Mechanism of Inhibition of Porcine Leukocyte 12-Lipoxygenase by the Isoform-Specific Inhibitor 4-(2-Oxapentadeca-4-yne)phenylpropanoic Acid†

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ABSTRACT: The mechanism of inhibition of porcine leukocyte 12-lipoxygenase by 4-(2-oxapentadeca-4-yne)phenylpropanoic acid (OPP) was investigated. This compound is selective for the leukocyte form of the 12-lipoxygenase and inhibits the purified recombinant enzyme with an IC_{50} value of approximately 2 μM. OPP induced a concentration-dependent lag phase in the oxygenation of arachidonic acid and decreased the maximal rate of reaction. Addition of the fatty acid hydroperoxide 13(*S*)-hydroperoxyoctadecadienoic acid (13-HPODE) to the reaction greatly reduced the OPP-induced lag. Lineweaver−Burk analysis of the effect of OPP on 12-lipoxygenase kinetics with arachidonic acid indicated that it was a mixed-type inhibitor. OPP was not metabolized by 12-lipoxygenase as evidenced by its quantitative recovery from incubations with stoichiometric amounts of enzyme and 13-HPODE or arachidonic acid. OPP inhibited the pseudoperoxidase activity of the enzyme with 13-HPODE and the reducing agent, BWA137C. Lineweaver−Burk analysis of the effect of OPP on pseudoperoxidase kinetics suggested that OPP was competitive with 13-HPODE. Single-turnover experiments indicated that OPP inhibited the reduction of 13-HPODE by a stoichiometric amount of ferrous 12-lipoxygenase. Addition of 13-HPODE shortened the OPP-induced lag phase but did not affect the maximal rate of enzyme activity. In addition, OPP had no effect on total product formation in either the presence or the absence of 5 μM 13-HPODE when the reaction was allowed to go to completion. All of these observations are consistent with a model for inhibition of 12-lipoxygenase activity in which OPP slows the oxidation of the inactive ferrous enzyme to the active ferric enzyme and competes with arachidonic acid for the ferric enzyme.

Plant and animal lipoxygenases catalyze the dioxygenation of polyunsaturated fatty acids at specific positions (*1*−*5*). For example, separate mammalian lipoxygenases oxidize arachidonic acid at the 5, 8, 12, or 15 positions to generate isomeric hydroperoxyeicosatetraenoic acids (Figure 1) (*4*). Two different 12-lipoxygenases have been described, but elucidation of their physiological function has been hampered by the lack of isoform-specific inhibitors (*6*). Therefore, our lab has undertaken efforts to synthesize candidate inhibitors. We recently reported the synthesis of oxaacetylenic acids as selective inhibitors of the leukocyte isoform of 12-lipoxygenase (*7*). The most potent inhibitor in this class is OPP[1] (4-[2-oxapentadeca-4-yne]phenylpropanoic acid) (Figure 1). This compound has an IC_{50} for inhibition of cytosolic preparations of leukocyte 12-lipoxygenase that is 3 orders of magnitude lower than the IC_{50}'s for inhibition of cytosolic preparations of platelet-type 12-lipoxygenase or rabbit reticulocyte 15-lipoxygenase (*7*). The structure−activity series for this compound suggests that the mechanism of inhibition may involve oxidative metabolism at the carbon that bridges the acetylenic and ether functional groups. Methylation of one or both of these methylene carbons dramatically reduces inhibitory potency.[2] Metabolism at a reactive methylene is analogous to the mechanism of action of acetylenic inhibitors

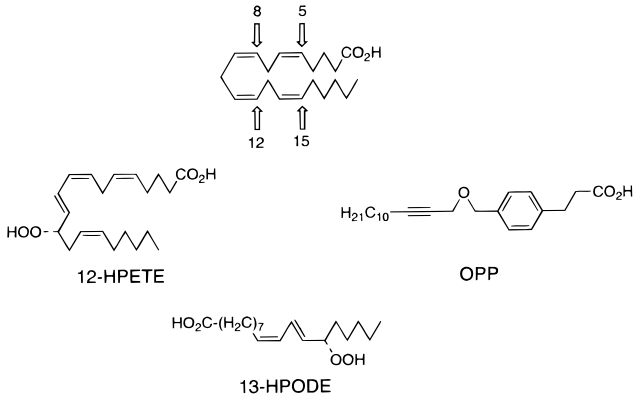

FIGURE 1: Positional specificities of mammalian arachidonate lipoxygenases and the structures of key molecules described in this paper.

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[1] Abbreviations: OPP, 4-[2-oxapentadeca-4-yne]phenylpropanoic acid; 13-HPODE, 13(*S*)-hydroperoxyoctadecadienoic acid; 13-HODE, 13(*S*)-hydroxyoctadecadienoic acid; 12-HPETE, 12(*S*)-hydroperoxyeicosatetraenoic acid.

[2] C. R. Johnson, G. Gorin, and L. J. Marnett, unpublished results.

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of lipoxygenases and suggested to us that OPP functions as a mechanism-based irreversible inactivator (*8*−*10*).

In the present study, we investigated the inhibition of purified leukocyte-type 12-lipoxygenase by OPP. The results indicate that, in contrast to our initial hypothesis, OPP does not act as a mechanism-based inactivator. Rather, it binds to both the ferrous and ferric forms of the enzyme and prevents hydroperoxide-dependent activation and oxidation of arachidonic acid. OPP elicits a pronounced lag phase prior to the achievement of the maximal rate of turnover of arachidonic acid by the enzyme, which is greatly reduced by the addition of a fatty acid hydroperoxide. In addition, OPP inhibits the pseudoperoxidase activity of the enzyme and is a reversible, non-redox-type inhibitor. Finally, OPP lowers the rate of maximal 12-lipoxygenase activity achieved independent of the length of the lag phase. This two-pronged mechanism of inhibition may serve as a prototype for the development of novel isoform-selective inhibitors of mammalian lipoxygenases.

MATERIALS AND METHODS

Arachidonic acid, linoleic acid, and 13-hydroxyoctadecadienoic acid were purchased from Cayman Chemical (Ann Arbor, MI). The expression vector pET-20b(+) and the *Escherichia coli* strain BL21(DE3) were purchased from Novagen (Milwaukee, WI). DNaseIwas from Boehringer Mannheim (Indianapolis, IN). IPTG was obtained from Gold Biotechnology (St. Louis, MO). The bead beater and 0.1 mm glass beads were purchased from Biospec Products (Bartlesville, OK). Econopac 10DG columns were purchased from Biorad (Hercules, CA). HiTrapQ columns, MonoP HR5/20 columns, and Polybuffer were from Pharmacia (Uppsala, Sweden). Tween-20 and the BCA protein assay kit were purchased from Pierce (Rockford, IL). Ammonium sulfate, ampicillin, Bis-Tris, Bis-Tris propane, chicken egg white type II-O trypsin inhibitor, catalase, dithiothreitol, EDTA, and phosphate-buffered saline were from Sigma (St. Louis, MO). BWA137C was a gift from Wellcome Research Laboratories (Beckenham, Kent, U.K.). 13(*S*)-Hydroperoxyoctadecadienoic acid (13-HPODE) was prepared by reacting soybean lipoxygenase-1 with linoleic acid as described (*11*). All other reagents were from common suppliers. UV assays were monitored using a Hewlett-Packard 8452A diode array spectrophotometer equipped with a water-jacketed cuvette. Enzyme purifications were performed using an FPLC system from Pharmacia operated at room temperature. This system consists of a GP-250 gradient programmer that controls two P-500 pumps.

Enzyme Preparation. The full-length cDNA for wild-type porcine 12-lipoxygenase (a generous gift of Shozo Yamamoto, Tokushima University, Japan) was subcloned from the pUC-19 plasmid into the pET-20b(+) expression vector through a series of subcloning steps. The C-terminal portion of the cDNA was transferred to the expression vector pET20b(+) via a *Sal*I/*Eco*RI digest. The N-terminal portion was transferred into the plasmid pBS+ using a *Xba*I/*Eco*RI digest. Site-directed mutagenesis was then used to add an *Age*I site to the cDNA through silent mutations. This new, unique *Age*I restriction site was used to insert a synthetic cassette at the N-terminus of the gene encoding wild-type protein but containing an *Nde*I site at the beginning of the cDNA. A *Nde*I/*Eco*RI digest was then used to move the N-terminus of the 12-lipoxygenase cDNA into the new expression vectors, resulting in wild-type 12-lipoxygenase cDNA in the expression vector pET 20b(+). The protocol used to express 12-lipoxygenase was the same as that used to express an allene oxide synthase−lipoxygenase fusion protein (*12*).

Cell pellets containing 12-lipoxygenase were resuspended in a total volume of 175 mL of lysis buffer (50 mM Bis-Tris propane, 1 mM DTT, 20 μg/mL catalase, 60 μg/mL chicken egg white type II-O trypsin inhibitor, pH 7.2) and lysed by mixing 6 times for 10 s with 0.1 mm glass beads with 2 min intervals between bursts. A dry ice/ethanol bath was used to cool the chamber during mixing. The lysate was then centrifuged at 5000*g* for 10 min at 4 °C, and the supernatant was then centrifuged at 100000*g* for 1 h at 4 °C. This supernatant was treated with 20 μg/mL DNaseI for 15 min at room temperature. Ammonium sulfate was added at 4 °C to all of the samples to yield 60% saturation, and the solution was centrifuged at 10 000 rpm for 15 min at 4 °C. The supernatant was removed, and the pellets were stored at −20 °C.

The ammonium sulfate pellets were thawed and resuspended in desalt buffer I (10 mM Bis-Tris propane, pH 7.2, 1 mM DTT, 60 μg/mL chicken egg white type II-O trypsin inhibitor, 20 μg/mL catalase). The solution was desalted using an EconoPac 10DG column. The desalted sample was then injected onto a HiTrapQ anion exchange column connected to the FPLC. Buffer A (10 mM Bis-Tris propane, 1 mM DTT, pH 7.2) was the initial buffer used to load the protein onto the column. Increasing the concentration of buffer B (10 mM Bis-Tris propane, 1 mM DTT, 1 M NaCl, pH 7.2) eluted the 12-lipoxygenase from the column. Catalase was added immediately to each fraction to a final concentration of 20 μg/mL. The fractions were assayed via the UV assay, and the active fractions were pooled. These fractions were then concentrated through a Centricon-30 device by centrifuging at 5000 rpm at 4 °C for 30 min. The concentrated sample was again desalted using an Econopac 10DG column. The sample was then injected onto a MonoP chromatofocusing column connected to the FPLC. Load buffer (25 mM Bis-Tris, pH 6.1) was used to load the protein onto the column while chromatofocusing buffer (10% Polybuffer 74, pH 4.8) was used to elute the protein from the column. EDTA was immediately added to the fractions to a final concentration of 100 μM, and the active fractions were pooled and stored at −80 °C. The enzyme used in these experiments had a specific activity of 8−12 μmol min^{-1} (mg of protein)$^{-1}$. A typical protein purification from 12 flasks of 280 mL each yielded approximately 15 mg of purified 12-lipoxygenase. Protein concentration was determined using an A_{280} extinction coefficient of 117 210 cm^{-1} M^{-1}.

Lipoxygenase Assay. 12-Lipoxygenase activity was detected by monitoring the absorbance at 236 nm of the conjugated diene product, 12(*S*)-hydroperoxyeicosatetraenoic acid (12-HPETE). The enzyme reactions included reaction buffer [50 mM Tris-HCl, 0.03% Tween 20 (w/v), pH 7.4], arachidonic acid, and enzyme (3−8 μg). Inhibitor and/or 13-HPODE was added to some assays by 500- or 1000-fold dilution from methanol stocks such that the total methanol concentration did not exceed 0.8% of the reaction volume. Reaction was initiated by the addition of enzyme to the

reaction cuvette followed by mixing. The reaction temperature was 30 °C, and the final reaction volume was 1 mL. Total product formation was calculated from the difference between the greatest and least values of A_{236} using an extinction coefficient of 23 000 $M^{-1}cm^{-1}$ (*13*).

Pseudoperoxidase Assay. Pseudoperoxidase activity was monitored by following the decrease in absorbance at 236 nm due to the consumption of 13-HPODE. The pseudoperoxidase reactions contained enzyme (3−8 μg), reaction buffer, 13-HPODE, and the reducing agent, BWA137C. The reaction was initiated by the addition of enzyme followed by mixing. The reaction temperature was 30 °C, and the final reaction volume was 1 mL.

Enzyme Kinetics. Enzyme kinetics were plotted by the Lineweaver−Burk method using the computer program Enzyme Kinetics 1.5 (Trinity Software). The velocity data were obtained by taking the slope of the reaction curve at the point of maximal reaction velocity. The lag phase was defined as the intersection of this tangential line at maximal reaction rate and a horizontal line defining the minimum absorbance obtained in the reaction (*14*).

Recovery of OPP from Reaction Mixtures. Enzyme (2 nmol) was reacted with equimolar amounts (2 nmol) of 13-HPODE, or OPP, arachidonic acid, or a combination thereof, for 10 min at 30 °C in reaction buffer (0.5 mL). Control reactions contained either no enzyme or boiled enzyme. The reactions were stopped by acidification with 50 μL of 1 N HCl and were extracted twice with 2 volumes of ether. The ether layer was evaporated under N_2. The residue was dissolved in HPLC solvent, and an aliquot was injected onto a 4.6 mm × 1.5 cm Zorbax Rx-SIL column (MacMod) and eluted isocratically with hexane/2-propanol/acetic acid (98.8:1.2:0.1, v/v/v). The eluate was monitored at 220 nm. The peak corresponding to OPP eluted at 8 min and was collected, dried under N_2, dissolved in methanol, and stored under N_2 in a glass vial at −20 °C. The recovery of OPP from the enzyme reaction was determined from comparison of the UV absorbance peak area with known amounts of standard.

Liquid Chromatography/Mass-Spectrometry. Liquid chromatography/mass-spectrometry was carried out using an HP 1090 series II liquid chromatograph interfaced to a Finnigan TSQ-7000 triple quadrupole mass spectrometer. Electrospray ionization (ESI) was carried out using nitrogen as sheath (70 psi) and auxiliary gas (20 psi) to assist with nebulization. A potential of 4.5 kV was applied to the ESI needle. The metal capillary was maintained at 200 °C in order to provide optimal desolvation of the ions generated by ESI. Mass spectrometer parameters (Finnigan TSQ-7000) were optimized to obtain maximum sensitivity without sacrificing unit resolution. Collision-induced dissociation was carried out using argon as the collision gas at a pressure of 2.25×10^{-3} Torr in the second quadrupole (Q_2). The skimmer pump pressure was held at 8.47×10^{-1} Torr.

Rapid Kinetics Assays. Consumption of 13-HPODE was monitored by the decrease in absorbance at 236 nm using an Applied Photophysics SX.18MV Stopped Flow Reaction Analyzer. Enzyme and substrate were prepared in separate syringes and combined in equal volumes of approximately 100 μL with a mixing time of 2 ms. The reactions contained final concentrations of 2 μM 12-lipoxygenase and 2 μM 13-HPODE (or 13-HODE as a control) in reaction buffer [50 mM Tris-HCl, 0.03% Tween 20 (w/v), pH 7.4]. Preincubation of 12-lipoxygenase with OPP in the same syringe for 10 min was also performed. The reaction temperature was 25 °C, and all reactions were monitored for 1 s. Reactions with OPP were also monitored for 50 s to allow completion of the reaction.

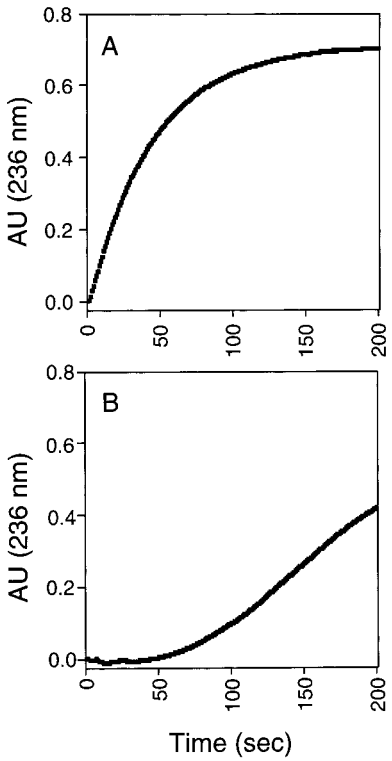


FIGURE 2: Effect of OPP on the dioxygenation of arachidonic acid by 12-lipoxygenase. The reaction conditions were as described for the lipoxygenase assay under Materials and Methods. Reactions contained 12-lipoxygenase (79 nM), arachidonic acid (40 μM), and (A) no inhibitor or (B) 10 μM OPP in reaction buffer [50 mM Tris-HCl, 0.03% Tween 20 (w/v), pH 7.4] and were initiated by addition of enzyme.

RESULTS

OPP Inhibition of the 12-Lipoxygenase Activity. To facilitate the study of the interaction of OPP with recombinant porcine leukocyte 12-lipoxygenase, an improved protein expression and purification protocol was employed which yielded significantly higher amounts of purified enzyme [approximately 4 mg/L vs approximately 250 μg/L in our original system (*15*)]. The expression of 12-lipoxygenase using the pET-20b(+) vector was performed according to a novel expression protocol developed by Boutaud and Brash which has been shown to result in high yields of lipoxygenases (*12*). The final protein product migrated as a single band on SDS−polyacrylamide gel electrophoresis and exhibited a specific activity of 8−12 μmol min^{-1} (mg of protein)$^{-1}$.

OPP potently inhibited the purified recombinant porcine leukocyte 12-lipoxygenase and exhibited an IC_{50} of approximately 2 μM. The effects of OPP on the time course of the reaction are shown in Figure 2. OPP increased the lag phase for attainment of maximal catalytic activity and decreased the rate of reaction once the lag phase was overcome. Neither effect required preincubation of OPP with enzyme before addition of substrate. The increase in lag

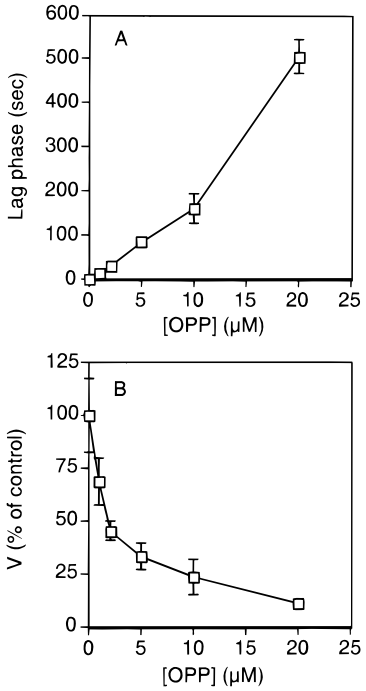


FIGURE 3: Effect of OPP on the dioxygenation of arachidonic acid by 12-lipoxygenase. (A) Plot of lag phase versus OPP concentration. (B) Plot of maximal reaction rate versus OPP concentration. The reaction conditions and definitions of lag phase and maximal rate of reaction were described under Materials and Methods. Reactions contained 12-lipoxygenase (52 nM) and arachidonic acid (60 μM) in reaction buffer [50 mM Tris-HCl, 0.03% Tween 20 (w/v), pH 7.4]. The results represent the average of four determinations ± standard deviations. The reactions were initiated by the addition of enzyme.

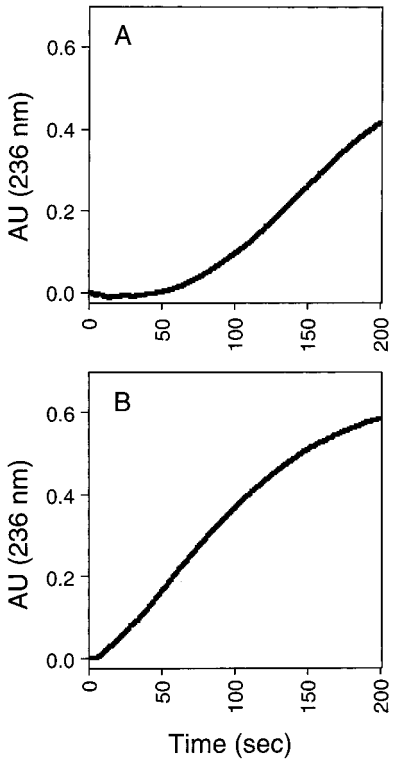


FIGURE 4: Effect of 13-HPODE on the OPP-induced lag in dioxygenation of arachidonic acid. The reaction conditions were as described under Materials and Methods. Reactions contained 12-lipoxygenase (79 nM), arachidonic acid (40 μM), and (A) 10 μM OPP and (B) 10 μM OPP and 10 μM 13-HPODE in reaction buffer [50 mM Tris-HCl, 0.03% Tween 20 (w/v), pH 7.4]. Both reactions were initiated by addition of enzyme.

phase and decrease in catalytic rate were concentration-dependent (Figure 3). To determine the effect of OPP on the kinetics of 12-lipoxygenase oxygenation of arachidonic acid, maximal rates of reaction were measured over a range of concentrations of arachidonic acid in the presence of increasing concentrations of OPP. The reactions were initiated by addition of enzyme. Lineweaver−Burk plots of the data suggested that OPP is a mixed-type inhibitor of 12-lipoxygenase (data not shown). However, the data were of limited utility because the plot was generated using the maximal velocities in the enzyme reactions. Due to the OPP-induced lag time in most of the reactions, the maximal velocity did not correspond to the initial enzyme velocity.

Figure 4 demonstrates that the fatty acid hydroperoxide 13-HPODE greatly reduced the lag phase created by OPP in the 12-lipoxygenase reaction. A concentration of 10 μM 13-HPODE virtually abolished the lag phase induced by an equivalent concentration of OPP. The corresponding alcohol (13-HODE) did not affect the inhibitor-induced lag phase (not shown). These data are consistent with the hypothesis that OPP functions as a redox-type inhibitor. Addition of hydroperoxide would lead to consumption of OPP, thereby eliminating the lag phase.

HPLC Analysis of the Reaction between OPP and 12-Lipoxygenase. The non-heme iron of lipoxygenase enzymes cycles between the ferrous and ferric states during catalysis (*16*, *17*). In most cases, lipoxygenases are isolated in the inactive, ferrous form following purification (*18*), so addition of oxidants such as fatty acid hydroperoxides activates them by generating ferric enzyme (*19*); this eliminates the lag phase (*20*, *21*). Thus, it is possible that the elimination of the OPP-induced lag phase in 12-lipoxygenase catalysis results from hydroperoxide-dependent oxidation of OPP by the ferric enzyme. A corollary of this hypothesis is that the lag phase induced by OPP corresponds to the time required for OPP oxidation. If this is the case, it should be possible to detect oxidation of OPP at the end of the lag phase or following addition of 13-HPODE.

Radiolabeled inhibitor was not available with which to monitor metabolism so we used HPLC to analyze extracts of incubation mixtures for consumption of OPP. To maximize the possibility that OPP was oxidized, we used a stoichiometric equivalent of enzyme to OPP (2 nmol of each). Each molecule of enzyme would only have to oxidize one molecule of inhibitor to result in total depletion of OPP. HPLC profiles of extracts of incubation mixtures are shown in Figure 5. OPP eluted at 8 min, and 13-HPODE eluted at 15 min. These peaks were well-separated from the other peaks in the chromatographic profile. The profiles demonstrate that OPP is not metabolized when incubated with enzyme and 13-HPODE. Possible metabolism of OPP also was tested in the absence of 13-HPODE, and in the presence of arachidonic acid. The recovery of OPP from these reaction mixtures was quantitated by HPLC by comparison to known amounts of standard. Although the recovery of OPP was variable in the different experiments, no reproducible disappearance of OPP was observed (Table 1). The peak coeluting with OPP was collected from the HPLC eluate and analyzed by liquid chromatography/mass spectrometry. The mass spectrum of the isolated material was identical to that of

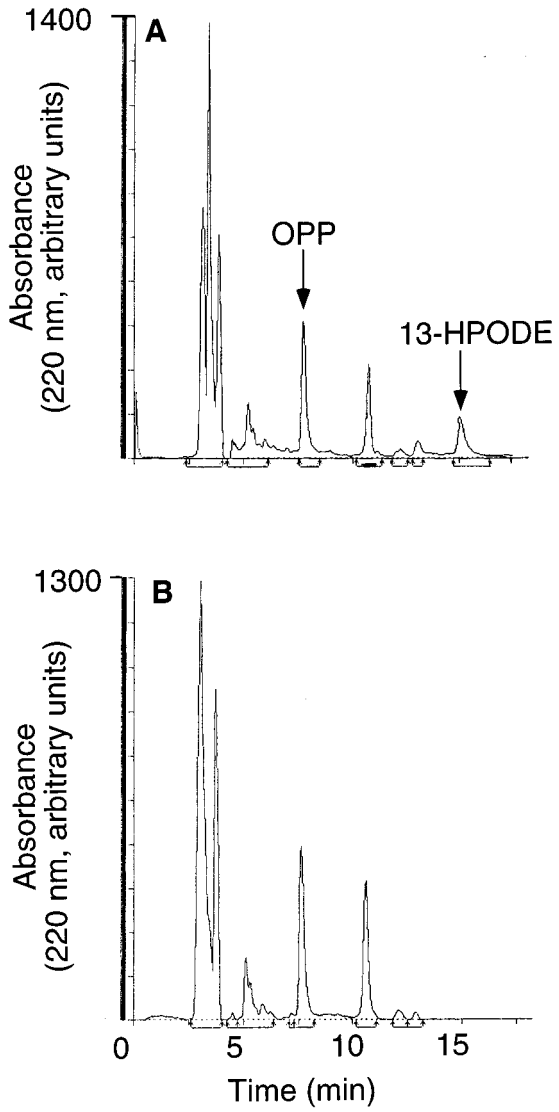


FIGURE 5: Straight phase HPLC of enzyme reaction extracts. Incubations contained equal amounts of OPP (2 nmol), 13-HPODE (2 nmol), and (A) boiled enzyme (control) or (B) active enzyme (2 nmol). The incubations were performed for 10 min at 30 °C in reaction buffer [50 mM Tris-HCl, 0.03% Tween 20 (w/v), pH 7.4]. The recovery of OPP and the HPLC conditions are described under Materials and Methods. The peak eluting at 11 min is an impurity in the enzyme preparation.

Table 1: HPLC Recoveries of OPP from Enzyme Incubations[a]

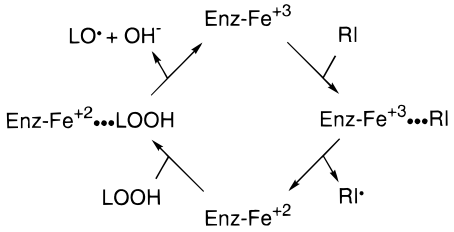
reactants	% OPP recovered
blank	91
boiled enzyme control	85
enzyme + OPP	83
enzyme + OPP + 13-HPODE[b]	120
enzyme + 13-HPODE + OPP[c]	100
enzyme + OPP + arachidonic acid	96

[a] Recoveries were determined by comparison of the UV absorbance peak area with known amounts of standard. [b] The enzyme (2 nmol) was first incubated with OPP (2 nmol) for 5 min before the addition of 13-HPODE. [c] The enzyme (2 nmol) was incubated with 13-HPODE (2 nmol) for 5 min before the addition of OPP.

authentic OPP (not shown). Thus, we conclude that 12-lipoxygenase does not oxidize OPP either in the absence or in the presence of the fatty acid hydroperoxide 13-HPODE.

Reversibility of OPP Inhibition. OPP competition with 13-HPODE suggested the formation of a reversible complex with the ferrous form of 12-lipoxygenase which inhibited the activation of the enzyme. Reversibility of the inhibition by OPP was confirmed in a dilution experiment. The enzyme (4.6 nM) was assayed in the lipoxygenase assay with a concentration of OPP (2 μM) resulting in 59% of enzyme activity. In a second incubation, 46 nM enzyme was incubated with the same concentration of inhibitor for 1 min prior to assay. An aliquot was diluted 10-fold for the standard lipoxygenase assay, and 95% of the enzyme activity was recovered.

Scheme 1: Pseudoperoxidase Reaction of Lipoxygenases[a]



[a] Enz-Fe^{2+}= ferrous enzyme, Enz-Fe^{3+} = ferric enzyme, LOOH = fatty acid hydroperoxide, LO• = fatty acid alkoxyl radical, RI = redox inhibitor, RI• = oxidized redox inhibitor.

OPP Inhibition of the Pseudoperoxidase Activity of 12-Lipoxygenase. It appeared that OPP was inhibiting the enzyme via a novel mechanism that created a lag phase in 12-lipoxygenase activity that could be overcome by fatty acid hydroperoxide. An alternative hypothesis to explain inhibition that does not involve OPP oxidation is that OPP inhibits the lipoxygenase reaction by preventing enzyme activation by fatty acid hydroperoxide. This possibility was tested by investigating the effect of OPP on the pseudoperoxidase activity of the enzyme (*22*). The pseudoperoxidase activity of the enzyme arises from oxidation of the ferrous form of the enzyme by a fatty acid hydroperoxide followed by reduction of the ferric form of the enzyme by a reducing agent (Scheme 1). The ferrous and ferric forms of the enzyme continually cycle to reduce fatty acid hydroperoxide by one electron to an alkoxyl radical. The alkoxyl radical cyclizes to an epoxy allylic radical that abolishes absorbance at 235 nm (*23*). Pseudoperoxidase activity was measured by monitoring the disappearance of the conjugated diene chromophore of 13-HPODE in the presence of a known redox-type inhibitor (BWA137C) (*24*). Figure 6 illustrates that OPP inhibited the pseudoperoxidase activity in a dose-dependent fashion. Thus, OPP did not serve as a substrate for the pseudoperoxidase activity (i.e., it did not stimulate 13-HPODE reduction) but inhibited it. This finding contrasts with observations made with other inhibitors that induce a lag phase by reducing active enzyme back to the inactive, ferrous state (*18*, *25*−*27*). Such inhibitors stimulate the pseudoperoxidase activity of lipoxygenases rather than inhibit this activity. To examine the kinetic effect of OPP on the pseudoperoxidase activity of the enzyme, the initial velocities of the reaction were obtained from the slope of the tangent to the linear portion of the reaction curve at different 13-HPODE and OPP concentrations. Lineweaver−Burk analysis of the data (Figure 7) suggests that OPP is a competitive inhibitor of the activity with regard to 13-HPODE. This is consistent with OPP binding to the ferrous form of 12-lipoxygenase, thereby preventing reduction of 13-HPODE. Because the pseudoperoxidase activity involves both the

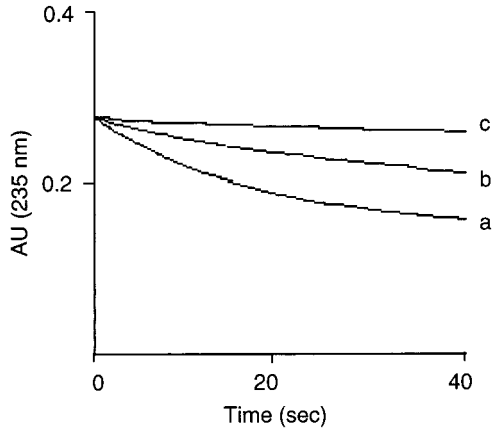


FIGURE 6: Inhibition of the pseudoperoxidase activity of 12-lipoxygenase by OPP. Reaction conditions were as described for the pseudoperoxidase assay. The substrates of the pseudoperoxidase activity were BWA137C (10 μM) and 13-HPODE (5 μM). The consumption of 13-HPODE (A_{236}) was measured in the absence (a) or in the presence of (b) 1 μM or (c) 10 μM OPP. Reactions were initiated by addition of enzyme.

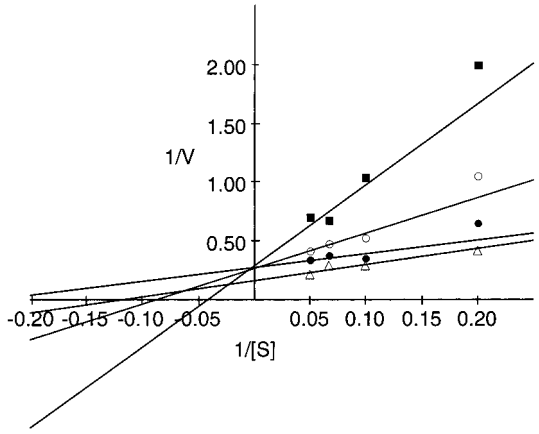


FIGURE 7: Lineweaver−Burk plot of pseudoperoxidase inhibition assay data with OPP. The substrates of the pseudoperoxidase activity were BWA137C (10 μM) and 13-HPODE. OPP concentrations were (■) 2.0 μM, (○) 1.0 μM, (●) 0.5 μM, and (△) 0.0. Reactions were initiated by addition of enzyme.

ferrous and the ferric forms of the enzyme, it is also possible that OPP inhibits the pseudoperoxidase activity by binding to the ferric form of the enzyme. Saturating concentrations of 13-HPODE would not be able to overcome OPP inhibition because of OPP's binding to the ferric form of the enzyme. This would manifest itself as a decreased apparent V_{max} for the pseudoperoxidase activity in the presence of OPP. Figure 8 indicates that the V_{max} does appear to decrease in the presence of OPP, suggesting that OPP may also bind to the ferric form of the enzyme. This would account for the fact that all of the curves in the Lineweaver−Burk plot do not intersect the *y*-axis at $1/V_{max}$.

OPP Inhibition of 13-HPODE Reduction by the Ferrous Form of 12-Lipoxygenase. The data presented thus far suggest that OPP prevents 12-lipoxygenase activation by acting as a reversible, competitive inhibitor of oxidation of the inactive ferrous form of the enzyme to the active ferric form. The concentration-dependent increase in lag phase and the competition with 13-HPODE in the pseudoperoxidase reaction by OPP strongly suggest that OPP binds to the ferrous form of the enzyme. To obtain direct evidence for

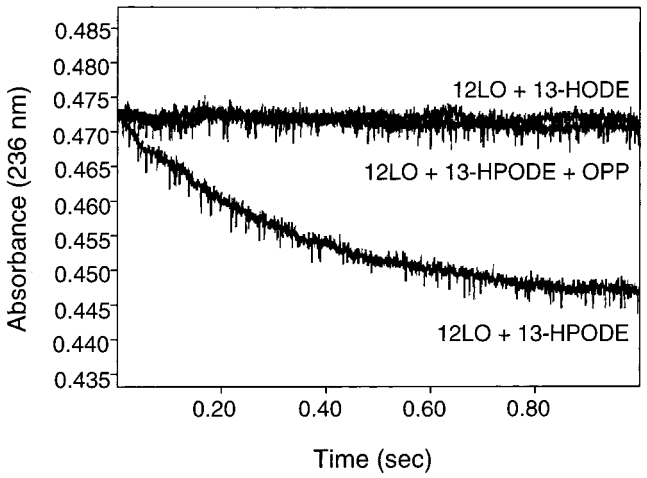


FIGURE 8: Time courses of rapid absorbance changes of 12-lipoxygenase incubated with various compounds. Enzyme and substrate were prepared in separate syringes and combined in equal volumes of approximately 100 μL with a mixing time of 2 ms. Stoichiometric amounts of 12-lipoxygenase (2 μM) and 13-HPODE (2 μM) or 13-HODE (2 μM) were present initially after mixing. For reactions with inhibitor, preincubation of 4 μM 12-lipoxygenase with 10 μM OPP (before stopped-flow mixing) was performed for 10 min. The temperature was 25 °C. The absorbance changes at 236 nm were monitored (1 cm path length, five consecutive experiments averaged). Because initial absorbances were slightly different due to differences in starting materials, all initial absorbances were set equal to the initial absorbance of 2 μM 12-lipoxygenase and 2 μM 13-HPODE.

this interaction, single-turnover stopped-flow experiments were undertaken to ascertain the effect of OPP on 13-HPODE reduction. Due to the unusually high reduction potential of the iron of lipoxygenases as well as the presence of reducing agents in the purification, lipoxygenases are isolated almost exclusively in the ferrous form (*18*, *25*). Therefore, an incubation of stoichiometric amounts of lipoxygenase and 13-HPODE allows one to monitor only the reduction of 13-HPODE without the complication of multiple enzyme forms affecting the observed reaction rates.

12-Lipoxygenase was rapidly mixed with 13-HPODE in a stopped-flow chamber to achieve final concentrations of 2 μM each (2 ms dead time). The disappearance of 13-HPODE was monitored by the decline in absorbance at 236 nm. Reducing agents were omitted from the incubation to prevent reduction of the enzyme after the single reaction of ferrous lipoxygenase with 13-HPODE. The time course of 13-HPODE reduction was rapid and was essentially complete in 1 s in the absence of OPP. The time course of the decrease in absorbance at 236 nm was consistent with a single-exponential decay. The magnitude of the absorbance change was 0.026 AU, corresponding to reduction of 57% of the 13-HPODE present. This incomplete reduction of 13-HPODE may reflect the presence of ferric enzyme or apoprotein in the 12-lipoxygenase preparation. We are unable to determine the distribution of iron between the ferric and ferrous states although the high reduction potential of the enzyme and the presence of high concentrations of dithiothreitol in the purification buffers suggest the enzyme is mainly ferrous. Quantitation of the iron content indicates that approximately 60−75% of the 12-lipoxygenase contains iron in a typical preparation. We did not correct the protein concentrations in the single-turnover experiments for the apoenzyme content.

Preincubation of 4 μM enzyme with 10 μM OPP (concentrations before mixing) greatly reduced the rate of reduction of 13-HPODE, providing direct evidence that OPP inhibits the 13-HPODE-dependent oxidation of ferrous 12-lipoxygenase. Consistent with a role for OPP as a reversible inhibitor, monitoring the reaction for 50 s revealed that 13-HPODE was eventually reduced to the same extent in the presence of OPP as in its absence (not shown). Kinetic analysis of the reactions indicated that preincubation with OPP decreased the initial velocity of reduction of 13-HPODE by approximately 98%. As a control, 12-lipoxygenase incubated with 13-HODE exhibited no decrease in absorbance at 236 nm over these time frames. 13-HPODE itself also did not display a decrease in absorbance at 236 nm (not shown).

OPP Inhibition of the Ferric Form of 12-Lipoxygenase. The question remains whether OPP also binds to the ferric form of the enzyme, thereby directly inhibiting arachidonic acid oxidation. As indicated above, Lineweaver−Burk analysis of the inhibition of the pseudoperoxidase reaction by OPP suggests that OPP may also bind to the oxidized form of the enzyme. If OPP binds to the active, ferric form, a concentration-dependent decrease in the maximal rate of reaction would be expected. Indeed, a decreased maximal velocity of arachidonic acid oxidation is observed after the lag phase is overcome (Figure 3). However, it is possible that the observed decrease in arachidonic acid oxidation reflects enzyme inactivation that might occur during hydroperoxide reduction associated with elimination of the lag phase (*28*). One-electron oxidation of ferrous enzyme should produce an alkoxyl radical that can cyclize to an epoxyallylic radical (*23*, *29*). An unspecified oxidant derived from the epoxyallylic radical could inactivate the enzyme.

Therefore, we attempted to find experimental conditions which dissociated the effect of OPP on enzyme activation from its effect on the maximal rates of reaction. Figure 9 displays the effect of OPP on lag phase and maximal rates of reaction in the presence and absence of 5 μM 13-HPODE. The OPP-induced lag phase was greatly reduced by the presence of 13-HPODE but still increased in a concentration-dependent manner with respect to OPP concentration. However, the decrease in maximal rates of arachidonate oxidation in the presence of 13-HPODE was virtually identical to the decrease seen in the absence of 13-HPODE. 13-HPODE greatly reduced the OPP-induced lag phase without affecting OPP's inhibition of the rate of arachidonic acid metabolism. These data, in conjunction with earlier results, suggest that 13-HPODE is able to shorten the OPP-induced lag phase by out-competing OPP for the ferrous form of the enzyme. However, 13-HPODE does not diminish OPP's competition with arachidonic acid for the active, ferric form of the enzyme.

OPP Does Not Affect Total Product Formation. If OPP acts solely as a reversible, competitive inhibitor, it would be expected that total product formation would be unaffected by OPP if the reaction was allowed to go to completion. Therefore, a final experiment was performed to ascertain the effect, if any, of OPP on total product formation. Figure 10 displays the yield of total products formed at different concentrations of OPP in the presence and absence of 5 μM 13-HPODE. It is clear from this figure that total product formation is not significantly affected by OPP in either circumstance. Therefore, reversible, competitive inhibition seems to account for all of OPP's inhibitory effects. The fact that total product formation is unaltered by OPP also argues against increased enzyme inactivation during the lag phase that was considered above as a possible alternative explanation for decreased rates of reaction in the presence of OPP. In addition, it suggests that OPP does not protect the enzyme from turnover-dependent inactivation.

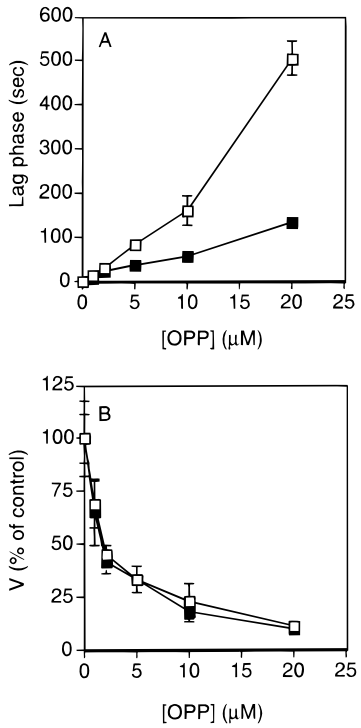


FIGURE 9: Effect of OPP on the dioxygenation of arachidonic acid by 12-lipoxygenase in the presence of 5 μM 13-HPODE. (A) Plot of lag phase versus OPP concentration. (B) Plot of maximal reaction rate versus OPP concentration. Reactions contained 12-lipoxygenase (52 nM), arachidonic acid (60 μM), and no 13-HPODE (□) or 5 μM 13-HPODE (■) and were initiated by addition of enzyme. The reaction conditions and definitions of lag phase and maximal rate of reaction are described under Materials and Methods. The results represent the average of four determinations ± standard deviations.

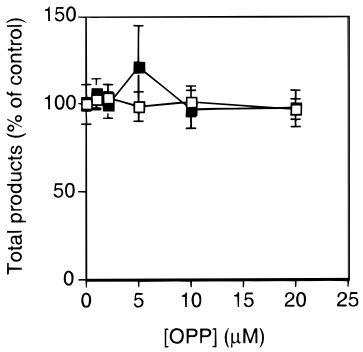


FIGURE 10: Effect of OPP on total product formation by 12-lipoxygenase. Reactions contained 12-lipoxygenase (52 nM), arachidonic acid (60 μM), and no 13-HPODE (□) or 5 μM 13-HPODE (■) and were initiated by addition of enzyme. The reaction conditions were described under Material and Methods. Total product formation was calculated from the difference between the greatest and least values of A_{236} using an extinction coefficient of 23 000 M^{-1} cm^{-1}. The results represent the average of four determinations ± standard deviations.

DISCUSSION

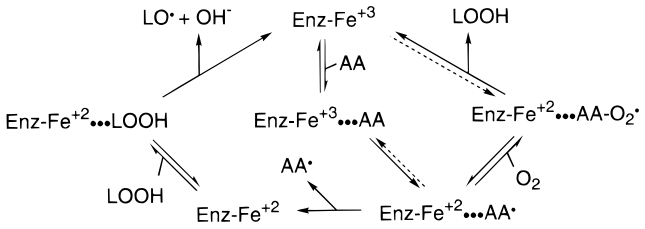
Many different structural classes of lipoxygenase inhibitors have been described, but their mechanisms of inhibition fall into either a redox or a nonredox category. According to the redox mechanism, the inhibitor reduces the active ferric enzyme to the inactive ferrous form and an oxidized derivative of the inhibitor is generated (*19*, *26*, *27*, *30*). The oxidized derivative of the inhibitor either diffuses from the enzyme or, in some cases, reacts with the enzyme to irreversibly inactivate it (*10*, *31*, *32*). The latter is believed to account for the irreversible inactivation of lipoxygenases by acetylenic fatty acid substrate analogues. If the oxidized derivative of the inhibitor does not covalently modify the protein, then enzyme inhibition can be reversed by oxidation of the ferrous form of the lipoxygenase to the active ferric form. This is accomplished by fatty acid hydroperoxides, either added exogenously or generated in situ by active lipoxygenase molecules in solution. Therefore, in the absence of covalent modification, enzyme inactivation by redox-type inhibitors is reversible, and the continued cycle of iron reduction and oxidation leads to consumption of inhibitor leaving active enzyme.

OPP was designed to be a redox-type inhibitor that would possibly covalently modify 12-lipoxygenase, leading to irreversible inactivation (*7*). A potentially oxidizable propargylic methylene group α to an oxygen was positioned nine carbons from the carboxylic acid to mimic the methylene group at carbon 10 of arachidonic acid that is oxidized by 12-lipoxygenase. Substitution of methyl for one of the methylene hydrogens significantly reduced inhibitory activity (in an enantioselective fashion), and dimethyl substitution abolished activity. By contrast, methyl substitution of the benzylic hydrogens did not reduce activity.[2] Thus, the preliminary structure−activity studies supported the hypothesis that OPP was a redox-type inhibitor. Further support for this hypothesis was provided by the induction of a concentration-dependent lag phase by inhibitory concentrations of OPP. The lag phase could be overcome by addition of the fatty acid hydroperoxide, 13-HPODE, in amounts comparable to OPP. Thus, all of the initial data were consistent with OPP acting as a redox-type inhibitor.

To confirm this mechanistic hypothesis, OPP was incubated with stoichiometric concentrations of purified 12-lipoxygenase alone or in the presence of arachidonic acid or 13-HPODE. Analysis of organic extracts of the incubation mixtures by HPLC and LC/MS revealed that OPP was not oxidized and could be recovered quantitatively. This observation was inconsistent with OPP acting as a redox-type inhibitor and was substantiated by the finding that dilutions of concentrated solutions of 12-lipoxygenase and OPP led to recovery of enzyme activity. A final experiment supported the conclusion that OPP is not a redox-type inhibitor and suggested a mechanism to explain its inhibitory action on 12-lipoxygenase. Incubation of OPP and 13-HPODE with 12-lipoxygenase did not support the decomposition of 13-HPODE according to the pseudoperoxidase activity, which is required for a redox-type inhibitor. In fact, OPP *inhibited* the pseudoperoxidase activity supported by the redox-type inhibitor BWA137C.

Kinetic analysis of the pseudoperoxidase reaction in the presence of OPP suggested that OPP acted competitively with 13-HPODE to inhibit the pseudoperoxidase activity. However, the kinetics were not cleanly competitive in that in the presence of increasing concentrations of OPP, all of the curves at high substrate concentration did not intersect at $1/V_{max}$. This suggests that OPP has other actions on the enzyme. A detailed kinetic mechanism has not been developed for leukocyte-type 12-lipoxygenase, but kinetic mechanisms are available for the soybean 15-lipoxygenase and the reticulocyte 15-lipoxygenase (Scheme 2). Several "sites" of action of OPP in this scheme are possible. Theoretically, all of its actions could result from binding to the ferric enzyme which would prevent oxidation of arachidonic acid (lipoxygenase activity) as well as inhibit the pseudoperoxidase activity by preventing the cycling of ferric to ferrous enzyme. We do not favor this hypothesis because the data in Figure 7 suggest that OPP is competitive with 13-HPODE. However, the lack of intersection of all of the curves at $1/V_{max}$ may indicate that a portion of the pseudoperoxidase inhibition derives from OPP binding to the ferric enzyme.

Scheme 2: Lipoxygenase Catalytic Cycle[a]

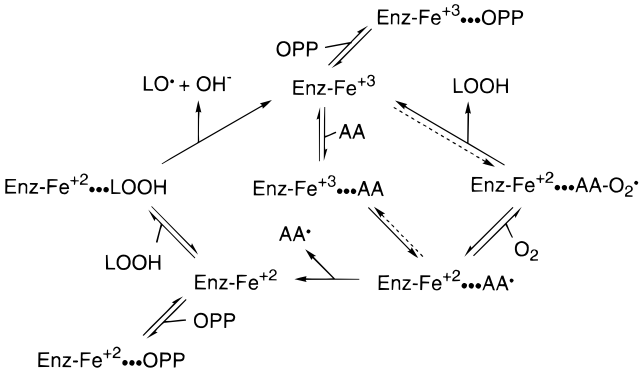


[a] The reactions shown occur in the absence of redox inhibitor. Enz-Fe^{2+} = ferrous enzyme, Enz-Fe^{3+} = ferric enzyme, LOOH = fatty acid hydroperoxide, LO• = fatty acid alkoxyl radical, AA = substrate fatty acid, AA• = substrate radical, AA/O_2• = substrate peroxyl radical. This scheme is adapted from Desmarais et al. (*26*).

To probe for the interaction of OPP with the ferrous form of 12-lipoxygenase in the absence of the involvement of ferric enzyme, we conducted a single-turnover experiment in which 12-lipoxygenase and 13-HPODE were rapidly mixed in the absence of a reducing agent. The rapid decrease in absorbance at 236 nm indicated that 13-HPODE reduction was rapid and complete within 1 s. Preincubation of enzyme with OPP clearly inhibited 13-HPODE reduction. This provides direct support for binding of OPP to the ferrous enzyme as an important mode of action. In addition, this is the first report of the detection of hydroperoxide reduction by a ferrous lipoxygenase in a single-turnover experiment. A previous attempt to detect single-turnover reduction by soybean 15-lipoxygenase was unsuccessful (*33*). This may have been due to differences in the enzyme isoform (mammalian 12-lipoxygenase versus plant 15-lipoxygenase) or differences in the monitoring of hydroperoxide reduction. We employed 236 nm where 13-HPODE absorbance is maximal. Even at this wavelength, the spectral changes of 13-HPODE reduction are small. Jones et al. monitored spectral changes at 250 nm where the sensitivity is significantly lower (*33*).

The ability of OPP to compete with 13-HPODE for binding to the 12-lipoxygenase active site provides a mechanism for the lag phase induced by the inhibitor. OPP is a nonredox inhibitor that prevents the redox changes at the iron center necessary to generate active enzyme. This is the first time such a role has been described for a non-redox-type inhibitor. As shown in Scheme 2, dissociation of

Scheme 3: Model of Inhibition of 12-Lipoxygenase by OPP[a]



[a] OPP binds to both the ferrous and ferric forms of 12-lipoxygenase. Enz-Fe^{2+} = ferrous enzyme, Enz-Fe^{3+} = ferric enzyme, Enz-Fe^{2+}···OPP = ferrous enzyme bound to OPP, Enz-Fe^{3+}···OPP = ferric enzyme bound to OPP, LOOH = fatty acid hydroperoxide, LO• = fatty acid alkoxyl radical, AA = substrate fatty acid, AA• = substrate radical, AA/O_2• = substrate peroxyl radical.

substrate radicals from the active site prior to reoxidation of the enzyme to the ferric form generates ferrous lipoxygenase throughout reactions with both soybean 15-lipoxygenase and reticulocyte 15-lipoxygenase (*13*, *14*). A significant fraction of enzyme exists in the ferrous state until sufficient hydroperoxide has been generated by the enzyme to quickly reoxidize ferrous enzyme. At this point, maximal rates are achieved. The "leakage" back to the ferrous form, if it occurs with leukocyte-type 12-lipoxygenase, should serve to magnify OPP's effect on the lag phase. Because multiple oxidation events are necessary to maintain the enzyme in the ferric state, OPP will inhibit the oxidation of the enzyme each time ferrous lipoxygenase is generated.

Several pieces of data suggest OPP also binds to the ferric enzyme. As stated above, Lineweaver−Burk analysis of the inhibition of pseudoperoxidase activity reveals that the reciprocal plots of activity in the presence of OPP do not intersect $1/V_{max}$ on the abscissa. This may be due to OPP binding to the ferric enzyme. More important is the inhibition of arachidonic acid oxygenation by OPP after the lag phase has been overcome. One anticipates that if OPP only inhibited lipoxygenase activation, the rate of arachidonate oxygenation would not be inhibited once the enzyme was fully active. This is clearly not the case. Even in the presence of concentrations of 13-HPODE that dramatically shorten the OPP-induced lag, the maximal rate of 12-lipoxygenase activity is less than the rate in the absence of OPP. The decrease in maximal rate is directly dependent on OPP concentration. At a given concentration of OPP, the maximal rate achieved is the same whether or not the enzyme is activated by 13-HPODE addition. These experiments indicate that OPP inhibits the oxygenation of arachidonic acid by the active ferric form of 12-lipoxygenase. Thus, OPP exerts two effects that combine to make it an effective inhibitor of 12-lipoxygenase (Scheme 3). It competes with fatty acid hydroperoxide to prevent the activation of ferrous enzyme, and it competes with arachidonic acid to prevent its oxygenation by the ferric enzyme. This dual effect may account for the mixed-type inhibition displayed in kinetic experiments.

The ability of OPP to prevent the activation of ferrous enzyme may be a more general phenomenon among nonredox inhibitors. Thorough characterization of enzyme−inhibitor interactions is difficult for lipoxygenases because of the complexity of the kinetics of arachidonic acid oxygenation even in the absence of an inhibitor (*34*, *35*). Thus, effects of inhibitors on enzyme activation and pseudoperoxidase activity are not routinely monitored. However, the induction of a lag phase is usually taken as diagnostic information that the inhibitor is redox-type, and, in fact, in many cases examined, the inhibitors support pseudoperoxidatic reduction of fatty acid hydroperoxide (*24*). In contrast, OPP induces prolonged lag phases but inhibits pseudoperoxidase activity.

The present results favor a reinterpretation of the structure−activity of OPP inhibition. The abolition of inhibitory activity by introduction of geminal methyl groups at the propargylic carbon was taken as supportive information for oxidation at this position. However, since OPP is not oxidized, the effect of methylation must relate to preventing binding to the enzyme. This interpretation is consistent with the absence of an isotope effect for inhibition of 12-lipoxygenase by [9,9-$^2H_2$]-OPP and by the absence of time-dependence in its inhibitory action. One might anticipate time-dependence if OPP was metabolized to a derivative that chemically inactivated the enzyme (*7*).

Since OPP exerts two separate actions to inhibit 12-lipoxygenase activity, it will be interesting to determine if both effects are mediated by binding to a common site or to separate sites on the enzyme. OPP is competitive with 13-HPODE binding to the ferrous enzyme and appears to be competitive with arachidonic acid binding to the ferric enzyme. Early models of lipoxygenases incorporated more than one lipid binding site to account for the observed nonlinear kinetics of lipoxygenases (*21*, *36*−*38*). However, it has been shown that a simpler, single binding site model for lipoxygenases is sufficient to account for the kinetics of lipoxygenase reactions (*14*, *39*, *40*). In addition, the crystal structure of a mammalian 15-lipoxygenase reveals a single hydrophobic binding site in close proximity to the non-heme iron (*41*). Therefore, 13-HPODE and arachidonic acid likely bind to the same site on the enzyme. Binding to this common site by OPP would account for its dual inhibitory properties without the need to invoke multiple binding sites. Additional studies are in progress to test this hypothesis.

There are many examples of position-specific lipoxygenase inhibitors. For example, a number of 5-lipoxygenase inhibitors have been synthesized that do not inhibit 12- or 15-lipoxygenases (*42*−*46*). However, OPP is the first example of a molecule that discriminates between two isoforms of a positional lipoxygenase. OPP is approximately 3 orders of magnitude more potent on leukocyte-type 12-lipoxygenase than on platelet-type 12-lipoxygenase (*7*). Now that the mechanism of enzyme inhibition by OPP has been established, it will be interesting to define the structural basis for its discrimination between the two lipoxygenase isoforms.

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