Hexanal Phenylhydrazone Is a Mechanism-Based Inactivator of Soybean Lipoxygenase 1

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ABSTRACT: Hexanal phenylhydrazone (**1**; 70:30 *E*:*Z* mixture) at micromolar concentration irreversibly inactivates soybean lipoxygenase 1 (L-1) in the presence of dioxygen. L-1 catalyzes the oxidation of **1** into its α-azo hydroperoxide **2** [$C_5H_{11}CH(OOH)N{=}NC_6H_5$]. **2** is an efficient inactivator of L-1. The aerobic reaction between **1** and L-1 follows a branched pathway leading to the release of **2** into the medium or to L-1 inactivation. The respective parameters corresponding to this inactivation by the (*E*)-**1** and (*Z*)-**1** isomers are K_i = 0.25 and 0.40 μM and k_{inact} = 0.8 and 2.1 min^{-1}. Linoleic acid protection agrees with a mechanism-based inactivation process. The oxidation of a minimum of 13 ± 3 molar equiv of **1** is required for complete L-1 inactivation, but up to 70 equiv is necessary in the presence of a very large excess of **1**. The inactivation is actually the result of two pathways: one is due to a reaction of **2** as soon as it is formed at the active site (20%); the other is due to **2** released into the medium and coming back to the active site (80%). The inactivation is accompanied by the oxidation of 1.8 ± 0.8 methionine residues of the protein into the corresponding sulfoxide. The inactivated L-1 is electron paramagnetic resonance (EPR) silent with an effective magnetic moment of μ = 5.0 ± 0.1 Bohr magnetons corresponding to an S = 2 spin state. An inactivation mechanism is proposed on the basis of EPR and magnetic susceptibility data obtained from the anaerobic and aerobic reactions of L-1 with **1** and **2**.

Lipoxygenases (EC 1.13.11.12) are non heme iron dioxygenases that catalyze the oxygenation of polyunsaturated fatty acids containing one or several 1(*Z*),4(*Z*)-pentadiene systems. Several phenylhydrazones are potent inhibitors of plant and mammalian lipoxygenases and of cyclooxygenases from different origins (Sun et al., 1980; Wallach & Brown, 1981; Baumann & Wurm, 1982; Le Breton et al., 1984; Bertez et al., 1984). In our laboratory we have investigated the "suicidal inactivation" of microsomal cytochrome P-450 by phenylhydrazones and shown that they lead to a destruction of the cytochrome and to the formation of a stable Fe(III)–σ-phenyl complex (Mansuy et al., 1985). Phenylhydrazones present an oxidizable NH in a pseudo-bis-allylic position and appeared to us as possible substrate analogues and candidates for a suicidal inactivation of lipoxygenases. As a part of a study of several hydrazones as inhibitors of plant and animal lipoxygenases, we have selected hexanal phenylhydrazone (**1**) to analyze the inhibition reaction of soybean lipoxygenase 1 (L-1).[1] L-1, which catalyzes the almost exclusive formation of 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid [13-(*S*)-HPOD] from linoleic acid [9(*Z*),12(*Z*)-octadecadienoic acid], is convenient for such as study because it is well-known [for reviews, see Axelrod (1974), Veldink et al. (1977), Galliard and Chan (1980), and Vliegenthart and Veldink (1982)] and several detailed proposals are available concerning its catalytic cycle (De Groot et al., 1975; Smith & Lands, 1972; Egmond et al., 1976; Lands, 1984; Petersson et al., 1985). Hexanal phenylhydrazone (**1**) has its NH group in a position comparable to that of the bis-allylic CH_2 of linoleic acid, relatively to the terminal CH_3 (Holman et al., 1969). Recently, Gibian and Singh (1986) reported that they dropped the study of the inhibition of L-1 by acetophenone phenylhydrazone because of nonreproducible results and concentrated on the case of phenylhydrazine, showing that its autoxidation, forming phenyldiazene, is responsible for the irreversible inhibition observed. In this paper we present the results of our study of the inactivation of L-1 by **1** [both (*E*)-**1** and (*Z*)-**1** isomers) and by its α-azo hydroperoxide **2** [$C_5H_{11}CH(OOH)N{=}NC_6H_5$].

Materials and Methods

Reagents used were of analytical grade. Ultrapure water was obtained through a reverse osmosis system (Elga, Millipore). The buffer used for all the experiments was 0.05 M Tris–acetate at pH 9 containing 0.1 mM DETAPAC.

Lipoxygenase activity was determined spectrophotometrically by monitoring the 234-nm absorbance of 13(*S*)-HPOD (ϵ_{max} = 25 000 $mol \cdot L^{-1} \cdot cm^{-1}$) formed from linoleic acid (Sigma, ~99% free acid) at pH 9 and 20 °C in 50 mM Tris–acetate buffer. One unit of activity is defined as the production of 1 μmol of 13(*S*)-HPOD/min at 20 °C.

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[1] Abbreviations: L-1, soybean lipoxygenase 1; 13(*S*)-HPOD, 13-(*S*)-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid; EPR, electron paramagnetic resonance; DETAPAC, diethylenetriaminepentaacetic acid; [$^2H_4$]TSP, sodium [2,2,3,3-$^2H_4$]-3-(trimethylsilyl)-1-propionate; NDGA, nordihydroguaiaretic acid; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate.

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Soybean lipoxygenase 1 was extracted from seeds (variety King Soy or Weber, grown by INRA, Castanet-Tolosan, France) and purified according to a method derived from that of Axelrod (Axelrod et al., 1981) with the following modifications: 100 g of seeds was swollen in pure water overnight before homogenization; after precipitations and dialysis, the enzyme was loaded on a DEAE-Trisacryl (IBF) column (2.7 cm × 30 cm) and eluted with 600 mL of a linear gradient formed from 300 mL of 20 mM Tris–acetate, pH 6.8, and 300 mL of 20 mM Tris in 0.15 M sodium acetate, pH 6.8, at a flow rate of 200 mL/h. Remaining contaminants were removed by a second fractionation on DEAE-Trisacryl with elution by 1.2 L of the same linear gradient at a flow rate of 60 mL/h. For all purification steps 0.1 mM DETAPAC was added to water and buffers. For the methionine sulfoxide quantification, the enzyme was purified under a nitrogen atmosphere. The enzyme contained about 0.95 Fe/mol (M_r = 98 000) as determined by atomic absorption spectrometry. The homogeneity of the preparation was better than 90% from SDS–polyacrylamide gel electrophoresis. The enzyme concentration was estimated spectrophotometrically with a coefficient $A^{1\%}_{280nm}$ = 14 obtained from protein determination by the Lowry method and identical with that found by Axelrod (Axelrod et al., 1981). The maximum specific activity obtained was 200 μmol·min^{-1}·mg^{-1} (corresponding to 3.33 μkat/mg or 333 turnovers/s), and the K_m for linoleic acid was 24 μM. All the experiments described in this paper have been run with a purified enzyme having a specific activity in the 100–200 μmol·min^{-1}·mg^{-1} range, depending on the various preparations. The ferric "yellow enzyme" was obtained by addition to native enzyme of 1 molar equiv of 13(*S*)-HPOD prepared enzymatically as described by Verhagen (Verhagen et al., 1977). The purity of the latter was checked by HPLC. When necessary, the Fe(III) enzyme was freed from excess 13(*S*)-HPOD by Sephadex G-25 filtration.

Hexanal phenylhydrazone [$nC_5H_{11}CH{=}NNHC_6H_5$ (**1**)] was prepared by reacting 10 mmol of phenylhydrazine with 10 mmol of hexanal (both freshly distilled) in 50 mL of methanol under argon atmosphere. After methanol evaporation, **1** was distilled under a reduced pressure of argon ($bp_{0.5}$ 160–161 °C) and stored under argon. **1** was characterized by UV (UV_{EtOH}:λ_{max} 275 nm, ϵ 17 000) and MS [*m/e* 190 (M), 161, 133, 93, 77]. ^{1}H NMR shows that one obtains a mixture of the *Z* (30%) and *E* (70%) isomers [–NH–N=C*H*– in $CDCl_3$ (relative to TMS)]: δ_Z 6.5 (0.3 H, t, J = 5 Hz); δ_E 6.8 (0.7 H, t, J = 8 Hz) (Karabatsos & Taller, 1963). These two isomers have been separated by semipreparative C_{18} reverse-phase HPLC [water (70%)–methanol (30%)–triethylamine (0.05%)]. They do not equilibrate either stored in degassed methanol at –80 °C or in the enzymatic reaction conditions at pH 9 during the time of an experiment. The (*Z*)-**1*** and (*E*)-**1*** isomers of hexanal [ring-U-^{14}C]phenylhydrazone having a specific activity of 28 mCi/mmol were synthesized from [ring-U-^{14}C]phenylhydrazine, prepared by SO_2 reduction of the diazonium salt derived from labeled aniline, and then condensed with hexanal to give a mixture of the *Z* (30%) and *E* (70%) isomers of the labeled hydrazone. These were purified and separated by preparative reverse-phase HPLC. Details of this synthesis are published elsewhere (Thang et al., 1987). The α-azo hydroperoxide $C_5H_{11}CH$(OOH)N=NC_6H_5 (**2**) was prepared by dioxygen oxidation of **1** in benzene (Bellamy & Guthrie, 1965) and characterized by UV (UV_{EtOH}: λ_{max} 268 nm, ϵ 10 500; λ_{max} 385 nm, ϵ 500) and ^{1}H NMR (in C_6D_6, relative to TMS): 0.8–1.0 (m, 3 H), 1.0–1.6 (m, 6 H), 1.7–1.9 (m, 2 H), 5.3 (t, J = 5 Hz, 1 H), 7.0–7.7 (m, 5 H), 9.1 (s, 1 H, exch D_2O). This α-azo hydroperoxide was synthesized just before use.

The inhibition experiments were run by measuring the loss of activity of 0.1 activity unit of L-1 (i.e., 1.6–3.2 nM lipoxygenase according to its specific activity) in 3 mL of 0.05 M Tris–acetate buffer at pH 9 and 20 °C, in the presence of various concentrations of **1** and **2**, added from a 1 mM degassed methanol solution [**1**: 0.035–0.230 μM and 0.002–14 μM for the determination of the partition ratio *r* (see text). **2**: 0.073–2.25 μM and 0.25–2.5 μM for the plot of (fractional activity remaining) vs $[\mathbf{2}]_0/[\text{L-1}]_0$]. Lipoxygenase activity was measured after addition of linoleic acid at 100 μM final concentration; 100 μM linoleic acid was checked to correspond to saturating conditions without perturbation from substrate inhibition (Egmond et al., 1976). The maximum amount of CH_3OH used (<0.1% and <5% for the millimolar experiments) was checked to have no inhibitory effect. The inhibition measurements were made for various preincubation times of the enzyme with **1** or **2** at room temperature. For comparative purpose, inhibitions by nordihydroguaiaretic acid (NDGA, Sigma) and acetone phenylhydrazone (prepared as **1**) were also evaluated.

Ultrafiltrations were performed with membrane cones (CF 25 Amicon System). After the first centrifugation of a 1-mL sample of 2 μM inactivated L-1, the residue was rinsed 5 times by addition of 6 mL of buffer and centrifugation for 10 min. The overall treatment lasted for 2 h. (The same treatment applied to L-1 inhibited by NDGA, K_i = 2.7 μM, led to the recovery of more than 95% of L-1 activity.)

Oxygen consumptions were measured on a Gilson Oxygraph equipped with a Clark electrode. The experiments were run at 1–100 μM enzyme in 0.05 M Tris–acetate buffer at pH 9 and 20 °C; autoxidation of the hydrazone was taken into account.

Trapping of **2** *in Solution by Glutathione (GSH) Peroxidase.* The activity of the GSH peroxidase for the conversion of the α-azohydroperoxide **2** was determined by the GSH reductase–NADPH coupled assay (Paglia & Valentine, 1967). We found a K_m value of 60 μM and a specific activity of 33.75 μmol of **2** transformed min^{-1} (mg of GSH peroxidase)$^{-1}$, in Tris–acetate buffer at pH 9 and 0 °C. The activity unit of GSH peroxidase is then defined as the amount of enzyme necessary to reduce 1 μmol of **2** per minute at saturating concentration of **2**. L-1 (0.47 μM) is completely inactivated by 16 equiv of **1** in 1-mL samples at 0 °C after 30 min. L-1 in the same conditions without **1** but with 475 μM GSH and 0.12–2.56 activity units of GSH peroxidase remains completely active (activity was measured after L-1 dilution to 4.5 nM, in the presence of 100 μM linoleic acid). In the conditions of the trapping experiment, 7.52 × 10^{-3} μmol of **2** was calculated to be released into the solution. In the presence of up to 2.56 activity units of GSH peroxidase (see text), no more than 78% of remaining activity could be obtained.

Labeling Experiments. Various amounts of ^{14}C-labeled **1*** or **2*** were respectively incubated at pH 9 with 0.2 mL of 10 μM enzyme during 15 min and 1 min at 0 °C. Aliquots were taken for remaining activity measurements. The enzyme solution was precipitated with an equal volume of 20% trichloroacetic acid, centrifuged, and washed twice with methanol. The protein precipitate was solubilized in Solulyte (Baker) before addition of scintillation liquid (Picofluor, Baker). Countings were performed on a Packard Tricarb 4530. Blanks were run with thermally inactivated enzyme or enzyme inactivated by unlabeled **1** or **2**. For a typical ex-

periment, protein determination by the Lowry method showed that the counting was performed on 94% of the initial protein. The total radioactivity recovered from the protein sample and washing solutions accounted for 90% of the initial radioactivity.

Quantification of Methionine Sulfoxide. A total of 5×10^{-8} mol of L-1 in 5 mL of Tris–acetate buffer was incubated during 15 min at 0 °C with 0 or 20 equiv of hexanal phenylhydrazone. Each solution was dialyzed against 10 mM ammonium bicarbonate during 24 h under N_2 and then against pure water during 24 h under N_2. Protein hydrolyses were carried out in 100 μL of twice-distilled 5.7 N HCl in evacuated sealed tubes at 110 °C for 24 h. For methionine sulfoxide determination, hydrolyses were carried out in 50 μL of 4 N NaOH in Eppendorf tubes, which were placed in Pyrex tubes for evacuation and sealing. After 24 h at 110 °C, 400 μL of 0.2 M citrate buffer, pH 2.2, was added and the pH adjusted to 1.8–2.2 with 5.6 N HCl (10–40 μL according to the case). The sample was then ready for injection.

Amino acid analyses were carried out with an LKB 4400 amino acid analyzer with ninhydrin detection. For normal analyses, the column was eluted with the pH 3.20 and 4.25 citrate buffers sold by the manufacturer, followed by a homemade 0.067 M citrate buffer, pH 6.35, containing 0.30 M NaCl. For methionine sulfoxide analyses, the pH 3.20 buffer was acidified to pH 3.00 with 5.7 N HCl. This resulted in increased resolution between the small methionine sulfoxide and the very large aspartic acid peaks. The resolution of other amino acids from the position of threonine to that of methionine was impaired, but in those analyses only the quantitation for methionine sulfoxide, aspartic acid, and leucine was made use of.

EPR spectra were recorded with a Bruker ER 220D at 9.4 GHz and 15 K, using an Oxford Instruments continuous-flow cryostat, a Hall probe, and a Hewlett-Packard frequency meter. The concentration of enzyme used was around 0.5 mM in Tris–acetate buffer, pH 9. This was obtained either by weighing 10 mg of lyophilized enzyme and addition of 200 μL of Tris–acetate buffer (enzymatic activity checked for each EPR sample) or by use of the following procedure: 10 μM enzyme solution in 10 mL of 50 mM ammonium bicarbonate–carbonate, pH 9, was incubated at 0 °C with inhibitor during 15 min. The enzyme activity was checked with an aliquot of the solution, and the latter was lyophilized. The enzyme was then solubilized in 150 μL of Tris–acetate buffer, pH 9. For every addition of reactant to an EPR sample, a 10-min incubation at room temperature preceded the new freezing. For quantification of iron, solutions contained also 0.2 mM cytochrome *c* (from Bovine Heart, Sigma). The zero field splittings of the $g = 6.2$ and $g = 4.3$ signals were obtained as described by Slappendel (Slappendel et al., 1980) from intensity versus temperature measurements between 25 K and 4 K with 2.14 mW as the power of radiation. Temperature was corrected by reference to the Curie law behavior of cytochrome *c*. Intensity was measured as the amplitude of the absorption-like signals at $g = 6.2$ for inhibited lipoxygenase and at $g = 3$ for cytochrome *c*; for the $g = 4.3$ signal, intensity was measured by double integration. The quantification of spin was done following Slappendel (Slappendel et al., 1981) but with the $g = 3$ signal of cytochrome *c* as a standard. For cytochrome *c*, we used the simple integral $T_{g=3}$ against field of the EPR derivative signal of the resonance at $g = 3$ because it is absorption-like (cytochrome *c* contains low-spin Fe(III) with $g_z = 3.0$, $g_x = 2.2$, and $g_y = 1.2$). For the $g = 6.2$ signal, the matter was more difficult; we used an approximate simple integral $T_{g=6.2}$ against field. Following Slappendel (Slappendel et al., 1981), we took $g_z = 6.20$, $g_x = 5.60$, and $g_y = 2.00$. This resonance occurs in the lowest Kramers' doublet, and at 11.4 K the occupancy of this doublet is 0.5. For the $g = 4.3$ signal, we used a double integral against field $I_{g=4.3}$. We considered this signal as isotropic. This resonance comes from the middle Kramers' doublet for which at 11.4 K the occupancy is 0.33. We used the theory of Aasa (Aasa et al., 1975): $T_{g=6.2} = KTC_{6.2}$; $T_{g=3} = KT'C_c$; $I_{g=4.3} = K(4.3)C_{4.3}$. $T_{g=6.2}$, $T_{g=3}$, and $I_{g=4.3}$ are defined above, K is a calibration constant, $C_{6.2}$ and $C_{4.3}$ are the concentrations of the species showing a signal at $g = 6.2$ and $g = 4.3$, C_c is the cytochrome *c* concentration, and

$$T = \frac{g_x^2 + g_y^2}{[(1 - \rho_x)(1 - \rho_y)]^{1/2}}$$

with $\rho_x = g_x^2/g_z^2$ and $\rho_y = g_y^2/g_z^2$. The results are then Fe(III) axial = 8% and Fe(III) rhombic = 2%. The figure for the axial species is not very accurate due to approximation of the integral $T_{g=6.2}$, approximation in the *g* factors, and neglect of the component at lower field. Nevertheless, the amount of axial Fe(III) species must not be higher than 10%.

Paramagnetic susceptibility measurements have been made by NMR (Bruker WM 250, 250 MHz) according to a modification of Evans' method (Evans, 1959). The [$^2H_4$]TSP reference in D_2O was in the inner capillary tube, and the paramagnetic compound ($NiCl_2$ or protein) in regular buffer containing reference ([$^2H_4$]TSP) was in the outer tube. By use of solutions of $NiCl_2$ in the buffer (1–10 mM), it was verified that the splitting of the [$^2H_4$]TSP peak was proportional to the concentration of paramagnetic species. The molar susceptibility of $NiCl_2$ was determined from

$$\Delta\delta = 4\pi/(3 \times 10^3)[\mathrm{P}]\left[\chi_P + \frac{\alpha - M_P}{M_R}\chi_R\right]$$

(Chopard, 1979).[2] It gave 4239×10^{-6} electromagnetic units (cgs) at 293 K, which is in very good agreement with the value published for this compound of 4240×10^{-6} (Foex et al., 1957). The difference between the splitting of the [$^2H_4$]TSP peak for solutions of the same concentration of native and yellow L-1 (2.51 Hz for a 1 mM solution) depends only on the magnetic susceptibility of iron in the enzyme. The effective moment of iron in the native Fe(II) and yellow Fe(III) forms of the enzyme can be calculated respectively by[3]

$$\mu_N^2 = (\chi_{Fe}^N + 3kT)/N \text{ and } \mu_Y^2 = (\chi_{Fe}^Y + 3kT)/N$$

The difference between atomic magnetic susceptibilities of iron in the native and yellow enzyme is equal to the difference of molar magnetic susceptibility between the two forms. Assuming that the effective moment of iron is 5.1 Bohr magnetons for native enzyme (Cheesbrough et al., 1983; Petersson et al., 1985), we can calculate for iron in the yellow enzyme an effective magnetic moment of 5.7 ± 0.1 Bohr magnetons. This value is in good agreement with the value of Cheesbrough (Cheesbrough et al., 1983). With this mod-

[2] In this equation $\Delta\delta$ is the splitting of the [$^2H_4$]TSP peak in ppm, [P] the concentration of the paramagnetic species in mol·L^{-1}, χ_P its molar susceptibility in electromagnetic units (cgs), M_P its molar weight, M_R the molar weight of the solvent, and χ_R its magnetic susceptibility in electromagnetic units (cgs). α correlates the variation of the specific mass of the paramagnetic solution with its concentration; $\alpha = 0.1158$ g·mol^{-1} for $NiCl_2$.

[3] μ, in Bohr magnetons; χ_{Fe}^N, molar susceptibility of iron in the native Fe(II) enzyme; χ_{Fe}^Y, molar susceptibility of iron in the yellow Fe(III) enzyme; k, Boltzmann's constant; T, temperature; N, Avogadro's number; β, Bohr magneton.

Table I: Concentrations (μM) for Half-Inhibition (IC_{50}) of Soybean Lipoxygenase 1 Catalyzed Hydroperoxidation of Linoleic Acid by Hexanal Phenylhydrazone (**1**), Its *E* and *Z* Isomers, Nordihydroguaiaretic Acid, and Acetone Phenylhydrazone

	inhibitor				
	1[a]	(*E*)-**1**	(*Z*)-**1**	NDGA[b]	ACPH[c]
without enzyme–inhibitor preincubation[d,e]	1.3 ± 0.5	1.8 ± 0.6	1.2 ± 0.5	2 ± 1	11 ± 5
after 1-min enzyme–inhibitor preincubation[d,e]	0.2 ± 0.1	0.8 ± 0.4	0.08 ± 0.04	2 ± 1	5 ± 2

[a] A 70:30 mixture of (*E*)-**1** and (*Z*)-**1**. [b] Nordihydroguaiaretic acid. [c] Acetone phenylhydrazone. [d] Some inhibition is observed without former enzyme–inhibitor preincubation. The IC_{50} values were obtained from a plot of the ln (L-1 residual activity) vs at least five concentrations of inhibitor, giving 10–90% inhibition of the initially 0.1 activity unit lipoxygenase sample. [e] The same IC_{50} values were obtained, within experimental error for both the native Fe(II) and yellow Fe(III) forms of the enzyme [3 nM in the presence of 0.04–2.7 μM **1**, (*E*)-**1** or (*Z*)-**1**].

ified Evans' method we can evaluate the magnetic moment of the iron in the inactivated enzyme. We have found that the splitting of the [$^3H_4$]TSP peak for a 1 mM solution of inactivated yellow enzyme is 2.3 Hz. The same value is obtained for a 1 mM anaerobic solution of yellow enzyme treated by 1 equiv of hexanal phenylhydrazone. The magnetic moment of iron is then 5.0 ± 0.1 Bohr magnetons, which is about the same value as that obtained for the native enzyme. Therefore, in those cases also, iron is in an $S = 2$ spin state.

Results

The results have been obtained with a purified enzyme used at different concentrations depending on the type of experiment: nanomolar range for the inhibition studies; micromolar for the oxygen consumption, product analysis, ^{14}C-labeling studies, and methionine sulfoxide quantification; millimolar for the EPR and magnetic susceptibility measurements. In the following, **1** stands for the 70:30 mixture of the *E* and *Z* isomers of hexanal phenylhydrazone, (*E*)-**1** and (*Z*)-**1**.

Type of Inhibition. Hexanal phenylhydrazone (**1**) gives a time-dependent inhibition of the hydroperoxidation of linoleic acid catalyzed by soybean lipoxygenase 1. The half-inhibitory concentrations (IC_{50} for 0.1 activity unit of native L-1 and 100 μM linoleic acid) after 0- and 1-min aerobic preincubation of the enzyme with the hydrazone are respectively 1.3 and 0.2 μM. Table I shows that **1** is a better inhibitor than NDGA and acetone phenylhydrazone (Baumann & Wurm, 1982) and that the *Z* isomer is more efficient than the *E* one, by a factor of 10 after 1-min preincubation. A 1:25 dilution or ultrafiltration (see Materials and Methods) of the preincubated samples does not overcome the inhibition of the enzyme (not shown), indicating an irreversible inactivation by the hydrazone.

No inhibition results from *anaerobic* preincubation of the enzyme with **1**,[4] showing that the inactivation reaction is oxygen dependent. Oxygen consumption studies show that before its complete inactivation L-1 catalyzes the oxidation of hexanal phenylhydrazone into its α-azo hydroperoxide **2** [$C_5H_{11}CH(OOH)N{=}NC_6H_5$] released into the medium. **2** was identified by its HPLC retention time and its UV spectrum, which were compared to those of an authentic sample (Bellamy & Guthrie, 1965). Addition of **1**, at final concentrations of 20–100 μM, to 100 μM native enzyme shows that only 1 mol of dioxygen is consumed per mole of **1**, to give **2**. When native L-1 is added, at a final concentration of 5 μM, to 185 μM hydrazone, the oxygen consumption, which lasts for about 10 min, shows that complete L-1 inactivation occurs after the conversion of 17 molar equiv of **1**.

When linoleic acid is added to the preincubation medium, it protects the lipoxygenase against aerobic inactivation by **1**. A 92% remaining activity was obtained after 1-min preincubation at 22 °C of 3.2 nM native L-1 with 0.43 μM **1** in the presence of 42 μM linoleic acid (the activity was measured after increasing the linoleic acid concentration to 166 μM). Higher concentrations of linoleic acid gave less protection because of the larger amounts of 13(*S*)-HPOD liberated in the medium (vide infra).

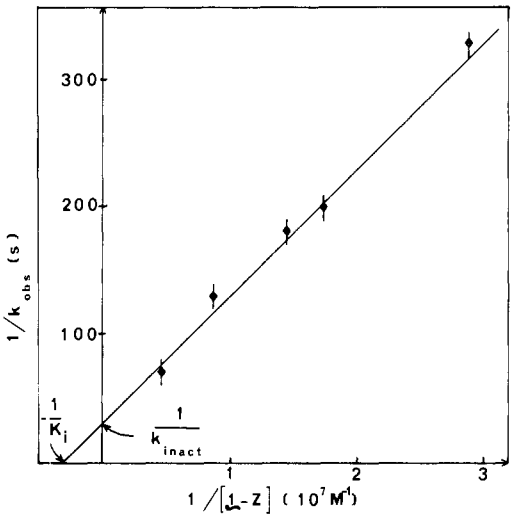
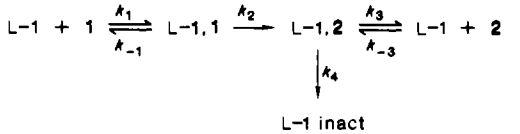


FIGURE 1: Kinetic analysis of the inactivation of soybean lipoxygenase 1 by hexanal phenylhydrazone (**1**). Double-reciprocal plot of k_{obsd} vs concentration of (*Z*)-**1**, giving the k_{inact} and K_i parameters. The k_{obsd} values were determined from the plots of ln (fractional activity remaining) vs preincubation time for various concentrations of (*Z*)-**1**. Because of the inhibition observed without L-1 preincubation with **1**, the L-1 fractional activity remaining was taken as the ratio of the measured activity to that of a sample to which the same concentration of **1** was added simultaneously with linoleic acid, i.e., without preincubation with **1**.

Scheme I: Inactivation Pathway of Soybean Lipoxygenase 1 (L-1) by Hexanal Phenylhydrazone (**1**)[a]



[a] **2** is the α-azo hydroperoxide $C_5H_{11}CH(OOH)N{=}NC_6H_5$.

All these results suggested a lipoxygenase-induced self-inactivation by **1**, according to the branched pathway shown in Scheme I (for the k_{-3} step vide infra). Plots of ln (fractional activity remaining) vs preincubation time gave pseudo-first-order rate constants of inactivation (k_{obsd}), for both the (*E*)-**1** and (*Z*)-**1** isomers in the concentration range 0.035–0.23 μM (not shown). The double-reciprocal plot of k_{obsd} vs inhibitor concentration was linear, with a positive ordinate intercept indicating that a reversible enzyme–inhibitor complex is formed prior to inactivation [shown for (*Z*)-**1** in Figure 1]. This allowed the determination of the dissociation constants K_i and the first-order inactivation rate constants k_{inact} (Walsh et al., 1978; Walsh, 1982), respectively, for (*E*)-**1**, 0.25 ± 0.15

[4] The sample of anaerobically preincubated enzyme has the same activity as that of a reference sample to which the same amount of **1** is added without preincubation.

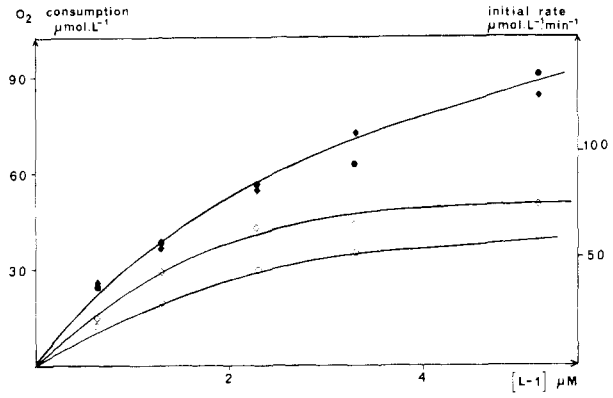


FIGURE 2: Oxygen consumption for L-1 inactivation by **1**. Plots of dioxygen consumption vs L-1 concentration (0.65–5.2 μM) for complete L-1 inactivation by **1** (185 μM) in 0.05 M Tris–acetate buffer at pH 9 and 25 °C: (●) native Fe(II) L-1 and (♦) Fe(III) L-1 (left ordinates). Initial rates of dioxygen consumption for native Fe(II) L-1 (○) and for Fe(III) L-1 (◇) (right ordinates).

μM and 0.8 ± 0.3 min^{-1}, and for (*Z*)-**1**, 0.40 ± 0.15 μM and 2.1 ± 0.7 min^{-1}.

More detailed oxygen consumption studies revealed that complete inactivation of 1 and 5 μM L-1, by 185 μM **1**, required respectively the conversion of 30 and 17 molar equiv of hydrazone, independently of the initial native Fe(II) or Fe(III) form of L-1 (Figure 2). The initial oxidation of **1** is faster with the Fe(III) than with the Fe(II) form of L-1 (Figure 2). Addition of 13(*S*)-HPOD (100–200 μM) to the reaction medium (1.3 μM L-1; 185 μM **1**) increases the initial oxidation rate of **1** by a factor of 10 and enhances the oxygen consumption by a factor of 2.5 before L-1 inactivation.

In order to determine the partition ratio r ($r = k_3/k_4$) for the branched inactivation pathway proposed in Scheme I (without the k_{-3} step), we plotted the fractional activity remaining vs the ratio of the initial concentration of **1** ($[\mathbf{1}]_0$) [as well as of pure (*E*)-**1** and (*Z*)-**1**] to that of L-1 ($[\text{L-1}]_0$) (Waley, 1985). None of the plots were linear. In these conditions, the intercepts of the curves on the abscissa could not be taken as (1 + r) (Knight & Waley, 1985). However, they indicated that for 2 nM to 1 μM concentrations of L-1, 13 ± 3 equiv of **1** is required to give a complete enzyme inactivation. One must note that for these experiments $[\mathbf{1}]_0/[\text{L-1}]_0$ ratios of 1–20 were used and the remaining activity plotted was that measured after all the time which was necessary to arrive at complete stabilization (up to 55 and 30 min, respectively, for 2 nM and 1 μM L-1).

These results together with the fact that 30 and 17 molar equiv of **1** were required to inactivate L-1 respectively at 1 and 5 μM concentrations could not be accounted for by the branched inactivation pathway proposed in Scheme I without the k_{-3} step. From the oxygen consumption data recorded for a series of experiments with various concentrations of L-1 (0.65–5.2 μM) and of **1** (2.6–1030 μM), one could plot the amount of hydrazone converted per mole of enzyme as a function of the ratio of their initial concentrations $[\mathbf{1}]_0/[\text{L-1}]_0$ (Figure 3). This shows that past the minimum ratio $[\mathbf{1}]_0/[\text{L-1}]_0$ necessary for complete enzyme inactivation (13 ± 3), the amount of **1** consumed to give total L-1 inactivation increases and levels off at about 70 mol of **1** per mole of L-1.

The α-azo hydroperoxide **2** is also an efficient inhibitor of L-1, with an IC_{50} value of 0.3 μM after 1 min of preincubation, in the same conditions as those of Table I. The plot of the fractional activity remaining vs the ratio of initial concentrations $[\mathbf{2}]_0/[\text{L-1}]_0$ is a straight line, and 4 equiv of **2** is required for complete L-1 inactivation [in these conditions,

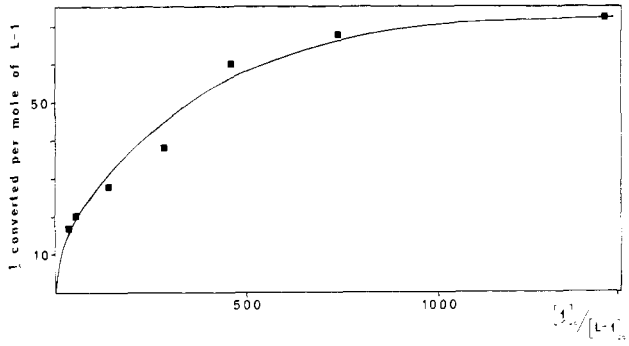


FIGURE 3: Conversion of **1** by L-1 as a function of the ratio of their initial concentrations. Plot of the number of moles of **1** converted per mole of L-1 vs the ratio of their initial concentrations $[\mathbf{1}]_0/[\text{L-1}]_0$. This plot was obtained from oxygen consumption data (see text).

no significant difference is observed between the Fe(II) and Fe(III) forms of L-1]. Whereas about 15 molar equiv of **1** completely inactivates 10 μM L-1 after 15 min of incubation, 1 min is sufficient for 15 and 5 molar equiv of **2** to respectively inactivate the Fe(III) and Fe(II) forms of the enzyme in aerobic or anaerobic conditions.

All these results suggested a lipoxygenase self-inactivation by **1**, according to the branched pathway of Scheme I involving the k_{-3} step. In order to confirm the occurrence of the k_{-3} step, a trapping experiment of **2** in solution was done with glutathione peroxidase.[5] The inactivation of L-1 (0.47 μM) by 16 equiv of **1** at 0 °C, in the presence of 475 μM of glutathione, was checked for various numbers of activity units of glutathione peroxidase (see Materials and Methods). The results are as follows (number of activity units of GSH peroxidase, % remaining activity of L-1): 0, 0%; 0.12, 55%; 0.60, 63%; 1.17, 78%; 2.56, 78%. They show that 22% of the inactivation of L-1 cannot be suppressed when all the released **2** is trapped in solution (see Materials and Methods).

Covalent Binding. Several decomposition reactions of the α-azo hydroperoxide **2** at the L-1 active site (Sheldon & Kochi, 1981; Baumstark, 1986) could lead to enzyme inactivation with the binding of a phenyl group to the protein. Hence labeling experiments were run with 10 μM L-1 (sp act. 170 μmol of $O_2 \cdot min^{-1} \cdot mg^{-1}$) with the (*E*)-**1*** and (*Z*)-**1*** isomers and the α-azo hydroperoxide **2*** uniformly labeled with ^{14}C on the phenyl ring (* indicates ring-U-^{14}C) (see Materials and Methods). No ^{14}C labeling of the enzyme is obtained when it is anaerobically incubated with (*E*)-**1*** or (*Z*)-**1***, conditions in which no inactivation occurs.

The plots of the inactivation and of the ^{14}C labeling of L-1 [10 μM, either Fe(II) or Fe(III)] vs the molar ratio of (*E*)-**1*** or (*Z*)-**1*** to L-1 (after 15-min incubation) show a clear decrease of the slope of the labeling curves, which become linear after the reaching of 100% inactivation. They show that complete inactivation of the enzyme by 15 equiv of (*E*)-**1*** or (*Z*)-**1*** is accompanied respectively by the covalent binding of 0.3 and 0.5 mol of [^{14}C]phenyl group (or a species containing it) per mole of protein. A first control experiment under the same conditions gave a straight line showing that 0.1 mol of phenyl group gets bound per mole of L-1 which had been previously inactivated by boiling or treatment with **1**. This labeling which is not dependent on L-1 activity is probably due to the α-azo hydroperoxide **2*** formed by autoxidation of **1***. Moreover, out of the 15 mol of **1*** used to inactivate 1 mol of L-1, 14 mol is converted into **2*** liberated into the medium (vide supra). A second control experiment showed

[5] We thank a referee for suggesting this experiment.

Table II: Quantification of Methionine Sulfoxide (MetSO) Formed during L-1 Inactivation by **1**

	native L-1[b]	inactivated L-1[b]	due to inactivation by **1**
MetSO relative to 87 Asp[a]	0.30 ± 0.11	1.42 ± 0.23	1.1 ± 0.3
MetSO relative to 82 Leu[a]	0.31 ± 0.11	1.51 ± 0.33	1.2 ± 0.4
average value of MetSO per mol of L-1[a]			1.15 ± 0.4
with correction factor for MetSO recovery[c]			1.8 ± 0.8

[a] Gel electrophoresis gave M_r 98 000 for our purified lipoxygenase 1; amino acid analysis led to 87 Asp, 82 Leu, and 17 Met for this molecular weight, in agreement with the composition reported by Feiters (Feiters et al., 1986). [b] Mean value from four analyses in each case. [c] An amino acid standard mixture including an equimolar amount of methionine sulfoxide was hydrolyzed under basic conditions along with the protein samples. The sulfoxide recovery compared to that of an unhydrolyzed standard gave the correction factor for destruction: 0.3 ± 0.06.

that these 14 equiv of **2*** lead to the binding of about 0.2 mol of phenyl group per mole of previously inactivated L-1. Therefore the ^{14}C labeling of L-1, observed after the inactivation by **1***, can be in great part assigned to a covalent binding due to the decomposition of the liberated **2***. This indicates that under the conditions of these experiments the binding of only 0.1 and 0.3 mol of phenyl group per mole of L-1 can be directly linked to the inactivation reaction by (*E*)-**1*** and (*Z*)-**1***. Complete inactivation of the native Fe(II) or Fe(III) forms of L-1 (10 μM) by the α-azo hydroperoxide **2*** (respectively 5 and 15 equiv), after 1-min incubation, gives the same labeling as that obtained with L-1 previously inactivated by boiling or treatment with **1** or **2**. Therefore, the corresponding covalent binding is clearly independent of the inactivation process. This labeling is the same as that observed after 15 min for the second control experiment with **2***.

Formation of Methionine Sulfoxide. α-Azohydroperoxides are efficient sulfide oxidants (Baumstark, 1986), and oxidation of an active site methionine could be an inactivation route of L-1 (Kühn et al., 1984). Hence, amino acid analysis was carried out after basic hydrolysis, for both native L-1 and L-1 completely inactivated by **1** (10 μM L-1, 20 equiv of **1**, see Materials and Methods). Table II gives the quantification of methionine sulfoxide, taking into account the correction factor for sulfoxide recovery. One finds 1.8 ± 0.8 mol of methionine sulfoxide formed per mole of inactivated L-1.

EPR and Magnetic Susceptibility Studies. EPR was used to investigate the involvement of the active site iron in the interaction of L-1 with **1** and **2**.

Anaerobic incubation of 0.5 mM Fe(II) native L-1 (S = 2; Slappendel et al., 1982; Cheesbrough & Axelrod, 1983; Petersson et al., 1985) with 1–5 equiv of **1** brings no appearance of any signal in the EPR spectrum of the enzyme that remains fully active.[4] *Anaerobic* incubation of the yellow Fe(III) L-1 ($S = {}^5/_2$; De Groot et al., 1975; Slappendel et al., 1981; Clapp et al., 1985) with 1 molar equiv of **1** leads to an EPR-silent species (Figure 4a). Magnetic susceptibility measurements, made by a modified Evans method (Evans, 1959; see Materials and Methods) gave an effective magnetic moment μ = 5.0 ± 0.1 Bohr magnetons corresponding to a high-spin state, S = 2. This new species obtained after addition of 1–5 equiv of **1** is still fully active[4] when put in the presence of linoleic acid and dioxygen. However, addition of 13(*S*)-HPOD alone (up to 5 equiv) does not regenerate the g = 6.2 signal, unless the EPR-silent species is previously put in the

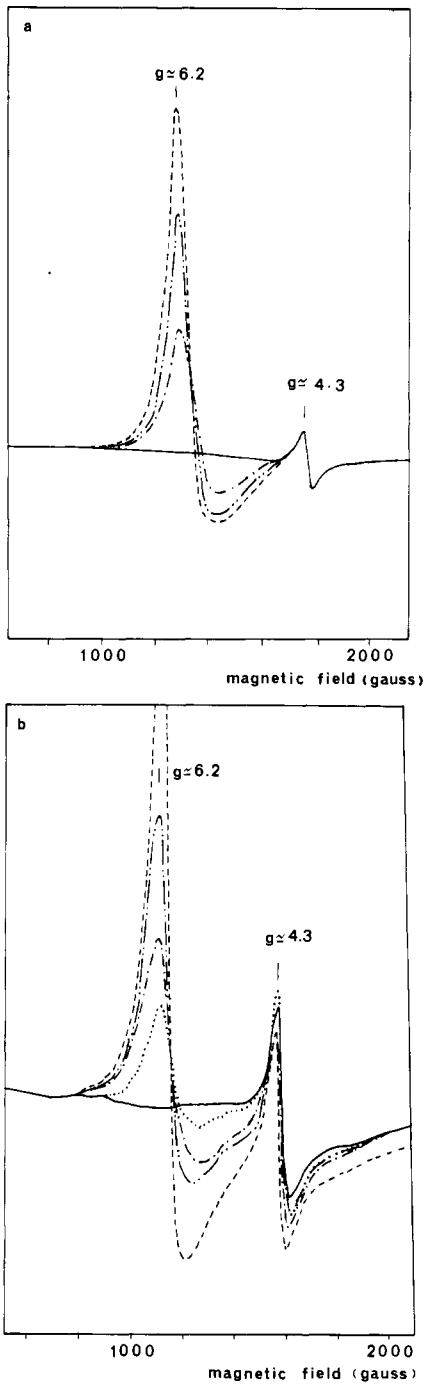


FIGURE 4: EPR monitoring of the interactions of the yellow Fe(III) and native Fe(II) forms of L-1 with **1** (a) and **2** (b) under *anaerobic* and *aerobic* conditions. (a) (1) Native Fe(II) L-1 (—); (2) yellow Fe(III) L-1 (– –); (3) *anaerobic* Fe(III) L-1 + 1 equiv of **1** (—) [identical to (1); unchanged upon 13(*S*)-HPOD addition]; (4) (3) + O_2 + 1 equiv of 13(*S*)-HPOD or (3) + 3 equiv of linoleic acid + 1 equiv of 13(*S*)-HPOD (– –) [identical to (2)]; (5) *aerobic* L-1 Fe(II) or Fe(III) + 8 equiv of **1** (—) [identical to (1)]; (6) *aerobic* L-1 Fe(III) + 1 equiv of **1** (–·–); (7) (6) + 1 equiv of 13(*S*)-HPOD (–··–). L-1 was ~0.5 mM in 0.05 M Tris–acetate buffer, pH 9, at 15 K: spectrometer frequency 9.43 GHz, microwave power 10 dB, modulation amplitude 10 Gpp, and gain 8 × 10^4. (b) (1) Native Fe(II) L-1 (—); (2) yellow Fe(III) L-1 (– –). The following results were obtained under both *anaerobic* and *aerobic* conditions: (3) Fe(III) L-1 + 1 equiv of **2** (–··–); (4) Fe(III) L-1 + 7 equiv of **2** (···); (5) Fe(II) L-1 + 2 equiv of **2** (–·–); (6) Fe(II) L-1 + 3 equiv of **2** (···) [identical to (4)]. The g = 2 signals are not shown because of overlapping with Mn(II) lines and because of poor reproducibility from one enzyme sample to another. A more thorough analysis is undertaken. L-1 was ~0.5 mM in 0.05 M Tris–acetate buffer, pH 9, at 15 K: spectrometer frequency 9.49 GHz, microwave power 10 dB, modulation amplitude 10 Gpp, and gain 10^5.

presence of either dioxygen or 3 equiv of linoleic acid (Figure 4a).

Aerobic inactivation of Fe(II) or Fe(III) L-1 at the 0.5 mM concentration used for the EPR experiments was obtained with about 10 equiv of **1** after 15–30-min incubation. The completely inactivated enzyme is EPR silent. It was checked, by atomic absorption spectroscopy, that it still contains 1 mol of iron per mole. No Fe(III) $g = 6.2$ signal can be (re)generated upon treatment with excess 13(*S*)-HPOD. Starting with Fe(III) L-1, *aerobic* incubation with increasing amounts of **1** leads to a decrease of the EPR $g = 6.2$[6] signal that disappears for 3 equiv of **1**. In these conditions the enzyme is only partially inactivated and the Fe(III) signal can be partly regenerated upon addition of 1 equiv of 13(*S*)-HPOD (it disappears again upon further addition of **1**) (Figure 4a). At this point it appeared necessary to know if the EPR-silent species obtained after complete aerobic inactivation of 0.5 mM L-1 was identical with the species formed at micromolar concentrations (which were used for dioxygen consumption and covalent binding experiments). Indeed, dioxygen concentration of an air-saturated aqueous solution is 0.24 mM at 25 °C, and an eventual lack of dissolved dioxygen, together with slow diffusion, could pertub the experiments. When 10 μM L-1 [either Fe(II) or Fe(III)] was completely inactivated by **1** under *aerobic* conditions (vide supra) and brought to 0.5 mM by lyophilization (see Materials and Methods), no signal at $g = 6.2$ was observed. The only modification was a slight increase of the $g = 4.3$ signal. Therefore, the enzyme *aerobically* inactivated by **1** is definitely EPR silent. Magnetic susceptibility measurements, by Evans' method, gave an effective magnetic moment $\mu = 5.0 \pm 0.1$ Bohr magnetons corresponding to a high-spin state, $S = 2$.

Inactivation of 0.5 mM Fe(II) or Fe(III) L-1 was obtained respectively with 4 and 7 equiv of the α-azo hydroperoxide **2** after 10-min incubation, both under *aerobic* or *anaerobic* conditions. Starting with Fe(III) L-1, treatment with 1 equiv of **2** decreases the intensity of the $g = 6.2$ signal. Complete inactivation leaves a small $g = 6.2$ resonance together with small sharp signals at $g = 4.3$ and 2.0 (Figure 4b). Starting with Fe(II) L-1, incubation with increasing amounts of **2** leads to the appearance of $g = 6.2$ and free radical type $g = 2.0$ resonances and to an increase of the $g = 4.3$ resonance. After addition of more than 2 equiv of **2**, the newly formed $g = 6.2$ signal decreases, and the spectrum of the completely inactivated enzyme is similar to that obtained from the Fe(III) form (Figure 4b). The same spectrum was also obtained with 10 μM native L-1 aerobically inactivated by **2** (vide supra) and then brought to EPR concentration. Because of the absence of any $g = 6.2$ signal in the spectrum of the enzyme inactivated by **1**, the quantification of the $g = 6.2$ and 4.3 resonances observed for L-1 inactivated by **2** was made. This quantification [according to Slappendel et al. (1981); see Materials and Methods] gave respectively about 8 and 2% of the total iron content for these two signals. Therefore, the major species appearing after L-1 inactivation by **2** is EPR silent like that obtained from aerobic inactivation by **1**.

Discussion

Hexanal phenylhydrazone **1** is a potent inhibitor of soybean lipoxygenase 1 (L-1) with IC_{50} values of 1.3 and 0.2 μM, after 0- and 1-min preincubation (Table I). The inhibition is actually a time- and oxygen-dependent irreversible inactivation (Figure 1).

That **1** is a mechanism-based inactivator of L-1 is shown by the following results:

(i) L-1 catalyzes the oxidation of **1** into its α-azo hydroperoxide **2** (Figure 2).

(ii) The kinetic treatment indicates that a complex is formed between L-1 and **1** prior to inactivation. The K_i values for the (*E*)-**1** and (*Z*)-**1** isomers, respectively 0.25 and 0.40 μM, compared to the K_m value of 24 μM for linoleic acid [in agreement with that of Allen (1968)] reveal a good binding of both isomers of **1** to L-1. However, the k_{inact} values, respectively 0.8 and 2.1 min^{-1}, are 4 orders of magnitude lower than the k_{cat} of linoleic acid dioxygenation (ca. 10^4 min^{-1} for our enzyme preparation). These results are comparable to the inactivation parameters of 5,8,11,14-eicosatetraynoic acid (K_i = 1.3 μM; k_{inact} = 0.36 min^{-1}) (Kühn et al., 1984) and of 13-thiaarachidonic acid (K_i = 0.8 μM; k_{inact} = 0.41 min^{-1}) (Corey et al., 1986).

(iii) Linoleic acid decreases the rate of inactivation of the enzyme by **1**. This suggests that both compounds compete for the fatty acid binding site of L-1.

The inactivation of L-1 by **1** follows a branched pathway as proposed in Scheme I. The kinetics of this type of pathway, without the k_{-3} step, have been worked out in detail (Waley, 1980; Tatsunami et al., 1981; Waley, 1985). They imply a constant partition ratio, $r = k_3/k_4$, for various inhibitor and enzyme concentrations. However, in our case, the number of moles of **1** whose oxidation is required to completely inactivate 1 mol of L-1 depends on the ratio of the initial concentrations of **1** to L-1 (Figure 3). It varies from a minimum of 13 ± 3 molar equiv of **1** to a maximum of 70 equiv when the hydrazone is in large excess over the enzyme. The α-azo hydroperoxide **2** is an efficient inactivator of L-1, with a minimum of 4 equiv required for complete L-1 inactivation. Therefore, our results show that an excess of **1** decreases the efficiency of the inactivation by precluding the k_{-3} step of Scheme I. They indicate that the observed inactivation is partly due to a direct inactivation by **2** as soon as it is formed at the active site and partly to an inactivation by **2** coming back from the medium to the active site. It is noteworthy that the same minimum number of moles of **1** necessary to inactivate 1 mol of L-1, i.e., 13 ± 3, was found all over the nanomolar to millimolar (EPR studies) range of concentrations of L-1. The increase of the apparent r value from 13 to 70 with increasing concentration of **1** suggests that about 13/70 = 19% of inactivation events occur before the product **2** dissociates from L-1. This is in agreement with the results of the trapping experiment of **2** by GSH peroxidase, which show that 22% of the inactivation of L-1 by **1** cannot be overcome by a complete removal of **2** released in the medium.[7]

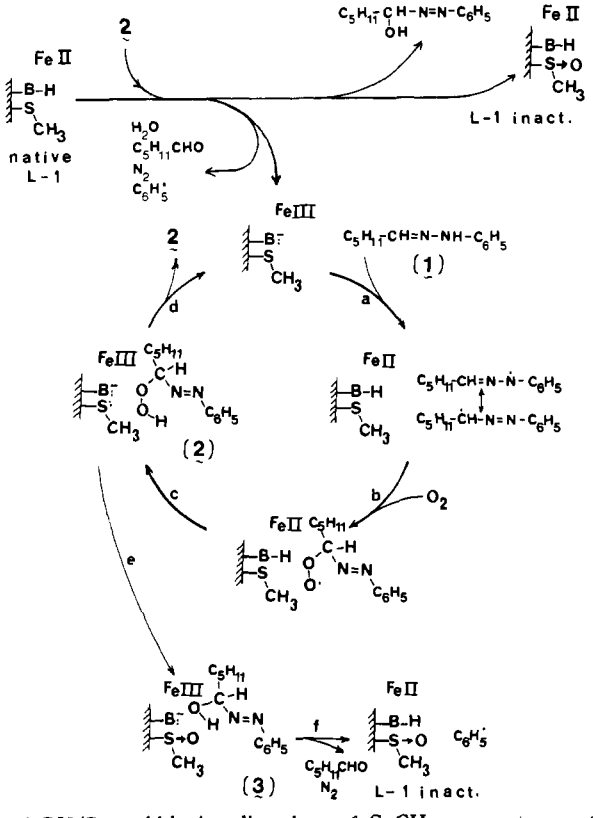
The spectroscopic data and the methionine sulfoxide formation support the inactivation mechansim proposed in Scheme II. The various steps are discussed in the following. The reactions between **1** and Fe(III) L-1 present common features with those involving fatty acid substrates.

(i) *Anaerobic* or *aerobic* reaction of Fe(III) L-1 with **1** leads to the disappearance of the characteristic Fe(III) EPR features (Figure 4a). For the *anaerobic* reaction, 1 molar equiv of **1** gives an EPR-silent species with an $S = 2$ spin state, as observed for the same *anaerobic* treatment with linoleic acid (De Groot et al., 1975; Egmond et al., 1977). This species is fully active[4] for the dioxygenation of linoleic acid. The fact that

[6] The absence of the $g = 5.8$ and 7.4 components of the Fe(III) signal is due to the presence of less than 5% methanol or ethanol in the sample, coming from the solutions of **1** and 13(*S*)-HPOD (Slappendel et al., 1982a).

[7] We thank a referee for a discussion of this point.

Scheme II: Proposed Mechanistic Scheme for Inactivation of Soybean Lipoxygenase 1 by Hexanal Phenylhydrazone[a]



[a] ⫽-BH/B⁻ could be iron ligands. ⫽-S-CH_3 represents an active site methionine.

it cannot be reoxidized by 13(*S*)-HPOD, unless it has been first put in the presence of dioxygen or linoleic acid, suggests that a radical derived from the monoelectronic reduction of the Fe(III) by **1** might remain at the active site. However, the presence of a free radical is not supported by the spectroscopic data, and further investigations are needed to determine the nature of the EPR-silent species, which could be an Fe(III)–σ-alkyl complex. For the *aerobic* reaction, when the number of molar equivalents of **1** is too small to lead to complete inactivation (e.g., 3 equiv), addition of 13(*S*)-HPOD can partly regenerate the Fe(III) enzyme (Figure 4a). This suggests that in step a of Scheme II **1** reduces Fe(III) L-1 to a Fe(II) species. A similar reduction, with no subsequent inactivation, has been described with *N*-alkylhydroxylamines (Clapp et al., 1985).

(ii) The initial oxidation rate of **1** into **2** is larger with the ferric than the native form of L-1 (Figure 2). This initial rate and the amount of **1** that is oxidized are increased in the presence of 13(*S*)-HPOD. This could be related to the role of 13(*S*)-HPOD in sustaining linoleic acid oxidation (Lands, 1984).

After steps b and c of Scheme II, the α-azo hydroperoxide **2** is formed at the active site. L-1 inactivated by **1** contains a little more than one methionine sulfoxide (1.8 ± 0.8), out of 17 methionines, per molecule of protein (Table II). α-Azo hydroperoxides are known to be very efficient sulfide oxidants in aprotic medium (Baumstark & Vasquez, 1983; Baumstark, 1986). It has also been reported that the formation of one methionine sulfoxide is involved in the self-inactivation of reticulocyte lipoxygenase (Rapoport et al., 1984; Schewe et al., 1986) and in the inactivation of soybean lipoxygenase by acetylenic fatty acids (Kühn et al., 1984). This suggests that the oxidation of an active site methionine (Zakut et al., 1976) is the inactivating reaction. In our case, the α-azo hydroperoxide **2** could give this reaction, generating the unstable α-azo alcohol **3** [$C_5H_{11}CH(OH)N{=}NC_6H_5$] (Baumstark & Vasquez, 1983) in the vicinity of the active site Fe(III) (step e, Scheme II). The too large 1.8 value could reflect some nonspecific oxidation of other methionines by the released **2**. The inactivated L-1 is EPR-silent (Figure 4a) with μ_{eff} = 5.0 Bohr magnetons corresponding to an $S = 2$ spin state. This suggests an Fe(II) form for the inactivated L-1, which could result from the oxidation of the α-azo alcohol **3** by the active site Fe(III) (step f, Scheme II). This reaction would generate a phenyl radical which could give the partial covalent binding observed with the (*E*)-**1*** and (*Z*)-**1*** isomers (respectively 0.1 and 0.3 mol of phenyl group per mole of L-1 under the conditions of the labeling experiments). As **2*** added to L-1 inactivates it without specific covalent binding, the labeling obtained with the (*E*)-**1*** and (*Z*)-**1*** isomers could be related to the 20% of direct inactivation by **2*** occurring as soon as it is formed at the active site (vide supra).

The native Fe(II) L-1 is also irreversibly inactivated by **1**. The question of the starting of the oxidation of **1** by the native L-1 is the same as that encountered for linoleic acid (Egmond et al., 1976). It could be due to a small proportion of the Fe(III) form present in the native enzyme (Slappendel et al., 1981). The Fe(III) L-1 is able to oxidize **1** into **2** (up to 70 mol per mole of L-1), and **2** could then either inactivate Fe(II) L-1 (to an EPR-silent species) or oxidize it into the Fe(III) form (as shown by EPR). The latter could then be inactivated by its reaction with excess **1** as discussed above.

In conclusion, hexanal phenylhydrazone (**1**) is a mechanism-based inactivator of soybean lipoxygenase 1 (L-1). The first step of the reaction involves Fe(III) L-1 reduction by **1** followed by the formation of the α-azo hydroperoxide **2** (Scheme II). Our results suggest that **2** is the inactivating species via the oxidation of an active site methionine. This inactivation is due to **2** as soon as it is formed at the active site and to **2** coming back to it. These two pathways respectively account for about 20 and 80% of L-1 inactivation. These results recall the self-inactivation of reticulocyte lipoxygenase (Rapoport et al., 1984), the inactivation of 5-lipoxygenase by 5-HPETE (Aharony et al., 1987), and the inactivation of soybean lipoxygenase by 5,8,11,14-eicosatetraynoic acid (Kühn et al., 1984). The α-azo hydroperoxide **2** is much more inactivating than the hydroperoxy fatty acids but less efficient than the allenic hydroperoxide derived from the acetylenic fatty acid. The latter leads to a stoichiometric inactivation of the enzyme before it can dissociate from it (Kühn et al., 1984) whereas **2** can leave the active site and the oxidation of 13–70 equiv of **1** is required for complete L-1 inactivation depending on the excess of **1**.

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We thank Dr. A. Bouniols (Centre de Recherche de Toulouse, Institut National de la Recherche Agronomique) for the supply of several varieties of soybean seeds. For the EPR studies not done at Orsay (J.J.G.), we are indebted to Pr. A. Vivien, Université Pierre et Marie Curie, for the access to the EPR facility of the Ecole Nationale Supérieure de Chimie de Paris. We thank A. Boutry, Laboratoire d'Electrochimie Interfaciale, CNRS, Meudon, for the iron atomic absorption measurements, S. Cortial for assistance with amino acid analyses, and M. Jaouen for assistance with the enzyme purifications.

Registry No. (*E*)-**1**, 111975-53-2; (*Z*)-**1**, 111975-54-3; **2**, 111975-55-4; L-1, 9029-60-1; Met, 63-68-3; $C_6H_5NHNH_2$, 100-63-0; $CH_3(CH_2)_4CHO$, 66-25-1.

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