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Inactivation of Lactoperoxidase by 4-Chloroaniline

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Chlorinated anilines are metabolites of a variety of herbicides, and these compounds are incorporated covalently into plant lignin. Recent studies have shown that chlorinated anilines in lignin become bioavailable in the digestive system of experimental animals. It is, therefore, of importance to assess the interaction of these compounds with potential target molecules. Bovine lactoperoxidase (LPX) oxidized 4-chloroaniline and was inactivated. Kinetic analysis revealed that inactivation was time dependent and exhibited saturation kinetics. The dissociation constant (K_I) for LPX and 4-chloroaniline was 1.5 mM, the pseudo-first-order rate constant for inactivation (k_{inact}) was 6.9×10^{-2} s^{-1}, and $t_{1/2}$ for inactivation was 10 s. The partition coefficient was found to be ~115 regardless of which of the cosubstrate (4-chloroaniline or hydrogen peroxide) concentrations was varied. Extensive (>40:1) incorporation of [^{14}C]-4-chloroaniline into LPX during turnover was demonstrated, and inactivation followed first-order kinetics only during the first 3 min of inactivation. These results show that 4-chloroaniline is a metabolically activated inactivator of LPX rather than a mechanism-based inactivator. Although LPX is inactivated by 4-chloroaniline *in vitro*, kinetic constants associated with this inactivation demonstrate that, prior to inactivation, LPX has only moderate affinity for 4-chloroaniline. Furthermore, these analyses suggest that it is improbable that the concentrations of 4-chloroaniline sufficient to cause inactivation of LPX would occur *in vivo* as a result of ingesting residual amounts of 4-chloroaniline present in foliage.

INTRODUCTION

Chlorinated anilines have been found as metabolites of a number of herbicides in soils (Bordeleau et al., 1972; Burge, 1973; Still and Herrett, 1976). In general, substituted anilines or their metabolites are recovered from soils as extractable compounds or as unextractable compounds bound to soil and insoluble material in plant residue. Lignin, in particular, is thought to be a major site of incorporation of these compounds in plants (Balba et al., 1979; Still, 1968; Still et al., 1981). As a consequence of their widespread use, a variety of animals may be exposed to chlorinated anilines or their metabolites. Of some concern is the report that even chlorinated anilines conjugated to lignin become biologically available in the digestive system of rats (Sandermann et al., 1990). It is unclear, however, how toxicologically significant these observations may be, especially for low concentrations of chemical, because mammals have adequate mechanisms for conjugation, detoxification, and elimination of aromatic amines (Fingl and Woodbury, 1975).

Considerable toxicological data are available concerning chlorinated anilines and related chemicals (Allen et al., 1991; McMillan et al., 1990a,b; Miller and Miller 1983; Smyth, 1962). However, little information is available concerning the effects of these compounds on individual enzymes. Lactoperoxidase (LPX) is a well-characterized enzyme found in milk, saliva, and tears (Kohler and Jenzer, 1989). *In vivo*, it functions to oxidize thiocyanate to hypothiocyanate, which has bactericidal properties (Kohler and Jenzer, 1989).

In the present study, we have demonstrated that 4-chloroaniline is a substrate for LPX and that it inactivates this enzyme during turnover. The kinetics of inactivation are also presented.

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MATERIALS AND METHODS

Chemicals. LPX ($A_{412/280}$ = 0.91) was purchased from Sigma Chemical Co. (St. Louis, MO). Radiolabeled [^{14}C]-4-chloroaniline (8.9 mCi/mmol, purity 98%) was also purchased from Sigma, while unlabeled 4-chloroaniline (purity >99%) was purchased from Fluka Chemical Corp. (Ronkonkoma, NY).

Analytical Procedures. Unless otherwise noted, oxidation of 4-chloroaniline by LPX was monitored, at room temperature (25 °C), at 435 nm in a reaction mixture (1 mL) containing 3.2 μM LPX, 1 mM 4-chloroaniline, and 1 mM H_2O_2 in 100 mM potassium phosphate, pH 7.0. Reactions were initiated by adding H_2O_2.

Kinetic parameters for LPX inactivation were determined in the presence of varied concentrations of 4-chloroaniline (0–1.5 mM) or H_2O_2 (0–1.0 mM). In these experiments, reaction mixtures (1 mL) were incubated; and at preselected times an aliquot was removed and diluted 100-fold, and its ability to oxidize guaiacol was assessed in reaction mixtures that contained 32 nM LPX, 5 mM guaiacol, and 100 μM H_2O_2 in 100 mM potassium phosphate, pH 7.0. Guaiacol oxidation was monitored at 470 nm.

Covalent modification of LPX was determined following incubations [1 mL of 3.2 μM LPX with 1 mM [^{14}C]-4-chloroaniline (1 nCi/nmol) and 0, 50, 100, 200, 500, and 1000 μM H_2O_2 in 100 mM potassium phosphate buffer, pH 7.0]. The reaction mixture was allowed to incubate for 5 min, after which time 0.4 g of urea and 100 μL of glacial acetic acid were added prior to gel exclusion column chromatography on PD-10 (G-25) columns (Pharmacia, Stockholm). The treatment with urea and glacial acetic acid was necessary because initial experiments revealed that substantial amounts of [^{14}C]-4-chloroaniline reaction products that comigrated with LPX were not covalently bound. Treatment of labeled LPX with denaturing agents (i.e., urea and glacial acetic acid) circumvented this problem. During the chromatography on PD-10 columns, 1-mL fractions were collected in scintillation vials and 10 mL of Safety Solve (Research Products International Corp., Mount Prospect, IL) was added. Samples were then assayed for radioactivity by liquid scintillation spectrometry. LPX eluted within the first 5 mL, and radioactivity associated with these fractions was considered to be covalently bound to LPX.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of [^{14}C]LPX was performed following chroma-

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tography on a PD-10 column and extensive dialysis against water. Cylindrical gels and a previously described electrophoretic system (Laemmli, 1970) were used. The length of the running gel (7.5% acrylamide, 2.7% cross-link) was 9 cm, while that of the stacking gel (4% acrylamide, 2.7% cross-link) was 1 cm. Electrophoresis buffers used were the same as previously described (Laemmli, 1970) except that 2-mercaptoethanol was omitted. Following SDS–PAGE, the running gel was stained in 0.125% Coomassie Blue R-250 in methanol/water/glacial acetic acid (50:40:10), destained in methanol/water/glacial acetic acid (50:40:10), and cut into 2-mm disks. Each disk was placed in a scintillation vial, and 600 μL of 85% perchloric acid/30% H_2O_2 (2:4) was added to each vial and incubated at 75 °C overnight to solubilize each gel slice (Mahin and Lofberg, 1966). Ten milliliters of Safety Solve was then added to each vial, and radioactivity was determined by liquid scintillation spectrometry.

Covalent modification of LPX was also documented by high-performance liquid chromatography. Following covalent modification, chromatography on a PD-10 column, and dialysis against water, aliquots were analyzed by gradient elution HPLC on a system equipped with a reversed-phase column (Protein C_4, 4.6 mm × 250 mm, Vydac 214TP104, The Sep/a/ra/tions Group, Hesparia, CA) suitable for protein separation. The HPLC elution program consisted of an isocratic elution in 20% acetonitrile in water containing 0.1% trifluoroacetic acid (TFA) for 5 min followed by a linear gradient from 20% acetonitrile (0.1% TFA) in water to 80% acetonitrile (0.1% TFA) in water for 20 min. The elution was then continued in 80% acetonitrile (0.1% TFA) in water for another 5 min. The flow rate was 1.5 mL/min, and the elution of LPX was monitored at 280 nm. Fractions (0.5 mL) were collected in scintillation vials. Ten milliliters of Safety Solve was added to each vial, and radioactivity was monitored by liquid scintillation spectrometry.

Several products formed during the oxidation of 4-chloroaniline by horseradish peroxidase have been identified by others (Simmons et al., 1986). Similar procedures were used to synthesize a number of 4-chloroaniline oxidation products for use as analytical standards (Chang and Bumpus, 1993). Products synthesized included 4,4′-dichloroazobenzene (**I**), 2-(4-chloroanilino)-*N*-(4-chlorophenyl)benzoquinone (**II**), 2-amino-5-chlorobenzoquinone di-4-chloroanil (**III**), 2-(4-chloroanilino)-5-hydroxybenzoquinone di-4-chloroanil (**IV**), 2-amino-5-(4-chloroanilino)benzoquinone di-4-chloroanil (**V**), and *N*-(4-chlorophenyl)benzoquinone monoimine (**VI**).

Spectroscopic studies were performed using a Cary 3 UV-visible spectrophotometer. The spectrum of the pyridine hemochromogen of LPX and the visible absorption spectrum (700–350 nm) of native LPX (40 μM) and of the well-characterized LPX intermediates, compounds 2 and 3, were obtained as previously described (Paul et al., 1953; Kohler et al., 1988). These same procedures were used to spectrally characterize covalently modified LPX. No attempt was made to generate LPX compound 1 as this intermediate has a half-life of only ~0.2 s, requiring special techniques such as stopped-flow analysis for characterization (Kohler and Jenzer, 1989).

RESULTS

Figure 1 shows that, upon addition of hydrogen peroxide to a reaction mixture containing LPX and 4-chloroaniline, 4-chloroaniline is oxidized and LPX is readily inactivated. As shown, the loss of activity was not due to depletion of either substrate (4-chloroaniline or hydrogen peroxide) because activity was restored only when additional LPX was supplied.

Oxidation of 4-chloroaniline by LPX resulted in a complex mixture of dimers, trimers, and tetramers. Among the products identified were 4,4′-dichloroazobenzene, 2-(4-chloroanilino)-*N*-(4-chlorophenyl)benzoquinone, 2-amino-5-chlorobenzoquinone di-4-chloroanil, 2-(4-chloroanilino)-5-hydroxybenzoquinone di-4-chloroanil, 2-amino-5-(4-chloroanilino)benzoquinone di-4-chloroanil, and *N*-(4-chlorophenyl)benzoquinone monoimine. Products were identified by comigration with authentic standards during

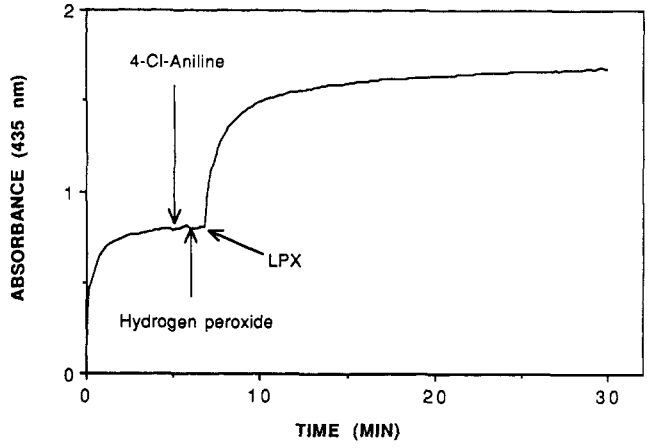

Figure 1. Oxidation of 4-chloroaniline by LPX. Initially the reaction mixture contained 1 mM 4-chloroaniline, 1.34 μM LPX, and 1 mM H_2O_2 in 100 mM potassium phosphate buffer, pH 7.0. The reaction was initiated by addition of H_2O_2. Equivalent supplemental amounts of 4-chloroaniline, H_2O_2, and LPX were added as indicated by the arrows.

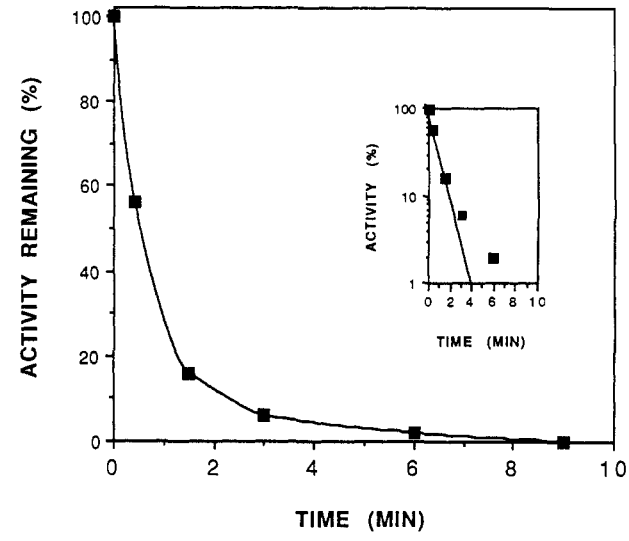

Figure 2. Time-dependent inactivation of LPX. The initial reaction mixture contained 3.2 μM LPX, 1 mM 4-chloroaniline, and 1 mM H_2O_2 in 100 mM potassium phosphate buffer, pH 7.0. At the times indicated, aliquots were removed and diluted 100-fold and their guaiacol oxidase activity was determined.

TLC and HPLC. Kinetic analysis (Figures 2 and 3) revealed that inactivation of LPX by 4-chloroaniline is a time-dependent process. It followed first-order kinetics for only the first ~3 min of the inactivation time course. The time required to inactivate half of the enzyme at infinite substrate concentration ($t_{1/2}$) was calculated to be 10 s. The pseudo-first-order rate constant for inactivation (k_{inact}) was 6.9 × 10^{-2} s^{-1}, and the dissociation constant (K_I) for LPX and 4-chloroaniline was 1.5 mM.

Partition coefficients (i.e., the theoretical number of turnovers required to inactivate one LPX molecule) were determined using both 4-chloroaniline and hydrogen peroxide as the limiting substrate. The partition coefficient was found to be ~115 regardless of which of the cosubstrates was varied.

To determine if 4-chloroaniline or its reaction products become covalently bound to LPX during the reaction, [^{14}C]-4-chloroaniline was incubated in reaction mixtures containing LPX and hydrogen peroxide followed by size exclusion column chromatography under denaturing conditions (i.e., in the presence of acetic acid and urea) on Sephadex PD-10 (G-25) columns to remove [^{14}C]-

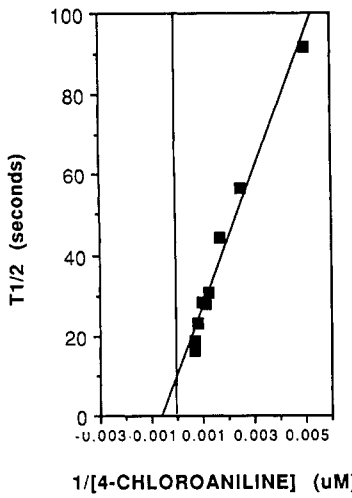

Figure 3. Kinetics of LPX inactivation of 4-chloroaniline. Reaction mixtures contained 3.2 μM LPX, variable concentrations of 4-chloroaniline, as indicated, and 1.0 mM H_2O_2 in 100 mM potassium phosphate buffer, pH 7.0. For each concentration of 4-chloroaniline, aliquots were removed at 0, 0.5, 1.0, 1.5, 3.0, 6.0, and 9.0 min and diluted 100-fold and their guaiacol oxidase activity was determined. The $t_{1/2}$ for inactivation of LPX was then calculated for each concentration of 4-chloroaniline tested using data obtained during the first 3 min of inactivation. In this figure, K_I is the negative reciprocal of the intercept on the abscissa, $t_{1/2}$ is the intercept on the ordinate, and $k_{inact} = \ln 2/t_{1/2}$.

4-chloroaniline and reaction products that were not covalently bound. Inactivation appeared to be linearly related to covalent binding. The greatest amount of covalent attachment was observed at a hydrogen peroxide to LPX ratio of 156:1. At this ratio, 48.5 mol of [^{14}C]-4-chloroaniline was bound/mol of LPX.

Because of the rather large amount of [^{14}C]-4-chloroaniline that was covalently bound to LPX and because of the tendency of the reaction products to nonspecifically bind LPX under nondenaturing conditions, further studies were performed to unequivocally demonstrate that covalent modification did, indeed, occur. Thus, in some experiments following oxidation of [^{14}C]-4-chloroaniline by LPX and subsequent column chromatography of the reaction mixture under denaturing conditions ^{14}C-labeled LPX ([^{14}C]LPX) was dialyzed extensively (10 000 MW exclusion limit) against water. In these experiments no radioactivity was lost by the [^{14}C]LPX. Dialyzed samples were then used for SDS–PAGE. Following SDS–PAGE of [^{14}C]LPX it was found that 46.8% of the recovered radioactivity comigrated with LPX, 24.4% was associated with material having a molecular weight greater than that of LPX, and 28.8% of the recovered radioactivity was found associated with material having a molecular weight lower than that of LPX. Although only about 50% of the radioactivity comigrated with LPX, this is regarded as further evidence that covalent incorporation of the radiolabel into LPX did, in fact, occur. The higher molecular weight material may indicate that multimers of LPX are formed as a consequence of covalent attachment of [^{14}C]-4-chloroaniline oxidation products. More likely, the high molecular weight material represents residual monomeric ^{14}C-labeled LPX that was nonspecifically trapped in the polyacrylamide matrix. The low molecular weight material may represent ^{14}C-labeled material that was initially covalently bound to LPX but was cleaved during electrophoresis. Taken together, these data confirm that LPX was covalently modified by [^{14}C]-4-chloroaniline. It should be noted that [^{14}C]LPX precipitated at slightly alkaline pH. This is further evidence that LPX wascovalently

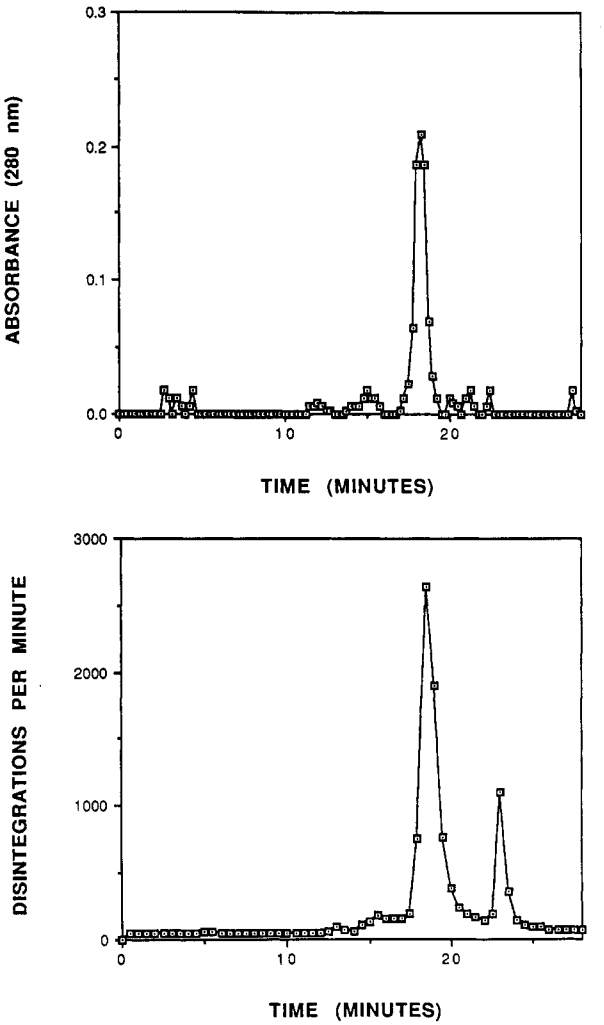

Figure 4. HPLC elution profile of [^{14}C]LPX. LPX was covalently modified with [^{14}C]-4-chloroaniline. Reaction mixtures (1 mL) contained 3.2 μM LPX, 1.0 mM [^{14}C]-4-chloroaniline, and 0.5 mM H_2O_2 in 100 mM potassium phosphate, pH 7.0. Following incubation for 5 min, 0.4 g of urea and 110 μL of glacial acetic acid were added and the mixture was chromatographed on a PD-10 column to remove noncovalently bound [^{14}C]-4-chloroaniline and [^{14}C]-4-chloroaniline reaction products. Radioactive material in the first 5 mL of the PD-10 chromatographic elution was dialyzed against water and used for HPLC. Fractions (0.5 mL) from HPLC were collected in scintillation vials, and radioactivity was determined by liquid scintillation spectrometry (bottom). Elution of [^{14}C]LPX was also monitored at 280 nm (top).

modified. However, this phenomenon made SDS–PAGE difficult since the alkaline loading buffer caused a substantial portion of the sample to remain at the top of the stacking gel, resulting in low (10–12%) recoveries of radioactivity following SDS–PAGE of [^{14}C]LPX.

In addition to size exclusion column chromatography on PD-10 columns, dialysis, and SDS–PAGE, HPLC was used as a fourth procedure in an attempt to unequivocally demonstrate covalent incorporation of [^{14}C]-4-chloroaniline into LPX (Figure 4). Prior to HPLC, covalently modified LPX was chromatographed under denaturing conditions on a PD-10 column and dialyzed against water. Figure 4 shows that during HPLC most of the recovered radioactivity comigrated with the dominant UV (280 nm) absorbing peak having a retention time of 18.15 min. Apparently covalently modified LPX ([^{14}C]LPX) is somewhat less polar than native LPX (retention time = 17.0). Several other minor peaks were also observed and

represent other forms of [^{14}C]LPX, or possibly, radiolabeled peptides formed during covalent modification or subsequent processing for HPLC. In any case, these results unequivocally demonstrate covalent incorporation of [^{14}C]-4-chloroaniline into LPX.

The effect of covalent modification of LPX by 4-chloroaniline on the heme binding site was studied by visible absorption spectroscopy. The absorption spectrum of covalently modified LPX was masked by the strong absorption of 4-chloroaniline oligomers that were covalently bound or otherwise tightly adsorbed to LPX following column chromatography on a PD-10 column under nondenaturing conditions. LPX intermediates (compounds **2** and **3**) were generated upon addition of hydrogen peroxide to native LPX as described (Kohler et al., 1989), but addition of hydrogen peroxide had virtually no effect on the visible absorption spectrum of covalently modified LPX. Difference spectroscopy confirmed our observation that covalently modified LPX did not form any of the well-characterized LPX intermediates. It was also not possible to form a pyridine hemochrome using covalently modified inactivated LPX.

DISCUSSION

Inactivation of LPX by 4-chloroaniline was shown to be a time-dependent process. Loss of activity was irreversible, and inactivation exhibited saturation kinetics. The relatively high partition coefficient observed is consistent with the fact that substantial turnover, resulting in product formation, occurred before inactivation, and the calculated dissociation constant indicates that LPX has only moderate reversible binding affinity for 4-chloroaniline prior to inactivation.

The inactivation of LPX by 4-chloroaniline during turnover fulfills many of the criteria established for mechanism-based suicide inactivation (Abeles and Maycock, 1976; Walsh, 1977; Waley, 1985; Silvermann, 1988). However, covalent incorporation of [^{14}C]-4-chloroaniline into LPX was shown to exhibit a much greater stoichiometry than the 1:1 ratio expected for mechanism-based suicide substrates. During enzyme inactivation, only one molecule (or fewer, in the case of negative cooperativity) of inactivator is required to be covalently incorporated at the active site per enzyme molecule for inactivation to occur. Despite this, greater ratios of covalent modification are often observed. This may come about because the activated intermediate formed from the substrate sometimes binds the enzyme at sites that are not critical for catalysis. This requires that the activated intermediate be sufficiently stable to migrate away from critical amino acid residues or prosthetic groups at the active site before binding the enzyme. Peroxidase-catalyzed oxidation of 4-chloroaniline is thought to occur by an initial one-electron oxidation to form an anilino free radical which is resonance stabilized by delocalization at the N, para, and ortho positions (Simmons et al., 1987). Such intermediates are thought to diffuse from the active site and further react with each other by radical coupling to form the oligomeric oxidation products that are observed (Simmons et al., 1986, 1987). Our data suggest that such radicals also covalently bind LPX, ultimately resulting in inactivation of the enzyme. Because inactivation is not exclusively (or necessarily) mechanism-based, 4-chloroaniline is more properly termed, in the nomenclature of Silverman (1988), a metabolically activated inactivator.

Previous studies (Ator and Ortiz de Montellano, 1987; Ortiz de Montellano et al., 1987, 1988; Ator et al., 1987; DePillis and Ortiz de Montellano, 1989; Doerge and Niemczura, 1989) have shown that several substrates inactivate peroxidases by covalent modification of the delta meso edge of the heme moiety, while others cause inactivation by covalent attachment to the protein portion of the enzyme. Given the magnitude of covalent incorporation by LPX of 4-chloroaniline, it seems that inactivation of LPX can probably be caused solely by nonspecific covalent modification of the protein portion of the enzyme. Several attempts were made to directly demonstrate covalent modification of the heme moiety by 4-chloroaniline. These efforts were unsuccessful due to the fact that the heme moiety of LPX is covalently bound to the protein moiety. Proteolytic and chemical cleavage extraction methods have been published documenting heme removal from LPX (Nichol et al., 1987). However, we have not been able to adapt them to successfully demonstrate extraction of covalently modified heme from [^{14}C]LPX. Thus, instead of direct determination of covalent binding to the heme moiety, absorption spectroscopy and difference spectroscopy were used to study the effect of covalent modification on the heme binding domain. Although our results cannot be used to confirm covalent modification of heme, they do show that extensive modification of heme or the heme binding domain must have occurred as evidenced by the fact that covalently modified LPX no longer has spectral characteristics of native LPX and is unable to form compounds **2** and **3** in the presence of hydrogen peroxide. Furthermore, it was not possible to form a pyridine hemochrome using covalently modified LPX.

Chlorinated anilines are not particularly persistent in the environment when compared to truly recalcitrant compounds such as DDT or dieldrin. Nevertheless, they are of environmental concern as these toxic compounds are often found in soil (Bordeleau et al., 1972; Burge, 1973; Still and Herrett, 1976). In addition to occurring free in soil, chlorinated anilines are also incorporated covalently into plant lignin (Balba et al., 1979; Still, 1968; Still et al., 1981). Although plant lignin is generally thought to be nondigestible, studies by Sandermann et al. (1990) showed that chlorinated anilines covalently bound to lignin become bioavailable in the rat digestive system. It is, therefore, important to understand how such chemicals interact with potential target molecules.

Our results show that 4-chloroaniline is a metabolically activated inactivator of LPX. However, kinetic constants associated with this inactivation demonstrate that, prior to inactivation, LPX has only moderate affinity for 4-chloroaniline. Furthermore, mammals have adequate mechanisms for detoxification and elimination of such compounds (Fingl and Woodbury, 1975). Thus, it is unlikely that concentrations of 4-chloroaniline sufficient to cause inactivation of LPX would occur *in vivo* as a consequence of ingesting residual concentrations (low micrograms per kilogram range or less) of this chemical in foliage.

The oral LD_{50} of 4-chloroaniline in rats is 310 mg/kg (Smyth et al., 1962), but the LD_{50} for this compound in cows is unknown. It is, however, reasonable to suggest that an acutely toxic dose in many mammals may be in the milligrams per kilogram range. In the event of accidental ingestion of large amounts of 4-chloroaniline, it is possible that the bactericidal activity of bovine lactoperoxidase would be compromised. However, given the more serious clinical consequences of ingesting large amounts of 4-chloroaniline, this would likely be a minor concern.

ACKNOWLEDGMENT

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LITERATURE CITED

Abeles, R. H.; Maycock, A. L. (1976) Suicide Enzyme Inactivators. *Acc. Chem. Res.* **1976**, *9*, 313–319.

Allen, R., Lyons, G., Hannant, M., Fletcher, C., Eds. *Chemical Safety Data Sheets—Toxic Chemicals (A–I) Vol. 4a*; Royal Society of Chemistry: Cambridge, U.K., 1991; pp 146–149.

Ator, M. A.; Ortiz de Montellano, P. R. Protein Control of Prosthetic Heme Reactivity: Reaction of Substrates with the Heme Edge of Horseradish Peroxidase. *J. Biol. Chem.* **1987**, *262*, 1542–1551.

Ator, M. A.; David, S. K.; Ortiz de Montellano, P. R. Structure and Catalytic Mechanism of Horseradish Peroxidase. Regiospecific Meso Alkylation of the Prosthetic Heme Group by Alkylhydrazines. *J. Biol. Chem.* **1987**, *262*, 14954–14960.

Balba, H. M.; Still, G. G.; Mansager, E. R. Pyrolytic Method for Estimation of Bound Residues of Chloroaniline Compounds in Plants. *J. Assoc. Off. Anal. Chem.* **1979**, *62*, 237–240.

Bordeleau, L. M.; Rosen, J. D.; Bartha, R. Herbicide-Derived Chlorobenzene Residues: Pathways of Formation. *J. Agric. Food Chem.* **1972**, *20*, 573–577.

Burge, W. D. Transformation of Propanil-Derived 3,4-Dichloroaniline in Soil to 3,3'4,4'-Tetrachloroazobenzene as Related to Soil Peroxidase Activity. *Soil Sci. Soc. Am. Proc.* **1973**, *37*, 392–395.

Chang, C-W.; Bumpus, J. A. Oligomers of 4-Chloroaniline are Intermediates Formed During its Biodegradation by *Phanerochaete chrysosporium*. *FEMS Microbiol. Lett.* **1993**, *107*, 337–342.

DePillis, G.; Ortiz de Montellano, P. R. Substrate Oxidation by the Heme Edge of Fungal Peroxidases. Reaction of Coprinus *macrorhizus* Peroxidase with Hydrazines and Sodium Azide. *Biochemistry* **1989**, *28*, 7947–7952.

Doerge, D. R.; Niemczura, W. P. Suicide Inactivation of Lactoperoxidase by 3-Amino-1,2,4-triazole. *Chem. Res. Toxicol.* **1989**, *2*, 100–103.

Fingl, E.; Woodbury, D. M. General Principles. In *The Pharmaceutical Basis of Therapeutics*, 5th ed.; Goodman, L. S., Gilman, A., Gilman, A. G., Koelle, G. B., Eds.; MacMillan Publishing: New York, 1975; pp 1–46.

Kohler, H.; Jenzer, H. Interaction of Lactoperoxidase with Hydrogen Peroxide. Formation of Enzyme Intermediates and Generation of Free Radicals. *Free Radical Biol. Med.* **1989**, *6*, 323–339.

Kohler, H.; Taurog, A.; Dunford, H. B. Spectral Studies with Lactoperoxidase and Thyroid Peroxidase: Interconversions between native enzyme, compound II and compound III. *Arch. Biochem. Biophys.* **1989**, *264*, 438–449.

Laemmli, U. K. Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4. *Nature* **1970**, *227*, 680–685.

Mahin, D. T.; Lofberg, R. T. A Simplified Method of Sample Preparation for Determination of Tritium, Carbon-14 or Sulfur-35 in Blood or Tissue by Liquid Scintillation Counting. *Anal. Biochem.* **1966**, *16*, 500–509.

McMillan, D. C.; Freeman, J. P.; Hinson, J. A. Metabolism of the Arylamide Herbicide Propanil I. Microsomal Metabolism and *in vitro* Methemaglobinemia. *Toxicol. Appl. Pharmacol.* **1990a**, *103*, 90–101.

McMillan, D. C.; Leakey, J. E. A.; Arlotto, M. P.; Millan, J. M.; Hinson, J. A. Metabolism of the Arylamide Herbicide Propanil II. Effects of Propanil and its Derivatives on Hepatic Microsomal Drug-Metabolizing Enzymes in the Rat. *Toxicol. Appl. Pharmacol.* **1990b**, *103*, 102–112.

Miller, J. A.; Miller, E. C. Some Historical Aspects of N-aryl Carcinogens and their Metabolic Activation. *Environ. Health Perspect.* **1983**, *49*, 3–12.

Nichol, A. W.; Angel, L. A.; Moon, T.; Clezy, P. S. Lactoperoxidase Haem, an Iron-Porphyrin Thiol. *Biochem. J.* **1987**, *247*, 147–150.

Ortiz, de Montellano, P. R.; Choe, Y. S.; DePillis, G.; Catalano, C. E. Structure-Mechanism Relationships in Hemeproteins. Oxygenations Catalyzed by Chloroperoxidase and Horseradish Peroxidase. *J. Biol. Chem.* **1987**, *262*, 11641–11646.

Ortiz de Montellano, P. R.; David, S. K.; Ator, M.; Tew, D. Mechanism-Based Inactivation of Horseradish Peroxidase by Sodium Azide. Formation of meso-Azidoprotoporphyrin IX. *Biochemistry* **1988**, *27*, 5470–5476.

Paul, K. G.; Theorell, H.; Akeson, A. The Molar Light Absorption of Pyridine Ferroprotoporphyrin (Pyridine Haemochromogen). *Acta Chem. Scand.* **1953**, *7*, 1284–1287.

Sandermann, H., Jr.; Arjmand, M.; Gennity, I.; Winkler, R.; Struble, C. B.; Aschbacher, P. W. Animal Bioavailability of Defined Xenobiotic Lignin Metabolites. *J. Agric. Food Chem.* **1990**, *38*, 1877–1880.

Silverman, R. B. *Mechanism-based enzyme inactivation: Chemistry and Enzymology*, CRC Press: Boca Raton, FL, 1988; Vol. 1, 210 pp.

Simmons, K. E.; Minard, R. D.; Freyer, A. J.; Bollag, J.-M. Structural and Quantitative Analyses of 4-Chloroaniline-Derived Oligomers. *Int. J. Environ. Anal.* **1986**, *Chem. 26*, 209–227.

Simmons, K. E.; Minard, R. D.; Bollag, J.-M. Oligomerization of 4-Chloroaniline by Oxidoreductases. *Environ. Sci. Technol.* **1987**, *21*, 999–1003.

Smyth, H. F. A Toxicologists's View of Thresehold Limits. *Am. Ind. Hyg. Assoc. J.* **1962**, *23*, 37–43.

Smyth, H. F.; Carpenter, C. P.; Weil, C. S.; Pozzani, U. C.; Streigel, J. A. Range Finding Toxicity Data: List VI. *Am. Ind. Hyg. Assoc. J.* **1962**, *23*, 95–108.

Still, G. G. Metabolism of 3,4-Dichloropropionanilide in Plants. The Metabolic Fate of the 3,4-Dichloroaniline Moiety. *Science* **1968**, *159*, 992–993.

Still, G. G.; Herrett, R. A. In *Herbicides. Chemistry, Degradation and Mode of Action*; Kearney, P. C., Kaufman, D. D., Eds.; Dekker: New York, 1976; Vol. 2, 609 pp.

Still, G. G.; Balba, H. M.; Mansager, E. R. Studies on the Nature and Identity of Bound Chloroaniline Residues in Plants. *J. Agric. Food Chem.* **1981**, *29*, 739–746.

Waley, S. G. Kinetics of Suicide Substrates: Practical Procedures for Determining Parameters. *Biochem. J.* **1985**, *227*, 843–849.

Walsh, C. Suicide Substrates: Mechanism-Based Enzyme Inactivators. *Tetrahedron* **1977**, *38*, 871–909.

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