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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 ($\lambda_{max}$ = 234 nm, $\epsilon$ = 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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chloromethane, and evaporation of the solvents gave a residue that was chromatographed on a silica gel Go-prepacked HPLC column (Lofar A., Merck) and eluted with a solution of dichloromethane and acetone 2:1 (v/v) to afford 0.22 mCi of 1-[U-$^{14}$C]phenyl-3-pyrazolidone. Its chemical and radiochemical purity examined by thin-layer chromatography were better than 98%. Its specific activity calculated from UV spectroscopy and liquid scintillation counting was 8.8 mCi/mmol.

*Soybean Lipoxygenase-1 (L-1) and Potato 5-Lipoxygenase (5-PLO) Activities.* These activities were determined spectrophotometrically by monitoring the 234-nm absorbance of the hydroperoxides from 100 $\mu$M linoleic acid at 20 °C in 50 mM Tris-acetate buffer, pH 9, containing 0.1 mM DETAPAC for L-1, and in 200 mM acetate buffer, pH 5.5, containing 0.5 mM DETAPAC for 5-PLO, in 3-mL cuvettes. One unit of activity is defined as the amount of enzyme giving an initial rate of formation of 1 $\mu$mol of hydroperoxide per minute at 20 °C.

L-1 was purified from soybean seeds (Weber variety) by a described method (Axelrod et al., 1981, Galey et al., 1988) ($\lambda_{max}$ = 280 nm, $\epsilon$ = 160 mM$^{-1}$ cm$^{-1}$). Its specific activity was about 120 units mg$^{-1}$ at 20 °C in 0.05 M Tris-acetate buffer, pH 9, containing 0.1 mM DETAPAC.

5-PLO was purified from potato tubers (Bintje variety) by a described method (Mulliez et al., 1987) ($\lambda_{max}$ = 280 nm, $\epsilon$ = 140 mM$^{-1}$ cm$^{-1}$). Its specific activity was about 60 units mg$^{-1}$ at 20 °C in 0.2 M acetate buffer, pH 5.5, containing 0.5 mM DETAPAC.

*Inhibition Experiments on L-1 and 5-PLO.* These experiments were performed by measurement of the initial rate of L-1 and 5-PLO reactions. They were done on pure lipoxygenase (0.1 unit) in 3 mL of buffer (pH 9 for L-1 and 5.5 for 5-PLO) containing 100 $\mu$M linoleic acid. Inhibitors were added from 10 mM solutions in $CH_3OH$ or DMSO. Reactions were started by the addition of linoleic acid and followed at 20 °C by the increase of absorbance at 234 nm during about 2 min.

Other inhibition experiments were performed by measurement of the hydroperoxides produced within 10 min by L-1 and 5-PLO. Conditions were identical except that 0.02 unit of lipoxygenase was used together with 10 $\mu$M linoleic acid in the case of L-1 and 10 $\mu$M arachidonic acid in the case of 5-PLO. Total amounts of hydroperoxides formed during 10 min incubations at 20 °C were determined.

*Inhibition Experiments on Human PMNL 5-Lipoxygenase.* PMNL from human blood were prepared as described previously (Boyüm, 1968). PMNL 5-lipoxygenase activity was measured on $10^7$ PMNL activated by ionophore A23187 (10 $\mu$M) from determination of the amounts of leukotriene B4 and its derived metabolites formed after a 10-min incubation at 37 °C in the presence or absence of the inhibitor, as described previously (Mansuy et al., 1989).

*Inactivation of L-1 by Phenidone (or BW755C) and 13-HPOD.* Most experiments were performed with 2 $\mu$M L-1, 100 $\mu$M 13-HPOD, and phenidone (0–100 $\mu$M) at 0 °C in 1 mL of 50mM Tris-acetate buffer, pH 9, containing 0.1 mM DETAPAC. The peroxidase-like activity of L-1 was followed by measurement of 13-HPOD consumption at 234 nm, by a previously described procedure (Mansuy et al., 1988). Measurement of the remaining lipoxygenase activity was done on 10-$\mu$l aliquots of the crude mixture.

*Covalent Binding Measurements in the Presence of Dioxygen.* 2 $\mu$M L-1 (2 nmol) and 100 $\mu$M 13-HPOD in 1 mL of Tris-acetate buffer, pH 9, were incubated during up to 60 min with 50 $\mu$M [$^{14}$C]phenidone (8.8 mCi per mmol) at 0 °C. Two methods were used to separate the protein from phenidone or its metabolites. The first one consisted of a precipitation of the protein by trichloroacetic acid, centrifugation, and several methanol washings according to a previously described method (Galey et al., 1988). The second method consisted in the gel filtration of 0.5-mL incubates with NAP-5 prepacked G 25 columns (Water Associates, Millipore). Fractions (0.5 mL) were collected; fractions 2 and 3 contained the protein; the next 15 fractions contained phenidone and its metabolites. Counting was performed on a Packard Tricarb 300 with an external standard quench correction after addition of a scintillation liquid (Picofluor, Baker). The levels of covalent binding of phenidone metabolites to L-1 determined by these two methods were in very good agreement. Determination of nonspecific covalent binding was done thanks to identical experiments but with thermally inactivated L-1 (10 min at 80 °C) or L-1 inactivated by unlabeled phenidone or with bovine serum albumin (2 mg for a 1-mL incubation) instead of active L-1. Protein determination by the Lowry method (Lowry et al., 1951) showed that at least 95% of the protein was recovered by the two methods.

*Covalent Binding Measurements in the Absence of Dioxygen.* Experiments were performed with 1 nmol of L-1, 75 molar equivalents of 13-HPOD and 37.5 molar equivalents of [$^{14}$C]phenidone in 0.5 mL of buffer previously deaerated by bubbling argon through the solution for at least 10 min. Determination of nonspecific covalent binding with thermally inactivated L-1 was run as above. After up to 120 min of incubation, solutions were filtered on G 25 columns and treated as described in the previous paragraph.

*EPR Measurements.* EPR spectra were recorded on a Brucker ER220D spectrometer, at 9.34 GHz and 4 K, equipped with an Oxford Instruments cryostat and using a Hewlett-Packard frequency meter (100 KHz modulation frequency).

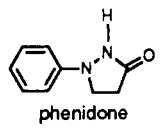
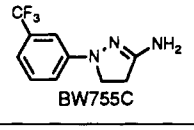
Experiments were done on 250 $\mu$L of Tris-acetate buffer, pH 9, containing 250 $\mu$M L-1 that was deaerated by five freeze-thawing cycles using argon. Phenidone and (or) 13-HPOD were added through a septum at room temperature, and the obtained solution was cooled to 4 K.

## Results

*Comparison of the Inhibitory Effects of Phenidone and BW755C on L-1, Potato 5-Lipoxygenase (5-PLO) and Human Leukocyte 5-Lipoxygenase, as a Function of the Conditions Used.* Phenidone and BW755C exhibited a very poor effect on the initial rate of linoleic acid dioxygenation by purified L-1 or 5-PLO, measured by following the appearance of the hydroperoxides (13- and 9-HPOD) as a function of time by UV spectroscopy (see Materials and Methods). Even after a 1-min preincubation of the enzymes with these compounds before the addition of linoleic acid, the $IC_{50}$ values determined from such experiments were above 100 $\mu$M (Table I). This showed that phenidone and BW755C were not inhibitors by themselves of these lipoxygenases.

In the above experiments, freshly prepared solutions of phenidone and BW755C in deaerated DMSO were used. However, when identical experiments were performed by using phenidone dissolved in aerated DMSO several hours before the experiment, a spectacular increase of its inhibitory effect was observed. As shown in Figure 1, the inhibitory effect on L-1 of a solution of phenidone previously dissolved in aerated DMSO increased as a function of the residence time of phenidone in aerated DMSO (in the absence of L-1) before the measurement. It increased from 0 to about 100% (for

Table I: Comparison of the Inhibitory Effects of Phenidone and BW755C on L-1, 5-PLO, and Human PMNL 5-Lipoxygenase, as a Function of the Assay Used[a]

| inhibitor | lipoxygenase $IC_{50}$ ($\mu$M) | | |
|---|---|---|---|
| | L-1 | 5-PLO | PMNL 5-LO |
|  phenidone | (>100)<br>5 ± 1 | (>100)<br>40 ± 5 | <br>60 ± 10 |
|  BW755C | (>100)<br>50 ± 5 | (>100)<br>40 ± 5 | <br>6 ± 2 |

[a] $IC_{50}$ values from measurements of the initial rates of linoleic acid dioxygenation are in parentheses, $IC_{50}$ values without parentheses are obtained from measurements of final amounts of dioxygenation products after a 10-min incubation (conditions are given under Materials and Methods). Mean values are ±SEM from 3–10 experiments.

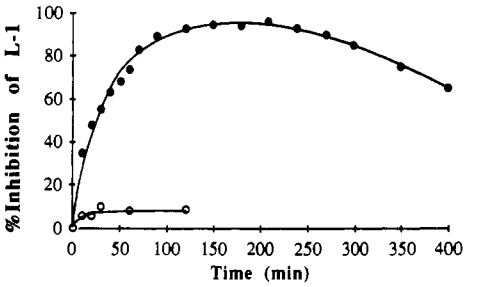



FIGURE 1: Effect of the time of preincubation of phenidone into aerated (or deaerated) DMSO on its inhibition of L-1: percent inhibition of the initial rate of linoleic acid dioxygenation by L-1 by phenidone as a function of the time of preincubation of phenidone in (●) aerated or (○) deaerated DMSO. Conditions: L-1 (5 nM), linoleic acid (100 $\mu$M), phenidone (5 $\mu$M); the assay was performed as described under Materials and Methods. The phenidone concentration for preincubation in DMSO was 10 mM.

concentrations of phenidone higher than 5 $\mu$M) when this time of preincubation of phenidone in aerated DMSO increased from 0 to 100 min and then decreased for preincubation times of phenidone in aerated DMSO longer than 200 min (Figure 1). We have checked that DMSO alone exhibited no inhibitory effect under identical conditions. These data suggested that compounds derived from a possible oxidation of phenidone by $O_2$ (Lee & Miller, 1966) in aerated DMSO could be responsible for the observed inhibitory effects. The intensity of the observed inhibitory effects should depend on the steady-state concentration of the active oxidized intermediates, which seems to reach a maximum level after about 100 min of preincubation of phenidone in aerated DMSO. Interestingly, solutions of phenidone in aerated methanol exhibited no inhibitory effect on L-1 under identical conditions, presumably because, in this solvent, the steady-state concentration of active oxidized intermediates derived from phenidone were very low. It is noteworthy that the $IC_{50}$ of phenidone, dissolved in aerated DMSO 2 h before the experiments, towards linoleic acid dioxygenation by L-1 was around 2 $\mu$M.

Another classical assay for the activity of lipoxygenases and particularly of lipoxygenases present in complex systems such as human PMNL consists in the measurement of the total amounts of the lipoxygenase products (hydroperoxides or leukotrienes) formed after a given incubation time (between 5 and 30 min) (Borgeat & Samuelsson, 1979). When this method was used either with purified L-1 or 5-PLO or with PMNL, phenidone and BW755C appeared as relatively good

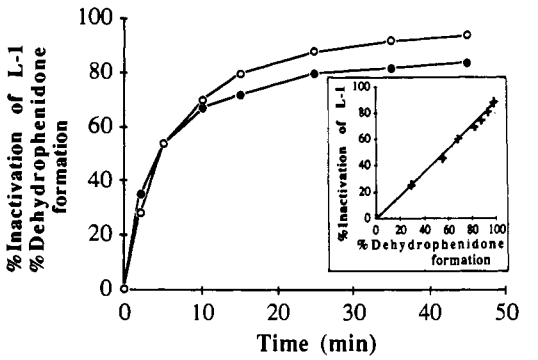



FIGURE 2: Kinetics of phenidone oxidation into 4,5-dehydrophenidone by L-1 and 13-HPOD and kinetics of L-1 inactivation. (○) Percent formation of 4,5-dehydrophenidone. (●) Percent inactivation of L-1. Incubations were done at 0 °C in 1 mL of Tris-acetate buffer, pH 9, containing 100 $\mu$M 13-HPOD, 50 $\mu$M phenidone, and 2 $\mu$M L-1. Formation of 4,5-dehydrophenidone was followed at 290 nm and by HPLC (Mansuy et al., 1988), and the remaining activity was measured by the usual assay of linoleic acid dioxygenation on 10-$\mu$L aliquots of the reaction mixture. Errors on the indicated values are ±5% (from 3 to 8 experiments). (Insert) Percent inactivation of L-1 versus percent 4,5-dehydrophenidone formation.

inhibitors with respective $IC_{50}$ values of 5, 40, and 60 $\mu$M for phenidone, and 50, 40, and 6 $\mu$M for BW755C, whatever the nature of the used phenidone solution (aerated or deaerated DMSO or methanol) (Table I). Considering our previous results, these data suggested that the observed inhibitory effects could be due to metabolite(s) formed in situ during a possible oxidation of phenidone by L-1.

Such an oxidation was likely as it has been previously shown that phenidone was oxidized into 4,5-dehydrophenidone by 13-HPOD in the presence of L-1, thanks to a peroxidase-like activity of L-1 (Mansuy et al., 1988). Accordingly, incubation of L-1 (2 $\mu$M) with phenidone (50 $\mu$M) and 13-HPOD (100 $\mu$M) led to three concomitant phenomena: the consumption of 13-HPOD and the appearance of 4,5-dehydrophenidone, as observed previously (Mansuy et al., 1988), and a time-dependent inactivation of L-1 (Figure 2). In the absence of either 13-HPOD or phenidone, no reaction occurred and L-1 was not inactivated. Moreover, there was a clear linear relationship between the appearance of 4,5-dehydrophenidone and L-1 inactivation (Figure 2). However, this inactivation of L-1 was not due to 4,5-dehydrophenidone as identical experiments performed with this compound instead of phenidone failed to inactive L-1. Nor was the inactivation due to products derived from the transformation of 13-HPOD since L-1 remained active after similar incubations performed with 13-HPOD and 4-aminophenol, another good substrate of the peroxidase-like activity of L-1 (Mansuy et al., 1988), instead of phenidone.

Interestingly, the inactivation of L-1 by phenidone and 13-HPOD was equally obtained when 13-HPOD was replaced by linoleic acid, probably because of its fast transformation into 13-HPOD under the conditions used. It was possible to measure the efficiency of phenidone by comparing the levels of inactivation of L-1 after 2-h incubations of L-1 and 13-HPOD at constant concentrations (2 and 100 $\mu$M, respectively) and phenidone at concentrations varying from 0 to 100 $\mu$M. The data obtained (Figure 3) show that about 11 mol of phenidone must be oxidized for the inactivation of 1 mol of L-1 (under aerobic conditions).

Very similar data about L-1 inactivation were obtained when BW755C was used instead of phenidone. The oxidation of 13 mol of BW755C was necessary for the inactivation of 1 mol of L-1 (data not shown).

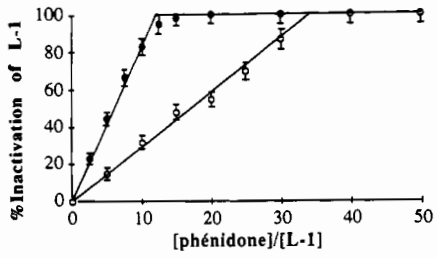



FIGURE 3: Determination of the number of moles of phenidone to be oxidized necessary for inactivation of 1 mol of L-1 in the presence of 13-HPOD. The percent inactivation of L-1 was measured as a function of the [phenidone]/[L-1] ratio, from incubations performed in the presence (●) or absence (○) of $O_2$, under the conditions of Figure 2, except that phenidone concentration varied from 5 to 100 μM. Errors on the indicated values are ±5% (from 3 to 6 experiments).

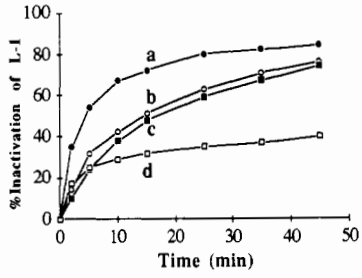



FIGURE 4: Effects of $O_2$, SOD, and tiron on the inactivation of L-1 by phenidone and 13-HPOD. (●) Incubations in aerated buffer as in Figure 2. (○) Incubations as in curve a but with SOD (1 mg/mL). (■) Incubations as in curve a but with 3 mM tiron. (□) Incubations as in curve a but in the absence of $O_2$. Errors on the indicated values are ±5% (2–3 experiments except for curve a).

*Irreversibility of L-1 Inactivation by Phenidone.* In the above experiments, we observed a loss of L-1 activity when the enzyme was incubated with phenidone and 13-HPOD. To measure the remaining enzymatic activity, we have taken aliquots (10 μL) from the incubation mixture as a source of L-1 and diluted it in the buffer (3 mL) containing linoleic acid. In spite of such a dilution (1:300), we observed a marked inhibition of L-1, which constituted a first clue in favor of the irreversibility of the enzyme inhibition. We further investigated this by separating inactivated L-1 by gel filtration in order to know whether the enzymatic activity was recovered after separation of free phenidone and its metabolites from the solution containing the enzyme. Indeed, L-1 (2 μM) incubated 45 min with 13-HPOD (100 μM) and phenidone (50 μM) remained inactive after gel filtration. A corresponding control sample of L-1 treated by the same procedure but without phenidone retained 90% of its starting activity.

*Role of Dioxygen on the Inactivation of L-1 by Phenidone and BW755C: Influence of Superoxide Dismutase, Tiron, and Catalase.* When similar oxidations of phenidone by 13-HPOD and L-1 were performed under anaerobic conditions, the rate of 13-HPOD consumption was found unchanged, showing that the peroxidase-like activity of L-1 was not affected by the presence of $O_2$. However, the rate of L-1 inactivation was markedly lowered, as shown in Figure 4. This decrease of the inhibitory efficiency of phenidone in the absence of $O_2$ was also revealed by the need of 34 mol of phenidone (45 mol in the case of BW755C) to be oxidized in order to inactivate 1 mol of L-1 in the presence of 13-HPOD, instead of 11 in the presence of $O_2$ (Figure 3). This shows that dioxygen is partially involved in the mechanism of L-1 inactivation.

In order to understand the role of dioxygen, we performed the same incubation of L-1 (2 μM), phenidone (50 μM) and 13-HPOD (100 μM) with dioxygen in the presence of superoxide dismutase (SOD) (1 mg/mL) or tiron (1–3 mM), which are known to catalyze the dismutation of $O_2^{\cdot-}$ (Halliwell & Gutteridge, 1985). These two compounds had no influence on the peroxidase-like activity of L-1 since the rate of 13-HPOD consumption by phenidone was unchanged in their presence. However, SOD and tiron decreased the rate of inactivation of L-1 at least during the first 10 min of the reaction (Figure 4). In fact, during the first minutes of the reaction, the rate of inactivation of L-1 under aerobic conditions but in the presence of SOD (or tiron) was very similar to that observed under anaerobic conditions. After this initial period, the rate of L-1 inactivation in the presence of $O_2$ and SOD became larger than that observed under anaerobic conditions, presumably because of the effects of $H_2O_2$ accumulated by dismutation of $O_2^{\cdot-}$. $H_2O_2$ has been described as a lipoxygenase inhibitor (Veldink et al., 1977), and, accordingly, in a control experiment, we observed a linear time-dependent inactivation of L-1 (2 μM) by $H_2O_2$ (20 μM) with a rate similar to that observed for L-1 in the presence of 13-HPOD, phenidone, and SOD (conditions of Figure 4, curve b, between 10 and 50 min).

In order to test this hypothesis, we performed experiments of L-1 inactivation by 13-HPOD, phenidone, and SOD in the presence of catalase. However, these experiments (data not shown) were not conclusive because of an unexpected secondary effect of catalase in the reaction medium. Actually, catalase increased the rate of L-1 inactivation by 13-HPOD and phenidone. Moreover, catalase was found to catalyze by itself the oxidation of phenidone by 13-HPOD as shown by the consumption of 13-HPOD and the appearance of 4,5-dehydrophenidone. These experiments (data not shown) indicated that catalase, as L-1, can catalyze a peroxidase-like oxidation of phenidone by 13-HPOD and, therefore, produce species able to inactivate L-1. This suggests that this species may be produced in solution outside the L-1 active site, in agreement with the experiments already described about the inhibitory effects of phenidone in aerated DMSO.

*Study by EPR of the Reduction of L-1($Fe^{III}$) by Phenidone.* EPR experiments about the oxidation of phenidone by L-1($Fe^{III}$) confirmed the possible existence outside the active site of L-1 of an oxidized metabolite, which could act as a precursor of the inactivating species. The addition of 1 mol of phenidone to L-1($Fe^{III}$) resulted in its fast reduction with the transient appearance of a signal at $g = 2.00$. This signal corresponded to the radical derived from a one-electron oxidation of phenidone, in agreement with previous results (Van der Zee et al., 1989). It did not appear in the corresponding control experiments (13-HPOD plus phenidone or L-1($Fe^{II}$) plus phenidone) but was also obtained, in the same way, from a solution of 10 mM phenidone incubated 2 h at 20 °C in aerated DMSO. Under these conditions, we observed the oxidation of phenidone with the appearance of the characteristic radical signal [ultraviolet bands at 472, 490, and 514 nm (Marnett et al., 1982)] (data not shown).

Addition of increasing amounts of phenidone to L-1($Fe^{III}$), previously obtained by treatment of native L-1 ($Fe^{II}$) by one molar equivalent of 13-HPOD and gel filtration to remove 13-HPOD or its derived products, led to a progressive disappearance of the signal at $g = 6$ corresponding to high-spin L-1($Fe^{III}$). Interestingly, this disappearance was already complete with about 0.4 mol of phenidone per mole of L-1. It has been shown that about 75% of resting L-1 was oxidized by one molar equivalent of 13-HPOD into high-spin L-1($Fe^{III}$) characterized by a signal at $g = 6$ (Slappendel et al., 1981). Thus, the complete reduction of L-1 ($Fe^{III}$) by only 0.4 mol

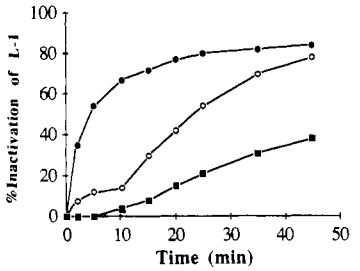



FIGURE 5: Effects of ascorbic acid on the inactivation of L-1 by phenidone in the presence of 13-HPOD. (●) Incubations as in Figure 2. (○) Identical conditions, but in the presence of 50 μM ascorbic acid. (■) Identical conditions, but in the presence of 500 μM ascorbic acid. Errors on the indicated values are ±5% (from 3 to 6 experiments).

of phenidone per mole of L-1 [0.53 mol per mole of L-1($Fe^{III}$)] indicated that the transient radical, created in a first step by a one-electron oxidation of phenidone by L-1($Fe^{III}$), was released in solution and was responsible for the reduction of the rest of the L-1($Fe^{III}$).

*Effects of Ascorbic Acid and Thiols on the Inactivation of L-1 by Phenidone.* From the aforementioned results, it appeared that phenidone was easily oxidized either by L-1($Fe^{III}$) or catalase and 13-HPOD, or simply by $O_2$ in DMSO to form the relatively stable (Lee & Miller, 1966) corresponding free radical derived from a one-electron oxidation of phenidone. This radical, or a species derived from its further oxidation by L-1, seemed responsible for L-1 inactivation. In order to further study the role of such species in L-1 inactivation, experiments were performed in the presence of reducing or trapping agents such as ascorbic acid and thiols.

Ascorbic acid was not oxidized by 13-HPOD in the presence of L-1 (Mansuy et al., 1988) and had no influence on the rate of consumption of 13-HPOD in the presence of phenidone and L-1 (data not shown). However, it delayed the oxidation of phenidone into 4,5-dehydrophenidone, because it was found able to reduce the phenidone-derived free radical back to phenidone [from experiments on phenidone and its radical followed by visible spectroscopy (data not shown)]. Therefore, the overall reaction observed under these conditions is the reduction of 13-HPOD by ascorbic acid catalyzed by L-1 and phenidone, presumably thanks to redox cycles shown in Figure 7, in which the free radical formed by a one-electron oxidation of phenidone by L-1($Fe^{III}$) is reduced back to phenidone by ascorbic acid. Accordingly, the addition of ascorbic acid to a solution of L-1 (2 μM), 13-HPOD (100 μM), and phenidone (50 μM) delayed the inactivation of L-1 until its complete oxidation by 13-HPOD (Figure 5).

Glutathione (GSH) and mercaptoethanol exhibited a behavior similar to that of ascorbic acid as they had no influence on the peroxidase-like activity of L-1 (at least at the beginning of the reaction), since they failed to modify the rate of consumption of 13-HPOD in the presence of phenidone, but delayed (or stopped at concentrations higher than 1 mM) the oxidation of phenidone into 4,5-dehydrophenidone. Morever, they also delayed or stopped the inactivation of L-1 caused by phenidone in the presence of 13-HPOD.

One could have expected that these good nucleophilic agents would protect L-1, at least in part, by trapping electrophilic metabolites of phenidone. Actually, when incubations were performed with [$^{14}$C]phenidone and analyzed by HPLC according to a previously described method (Mansuy et al., 1988), no labeled adduct could be detected. Thus, it seems that the protecting effects of GSH and mercaptoethanol are mainly due to redox cycles similar to those proposed in the case of ascorbic acid (Figure 7).

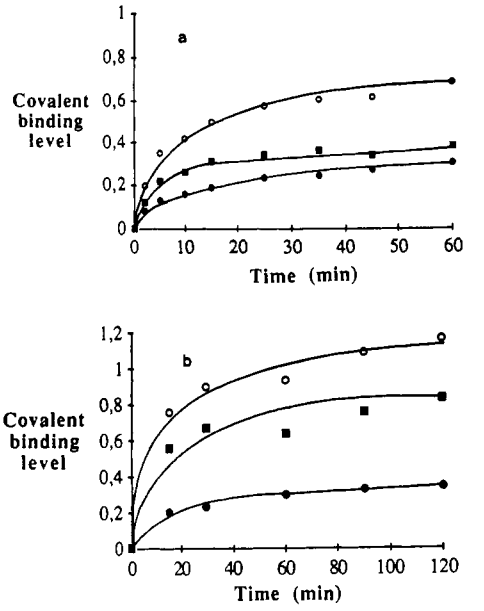



FIGURE 6: Covalent binding of phenidone metabolites to L-1 during its inactivation by phenidone in the presence of 13-HPOD. (a) Levels (in moles of phenidone covalently bound per mole of L-1) of the total (○), nonspecific (●), and specific (■) covalent binding of [$^{14}$C]-phenidone to L-1, under the conditions of Figure 2 (in the presence of $O_2$). (b) Identical measurements, but on experiments performed under anaerobic conditions. Errors on the indicated values are ±0.02 (from 3 to 5 experiments).

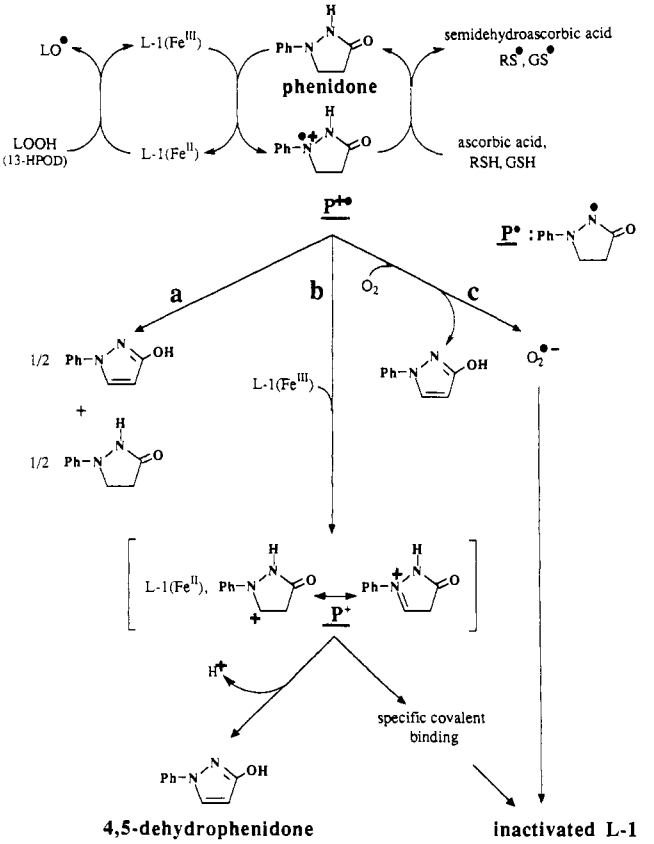



FIGURE 7: Possible mechanisms for the oxidation of phenidone by L-1 and for the inactivation of L-1 during this reaction.

*Covalent Binding of [$^{14}$C]Phenidone Metabolites to L-1 during Its Inactivation.* After incubation of L-1 with 13-HPOD and [$^{14}$C]phenidone labeled on all the carbons of its phenyl moiety and separation of the protein either by precipitation and extensive washing, or by gel filtration, pheni-

done-derived moieties remained irreversibly bound to the protein. Nonspecific covalent binding was similarly determined by using thermally inactivated L-1 or L-1 previously inactivated by unlabeled phenidone and 13-HPOD (Figure 6). These experiments allowed us to determine the covalent binding specifically due to the oxidation of phenidone by L-1 (in the presence or absence of dioxygen) (Figure 6). For the same incubation, the time-dependent specific covalent binding of phenidone to L-1 (Figure 6a) and the time-dependent inactivation of L-1 (Figure 2) were linearly correlated. This showed that 0.41 ± 0.05 mol of phenidone was specifically bound to 1 mol of L-1 in aerobic reactions, whereas 0.8 ± 0.1 mol of phenidone was bound to L-1 when the experiments were done under anaerobic conditions (Figure 6b).

## Discussion

Phenidone and BW755C are not inhibitors of L-1 by themselves but become inhibitors in long-term incubations when L-1 or 5-PLO transform their fatty acid substrates into the corresponding hydroperoxides. These data show that classical enzymatic tests of measurements of initial rate alone are not adapted to the study of the inhibitory effects of phenidone and BW755C. In fact, the research of good conditions to observe inhibitory effects of phenidone and BW755C shows that these effects occur when the dioxygenation reaction is largely advanced. In this manner, we observed a good inhibitory response whatever the source of lipoxygenases (soybean, potato, and human leukocytes).

In fact, our data show that an irreversible inactivation of L-1 only occurs if phenidone is oxidized and that there is a clear relationship between the oxidation of phenidone by L-1 and 13-HPOD and the inactivation of L-1 (Figure 2). The final product of phenidone oxidation, 4,5-dehydrophenidone, is not responsible of the observed inhibitory effects, and the species formed by a one-electron oxidation of phenidone, the radical-cation $\underline{P}^{+\bullet}$ or the radical derived from it by a proton loss $\underline{P}^{\bullet}$ (Figure 7), seems to play a key role in L-1 inactivation. This species is well-known, easily produced during oxidation of phenidone by a wide variety of oxidizing agents and easily followed by UV–visible and EPR spectroscopy (Lee & Miller, 1966; Marnett et al., 1982; Van der Zee et al., 1989). We showed its formation in incubations leading to L-1 inactivation (use of aerated DMSO solutions of phenidone, incubations of L-1, 13-HPOD, and phenidone). Its role in L-1 inactivation was shown by trapping experiments using reducing agents such as ascorbic acid or thiols. This radical is produced by oxidation of phenidone by L-1($Fe^{III}$) and is released in the medium as about 0.5 mol of phenidone is able to reduce 1 mol of L-1-($Fe^{III}$). It is known to be relatively stable (Lee & Miller, 1966) and does not seem to be very reactive toward L-1 since the inactivation of 1 mol of L-1 requires the oxidation of more than 34 mol of phenidone under anaerobic conditions (11 mol in the presence of air). The further oxidation of $\underline{P}^{\bullet}$ or $\underline{P}^{+\bullet}$ into 4,5-dehydrophenidone should be due to different reactions. First, its dismutation in solution is well-known and leads to phenidone and its dehydro derivative (Lee & Miller, 1966) (Figure 7, pathway a). Second, its oxidation by L-1$Fe^{(III)}$ should lead to dehydrophenidone via a possible carbocation or immonium ion, $\underline{P}^{+}$ (Figure 7, pathway b), which could be responsible for the covalent binding to the protein by reaction with a nucleophilic residue of a protein amino acid. This is supported by the linear correlation observed between the specific covalent binding and the inactivation of L-1 (data obtained from Figures 6a and 2). The role of dioxygen in L-1 inactivation suggests a third possible reaction of $\underline{P}^{\bullet}$ (or $\underline{P}^{+\bullet}$), its oxidation by $O_2$ itself leading to dehydrophenidone and $O_2^{\bullet-}$ (Figure 7, pathway c). In the presence of dioxygen, the increased inactivation of L-1 should be due to $O_2^{\bullet-}$ itself as SOD and tiron, which dismute $O_2^{\bullet-}$, decrease the rate of L-1 inactivation (Figure 4). In fact, under these conditions, $H_2O_2$ could also be responsible for a slow inactivation of L-1. This $O_2^{\bullet-}$- and $H_2O_2$-dependent inactivation of L-1 could be related to the oxidation of an amino acid residue important for the L-1 activity, such as the methionine residue whose oxidation has been found to play a crucial role in L-1 inactivation by acetylenic fatty acids (Kühn et al., 1984) and arylhydrazones (Galey et al., 1988). A comparison of the data obtained for L-1 inactivation in the presence or absence of $O_2$ is in complete agreement with these two modes of L-1 inactivation (oxidation and alkylation of the protein). In the absence of $O_2$, inactivation of L-1 is linked to an almost stoichiometric specific covalent binding of phenidone to L-1, in agreement with the only involvement of pathway b (Figure 7) under these conditions. In the presence of $O_2$, L-1 inactivation is also due to pathway c, explaining why it is more efficient (11 mol of phenidone to be oxidized for 1 mol of L-1 inactivated, instead of 34 mol of phenidone in the absence of $O_2$) and why only 0.4 mol of phenidone is specifically bound to the protein.

The data described in this paper indicate that reactive species formed during the oxidation of phenidone or BW755C by the peroxidase-like activity of L-1 are responsible for an irreversible inactivation of L-1. In a more general manner, other substrates of this peroxidase-like activity of L-1 could act as L-1 inhibitors by similar mechanisms. Many substrates have been found for this activity, which was demonstrated not only for L-1 (Mansuy et al., 1988; Kulkarni & Cook, 1988a,b) but also for 5-PLO (Cucurou et al, 1991). Some of them, such as hydroxamic acids, have been found to inactivate L-1 irreversibly (Reynolds, 1988). The relevance of such inactivation processes of mammalian lipoxygenases by some inhibitors, acting as substrates for the peroxidase-like activity of these lipoxygenases, appears likely but remains to be demonstrated.

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**Registry No.** BW755C, 66000-40-6; PLO, 9029-60-1; phenidone, 92-43-3; 4,5-dehydrophenidone, 1008-79-3.

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# Glycolysis and Glucose Uptake in Intact Outer Segments Isolated from Bovine Retinal Rods†

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ABSTRACT: Glucose transport across the plasma membrane of isolated bovine rod outer segments (ROS) was measured by uptake of $^{14}$C-labeled 3-*O*-methylglucose and 2-deoxyglucose and was inferred from deenergization of ROS with 2-deoxyglucose. Glucose transport was mediated by a facilitated diffusion glucose transporter that equilibrated external and internal free hexose concentrations. Glucose transport in ROS displayed two components as judged from kinetic analysis of hexose equilibration and as judged from inhibition by cytochalasin B and phloretin. Transport under exchange conditions was considerably faster as compared with net hexose uptake, similar to that observed for the erythrocyte glucose transporter. Sensitivity to cytochalasin B and affinity to 3-*O*-methylglucose were similar to those observed for the hepatocyte glucose transporter. The cytochalasin-insensitive component appears unique to ROS and did not reflect leakage transport as judged from a comparison with L-glucose uptake. Glucose transport feeds glycolysis localized to ROS. We suggest that a major role for glycolysis in ROS is phosphorylation of GDP to GTP via pyruvate kinase and PEP, while phosphorylation of ADP to ATP can use the creatine kinase/phosphocreatine pathway as well.

The outer segments of retinal rod cells (ROS) are separated from the rest of the rod photoreceptor cell by a narrow cilium; ROS are the site of visual transduction, both visual excitation and adaptation. The energy demand of ROS arises from the use of GTP by enzymes involved in the metabolism of the excitatory messenger cGMP (i.e., guanylate cyclase and the G-protein transducin) and from the use of ATP for multiple phosphorylation of rhodopsin by rhodopsin kinase and for the reduction of the chromophore retinal to retinol. The metabolic demand in ROS for GTP by enzymes of the cGMP cascade is reflected by the presence in ROS of equimolar concentrations of GTP and ATP (Berger et al., 1980; Biernbaum & Bownds, 1985; Schnetkamp, 1986). It is generally believed that the energy demand of ROS is provided for in its entirety by a dense cluster of mitochondria located in the rod inner

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‡This paper is dedicated to the memory of Ricardo Lopez-Escalera who sadly passed away on February 21, 1991.

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