Determination of the Spin State of Iron in Native and Activated Soybean Lipoxygenase 1 by Paramagnetic Susceptibility†

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ABSTRACT: The paramagnetic susceptibility of the iron present in soybean lipoxygenase 1 was measured by proton nuclear magnetic resonance. Paramagnetic iron was found in both the native and activated forms of the enzyme. No paramagnetic shifts were seen with the apoenzyme nor when the native enzyme was inactivated by incubation with excess linoleic acid under anaerobic conditions. Evaluation of the observed shifts indicates the enzyme contains high-spin Fe(III) in the activated state, as expected from its known electron paramagnetic resonance characteristics, and high-spin Fe(II) in the native state.

Lipoxygenase is a dioxygenase that catalyzes the hydroperoxidation of lipids containing a *cis,cis*-1,4-pentadiene moiety, as present, e.g., in the essential polyunsaturated fatty acids. Although lipoxygenases occur in both plants and animals, the enzymes most extensively studied have been those obtained from higher plants, especially soybean seeds. [For reviews, see Axelrod (1974) and Veldink et al. (1977).] Three distinct soybean isoenzymes have been purified, lipoxygenases 1, 2, and 3 (L-1, L-2, L-3).[1] L-1 has a molecular weight of about 100 000 (Theorell et al., 1947) and contains 1 atom of iron per molecule (Chan, 1973; Rosa & Francke, 1973; Pistorius & Axelrod, 1973). L-2 and L-3, although they differ kinetically, immunologically, and in amino acid composition from L-1, have similar molecular weights and iron content (Axelrod et al., 1972; Christopher, 1972; Christopher et al., 1972a,b; Pistorius, 1974; Bild, 1978). That iron is essential for the activity of all three isozymes has been demonstrated by showing that removal of iron is accompanied by a parallel loss of enzyme activity (Pistorius & Axelrod, 1974).

To understand the mechanism of lipoxygenase action, it is important to know what electronic changes occur in the iron during the reaction. The functional participation of iron in soybean catalysis lipoxygenase was demonstrated by EPR studies at liquid He temperatures (Pistorius & Axelrod, 1974; Axelrod et al., 1975; Pistorius et al., 1976). Although the native enzyme was EPR silent, a prominent EPR resonance at $g = 6$, indicative of high-spin Fe(III) ($S = {}^5/_2$), appeared when it was treated with a small molar excess of the primary dioxygenation product, L-13-hydroperoxylinoleic acid (LAOOH). Similar results were obtained when linoleic acid (LA) was used, since it is rapidly and quantitatively converted to LAOOH. Detailed analysis of the spectrum indicated that 75% of the iron in L-1 is in high-spin Fe(III) in a mixture of three different ligand structures (Slappendel et al., 1981).

The conversion of the native, EPR-silent form to the EPR-active form of the enzyme by the reaction product can be related to an earlier discovery in our laboratory showing that the lipoxygenase reaction occurs with a lag that is overcome by the product hydroperoxide (Haining & Axelrod, 1958). This phenomenon has been confirmed by others (Smith & Lands, 1972). It has been proposed that iron occurs in the native enzyme as Fe(II) and is converted to Fe(III) by the action of the LAOOH (De Groot et al., 1975a). While there is little question that Fe(III) is present in the activated enzyme, the state of the iron in the resting state is not established. Chelator studies that suggested that the iron in the native enzyme was Fe(III) are equivocal (Pistorius et al., 1976). Indeed, this suggestion was questioned in a later review on the basis of unpublished work (Veldink et al., 1977). The failure to detect an EPR signal lead to the proposal that the iron was in a low-spin, diamagnetic, Fe(II) form (Veldink et al., 1977; De Groot et al., 1975a). Since complexes containing paramagnetic iron can sometimes be EPR silent, the failure to detect an EPR signal is not sufficient evidence for assigning the electronic state of the iron.

Evans has developed a method employing a coaxial NMR tube for studying susceptibility in proteins using ^{1}H NMR (Evans, 1959). The inner cylinder of the tube contains the sample protein mixed with an inert reference compound that displays a prominent shift signal. The annular space of the tube contains the reference compound but no protein. Thus the spectrum displays at once the normal line and the shifted line. The chemical shift difference is a function of the intrinsic paramagnetic susceptibility of the enzyme and its concentration. This method allows simple, nondestructive analysis of metalloproteins (Bartle et al., 1968). Paramagnetic metal complexes whose relaxation rates are too fast for EPR detection can be measured by this method. Using this procedure, we show in this paper that the iron in the native enzyme is high-spin Fe(II).

Experimental Procedures

Materials. The methyl α-D-glucoside (MEG) was obtained from Aldrich Chemical Co. Sodium tetraborate and boric acid, analytical reagent grade, were from Mallinckrodt, Inc. Deuterium oxide, 99.75% ^{2}H, was from J. T. Baker Chemical Co. The linoleic acid (>99%) (LA) was purchased from Nu-Chek Preps Inc. All other chemicals were analytical

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[1] Abbreviations: L-1, L-2, and L-3, lipoxygenase isozymes 1, 2, and 3, respectively; LAOOH, L-13-hydroperoxy-*cis,trans*-octadecadienoic acid; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; LA, linoleic acid; MEG, methyl α-D-glucoside; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; DSS, sodium 4,4-dimethyl-4-silapentanesulfonic acid.

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reagent grade and were obtained from Fisher Scientific Co. or MCB Manufacturing Chemists, Inc. The 5-mm, high-precision, coaxial NMR tubes (5 mm) were obtained from Wilmad Glass.

Purification of Lipoxygenase 1. L-1 was prepared as described earlier (Axelrod et al., 1981). The purified L-1 (sp act. 261 μmol min/mg) was dialyzed against glass-distilled deionized water and lyophilized.

Preparation of Iron-Free Lipoxygenase. A total of 75 mg of L-1 was treated with *o*-phenanthroline and β-mercaptoethanol in 50 mM Tris buffer (pH 8.0) to remove the iron (Pistorius & Axelrod, 1974). The reaction was monitored by following the absorbance of the iron–*o*-phenanthroline complex at 512 nm. A standard curve was prepared from ferrous chloride. The apo-L-1 was dialyzed against two changes (24 volumes each) of 50 mM Tris (pH 8.0) and six changes (100 volumes each) of 200 mM Tris (pH 8.0) in 30% (v/v) 1-propanol. Finally, the apoenzyme was dialyzed against six changes (100 volumes each) of glass-distilled water and lyophilized.

Atomic Absorbance Analysis. After analysis by NMR, samples were dialyzed against two changes (3000 volumes) of glass-distilled water and lyophilized. Samples of 3 mg were analyzed on a Perkin-Elmer Model 403 atomic absorption spectrophotometer.

Assay Buffer. Borate buffer, 50 mM, was prepared in D_2O by addition of solid boric acid and sodium tetraborate. An aliquot was removed and the pH determined with a hydrogen electrode. All the solutions used gave an apparent pH of 9.0 (or a pD of 8.6). Where indicated, sufficient solid MEG was added to give a concentration of 10 mM.

Linoleic Acid and L-13-Hydroperoxylinoleic Acid Solutions. Linoleic acid was added directly to the above buffer, which contained 0.16% Tween-20, to give a final concentration of 2 mM. When the LA was to be used in studies of the enzyme–LA mixture, the buffer was degassed and purged with dry N_2, before adding the LA. When the LA was to be converted to LAOOH, the buffer was saturated with O_2 by bubbling for 60 min. Formation of LAOOH was monitored by measuring the increase in absorbance at 234 nm. LAOOH formation was 100% of theory. If the reaction mixture is allowed to go anaerobic, there is a possibility of the formation of 13-oxo-9,11-octadecadienoic acid. Failure to observe absorption at 280 nm showed that this ketodiene was not formed. When LAOOH was added to the enzyme prior to NMR analysis, the mixture became blue (Axelrod et al., 1975; De Groot et al., 1975b) but then rapidly turned to pale yellow on standing. NMR measurements were made on the yellow form of activated L-1.

NMR Determinations. NMR studies were made on the Nicolet NT-470 and -360 spectrometers in the Purdue Magnetic Research Laboratory. Samples were placed in 5-mm coaxial tubes and analyzed according to the Evans procedure (Evans, 1959; Bartle et al., 1968). Probe temperature was 25 °C. The pulse Fourier-transform runs were performed with a 90° one pulse sequence as described in the figure legends. A total of 128 transients was averaged and Fourier transformed to yield the spectrum. Splittings were determined from the analysis of the methyl proton peak of MEG.

Results

Native L-1 in the presence of MEG showed splitting of the methyl proton peak of MEG (compare panels A and B, Figure 1). Since any material placed in a magnetic field can alter the effective local field, a theoretical calculation was made to determine the effect of the amino acids in L-1 on the spectra.

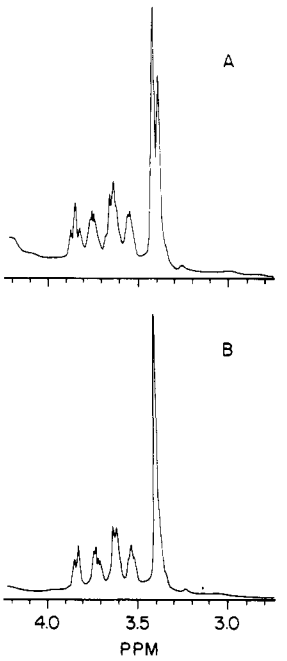

FIGURE 1: NMR spectrum of MEG in the presence of native L-1 under aerobic conditions. The studies were made on the NT-470 spectrometer with a 5-mm coaxial tube. The spectrum of MEG in the presence (A) and the absence (B) of 0.5 mM native L-1. The spectra were obtained by the Fourier transform of 128 scans. Spectral parameters are as follows: 90° one pulse sequence; radio frequency pulse width, 8.5 μs; acquisition delay, 3.00 s; acquisition time, 1.64 s; spectral width, ±2500.00 Hz; offset, 2282.8 Hz; number of bits digitized, 16384; spectrometer frequency, 470.081840 MHz. Quadrature phase detection and automatic base-line correction were used.

Table I: Effect of Iron-Free Protein on the Shift of the Methyl Proton Peak of Methyl α-D-Glucoside

sample[a]	concn (mg/mL)	chemical shift (ppm)[b]	Δf (Hz)[c]
apo-L-1	50	3.359	8.04
apo-L-1	25	3.351	5.02
BSA	50	3.352	5.62
no protein		3.340	

[a] The samples were analyzed by sample substitution on the Nicolet NT-470 spectrometer, using the pulse sequence described in the legend for Figure 1. [b] Shifts are assigned to the methyl protons of MEG. [c] The change in chemical shift (Δf) was obtained by subtraction of the shift in the absence of protein from the shift in the presence of protein sample.

On the basis of the amino acid composition of L-1 (Pistorius, 1974), a mass susceptibility of -0.5245×10^{-7} was obtained by calculation from Pascal's constants (Selwood, 1960). This value indicates that the contribution of the amino acids should cause a shift in the reference spectrum opposite in sign to that seen in Figure 1. The calculated susceptibility, not sufficiently small to ignore, was applied as a correction to all of the mass susceptibilities determined with the holoenzyme. By way of confirmation, the apoenzyme was also tested by the Evans method. The spectra of apo-L-1 showed a definite broadening of the reference peak but no splitting. Since it was not possible to conclude whether the broadening was the result of an unresolved splitting or the increased viscosity of the sample, the apoenzyme was reexamined by the sample-substitution method (Bartle et al., 1968) in a standard, single-chamber NMR tube. The spectra revealed a definite shift as a function of increasing apo-L-1 concentrations. The chemical shift of the methyl proton peak was calculated for each sample (see Table I). The broadening seen with 0.5 mM apo-L-1 may account for the

Table II: Paramagnetic Susceptibility of L-1[a]

sample[b]	reference	concn (mM)	Δf (Hz)	χ_0 ($\times 10^{-7}$)	χ ($\times 10^{-6}$)	μ
active L-1[c]	MEG	0.50	−12.28	0.8658	13 903	5.76
	MEG	0.33	−7.86	0.8281	13 526	5.68
	MEG	0.25	−6.14	0.8658	13 903	5.76
active L-1[d]	DSS	0.50	−9.60	0.8914	14 159	5.81
	DSS	0.25	−5.20	0.9975	15 220	6.02
native L-1[c]	MEG	0.50	−9.83	0.6226	11 470	5.22
	MEG	0.33	−6.39	0.6134	11 379	5.20
	MEG	0.25	−4.91	0.6216	11 461	5.22
native L-1[d]	DSS	0.50	−6.00	0.4198	9 443	4.74
	DSS	0.25	−3.60	0.5789	11 034	5.12
apo-L-1	MEG	0.50	8.04	−0.4408		
	MEG	0.25	5.02	−0.6439		

[a] The data for χ_g and μ were calculated from eq 1 and 2. The following abbreviations are used: L-1, lipoxygenase 1; MEG, methyl α-D-glucoside; DSS, sodium 4,4-dimethyl-4-silapentanesulfonic acid; Δf, change in chemical shift; χ_0, mass susceptibility; χ, molar susceptibility; μ, effective magnetic moment (in Bohr magnetons). [b] Samples were prepared as described under Experimental Procedures. [c] Data obtained on Nicolet NT-470 NMR spectrometer, with probe at 25 °C, as described in Figure 1. [d] Data obtained on a Nicolet NT-360 NMR at 25 °C with a Fourier-transform 90° one pulse sequence. Spectral parameters are as follows: pulse width, 10.0 μs; spectrometer frequency, 460.080411 MHz; acquisition time, 2.05 s; delay between pulses, 2 s; number of acquisitions, 256; spectral width, ±2000.00 Hz; number of bits digitized, 16384. Quadrature detection was used.

failure to find any detectable splitting with the coaxial tube.

Tappel et al. (1952) found that L-1 was inactivated by treatment with high concentrations of LA under anaerobic conditions. In order to see how the electronic state of the iron was affected, analyses were also performed on anaerobic solutions of L-1 plus LA. The methyl proton resonance of MEG showed no apparent splitting. As with apo-L-1, the signal was broadened, but the cause of this broadening was not investigated further.

The iron content of the L-1 samples was determined colorimetrically by the *o*-phenanthroline method (Pistorius & Axelrod, 1974) and by atomic absorption. The native, activated and LA-inactivated samples contained 1.03, 1.03, and 1.05 mol of Fe/mol of enzyme, respectively. The apoenzyme appears to contain ≤0.15 mol of Fe/mol of enzyme. Since the L-1 samples did not contain excess iron, the paramagnetic splittings must be attributed to enzymic iron.

Determinations of magnetic susceptibilities on the basis of NMR shifts in the reference signal are valid only for pseudocontact shifts (Bartle et al., 1968). One can differentiate pseudocontact from contact shifts by their concentration dependence. Pseudocontact shifts show a first-order concentration dependence, while contact shifts exhibit a more complex behavior (Bartle et al., 1968). It is evident from Table II that the observed shifts are linearly dependent on L-1 concentration. The results of the shift experiments may be properly used to calculate magnetic susceptibility.

The experiments shown in Figure 1 were performed with MEG as the reference. Studies were also made with native and active L-1, with sodium 4,4-dimethyl-4-silapentanesulfonic acid as a reference with similar results.

The mass susceptibility (χ_g) of the samples was determined by using the equation (Bartle et al., 1968)

$$\chi_g = \frac{3\Delta f}{4fm} + \chi_0 + \chi_0\left(\frac{d_0 - d_s}{m}\right) \quad (1)$$

where Δf is the signal shift, f is the spectrometer frequency in hertz, m is the concentration of solute (g/cm^3), χ_0 is the mass susceptibility of the solvent, d_0 is the density of the solvent, and d_s is the density of the sample (g/cm^3). These values (see Table II) were converted to the effective magnetic moments (μ in Bohr magnetons) by the following equation:

$$\mu^2 = \frac{\chi + 3kT}{N_n\beta^2} \quad (2)$$

where χ is the molar susceptibility, k is Boltzmann's constant, T is the temperature in degrees Kelvin, N is Avagadro's number, n is the number of paramagnetic centers per molecule, and β is the Bohr magneton.

The average values for μ of native and activated L-1 are 5.10 and 5.81, respectively. The theoretical values of μ for Fe(III) are 5.90, 3.87, and 1.87 for high spin ($S = {}^5/_2$), intermediate spin ($S = {}^3/_2$), and low spin ($S = {}^1/_2$), respectively. For Fe(II), the theoretical values for μ are 4.9 and 0, corresponding to high spin ($S = {}^4/_2$) and low spin ($S = 0$) (Iizuka & Yonetani, 1972).

Discussion

That the iron present in L-1 undergoes electronic changes in the course of optical activity was originally shown by optical and EPR studies (Pistorius & Axelrod, 1974; Pistorius et al., 1976). It was found that the enzyme that was EPR silent in the native (resting) form was altered by treatment with the reaction product, LAOOH, to a form exhibiting resonance characteristic of high-spin ($S = {}^5/_2$) Fe(III). Molar equivalents of LAOOH were sufficient to effect the transformation. The appearance of the EPR-positive state was accompanied by an increase in absorption in the range of 360–700 nm. When L-1 was titrated with LAOOH, 1 equiv of LAOOH was required to achieve the maximum increase, when absorbance was measured at 360 nm. The existence of an activated state of the enzyme was foreshadowed by the observation of Haining & Axelrod (1958) that the kinetic lag exhibited by the enzyme was specifically abolished by small amounts of LAOOH. When LAOOH was added to the enzyme under these circumstances (at 25 °C), the absorption change was visible to the eye, as a change from a weak straw color to a more yellow color. When the additions were done at 0 °C prior to the EPR studies, a deep blue color was seen with a pronounced absorption peak at about 570 nm. This color was unstable above 15 °C (Axelrod et al., 1975).

It was shown by Garssen et al. (1971) that LAOOH is destroyed by excess LA in the presence of L-1, if O_2 is excluded. Thus when linoleic acid was added to L-1, in an amount in excess to the O_2 present, it was possible by stopped-flow studies to observe the formation of the LAOOH-activated enzyme by the increase in absorption at 360 nm, followed by its decrease as the LAOOH was destroyed (Pistorius et al., 1976). The effect of LAOOH on the EPR and optical characteristics of L-1 has also been noted by others (De Groot et al., 1975a,b).

From the evidence presented above, it is now clear that Fe in the native enzyme is paramagnetic Fe(II). If the iron were low-spin Fe(II), there should have been be no splitting of the reference peak. On the basis of theoretical calculations and by taking into account the requisite controls, one must conclude that any splitting seen is due to the iron in L-1. Failure to see splitting of the MEG-methyl proton signal in the apoenzyme or the anaerobic LA plus L-1 sample reinforces this view.

The calculated magnetic moments indicate that the iron in L-1 has an apparent valence of 3+ (corresponding to $S = {}^5/_2$) in the activated enzyme and 2+ ($S = {}^4/_2$) (Iizuka & Yonetani, 1972) in the native enzyme (see Table II). The value found for the active enzyme is in agreement with those obtained in previous EPR studies (De Groot et al., 1975a; Pistorius et al., 1976; Slappendel et al., 1981). The addition of LAOOH to the native enzyme converts the iron from high-spin Fe(II) to high-spin Fe(III).

The iron appears to be dimagnetic in anerobic mixtures of L-1 and LA and thus differs from that in the native enzyme (spectra not shown). This finding is not surprising in view of earlier reports of anerobic inactivation of L-1 by LA (Tappel et al., 1952) and of fluorescence quenching upon addition of LA to anaerobic L-1 solutions (Finazzi-Agro et al., 1973). The enzyme recovered from the anaerobic study was inactive. In contrast, the native and activated L-1 samples retained more than 90% of their activity.

A recent paper from the Chalmers Institute of Technology, Göteborg, The University of Stockholm, and The State University of Utrecht also reports that the iron in native L-1 is high-spin Fe(II) (Slappendel et al., 1982).

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Registry No. Lipoxygenase, 9029-60-1; Fe, 7439-89-6.

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