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

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ABSTRACT: Lipoxygenases (LOXs) are a ubiquitous family of enzymes that catalyze the dioxygenation of polyunsaturated fatty acids. Their role in a diverse range of biological processes has prompted the development of a large number of lipoxygenase inhibitors of possible therapeutic and probative value. The isoform-selective inhibitor 4-(2-oxapentadeca-4-yne)phenylpropanoic acid (OPP) was previously shown to inhibit leukocyte-type 12-LOX by a novel mechanism in which it binds to both the ferrous and ferric forms of the enzyme. The current study provides a detailed kinetic model of this inhibition. Nonlinear regression analysis of OPP's inhibition of arachidonic acid dioxygenation indicated mixed inhibition toward the ferric form of 12-LOX with apparent K_I values in the low micromolar range: 2.0 ± 0.2 μM for the free enzyme and 4.5 ± 0.7 μM for the substrate-bound form of the enzyme. Rapid kinetic techniques allowed OPP's inhibition of the activation of the enzyme from the ferrous to the ferric form to be investigated. Titration of ferrous 12-LOX with OPP indicated that it bound to the ferrous form with an apparent K_I value of 70 ± 20 nM, suggesting a significantly higher affinity for the ferrous form than for the ferric form of the enzyme. Investigation of the LOX inhibitors nordihydroguaiaretic acid, *N*-(4-chlorophenyl)-*N*-hydroxy-*N*′-(3-chlorophenyl)urea, BWA137C, and eicosatetraynoic acid revealed that eicosatetraynoic acid also inhibited the activation of 12-LOX. These results demonstrate that LOX inhibitors are capable of binding to multiple forms of LOXs with high affinity and suggest that inhibition of enzyme activation may be an unrecognized mechanism of inhibition of additional LOX inhibitors.

Lipoxygenases (LOXs)[1] are a family of non-heme iron-containing proteins that are present throughout the plant and animal kingdoms. They catalyze the regio- and stereospecific dioxygenation of polyunsaturated fatty acids to generate conjugated diene-containing hydroperoxy fatty acids (*1*−*6*). The catalytic cycle of LOXs is shown in Figure 1. The resting, ferrous form of the enzyme is oxidized to the active, ferric form of the enzyme by lipid hydroperoxides (*7*). The ferric form then oxidizes the polyunsaturated fatty acids (e.g., linoleic acid and arachidonic acid). Separate mammalian LOXs have been shown to oxidize arachidonic acid at the 5, 8, 12, and 15 positions (*4*). The 5-LOX pathway is an important contributor to inflammatory and anaphylactic diseases, and roles for other LOXs have been suggested in such diverse disease states as atherosclerosis, diabetes, and cancer (*6*). Therefore, considerable interest exists in the development of LOX inhibitors as potential therapeutic agents and as aids in the elucidation of the biological roles of LOXs (*8*, *9*).

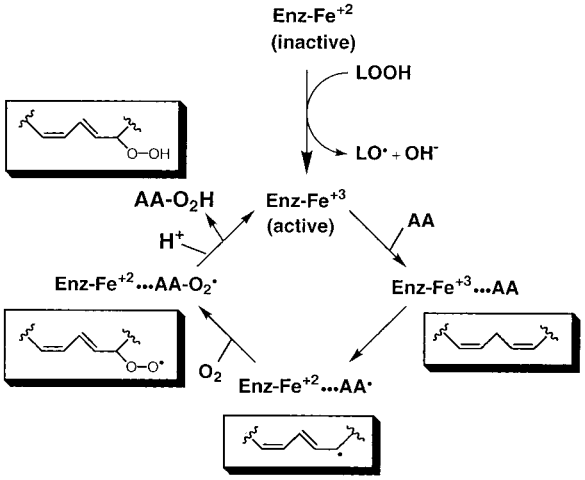

FIGURE 1: Catalytic cycle of LOXs. Structures of functional groups indicated in boxes.

Two different 12-LOXs have been extensively studied (platelet-type and leukocyte-type) which catalyze essentially the same reaction but differ in terms of tissue distribution, substrate specificity, and inactivation kinetics (*8*). We have previously reported the synthesis of a series of oxaacetylenic acids that selectively inhibit leukocyte-type 12-LOX (*10*). The most potent compound in this series, 4-(2-oxapentadeca-4-yne)phenylpropanoic acid (OPP), is shown in Figure 2. It inhibits 12-LOX through a novel mechanism in which it binds to both the inactive and active forms of the enzyme

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[1] Abbreviations: LOX, lipoxygenase; OPP, 4-(2-oxapentadeca-4-yne)phenylpropanoic acid; 13-HPODE, 13(*S*)-hydroperoxyoctadecadienoic acid; NDGA, nordihydroguaiaretic acid; CPHU, *N*-(4-chlorophenyl)-*N*-hydroxy-*N*′-(3-chlorophenyl)urea; ETYA, eicosatetraynoic acid.

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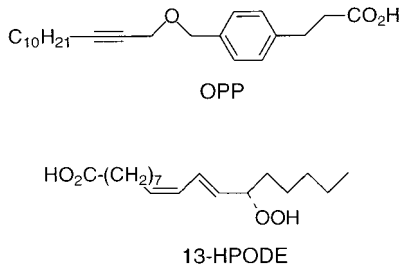

FIGURE 2: Structures of OPP and 13-HPODE.

(*11*). The former interaction slows enzyme activation, whereas the latter interaction inhibits the hydroperoxidation of the substrate.

In the study presented here, we investigated the kinetics of OPP inhibition of purified leukocyte-type 12-LOX. OPP bound to the ferric form of the enzyme both competitively and uncompetitively with K_I values in the low micromolar range. OPP also bound to the ferrous form with an affinity significantly higher than that for the ferric form, with a K_I value in the low nanomolar range. Studies of additional LOX inhibitors indicated that eicosatetraynoic acid (ETYA) also bound to the ferrous form of 12-LOX. Inhibition of LOX activation may represent an unrecognized aspect of inhibition of additional nonredox LOX inhibitors.

MATERIALS AND METHODS

Arachidonic acid and 13-hydroperoxyoctadecadienoic acid were purchased from Cayman Chemical (Ann Arbor, MI). The expression vector pET-20b(+) and *Escherichia coli* strain BL21(DE3) were purchased from Novagen (Milwaukee, WI). DNaseI was from Boehringer Mannheim (Indianapolis, IN). The bead beater and 0.1 mm glass beads were purchased from Biospec Products (Bartlesville, OK). Econopac 10DG columns were purchased from Bio-Rad (Hercules, CA). HiTrapQ columns, MonoP HR5/20 columns, and Polybuffer were from Pharmacia (Uppsala, Sweden). Tween 20 was 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.). All other reagents were from common suppliers. Enzyme purifications were performed using an FPLC system (Pharmacia) operated at room temperature. This system consists of a GP-250 gradient programmer that controls two P-500 pumps. Recombinant porcine leukocyte-type 12-LOX was purified to homogeneity as previously described (*11*).

Lipoxygenase Assays. Lipoxygenase activity was detected by monitoring the absorbance of the conjugated diene product at 236 nm. UV assays were monitored using a Hewlett-Packard 8452A diode array spectrophotometer equipped with a water-jacketed cuvette. The enzyme reactions included reaction buffer [50 mM Tris-HCl and 0.03% Tween 20 (pH 7.4)], arachidonic acid, and enzyme. Inhibitor was added to some assays by 500- or 1000-fold dilution from methanol stocks, with the total concentration of methanol held constant for a given experiment. All reactions were initiated by the addition of enzyme to the reaction cuvette followed by mixing. The reaction temperature was 25.0 °C, and the final reaction volume was 1 mL. The extent of product formation was calculated using an extinction coefficient of 23 000 M^{-1} cm^{-1} for the conjugated diene product (*12*).

Rapid Kinetics Assays. Reduction of 13-HPODE by ferrous 12-LOX was monitored using an Applied Photophysics SX.18MV stopped-flow reaction analyzer. The enzyme and substrate were prepared in separate syringes and combined in equal volumes of approximately 100 μL with a mixing time of 2.5 ms. The inhibitor OPP was preincubated with the enzyme for 10 min. The reaction mixtures contained final concentrations of 12-LOX (2.8 μM), OPP (0−6 μM), and 13-HPODE (3.0−15.5 μM) in reaction buffer [50 mM Tris-HCl and 0.03% Tween 20 (pH 7.4)]. The reaction temperature for all reactions was 25.0 °C. The extent of reduction of 13-HPODE was measured as a decrease in absorbance at 236 nm.

Enzyme Kinetics. UV assay results were assessed using the computer programs Prism (Graphpad Software) and Enzyme Kinetics 1.5 (Trinity Software). Velocity data were obtained by taking the slope of the reaction curve at the point of maximal reaction velocity. Due to the lag phase caused by OPP in some reactions, this rate was not necessarily the initial rate. Therefore, the substrate concentration was corrected to correspond to the actual concentration present at the time of the maximal reaction velocity. This was done by using the A_{236} value at the time of the maximal rate of reaction as an indication of the amount of substrate that had been metabolized. The corrected kinetic data were analyzed through nonlinear regression to determine the Michaelis−Menten kinetic parameters. Secondary plots ($1/v$ vs [OPP] and s/v vs [OPP]) were used to graphically determine the type of inhibition of OPP (*13*, *14*). Because these plots require common substrate concentrations across different inhibitor concentrations, the nonlinear regression fits of the corrected data were used to plot the rate data across the substrate and inhibitor concentrations that were studied. To determine the K_I values for the free and substrate-bound forms of the enzyme, plots of K_M/V_{max} versus [OPP] and $1/V_{max}$ versus [OPP] were also constructed as described previously (*13*, *14*).

Stopped-flow results were analyzed using the computer program Prism. The time course of each reaction was analyzed by linear regression using the least-squares method to obtain initial rate data. Data were analyzed until 10% of the enzyme present had been oxidized to the ferric form (5−438 ms). For each set of conditions, the reactions were performed in triplicate to yield an average rate of reaction. For each concentration of OPP, the mean initial rate was determined from six concentrations of 13-HPODE (3.0, 5.5, 8.0, 10.5, 13.0, and 15.5 μM). The relationship between initial rates and the concentrations of 12-LOX and OPP is expressed in eq 5, which is developed below in Results. Nonlinear regression analysis was used to fit this equation to the data in Figure 8 to obtain the inhibitor constant (K_I) of OPP for the ferrous form of the enzyme.

RESULTS

OPP Inhibition of Arachidonic Acid Dioxygenation. As previously described, OPP inhibits leukocyte 12-LOX by causing both a pronounced lag phase and a decrease in the maximal rate of reaction after the lag phase (*11*). The duration of the lag phase does not influence the maximum

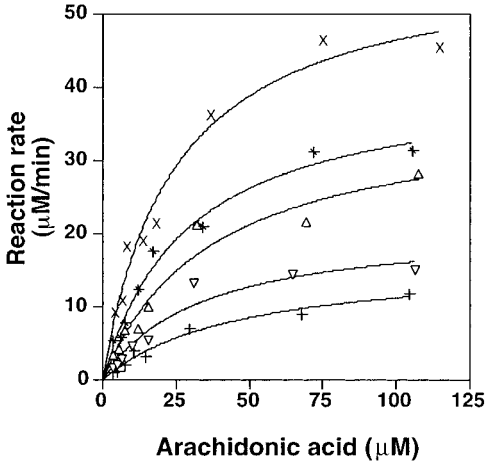

FIGURE 3: Nonlinear regression analysis of OPP inhibition of arachidonic acid dioxygenation by 12-LOX. The reaction conditions are described for the LOX assay in Materials and Methods. All reaction mixtures contained enzyme (44 nM) and arachidonic acid (5−120 μM) in reaction buffer [50 mM Tris-HCl and 0.03% Tween 20 (pH 7.4)]. OPP concentrations were (×) 0.0, (∗) 1.0, (△) 2.0, (◇) 5.0, and (+) 10 μM. The temperature was 25 °C, and the reactions were initiated by addition of enzyme.

rates of reaction; for example, reducing the length of the OPP-induced lag phase by addition of lipid hydroperoxides does not alter either the maximum rates of reaction or the level of total product formation (*11*). Because the ferric form of the enzyme is responsible for dioxygenation of arachidonic acid, a decrease in the maximum rate of reaction after the lag phase is an indication of OPP's ability to inhibit the ferric form. Therefore, the maximal rates were used to quantify OPP's binding to the ferric form. Nonlinear regression analysis was performed on reaction mixtures containing varying amounts of substrate (5−120 μM) and inhibitor (0−10 μM) (Figure 3). As discussed in Materials and Methods, the amount of substrate present at the time of the measured rate of reaction was corrected for the amount of substrate utilized during the lag phase. Secondary plots of the data were used to determine the type of inhibition of OPP (Figure 4). As discussed in Materials and Methods, the nonlinear regression lines were plotted as opposed to the corrected data because these secondary plots require common substrate concentrations across different experiments. The pattern of intersecting lines in both panels A and B of Figure 4 indicates that OPP acted as a mixed inhibitor (*13*, *14*). To determine the K_I values of OPP binding, additional secondary plots shown in Figure 5 were constructed; OPP bound to free enzyme with an apparent K_I of 2.0 ± 0.2 μM and bound to the enzyme−substrate complex with an apparent K_I' of 4.5 ± 0.7 μM (*13*, *14*).

OPP Inhibition of 13-HPODE Reduction. We have previously shown that the activation of 12-LOX by the fatty acid 13-HPODE (shown in Figure 1) can be monitored using rapid kinetic techniques (*11*). The one-electron oxidation of ferrous 12-LOX to the active, ferric form is concomitant with the one-electron reduction of 13-HPODE (Figure 2). Rearrangement of the resulting fatty acid alkoxyl radical results in a decrease in A_{236} due to a loss of the conjugated diene (*15*). This serves as an indirect measure of the reaction between 13-HPODE and 12-LOX, and OPP (10 μM) was shown to inhibit this reaction (*11*). Consistent with our earlier studies, OPP caused a concentration-dependent decrease in the rate

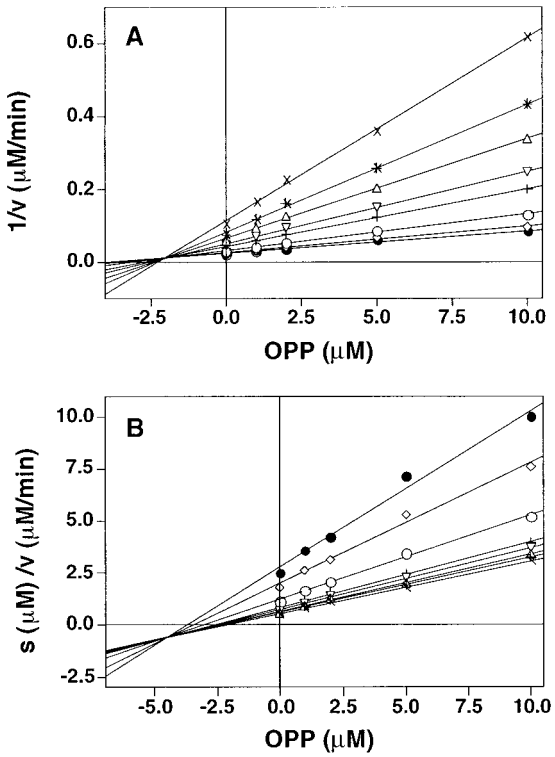

FIGURE 4: Determination of the type of inhibition of OPP for ferric 12-LOX. Plot of (A) $1/v$ or (B) s/v vs [OPP]. Because the experiments at different inhibitor concentrations must have common substrate values, the rate data for these plots are the values determined from the nonlinear regression lines in Figure 3. Rates for the following arachidonic acid concentrations were calculated: (×) 5.0, (∗) 7.5, (△) 10, (▽) 15, (+) 20, (○) 40, (◇) 80, and (●) 120 μM.

of 13-HPODE reduction by 12-LOX (Figure 6). To develop a detailed understanding of this interaction, reduction of 13-HPODE by the ferrous enzyme was monitored in the absence of OPP under a variety of conditions (0−2.8 μM 12-LOX and 3.0−15.5 μM 13-HPODE). The initial rate data were derived from the time courses up to the point where 10% of the enzyme was oxidized to the ferric form. As shown in Figure 7, the initial rate of 13-HPODE reduction was linear with respect to both 12-LOX and 13-HPODE. For both lines, the 95% confidence intervals of the *y*-intercepts include zero as would be expected in the absence of either substrate or enzyme. Higher concentrations of 13-HPODE could not be used due to their absorbance at 236 nm. Because saturating substrate concentrations were not achieved, the Michaelis−Menten kinetic parameters of this reaction could not be calculated.

The linear relationship between 13-HPODE reduction and the concentration of the free, ferrous enzyme allows one to calculate the concentration of free 12-LOX by comparing initial rate data to those of control experiments.

$$\frac{v_o}{v_{o-con}} = \frac{[Enz_{free}]}{[Enz_{free-con}]} \quad (1)$$

In the absence of inhibitor, the free enzyme concentration in control experiments equals the total enzyme concentration. For eq 1 to be valid in the presence of inhibitor, one must assume that the inhibitor-bound enzyme has no activity. This appears to be valid in the case of ferrous 12-LOX and OPP

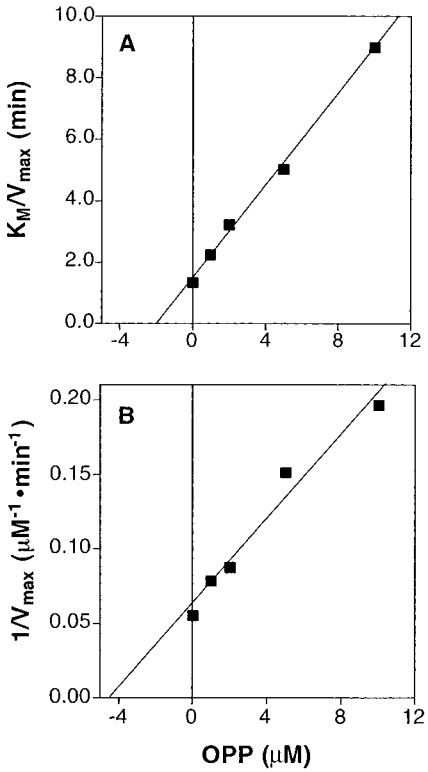

FIGURE 5: Determination of K_I values of OPP for ferric 12-LOX. Plot of (A) K_M/V_{max} or (B) $1/V_{max}$ vs [OPP] to determine the K_I values of competitive and uncompetitive inhibition of OPP, respectively. As previously described (*13*, *14*), the K_I values of the free enzyme and substrate-bound enzyme equal the negative of the *x*-intercepts of plots A and B, respectively.

because the remaining enzyme activity decreases on an almost stoichiometric basis with the addition of OPP under these conditions (see below). Therefore, the remaining concentration of free 12-LOX can be ascertained after incubations of the enzyme with OPP. The following three equations were used with eq 1 to analyze the interaction of OPP and 12-LOX.

$$[Enz_{total}] = [Enz_{free}] + [Enz \cdot OPP] \quad (2)$$

$$[OPP_{total}] = [OPP_{free}] + [Enz \cdot OPP] \quad (3)$$

$$K_I = \frac{[Enz_{free}] \cdot [OPP_{free}]}{[Enz \cdot OPP]} \quad (4)$$

Rearrangement of eqs 1−4 using the quadratic equation allows one to solve for the remaining enzyme activity in terms of the K_I value of OPP for ferrous 12-LOX and the known variables $[Enz_{total}]$ and $[OPP_{total}]$.

$$\frac{v_o}{v_{o-con}} = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a} \quad (5)$$

where $a = [Enz_{total}]^2$, $b = [Enz_{total}][OPP_{total}] + K_I[Enz_{total}] - [Enz_{total}]^2$, and $c = -K_I[Enz_{total}]$.

Various concentrations of OPP (0−6 μM) were preincubated with 12-LOX (2.8 μM) and then reacted with 13-HPODE (3.0−15.5 μM). The means of the initial rates of the reaction versus OPP concentration are shown in Figure 8. Longer times of preincubation of 12-LOX with OPP did not affect the initial rates of reaction, indicating that equilibrium had been achieved (data not shown). As above, time course data were used up to the point where 10% of

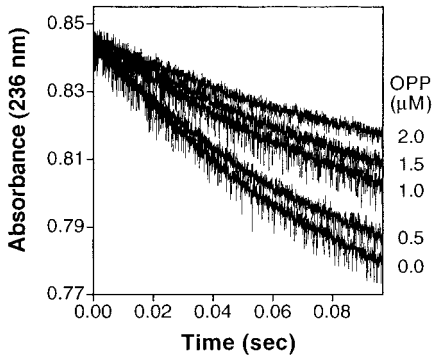

FIGURE 6: Effect of OPP on 13-HPODE reduction by ferrous 12-LOX. The reaction conditions are as described in Materials and Methods. Final concentrations of 2.8 μM 12-LOX and 8 μM 13-HPODE were present initially after mixing. For reactions with inhibitor, preincubation of 12-lipoxygenase and OPP was performed for 10 min. Because initial absorbances were slightly different due to differences in starting materials, all initial absorbances were set equal to the initial absorbance in the absence of inhibitor.

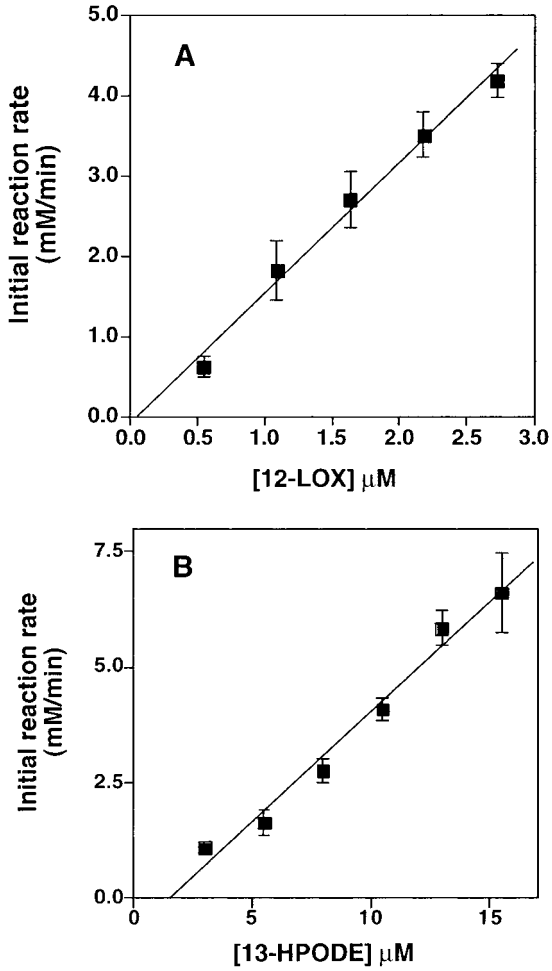

FIGURE 7: Effect of 12-LOX and 13-HPODE concentrations on the rate of 13-HPODE reduction by ferrous 12-LOX. The reaction conditions are as described in Materials and Methods. (A) Plot of the initial reaction rate vs [12-LOX]. Reaction mixtures contained enzyme (0.5−2.8 μM) and 8 μM 13-HPODE immediately after mixing. (B) Plot of the initial reaction rate vs [13-HPODE]. Reaction mixtures contained 2.7 μM 12-LOX and 13-HPODE (3.0−15.5 μM) immediately after mixing.

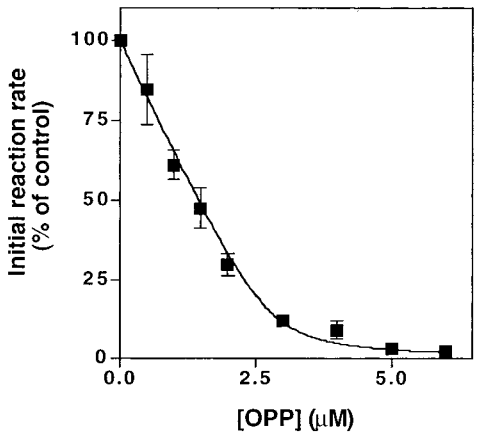

FIGURE 8: Dependence of initial reaction rates of 13-HPODE reduction by ferrous 12-LOX on [OPP]. The reaction conditions are as described in Materials and Methods. The data represent the mean initial reaction rates of six incubations with 2.8 μM 12-LOX and varying concentrations of 13-HPODE (3.0−15.5 μM) for each concentration of OPP. The solid line is a fit of the experimental data to eq 5, yielding an inhibitor constant K_I for OPP of 70 ± 20 nM.

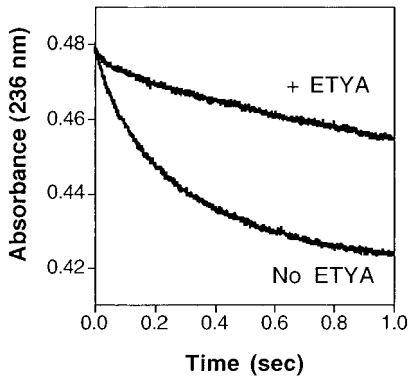

FIGURE 9: Effect of ETYA on 13-HPODE reduction by ferrous 12-LOX. The reaction conditions are as described in Materials and Methods. The enzyme was prepared in one syringe, while the substrate and inhibitor were prepared in a separate syringe. Final concentrations of 1.5 μM 12-LOX, 5 μM 13-HPODE, and 10 μM ETYA were present immediately after mixing. Because initial absorbances were slightly different due to differences in starting materials, the initial absorbances were set equal to the initial absorbance in the absence of inhibitor.

the enzyme had been oxidized to the ferric form. The initial rate data were then fit to eq 5 to yield an apparent K_I value of 70 ± 20 nM. Because the experiment was designed as a titration of the free ferrous enzyme to the OPP-bound enzyme, this inhibitor constant corresponds to OPP acting as a competitive inhibitor of 13-HPODE reduction. In the absence of saturating substrate concentrations, no conclusions can be reached regarding the possibility that OPP also acts as an uncompetitive inhibitor of this reaction. Of note, OPP appears to bind much more tightly to ferrous 12-LOX than ferric 12-LOX, with an apparent K_I value 29-fold lower for the ferrous form.

ETYA Inhibition of 13-HPODE Reduction. With the ability to monitor the inhibition of the activation of 12-LOX, we explored the possibility that other LOX inhibitors act in a similar manner. The LOX inhibitors eicosatetrayenoic acid (ETYA), nordihydroguaiaretic acid (NDGA), *N*-(4-chlorophenyl)-*N*-hydroxy-*N*′-(3-chlorophenyl)urea (CPHU), and BWA137C were tested for their ability to inhibit 13-HPODE reduction by ferrous 12-LOX. As shown in Figure 9, rapid kinetic techniques indicate that 10 μM ETYA does inhibit this reaction. None of the other LOX inhibitors had an effect on 13-HPODE reduction at 10 μM (data not shown).

DISCUSSION

Lipoxygenases are ubiquitous, non-heme iron-containing enzymes whose physiological roles are still under investigation. Fueling interest in this field, however, is the fact that LOXs have been implicated in aspects of atherosclerosis, carcinogenesis, diabetes, nociception, and hypersensitivity reactions (*6*). Because of the possible importance of these enzymes, a large number of LOX inhibitors have been described which fall into two general categories: redox and nonredox inhibitors. Inhibitors acting by redox mechanisms reduce the enzyme to the ferrous form while being converted to an oxidized derivative. This conversion of LOXs to the inactive ferrous form causes a lag phase in product formation and is responsible for the inhibitory properties of many LOX inhibitors (*16*−*20*). Some redox inhibitors also cause the covalent modification of the enzyme, thereby inactivating the enzyme, through covalent attachment of the oxidized derivative of the inhibitor to the enzyme or through the generation of a hydroxyl radical that leads to inactivation (*21*−*23*). Nonredox inhibitors generally act as competitive inhibitors, presumably by competing with the substrate for binding to the active site. Due to toxic side effects associated with many redox-type inhibitors, nonredox inhibitors are increasingly viewed as the most attractive means of inhibiting LOXs (*24*−*28*).

Despite much progress in developing LOX inhibitors, most are not specific, inhibiting other LOXs and sometimes other families of enzymes. The inhibitor OPP, however, displays exquisite specificity by inhibiting leukocyte-type 12-LOX with an IC_{50} value 3 orders of magnitude lower than those of other closely related LOXs, including platelet-type 12-LOX (*10*). Thus, it is the only isoform-specific LOX inhibitor described to date. OPP was designed to be a redox-type inhibitor of leukocyte 12-LOX, but studies have demonstrated that OPP is not metabolized by the enzyme (*11*). Rather, it inhibits 12-LOX by binding both to the ferrous form, which slows activation and causes a lag phase, and to the ferric form, which decreases the maximum rates of reaction achieved after the initial lag phase (*11*). Because OPP is a nonredox, isoform-selective LOX inhibitor, a detailed understanding of its inhibition should aid in the development of additional inhibitors with similar properties.

To understand in greater detail the inhibition of leukocyte 12-LOX by OPP, incubations of various amounts of arachidonic acid substrate and OPP with enzyme were monitored for product formation. Because enzyme inactivation does not occur to a significant degree during the lag phase (*11*), the decreased maximum rates of reaction reflect the potency of OPP's binding to the ferric form. Nonlinear regression analysis of these reactions, corrected for substrate utilization during the lag phase, combined with secondary plots of the data indicated that OPP acts as a mixed inhibitor with respect to the ferric form (Figures 3 and 4). The apparent K_I's for the free and substrate-bound forms of the enzyme are 2.0 ± 0.2 and 4.5 ± 0.7 μM, respectively (Figure 5). Somewhat surprising was the clear binding of OPP to the substrate-bound form of 12-LOX.

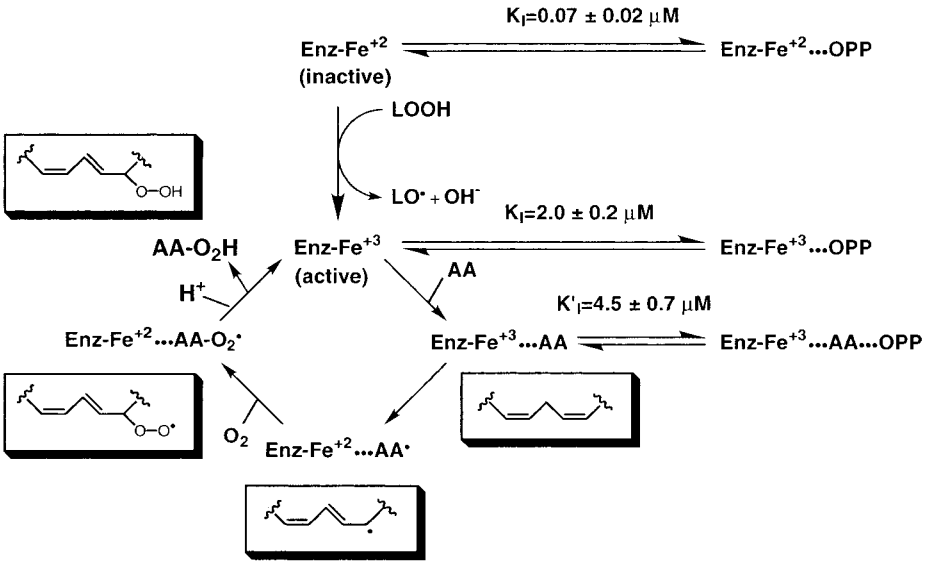

FIGURE 10: Model of OPP inhibition of 12-LOX.

Because OPP is structurally similar to the substrate arachidonic acid but is not metabolized by 12-LOX, OPP was expected to act as a simple competitive inhibitor. The crystal structure of a very similar LOX, rabbit reticulocyte 15-LOX, allows some conclusions to be reached regarding possible sites of OPP binding (*29*). 15-LOX has only one open area in the proximity of the non-heme iron. Therefore, this site is considered to be the binding site of both lipid hydroperoxides and the substrate arachidonic acid. Such a conclusion is supported by both site-directed mutagenesis and kinetic studies (*30*−*32*). The structural similarities of OPP to these fatty acids and its demonstrated competition with them suggest that OPP binds to this site as well. However, the active site does not appear to be large enough to accommodate both arachidonic acid and OPP. Therefore, a binding site for OPP distinct from the active site may also exist. At least one other site capable of exerting an inhibitory influence on LOXs has been proposed. The inhibitor acetyl-11-keto-β-boswellic acid was shown to act as an allosteric inhibitor of 5-LOX by binding to a site distinct from the active site (*33*, *34*). Kinetic studies have suggested that oleyl sulfate also acts as an allosteric inhibitor of certain LOXs (*35*). OPP may bind to one of these sites, or a different site, to uncompetitively inhibit the enzyme. The study of uncompetitive inhibition of LOXs by OPP and other inhibitors appears to be fertile ground for future mechanistic and structural studies.

In addition to OPP binding to the ferric form of 12-LOX, we previously showed that OPP binds to the ferrous form of the enzyme (*11*). Indeed, OPP caused a concentration-dependent decrease in the rate of 13-HPODE reduction by ferrous 12-LOX (Figure 6). Incubations with varying amounts of 12-LOX and 13-HPODE demonstrated that the rate of 13-HPODE reduction by ferrous 12-LOX increased linearly with respect to both enzyme and substrate over the concentrations that were used (Figure 7). This relationship between the free enzyme concentration and the rate of 13-HPODE reduction allowed the quantification of free enzyme after OPP was incubated with 12-LOX (Figure 8). The resulting relative rates of the reaction were fit to eq 5, which describes the initial rates in terms of the K_I value and the known values of [Enz_{total}] and [OPP_{total}]. This yielded a K_I value of OPP for the ferrous form of 70 ± 20 nM. Because of the conditions of the experiment, this value reflects OPP's competitive inhibition of enzyme activation. In the absence of saturating conditions of the substrate, which were prohibited due to the strong absorbance of 13-HPODE at 236 nm, no conclusions could be reached regarding an uncompetitive aspect of OPP inhibition of enzyme oxidation. Interestingly, this value for OPP binding is 29-fold lower than OPP's apparent constant for binding to the free ferric form. Therefore, it appears that OPP not only is selective for leukocyte-type 12-LOX but also has a higher affinity for the inactive ferrous form of the enzyme than for the active ferric form. A model of OPP inhibition of 12-LOX is shown in Figure 10 which demonstrates the affinities of OPP for different forms of the enzyme.

The implication of these findings is that significant structural differences exist between ferrous and ferric 12-LOX which affect OPP binding. Until recently, each of the determined LOX crystal structures was of the ferrous enzyme (*35*). However, the structure of a metastable oxidized form of soybean lipoxygenase-3 was determined recently (*36*). The formation of an iron−peroxide complex in this "purple enzyme" yielded a significant alteration of the iron site compared to the previously determined ferrous form of the enzyme. Interestingly, little change was noted in the overall topology of the enzyme. Presumably, this would also be the case in the absence of bound fatty acid. However, it is difficult to extend these observations to mammalian LOXs because of differences that exist between the structures of the mammalian and plant LOXs. This is particularly true with regard to channels to the active site. Whether OPP binding is affected by subtle structural differences or whether leukocyte-type 12-LOX undergoes significantly more change than is seen with soybean LOX-3 is not clear at this time.

OPP was the first LOX inhibitor shown to slow enzyme activation by binding to the ferrous form of the enzyme (*11*). Therefore, we examined other inhibitors to determine if this novel mechanism of inhibition might be more general. Rapid kinetic techniques indicated that 10 μM ETYA also inhibited the reduction of 13-HPODE by ferrous 12-LOX (Figure 9),

whereas NDGA, CPHU, and BWA137C (10 μM) had no effect on the rates of 13-HPODE reduction (data not shown). ETYA has been shown previously to inhibit LOXs through several mechanisms, including competitive inhibition and enzyme inactivation (*4*, *37*, *38*). Like that of OPP, inhibition of LOX activation by ETYA appears to represent another way in which product formation is reduced.

The relative importance of these multiple inhibitory mechanisms will vary depending on the context of the assay. For example, in situations with low levels of lipid hydroperoxides, as is expected to occur in vivo, inhibition of enzyme activation would be expected to be more important due to an enhanced ability to slow LOX oxidation. Indeed, a discrepancy between the inhibitory potency of nonredox 5-LOX inhibitors in cellular and noncellular assays has been noted (*39*). It was concluded that low hydroperoxide concentrations are important for the inhibitory potency of the nonredox inhibitors that were tested. Based on the data with OPP, one explanation for these results is that inhibition of enzyme activation is an important aspect of their overall activity. Through detailed kinetic studies, using both traditional and rapid kinetic techniques, it should be possible to understand the basis for differences in activity in different contexts and develop assays that more accurately predict in vivo LOX inhibitory activity.

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