Peroxidase-Catalyzed S-Oxygenation: Mechanism of Oxygen Transfer for Lactoperoxidase†

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ABSTRACT: The mechanism of organosulfur oxygenation by peroxidases [lactoperoxidase (LPX), chloroperoxidase, thyroid peroxidase, and horseradish peroxidase] and hydrogen peroxide was investigated by use of para-substituted thiobenzamides and thioanisoles. The rate constants for thiobenzamide oxygenation by LPX/H_2O_2 were found to correlate with calculated vertical ionization potentials, suggesting rate-limiting single-electron transfer between LPX compound I and the organosulfur substrate. The incorporation of oxygen from ^{18}O-labeled hydrogen peroxide, water, and molecular oxygen into sulfoxides during peroxidase-catalyzed S-oxygenation reactions was determined by LC– and GC–MS. All peroxidases tested catalyzed essentially quantitative oxygen transfer from ^{18}O-labeled hydrogen peroxide into thiobenzamide *S*-oxide, suggesting that oxygen rebound from the oxoferryl heme is tightly coupled with the initial electron transfer in the active site. Experiments using $H_2{}^{18}O_2$, $^{18}O_2$, and $H_2{}^{18}O$ showed that LPX catalyzed approximately 85, 22, and 0% ^{18}O-incorporation into thioanisole sulfoxide oxygen, respectively. These results are consistent with a active site controlled mechanism in which the protein radical form of LPX compound I is an intermediate in LPX-mediated sulfoxidation reactions.

Organosulfur compounds are an important class of environmental chemicals whose activity is often modified by oxidative metabolism (Zeigler, 1980). Enzymatic sulfoxidation produces metabolites that possess increased water solubility leading to enhanced excretion. Sulfoxidation can also produce chemically reactive species, e.g., those responsible for the toxicity elicited by thionosulfur compounds (Neal & Halpert, 1982).

Peroxidases are heme-containing enzymes that catalyze the oxygenation of organosulfur compounds (Doerge, 1986a; Kobayashi et al., 1986). Previous work from this laboratory on the S-oxygenase activity of LPX[1] and the closely related TPX elucidated the mechanism of suicide inactivation by derivatives of imidazoline-2-thiones that are used therapeutically as antihyperthyroid drugs (Doerge, 1986b, 1988; Doerge et al., 1987; Doerge & Takazawa, 1990). The proposed mechanism involves S-oxygenation of the thione group to form a reactive imidazole-2-sulfenic acid, which binds covalently to the prosthetic heme and irreversibly blocks enzyme activity.

This report describes the kinetics of LPX-catalyzed organosulfur oxygenation and the source of oxygen in product sulfoxides. These results are compared to those from other peroxidases in order to define catalytic mechanisms.

Materials and Methods

Para-substituted thiobenzamides (H, F, Cl, OCH_3, CH_3) (Fairful et al., 1952) and the respective sulfoxides (Cashman & Hanzlik, 1982) were synthesized by literature procedures. Thioanisoles were purchased from Aldrich Chemical Co., and thioanisole sulfoxide was prepared as previously described (Doerge, 1986a). Compound purity was determined with TLC. LPX, CPX, and HRP were obtained from Sigma Chemical Co., and concentrations were determined spectrophotometrically as previously described (Doerge, 1986a). Hog TPX was isolated and purified as previously described (Doerge & Takazawa, 1990). $H_2{}^{18}O$ (98.9% atom excess) and $^{18}O_2$ (97.0% atom excess) were purchased from Isotec Co., Miamisburg, OH. Extinction coefficients for thiobenzamide sulfoxides were determined experimentally in 0.1 M phosphate (pH 7.0). Initial rates of LPX-catalyzed sulfoxide formation from para-substituted thiobenzamides were determined in duplicate at 340 nm with an HP 8452A diode array spectrophotometer at 22 °C. The second-order rate constants for the reaction between LPX (62 nM) and thiobenzamides (50–500 μM) with hydrogen peroxide (200 μM) in 0.1 M phosphate (pH 7.0) were calculated from the linear plots of initial rate vs thiobenzamide concentration. The rates of oxidation in the absence of LPX were negligible. Determination of labeling during oxidation of thiobenzamide by peroxidases was conducted as previously described (Doerge, 1986a) with [CPX] = 2nM and [H_2O_2] = 2 mM, pH 4.5; [TPX] = 0.37 μM and [H_2O_2] = 0.2 mM, pH 7.0; [HRP] = 12.5 μM and [H_2O_2] = 1 mM, pH 7.0; and [LPX] = 62 nM and [H_2O_2] = 0.2 mM, pH 7.0.

Hydrogen peroxide labeled with ^{18}O was synthesized as previously described from $^{18}O_2$ (Sitter & Turner, 1985), and the isotopic content was determined by measuring the base-catalyzed incorporation of ^{18}O into menadione epoxide (Ortiz de Montellano & Catalano, 1985). Peroxidase-catalyzed formation of thiobenzamide sulfoxide from ^{18}O-labeled hydrogen peroxide (96.0 atom %) was determined after a 1-min incubation at room temperature, followed by quenching with catalase, with TSP LC-MS (VG Trio 2A) on a Hamilton PRP column with 50% acetonitrile/0.1 M ammonium acetate, pH 7.0, as eluant at a 0.75 mL/min flow rate. Retention times

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[1] Abbreviations: CPX, chloroperoxidase; DOPA, 3,4-dihydroxyphenylalanine; HOMO, highest occupied molecular orbital; HRP, horseradish peroxidase; LPX, lactoperoxidase; MNDO, modified neglect of differential overlap; TPX, thyroid peroxidase; TSP, thermospray.

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Table I: LPX-Catalyzed Incorporation of Peroxide Oxygen into Thiobenzamide and Thioanisole Sulfoxides

incubation[a]	M^+ area ($\times 10^{-6}$)	$(M + 2)^+$ area ($\times 10^{-6}$)
LPX + TB + $H_2{}^{16}O_2$	338	18.0
LPX + TB + $H_2{}^{18}O_2$	12.0	189
LPX + TA + $H_2{}^{16}O_2$	7.18	0.390
LPX + TA + $H_2{}^{18}O_2$	0.155	0.597

[a] Incubations with LPX and mass spectrometric analysis of thiobenzamide (TB) and thioanisole (TA) sulfoxides were performed as described under Materials and Methods. Representative integrated area counts are listed from mass chromatograms of the molecular ions for ^{16}O- and ^{18}O-labeled sulfoxides.

were 1.98 and 3.93 min for thiobenzamide sulfoxide and thiobenzamide, respectively. The TSP capillary temperature was 190 °C, source temperature was 270 °C, and the discharge electrode was on (200 V). Positive ion spectra were recorded under full scan (100–300 m/z in 1 s) or selected ion monitoring (154 and 156 m/z) conditions. The efficiency of ^{18}O incorporation into thiobenzamide sulfoxide was calculated from the area under mass chromatograms of the molecular ion [($^{16}O - M + 1)^+ = 154$ m/z and ($^{18}O - M + 1)^+ = 156$ m/z] (see Table I). A correction for the contribution to the 156 m/z ion from *unlabeled* sulfoxide was calculated with use of the VG ISO algorithm that computes molecular weight distributions on the basis of the natural isotopic composition of component atoms. Incorporation efficiency was calculated from the ratio of excess ^{18}O in the sulfoxide to that in the hydrogen peroxide (96.0%). Unless specified, values reported represent the average of at least two determinations of a single incubation. Undetectable amounts of oxidized products were obtained from thiobenzamide in the absence of peroxidase.

Thioanisoles (1 mM) were incubated with LPX (2 μM) and hydrogen peroxide (1 mM) in 0.1 M phosphate (pH 7.0). Incubations with CPX and HRP were made as described above for thiobenzamides. After a 5-min incubation at room temperature the reaction was terminated by extraction with a 2-mL aliquot of chloroform that was then dried over sodium sulfate and analyzed by electron impact GC–MS, and positive ion spectra were obtained from full scans (50–400 m/z in 1 s) with a VG Trio 2A mass spectrometer with a 25-m DB5 column (0.25 μM, 1 mL/min He flow, splitless injection at 40 °C, temperature program 40–240 °C at 40 deg/min, source and injector temperature of 200 °C). Thioanisole incubations were performed with $H_2{}^{16}O_2$, 96.0% $H_2{}^{18}O_2$, 50% $H_2{}^{18}O$, and 97.0% $^{18}O_2$. Incubation with $^{18}O_2$ was performed with use of a Firestone valve (Aldrich Chemical Co.) to remove atmospheric oxygen by a 5-cycle vacuum, nitrogen flush procedure followed by introduction of the $^{18}O_2$ gas above the reaction mixture in a 5-mL round-bottom flask at room temperature. The incorporation of ^{18}O into para-substituted thioanisole sulfoxide and the correction for unlabeled contributions were determined from the area under mass chromatograms for the molecular ions (142 m/z and 140 m/z, respectively) as described above (see Table I). Incubation of [^{16}O]thioanisole sulfoxide with LPX and $^{18}O_2$ resulted in no ^{18}O incorporation, and no detectable thioanisole sulfoxide was formed in the absence of peroxidase.

Vertical ionization potentials for para-substituted thiobenzamides were calculated according to the AM1 semiempirical molecular orbital approach (Brewster et al., 1991). Calculations were performed with use of a Tektronix CAChe computer-aided chemistry workstation fitted with a Motorola 20-MHz 88000 RISC processor that operated at 17 MIPS. Molecular geometries were obtained by global energy minimization according to the Bruyden–Fletcher–Goldfarb–Shanno procedure and vertical ionization potentials estimated as the negative of HOMO energies (Koopman's Theorem).

Results

Under catalytic conditions, LPX effected the S-oxygenation of para-substituted thiobenzamides to the corresponding *S*-oxide. The logarithm of the second-order rate constants showed significant correlation ($r^2 = 0.95$) with the vertical ionization potentials for thiobenzamides calculated by use of the AM1 semiempirical molecular orbital methods.

The source of *S*-oxide oxygen for thiobenzamide oxygenation by several peroxidases was investigated under catalytic conditions. Thiobenzamide *S*-oxide formation was measured by TSP–MS because of its polar, nonvolatile nature. CPX, LPX, TPX, and HRP all produced thiobenzamide *S*-oxide with essentially quantitative incorporation of peroxide oxygen (100, 98 [$n = 3$], 96, and 95%, respectively) from ^{18}O-labeled hydrogen peroxide (in the presence of $H_2{}^{16}O$ and $^{16}O_2$). Since essentially all sulfoxide oxygen was derived from hydrogen peroxide, incorporation from other possible sources (water and atmospheric oxygen) was not investigated further.

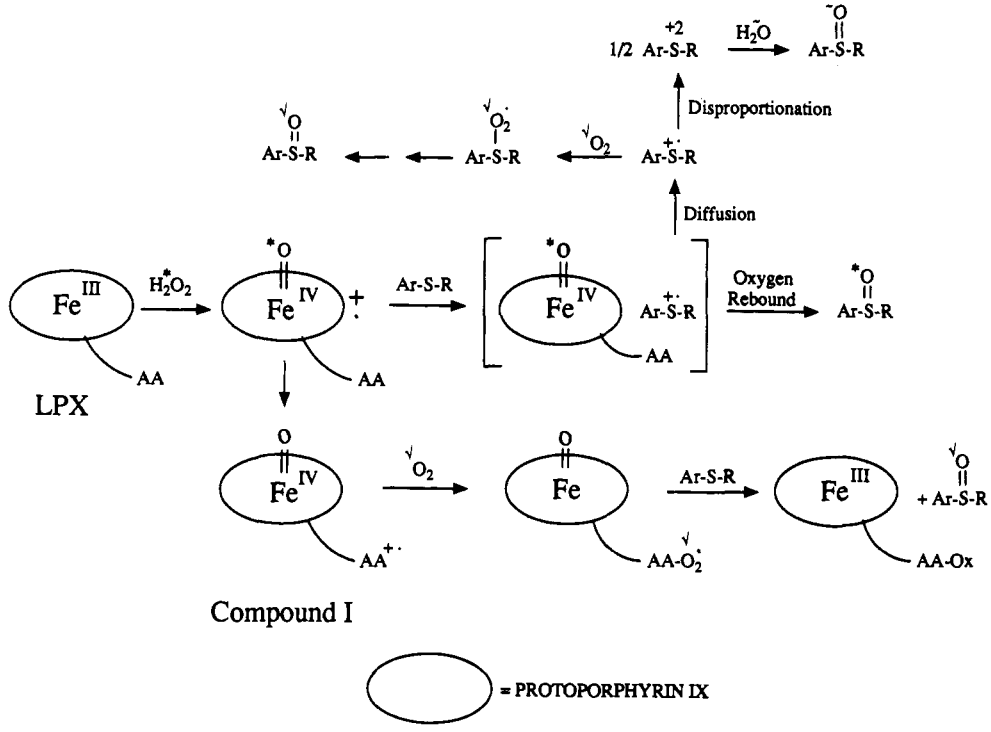
Thioanisole oxidation catalyzed by LPX and ^{18}O-labeled hydrogen peroxide resulted in 85 ± 5% ($n = 4$) incorporation into thioanisole sulfoxide. When the reaction was conducted in $H_2{}^{18}O$ using $H_2{}^{16}O_2$, 0.5% incorporation of ^{18}O into the sulfoxide was observed. When thioanisole was incubated with LPX and $H_2{}^{16}O_2/H_2{}^{16}O$ under an atmosphere of $^{18}O_2$ gas, 22% of the sulfoxide oxygen was labeled with ^{18}O. Incorporation of peroxide oxygen into thioanisole sulfoxide was determined to be 99 and 90% for CPX and HRP, respectively. When *p*-bromo-, *p*-methyl-, and *p*-methoxythioanisole were used as substrates for LPX, 99, 98, and 96% peroxide oxygen incorporation was observed, respectively.

Discussion

The results from AM1 treatment of the para-substituted thiobenzamide substrates indicate that the HOMO, i.e., the orbital that loses an electron in the oxidation reaction, is centered primarily on sulfur. Thus, the observed correlation between AM1-derived vertical ionization potentials and second-order rate constants for LPX-catalyzed S-oxygenation is consistent with rate-determining formation of the sulfur radical cation. However, while information provided by substrate properties and reactivities is useful in probing mechanistic proclivities, it cannot yield the exact mechanism of enzymatic electron transfer, i.e., whether significant π-overlap exists between the substrate and the heme edge or the hybridized d_{xz}–p_x orbital system of the oxoferryl group. While semiempirical and other molecular orbital techniques have been applied to the study of enzymatic reactions, systematic errors may be introduced by neglecting the effects of bulk water. These effects result from electrostatic stabilization of incipient polar intermediates (Warshel, 1981; Warshel et al., 1989). On the other hand, it has been suggested that enzyme–substrate interactions, by their nature, exclude water from the associating components, and this is better modeled using gas-phase simulation (Dewar & Storch, 1985). In the present study, the relationship generated between semiempirical and reactivity parameters suggests that matrix effects need not be explicitly considered in generating a predictive model of LPX-catalyzed S-oxidation.

LPX-mediated oxidation is most likely effected by compound I, the intermediate formed upon two-electron oxidation of ferric LPX by hydrogen peroxide (Ohtaki et al., 1982; Virion et al., 1985). The observed relationship between para-substituted thiobenzamide HOMO energies and rate

Scheme I: Proposed Mechanisms for Oxygen Incorporation into Sulfoxides Catalyzed by LPX



constants is in good agreement with previous studies that showed correlation of LPX substrate activity (k_{cat}/K_m) with the electrochemical oxidation potential for a series of organosulfur compounds including thiobenzamide (Doerge et al., 1987). In this previous study, the reaction kinetics indicated positive charge development on sulfur in the transition state and the formation of an S-dealkylated product was consistent with a sulfur cation radical intermediate. In addition, the formation of a sulfur cation radical intermediate during the HRP-mediated sulfoxidation of thioanisoles has been supported by Hammett correlations, product stereochemistry (Kobayashi et al., 1987), and reaction kinetics (Perez & Dunford, 1990a).

The further reactions of peroxidase compound II with the sulfur cation radical determine the source of the oxygen atom in the sulfoxide product. Some possible pathways with experimentally distinguishable outcomes are identified in Scheme I. (1) Reaction of the oxoferryl group with the sulfur cation radical produces the sulfoxide product and regenerates native peroxidase. This rebound mechanism requires transfer of the peroxide oxygen to the sulfoxide since it is the source of the oxygen in compound I (Hewson & Hager, 1979). Previous research suggests that although the oxoferryl oxygen in HRP compound II does exchange with solvent water under similar conditions (Hashimoto et al., 1986), the analogous species in ferryl-myoglobin and ferryl-hemoglobin does not exchange (Catalano & Ortiz de Montellano, 1987). Under the catalytic conditions used in the present study with LPX, significant oxygen exchange with solvent of the oxoferryl species can be excluded during oxidation of thiobenzamides and thioanisoles. (2) Diffusion of the sulfur cation radical from the active site and subsequent disproportionation would yield the sulfur dication. Reaction of compound II with a second equivalent of sulfide substrate [the classical peroxidase mechanism (Metodiewa & Dunford, 1990)] would yield a second equivalent of sulfur cation radical and native LPX. Reaction of the dication with water would yield sulfoxide product containing solvent oxygen. (3) LPX and TPX are unusual peroxidases since they have an isomeric form of compound I that has one of the two oxidizing equivalents located on an unidentified amino acid residue (Ohtaki et al., 1982; Virion et al., 1985), probably a tyrosine residue (King et al., 1967). The formation of an amino acid based radical intermediate makes possible a reaction with molecular oxygen to form a peroxy radical. Reaction of organosulfur substrate with a peroxy radical at the active site would lead to incorporation of molecular oxygen into the sulfoxide and the formation of an oxidized amino acid residue. Formation of DOPA residues during the formation of ferryl-myoglobin has been reported (King et al., 1967). This mechanism is similar to that proposed for the epoxidation of olefins by a protein radical form of ferryl-myoglobin (Ortiz de Montellano & Catalano, 1985).

Quantitative peroxide oxygen incorporation into thiobenzamide *S*-oxide was catalyzed by all peroxidases tested. This suggests that the oxygen-transfer reaction from compound II to the thiobenzamide sulfur cation radical is tightly coupled with electron transfer at the active site of the peroxidases such that release of the sulfur radical cation and subsequent disproportionation to the sulfur dication is precluded. Current information supports differences in mechanism between HRP- and CPX-catalyzed reactions (Ortiz de Montellano, 1987). Access to the active site of HRP compound I is restricted with the result that approach of substrates to the oxoferryl group is not possible. Because of this, only one-electron oxidations take place at the heme edge to generate radical species. This contrasts CPX compound I where the oxoferryl group is accessible for substrate oxidation, and predominately two-electron reactions ensue (Doerge & Corbett, 1990). Since all peroxidases tested, including HRP and CPX, gave essentially quantitative peroxide oxygen transfer to thiobenzamide, the oxygen of compounds II must be available for transfer to the sulfur radical cation in an oxygen rebound mechanism. Since substrate accessibility to the active site is thought to be limiting in the case of HRP (Ortiz de Montellano, 1987), oxygen rebound involving reaction of $OH^{\bullet}$ from compound II with the sulfur cation radical by a mechanism previously proposed (Perez & Dunford, 1990) is consistent with the present labeling

results.

The oxygenation of thioanisole by these peroxidases gave predominately peroxide oxygen incorporation into thioanisole sulfoxide. The efficiency of peroxide transfer for HRP is higher than previously reported (Kobayashi et al., 1986), but the present study used shorter incubations (5 min vs 12 h) to avoid potential competing side reactions. Thioanisole oxidation by LPX was the least efficient in catalyzing peroxide oxygen transfer (85%). In this limiting case, incubation in the presence of $H_2{}^{16}O_2$ and $H_2{}^{18}O$ produced essentially no ^{18}O-labeled sulfoxide. This observation gives strong support to the conclusion that the formation of a sulfur dication and its subsequent trapping by water does not occur in the LPX-catalyzed reactions studied. The 90% efficiency of peroxide oxygen transfer from HRP to thioanisole sulfoxide also suggests a minimal role for the sulfur dication in agreement with previous studies where approximately 10% of the *p*-methoxythioanisole sulfoxide oxygen was derived from water (Kobayshi et al., 1986). CPX catalyzed quantitative peroxide oxygen incorporation in agreement with a previous study and the proposed peroxygenase mechanism in the N-oxygenation of arylamines (Doerge & Corbett, 1990).

When LPX-catalyzed oxygenation of thioanisole was carried out in the presence of $^{18}O_2$, significant incorporation (22%) into the sulfoxide was observed. Two possible mechanisms have been considered in Scheme I. (1) Release of the sulfur cation radical from the active site and subsequent reaction with molecular oxygen produces a peroxyl species that could be converted to the sulfoxide by unknown reductants in solution or on the enzyme. The oxidation of benzylpenicillin G by LPX produced superoxide anion and an unidentified oxidation product by a mechanism that may involve release of sulfur-based radicals (Metodiewa & Dunford, 1990). However, the lack of $H_2{}^{18}O$ labeling via disproportionation to the dication during thioanisole oxidation catalyzed by LPX suggests that egress of the radical cation from the active site does not occur. The observation of ca. 10% $H_2{}^{18}O$ incorporation during HRP-catalyzed oxygenation of *p*-methoxythioanisole (Kobayashi et al., 1986) suggests that dication formation *can* occur under aerobic conditions. In addition, the lack of oxygen consumption observed during oxidation of thioanisole by HRP (Doerge, 1986a) suggests that radical reactions leading to molecular oxygen incorporation into sulfoxide products do not occur. This lack of molecular oxygen incorporation is noteworthy in that HRP-catalyzed reactions appear to produce both sulfur radical cation and dication intermediates. For these reasons, and in the absence of further experimental data, this possible mechanism is discounted.

(2) LPX and TPX are different from HRP and CPX in that compound I can exist in both an oxoferryl π-cation radical and an oxoferryl protein radical form (Ohtaki et al., 1982; Virion et al., 1985). In the oxidation of benzylpenicillin G, both forms of LPX compound I appear catalytically competent (Metodiewa & Dunford, 1990). The formation of a protein radical makes possible a reaction with molecular oxygen to form a reactive peroxyl radical (see Scheme I). Reaction of thioanisole with the peroxy radical would yield sulfoxide labeled with molecular oxygen and an oxidized amino acid on the enzyme. Precedent for this mechanism is the incorporation of oxygen into styrene epoxide from both H_2O_2 and O_2 (Ortiz de Montellano & Catalano, 1985) effected by the oxoferryl protein radical formed upon reaction of metmyoglobin with H_2O_2 (King et al., 1967). This mechanism does not appear significant for any of the other thioanisoles tested here. The p-Br, p-CH_3, and p-OCH_3 derivatives were chosen to provide a range of one-electron oxidation potentials and hence a range of reactivity with LPX compound I. The essentially quantitative peroxide oxygen transfer observed into all but the unsubstituted derivative suggests that other factors, e.g., steric effects, were the cause of the exceptional behavior. These findings suggest that the protein radical form of LPX compound I may participate in S-oxygenation reactions. However, the role of this intermediate in the predominant reaction, peroxide oxygen transfer, is currently not known.

These results are applicable to the enzymatic S-oxygenation of imidazoline-2-thione derivatives that are suicide inhibitors of LPX and TPX (Doerge, 1986b) since it is likely that catalytic oxygenation of the thione moiety of substituted thioureas proceeds by a mechanism similar to that described here for thioamides. The present results support the previous proposal that the mechanism of enzyme inactivation involves S-oxygenation of suicide substrates to reactive imidazoline-2-sulfenic acids (Doerge, 1988). The significance of these findings is underscored by the many similarities between LPX- and TPX-catalyzed organosulfur oxidation reactions. Further research to define the consequences of thione oxygenation and the identity of reactive intermediates responsible for suicide inactivation is ongoing.

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Mechanisms of Inactivation of Lipoxygenases by Phenidone and BW755C

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ABSTRACT: Inhibition of soybean lipoxygenase (L-1) and potato 5-lipoxygenase (5-PLO) by the pyrazoline derivatives phenidone and BW755C only occurs after oxidation of these compounds by the peroxidase-like activity of the lipoxygenases. There is a clear relationship between this oxidation and the irreversible inactivation of L-1. The final product of phenidone oxidation by L-1, 4,5-didehydrophenidone, is not responsible of this inactivation, but the species derived from a one-electron oxidation of phenidone plays a key role in L-1 inactivation. In the absence of O_2, inactivation of 1 mol of L-1 occurs after the oxidation of 34 mol of phenidone and the covalent binding of 0.8 mol of phenidone-derived metabolite(s) to L-1. In the presence of O_2, inactivation of 1 mol of L-1 occurs already after oxidation of 11 mol of phenidone and only involves the covalent binding of 0.4 mol of phenidone-derived metabolite(s) to L-1. A mechanism is proposed for L-1 inactivation by phenidone, which involves the irreversible binding of a phenidone metabolite to the protein and the oxidation of an L-1 amino acid residue (in the presence of O_2).

Lipoxygenases are nonheme iron enzymes that catalyze the dioxygenation of polyunsaturated fatty acids containing a 1,4-*cis*,*cis*-pentadiene moiety into 1,3-*cis*,*trans*-diene-5-hydroperoxides (Vliegenthart & Veldink, 1982). Since the discovery of the role of these enzymes in the biosynthesis of leukotrienes and lipoxins (Samuelsson et al., 1987), a huge amount of research has been done to find efficient inhibitors of lipoxygenases. Many compounds have been found to act as good inhibitors in various in vitro systems and even in vivo (Cashman, 1985). However, only very few data are presently available on the mechanisms of action of these inhibitors. For many lipoxygenases, this is presumably due to a difficult access to the purified enzymes and to our limited knowledge of the detailed nature of their active site and catalytic mechanisms. Most studies of inhibition mechanisms have been done on soybean lipoxygenase-1 (L-1),[1] which is readily available in a pure form and whose catalytic mechanism is relatively well-known (Vliegenthart & Veldink, 1982; Vliegenthart et al., 1983; Kühn et al, 1986; Schewe et al., 1986). Some arachidonic acid derivatives (Kühn et al., 1984; Corey, 1987) and arylhydrazones (Galey et al., 1988) have been demonstrated as mechanism-based inhibitors acting via an irreversible oxidation or alkylation of the protein. Moreover, catechols (Kemal et al., 1987; Nelson, 1988) and *N*-alkylhydroxylamines (Clapp et al., 1985) have been found to act by reducing and/or chelating the iron of the active site of L-1.

Some pyrazoline derivatives, such as phenidone and BW755C, have long been found to be good inhibitors of lipoxygenases (Higgs et al., 1979; Blackwell & Flower, 1978) and are widely used as reference inhibitors in in vitro systems. However, their mechanism of inhibition of lipoxygenases remains almost unknown.

This paper describes a detailed study of the mechanism of inhibition of L-1 by phenidone and BW755C and shows that metabolites formed by the oxidation of these compounds by L-1 are at the origin of the inhibitory effects by producing irreversible modifications of the protein.

Materials and Methods

Chemicals. BW755C was a gift from Wellcome Laboratories. All other chemicals were purchased from commercial sources at the highest level of purity available. Compounds were used from fresh solutions in methanol. The maximum amount of methanol (1%) was checked to have no inhibitory effect. Linoleic and arachidonic acid (99%, from Sigma) were purified under argon as reported previously (Galey et al., 1988). 13-HPOD (λ_{max} = 234 nm, ϵ = 25 mM^{-1} cm^{-1}) was prepared enzymatically by a described procedure (Gibian & Gallaway, 1976) and stored under argon in isopropanol at −18 °C.

Synthesis of 1-[U-^{14}C]Phenyl-3-pyrazolidone ([^{14}C]-Phenidone). To a solution of phenylhydrazine labeled on all the carbons of the phenyl ring, [ring-U-^{14}C]phenylhydrazine (Thang et al., 1987) (0.2 mmol, 1.76 mCi), in dry ethanol (0.3 mL) at room temperature, under nitrogen, 3-chloropropionic acid (0.25 mmol) in dry ethanol (0.4 mL) was added. The mixture was heated at 120 °C for 12 h. Cooling, addition of hot water (3 mL), extraction of the cooled solution with di-

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[1] Abbreviations: BW755C, 3-amino-1-[3′-(trifluoromethyl)phenyl]-2-pyrazoline; DETAPAC, diethylenetriaminepentaacetic acid; DMSO, dimethyl sulfoxide; 13-HPOD, 13(*S*)-hydroperoxy-9*Z*,11*E*-octadecadienoic acid; L-1, soybean lipoxygenase-1; phenidone, 1-phenyl-3-pyrazolidone; 5-PLO, potato 5-lipoxygenase; PMNL, polymorphonuclear leukocytes; SOD, superoxide dismutase; tiron, 4,5-dihydroxy-1,3-benzenedisulfonic acid, disodium salt monohydrate.

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