Reductive Inactivation of Soybean Lipoxygenase 1 by Catechols: A Possible Mechanism for Regulation of Lipoxygenase Activity†

Cemal Kemal,* Pearl Louis-Flamberg, Rosita Krupinski-Olsen, and Andrew L. Shorter

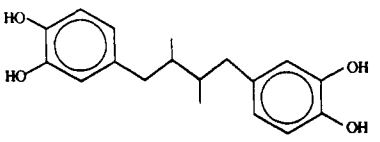
Smith Kline and French Laboratories, Swedeland, Pennsylvania 19479

Received March 23, 1987; Revised Manuscript Received May 14, 1987

ABSTRACT: Nordihydroguaiaretic acid (NDGA), one of the most efficient inhibitors of lipoxygenases, is shown, by electron paramagnetic resonance, circular dichroism, and fluorescence studies, to reduce the catalytically *active* ferric soybean lipoxygenase 1 (E_{ox}) to the *inactive* ferrous form (E_{red}). In decreasing order of reactivity, the following also reduce E_{ox}: catechol > hydroquinone > 2,6-di-*tert*-butyl-4-methylphenol > esculetin > caffeic acid ≈ α-tocopherol > norepinephrine > dithiothreitol. The reduction of E_{ox} by NDGA ($k = 8.1 \times 10^6$ M^{-1} s^{-1}, pH 9.0, 25 °C) is almost as fast as the E_{ox}-catalyzed conversion of linoleate (LH) to 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoate (LOOH) and the oxidation of E_{red} by LOOH to give E_{ox}. Thus, NDGA can efficiently inhibit the E_{ox}-catalyzed conversion of LH to LOOH by reducing E_{ox} to the inactive E_{red}, thereby diminishing the turnover rate. Lipoxygenase catalyzes the oxidation of NDGA by LOOH at a rate that is consistent with the independently determined rate constant for the reduction of E_{ox} by NDGA. All four reducing equivalents from the two catechol groups in NDGA can be utilized in the reduction of E_{ox}, leading to the consumption of 4 mol of LOOH/mol of NDGA initially present. Because the catalytically inactive E_{red} is oxidized by fatty acid hydroperoxides (e.g., LOOH) to give the active E_{ox}, reducing agents such as NDGA are most effective as lipoxygenase inhibitors at low hydroperoxide concentrations. Our results suggest that in vivo, where lipid hydroperoxides are maintained at low steady-state levels, reduction of lipoxygenase from the ferric to ferrous state may be important in the regulation of lipoxygenase activity and hence leukotriene biosynthesis.

Lipoxygenases (EC 1.13.11.12) catalyze dioxygenation of polyunsaturated fatty acids possessing a *cis,cis*-1,4-pentadiene unit to yield cis,*trans* conjugated diene hydroperoxides [for reviews, see Vliegenthart and Veldink (1982) and Papatheofanis and Lands (1985)]. Conversion of arachidonate to a hydroperoxide, 5-HPETE,[1] is the first step in leukotriene biosynthesis; arachidonate 5-lipoxygenase, which catalyzes this reaction, has recently been shown to also catalyze the second step of this pathway, i.e., conversion of 5-HPETE to leukotriene A_4 (Rouzer et al., 1986).

Due to the proposed role of leukotrienes in inflammation and immediate hypersensitivity (Samuelsson, 1983), there is much current interest in inhibiting lipoxygenases (Gleason et al., 1986). NDGA, a catecholic antioxidant, is one of the most



NDGA

effective inhibitors of these enzymes. Lipoxygenases from a wide variety of sources are inhibited by NDGA, e.g., 5-lipoxygenases from human leukocytes, rabbit peritoneal polymorphonuclear leukocytes, rat basophilic leukemia cells, and potatoes (McMillan et al., 1985; Walker et al., 1980; Falkenhein et al., 1980; Reddanna et al., 1985), 12-lipoxygenase from guinea pig tissues (Hamberg, 1976); and 15-lipoxygenases from soybeans and rabbit reticulocytes (Tappel et al., 1953; Ishiura et al., 1986). Within the past few years, a number of other catecholic compounds of plant origin have been shown to inhibit lipoxygenases, e.g., caffeic acid (Koshihara et al., 1984), esculetin (Murota & Koshihara, 1985), gossypol (Hamasaki & Tai, 1985), flavonoids (Alcaraz & Hoult, 1985; Wheeler & Berry, 1986; Takahama, 1985), and diarylheptanoids (Flynn et al., 1986).

Although a role for the catechol functionality is clearly implicated in the inhibition of lipoxygenase, the mechanism of this process has not yet been established. Much of the available information on the mode of action of catechols has come from work done with soybean lipoxygenase 1. The following have been suggested to account for inhibition of this enzyme by catechols: (i) inhibition via reduction of enzyme-bound radical intermediates generated from the fatty acid substrate during turnover (Tappel et al., 1953; Papatheofanis & Lands, 1985; Takahama, 1985); (ii) competitive reversible inhibition (Yasumoto et al., 1970); and (iii) irreversible inhibition resulting from coordination of the catechol to the ferric cofactor (Galpin et al., 1976).

We describe herein the results of our studies on the inhibition of soybean lipoxygenase 1 by NDGA and demonstrate that this catecholic compound, as well as several others, inhibits the enzyme by reducing the catalytically *active* ferric enzyme to the catalytically *inactive* ferrous form.

† This work was supported by National Institutes of Health Grant GM3[illegible].

* Author to whom correspondence should be addressed. Present address: Chevron Chemical Co., P.O. Box 4010, Richmond, CA 94804.

[1] Abbreviations: 5-HPETE, 5(*S*)-hydroperoxy-6(*E*),8(*Z*),11(*Z*),14(*Z*)-eicosatetraenoic acid; E_{red}, the native reduced (ferrous) form of soybean lipoxygenase 1; E_{ox}, the oxidized (ferric) form of soybean lipoxygenase 1; LH, linoleate; LOOH, 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoate; BHT, 2,6-di-*tert*-butyl-4-methylphenol; NDGA, nordihydroguaiaretic acid; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]-propanesulfonic acid; DTT, dithiothreitol; IC_{50}, concentration of inhibitor necessary for 50% inhibition of an enzymic reaction; EPR, electron paramagnetic resonance; CD, circular dichroism.

0006-2960/87/0426-7064$01.50/0

Experimental Procedures

Materials

Soybean lipoxygenase 1 was purified as described by Galpin et al. (1976) from type I lipoxidase supplied by Sigma Chemical Co. Linoleic acid (>99%) was obtained from Nu Chek Prep, Inc., and stored under a nitrogen atmosphere (glovebox). Catalase (bovine liver), NDGA, pyrocatechol, hydroquinone, esculetin, norepinephrine, caffeic acid, and TAPS were purchased from Sigma. NDGA was recrystallized from dilute acetic acid. BHT (Aldrich Chemical Co.) was purified by sublimation. α-Tocopherol (Eastman Kodak Co.) stock solutions were prepared in methanol and stored at −70 °C. All other chemicals were purchased from commercial sources at the highest level of purity available.

Methods

Spectrophotometric assays were done with a Perkin-Elmer Lambda 7 UV–vis spectrophotometer. Fluorescence changes were monitored by using either a Kinetic Instruments, Inc., stopped-flow spectrophotometer interfaced to an On-Line Instruments data acquisition system or a Perkin-Elmer Model MPF-66 spectrofluorometer interfaced to a Perkin-Elmer Model 7500 data station. EPR spectra were recorded on a Varian E109 spectrometer. CD spectra were recorded with a Jasco J-500A spectropolarimeter. Concentrated solutions of lipoxygenase needed for EPR and CD experiments were prepared by using a Micro-ProDicon dialysis/concentrator (Bio-Molecular Dynamics). Oxygen uptake was measured with a Yellow Springs Instruments Model 53 dissolved oxygen meter. All kinetic experiments were done at 25 °C.

Assays. The concentration of soybean lipoxygenase 1 was calculated from A_{280} by using an extinction coefficient of 1.6×10^5 M^{-1} cm^{-1} (Spaapen et al., 1979). Activities were determined in air-saturated solutions, both by spectrophotometry and by monitoring oxygen consumption. With the former method, the formation of LOOH was followed at 234 nm ($\epsilon = 2.5 \times 10^4$ M^{-1} cm^{-1}) in a solution of pH 9.0 containing 320 μM LH [0.1 M borate, μ = 0.2 with KCl, 5% methanol (v/v)]. For oxygen consumption measurements, the standard polarographic procedure of Vliegenthart and Veldink (1982) (1.8 mM LH, 0.1 M borate, pH 9.0) was modified by inclusion of 40 μM BHT or α-tocopherol in assay solutions [5% methanol (v/v)]. The purpose of including these antioxidants is to inhibit the nonenzymic autoxidation of LH, which is initiated by LH-derived radicals released by the enzyme during turnover and contributes ca. 30% to the overall O_2 uptake in the micellar 1.8 mM LH solutions.[2] With this modification, specific activities determined by spectrophotometric and polarographic measurements agreed with each other and were in the range of 150–180 μmol min^{-1} mg^{-1}. It should be noted that the specific activity reported by Vliegenthart and Veldink (1982) for pure soybean lipoxygenase 1 (240 μmol min^{-1} mg^{-1}) has *not* been corrected for autoxidation. In agreement with the reported value, we obtained an *apparent* specific activity of 225–260 μmol min^{-1} mg^{-1}, using the polarographic procedure of these workers without modification.

Fluorescence Studies of the Reduction of E_{ox} to E_{red}. The ferric enzyme was prepared, just before use, by adding sufficient LOOH or LH (0.7–1.0 molar equiv, depending on the enzyme preparation) to the native ferrous enzyme to produce the maximum drop in fluorescence (Galpin et al., 1976). Adding LH is equivalent to adding LOOH because LH is rapidly converted to LOOH by traces of ferric enzyme present. The reduction of the iron cofactor by catechols and other reductants was monitored by following the increase in enzyme fluorescence that occurs as the ferric enzyme is converted to the ferrous form. Excitation was at 280 nm. In the stopped-flow experiments, either a 75-W xenon lamp or a 100-W xenon–mercury lamp was used, and fluorescence was observed through two filters (Corning 0-54 and 7-54), which allowed emitted light in the range of 300–420 nm to reach the detector. In a typical experiment, a solution of ferric soybean lipoxygenase 1 in a buffer containing 5% methanol (v/v) was mixed with a solution of the reductant in the same solvent system. Solutions of NDGA in pH 9.0 TAPS buffer were prepared under anaerobic conditions in a glovebox. All other solutions were made in air-saturated buffers just prior to use.

Irreversible Inactivation of Lipoxygenase. Irreversible loss of lipoxygenase activity was followed by diluting a small portion of an incubation of the enzyme and the inactivating agent into a standard assay solution containing 320 μM LH and monitoring conjugated diene hydroperoxide formation at 234 nm. The enzymic activity was calculated by using maximal rates attained after an initial lag phase.

Results

Reduction of E_{ox} by NDGA. As shown in Figure 1A, upon addition of NDGA to E_{ox}, the EPR signal at $g = 6.1$ due to the ferric cofactor (Slappendel et al., 1982) disappears. Since the ferrous enzyme is EPR silent, this result is consistent with reduction of the ferric cofactor to ferrous. The signal at $g = 6.1$ can be regenerated by the addition of LOOH, which oxidizes E_{red} to E_{ox}.

Reduction of E_{ox} to E_{red} by NDGA can also be demonstrated by exploiting a difference in the CD spectra of these two enzyme forms. Spaapen et al. (1979) have shown that E_{ox} exhibits a positive dichroic band at 425 nm, while E_{red} does not have any bands in the visible region. Addition of NDGA to E_{ox} leads to the disappearance of the 425 nm band, which is regenerated upon addition of LOOH (Figure 1B).

A third indication of the reduction of E_{ox} by NDGA comes from fluorescence experiments. E_{ox} is ca. 35% less fluorescent than E_{red} (Figure 2, inset) (Egmond et al., 1975). Upon addition of NDGA to E_{ox}, the fluorescence intensity increases in a time-dependent manner to nearly the level of that of E_{red}. This change is also reversed on addition of LOOH (see below).

Determination of Reduction Rates Using Fluorescence. Rate constants for the reduction of E_{ox} by NDGA were determined by monitoring the increase in enzyme fluorescence that occurs as E_{ox} is reduced to E_{red}. Under pseudo-first-order conditions ([NDGA] $\geq 10[E_{ox}]$), the rate of reduction of E_{ox} by NDGA was found to obey the first-order rate law to greater than 4 half-lives. At pH 9.0, this process was studied in two different buffers, borate and TAPS. In the range of NDGA concentrations used (5–500 μM in borate, 1–20 μM in TAPS), saturation kinetics were not observed in either buffer. However, the second-order rate constants were significantly different: 2.4×10^4 M^{-1} s^{-1} in borate (Figure 2) and 8.1×10^6 M^{-1} s^{-1} in TAPS (Figure 3A). The slower rate in borate can be ascribed to complexation of NDGA by boric acid. Using the literature value for the association constant between catechol and boric acid (K = [complex]/[catechol][borate] = 7.8×10^3 M^{-1}) (Roy et al., 1957), we calculate that under our conditions the fractions of free NDGA, complex **1**, and complex **2** would be 1.2×10^{-5}, 3.5×10^{-3}, and 0.996, respectively; i.e., only 0.35% of the total catechol groups will be in the uncomplexed state. This is in good agreement with our finding that the value for the rate constant for the reduction of E_{ox} by NDGA in borate buffer is 0.29% of that observed

[2] C. Kemal, unpublished results.

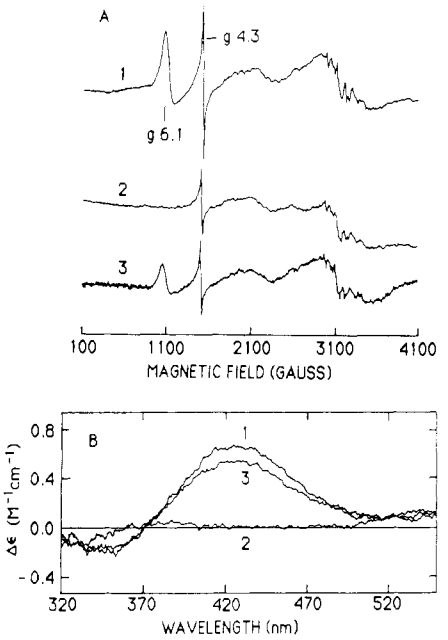
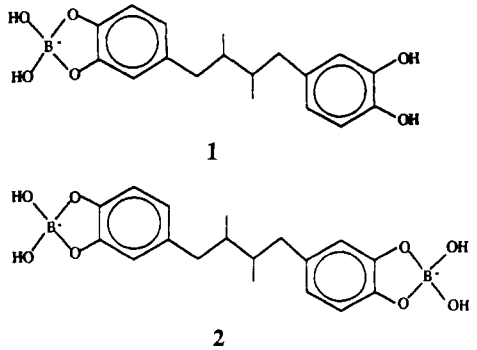
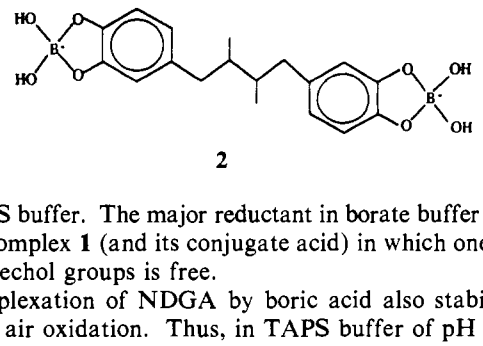


FIGURE 1: (A) Effect of NDGA on the EPR spectrum of ferric soybean lipoxygenase 1: (1) ferric enzyme; (2) ferric enzyme treated with 0.5 molar equiv of NDGA; (3) ferric enzyme treated with 0.5 molar equiv of NDGA and, 1 min later (25 °C), with 2 molar equiv of LOOH. The three traces shown were obtained with three separately prepared samples; the concentration of the enzyme was higher in the first sample (300 μM) than in the last two (200 μM). All three solutions were at pH 9.0 (110 mM borate) and contained 5% methanol (v/v) in a total volume of 210 μL. The signal at $g = 4.3$ is due to denatured lipoxygenase and/or contaminating iron; in the range 1700–3000 G, the base line was not flat due to a signal given by the sample cavity. The spectra were recorded at 103 K under the following conditions: spectrometer frequency 9.135 Hz; modulation frequency 100 KHz; modulation amplitude 5 G; microwave power 4.78 mW. (B) Effect of NDGA on the CD spectrum of ferric lipoxygenase 1: (1) CD spectrum of ferric lipoxygenase 1 (225 μM) in pH 9.0 borate buffer; (2) solution 1 after the addition of 0.25 molar equiv of NDGA; (3) solution 2 after the addition of 1.5 molar equiv of LOOH. Spectrum 3 has not been corrected for a 15% dilution that occurred on addition of LOOH. Solutions of NDGA and LOOH were in methanol; the final methanol concentration in the enzyme solution was 6% (v/v). The spectra were recorded at room temperature.



1



2

in TAPS buffer. The major reductant in borate buffer is most likely complex **1** (and its conjugate acid) in which one of the two catechol groups is free.

Complexation of NDGA by boric acid also stabilizes it against air oxidation. Thus, in TAPS buffer of pH 9.0, 10 μM NDGA was found to air oxidize with a half-life of 12.5 min. In borate buffer of the same pH, reproducible results were obtained when NDGA solutions were used within 1 h, indicating that no significant oxidation had occurred in this amount of time.

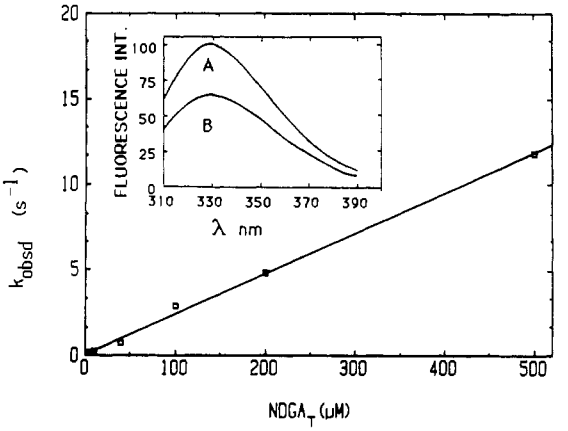


FIGURE 2: Dependence of k_{obsd} for the reduction of ferric soybean lipoxygenase 1 on the total NDGA concentration ([NDGA] + [**1**] + [**2**]) in *borate* buffer, pH 9.0. Rate constants were determined at 25 °C by monitoring the increase in enzyme fluorescence that occurs as the ferric enzyme is reduced to ferrous. Each rate constant is the average of four to eight determinations. Inset: Comparison of the fluorescence spectra of the ferrous (A) and the ferric (B) enzymes (λ_{ex} = 280 nm).

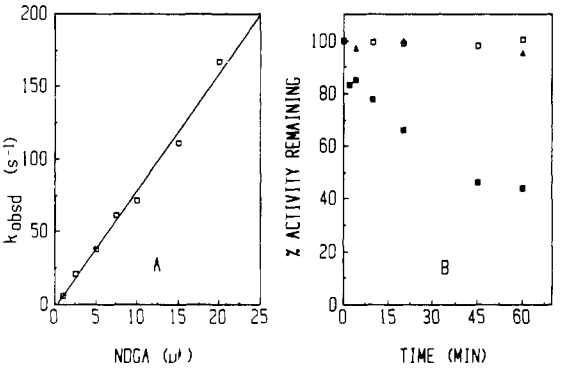


FIGURE 3: (A) Dependence of k_{obsd} for the reduction of ferric soybean lipoxygenase 1 on NDGA concentration in *TAPS* buffer, pH 9.0. Rate constants were determined at 25 °C as described in the legend of Figure 2. Solutions of NDGA were prepared under anaerobic conditions. Each rate constant is the average of three to five determinations. (B) Irreversible inactivation of lipoxygenase (1.0 μM) by H_2O_2 produced from air oxidation of NDGA: (□) lipoxygenase by itself; (■) lipoxygenase plus NDGA (100 μM); (▲) lipoxygenase plus NDGA (100 μM) plus catalase (1.6 μM). Incubations were at 25 °C in phosphate buffer, pH 8.0. Similar results were obtained at pH 9.0 in borate buffer.

Using the fluorescence method, we determined the rate constants for the reduction of E_{ox} by several other reductants. The data, summarized in Table I, show that E_{ox} may be reduced by a variety of compounds, including catechols, hydroquinone, BHT, α-tocopherol, and DTT (see Discussion). The increase in enzyme fluorescence that resulted on addition of each reductant to E_{ox} was reversed on addition of LOOH. The reduction of E_{ox} by BHT was also confirmed by EPR experiments (not shown).

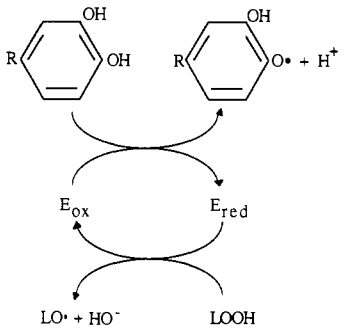
Irreversible Inactivation. During the time required for the reduction of E_{ox} by NDGA, there is little or no *irreversible* inactivation of the enzyme. Greater than 95% of the enzyme is converted to the ferrous form, which, although catalytically inactive, can be easily converted to the active ferric form under assay conditions by trace levels of LOOH. Incubation of the enzyme (ferrous or ferric) with NDGA for long periods of time does, however, lead to irreversible inactivation. For example, incubation of 1.0 μM E_{ox} with 100 μM NDGA results in 60% irreversible loss of activity in 1 h (Figure 3B). This inactivation is unrelated to the reduction of E_{ox}, which, under the above conditions, goes to completion in less than 1 s. In agreement

Table I: Rate Constants at 25 °C for Reduction of Ferric Soybean Lipoxygenase 1 by 10 μM Reductant[a]

reductant	k_{obsd} (s^{-1}) (pH 8.0)[b]	k_{obsd} (s^{-1}) (pH 9.0)[c]
NDGA	5.0	8.1×10^{1}
catechol	1.7×10^{-1}	
hydroquinone	7.5×10^{-2}	
esculetin	8.5×10^{-4}	
caffeic acid	4.2×10^{-4}	
norepinephrine	1.9×10^{-4}	
BHT		1.8×10^{-2}
α-tocopherol[d]		$\sim 5. \times 10^{-4}$
DTT[e]	$\sim 1.3 \times 10^{-4}$	$\sim 1. \times 10^{-4}$

[a] Determined from the increase in enzyme fluorescence during reduction of E_{ox} (100–800 nM). Rate constants (except those for α-tocopherol and DTT) are averages of four to eight determinations. [b] In 70 mM phosphate [5% methanol (v/v)]. [c] In 100 mM TAPS (NDGA) or 100 mM borate [μ = 0.2, 5% methanol (v/v)]. [d] The rate constant shown is the average of two determinations. [e] The rate constant at pH 8.0 is one-tenth the rate constant obtained when 100 μM DTT was used in the presence of 1.6 μM catalase. The rate constant at pH 9.0 was calculated from a second-order rate constant determined with five DTT concentrations in the range 0.3–3.0 mM.

Scheme I



with Yasumoto et al. (1970), we find that the slow, irreversible inactivation can be completely eliminated by catalase (Figure 3B), suggesting that inactivation is caused by hydrogen peroxide, a known potent irreversible inactivator of soybean lipoxygenase 1 (Mitsuda et al., 1967), formed by air oxidation of NDGA.

Reduction of E_{ox} by the other catecholic compounds used in this study was accompanied by little (<10%) or no irreversible inactivation. In contrast to Galpin et al. (1976), who reported that at pH 7.0 catechol instantaneously inactivated E_{ox} (0 °C), we found no irreversible loss of activity when E_{ox} (10 μM) was incubated with 1.7 molar equiv of catechol at 0 °C for a period of 3 h (pH 7.0). Incubation of the enzyme with hydroquinone for long periods of time (hours) led to irreversible inactivation that could be attributed to two air-oxidation products of hydroquinone, hydrogen peroxide (preventable by 1.6 μM catalase) and *p*-benzoquinone. Incubation of ferrous lipoxygenase 1 with 100 μM *p*-benzoquinone resulted in irreversible loss of enzyme activity with a half-life of ca. 11 min (pH 8.0, not shown). With NDGA, irreversible inactivation attributable to an *o*-quinone product was not detected (Figure 3B), presumably because the *o*-quinone is unstable in aqueous solution. During the reduction of E_{ox} by DTT (0.1–3 mM) ca. 40–70% of the enzyme was irreversibly inactivated. This inactivation was completely eliminated by catalase (1.6 μM), suggesting that it was caused by hydrogen peroxide formed as a result of the air oxidation of DTT.

Lipoxygenase-Catalyzed Oxidation of NDGA by LOOH. If NDGA is capable of reducing E_{ox}, then lipoxygenase should catalyze the oxidation of NDGA by LOOH as depicted in Scheme I. The results shown in Figure 4 and Table II demonstrate that lipoxygenase does indeed catalyze this reaction. These experiments were done in borate buffer in order to minimize the contribution to absorbance changes from air oxidation of NDGA (see above). The initial rate of disappearance of LOOH (10 μM), monitored by following the decrease in absorbance at 234 nm (the λ_{max} of LOOH), was directly proportional to the concentration of both NDGA and the enzyme (Table II). In these experiments, the rate of NDGA-independent decomposition of LOOH (catalyzed by the enzyme) was negligible (<1% of the rates seen in the presence of NDGA, Table II).

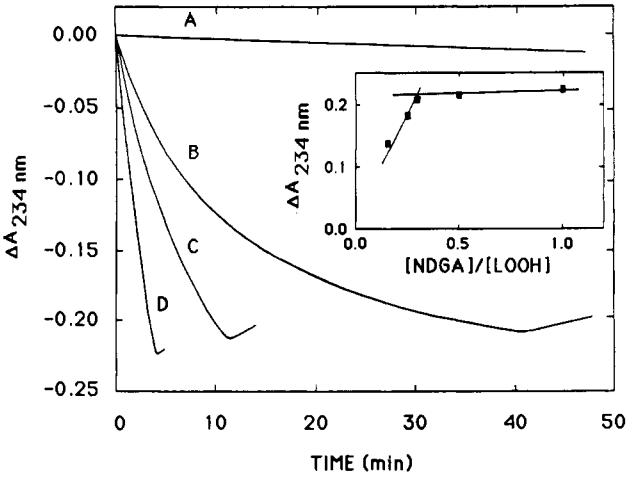


FIGURE 4: Dependence of the time course of lipoxygenase (320 nM) catalyzed reaction between NDGA and LOOH (10 μM) on NDGA concentration. NDGA concentrations were 0 (A), 3.0 (B), 5.0 (C), and 10.0 (D) μM. Incubations were at 25 °C in *borate* buffer, pH 9.0. The increase in absorbance seen after the depletion of LOOH is due to air oxidation of NDGA. Inset: Stoichiometry of the lipoxygenase-catalyzed reaction of LOOH with NDGA. Maximal decrease in A_{234nm} was achieved at [NDGA]/[LOOH] ~ 0.25–0.3.

Table II: Dependence of Rate of Disappearance of LOOH on Concentrations of Lipoxygenase and NDGA[a]

[E] (nM)	[NDGA] (μM)	obsd initial rate[b] (nM/s)	calcd initial rate[c] (nM/s)
320	0	0.2	
320	10.0	60.1	76.8 (60.7)
320	5.0	30.0	38.4 (30.3)
320	3.0	17.7	23.0 (18.2)
640	10.0	119.1	153.6 (121.3)
640	2.5	35.5	38.4 (30.3)
640	1.5	22.5	23.0 (18.2)

[a] The concentration of LOOH was 10 μM in all of the experiments. Reactions were carried out at pH 9.0 in 100 mM borate buffer (μ = 0.2 with KCl) containing 1% methanol (v/v). [b] Determined at 234 nm. [c] Calculated from the equation $-d[LOOH]/dt = k_r[E][NDGA]$, where k_r is the independently determined second-order rate constant for the reduction of E_{ox} by NDGA. The values in parentheses are obtained by multiplying the term $k_r[E][NDGA]$ by $K_I/(K_I + [LOOH])$ to correct for competitive inhibition, by LOOH, of the reduction of E_{ox} by NDGA. A K_I value of 37.5 μM was used (Gibian & Galaway, 1976).

The stoichiometry of lipoxygenase-catalyzed oxidation of NDGA by LOOH was determined from the dependence of the total change in absorbance at 234 nm on [NDGA] (in borate buffer), as well as from spectrofluorometric titration of E_{ox} with NDGA (in TAPS buffer). From the dependence of ΔA_{234} on [NDGA] (Figure 4, inset), it was estimated that ca. 0.25–0.3 mol of NDGA is oxidized per mol of LOOH reduced. In close agreement with this result, spectrofluorometric titration of E_{ox} with NDGA in TAPS buffer indicated that 0.31 molar equiv of NDGA was sufficient to completely reduce the enzyme (Figure 5). These results suggest that all four reducing equivalents from the two catechol groups of

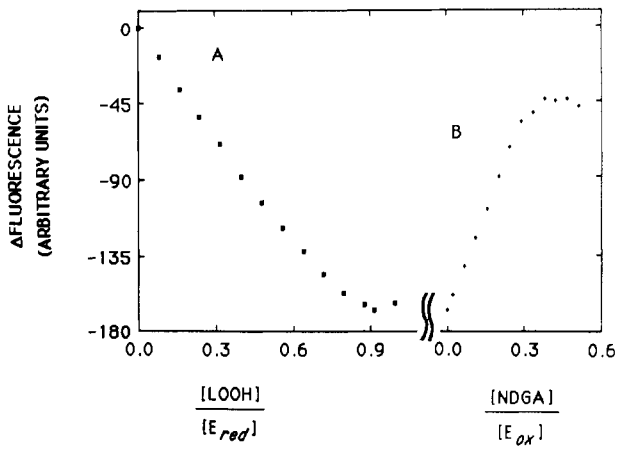


FIGURE 5: Oxidative titration of ferrous lipoxygenase (920 nM) with LOOH (A) and reductive titration of ferric lipoxygenase (920 nM) with NDGA (B) by monitoring changes in enzyme fluorescence. Both titrations were done with a single enzyme solution in *TAPS* buffer, pH 9.0.

NDGA can be utilized in the reduction of E_{ox}. The deviation from 0.25:1 stoichiometry may be caused by side reactions (e.g., air oxidation of semiquinone intermediates). There was no *irreversible* activity loss during the titration of E_{ox} with NDGA. However, after the addition of a 0.31 molar equiv of NDGA to E_{ox}, the fluorescence intensity returned to 95% of the level of the ferrous enzyme. Reoxidation of the reduced enzyme resulted in a fluorescence intensity that was 5% lower than that of the ferric enzyme. Thus, it appears that oxidation products of NDGA may be quenching the fluorescence of both enzyme forms. After five cycles of oxidation/reduction, covering a period of 4 h, there was ca. 25% loss in enzyme activity.

Although no attempt was made to titrate E_{ox} with NDGA by monitoring the disappearance of the CD and EPR signals of E_{ox}, the results reported in Figure 1 are consistent with close to all four reducing equivalents of NDGA being utilized in the reduction of E_{ox}. Thus the CD and EPR signals of E_{ox} completely disappeared on addition of 0.25 and 0.5 molar equiv of NDGA, respectively. (No attempt was made to use a lesser amount in the EPR experiments.)

At pH 9.0, the rate constant for the oxidation of E_{red} by 10 μM LOOH has been reported to be ca. 100 s^{-1} (Aoshima et al., 1977). Comparison of this rate constant to that determined in this study for the reduction of the ferric enzyme by 10 μM NDGA (i.e., 0.24 s^{-1}, Figure 2) reveals that in *borate* buffer the rate-determining step in Scheme I should be the reduction of E_{ox} by NDGA. That this is so is indicated by the dependence of the initial rate of LOOH disappearance on [NDGA] (Table II), as well as by the influence of LOOH on the time course of the reaction of NDGA with E_{ox}. As shown in Figure 6, when the reduction of E_{ox} (200 nM) by NDGA (10 μM) was studied in the presence of LOOH, a kinetic lag phase appeared in the fluorescence traces; the duration of the lag phase increased as [LOOH] was increased. If the rate of oxidation of E_{red} by LOOH is much faster than the rate of reduction of E_{ox} by NDGA, then in the presence of LOOH the enzyme will remain in the ferric form until all the LOOH is consumed. Consequently, as long as LOOH is present, the fluorescence intensity will remain at the level of E_{ox}, giving the observed lag phase. Furthermore, the duration of this lag phase is consistent with the independently determined second-order rate constant for the reduction of E_{ox} by NDGA in borate buffer. The length of the lag phase may be estimated from the ratio $[LOOH]/(k_r[E][NDGA])$. Using this expression, we calculate that in the presence of 10 μM NDGA and 200 nM E_{ox} it would take ca. 3.3 and 14.6 s to consume 160 and 700 nM LOOH, respectively. These values are in excellent agreement with the experimentally observed lag phases of ca. 3 and 15 s (Figure 6).

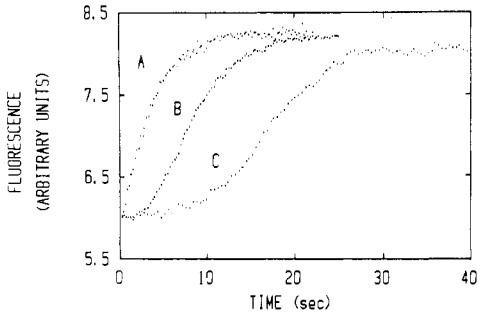


FIGURE 6: Influence of LOOH on the reduction of E_{ox} (200 nM) by NDGA (10 μM) in *borate* buffer, pH 9.0. LOOH concentrations were 0 (A), 160 (B), and 700 (C) nM. Each trace is the average of four to six determinations.

The rate of disappearance of LOOH (Figure 4) was also consistent with the second-order rate constant for the reduction of E_{ox} by NDGA, determined independently by monitoring changes in enzyme fluorescence (Figure 2). As shown in Table II, the rates of LOOH disappearance, calculated from the expression $-d[LOOH]/dt = k_r[E][NDGA]$, are ca. 25% larger than the experimental values determined by monitoring the absorbance decrease at 234 nm ($\Delta\epsilon = 2.2 \times 10^4$ M^{-1} cm^{-1}). This small difference can be accounted for by applying a correction factor to the calculated rate to reflect competitive inhibition, by LOOH, of the reduction of E_{ox} by NDGA (Table II).

When the concentration of NDGA was kept in the micromolar range, lipoxygenase catalyzed the reduction of more than 50 molar equiv of LOOH without any significant loss of enzyme activity. For example, no loss of activity was detected during the conversion of 2.5 μM LOOH by 50 nM lipoxygenase in the presence of 1.0 μM NDGA. On incubation of 1.0 μM enzyme with NDGA (100 μM) and LOOH (25 μM), irreversible inactivation did occur, but on a much longer time frame than needed for the consumption of LOOH. This inactivation was preventable by catalase (as in the experiments of Figure 3B), indicating that it was caused by H_2O_2 produced from the air oxidation of NDGA.

Products from Lipoxygenase-Catalyzed Oxidation of NDGA by LOOH. It has been reported that the major fate of LO•, produced from the one-electron reduction of LOOH by E_{red} (Scheme I), is cyclization to an epoxyallylic radical, which, after O_2 addition, would give products that do not absorb in the near-UV (Dix & Marnett, 1983; Clapp et al., 1985). Consistent with this are the results of Figure 4, which show a large change in absorbance at 234 nm during the lipoxygenase-catalyzed reaction of LOOH with NDGA. The extinction coefficients (M^{-1} cm^{-1}) of LOOH and NDGA at this wavelength are 2.5×10^4 and 8.4×10^3, respectively, while the $\Delta\epsilon$ for the reaction is ca. 2.2×10^4 M^{-1} cm^{-1}. Therefore, conversion of LO• to LOH, the corresponding conjugated diene alcohol (which has the same extinction coefficient at 234 nm as LOOH), cannot be a major reaction; i.e., most of LO• is converted to products that do not absorb at this wavelength. As would be expected if LO• decomposes mainly by cyclization to an epoxyallylic radical, consumption of O_2, corresponding to ca. half of the initial LOOH concentration of 10 μM, could be detected under the conditions of the experiments of Figure 4 (not shown). The decomposition of LO• has previously been shown to also give, via β-cleavage, two minor products: 13-

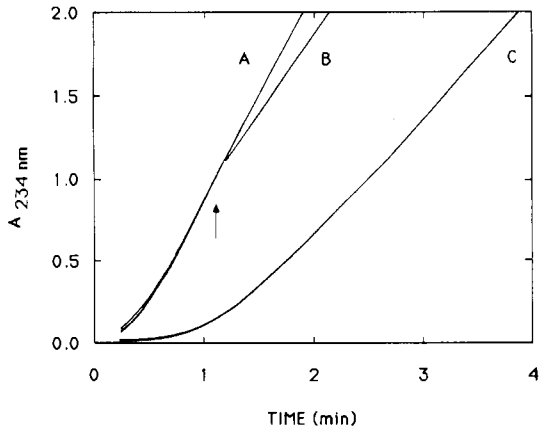


FIGURE 7: Effect of NDGA (2 μM) on lipoxygenase-catalyzed conversion of LH to LOOH in *TAPS* buffer, pH 9.0: (A) LH (320 μM) plus lipoxygenase (3.0 nM); (B) same as (A) except for the addition of 2.0 μM NDGA at the point indicated by the arrow; (C) same as (A) except for the presence of 2.0 μM NDGA prior to the initiation of the reaction.

keto-9,11-octadecadienoic and 13-oxo-9,11-tridecadienoic acids (total yield 10–20%) (Spaapen et al., 1979; Clapp et al., 1985).

On the basis of earlier work on the oxidation of catechols by aqueous ferric ion (Mentasti et al., 1976a; Mentasti et al., 1976b) and by periodate (Weidman & Kaiser, 1966), it is reasonable to assume that, in the presence of lipoxygenase and LOOH, NDGA is oxidized to the corresponding di-*o*-quinone which then polymerizes.

Effect of NDGA on Lipoxygenase-Catalyzed Oxygenation of LH. Figure 7 illustrates the effect of NDGA on lipoxygenase-catalyzed conversion of LH to LOOH in pH 9.0 TAPS buffer. When NDGA (final concentration 2 μM) was added to a reaction in progress (after ca. 40 μM LOOH had been produced), there was an instantaneous decrease of 25% in the rate of absorbance increase at 234 nm.[3] However, when 2 μM NDGA was present prior to the initiation of the enzymic reaction, the *initial rate* was decreased by more than 98% compared to the control run. The *maximal velocity* attained was about the same as the maximal velocity seen when NDGA was added to a reaction already in progress. Consequently, the inclusion of NDGA in the incubation solution resulted in a lag phase of considerably longer duration. These results are consistent with a mechanism of inhibition involving reduction of the catalytically *active* E_{ox} to the *inactive* E_{red} by NDGA.

Discussion

Reduction of Ferric Soybean Lipoxygenase 1 by NDGA. Native soybean lipoxygenase 1 contains a nonheme ferrous ion that must be oxidized to yield the catalytically active ferric enzyme (Feiters et al., 1985; de Groot et al., 1975; Pistorius et al., 1976). Interestingly, the fatty acid hydroperoxide product generated by the enzyme (e.g., LOOH) is utilized as an oxidant in this activation process. The ferric enzyme can be distinguished from the ferrous form by its unique EPR and CD features, as well as by its decreased fluorescence (Slappendel et al., 1982; Spaapen et al., 1979; Egmond et al., 1975). By exploiting these differences, we have shown that NDGA reduces the ferric enzyme (E_{ox}) to the ferrous state (E_{red}), and by monitoring fluorescence changes, we have kinetically characterized this reaction.

Inhibition of lipoxygenase by catechols had not previously been attributed to the ability of these compounds to reduce the iron cofactor (Vliegenthart & Veldink, 1982).[4] Galpin et al. (1976) reported that catechol, 4-nitrocatechol, protocatechualdehyde, 2,3-dihydroxyanthraquinone, and 3,4-dihydroxybenzonitrile *irreversibly* inhibited E_{ox} (but not E_{red}) by coordinating to the iron. 4-Nitrocatechol was later shown to reversibly form a green complex with E_{ox}, which slowly and irreversibly converted to a brown species (Spaapen et al., 1980). While it is likely that, due to an unfavorable redox potential, 4-nitrocatechol might not reduce E_{ox}, it is not clear why, in contrast to our results (Table I), Galpin et al. (1976) did not detect reduction of the enzyme by unsubstituted catechol. These workers reported that, at pH 7.0, treatment of 10 μM E_{ox} with slightly more than a stoichiometric amount of catechol led to essentially instantaneous irreversible inhibition (>99%) of the enzyme at 0 °C. Under these conditions we have found no irreversible inactivation by catechol in a period of 3 h. Galpin et al. also reported that on addition of catechol the characteristic EPR signal at $g = 6.1$ of the ferric enzyme was rapidly transformed to a $g = 4.3$ signal, presumed to be due to inactivated enzyme. In contrast, our EPR results show that E_{ox} is reduced by the substituted catechol NDGA (Figure 1A). This reduction is not accompanied by any irreversible loss of enzyme activity. In agreement with Yasumoto et al. (1970), we find that irreversible inactivation of soybean lipoxygenase 1 takes place only when the enzyme is incubated with excess NDGA for long periods of time (hours) and is caused by H_2O_2 produced by air oxidation of NDGA (Figure 3B).

It is interesting to note that the behavior of ferric soybean lipoxygenase 1 toward catechols is similar to that of ferric ion in aqueous solution (pH 1–2). Catechols with strongly electron-withdrawing substituents (e.g., 4-cyano-1,2-dihydroxybenzene) have been shown to form a bidentate complex with Fe(III) without reducing it (Mentasti et al., 1976a). Catechol and catechols with electron-releasing substituents form a complex, but this is followed by a redox decomposition leading to Fe(II) (Mentasti et al., 1976b). Similarly, catechol reduces E_{ox} (Table I), but 4-nitrocatechol only forms a complex, without reducing the ferric cofactor (Spaapen et al., 1980). Evidently E_{ox} *can* be reduced without forming a bidentate complex, because hydroquinone, which cannot form such a complex, is nearly as effective as catechol as a reductant (Table I). One of the hydroxyl groups may, however, coordinate to the iron prior to electron transfer, or it may be that the reduction of E_{ox} occurs by an outer-sphere electron transfer, as in the reduction of tris(1,10-phenanthroline)iron(III) by catechols (Pelizzetti & Mentasti, 1977).

The fact that, per catechol unit, NDGA is a 15-fold faster reductant than catechol (Table I) can be attributed to electron-releasing properties of the alkyl group bridging the two catechols of NDGA; e.g., 4-methyl-1,2-benzenediol is a better reducing agent than catechol (Pelizzetti & Mentasti, 1977). Due to the widely differing structures of the reductants used in this study, it is not expected that the order of reactivity shown in Table I would necessarily hold for other lipoxygenases. For example, caffeic acid, one of the poorest catecholic reductants in this study, appears to be more effective in inhibiting 5-lipoxygenase (Koshihara et al., 1984) than the soybean enzyme (an arachidonate 15-lipoxygenase). Because the distance between the carboxylate and catechol groups of

[3] A small percentage of this decrease is due to consumption of LOOH according to Scheme I. Another ~10% is due to inhibition, by NDGA, of autoxidation initiated by LH-derived radicals released from the active site during turnover (C. Kemal, unpublished results).

[4] Clapp et al. (1985), however, have shown that *N*-alkylhydroxylamines inhibit soybean lipoxygenase 1 by reducing E_{ox} to E_{red}.

caffeic acid is comparable to the distance between the carboxylate group and C-7 of arachidonic acid (from which a hydrogen atom is abstracted in the 5-lipoxygenase reaction), the catechol unit of caffeic acid may bind closer to the iron site in 5-lipoxygenase than in 15-lipoxygenase. Therefore, on the basis of structural considerations, caffeic acid might be expected to reduce ferric 5-lipoxygenase faster than ferric 15-lipoxygenase.

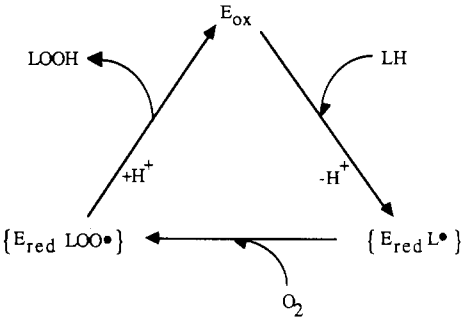
Since norepinephrine and hydroquinone are reductants of ferric soybean lipoxygenase 1 (Table I), it seems likely that the inhibition of human platelet 12-lipoxygenase by epinephrine and hydroquinone (Ho et al., 1977) is due to the reduction of the ferric form of this enzyme by these compounds. Ferric soybean lipoxygenase 1 is also reduced by the phenolic compounds α-tocopherol and BHT as well as by thiols (Table I). Our finding that DTT reduces E_{ox} may be of significance in relation to studies on human leukocyte 5-lipoxygenase. According to a recent report, DTT, which is used as a stabilizer of this enzyme during purification, causes irreproducible or unexpectedly low activities during assays, which may be remedied by addition of fatty acid hydroperoxides (Rouzer & Samuelsson, 1986). Perhaps the low activities observed are related to the reduction of the ferric form of this lipoxygenase by DTT, which would lead to consumption of hydroperoxide as in Scheme I. Also, hydrogen peroxide produced by air oxidation of DTT might irreversibly inactivate this lipoxygenase as we have observed with the soybean enzyme.

Inhibition of Lipoxygenase-Catalyzed Oxygenation of LH to LOOH by NDGA. The reduction of E_{ox} by NDGA (k = 8.1×10^6 M^{-1} s^{-1} at pH 9.0) is almost as facile as the conversion of LH to LOOH by E_{ox} ($k_{cat}/K_m \sim 2 \times 10^7$ M^{-1} s^{-1}) (Egmond et al., 1976) and the oxidation of E_{red} by LOOH ($k_{ox}/K_s \sim 2 \times 10^7$ M^{-1} s^{-1}) (Aoshima et al., 1977). This comparison shows that the reduction of E_{ox} by NDGA is fast enough for NDGA to reductively inhibit lipoxygenase-catalyzed oxygenation of LH. Thus, in the presence of NDGA, the steady-state concentration of the inactive E_{red} will increase at the expense of that of E_{ox}, resulting in a diminished turnover rate. Because the actions of NDGA and LOOH oppose each other in interconverting E_{ox} and E_{red} (Scheme I), the effectiveness of NDGA as an inhibitor of the conversion of LH to LOOH *must* depend on the concentration of the product hydroperoxide. Our results clearly show this; NDGA is most effective as an inhibitor when LOOH levels are low, as is the case when NDGA is present in solution before the enzymic conversion of LH to LOOH is initiated (Figure 7). When NDGA is added to a reaction in progress, it is much less effective as an inhibitor, because the product LOOH, now present at high levels, can more readily reactivate E_{red} generated by NDGA.

It has been a common practice to define the inhibitory efficiency of NDGA toward lipoxygenases in terms of an IC_{50} value. An inherent assumption in this approach is that inhibition is of the competitive type. Inhibition of lipoxygenase by NDGA cannot, however, be described as competitive. This is most readily apparent from a comparison of the results of Figure 3A, which show that the dissociation constant of NDGA from E_{ox} is higher than 20 μM, to the results of Figure 7, which show that NDGA inhibits turnover substantially at a concentration of only 2 μM, despite the presence of saturating levels of the substrate.

It should be noted that there are practical difficulties in obtaining accurate IC_{50} values because the inhibitory action of NDGA results in the consumption of both hydroperoxide and oxygen. The latter is consumed during the conversion of

Scheme II



an alkoxyl radical, produced from one-electron reduction of the hydroperoxide, to stable products (Dix & Marnett, 1983). Thus, regardless of the assay procedure used (e.g., spectrophotometry, O_2 consumption, or analysis of radiolabeled products), it is necessary to account for changes due to the cycle shown in Scheme I. Also, because lipoxygenase reductively inactivated by NDGA is reactivated by the product hydroperoxide, an IC_{50} value is not meaningful unless it is expressed as a function of the concentration of the latter. In the assays carried out in vitro to determine IC_{50} values of NDGA with different lipoxygenases, the amount of hydroperoxide has been variable and not well-defined. In many cases, the influence of NDGA on the rate of lipoxygenase-catalyzed oxygenation of a fatty acid substrate is assessed after the completion of a lag phase, at which time there is a substantial amount of product hydroperoxide (10 μM or more) present (see, for example, Figure 7, traces A and B). This situation does not correspond to that in vivo, where lipid hydroperoxides are maintained at low steady-state levels. Because the in vivo concentration of hydroperoxides is low (e.g., $\sim$0.4 μM in rabbit plasma; Miller et al., 1986), the amount of NDGA needed for 50% inhibition of lipoxygenase should be significantly (10–100-fold) lower than that determined in in vitro assays; i.e., NDGA should be more effective as an inhibitor in vivo than suggested by the IC_{50} values determined in vitro.

NDGA Does Not Inhibit Lipoxygenase by Trapping Enzyme-Bound Radical Intermediates. Inhibition of lipoxygenase by antioxidants has often been attributed to the ability of these compounds to reduce the *enzyme-bound* fatty acid radical intermediates, L$^\bullet$ and/or LOO$^\bullet$ (Tappel et al., 1953; Papatheofanis & Lands, 1985; Takahama, 1985). According to the mechanism for soybean lipoxygenase 1 (Scheme II)[2] (Feiters et al., 1985), both radicals are bound to the ferrous enzyme; hence, the reduction of these intermediates is equivalent to the reduction of the ferric enzyme in that in all three cases the enzyme is converted to the catalytically inactive ferrous state. A consideration of the rates of formation and disappearance of $E_{red}L^\bullet$ and $E_{red}LOO^\bullet$ reveals, however, that it is highly unlikely that NDGA inhibits lipoxygenase by trapping these enzyme-bound radicals. Since a large primary deuterium kinetic isotope effect ($k_H/k_D \sim$ 9.0) (Egmond et al., 1973) is observed with LH labeled with deuterium at C-11, the predominant catalytic cycle intermediate during turnover is E_{ox}; i.e., the complexes $E_{red}L^\bullet$ and $E_{red}LOO^\bullet$ must exist at low steady-state concentrations. Therefore, the conversion of $E_{red}L^\bullet$ to $E_{red}LOO^\bullet$ and of the latter to E_{ox} and LOOH (via electron transfer from the ferrous cofactor to LOO$^\bullet$) must be significantly faster than the reaction of E_{ox} and LH to produce $E_{red}L^\bullet$ ($k_{cat} \sim 300$ s^{-1}; Egmond et al., 1976). A minimum rate constant for the conversions of both $E_{red}L^\bullet$ and $E_{red}LOO^\bullet$ may be estimated as 10 times the turnover number, or 3×10^3 s^{-1}. For NDGA (1 μM)

to effectively compete with these processes, it would have to react with $E_{red}L^{\bullet}$ or $E_{red}LOO^{\bullet}$ with a rate constant of $\sim 10^9$ M^{-1} s^{-1}, i.e., at a rate near or exceeding the diffusion-controlled limit.

In air-saturated solution ($[O_2]$ = 240 μM), the pseudo-first-order rate constant for the *nonenzymic* reaction of $L^{\bullet}$ with O_2 to give $LOO^{\bullet}$ is $\sim 7 \times 10^4$ s^{-1} ($k \sim 3 \times 10^8$ M^{-1} s^{-1}; Hasegawa & Patterson, 1978). Therefore, the above estimate of 3×10^3 s^{-1} as the rate constant for the conversion of $E_{red}L^{\bullet}$ to $E_{red}LOO^{\bullet}$ may be too low by more than an order of magnitude. If the rate of the reaction of $E_{red}L^{\bullet}$ with O_2 is comparable to that of the analogous *nonenzymic* reaction, NDGA (1 μM) would not be able to compete with O_2 in reacting with $E_{red}L^{\bullet}$ unless it reduced the latter with a second-order rate constant exceeding 10^{10} M^{-1} s^{-1}. Since this rate constant is certainly greater than the diffusion-controlled limit, inhibition of turnover cannot be attributed to the reduction of $E_{red}L^{\bullet}$ by NDGA. Consistent with this conclusion is the well-known fact that, in inhibiting autoxidation, phenolic antioxidants are able to trap peroxyl radicals, not alkyl radicals, because the latter react with O_2 at near-diffusion-controlled rates (Ingold, 1969). By analogy, it would seem more likely for NDGA to reduce $E_{red}LOO^{\bullet}$ rather than $E_{red}L^{\bullet}$. However, we have recently obtained evidence that the first-order rate constant for the conversion of $E_{red}LOO^{\bullet}$ to $E_{ox}LOOH$ is greater than the pseudo-first-order rate constant for O_2 addition to $E_{red}L^{\bullet}$ (i.e., $k[O_2]$, where k is a second-order rate constant) to give $E_{red}LOO^{\bullet}$; this is indicated by our finding that O_2 suppresses leakage of linoleate radicals (from $E_{red}L^{\bullet}$) during turnover.[5] On the basis of these considerations, it seems certain that inhibition of lipoxygenase 1 by NDGA is due to the reduction of E_{ox} and not of the intermediates $E_{red}L^{\bullet}$ or $E_{red}LOO^{\bullet}$.

Reductive Regulation of Lipoxygenase Activity. The requirement of fatty acid hydroperoxide for enzymic activity is a common feature of lipoxygenases derived from both plant and mammalian tissues (Vliegenthart & Veldink, 1982; Croset & Lagarde, 1985; Rouzer & Samuelsson, 1986; Yokoyama et al., 1986; Bryant et al., 1986). Given that fatty acid hydroperoxides *turn on* lipoxygenase by oxidizing ferrous enzyme to ferric, the hydroperoxide requirement means that the enzyme does not remain in this active state; i.e., *there must be pathways for the conversion of the ferric enzyme to the inactive ferrous form.* One such pathway exists in the normal enzymic reaction: in air-saturated solution, ferric soybean lipoxygenase 1 reverts to the inactive ferrous form at a rate that is ca. 30-fold slower than the turnover rate, as a result of the leakage of linoleate radical from the catalytic cycle intermediate $E_{red}L^{\bullet}$.[2] The results of this study show another pathway for *turning off* the enzyme, i.e., reduction of the ferric enzyme to the ferrous state utilizing a variety of biologically relevant reducing agents.

Assuming that endogenous compounds which can affect the oxidation state of lipoxygenases exist at subsaturation levels, it can be shown that at steady state the fraction of lipoxygenase in the catalytically active ferric form is

$$\frac{[E_{ox}]}{[E_{total}]} = \frac{\sum_i k_{ox(i)}[\text{oxidant}_i]}{\sum_i k_{ox(i)}[\text{oxidant}_i] + \sum_i k_{red(i)}[\text{reductant}_i]} \quad (1)$$

In this equation, $k_{red(i)}$ and $k_{ox(i)}$ are the second-order rate constants for the reduction of E_{ox} by $(\text{reductant})_i$ and oxidation of E_{red} by $(\text{oxidant})_i$, respectively. From this equation it may be seen that, at a constant oxidant level, increasing concentration of reductants will result in a decrease in the fraction of the enzyme in the ferric state. Because fatty acid hydroperoxides are produced by the catalytic action of E_{ox}, decreasing E_{ox} levels (via reduction to E_{red}) will also result in lower fatty acid hydroperoxide levels. If in vivo the only oxidants of E_{red} are hydroperoxides derived from fatty acids, then increasing the reductant level will also result in a decrease in the steady-state concentrations of the oxidants capable of converting E_{red} to E_{ox}. Therefore, reductants would be able to decrease the fraction of the catalytically active ferric form of the enzyme both by direct reduction of the latter and by simultaneous suppression of the levels of the only known oxidants of ferrous lipoxygenase, fatty acid hydroperoxides.

In conclusion, the results of this paper serve to emphasize that ferric lipoxygenase can be inactivated easily by reduction. As noted by Clapp et al. (1985), design of inhibitors that would inactivate lipoxygenase by reduction may be a worthwhile strategy. Our results also raise the interesting question of whether reduction of lipoxygenases from the ferric to ferrous state is important for in vivo regulation of lipoxygenase activity and hence leukotriene biosynthesis.

[5] In the range of 2–100% O_2, the rate of radical leakage is inversely related to the concentration of O_2 (R. Krupinski-Olsen, L.-C. Yuan, and C. Kemal, unpublished results).

ACKNOWLEDGMENTS

We are extremely grateful to Drs. George Markham and Thomas Leyh of The Fox Chase Institute for Cancer Research for their assistance with the EPR experiments and to Dr. C. Michael Dicken for helpful comments.

REFERENCES

Alcaraz, M. J., & Hoult, J. R. S. (1985) *Arch. Int. Pharmacodyn. Ther. 278*, 4–12.

Aoshima, H., Kajiwara, T., Hatanaka, A., Nakatani, H., & Hiromi, K. (1977) *Biochim. Biophys. Acta 486*, 121–126.

Bryant, R. W., She, H. S., Ng, K. J., & Siegel, M. I. (1986) *Prostaglandins 32*, 615–627.

Clapp, C. H., Banerjee, A., & Rotenberg, S. A. (1985) *Biochemistry 24*, 1826–1830.

Croset, M., & Lagarde, M. (1985) *Lipids 20*, 743–750.

De Groot, J. J. M. C., Veldink, G. A., Vliegenthart, J. F. G., Bolding, J., Wever, R., & van Gelder, B. F. (1975) *Biochim. Biophys. Acta 377*, 71–79.

Dix, T. A., & Marnett, L. J. (1983) *J. Am. Chem. Soc. 105*, 7001–7002.

Egmond, M. R., Veldink, G. A., Vliegenthart, J. F. G., & Bolding, J. (1973) *Biochem. Biophys. Res. Commun. 54*, 1178–1184.

Egmond, M. R., Finazzi-Agrô, A., Fasella, P. M., Veldink, G. A., & Vliegenthart, J. F. G. (1975) *Biochim. Biophys. Acta 397*, 43–49.

Egmond, M. R., Brunori, M., & Fasella, P. M. (1976) *Eur. J. Biochem. 61*, 93–100.

Falkenhein, S. F., MacDonald, H., Huber, M. M., Koch, D., & Parker, C. W. (1980) *J. Immunol. 125*, 163–168.

Feiters, M. C., Aasa, R., Malmström, B. G., Slappendel, S., Veldink, G. A., & Vliegenthart, J. F. G. (1985) *Biochim. Biophys. Acta 831*, 302–305.

Flynn, D. L., Rafferty, M. F., & Boctor, A. M. (1986) *Prostaglandins, Leukotrienes Med. 22*, 357–360.

Galpin, J. R., Tielens, L. G. M., Veldink, G. A., Vliegenthart, J. F. G., & Boldingh, J. (1976) *FEBS Lett. 69*, 179–182.

Gibian, M. J., & Galaway, R. A. (1976) *Biochemistry 15*, 4209–4214.

Gleason, J. G., Perchonock, C. D., & Torphy, T. J. (1986) *Annu. Rep. Med. Chem. 21*, 73–83.

Hamasaki, Y., & Tai, H. H. (1985) *Biochim. Biophys. Acta 834*, 37–41.
Hamberg, M. (1976) *Biochim. Biophys. Acta 431*, 651–654.
Hasegawa, K., & Patterson, L. K. (1978) *Photochem. Photobiol. 28*, 817–823.
Ho, P. P. K., Walters, C. P., & Sullivan, H. R. (1977) *Biochem. Biophys. Res. Commun. 76*, 398–405.
Ingold, K. U. (1969) *Acc. Chem. Res. 2*, 1–9.
Ishiura, S., Tanihiro, Y., & Villee, C. A. (1986) *FEBS Lett. 201*, 87–93.
Koshihara, Y., Neichi, T., Murota, S., Lao, P., & Tatsuno, T. (1984) *Biochim. Biophys. Acta 792*, 92–97.
McMillan, R. M., Masters, D. J., Sterling, W. W., & Bernstein, P. R. (1985) in *Prostaglandins, Leukotrienes, and Lipoxins* (Bailey, J. M., Ed.) pp 655–668, Plenum, New York.
Mentasti, E., Pelizzetti, E., & Saini, G. (1976a) *J. Inorg. Nucl. Chem. 38*, 785–788.
Mentasti, E., Pelizzetti, E., & Saini, G. (1976b) *J. Inorg. Nucl. Chem. 38*, 2017–2021.
Miller, J. F., Jr., Rich, S., & Lands, W. E. M. (1986) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 45*, 1936.
Mitsuda, H., Yasumoto, K., & Yamamoto, A. (1967) *Agric. Biol. Chem. 31*, 853–860.
Murota, S., & Koshihara, Y. (1985) *Drugs Exp. Clin. Res. 11*, 641–644.
Papatheofanis, F. J., & Lands, W. E. M. (1985) in *Biochemistry of Arachidonic Acid Metabolism* (Lands, W. E. M., Ed.) pp 9–39, Martinus Nijhoff, Boston.
Pelizzetti, E., & Mentasti, E. (1977) *Z. Phys. Chem. (Munich) 105*, 21–34.
Pistorius, E. K., Axelrod, B., & Palmer, G. (1976) *J. Biol. Chem. 251*, 7144–7148.
Reddanna, P., Rao, M. K., & Reddy, C. C. (1985) *FEBS Lett. 193*, 39–43.
Rouzer, C. A., & Samuelsson, B. (1986) *FEBS Lett. 204*, 293–296.
Rouzer, C. A., Matsumoto, T., & Samuelsson, B. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 857–861.
Roy, G. L., Laferriere, A. L., & Edwards, J. O. (1957) *J. Inorg. Nucl. Chem. 4*, 106–114.
Samuelsson, B. (1983) *Science (Washington, D.C.) 220*, 568–575.
Slappendel, S., Aasa, R., Malmström, B. G., Verhagen, J., Veldink, G. A., & Vliegenthart, J. F. G. (1982) *Biochim. Biophys. Acta 708*, 259–265.
Spaapen, L. J. M., Veldink, G. A., Liefkens, T. J., Vliegenthart, J. F. G., & Kay, C. M. (1979) *Biochim. Biophys. Acta 574*, 301–311.
Spaapen, L. J. M., Verhagen, J., Veldink, G. A., & Vliegenthart, J. F. G. (1980) *Biochim. Biophys. Acta 617*, 132–140.
Takahama, U. (1985) *Phytochemistry 24*, 1443–1446.
Tappel, A. L., Lundberg, W. O., & Boyer, P. D. (1953) *Arch. Biochem. Biophys. 42*, 293–304.
Vliegenthart, J. F. G., & Veldink, G. A. (1982) in *Free Radicals in Biology* (Pryor, W. A., Ed.) Vol. V, pp 29–64, Academic, New York.
Walker, J. R., Boot, J. R., Beverley, C., & Dawson, W. (1980) *J. Pharm. Pharmacol. 32*, 866–867.
Weidman, S. W., & Kaiser, E. T. (1966) *J. Am. Chem. Soc. 88*, 5820–5827.
Wheeler, E. L., & Berry, D. L. (1986) *Carcinogenesis 7*, 33–36.
Yasumoto, K., Aijiro, Y., & Mitsuda, H. (1970) *Agric. Biol. Chem. 34*, 1162–1168.
Yokoyama, C., Shinjo, F., Yoshimoto, T., Yamamoto, S., Oates, J. A., & Brash, A. R. (1986) *J. Biol. Chem. 261*, 16714–16721.