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EPR SPECTROSCOPY OF SOYBEAN LIPOXYGENASE-1

DETERMINATION OF THE ZERO-FIELD SPLITTING CONSTANTS OF HIGH-SPIN Fe(III) SIGNALS FROM TEMPERATURE AND MICROWAVE FREQUENCY DEPENDENCE

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Summary

The zero-field splitting constants (D) of the different components building up the high-spin Fe(III) EPR spectrum of lipoxygenase from soybeans were determined by two methods: (1) temperature dependence studies using the low-spin Fe(III) signal of cytochrome *c* at g 3 for accurate measuring of the temperature in the sample; (2) by establishing g-shift upon increasing the microwave frequency. The ranges of D for the axial and rhombic species contributing to the complex signal at g 6 are found to be 1.5—3.0 K and 1.8—4.4 K, respectively. The occurrence of such large ranges is attributed to variations in amount and number of species in the different samples. The combination of the applied methods offers a more generally applicable approach to the determination of zero-field splitting constants.

Introduction

Lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12) catalyzes the hydroperoxidation of polyunsaturated fatty acids containing a 1,4-*cis*,*cis*-pentadiene system [1].

Incubation of soybean lipoxygenase-1 with linoleic acid at pH 9.0 results in

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Abbreviation: 13-L-ROOH, 13-L-hydroperoxy-9-*cis*,11-*trans*-octadecadienoic acid.

the formation of mainly 13-L-ROOH [2]. This enzyme contains one atom of non-heme iron per molecule (M_r 98 500) [3—5]. An EPR study of the interaction of substrate and product with the enzyme clearly showed the functionality of the iron in the catalytic cycle [6,7]. Addition of an equimolar amount of 13-L-ROOH to the native, colourless and virtually EPR-silent enzyme causes a change to yellow [8], while in the EPR spectrum a complex signal around *g* 6 appears attributed to at least two high-spin Fe(III) species with different symmetries [7]. When a molar excess of 13-L-ROOH is added to the yellow Fe(III) enzyme, the colour turns into purple and in the EPR spectrum the signal around *g* 6 is perturbed, while a strong increase of the signal at *g* 4.3 is observed [9].

Little is known about the zero-field splitting constants of the iron in lipoxygenase. In an attempt to interpret the EPR spectra of lipoxygenase, a negative sign of *D* has been suggested [6]. Furthermore, different zero-field splitting constants of the lipoxygenase-NO complex have been reported [10,11].

The present paper describes the determination of the zero-field splitting constants of the different Fe(III) species of lipoxygenase by studying the temperature dependence of the signals and the microwave frequency dependence of the *g* value. These values are necessary for the estimation of the amount of EPR-visible iron of lipoxygenase.

Materials and Methods

Lipoxygenase-1 was isolated from soybeans and purified according to Finazzi-Agrò et al. [3]. Metal chelators were used as described by Galpin et al. [12]. The specific activity was found to be 235 μmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ as measured polarographically on a Gilson oxygraph equipped with a Clark oxygen electrode (1.8 mM NH_4^+ linoleate/0.1 M sodium borate buffer, pH 9.0, 25°C).

The iron and manganese content determined by flameless atomic absorption spectroscopy were 0.98 and 0.07 mol per mol of enzyme, respectively. Linoleic acid (purity above 99%) was purchased from Lipid Supplies (St. Andrews University, St. Andrews, U.K.). 13-L-ROOH was prepared by incubation of linoleic acid with soybean lipoxygenase-1 in oxygen-saturated 0.1 M sodium borate buffer, pH 9.0, at 4°C and purified by high performance liquid chromatography [13]. Cytochrome *c* (Sigma Type VI), further purified by ion-exchange chromatography [14] and freeze dried, was a generous gift of Jonas Ångström.

EPR spectra at 9 GHz were recorded with a Varian E-3 spectrometer at liquid nitrogen temperature and with a Varian E-9 spectrometer at temperatures between 3 and 30 K using an Oxford Instruments liquid helium cryostat and control system. For temperature measurements a calibrated carbon resistor was available. Spectra at 35 GHz were obtained by a Varian V-4503 spectrometer equipped with a helium gas flow system [15]. Cytochrome *c* was added as a solid and the concentration was determined using a molar absorption of 21 100 $M^{-1} \cdot cm^{-1}$ at 550 nm (reduced minus oxidized) [16].

Results and Discussion

The EPR spectra of lipoxygenase

The 9 GHz EPR spectrum of yellow-coloured lipoxygenase-1, obtained by

addition of 1.3 equiv. 13-L-ROOH to native enzyme, is shown in Fig. 1. The high-field part of the spectrum is presented in the insert. A radical type of signal is superimposed on the signal from contaminating manganese. The g_z-parts of the high-spin Fe(III) signals near g 2 are not resolved and therefore cannot contribute directly to the elucidation of the spectrum. The low-field part shows EPR lines at g 7.5 and 6.1, a weak shoulder at g 5.8 and a line at g 4.3. De Groot et al. [7] attribute the signal at g 4.3 to contaminating high-spin Fe(III) in a ligand field of rhombic symmetry and the complex signal near g 6 to at least two species of high-spin Fe(III) with axial and rhombic symmetry, respectively.

The EPR spectrum of the sample recorded at 35 GHz is shown in Fig. 2. Because of line broadening at higher frequency the resolution of the spectrum has not improved. However, a change in g values is observed which can be used for the determination of the zero-field splitting parameters.

Zero-field splitting constants from g-shift

High-spin Fe(III) has six low-lying isolated energy levels forming three so-called Kramers' doublets. The EPR spectrum can be described by the spin Hamiltonian

$$H = D[S_z^2 - \tfrac{1}{3}S(S+1)] + E[S_x^2 - S_y^2] + g\mu_B \vec{B} \cdot \vec{S} \quad (1)$$

wherein D and E are the axial and rhombic zero-field splitting constants,

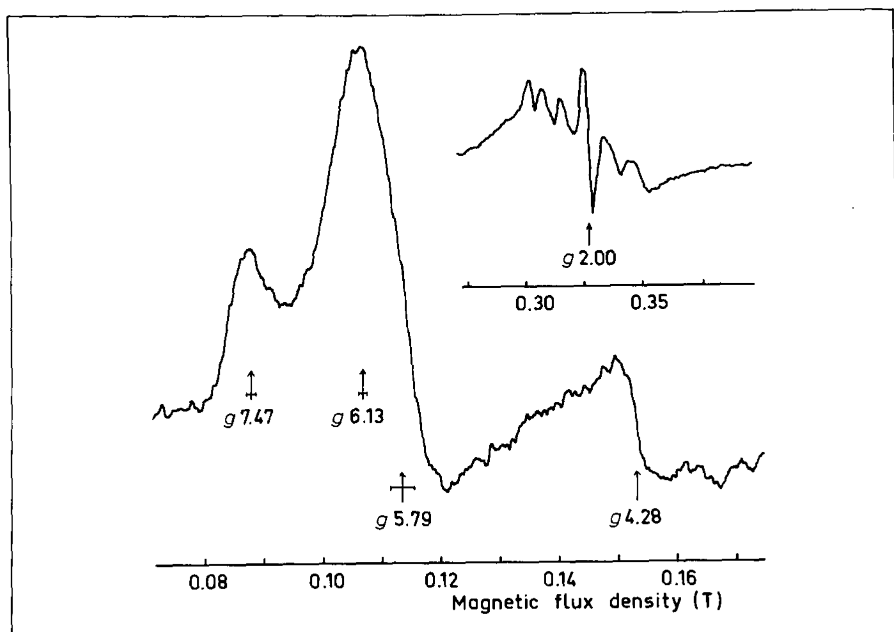

Fig. 1. EPR spectrum of Fe(III) yellow soybean lipoxygenase-1 at 9.195 GHz. 100 μl native lipoxygenase solution (138 g/l) were incubated with 6.7 μl of a 13-L-ROOH solution (27.6 mM); final concentrations: 1.31 mM and 1.73 mM, respectively, in 0.1 M borate buffer, pH 9.0. Microwave power 20 mW; modulation amplitude 2 mT; gain $5 \cdot 10^4$; temperature 77 K. Insert: High field part of the spectrum. Gain 10^5. Apparent g-values are indicated by arrows with estimated errors marked by horizontal bars.

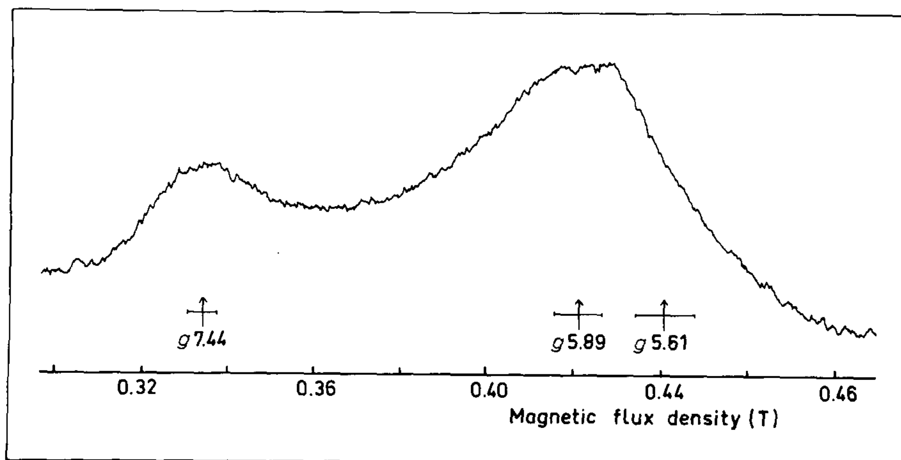

Fig. 2. EPR spectrum of Fe(III) yellow soybean lipoxygenase-1 (same sample as described in Fig. 1) at 34.66 GHz. Microwave power 10 mW; modulation amplitude 1 mT; gain $2 \cdot 10^3$; temperature 14 K.

respectively. Signals in the g 6 region, as found in lipoxygenase, are obtained from the doublet having $M_s = \pm 1/2$ if $D >> g\, \mu_B\, B$ and $D >> E$.

Third-order perturbation calculation using the spin Hamiltonian (Eqn. 1) gives the apparent g-values in the g 6 region as

$$g_{x,y} = 3g\left[1 \pm 4\left(\frac{E}{D}\right) - \frac{118}{9}\left(\frac{E}{D}\right)^2 - \frac{1}{2}\left(\frac{g\mu_B B}{D}\right)^2\right]$$

The first three terms are magnetic field-independent. However, the last contains the dependence of the g values on the microwave frequency. This shift (Δg) is identical for g_x and g_y and simple arithmetics give

$$\Delta g = g_{\nu_1} - g_{\nu_2} = \frac{3}{2}\frac{g^3}{D^2}\left[\left(\frac{h\nu_2}{g_{\nu_2}}\right)^2 - \left(\frac{h\nu_1}{g_{\nu_1}}\right)^2\right] \tag{2}$$

wherein ν_1 and ν_2 are two microwave frequencies with the corresponding apparent g-values g_{ν_1} and g_{ν_2}. If D is measured in kelvins, $g = 2.00$ and ν_1 and ν_2 are 9 and 35 GHz, respectively, only slight approximations reduce Eqn. 2 to

$$D \approx \sqrt{\frac{1}{\Delta g}} \tag{3}$$

which is particularly suitable form for a rough determination of D-values from experimental g-shifts. Earlier Eisenberger and Pershan [17] used a similar method for the determination of D of met-myoglobin.

From Figs. 1 and 2 the range of the g-shift can be obtained using the estimated maximal errors in the g-values. Δg_{min} and Δg_{max} are 0 and 0.30 for the rhombic species (approx. g 7.5) and 0.11 and 0.42 for the axial species (approx. g 6.1), respectively. Application of Eqn. 3 leads then to $D > 1.8$ K and 3.0 K $> D > 1.5$ K for the rhombic and axial species, respectively. These results are obtained from measurements of five different samples. No significant difference in g-shift between yellow and purple form is observed. The g-shift of the axial species is the same whether the g-values are measured at the top or at the baseline-crossing point of the signal (Figs. 1 and 2).

Temperature measurements using cytochrome c as thermometer

The population of the $M_s = \pm 1/2$ doublet determines the intensity of the g 6 signal. By measuring the intensity of the EPR signals at various temperatures the order of the doublets and the energy differences between the doublets can be derived. In order to be able to determine accurately a value for D the temperature in the sample should be known exactly.

A difficulty in the measurements of the temperature in a gas flow system, like that from Oxford Instruments, is the temperature gradient between sample and thermocouple, the latter being placed directly above the helium outlet in the cryostat. The gradient is not constant because of variations in the gas flow, necessary to change the temperature. This problem can be circumvented by using an internal standard, e.g. cytochrome c, as a thermometer. Fig. 3 gives EPR spectra of samples containing both lipoxygenase and cytochrome c. There is no observable interaction between these proteins. The interesting parts of the EPR spectra show no overlap, except for the g 2 signals of lipoxygenase which are almost buried in the cytochrome c features. The low-spin Fe(III) signal of cytochrome c at g 3 can act as a temperature indicator because excited states can be neglected in the temperature range used so that this signal follows Curie's law, i.e. intensity (I) × temperature (T) is independent of the temperature [18]. Fig. 4A shows $I \cdot T$ plotted against T using the temperature indication of the thermocouple. Because the linewidth of the g 3 signal of cyto-

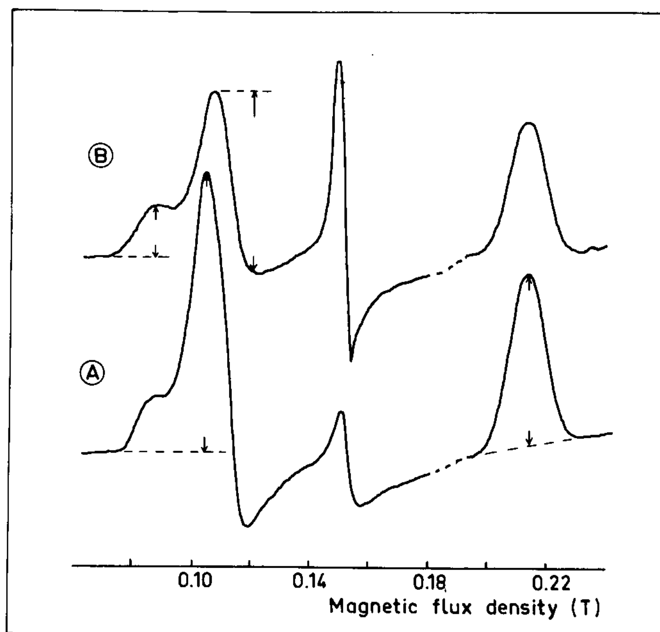

Fig. 3. EPR spectra of lipoxygenase-1 samples containing cytochrome c. A. Fe(III) yellow lipoxygenase. B. Fe(III) purple lipoxygenase. Concentrations in 0.1 M borate buffer pH 9.0: lipoxygenase 0.47 mM (A, B); 13-L-ROOH 0.44 mM (A), 1.76 mM (B); cytochrome c 0.81 mM (A), 0.64 mM (B). Microwave frequency 9.12 GHz; microwave power 2 mW; modulation amplitude 2 mT; gain $2 \cdot 10^3$ (lipoxygenase), $3.2 \cdot 10^3$ (cytochrome c); temperature 16.0 K. The signal at 0.21 T is the g 3 line of cytochrome c. The arrows indicate the way of measuring the various amplitudes.

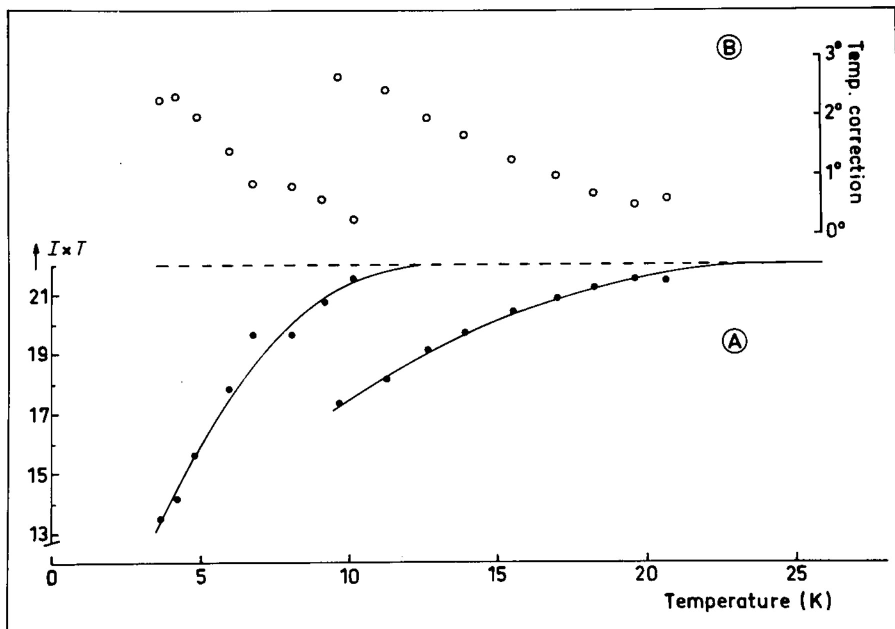

Fig. 4. A. Apparent deviation from Curie's law of the *g* 3 signal of cytochrome *c* caused by the use of the thermocouple temperature. The measurements are started at the lowest temperature keeping the flow constant and using the heating system to increase the temperature. At about 10 K the flow is decreased. The straight dashed horizontal line shows Curie's law behaviour. B. Corrections of the thermocouple temperature necessary to obtain Curie's law behaviour for the cytochrome *c* signals. Samples as described in Fig. 3B.

chrome *c* is constant below 25 K the amplitude can be taken as a measure of the signal intensity. The microwave power was always kept well below saturation.

The deviation from the straight line at low temperatures reflects the errors in the temperature indication by the thermocouple. A change in the gas flow gives a corresponding change in the temperature gradient of the cryostat as is demonstrated by the discontinuity observed at about 10 K. The constant value of $I \cdot T$ of cytochrome *c* above 20 K can be used for calculation of the correct temperatures below 20 K. In a separate experiment a calibrated carbon resistor placed inside the sample tube was used to confirm that the temperatures of the thermocouple above 20 K are reliable, thereby providing a correct value of $I \cdot T$. It was not possible to measure temperatures below 8 K in the sample tube with the carbon resistor because of instabilities probably caused by superconducting phenomena. In view of the problems arising from variations in the gas flow the addition of cytochrome *c* to the lipoxygenase samples is obligatory. Therefore, a separate calibration of the thermocouple with cytochrome *c* is not sufficient.

The observed temperatures of the thermocouple are systematically too low. The necessary corrections can be derived from a plot of $I \cdot T$ against T of the cytochrome *c* low-spin Fe(III) signal, using the thermocouple temperature (Fig. 4). The best results are obtained by correcting each point individually.

Zero-field splitting constants from temperature dependence

The $M_s = \pm 1/2$ doublet can be either the lowest or the highest of the three Kramers' doublets, the zero-field splitting constant D then being positive and negative, respectively. If the $M_s = \pm 1/2$ doublet is the lowest, the middle and the highest doublets are approx. 2 D and 6 D higher in energy, respectively. This holds provided $E << D$ and all fourth rank terms in the spin Hamiltonian are negligible. The latter seems to be true for lipoxygenase because all experimental g values are consistent with the spin Hamiltonian in Eqn. 1. Mössbauer spectroscopy [19] can in principle give more information about possible fourth-order terms. However, serious difficulties with the incorporation of ^{57}Fe in soybean lipoxygenase make such studies virtually impossible. The relation between the intensity (I) of the EPR signal and the temperature is thus given by:

$$I \propto \frac{1}{T} \cdot \frac{1}{1 + \exp(-2D/kT) + \exp(-6D/kT)} \tag{4}$$

The same equation is also valid if the $M_s = \pm 1/2$ doublet is the highest one. Typical results of the temperature dependence of the EPR signals of lipoxygenase are plotted in Fig. 5 for the rhombic and axial parts. The temperature scale was derived from the cytochrome c signal as described above. The EPR spectrum of lipoxygenase around g 6 appears to be unaltered (i.e. no change in linewidth) over the temperature range from 4 to 77 K. Therefore, amplitudes can be taken as a measure of intensities as illustrated in Fig. 3. The two different ways of measuring the amplitude of the axial signals gave the same results within experimental error.

The increase of the product of amplitude and corrected temperature at lower temperatures directly points to a positive D value. In contrast, a negative value of D has been reported by Que et al. [20] for the high-spin Fe(III) signal of protocatechuate 3,4-dioxygenase ($D \approx -2$ cm^{-1}).

The experimental data were fitted to Eqn. 3 by means of a non-linear least-squares procedure. The best fits are presented in Fig. 5 as solid lines.

For the g 4.3 signal only the upper limit of the zero-field splitting (Z) could be estimated: $Z < 3$ K. A more accurate determination would require much lower temperatures.

The results of the temperature dependence studies and the combination of both methods applied are summarized in Table I. The table contains results of measurements obtained from several samples. Although a considerable overlap between the ranges of the D values of the rhombic and axial species is found, in the same sample the D value of the latter is always smaller. The width of the range partly reflecting statistical errors must also be attributed to differences in the samples due to variations in amount and number of species building up the complex signal around g 6. The possibility of more than two species has been suggested [7] and seems to be substantiated further by the shape and g values of the spectra in Figs. 1 and 3. This is even more evident in Fig. 6 which shows the spectra of two different samples of yellow lipoxygenase prepared in the same way. The origin of this large variation in shape is as yet not clear. It is interesting to note that also for protocatechuate 3,4-dixoygenase large differences in the EPR spectra are reported upon addition of substrate to the enzyme

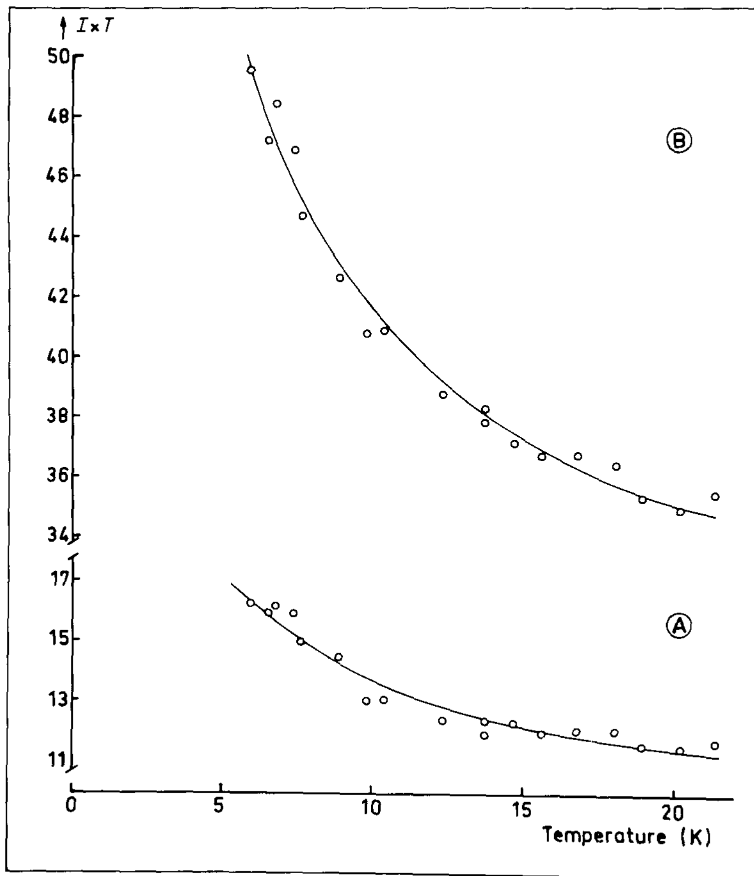

Fig. 5. Temperature dependence of the rhombic (A) and axial (B) Fe(III) species of the lipoxygenase sample described in Fig. 3B. The temperature has been corrected using the data of Fig. 4B. The solid lines are calculated with $D = 2.0$ K (A) and $D = 1.7$ K (B), respectively.

in an aerobic system, possibly due to the absence of a rigid coordination sphere for iron like porphyrin [20].

It is not possible to draw definite conclusions about the nature of the ligands of iron in lipoxygenase from the magnitude and sign of the zero-field splitting parameters. Zero-field splitting constants have been used in attempts

TABLE I

ZERO-FIELD SPLITTING CONSTANTS (D in K)

D values obtained from temperature dependence were calculated using the results from six different samples.

Species	D value determined by temperature dependence	Combination with results from g-shift method
Rhombic	$4.4 > D > 1.5$	$4.4 > D > 1.8$
Axial	$3.8 > D > 0.8$	$3.0 > D > 1.5$

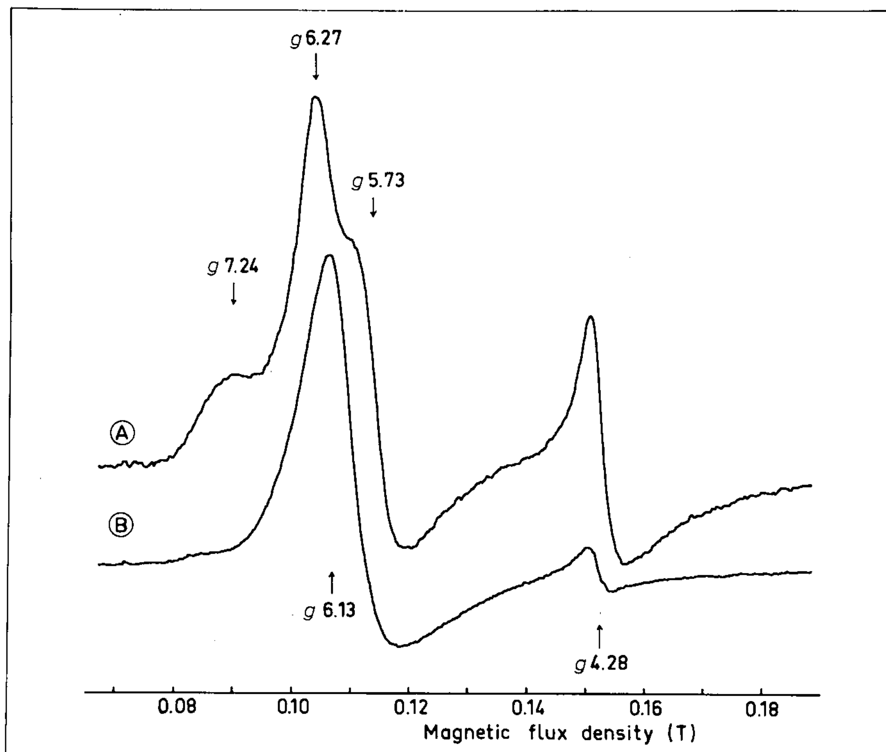

Fig. 6. EPR spectra of Fe(III) lipoxygenase demonstrating the variation in the shape of the low field signal. Concentrations in 0.1 M borate buffer pH 9.0: lipoxygenase 0.26 mM (A), 0.40 mM (B); 13-L-ROOH 0.24 mM (A), 0.38 mM (B). Microwave frequency 9.12 GHz; gain $6.3 \cdot 10^3$ (A), $2.5 \cdot 10^3$ (B); microwave power 2 mW, modulation amplitude 2 mT, temperature 15.0 K.

in identifying the ligands of iron in proteins [21] but recent work [20] on protocatechuate-3,4-dioxygenase has cast some doubt on this practice.

For quantitative EPR work, on the other hand, knowledge of the zero-field splitting parameters is obligatory. The methods presented here are applicable to all high-spin Fe(III) systems as well as to other ions having $S > \frac{1}{2}$. For lipoxygenase information on the amounts of the different Fe(III) species found in the various coloured forms of the enzyme is important for the understanding of the catalytic mechanisms. Quantitative EPR-studies on lipoxygenase based on the present results are in progress.

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