Inhibition of Soybean Lipoxygenase-1 by a Diaryl-*N*-hydroxyurea by Reduction of the Ferric Enzyme

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Received June 8, 1994[⊗]

ABSTRACT: It has been proposed that catechols and other antioxidants inhibit lipoxygenase activity by reducing the active Fe^{3+} form of the enzyme [Kemal *et al.* (1987) Biochemistry *26*, 7064−7072]. In this model, reductively inactivated lipoxygenase can be reactivated by reaction with the hydroperoxide product in a pseudoperoxidase reaction. The contribution of enzyme reduction in the inhibition of the activity of soybean lipoxygenase-1 by the reducing inhibitor *N*-(4-chlorophenyl)-*N*-hydroxy-*N'*-(3-chlorophenyl)-urea (CPHU) has been evaluated quantitatively. The inhibition by CPHU of the oxygenation of linoleic acid to 13-hydroperoxy-9,11-octadecadienoic acid (13-HpODE) was accompanied by an initial lag phase which could be eliminated by the presence of exogenous 13-HpODE at the initiation of the reaction. In addition, both 13-HpODE and CPHU were found to be consumed during the lipoxygenase reaction, indicating occurrence of both oxygenase and pseudoperoxidase reactions. When analyzed individually, both the oxygenase reaction at different linoleic acid and O_2 concentrations and the pseudoperoxidase reaction at different 13-HpODE and CPHU concentrations were found to follow ping-pong kinetics. A rate equation for the lipoxygenase-catalyzed reaction in the presence of reducing agent was derived considering that the inhibition of the oxygenase reaction is the combined result of 13-HpODE consumption and formation of inactive Fe^{2+} enzyme due to occurrence of the pseudoperoxidase reaction. By comparing the experimental data with those predicted by the rate equation, it is concluded that the inactivation of the enzyme by reduction can quantitatively account for the inhibition caused by CPHU.

Hydroxamic acids and *N*-hydroxyureas are known to inhibit the catalytic activities of nonheme iron proteins such as ribonucleotide reductase (Kjoeller Larsen *et al.*, 1982), ascorbate peroxidase (Chen & Asada, 1990), and lipoxygenase (Garland & Salmon, 1991). Since these compounds are able to act as iron chelators, radical scavengers, and reductants, several different mechanisms have been considered to account for the role of the NOH functional group in the inhibition of enzymatic reactions.

Hydroxamic acid and *N*-hydroxyurea inhibitors could form reversible dead-end complexes with the enzyme by coordination of the iron at the active site. This was the initial hypothesis in the development of a series of potent inhibitors of 5-lipoxygenase, and thus of leukotriene biosynthesis, for potential therapeutic applications in inflammatory and allergic disorders (Salmon *et al.*, 1989; Summers *et al.*, 1987, 1990; Batt, 1992). Because of the difficulties in dealing with the kinetics of the 5-lipoxygenase reaction, little data have been available on the mechanism of inhibition of the enzyme by this class of compounds. The reversibility of 5-lipoxygenase inhibition has been demonstrated for at least one *N*-hydroxyurea derivative (zileuton) using both cell-free and intact cell assays (Carter *et al.*, 1991).

Another mechanism by which NOH compounds can inhibit the enzyme activity is the scavenging of free radical intermediates which are formed during the reaction. Hydroxamate iron chelators, including the lipoxygenase inhibitor BW A4C, have been shown to scavenge peroxyl radicals in a model system of lipid peroxidation based on the oxidation of linoleic acid by a thermolabile azo compound (Darley-Usmar *et al.*, 1989). This observation and studies on other types of inhibitors have led to the proposal that site-directed peroxyl radical scavenging in the lipoxygenase may be an important mechanism of inhibition of this protein (Roberfroid *et al.*, 1987). Other inhibitory effects, such as quenching of the tyrosyl radical (Atkin *et al.*, 1973) or suicide inactivation of the enzyme by oxidation products of *N*-hydroxyureas (Chen & Asada, 1990), have been described for other proteins but not observed or investigated in the case of lipoxygenases.

In addition, *N*-hydroxyureas could inhibit lipoxygenase activity by converting the ferric enzyme to an inactive ferrous form as illustrated in the reaction scheme of Figure 1. This model has been proposed initially to account for the inhibition of soybean lipoxygenase by alkylhydroxylamines (Clapp *et al.*, 1985) and NDGA[1] (Kemal *et al.*, 1987). Indirect evidence for the reduction of the enzyme to a form which could be reoxidized by hydroperoxides has been obtained for the soybean lipoxygenase-1 using acetohydroxamic acids (Reynolds, 1988) and for leukocyte 5-lipoxygenase using *N*-hydroxyureas (Riendeau *et al.*, 1991b). EPR spectroscopy studies have recently provided direct measurements for the reduction of the ferric center of the soybean enzyme (Nelson *et al.*, 1991) and the human 5-lipoxygenase

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[⊗] Abstract published in *Advance ACS Abstracts,* October 15, 1994.

[1] Abbreviations: CPHU, *N*-(4-chlorophenyl)-*N*-hydroxy-*N'*-(3-chlorophenyl)urea; 13-HpODE, 13-hydroperoxy-9,11-octadecadienoic acid; NGDA, nordihydroguaiaretic acid; DTT, dithiothreitol; TAPS, *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid; RP-HPLC, reverse-phase high-pressure liquid chromatography.

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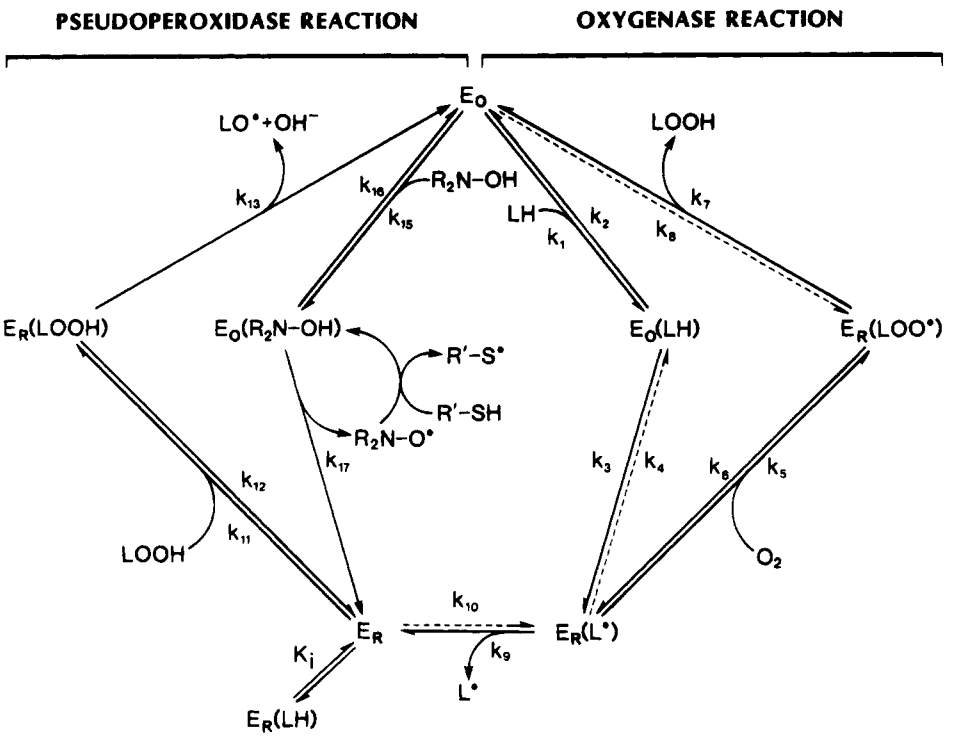

FIGURE 1: Proposed scheme for lipoxygenase-catalyzed reactions and inhibition by reducing agent. The oxygenase activity (right) results from the reaction of the oxidized enzyme form (E_O) with linoleic acid (LH) and oxygen (O_2) to generate the lipid hydroperoxide (LOOH). The pseudoperoxidase activity (left) results from the reaction of the lipid hydroperoxide (LOOH) with the reduced enzyme from (E_R) to generate the unstable alkoxide radical ($LO^{\bullet}$) and the oxidized enzyme. Enzyme recycling to the reduced form occurs by reaction with the *N*-hydroxyurea (R_2NOH). The N-hydroxyurea is oxidized to the nitroxide radial ($R_2NO^{\bullet}$), which can either decompose to other species or be reduced by thiols (R'SH) to regenerate the *N*-hydroxyurea.

(Chasteen *et al.*, 1993) by *N*-hydroxy derivatives. Furthemore, the production of nitroxide free radicals during the enzyme-catalyzed oxidation of hydroxamic acids and *N*-hydroxyureas has been demonstrated (Chamulitrat *et al.*, 1992), in agreement with a one-electron reduction of the ferric center of the enzyme by these compounds.

Although the ability of *N*-hydroxyureas and other types of inhibitors to reduce lipoxygenase is well established, the relative importance of reductive inactivation versus the other possible mechanisms of inhibition has not been evaluated quantitatively. In the present study, we have used a steady-state kinetic approach to further examine the mechanism of inhibition of the soybean lipoxygenase by *N*-(4-chlorophenyl)-*N*-hydroxy-*N'*-(3-chlorophenyl)urea (CPHU), a diaryl-*N*-hydroxyurea which is an effective reducing agent of various types of lipoxygenases (Falgueyret *et al.*, 1992). CPHU is a useful inhibitor for such studies since its degradation during enzyme reduction can be prevented by the presence of a low concentration of thiol, allowing measurements of enzymatic activity at fixed concentrations of reducing inhibitor. The experimental data have been compared to those calculated on the basis of a kinetic model for the inhibition of the enzyme where both the oxygenase reaction and the pseudoperoxidase reaction resulting from enzyme reduction were considered to occur simultaneously in the presence of the inhibitor. The results are consistent with enzyme reduction accounting for the inhibition of the lipoxygenase reaction by CPHU.

MATERIALS AND METHODS

Materials. Linoleic acid, dithiothreitol, and TAPS were purchased from Sigma Co. 13-HpODE was obtained from Biomol Research Laboratories (Plymouth Meeting, PA). CPHU was synthesized at the Department of Medicinal Chemistry, Merck Frosst Centre for Therapeutic Research, as previously described (Falgueyret *et al.*, 1992). Soybean lipoxygenase-1 from Sigma Co. (type V) was further purified by anion exchange chromatography. The enzyme solution was initially centrifuged at 10000*g* for 10 min at 5 °C to lower the ammonium sulfate concentration. The diluted supernatant was applied to a Mono-Q column (Pharmacia) and eluted with a 0−0.5 M NaCl gradient in 20 mM Tris-HCl, pH 7.6. The solution of the major protein peak corresponding to soybean lipoxygenase-1 was collected, concentrated to 3 mg/mL by adsorption on a Mono-Q column, and eluted with 0.5 M NaCl in 20 mM Tris-HCl, pH 7.6. The concentration of purified enzyme was calculated from the absorbance at 280 nm by using a molar extinction coefficient of 1.6×10^5 M^{-1} cm^{-1} (Spaapen *et al.*, 1979). The enzyme was stored in liquid nitrogen. Immediately after thawing, the enzyme was diluted to 0.3 mg/mL in 0.1 M TAPS buffer, pH 9.0, and kept in an ice bath. The apparent initial velocities of product formation were corrected for the small loss of enzyme activity (~15%/h) with each set of experiments not exceeding a period of 50 min.

Spectrophotometric Assay of the Oxygenase Reaction. The oxygenase activity of the purified soybean lipoxygenase-1 was determined from the initial rate of conjugated diene hydroperoxide (13-HpODE) formation by continuously monitoring the absorbance at 234 nm on a Hewlett-Packard diode array spectrophotometer (Model 8452A). A molar extinction coefficient of 23 000 M^{-1} cm^{-1} (Gibian & Vandenberg, 1987) was used to convert the absorbance values to moles of the hydroperoxide product. Initial

velocities were determined from the highest linear slope using Hewlett-Packard UV−visible kinetic software. The standard assay was performed in a 1 cm path length cuvette containing 100 μM linoleic acid, 0.4 μM 13-HpODE, and soybean lipoxygenase-1 (0.3 mg/mL) in air-saturated 0.1 M TAPS buffer, pH 9.0, at room temperature. Reaction mixtures (2.0 mL final volume) were mixed by a magnetic stirrer operating at 250 rpm.

Measurement of the Lipoxygenase Reaction at Various Oxygen Concentrations. The assay was performed at various concentrations of oxygen and linoleic acid under the conditions of the standard spectrophotometric assay in the presence of 0.6 μM 13-HpODE except that it was carried out in a final volume of 2.5 mL in a 3 mL cuvette provided with a rubber sealed cap. The oxygen concentration of the solution was varied by mixing in the cuvette known amounts of a buffer solution equilibrated with air at room temperature (240 μM O_2) with a buffer solution from which oxygen was carefully removed by flushing with argon for a minimum of 15 min. Control experiments indicated that no significant level of lipoxygenase reaction occurred with argon-purged buffer, and these were routinely performed to establish the dependence on added O_2 in each set of experiments.

Spectrophotometric Assay of the Pseudoperoxidase Reaction. This assay was performed in a reaction mixture containing various concentrations of 13-HpODE and CPHU in the presence of 50 μM DTT and 0.4 μg/mL enzyme in 0.1 M TAPS air-saturated buffer, pH 9.0 (1 mL final volume). CPHU and 13-HpODE were added from concentrated solutions in ethanol with the final concentration of ethanol not exceeding 0.8%. The enzyme activity was determined from the initial rate of 13-HpODE consumption as monitored by the decrease in absorbance at 234 nm.

Measurement of CPHU Levels. The variation in the level of CPHU was measured by RP-HPLC on a Nova-Pak C18 column (Millipore) using a mixture of acetonitrile/water/acetic acid (55:45:0.1) as eluent (2 mL/min). Incubation mixtures were prepared as described for the spectrophotometric assay in the presence of CPHU. The reaction was stopped by the addition of 1 volume of the RP-HPLC elution solvent prior to injection of an aliquot (100 μL) on the HPLC column. The elution of CPHU (t_R = 2.6 min) was monitored by absorbance at 260 nm. The recoveries of CPHU after different periods of time were quantitated by comparison of the peak area with that of known amounts of the synthetic standard.

Determination of Kinetic Parameters for Rate Equations. Estimates of values for kinetic parameters obtained from analysis of the Lineweaver−Burk plots of the appropriate data according to eqs 1−3 (see Results) were used as first approximations in fitting eq 4 and the reciprocal forms of eqs 1−3 to the data using the nonlinear least-squares curve-fitting program BMDP. Unless otherwise indicated, the data points represent an average of two determinations of the rates under the indicated conditions. The ± values given with the kinetic parameters are the standard deviations calculated using the program BMDP.

RESULTS

Effect of O_2 and Linoleic Acid Concentrations on the Oxygenase Activity of Soybean Lipoxygenase-1. The characteristics of the dioxygenation of linoleic acid by purified soybean lipoxygenase-1 were investigated at various concentrations of linoleic acid, O_2, and CPHU in order to determine whether the kinetic mechanism shown in Figure 1 can quantitatively account for the inhibition of the enzyme by CPHU. This mechanism is described in the figure legend, and the inhibition by CPHU is attributed entirely to its ability to convert the active ferric enzyme (E_O) to the inactive ferrous enzyme (E_R). Enzyme activity was measured using a continuous spectrophotometric assay at 234 nm to determine the rate of 13-HpODE formation. Figure 2 shows that a linear relationship between the initial rate of the reaction and the enzyme concentration was obtained for representative sets of experimental conditions used in the present study such as low O_2 concentration and the presence of CPHU.

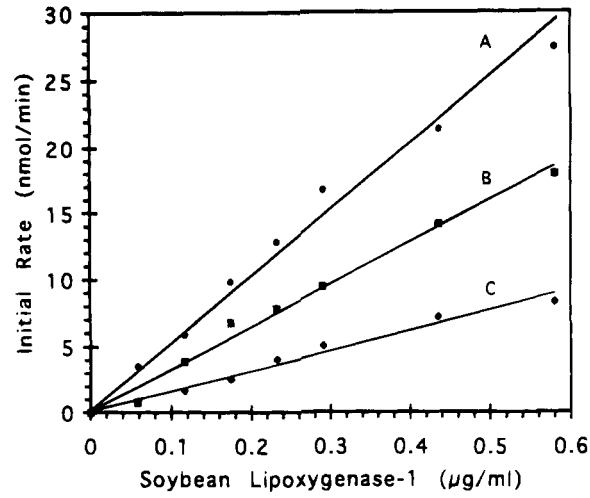

FIGURE 2: Linear dependence of the oxygenase activity of soybean lipoxygenase-1 on enzyme concentration under various conditions. Enzyme activities were measured using initial concentrations of 5 μM linoleic acid (A) 0.4 μM 13-HpODE in air-saturated buffer, (B) 0.6 μM 13-HpODE and 20 μM oxygen, and (C) 2 μM 13-HpODE, 4.3 μM CPHU, and 50 μM DTT in air-saturated buffer.

The data of Figure 2 were obtained under conditions of initial substrate and 13-HpODE concentrations such that lag phases at the beginning of the reaction were minimal in order to facilitate the measurement of steady-state velocities. The lag phases have been attributed to the time required for oxidation of the native ferrous enzyme to the catalytically active ferric form and to build up a sufficient concentration of 13-HpODE to maintain the enzyme in the active form (De Groot *et al.*, 1975). The lags can be considerably reduced by the addition of a low amount of 13-HpODE to the assay mixture before initiation of the reaction (Figure 3A, curves A and B). The duration of the lag phase was found to be increased by lowering the concentration of O_2, as observed for the reticulocyte lipoxygenase (Ludwig *et al.*, 1987) and illustrated in Figure 3A for an initial O_2 concentration of 10 μM (curve C). In this case, the production of 13-HpODE occurred at a slower rate than in air-saturated solutions and proceeded to complete consumption of oxygen. The extension of the lag phase is consistent with the present kinetic model since, at low O_2 concentration, dissociation of the linoleyl radical to generate the inactive ferrous enzyme, E_R (see Figure 1), competes more successfully with trapping of $E_R(L^\bullet)$ by O_2 followed by generation of product.

Lowering the concentration of oxygen also increased the sensitivity of the reaction to substrate inhibition by linoleic

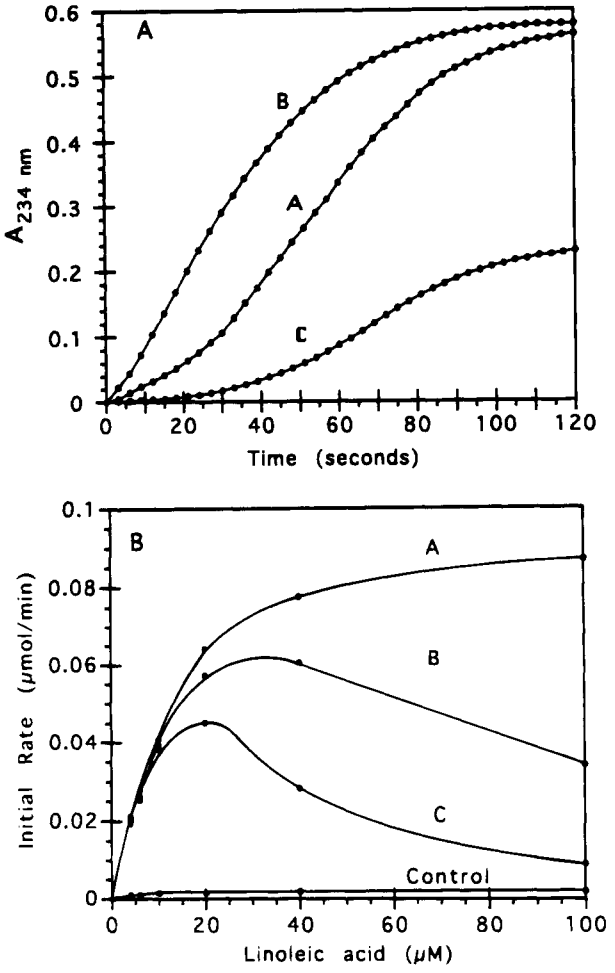

FIGURE 3: Effect of lowering the concentration of oxygen on progress curves of the oxygenase reaction (A) and on the substrate inhibition by linoleic acid (B). (A) The production of 13-HpODE (A_{234}) was measured as function of time (every 3 s) using 25 μM linoleic acid as the initial substrate concentration and 0.3 μg/mL soybean lipoxygenase-1 in air-saturated buffer (curve A), air-saturated buffer containing 0.4 μM 13-HpODE (curve B), or buffer containing 10 μM oxygen and 0.4 μM 13-HpODE (curve C). (B) Enzyme activity was measured as function of the concentration of linoleic acid using 0.4 μg/mL enzyme and 0.6 μM 13-HpODE in air-saturated buffer (curve A), buffer containing 50 μM oxygen (curve B), or buffer containing 25 μM oxygen (curve C). No significant level of 13-HpODE formation could be measured under these assay conditions using deoxygenated solutions with argon flushing (control).

acid. Figure 3B shows that no inhibition by linoleic acid was observed up to a concentration of 100 μM in air-saturated buffer, whereas significant substrate inhibition occurred at oxygen concentrations of 50 and 25 μM, as seen in curves B and C, respectively. This effect is also in agreement with the reaction scheme of Figure 1, with substrate inhibition resulting from the generation of increased steady-state levels of E_R at low oxygen concentrations and the binding of linoleic acid to E_R.

Inhibition of the Oxygenase Activity by CPHU. The effect of CPHU on the lipoxygenase-catalyzed oxygenation of linoleic acid is illustrated in Figure 4. Curve A shows the time course of the reaction with 10 μM linoleic acid and 0.4 μM 13-HpODE. Under these conditions, no lag phase was apparent and the reaction proceeded to completion within about 1 min. Addition of 4.3 or 8.6 μM CPHU (curves B and D) resulted in lower initial rates, increased lag phases, and lower maximum absorbances followed by absorbance

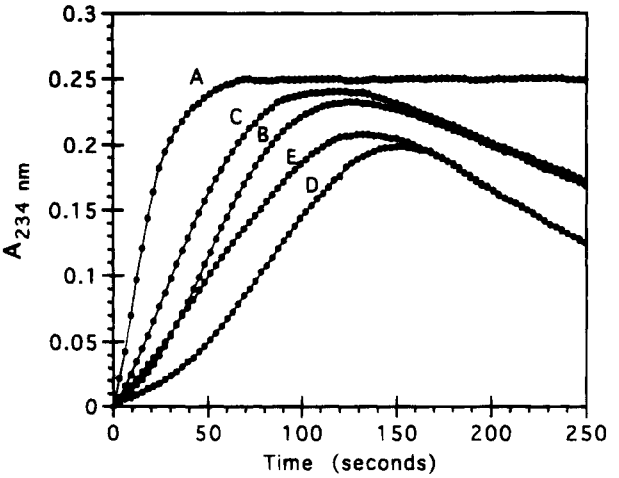

FIGURE 4: Effect of CPHU on the initial time course of 13−HpODE formation. The variation in A_{234} was followed as a function of time after addition of soybean lipoxygenase-1 (0.3 μg/mL) to the reaction mixtures containing 10 μM linoleic acid, 50 μM DTT, and (A) 0.4 μM 13-HpODE; (B) 4.3 μM CPHU and 0.4 μM 13-HpODE; (C) 4.3 μM CPHU and 2 μM 13-HpODE; (D) 8.6 μM CPHU and 0.4 μM 13-HpODE; and (E) 8.6 μM CPHU and 2 μM 13-HpODE.

decreases. Increasing the initial 13-HpODE concentration to 2 μM (curves C and E for 4.3 and 8.6 μM CPHU, respectively) eliminated the lag phases but had little effect on either the maximum initial rate or the maximum absorbance reached during the time course. These results are qualitatively consistent with simultaneous occurrence of the lipoxygenase-catalyzed oxygenase and pseudoperoxidase reactions as indicated in Figure 1. When CPHU is present, the pseudoperoxidase reaction consumes some of the 13-HpODE produced from the oxygenase reaction, and the pseudoperoxidase reaction becomes dominant when most or all of the linoleic acid has been consumed. The reduction in the rate of 13-HpODE production caused by CPHU early in the time course can be partially overcome by higher initial 13-HpODE concentrations, which results in a more efficient reoxidation of the ferrous enzyme, E_R, which is generated by reaction of E_O with CPHU. Higher 13-HpODE concentrations cannot restore the oxygenation reaction to its uninhibited rate because it does not influence the relative rates of reaction of E_O with CPHU and linoleic acid.

Detection of the Pseudoperoxidase Reaction during the Inhibition of the Oxygenase Reaction by CPHU. If the pseudoperoxidase reaction is responsible for 13-HpODE consumption as suggested by the data of Figure 4, it should be possible to detect both the degradation products of 13-HpODE and the consumption of inhibitor during the course of the inhibited reaction. It has been shown previously that 13-HpODE is converted in part to products which absorb at 270−285 nm, presumably corresponding to the oxodiene cleavage products of the alkoxide radical of 13-HpODE. (Garssen *et al.*, 1971, 1972; Streckert & Stan, 1975; Kemal *et al.*, 1987). The A_{285} value was found to increase during the entire time course of the reaction in the presence of 8.6 μM CPHU, consistent with occurrence of the pseudoperoxidase activity (Figure 5, curve B). This activity resulted mainly from enzyme reduction by CPHU, rather than leaking out of E_R from the oxygenase reaction cycle or 13-HpODE self-degradation since a considerably smaller rate of increase in A_{285} was observed during the reaction in the absence of inhibitor under the same assay conditions (Figure 5, curve A) in spite of a severalfold larger rate of production of 13-

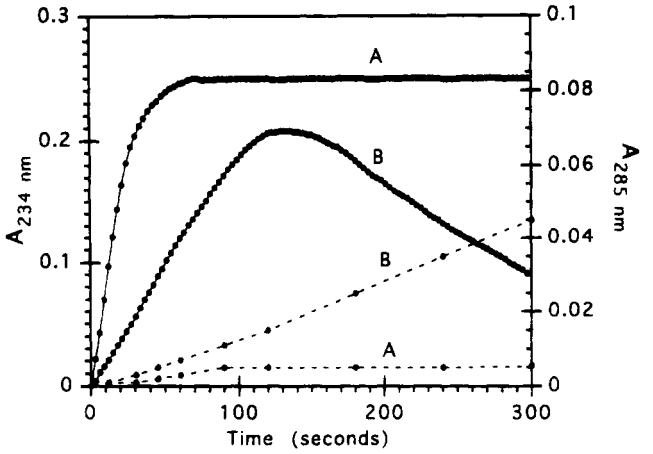

FIGURE 5: Consumption of 13-HpODE during the inhibition of the oxygenase reaction by CPHU. The variation in A_{234} (—) and A_{285} (— — —) were monitored in reaction mixtures containing 0.3 μg/mL soybean lipoxygenase-1, 10 μM linoleic acid, and 50 μM DTT in the absence (A) and presence (B) of 8.6 μM CPHU.

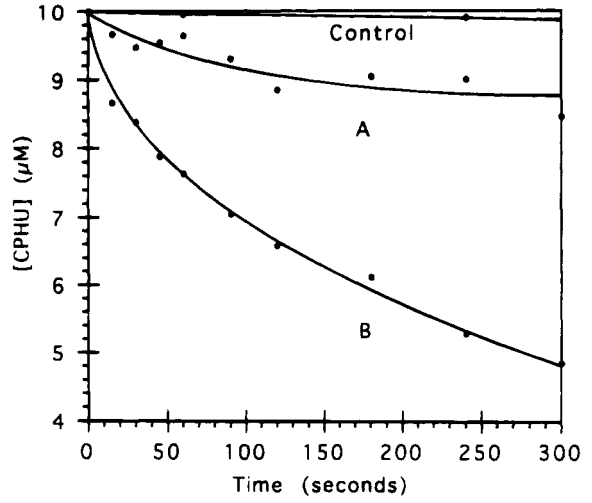

FIGURE 6: Degradation of CPHU during the inhibition of the oxygenase reaction. The reaction was performed under the conditions of the spectrophotometric assay using initial concentrations of 20 μM linoleic acid, 10 μM CPHU, and 0.4 μM 13-HpODE in the presence of 50 μM DTT (A) or in its absence (B). The recovery of CPHU was quantitated by RP-HPLC at different times during the reaction. Control experiment was performed with heat-inactivated enzyme. The results are expressed as the mean of the CPHU concentration from three independent experiments.

HpODE. Additional results were obtained consistent with generation of E_R via the k_9 step of Figure 1 being a relatively rare event except at extremely low oxygen concentrations. When O_2 concentration was varied from 240 to 10 μM, the yield of 13-HpODE from a given concentration of linoleic acid remained constant and close to the theoretical maximum as assessed by the absorbance at 234 nm. This is consistent with evidence in the literature that trapping of $E_R(L^\bullet)$ by O_2 is very efficient except at submicromolar concentrations of O_2. For example, the soybean lipoxygenase-catalyzed decomposition of 13-HpODE (no reducing agent) is observed only at submicromolar O_2 concentrations (Verhagen *et al.*, 1976). Other investigators have also reported that under aerobic conditions and in the absence of added reducing agents, products of 13-HpODE decomposition are not produced to a significant extent during the oxygenase reaction (Kuhn *et al.*, 1986; Wang *et al.*, 1993). Consumption of CPHU during the lipoxygenase reaction was quantitated by

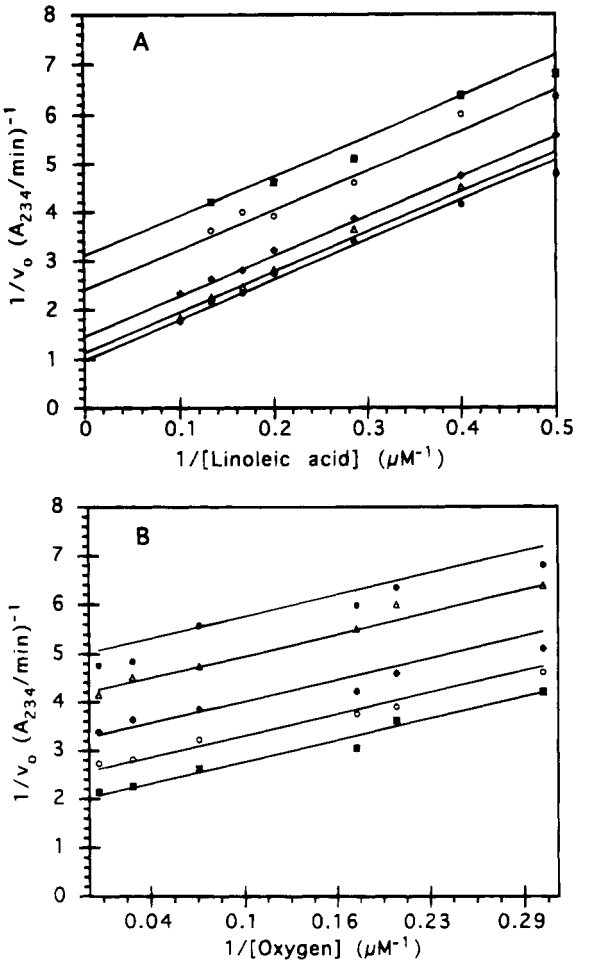

FIGURE 7: Ping-pong kinetic profile of the oxygenase reaction catalyzed by the soybean lipoxygenase-1 in the presence of linoleic acid and oxygen. Initial velocities (v_o) of 13-HpODE formation were measured under the conditions of the spectrophotometric assay at various O_2 concentrations in the presence of 0.6 μM 13-HpODE and 0.3 μg/mL enzyme. (A) Double-reciprocal plots of the dependence of v_o on linoleic acid (2−10 μM) at different O_2 concentrations: (■) 3 μM, (○) 5 μM, (◆) 14 μM, (△) 35 μM, and (●) 240 μM. (B) Double-reciprocal plots of the dependence of v_o on O_2 (3−240 μM) at different linoleic acid concentrations: (●) 2 μM, (△) 2.5 μM, (◆) 3.5 μM, (○) 5 μM, and (■) 7.5 μM. Results are expressed as the mean of two experiments. The solid lines were calculated by fitting the experimental initial rates of 13-HpODE formation to the reciprocal form of eq 1. Parameter values: V_m = 0.12 μmol/min; k_{cat} = 267 ± 10 s^{-1}; K_{LH} = 8.7 ± 0.1 μM; K_{O_2} = 7.7 ± 0.1 μM; specific activity = 160 ± 6 μmol/min/mg.

RP-HPLC and is shown in Figure 6. A time-dependent degradation of CPHU (up to 55% of a 10 μM initial concentration) was observed for the reaction performed in the absence of DTT (curve B). Much less CPHU was consumed when DTT was present (curve A) and none when heat-denatured enzyme was used (control), in agreement with previous observation on CPHU recycling by DTT during the pseudoperoxidase reaction (Falgueyret *et al.*, 1992).

Steady-State Kinetics of the Lipoxygenase-Catalyzed Oxygenase Reaction. In order to quantitatively test the mechanism of Figure 1, studies were done under a variety of conditions. Unless otherwise indicated, O_2 and 13-HpODE concentrations were such that lags were minimal and substrate inhibition was not observed. Figure 7 shows the double-reciprocal plots for the initial rates of 13-HpODE formation as a function of the linoleic acid and O_2 concentra-

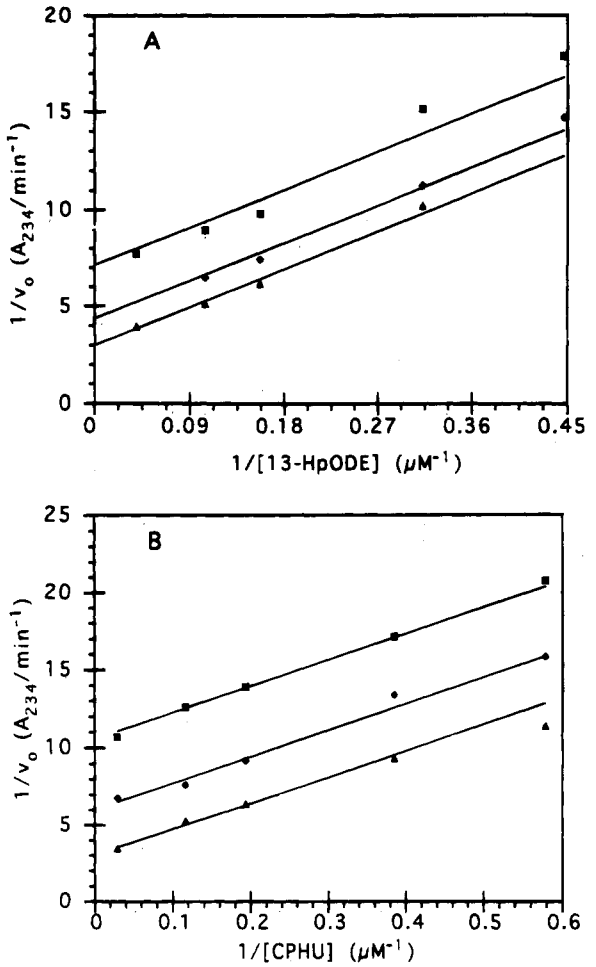

FIGURE 8: Ping-pong kinetic profile of the pseudoperoxidase reaction catalyzed by the soybean lipoxygenase-1 in the presence of 13-HpODE and CPHU. Initial velocities of 13-HpODE consumption were measured under the conditions of the spectrophotometric assay for the pseudoperoxidase reaction. (A) Double-reciprocal plots of the dependence of v_o on 13-HpODE (2.2−26 μM) at different CPHU concentrations: (■) 4.3 μM, (◆) 8.6 μM, and (▲) 17 μM. (B) Double-reciprocal plots of the dependence of v_o on CPHU concentration (1.7−35 μM) at different 13-HpODE concentrations: (■) 3.2 μM, (◆) 6.4 μM, and (▲) 19 μM. Oxygen concentration was fixed at 240 μM. Results are expressed as the mean of two experiments. The solid lines were calculated by fitting the experimental initial rates of 13-HpODE formation to the reciprocal form of eq 2. Parameter values: $k_{cat}' = 125 \pm 13$ s^{-1}; $K_I = 14.9 \pm 0.1$ μM; $K_P' = 13.6 \pm 0.1$ μM; specific activity = 75 ± 8 μmol/min/mg.

tions. In terms of the mechanism shown in Figure 1, this reaction proceeds through the steps involving k_1, k_3, k_5, and k_7. A parallel line pattern characteristic of a ping-pong mechanism was observed, suggesting that the formation of the $E_R(L^\bullet)$ complex is an irreversible process ($k_4 = 0$) as previously proposed for the reticulocyte 15-lipoxygenase (Ludwig *et al.*, 1987). Analyzing the data in terms of eq 1 for a two-substrate ping-pong mechanism, the K_m values for linoleic acid and O_2 were 8.7 ± 0.1 μM (K_{LH}) and 7.7 ± 0.1 μM (K_{O_2}), respectively. The k_{cat} value of the oxygenase reaction was 267 ± 10 s^{-1}, corresponding to a specific activity of 160 ± 6 μmol/min/mg of protein.

$$\frac{1}{v_o} = \frac{1}{V_m}\left(1 + \frac{K_{LH}}{[LH]} + \frac{K_{O_2}}{[O_2]}\right) \quad (1)$$

Steady-State Kinetics of the Lipoxygenase-Catalyzed

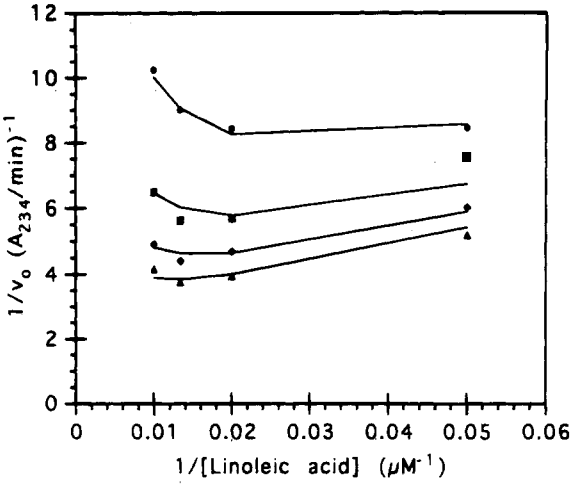

FIGURE 9: Substrate inhibition of the linoleate-dependent anaerobic pseudoperoxidase reaction. The reaction was performed in sealed cuvettes containing 2 mL of argon-flushed buffer, lipoxygenase-1 (1.9 μg/mL), linoleic acid (20−100 μM), and different 13-HpODE concentrations: (●) 12 μM, (■) 24 μM, (◆) 40 μM, and (▲) 64 μM. Results are expressed as the mean of two determinations of the initial rate of 13-HpODE consumption. Parameter values: $k_{cat}'' = 21 \pm 4$ s^{-1}; $K_{LH}'' = 31.3 \pm 0.3$ μM; $K_P'' = 21.3 \pm 0.2$ μM; $K_i = 65 \pm 1$ μM; specific activity = 13 ± 3 μmol/min/mg.

Pseudoperoxidase Reaction with CPHU as Reducing Substrate. Figure 8 shows the double-reciprocal plots for the initial rates of 13-HpODE consumption by the pseudoperoxidase reaction as a function of CPHU and 13-HpODE concentrations. This reaction is viewed as proceeding via the steps involving k_{15}, k_{17}, k_{11}, and k_{13} as indicated in Figure 1. A parallel line pattern was observed, which is not surprising since both substrates are converted to radical products which are not considered to accumulate in the reaction mixture. Analyzing the data in terms of eq 2 for a two-substrate ping-pong mechanism, the K_m values for CPHU and 13-HpODE were 14.9 ± 0.1 μM (K_I) and 13.6 ± 0.1 μM (K_P'), respectively. The k_{cat}' value of the pseudoperoxidase reaction was 125 ± 13 s^{-1}, corresponding to a specific activity of 75 ± 8 μmol/min/mg of protein.

$$\frac{1}{v_o} = \frac{1}{V_m'}\left(1 + \frac{K_I}{[I]} + \frac{K_P'}{[P]}\right) \quad (2)$$

Steady-State Kinetics of the Lipoxygenase-Catalyzed Anaerobic Pseudoperoxidase Reaction. Figure 9 shows the double-reciprocal plots for the rates of the anaerobic pseudoperoxidase reaction with linoleic acid and 13-HpODE as substrates. In terms of the mechanism shown in Figure 1, this reaction proceeds via the steps involving k_1, k_3, k_9, k_{11}, and k_{13}, with the substrate inhibition being accounted for by binding of linoleic acid to E_R. The data of Figure 9 were analyzed in terms of eq 3, which is in the form of a two-substrate ping-pong mechanism, with substrate inhibition by the first substrate binding as a dead-end inhibitor to the same state of the enzyme (E_R) to which the second substrate binds.

$$\frac{1}{v_o} = \frac{1}{V_m''}\left(1 + \frac{K_{LH}''}{[LH]} + \frac{K_P''\left(1 + \frac{[LH]}{K_i}\right)}{[P]}\right) \quad (3)$$

The K_m values for linoleic acid and 13-HpODE in the

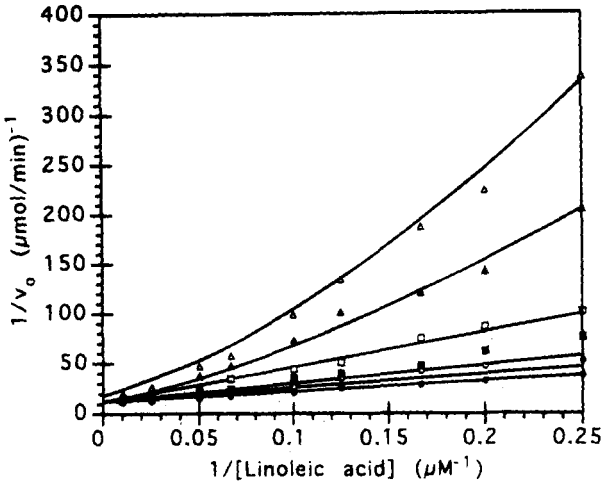

FIGURE 10: Double-reciprocal plot of the inhibition of the oxygenase reaction by CPHU. The initial rate of 13-HpODE formation was measured at different linoleic acid concentrations (4−100 μM) in the presence of CPHU: (●) 0 μM, (○) 0.86 μM, (■) 1.7 μM, (□) 4.3 μM, (▲) 8.6 μM, and (△) 13 μM. Oxygen concentration was fixed at 240 μM. The assays were done in duplicate under the conditions of the spectrophotometric assay using 0.3 μg/mL soybean lipoxygenase-1, 50 μM DTT, and 2 μM 13-HpODE (2 mL final volume). The solid lines were calculated by using eq 4 and the parameters described in the text.

anaerobic pseudoperoxidase reaction were 31.3 ± 0.3 μM (K_{LH}'') and 21.3 ± 0.2 μM (K_P''), respectively. The K_i value for linoleic acid (the dissociation constant for linoleic acid binding to E_R as shown in Figure 1) was 65 ± 1 μM. The k_{cat}'' value (21 ± 4 s^{-1}) for the linoleate-dependent pseudoperoxidase reaction (specific activity = 13 ± 3 μmol/min/mg of protein) was about 6-fold lower than for the CPHU-dependent reaction and about 13-fold lower than for the oxygenase reaction.

Steady-State Kinetic Analysis of the Lipoxygenase-Catalyzed Oxygenase Reaction in the Presence of CPHU. Figure 10 shows double-reciprocal plots of the rate of 13-HpODE production as a function of linoleic acid concentration at the single fixed oxygen concentration of 240 μM, in the absence of CPHU and at several different fixed concentrations of CPHU. The data were analyzed in terms of eq 4, which was derived on the basis of the mechanism shown in Figure 1.

$$v_o = \frac{V_m}{1 + \frac{K_{O_2}}{[O_2]} + \frac{K_{LH}}{[LH]}\left(1 + \frac{[I]}{K_I} + \frac{[I]K_P'(1 + [LH]/K_i)}{[P]K_I}\right)} - \frac{V_m'}{1 + \frac{K_P'(1 + [LH]/K_i)}{[P]} + \frac{K_I}{[I]}\left(1 + \frac{[LH]}{K_{LH}} + \frac{[LH]K_{O_2}}{[O_2]K_{LH}}\right)} \quad (4)$$

The definitions of the constants in eqs 1−4, in terms of the rate constants indicated in Figure 1, are given in eqs 5−13. For the oxygenase reaction:

$$V_m = \frac{k_3k_7E_T}{k_3 + k_7} \quad (5)$$

where E_T corresponds to the total enzyme concentration.

$$K_{LH} = \frac{k_7(k_2 + k_3)}{k_1(k_3 + k_7)} \quad (6)$$

$$K_{O_2} = \frac{(k_3 + k_4)(k_6 + k_7)}{k_5(k_3 + k_7)} \quad (7)$$

For the pseudoperoxidase reaction:

$$V_m' = \frac{k_{13}k_{17}E_T}{(k_{13} + k_{17})} \quad (8)$$

$$K_P' = \frac{k_{17}(k_{12} + k_{13})}{k_{11}(k_{13} + k_{17})} \quad (9)$$

$$K_I = \frac{k_{13}(k_{16} + k_{17})}{k_{15}(k_{13} + k_{17})} \quad (10)$$

For the anaerobic pseudoperoxidase reaction:

$$V_m'' = \frac{k_3k_9k_{13}E_T}{(k_9k_{13} + k_9k_3 + k_3k_{13})} \quad (11)$$

$$K_P'' = \frac{k_3k_9(k_{12} + k_{13})}{k_{11}(k_9k_{13} + k_9k_3 + k_3k_{13})} \quad (12)$$

$$K_{LH}'' = \frac{k_{13}k_9(k_2 + k_3)}{k_1(k_9k_{13} + k_9k_3 + k_3k_{13})} \quad (13)$$

Equations 1−4 were obtained by applying the method of King and Altman (1956) to Figure 1. Several simplifying assumptions were made on the basis of the results presented above, which led to a rate equation (4) in which all of the constants are identical to those obtained in deriving rate eqs 1−3, for the cases in which only either the oxygenase or the pseudoperoxidase reaction is occurring. The study of the anaerobic pseudoperoxidase reaction of linoleic acid and 13-HpODE was done mainly to obtain a value of K_i for linoleic acid as a substrate inhibitor. Although substrate inhibition by linoleic acid could be seen in the oxygenase reaction at lower oxygen concentrations, it was more convenient to quantitate in the anaerobic pseudoperoxidase reaction. Another goal in studying this reaction was to gain information on what limitation the k_9 step might place on the rate of the pseudoperoxidase reaction since this is the only process studied in this work which is viewed as proceeding via a mechanism involving the k_9 step. Although inhibition of the soybean lipoxygenase by 13-HpODE has been observed under certain conditions (Smith & Lands, 1972; Wang *et al.*, 1993), there is no evidence that it was significant under the conditions of the studies reported here. A value of K_i = 59 μM has been reported for 13-HpODE (Wang *et al.*, 1993) as an inhibitor of soybean lipoxygenase-1, so it is not surprising that no significant inhibition by 13-HpODE was observed under the conditions of the present work. Also consistent with the relative insignificance under the conditions used here of any process involving the k_8 reaction is the observation that the soybean lipoxygenase-1-catalyzed anaerobic pseudoperoxidase reaction of 13-HpODE alone is about 1500-fold slower than the anaerobic pseudoperoxidase reaction of linoleic acid and 13-HpODE

(Verhagen *et al.*, 1976). Therefore k_8 was set equal to zero which simplified the rate equation. A value of zero was also assigned to k_9 because, as described earlier, there was no evidence for a significant effect of oxygen concentration on the yield of 13-HpODE from a given amount of linoleic acid over the range of conditions used in the experiment whose results are shown in Figure 10. Again, it is certain that reaction steps corresponding to k_8 and k_9 do occur. For example, in terms of the mechanism of Figure 1, the enzyme-catalyzed anaerobic decomposition of 13-HpODE occurs via k_8, k_6, k_9, k_{11}, and k_{13} and the anaerobic lineolic acid-driven pseudoperoxidase reaction described earlier proceeds via k_1, k_3, k_9, k_{11}, and k_{13}. However, it appears that in the presence of oxygen and under the conditions used in the experiment of Figure 10, the reactions with rate constants k_8 and k_9 did not impinge significantly on the rates which were observed. The justifications for setting $k_4 = 0$ and for considering the reactions of k_{13} and k_{17} to be essentially irreversible were given earlier.

The solid lines shown in Figure 10 were calculated using eq 4 with the following values for the constants: $K_{LH} = 10.3 \pm 0.1$ μM; $K_{O_2} = 9.4 \pm 0.1$ μM; $K_I = 20 + 0.2$ μM; $K_P' = 15 \pm 0.2$ μM: $K_i = 70 \pm 2$ μM; $k_{cat} = 284 \pm 5$ s^{-1}; and $k_{cat}' = 83 \pm 2$ s^{-1}. The values for these constants were obtained by nonlinear regression analysis of the data points as described under Materials and Methods. The values of these constants are reasonably close to those obtained by analysis of the data shown in Figures 7–9. The differences are for the most part within the range one expects to see from analysis of different data sets obtained when a given experiment is repeated on different days with different batches of purified enzyme and by the fact that the experiments whose results are shown in Figures 7–10 were of necessity performed under slightly different conditions. For example, when only the pseudoperoxidase reaction of CPHU and 13-HpODE occurs, no linoleic acid is present, and when only the oxygenase reaction occurs, there is very little accumulation of the products of 13-HpODE decomposition which are generated in the pseudoperoxidase reaction. These differences may affect the system in ways not entirely accounted for in the scheme shown in Figure 1. For example, it is possible that products of the pseudoperoxidase reaction could either activate the enzyme or stimulate some nonenzymic consumption of 13-HpODE accounting for the difference in V_m values obtained from the data of Figures 8 and 10. The main conclusions from the results shown in Figure 10, and their analysis, are that (1) eq 4 provides a good fit of calculated to experimental rates of soybean lipoxygenase-catalyzed formation of 13-HpODE in the presence of various concentrations of the inhibitor CPHU and (2) the constants used in this fit are similar to those obtained from analogous studies of the oxygenase and pseudoperoxidase reactions occurring in isolation from each other. On the basis of these conclusions, it appears clear that inhibition of the oxygenase activity of the soybean lipoxygenase by CPHU can be entirely accounted for by the action of CPHU as a substrate in the pseudoperoxidase activity of the enzyme.

As an additional test of the mechanism shown in Figure 1, it was determined whether numerical integration of eq 4 could be used to fit the entire time course of the enzyme-catalyzed production and consumption of 13-HpODE when the reaction is initiated in the presence of CPHU and linoleic acid. The results from such an experiment, and the calculated lines, are shown in Figure 11. The calculated lines were obtained by a series of manual iterations using eq 4. Initial velocities were calculated from the kinetic parameters and the initial concentrations of linoleic acid, CPHU, 13-HpODE, and O_2. The changes in concentrations calculated after 3 s of reaction at the initial rate were used to obtain the amount of 13-HpODE formed for plotting on the y axis of Figure 11 and the new concentrations of linoleic acid and 13-HpODE for use in the next iteration. Since O_2 was present in large excess and CPHU was regenerated by DTT, the concentrations of these two reactants were assumed to be constant. The fits of the calculated lines to the experimental results are reasonably close. The deviations that do occur might be accounted for by a small amount of enzyme inactivation during the time course of the reaction and by some of the reasons discussed above for Figure 10.

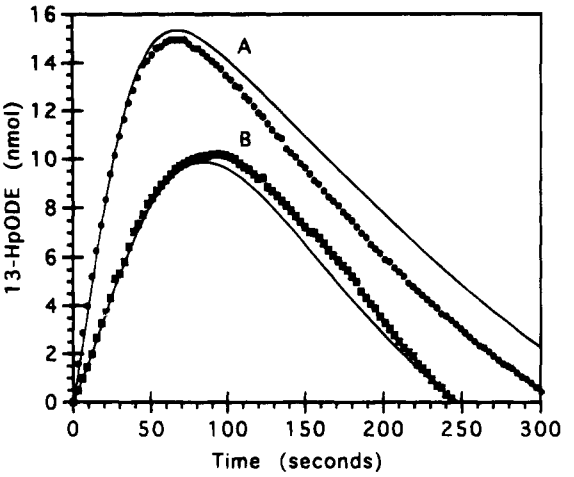

FIGURE 11: Comparison of the experimental and simulated progress curves for the inhibition of the oxygenase activity by CPHU. Experimental (●, ■) and simulated (—) curves are shown for reactions of the lipoxygenase-1 with 10 μM linoleic acid, 2 μM 13-HpODE, and 50 μM DTT in the presence of (A) 4.3 μM CPHU or (B) 8.6 μM CPHU. Simulated 13-HpODE formation was generated using the same kinetic parameters used for the calculated lines in Figure 10.

DISCUSSION

The mechanism shown in Figure 1 for the oxygenase reaction is very similar to that proposed previously by other investigators (Schilstra *et al.*, 1992, 1993; Ludwig *et al.*, 1987), differing mainly by the addition of the k_{15}, k_{17} pathway involving reduction of E_O by the *N*-hydroxyurea. The present mechanism also involves a compulsory order of binding of linoleic acid and oxygen, as required by the results of Figure 7. Schilstra *et al.* (1992) have assumed that dissociation of the pentadienyl radical from E_R, k_9 in Figure 1, is very fast compared with the process corresponding to k_3 in Figure 1. In the present work, the opposite relationship in the relative rates of these two processes is supported by the results presented above and by the approximately 13-fold smaller value of k_{cat}'' than k_{cat}. However, the data of Schilstra *et al.* (1992) are also consistent with a relatively slow rate of the k_9 process, compared with the reaction steps involved in the oxygenase reaction. For example, when the reaction is initiated with E_O, the rate is initially high, then decreases due to formation of E_R which can be stabilized by binding linoleic acid, and then increases again as the 13-HpODE

concentration increases to a level sufficient to maintain the steady-state E_R levels very low. The time necessary for the initial rate to reach its minimum value in these experiments and the amount of 13-HpODE produced during this time [Figure 1 in Schilstra *et al.* (1992)] are sufficient for more than 10 enzyme turnovers in the oxygenase cycle, consistent with a relatively low value of k_9 and with the relative values of k_{cat} and k_{cat}'' reported here. In a more recent study, Schilstra *et al.* (1993) have interpreted results in terms of what corresponds to a relatively low value for the k_9 step.

The effects of linoleic acid and 13-HpODE concentrations on the initial rates of the lipoxygenase-catalyzed oxygenase reaction are rationalized partly in terms of linoleic acid binding as a dead-end inhibitor to E_R in competition with binding of 13-HpODE to E_R followed by regeneration of the active E_O. An alternative mechanism which involves the competition of linoleic acid and 13-HpODE for binding at one or more regulatory sites and does not involve binding of linoleic acid as a dead-end inhibitor at the catalytic site has also been proposed (Egmond *et al.*, 1976; Wang *et al.*, 1993). However, as summarized elsewhere (Ford-Hutchinson *et al.*, 1994), the mechanism shown in Figure 1 accounts for all of the known lipoxygenase activities with only one ligand-binding site and two oxidation states of the nonheme iron center. This mechanism is actually simpler than one requiring multiple allosteric sites and is adequate to accommodate all of the present data.

Although a large amount of evidence provides strong support for redox cycling of the iron center in lipoxygenases during each catalytic turnover (Ford-Hutchinson *et al.*, 1994), it has been reported that the Fe(II) enzyme can be activated at negligible concentrations of lipid hydroperoxides (Wiseman *et al.*, 1988) and that there is a discrepancy between the time courses for the appearance of product and the conversion of E-Fe^{2+} to E-Fe^{3+} during lipoxygenase activation (Wang *et al.*, 1993). A few of the points made by Wang *et al.* (1993) against redox cycling during catalysis require comment at this time. First, it is argued that the low yield of products of decomposition of the radical LO•, arising from the k_{13} step in terms of Figure 1, "eliminates the possibility that repetitive conversion between stable E-Fe^{2+} and E-Fe^{3+} would have taken place during the catalytic process". Evidence has been presented here and in cited work that, because of the k_9 step being slow, products of LO• are expected to be produced in very small quantity during catalysis except at very low oxygen concentrations or in the presence of a reducing agent such as an *N*-hydroxyurea. Second, it was shown that the time courses for product formation catalyzed by 430 nM lipoxygenase are very similar whether the enzyme is added as the native enzyme or as the Fe^{3+} form [Figure 1 in Wang *et al.* (1993)]. In their detailed studies, Schilstra *et al.* (1992, 1993) have shown that whether one initiates catalysis with ferrous or ferric lipoxygenase, essentially identical steady-state rates are quickly reached and that the nature and duration of the pre-steady-state time course depends critically upon conditions. At the high concentrations (430 nM) of lipoxygenase used by Wang and co-workers, as little as 5% of the native enzyme existing in the ferric form could have a very large effect on the initial rate. Third, the fact that there is a lag in the rate of decrease in fluorescence of the lipoxygenase following initiation of reaction by the native enzyme, which is not paralleled by a lag in the reaction progress time course [(Figure 1 in Wang *et al.* (1993)], is taken as evidence that catalysis by the Fe^{2+} form of the enzyme has occurred. While the intrinsic fluorescence yields of the unliganded ferric and ferrous forms of lipoxygenase are known, it is not known what the fluorescence yields are for the ferric and ferrous forms which are intermediates in the catalytic mechanism during turnover. In the absence of this information, it is difficult to interpret the fluorescence data being discussed in terms of the catalytic mechanism. The fact that inhibition of lipoxygenase by an *N*-hydroxyurea can be entirely accounted for by reduction of the ferric enzyme provides additional support for the essential role of redox cycling during catalysis.

CPHU is an efficient and rather nonselective reducing agent of lipoxygenases (Falgueyret *et al.*, 1992), and it should be noted that, although a variety of other NOH inhibitors have been shown to react with these enzymes (Reynolds, 1988; Riendeau *et al.*, 1991a,b; Chamulitrat *et al.*, 1992), the contribution of reductive inactivation to the mechanism of inhibition would be expected to vary according to the reactivity and affinity of a particular hydroxyurea for the active site. In a previous study with leukocyte 5-lipoxygenase, the ability of various compounds to function as reducing substrates for the enzyme was found to be related to their potency as inhibitors (Riendeau *et al.*, 1991b). The reversible inhibition of 5-lipoxygenase by the hydroxyurea zileuton has been reported previously (Carter *et al.*, 1991), and the relative importance of enzyme reduction in the mechanism of inhibition of 5-lipoxygenase by this compound has not yet been assessed. The application of the present model to other inhibitors and mammalian lipoxygenases should allow a better definition of the mechanisms involved in lipoxygenase inhibition.

ACKNOWLEDGMENT

We would like to thank Patrick J. Roy for the synthesis of CPHU and M. David Percival and J.-P. Falgueyret for valuable discussions during the course of this work.

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