their different cyanide ion reactivities is unknown at the present time.

LPX-catalyzed metabolism of thiocarbamides is characterized by a unique pattern of binding by both ^{14}C and ^{35}S portions of the thiono-sulfur inhibitor to the inactivated enzyme and a 1:1 relationship between loss of enzymatic activity and the moles of bound inhibitor. These observations suggest the reactions of a novel intermediate, although its identity cannot be unambiguously determined at this time since both sulfenic and sulfinic acids possess a reactive sulfur center that is known to add to olefins (Scheme II) (Stirling, 1971; Shelton & Davis, 1973). The binding of 1 mol of inhibitor to the heme of LPX suggests confinement of the reactive intermediate to the enzyme active site, as predicted for a true mechanism-based inhibitor (Ables & Maycock, 1976).

This study provides strong support for the previously proposed mechanism of LPX suicide inactivation by thiocarbamide goitrogens. Since thiocarbamides are used clinically in the treatment of hyperthyroidism, details on the mechanism of action are essential for their continued safe and effective use.

Registry No. MBI, 583-39-1; MMI, 2360-22-7; LPX, 9003-99-0.

References

Ables, R. H., & Maycock, A. L. (1976) *Acc. Chem. Res. 9*, 313.

Doerge, D. R. (1986a) *Biochemistry 25*, 4724.

Doerge, D. R. (1986b) *Arch. Biochem. Biophys. 244*, 678.

Doerge, D. R. (1988) *J. Labelled Compd. Radiopharm.* (in press).

Doerge, D. R., & Ingraham, L. L. (1980) *J. Am. Chem. Soc. 102*, 4828.

Doerge, D. R., Pitz, G. L., & Root, D. P. (1987) *Biochem. Pharmacol. 36*, 972.

Gilman, A. G., & Murad, F. (1975) in *Pharmaceutical Basis of Therapeutics* (Goodman, L. S., & Gilman, A., Eds.) pp 1410–1415, Macmillan, New York.

Halpert, J., Hammond, D., & Neal, R. A. (1980) *J. Biol. Chem. 255*, 1080.

Harrison, D., & Ralph, J. T. (1965) *J. Chem. Soc.*, 3132.

Hewson, W. D., & Hager, L. P. (1979) *Porphyrins 7*, 295–325.

Kikugawa, Y. (1981) *Synthesis*, 124.

Kolthoff, I. M., Sandell, E. B., Meehan, E. J., & Bruckenstein, S. (1969) in *Quantitative Chemical Analysis*, pp 842–860, Macmillan, London.

Marnett, L. J., & Weller, P. E. (1986) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 45*, 1642.

Neal, R. A. (1980) *Rev. Biochem. Toxicol. 2*, 131–171.

Parker, A. J., & Kharasch, N. (1960) *J. Am. Chem. Soc. 82*, 3071.

Shelton, J. R., & Davis, K. E. (1973) *Int. J. Sulfur Chem. 8*, 205.

Stirling, C. J. M. (1971) *Int. J. Sulfur Chem., Part B 6*, 277.

Vogel, A. (1978) in *Textbook of Quantitative Inorganic Analysis*, pp 504–507, Longman, New York.

Wagner, E. C., & Millet, W. H. (1943) *Org. Synth. 2*, 65–66.

Waley, S. G. (1985) *Biochem. J. 227*, 843.

Weller, P. E., Markey, C. M., & Marnett, L. J. (1985) *Arch. Biochem. Biophys. 243*, 633.