Chemical and Enzymatic Oxidation of Benzimidazoline-2-thiones: A Dichotomy in the Mechanism of Peroxidase Inhibition

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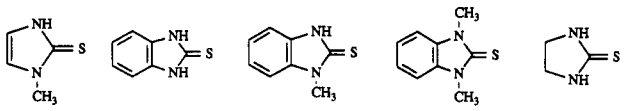
ABSTRACT: Derivatives of imidazole-2-thiones block reactions catalyzed by thyroid peroxidase (TPX) and the closely related lactoperoxidase (LPX), and this property is used therapeutically to treat hyperthyrodism. The reactions of a series of benzimidazoline-2-thiones with chemical and enzymatic oxidants were investigated to probe systematically the mechanism of inhbition. Oxidation of benzimidazoline-2-thione (I) and 1-methylbenzimidazoline-2-thione (II) with 3-chloroperbenzoic acid (PBA) yielded reaction products and stoichiometry consistent with benzimidazole-2-sulfenic acids as reactive intermediates. The N,N′-disubstituted nature of 1,3-dimethylbenzimidazoline-2-thione (III) precludes sulfenic acid formation by tautomerization, and the oxidation of III with PBA yielded products and stoichiometry that were consistent with a benzimidazole-2-sulfonyl ylide as the reactive intermediate. I and II are suicide inhibitors of LPX and TPX, but III was found to inhibit only peroxidase-catalyzed iodination reactions by an alternate substrate mechanism. These results provide support for the hypothesis that imidazole-2-sulfenic acids are important reactive intermediates in the suicide inactivation of TPX and LPX and relate the chemical reactivity of the inhibitor with both the potency and mechanism of inhibition. These results suggest that 1,3-disubstituted thiourea derivatives represent a new class of potential antihyperthyroid drugs that block TPX-catalyzed tyrosine iodination but do not cause irreversible enzyme inactivation.

Derivatives of thiourea (thiocarbamides) depress thyroid function by inhibiting the iodination and coupling of tyrosine residues required in the TPX[1]-catalyzed synthesis of thyroid hormones (DeGroot et al., 1984). Imidazoline-2-thione derivatives are suicide inhibitors of TPX and the closely related LPX (Engler et al., 1982; Doerge, 1986, 1988a). Irreversible inactivation proceeds by kinetics consistent with a suicide mechanism, covalent binding of 1 mol of inhibitor/equiv of enzyme inactivated, and changes in the visible spectrum of LPX consistent with covalent binding to the heme prosthetic group (Doerge, 1986, 1988a). Suicide inhibition of TPX is utilized therapeutically by drugs (e.g., MMI; see Chart I) that reduce excessive levels of thyroid hormones found in patients with Graves' disease (Cooper, 1984).

Formation and reactions of unstable S-oxygenated intermediates are central to the proposed mechanism for suicide inactivation of LPX by benzimidazoline-2-thiones (Doerge, 1986, 1988a). The present study used a series of N-substituted benzimidazoline-2-thiones (Chart I) to probe systematically the identity of reactive intermediates formed by chemical and enzymatic S-oxidation. Chemical studies with hydroperoxides were used to model the S-oxidation reactions of peroxidase compound I because it was shown that the same sulfoxide

Chart I: Benzimidazoline-2-thiones



products are formed (Doerge et al., 1986). Enzymatic studies using LPX were conducted either without iodide ion to model suicide inactivation conditions (Doerge, 1988a) or in the presence of high concentrations of iodide ion to model the possible reaction conditions that occur in the thyroid (Engler et al., 1983). This study demonstrates that structural modifications of benzimidazoline-2-thiones not only affect the relative potency of suicide inhibitors but also can determine the *mechanism* of inhibition.

MATERIALS AND METHODS

Bovine LPX, bovine liver catalase, glucose oxidase, 30% hydrogen peroxide, glucose, guaiacol, ABTS, and deuterium oxide were purchased from Sigma Chemical Co. Selenium dioxide, [^{13}C]methyl iodide, and PBA were purchased from Aldrich Chemical Co. and PBA was purified as described (Fieser & Fieser, 1967). Purity of LPX was checked by PAGE (Doerge et al., 1986), and hydroperoxide concentrations were determined by iodometric titration (Koltoff et al., 1969). Aldrich Chemical Co. supplied I, which was recrystallized from aqueous ethanol as were II (Duffin & Kendall, 1956), III (Duffin & Kendall, 1956), and Ia (Wagner & Millet, 1943). Distillation was used to purify IIa (Kikugawa, 1981). IIIa (iodide salt) was synthesized and purified by recrystallization from ethyl acetate/methanol (Karkhanis & Field, 1985). A published method was used to prepare [1-^{13}C]II from [^{13}C]methyl iodide (Doerge, 1988b). Preparation, purification, and analysis of PPHP and PPA were accom-

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[1] Abbreviations: ABTS, 2,2′-azinobis(benzothiazoline-6-sulfonic acid); DSS, 2,2′-dimethyl-2-silapentane-5-sulfonic acid; EI, electron impact; ETU, imidazolidine-2-thione; FAB, fast atom bombardment; I, benzimidazoline-2-thione; Ia, benzimidazole; II, 1-methylbenzimidazoline-2-thione; IIa, 1-methylbenzimidazole; III, 1,3-dimethylbenzimidazoline-2-thione; IIIa, 1,3-dimethylbenzimidazolium; LPX, lactoperoxidase; MMI: 1-methylimidazoline-2-thione (methimazole); PAGE, polyacrylamide gel electrophoresis; PBA, 3-chloroperbenzoic acid; PPA, 5-phenylpentenyl alcohol; PPHP, 5-phenylpentenyl hydroperoxide; TFA, trifluoroacetic acid; TPX, thyroid peroxidase; TSP, thermospray.

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plished as described (Weller et al., 1985). The methyl ester of 1-methylbenzimidazole-2-sulfinic acid was prepared by addition of hydrogen peroxide (5 equiv of a 10 M solution) to II (0.1 M) in methanol. The products, IIa and the sulfinic acid methyl ester, were obtained following preparative TLC on silica (Uniplate; Analtech Co., Newark, DE) using 5% methanol/chloroform as eluant.

Oxidation products were identified by UV spectra (Hewlett Packard 8452A diode array spectrophotometer), NMR spectra at 300 and 76 MHz for ^1H and ^{13}C, respectively (Nicolet NT300 or General Electric QE300 spectrometer), using DSS (^1H) and dioxane (^{13}C) as references, and mass spectrometry (VG Trio 2A). FAB-MS was performed using a continuous-flow (dynamic) probe with the mobile phase methanol/water/glycerol/TFA (45/45/10/0.1) and an 8-kV Xe atom beam. Products and starting materials were quantitated by HPLC using a Perkin Elmer 410 pump and LC95 UV detector by comparing peak heights with those generated by known amounts of standards. I was chromatographed on Novapak silica (4 μm, 8 × 100 mm cartridge; Waters Associates, Milford, MA) with 25% acetonitrile/water containing 0.01% triethylamine, a flow rate of 1.5 mL/min, and detection at 305 nm. Chromatography of II and III was performed using a Novapak C18 cartridge (4 μm, 8 × 100 mm; Waters Associates) with 35% or 60% acetonitrile/water, respectively, a 1.5 mL/min flow rate, and detection at 305 nm. For TSP LC-MS analysis, a Hamilton PRP column was used with 50% acetonitrile/0.1 M ammonium acetate, pH 7.0, at a flow rate of 0.5 mL/min with the TSP capillary temperature at 190 °C and source temperature at 250 °C. The production of bisulfite ion was measured colorimetrically (5) and sulfate ion was determined by ion chromatography using an AS4A column (Dionex Co., Sunnyvale, CA) with a Dionex Gradient Pump Module and a Dionex CDM II conductivity detector equipped with an AMMS suppressor using 3 mM sodium carbonate/bicarbonate buffer, pH 10, as eluant at 2.0 mL/min.

Oxidation stoichiometries with PBA were determined in methanol by measuring thione concentration spectrophotometrically at 305 nm after addition of limiting amounts of PBA to 100 μM I–III. Stoichiometries of substrate consumption/product formation were also determined by addition of limiting amounts of PBA to 100 μM I or II in 50% methanol pH 7/potassium phosphate, 100 mM. In this case, disappearance of I and II and production of Ia, IIa, and bisulfite ion were determined after 1 h at 25 °C. Reaction products were also monitored by ^1H and ^{13}C NMR.

The oxidation kinetics of I–III were measured spectrophotometrically (305 nm) at 22 ± 1 °C by addition of thione (final concentration 25 μM) to hydrogen peroxide (34 mM) and TFA (163 mM) in methanol. Relative rates were calculated from the initial slopes.

Inhibition of LPX activity by I–III was determined from the kinetics of oxidation of iodide ion and guaiacol (Doerge, 1986) or ABTS (Mansson-Rahemtulla et al., 1988; Doerge & Takazawa, 1990) assays conducted at 25 ± 0.2 °C. Kinetic parameters of suicide inactivation were determined as previously described (Doerge, 1986).

Radiolabeled I, II, and III were synthesized with ^{14}C and ^{35}S and the specific activities and purity were determined as previously described (Decker & Doerge, 1992). Covalent binding of radiolabeled thiocarbamides was determined in incubations containing LPX (5 μM) and thiocarbamide (250 or 500 μM, ca. 0.03–0.4 μCi) with either no hydrogen peroxide, hydrogen peroxide (200 μM), or hydrogen peroxide + iodide ion (5 mM) in 50 mM potassium phosphate buffer, pH 7.0.

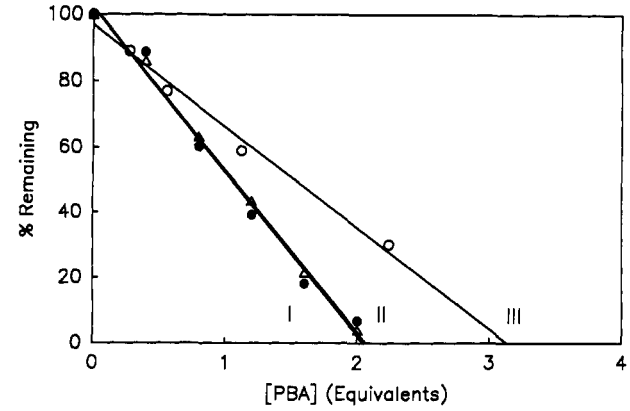


FIGURE 1: Stoichiometry of thione oxidation by PBA. Thione (I–III, 100 μM) was incubated with varying amounts of PBA (0–250 μM) as described in Materials and Methods, and the amount of thione remaining was determined spectrophotometrically at 305 nm.

Table I: Reactant/Product Stoichiometry for PBA Oxidations[a]

product or reactant	stoichiometry I	stoichiometry II
PBA	1.89	1.98
benzimidazole	0.89	0.87
bisulfite/thione	0.72	0.72

[a] Thione (100 μM) was incubated with varying concentrations of PBA (0–200 μM); reactants and products were determined by LC as described in the Materials and Methods section; and the ratios of products formed or PBA consumed to thione consumed were calculated. Values are the average of two slope determinations (moles of product formed or reactant consumed vs moles of oxidant added) from lines containing at least five data points.

The reactions were conducted at 22 ± 1 °C for 1 min and terminated by addition of catalase (500 units). The radioactivity bound to LPX was determined by LSC, and LPX heme concentration was determined spectrophotometrically at 412 nm before and after dialysis in phosphate buffer (4000× volume with two changes, 50 mM, pH 7.0).

The products from enzymatic oxidation of III were determined by ^1H NMR. To 10 mL of a solution containing potassium phoshate (10 mM), pH 7, potassium iodide (5 mM), III (100 μM), and LPX (50 nM) was added hydrogen peroxide (300 μM) to initiate the reaction. After 1 min at 22 ± 1 °C, the reaction was terminated by addition of 500 units of catalase and the solution was lyophilized. The solids were dissolved in 1 mL of D_2O and lyophilized again. Proton–deuterium exchange was repeated twice more, and the solids were dissolved in 0.5 mL of D_2O containing DSS prior to NMR analysis. The HOD peak was suppressed in a one-pulse presaturation experiment where the HOD peak was irradiated for 2 s prior to acquisition of sample resonances. The ^1H NMR spectra of III and IIIa were similarly recorded in D_2O.

RESULTS

Chemical Oxygenation Studies. The kinetics and products from reactions of hydroperoxide with I–III were used as models for enzymatic reactions under suicide inactivation conditions, i.e., no iodide ion present (Doerge, 1988a). The rapid reaction ($t_{1/2}$ < 10 s at 25 °C) of benzimidazole-2-thiones with PBA in methanol provided a convenient method for determining oxidation stoichiometry. The disappearance of thione upon addition of limiting amounts of peracid is shown in Figure 1. The slopes and intercepts showed that I and II required 2 mol of oxidant for total reaction whereas III required 3 mol. The reaction of I and II with PBA was also examined in aqueous-

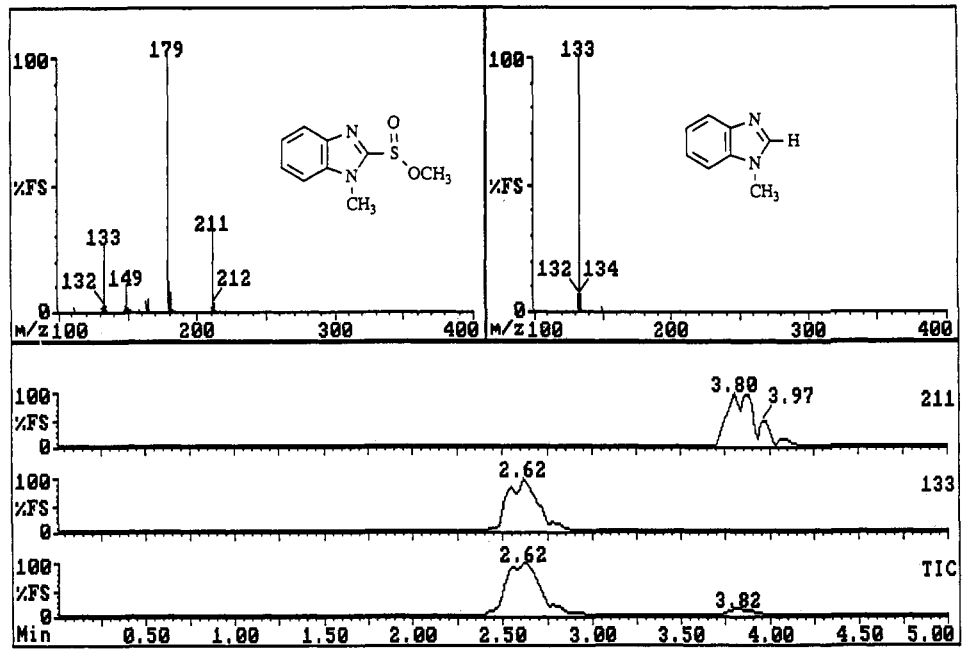


FIGURE 2: TSP MS analysis of the oxidation product from II. II was oxidized with excess hydrogen peroxide/SeO_2 in methanol at 0 °C and analyzed by TSP MS as described in Materials and Methods. Mass chromatograms for the total ion current (TIC), IIa (M + H^+) = 133 m/z, and the corresponding sulfinic acid methyl ester (M + H^+) = 211 m/z, are displayed along with the corresponding mass spectra.

buffered methanol (Table I). In this solvent the consumption of I and II was accompanied by the nearly quantitative production of Ia and IIa, respectively, and bisulfite ion. None of the corresponding benzimidazoline-2-one products were observed by LC/UV or ^1H or ^{13}C NMR (limit of detection = ca. 1% yield). Approximately 2 mol of PBA was also required for consumption of I and II in aqueous solution. In a aqueous medium, 3 equiv of PBA converted III into IIIa and sulfate ions in quantitative yield as determined by ^1H NMR and ion chromatography, respectively (data not shown). No bisulfite ion was detected from chemical oxidation of III (limit of detection = ca. 1% yield).

The oxidative reagent produced from hydrogen peroxide/selenium dioxide in methanol (Drabowitz & Mikolajczak, 1978) was found to oxidize cleanly benzimidazole-2-thione derivatives. The oxidation products from II were analyzed using GC-MS (data not shown) and TSP LC-MS (Figure 2). In both cases, two distinct chromatographic peaks were observed with protonated molecular ions corresponding to IIa and 1-methylbenzimidazole-2-sulfinic acid methyl ester. To characterize further the reaction products, II was synthesized with ^{13}C as an NMR reporter group. In contrast to the chromatographic results, ^{13}C NMR showed only one resonance at δ = 33.6 ppm. This resonance was different from those determined for the chromatographically observed products, IIa and the sulfinic acid methyl ester (31.2 and 30.9 ppm, respectively). The primary product was stable indefinitely at 5 °C in methanol. When less than 2 equiv of oxidant were added, NMR analysis showed only product and starting material resonances and no intermediates were observed (data not shown). Since the initial oxidation product is stable in aqueous methanol, it is likely that surface interactions during chromatography catalyze its decomposition, especially at elevated temperatures.

The identity of the primary product was investigated using continuous-flow FAB-MS in positive and negative ion modes. This FAB-MS method facilitated the observation of weak signals because spectra were obtained after background subtraction of the intense matrix peaks. The reliability of mass assignments was further enhanced by using two isotopic derivatives of II for the oxidation reaction, native and 1-CD_3-[2-^{13}C]I. The isotopic shifts of 4 mass units for monomeric and 8 mass units for dimeric products aided in making product assignments. In positive ion mode, low intensity ions were observed at 405/413 m/z that corresponded to those predicted for a methanol adduct of the sulfinylsulfone. Ions at 327/335 m/z corresponded to those for a disulfide fragment. It should be noted that all these ions were of low abundance as the base peak (m/z 133) was due to protonated IIA, a fragmentation product. Analysis by FAB-MS in the negative ion mode showed ions at 111 and 97 m/z with both isotopic variants of II. However, when the reaction was performed in CD_3OD instead of CH_3OH, the observed masses were 114 and 97 m/z. These masses are consistent with $^-$O-S-S-OCH_3 or $^-$S-SO-OCH_3, methanolysis products derived from a compound containing an oxidized disulfide linkage.

Kinetics of thione oxygenation were determined and the order of reactivity was I > II > III (relative rates = 2.3:1.3:1, respectively).

Enzymatic Oxidation Studies. In the absence of iodide ion, I and II caused irreversible inactivation of LPX (Doerge, 1986, 1988a) and TPX (Doerge & Takazawa, 1990) as measured by guaiacol, iodide, or ABTS oxidation assays. The LPX suicide inactivation kinetic parameters for I and II were determined to be K_i = 0.1 and 274 μM and the partition ratios were 3 and 35, respectively. However, when I and II were incubated with LPX/hydrogen peroxide *in the presence of iodide ion*, no loss of enzyme activity was observed (data not shown).

The presence of III in assay mixtures containing guaiacol or ABTS (without iodide ion) and LPX plus hydrogen peroxide did not affect the rate of enzymatic oxidation of these two typical peroxidase substrates. However, the presence of III in assay media *containing iodide ion* caused marked inhibition of LPX-mediated iodide ion oxidation as determined by triiodide ion formation. The inhibition was characterized by a lag phase during which triiodide ion formation was retarded followed by an increased rate approaching the uninhibited oxidation rate (Figure 3). Control experiments showed that hydrogen peroxide depletion and not enzyme inactivation was

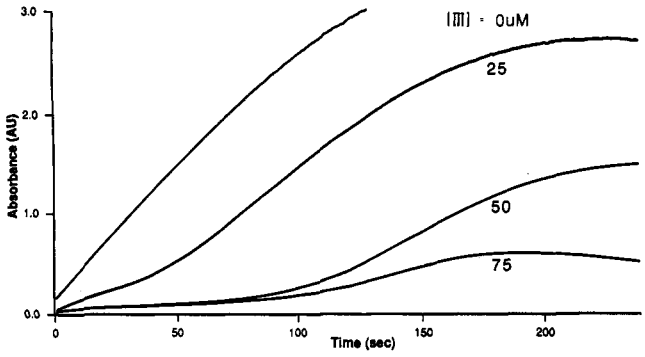


FIGURE 3: Inhibition of iodide ion oxidation by III. LPX (25 nM final concentration) was incubated with iodide ion (5 mM) and a hydrogen peroxide generating system (10 μg/mL glucose oxidase plus 1 mg/mL glucose) at 25 °C with III at the indicated concentrations. Iodide ion oxidation was monitored spectrophotometrically at 352 nm.

responsible for the deviations from control rates. This lag period was directly related to the concentration of III but inversely related to the concentration of iodide ion or hydrogen peroxide in the assay mixture (data not shown). Figure 4 shows the results of repetitive scanning during the lag phase of III-inhibited LPX-mediated iodide ion oxidation. This showed that the lag in triiodide ion formation (290- and 352-nm absorbance bands) is accompanied by disappearance of III (305-nm band). Production of triiodide ion began *only* after III had been consumed, and the absorption bands at 270 and 278 nm indicated the formation of IIIa as an oxidation product.

Incubation of LPX/hydrogen peroxide with III in the *absence* of iodide ion did not cause any loss of enzymatic activity (see Table II). This is in contrast to the action of I and II which produced rapid, irreversible loss of LPX activity under similar conditions (Doerge, 1988a). Under conditions where III is converted to IIIa and iodide ion oxidation is completely blocked, no loss of enzymatic activity is detected following 1/200 dilution (Table II). Enzymatic oxidation of III by LPX/hydrogen peroxide occurs only in the presence of iodide ion (data not shown).

The diagnostic methyl and aromatic ^{1}H NMR resonances of III and IIIa provided a selective method to detect enzymatic

Table II: Invariance of LPX Activity in Incubations Containing III[a]

incubation conditions	rate (% control)	incubation conditions	rate (% control)
LPX + III	100 ± 4	LPX + H_2O_2 + III + I^-	107 ± 6
LPX + H_2O_2	99 ± 4	LPX + H_2O_2 + I^-	104 ± 6
LPX + H_2O_2 + III	104 ± 6		

[a] LPX (0.1 μM, final concentration) was incubated with hydrogen peroxide (100 μM), iodide ion (5 mM), and III (100 μM) as indicated. After a 100-s incubation at 25 °C, an aliquot was diluted 200-fold and the rate of iodide ion oxidation was determined. Values shown are averages ± standard deviations (n = 4) of the percentage relative to untreated LPX.

transformation of III. Figure 5 shows the ^{1}H NMR spectra of III, IIIa, and a reaction mixture containing LPX, III, and iodide ion after addition of 3 equiv of hydrogen peroxide. The only product observed was IIIa. Control experiments were conducted under the same conditions except that (a) no iodide ion was present, (b) no LPX was present, and (c) no hydrogen peroxide was present. In all cases, no conversion of III to IIIa was observed. Formation of IIIa was also confirmed spectrophotometrically (see Figure 4).

When triiodide ion was formed by addition of hydrogen peroxide to LPX and iodide ion, subsequent addition of III caused rapid quenching of triiodide ion (ca. 1 s) and IIIa was the sole product. Triiodide ion formed by addition of I_2 to aqueous iodide ion reacted identically.

PPHP is a chromophoric hydroperoxide interchangeable with hydrogen peroxide in many peroxidase-catalyzed reactions (Weller et al., 1985). LPX-catalyzed turnover of PPHP gives PPA, the corresponding alcohol, that can be quantitated by LC (Doerge, 1988a). The use of PPHP permitted a direct correlation of LPX-catalyzed substrate consumption and product formation with actual hydroperoxide turnover. Under conditions where PPHP-supported LPX-catalyzed iodination was inhibited by III, 3.2 ± 0.2 mol of PPA was formed and 3.2 mol of PPHP was consumed/mol of III consumed. It was also determined that 3.2 mol of sulfate ion were formed/mol of hydrogen peroxide added to a mixture of LPX, iodide ion, and III.

The covalent binding of [^{14}C]- and [^{35}S]I, -II, and -III to LPX was measured in the presence of hydrogen peroxide

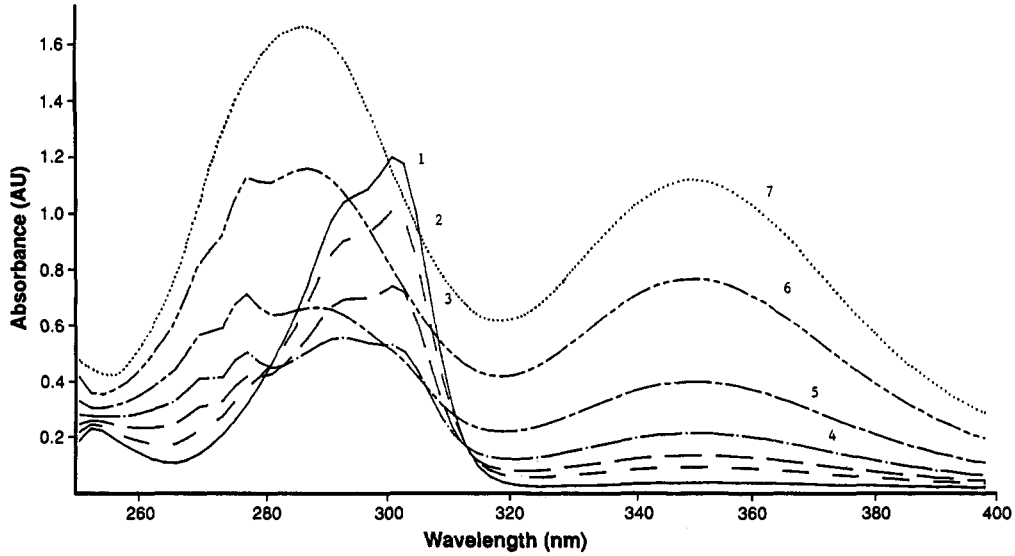


FIGURE 4: Repetitive UV scans showing oxidation of III during the lag phase of iodide ion oxidation. LPX was incubated with III (100 μM) and a hydrogen peroxide generating system as shown in Figure 3. Scans were taken at 20-s intervals over the wavelength range 250–400 nm. The absorption maxima are III, 305 nm; triiodide ion, 290 and 352 nm; IIIa, 270 and 278 nm. The scan numbers are inset alongside the spectra.

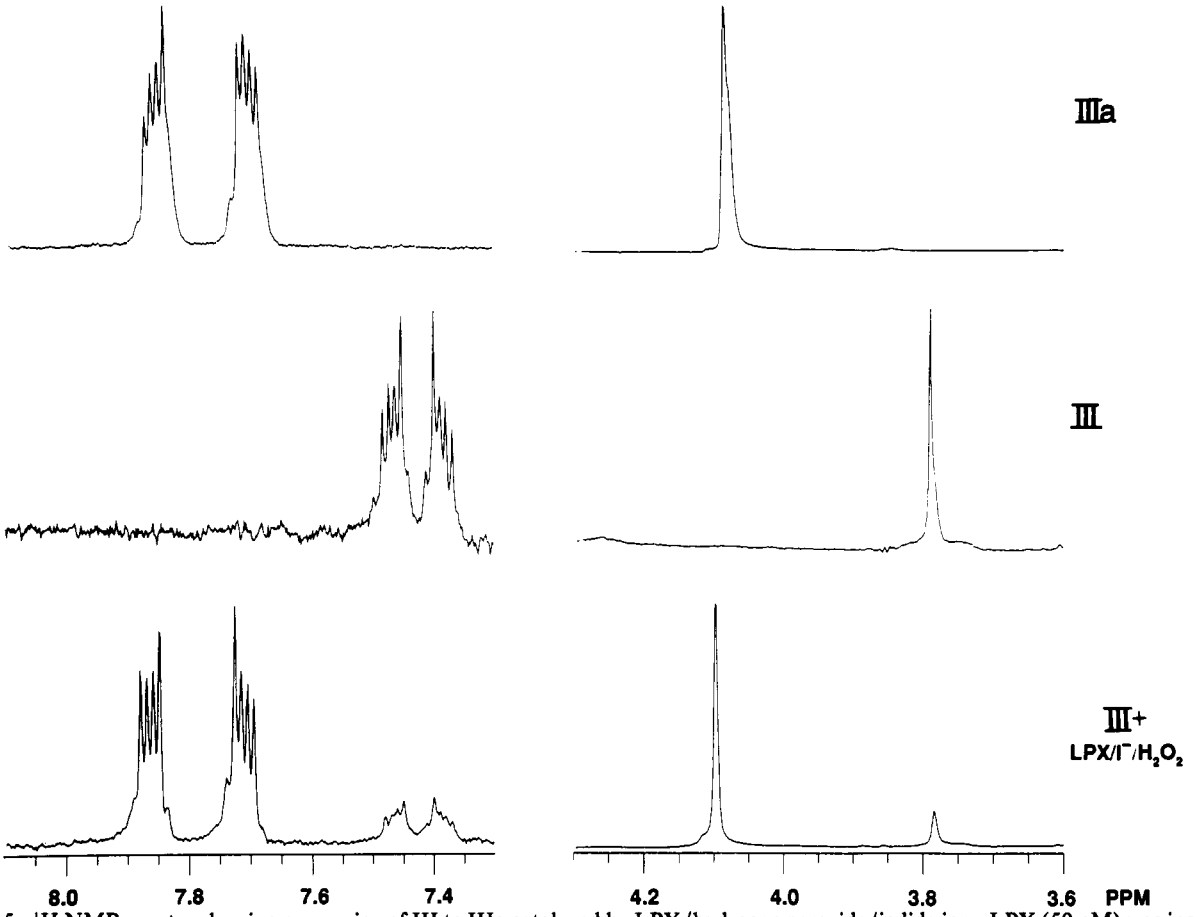


FIGURE 5: ^{1}H NMR spectra showing conversion of III to IIIa catalyzed by LPX/hydrogen peroxide/iodide ion. LPX (50 nM) was incubated with III (100 μM), iodide ion (5 mM), and hydrogen peroxide (300 μM) in 0.01 M potassium phosphate, pH 7.0, in a 10-mL reaction. The mixture was incubated at 25 °C for 1 min and catalase was added. The sample was lyophilized and analyzed by ^{1}H NMR as described in Materials and Methods. The methyl and aromatic regions are expanded for clarity, and no other resonances were observed.

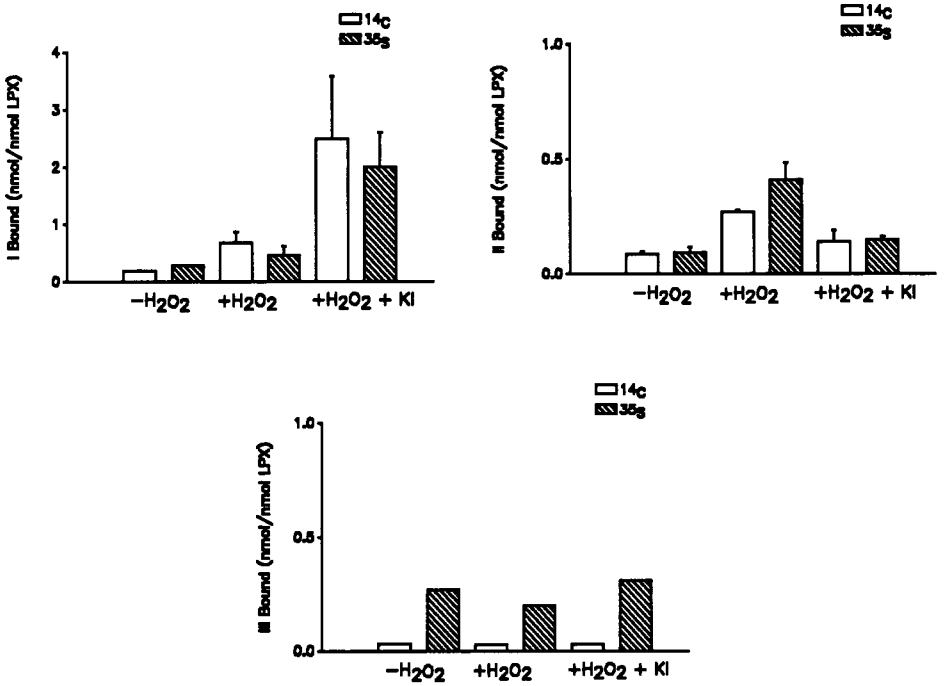
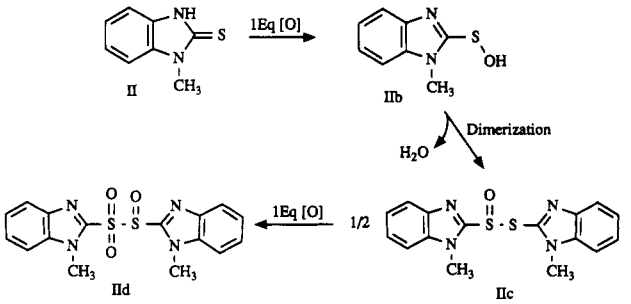


FIGURE 6: Covalent binding of [^{14}C]- and [^{35}S]benzimidazoline-2-thiones to LPX. Radiolabeled compound (250 μM) was incubated with LPX (5 μM) with hydrogen peroxide (200 μM) and iodide ion (5 mM) in potassium phosphate buffer (50 mM, pH 7.0) as noted. Covalent binding to LPX was measured after extensive dialysis in the same buffer. Values listed are averages of three (I and II, error bars = standard deviation) or two (III) determinations.

(suicide inactivation conditions) and iodide ion plus hydrogen peroxide (alternate substrate inhibition conditions) as shown in Figure 6. The covalent binding of I under suicide inactivation conditions required hydrogen peroxide and approached 1 mol/mol LPX as previously described (Doerge, 1988a). When iodide ion was included in the incubation

Scheme I: Proposed Mechanism for Oxidation of II by Hydroperoxides



mixture, a ca. 5-fold enhancement in binding of [^{14}C]- and [^{35}S]I occurred. Covalent binding of II under suicide inactivation conditions was also hydrogen peroxide-dependent and approximately equal for ^{14}C and ^{35}S but was ca. 50% that observed with I. In this case, however, the addition of iodide ion caused a decrease in binding to near control levels. No covalent binding *above background* was observed for [^{14}C]- and [^{35}S]III under any conditions.

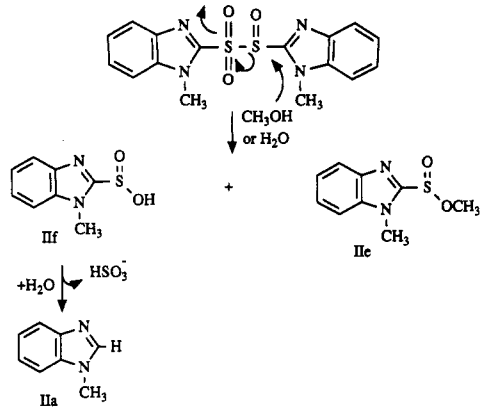
DISCUSSION

Oxidation of a series of N-substituted benzimidazole-2-thiones by chemical and enzymatic reagents was investigated. Although the rate constants for reaction of chemical oxidants with the three compounds are similar, there exist significant differences in the chemical and enzymatic oxidation products and stoichiometries. From the qualitative and quantitative differences observed, a pattern linking mechanisms of chemical and enzymatic oxidation emerged.

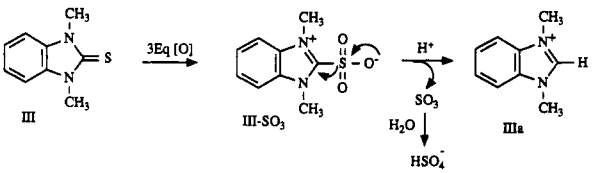
In the absence of iodide ion, I and II are suicide inhibitors of LPX and TPX (Doerge, 1986, 1988a; Doerge & Takazawa, 1990) although the potency diminishes markedly upon N-methylation. In the chemical model reactions, these suicide inhibitors were completely oxidized by 2 equiv of peroxidative reagents (see Figure 1). Since similar oxidation products were seen for I, II, and MMI (data not shown), II was used in these studies to model the reactivity of the suicide inhibitors used therapeutically in the treatment of hyperthyroidism. The reaction of II with hydroperoxides is proposed to proceed first to the benzimidazole-2-sulfenic acid (IIb, see Scheme I). This intermediate has been proposed as the one responsible for binding covalently to the prosthetic heme of LPX and TPX concomitant to suicide inactivation (Doerge, 1988a; Doerge & Takazawa, 1990). This intermediate is highly reactive, and a very facile reaction is dimerization to the thiolsulfinate (IIc) (Davis et al., 1986). Subsequent oxidation by hydroperoxide would form the sulfinylsulfone (IId) as the terminal product (Kice, 1980). Under conditions where excess II were present, the reaction of sulfenic acid with II would produce the disulfide. Further oxidation of the disulfide would also yield the same end product. Disulfides have been observed during peroxidase-mediated oxidation of MMI and propylthiouracil (Taurog et al., 1989b,c).

Experimentally, the chemical oxidation of II by hydroperoxides consumed 2 equiv of oxidant/mol of thione, formed a single product that was stable in the presence of excess oxidant, and produced no intermediates under conditions of incomplete oxidation. Chromatographic separation and detection of the product by GC-MS, TSP LC-MS, or TLC resulted in decomposition to IIa, the sulfinic acid methyl ester (IIe), and bisulfite ion (Figure 2). These products are consistent with solvolysis of the sulfinylsulfone (IId) as proposed in Scheme II. Nucleophilic attack of methanol or

Scheme II: Proposed Mechanism of Sulfinylsulfone Solvolysis



Scheme III: Proposed Mechanism for Oxidation of III by Hydroperoxides



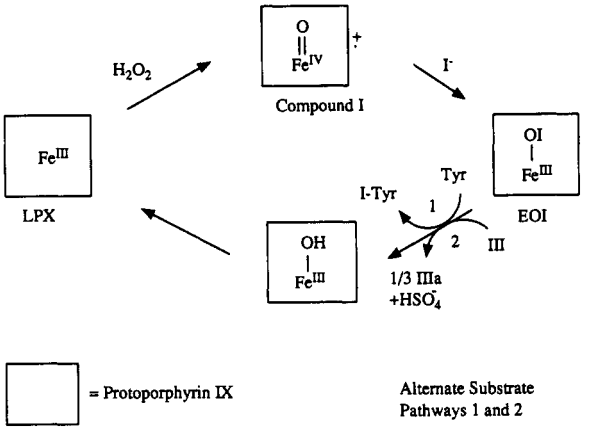
water at the sulfinyl sulfur forms 1 mol each of the sulfinic acid methyl ester (IIe) and the sulfinic acid (IIf) or 2 mol of IIf, respectively. Treatment of reaction mixtures with diazomethane prior to chromatography did not produce detectable amounts of the sulfinic acid methyl ester, and NMR showed no additional resonance. This suggests that elimination of SO_2 from IIf is a facile reaction that produces IIa and bisulfite ion as previously described for sulfinates possessing good leaving groups attached to the sulfur center (Kice, 1980).

The single primary oxidation product from II was also analyzed by FAB-MS, and its reactivity lead to several diagnostic decomposition products. Tentative assignment of a sulfinylsulfone structure was made on the basis of the observed fragmentation pattern from FAB-MS (see Results). While limited *direct* evidence for the sulfinylsulfone structure was seen, the results taken together with those from TSP-MS and GC-MS provide strong support for the proposed structure of a very labile product.

Formation of a sulfinylsulfone product requires 1-methylbenzimidazoline-2-sulfenic acid (IIb) as a reactive intermediate from oxidation of the parent thione. This type of reactive intermediate (Davis et al., 1986) was proposed as the species responsible for covalent binding to the prosthetic heme of LPX and TPX during suicide inactivation by imidazoline-2-thiones (Doerge, 1986, 1988a). This mechanism is also consistent with the observed products which form concomitant to inactivation of LPX by II (Doerge, 1988a). LPX-catalyzed formation of a sulfenic acid (e.g., IIb) from II and subsequent dimerization to the thiolsulfinate (as in Scheme I, IIc) in an aqueous environment could be followed by hydrolysis of the sulfinic acid to yield IIa and bisulfide ion. Alternatively, the sulfenic acid could undergo a second oxidation by LPX compound I to directly form IIf.

In contrast to the suicide inhibitors, oxidation of III in aqueous methanol required 3 equiv of hydroperoxide and the sole products were IIIa and sulfate ions (see Scheme III). Analogous reactivity was reported for *N,N*-dimethylimidazole-2-thione and hydrogen peroxide in aqueous methanol (Karkhanis & Field, 1985). Since III is an N,N′-disubstituted thiourea derivative, sulfenic acid formation cannot occur since tau-

Scheme IV: Proposed Alternate Substrate Mechanism for LPX Inhibition by III



tomerization is not possible as it is with unsubstituted or monosubstituted derivatives like I and II. It is proposed that elimination of this reaction pathway results in complete S-oxidation to the sulfonyl functional group. The resulting sulfonyl ylide (III-SO_3) formed in aqueous media would yield IIIa and sulfate ions by elimination of sulfur trioxide as shown in Scheme III.

The differences between III and suicide inhibitors like I and II were equally apparent when the effects on LPX-catalyzed reactions were examined. In the presence of LPX/hydrogen peroxide but in the absence of iodide ion, I and II caused rapid irreversible enzyme inactivation, covalent binding to prosthetic heme, and the formation of small amounts of turnover products (Doerge, 1986, 1988a). The binding of 1 mol of inhibitor/mol of LPX inactivated was strong support for the proposed suicide mechanism. In addition, the equal binding of ^{14}C- and ^{35}S-labeled I and II provided convincing evidence that the entire suicide substrate became bound as opposed to the binding of reactive fragments (Doerge, 1988a). However, III did not inactive LPX, bind covalently, or undergo oxidation under analogous conditions (see Table II and Figure 6). Unlike the suicide inhibitors, III did not affect LPX/hydrogen peroxide-catalyzed oxidation of the typical peroxide substrates, ABTS and guaiacol. These substrates are oxidized by the compound I and II forms, respectively, of peroxidases (Shindler et al., 1976; Nakamura et al., 1985).

III inhibited only iodination reactions that are catalyzed by LPX/hydrogen peroxide (see Figure 4) and TPX-catalyzed iodination of tyrosine (data not shown) through reaction with an enzyme-produced oxidized form of iodine. In the presence of iodide ion, III did not cause any loss of enzymatic activity (see Table II) or bind covalently to LPX above background levels (see Figure 6) under conditions where >300 equiv of III are consumed (cf. Table II). These results are similar to the alternate substrate mechanism by which ETU inhibits TPX- and LPX-catalyzed iodination reactions (Doerge & Takazawa, 1990). Similar experimental observations were also made for the reactions of TPX with carbimazole (1-carboxyethyl-3-methylimidazoline-2-thione) (Engler et al., 1983). As previously proposed for ETU, in the presence of iodide ion, III does not react with LPX compound I or II but instead reacts with the enzymatic iodinating intermediate (EOI) as shown in Scheme IV (Magnusson et al., 1984). The reaction of III with LPX in the presence of 3 equiv of hydrogen peroxide and iodide ion results in the quantitative conversion to IIIa and sulfate ions (see Figures 4 and 5). The stoichiometry of hydrogen peroxide consumption observed in the presence of iodide ion is 3 mol/mol of III, and the products formed are identical to those observed in the chemical oxidation studies. This stoichiometry is identical even though the oxidant in the chemical studies was a hydroperoxide and the enzymatic oxidant is an oxidized iodine species (e.g., EOI, hypoiodous acid, or triiodide ion). This suggests that both oxidants effect the conversion of III to the sulfonyl state prior to elimination of sulfate and IIIa ions (see Scheme III).

These studies provide further support for the importance of imidazole-2-sulfenic acids as reactive intermediates in the suicide inactivation of LPX and TPX by antihyperthyroid drugs. The proposed mechanism for peroxidase inactivation in the absence of iodide ion requires thione oxidation to the sulfenic acid by compound I with subsequent covalent binding of 1 mol of inhibitor/mol of the prosthetic heme (Doerge, 1986, 1988a). Compounds like I, II, and MMI which are capable of forming sulfenic acids are suicide inhibitors in the absence of iodide ion. Disubstituted derivatives like III, which cannot form a sulfenic acid, are not suicide inhibitors and block *only* iodination reactions via an alternate substrate mechanism. The requirement for a thiourea group contained in an aromatic nucleus is underscored by the fact that ETU, which can form a sulfenic acid, is not a suicide inhibitor but an alternate substrate inhibitor like III (Doerge & Takazawa, 1990). The lack of reactivity for ETU is probably caused by a lower driving force for S-oxidation due to the much higher one-electron oxidation potential for ETU (ca. 1 V vs Ag/Ag^+) as opposed to ca. 0.5 V for I–III (Doerge et al., 1986). This should have the effect of greatly retarding or abolishing sulfenic acid formation from the reaction of LPX compound I and ETU relative to I or II.

In the presence of high concentrations of iodide ion, the reaction of I and II with enzymatically generated oxidized iodine species likely proceed via sulfenyl iodide intermediates. Since both iodide ion and antithyroid drugs are concentrated in the thyroid, these observations could be relevant to the normal state in vivo (Taurog & Dorris, 1989). In the presence of high concentrations of iodide ion, inactivation of LPX or TPX by I or II did not occur. However, as shown in Figure 6, additional binding occurs on the apoprotein portion, and not the heme, of LPX because no changes in the visible spectrum or losses of activity were observed. Although the binding of II in the presence of iodide ion was lower than that seen in the absence of iodide ion, it was above background levels. However, the lack of covalent binding above background for III, in the presence or absence of iodide ion, suggests that no reactive metabolites were generated from oxidation of III. These findings could be significant in light of the proposals that neoantigen formation from covalent binding of reactive drug metabolites may lead to allergic hypothyroid responses as well as nontarget organ toxicity (Uetrecht, 1988; Gupta et al., 1992). In this case, the possibility of drug-induced allergic reactions should increase in the order III < II < I.

These studies provide a chemical basis for a hypothesis that distinguishes between reversible (high iodide ion) and irreversible (low iodide ion) inhibition of LPX and TPX by thiourea derivatives. These results suggest the potential use of 1,3-disubstituted thiourea derivatives, like III, as novel antihyperthyroid drugs that can effectively block TPX-catalyzed tyrosine iodination via an alternate substrate inhibition mechanism in the presence of high concentrations of iodide ion. However, these compounds are incapable of causing irreversible enzyme inactivation via formation and covalent binding of reactive sulfenic acid intermediates under conditions of low iodide ion concentration. Because iodide

ion is normally present in the thyroid, it has been proposed that all thiourea derivatives block TPX-catalyzed iodination reactions by the alternate substrate mechanism (Taurog & Dorris, 1989). This proposed was based on intrathyroidal metabolites from antihyperthyroid drugs; however, the complex milieu of the thyroid and the destructive conditions required for extraction of metabolites complicated these conclusions. Resolution of these hypotheses could aid in the design of effective antihyperthyroid drugs with minimal side effects.

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