Biochimica et Biophysica Acta, 667 (1981) 77—86

BBA 38586

EPR SPECTROSCOPY OF SOYBEAN LIPOXYGENASE-1

DESCRIPTION AND QUANTIFICATION OF THE HIGH-SPIN Fe(III) SIGNALS

STEVEN SLAPPENDEL [a], GERRIT A. VELDINK [a], JOHANNES F.G. VLIEGENTHART [a], ROLAND AASA [b] and BO G. MALMSTRÖM [b]

[a] *State University of Utrecht, Department of Bio-organic Chemistry, Croesestraat 79, 3522 AD Utrecht (The Netherlands) and* [b] *Chalmers Institute of Technology and University of Göteborg, Department of Biochemistry and Biophysics, 412 96 Göteborg (Sweden)*

(Received June 18th, 1980)

Key words: Lipoxygenase-1; ESR; Simulation of line-shape; High spin Fe(III) signal; Signal intensity; (Soybean)

Summary

The interaction of soybean lipoxygenase-1 with 13-L_S-hydroperoxy-9-*cis*,11-*trans*-octadecadienoic acid, the product of the enzymic dioxygenation of linoleic acid, results in the formation of either a yellow or a purple coloured enzyme form depending on the amount of product used. The composition of the high-spin Fe(III) signals in the EPR spectra of both enzyme forms has been studied and the amount of EPR-visible iron determined by integration and simulation. Sets of g values of the species building up the high-spin Fe(III) signal around g 6 are derived from both third-order perturbation calculation and exact numerical diagonalization of the spin Hamiltonian describing the system. The results of these calculations are generally applicable to systems having $S = 5/2$. The iron in the native, colourless enzyme is almost EPR-nondetectable. The yellow form of the enzyme shows a complex EPR signal around g 6 which consists of contributions of at least three species with different ligand symmetry. The signal corresponds to approx. 75% of the total iron content. The g 6 signal of the purple Fe(III) enzyme corresponds roughly to the same amount of iron but the ratio between the different species is not the same as in the yellow enzyme. This enzyme form also shows an additional g 4.3 signal with a large amplitude but a relatively low integrated intensity (approx. 10% of the total iron content). The results are consistent with the suggested mechan-

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Abbreviation: 13-HPOD, 13-L_S-hydroperoxy-9-*cis*,11-*trans*-octadecadienoic acid.

ism of the catalytic function of iron in lipoxygenase which was based on qualitative EPR results (De Groot, J.J.M.C., Veldink, G.A., Vliegenthart, J.F.G., Boldingh, J., Wever, R. and van Gelder, B.F. (1975) Biochim. Biophys. Acta 377, 71—79).

Introduction

Lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12) is a non-heme iron dioxygenase catalyzing the hydroperoxidation of polyunsaturated fatty acids with a methylene-interrupted *cis,cis*-diene system, like linoleic acid [1]. 13-L$_S$-Hydroperoxy-9-*cis*,11-*trans*-octadecadienoic acid (13-HPOD) is formed almost exclusively (95% [2]) upon incubation of soybean lipoxygenase-1 with linoleic acid at pH 9.0. In an anaerobic system this enzyme is capable of converting 13-HPOD to a variety of products including oxodienoic acids and epoxyhydroxy-enoic acids [3]. If both linoleic acid and 13-HPOD are present in the reaction medium, dimerisation products of radicals derived from these substrates are formed in addition to oxodienoic acids and *n*-pentane [4].

Soybean lipoxygenase-1 (M_r 98 500) contains one atom of iron per molecule [5—7]. The involvement of iron in the catalytic activities of lipoxygenase has been demonstrated by EPR [8,9]. The native, colourless and virtually EPR-nondetectable enzyme is converted into a yellow form by addition of a molar equivalent of 13-HPOD [10]. This enzyme form shows a complex EPR signal around *g* 6, which has been attributed to at least two high-spin Fe(III) species with different symmetry [8]. Upon addition of linoleic acid to the yellow enzyme in an anaerobic system a colourless enzyme form is obtained, this being almost EPR-nondetectable, as is the native enzyme. A purple enzyme form is obtained when a molar excess of 13-HPOD is added to the yellow enzyme [11]. The signal around *g* 6 in the EPR spectrum of the purple enzyme has changed compared to the corresponding signal of the yellow enzyme and also a strong signal at *g* 4.3 is present, which has been attributed to high-spin Fe(III) in a ligand field of rhombic symmetry [12,13].

Based on qualitative EPR results, De Groot et al. [8] have proposed a reaction scheme in which iron is switching between the ferric and ferrous state during catalysis. For a more stringent test of the proposed functional role of iron knowledge of the amount of iron visible in the EPR spectra of the different enzyme forms is obligatory. So far, only a preliminary estimation of the amount of EPR-visible iron has been reported [9] without, however, having available the zero-field splitting constants necessary for the calculation. We have recently determined the zero-field splitting constants of soybean lipoxygenase-1 [14], and in this paper we describe the structure of the EPR signals in more detail and present the results of a quantitative EPR study using integration and simulation procedures.

Materials and Methods

Lipoxygenase-1 from soybeans was isolated according to Finazzi-Agrò et al. [5], with metal chelators present in all purification steps [15]. Specific activ-

ity: 235 μmol $O_2 \cdot min^{-1} \cdot mg^{-1}$, corresponding to 3.92 μkat $\cdot mg^{-1}$. For the determination of iron both atomic absorption spectroscopy and a colorimetric method [16] were used: 0.97 ± 0.05 mol Fe per mol enzyme. The amounts of Mn and Cu were found to be 0.07 and 0.006 mol per mol enzyme, respectively, as determined by atomic absorption. Linoleic acid was obtained from Lipids Supplies (St. Andrews University, St. Andrews, U.K.) and 13-HPOD was prepared and purified according to the method of Verhagen et al. [17].

EPR spectra at 9.12 GHz were recorded on a Varian E-9 spectrometer with 100 kHz field modulation. All measurements were carried out with a modulation amplitude of 2 mT, at a temperature of 15 K using an Oxford Instruments liquid helium cryostat [14]. Unless stated otherwise, a microwave power of 2 mW was used. Integrations and simulations of the EPR spectra were performed with a Nova 3 minicomputer using methods described earlier [18,19]. As references a Cu^{2+} solution in 2 M $NaClO_4$, pH 2, and a solution of human serum transferrin (an iron-transporting protein) [20] were used, always recorded at non-saturating conditions.

Theory

The EPR spectrum of high-spin Fe(III), like that obtained from lipoxygenase, can be described by the spin Hamiltonian

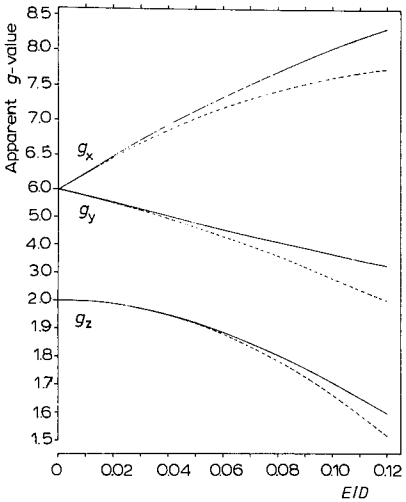
$$H = D[S_z^2 - \tfrac{1}{3}S(S+1)] + E[S_x^2 - S_y^2] + g\mu_B \vec{B} \cdot \vec{S} \qquad (1)$$

where $S = 5/2$, g approx. 2.00 and D and E are the axial and rhombic zero-field splitting constants, respectively. The six low-lying isolated energy levels of the high-spin Fe(III) form three non-degenerated doublets (Kramers' doublets). High-spin Fe(III) in a strong ligand field of axial symmetry gives EPR signals in the $g = 6$ region arising from the doublet having $M_s = \pm\frac{1}{2}$. If $D >> E$ and $D >> g\mu_B B$, quantum mechanical perturbation calculation can be applied to determine the apparent g values. Using the spin Hamiltonian (Eqn. 1) such a calculation brought up to third order gives

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

$$g_z = g\left[1 - \frac{152}{9}\left(\frac{E}{D}\right)^2\right]$$

If the conditions for the perturbation calculation are not fulfilled, i.e. E and/or $g\mu_B B$ are not very small compared to D, an exact numerical diagonalization of the spin Hamiltonian (Eqn. 1) has to be made. The apparent g values for the $M_s = \pm\frac{1}{2}$ doublet obtained by both third-order perturbation calculation and by an exact treatment are presented as a function of E/D in Fig. 1. A similar plot using only perturbation calculation has been derived by Wickman et al. [21]. Fig. 1 shows g values calculated with D approx. 3 K [14] and B corresponding to X-band microwave frequency (9 GHz), i.e. $g\mu_B B/D = 0.03$—0.06. This is, however, not a very serious limitation for the use of Fig. 1. If $D > 2.2$ K, the g values shown in Fig. 1 (exact treatment) are correct within 1‰ for g_x and within 1% for g_y and g_z. As an example of the use of Fig. 1, all three observed g values



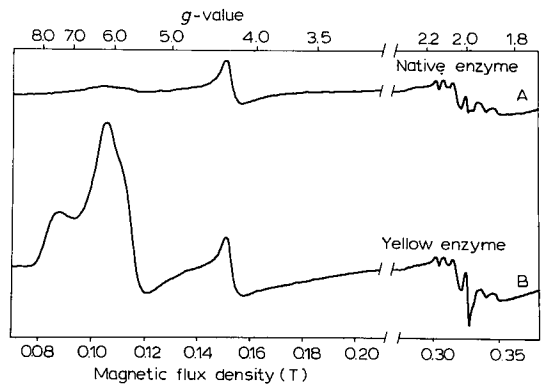


Fig. 1. Apparent g values within the $M_S = \pm\frac{1}{2}$ doublet for an $S = 5/2$ ion in a strong, nearly axial ligand field. The curves are obtained from the spin Hamiltonian (Eqn. 1) by an exact numerical diagonalization (———) or a third-order perturbation calculation (- - - - -). Note the changes of the scale on the ordinate.

Fig. 2. EPR spectra of native and yellow lipoxygenase-1. A. Native lipoxygenase: 0.54 mM in 0.1 M borate buffer pH 9.0. B. Yellow lipoxygenase: 2.8 µl of a 13-HPOD solution (44.3 mM) was added to 250 µl of a native lipoxygenase solution (0.54 mM); final concentrations: 0.49 and 0.53 mM, respectively, in 0.1 M borate buffer pH 9.0.

(8.0, 3.7, 1.7) for high-spin cytochrome *P*-450 [22] can be obtained from this graph for $E/D = 0.10$.

We have used Fig. 1 for the assignment of the EPR signals to different species of lipoxygenase. Thus, in this paper, with only one exception, all sets of g values used for the simulation of the EPR spectra of lipoxygenase are consistent with those derived from Fig. 1.

Results and Discussion

Description of the signals

The low-field part of the EPR spectrum of native lipoxygenase, presented in Fig. 2A, shows in addition to the g 4.3 signal a small EPR feature around g 6 attributed to high-spin Fe(III) in an axial ligand field. The conversion of the native into yellow enzyme upon addition of a molar equivalent of 13-HPOD results in the formation of a complex signal around g 6 (Fig. 2B). EPR lines at g 7.4, 6.2 and a shoulder at approx. g 5.8 are observed, which are attributed to high-spin Fe(III) [8]. Based on the effect of oxygen on the intensities of the resonances at g 6.2 and those at g 7.4 and 5.8 De Groot et al. [8] postulated that this signal is built up of at least two species with different symmetry. For the least axial species having g_x 7.4, Fig. 1 gives g_y 4.5 and, therefore, the signals at g 7.4, 6.2 and 5.8 cannot stem from merely one species. The signals at g 6.2 and 5.8 might then be g_x and g_y of the same species, respectively. However, upon increasing the microwave power the shoulder at

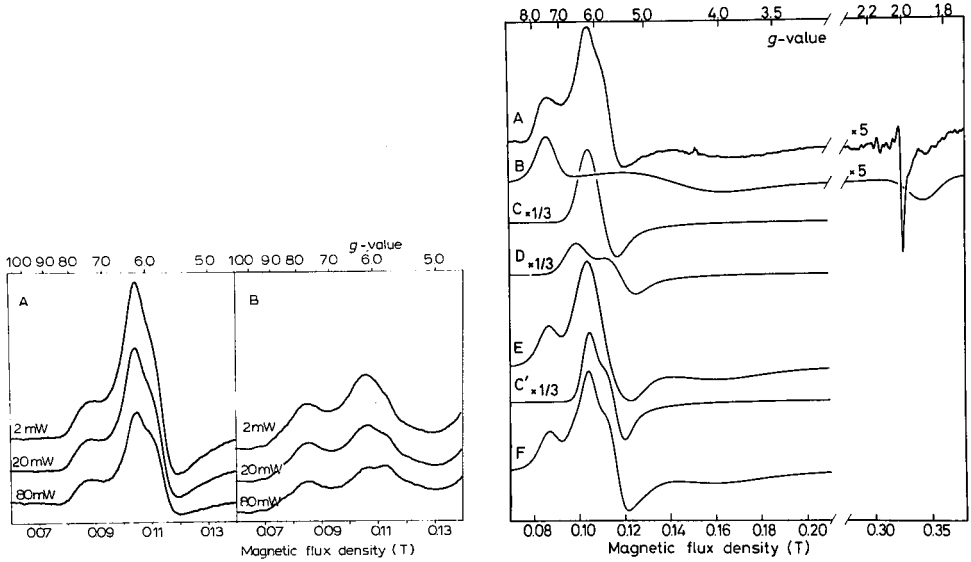


Fig. 3. Effect of the microwave power on the signals around g 6. A. Yellow lipoxygenase B. Purple lipoxygenase. Concentrations in 0.1 M borate buffer pH 9.0: lipoxygenase 0.63 mM (A, B); 13-HPOD 0.59 mM (A) and 2.36 mM (B). The gain has been corrected for the change in microwave power.

Fig. 4. Experimental and simulated EPR spectra of yellow lipoxygenase. A. Spectrum of yellow lipoxygenase obtained by subtraction of spectrum A from B (Fig. 2). B, C, D and C′. Simulated spectra giving the sum spectra E (B + C + D) and F (B + C′ + D). g values, linewidths and relative weights are presented in Table I. Unless stated otherwise, the simulated spectra of the components are recorded with a gain making their total integrated intensities identical.

g 5.8 becomes more pronounced (Fig. 3), a behaviour pointing to the presence of more than one species contributing to the signals at g 6.2 and 5.8. This implies that the complex signal around g 6 consists of at least three species with different symmetry.

In order to obtain more information on the number, nature and amount of different species building up the signal around g 6, the g 4.3 signal and the signals around g 2 are eliminated by subtraction of the spectrum of the native enzyme from that of the yellow enzyme. The difference spectrum is presented in Fig. 4A. For the simulation of this spectrum three species with dif-

TABLE I

SIMULATION DATA OF YELLOW LIPOXYGENASE

The letters refer to the spectra presented in Fig. 4.

Component	g value			Linewidth (mT)			Relative weight	
	x	y	z	x	y	z	Spectrum E	Spectrum F
1 (B)	7.35	4.55	1.88	7	35	25	1.00	1.00
2 (C)	6.20	5.80	2.00	8	9	25	0.40	
2′ (C′)	6.20	5.60	2.00	5.5	6.5	25		0.40
3 (D)	6.55	5.45	1.98	9	10	25	0.45	0.50

ferent symmetry had to be used to obtain a reasonable fit (Fig. 4). The g values, linewidths and relative weights of each of the components are listed in Table I. The g_z part near g 2 of the signal of the high-spin Fe(III) species having least axial symmetry has also been simulated. The computed spectrum agrees well with the corresponding part of the difference spectrum (Fig. 4A and 4B). The radical type of signal at g 2 makes it impossible to compare experimental and simulated g_z parts of the other species. The shape of the signal around g 5.8 in the experimental spectrum is somewhat peculiar. Simulation of the shoulder at g 5.8 is not possible with theoretically determined sets of g values. However, this shoulder can be simulated by using spectrum C′ instead of spectrum C, leading to the sum spectrum F, which agrees better with the experimental spectrum A (Fig. 4). Spectrum C′ has g_x and g_y equal to 6.2 and 5.6, respectively, which give a mean value of 5.9. Small deviations from the theoretical value of 6.0 (cf. Fig. 1) have been observed earlier (see, for example, Ref. 18).

The spectrum of yellow lipoxygenase can show different shapes upon addition of 1 molar equivalent of 13-HPOD [14]. An 'axial' type of spectrum of the yellow enzyme at pH 7 is presented in Fig. 5A. Almost the same type of spec-

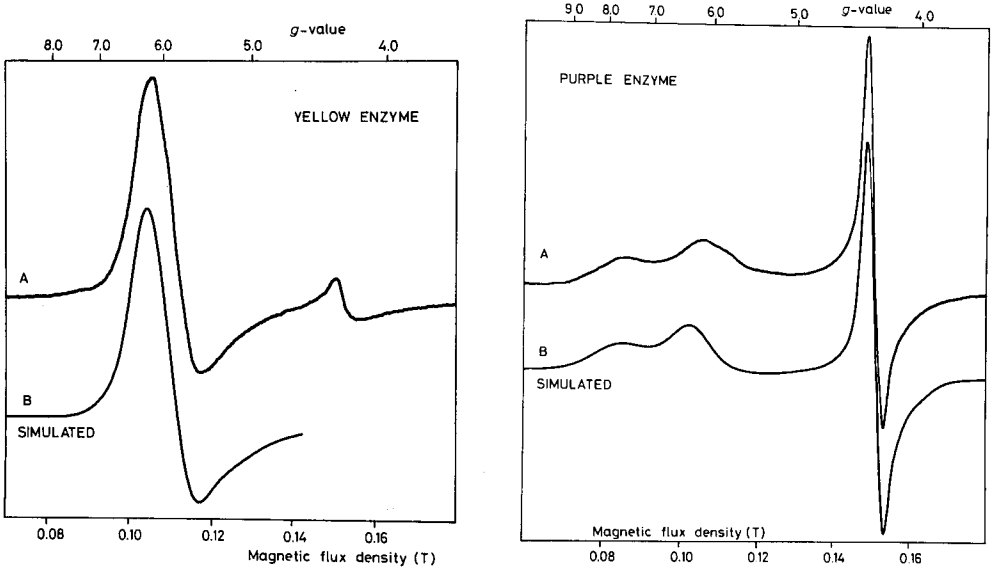


Fig. 5. 'Axial' type of EPR spectrum of yellow lipoxygenase-1. A. Experimental spectrum. Concentrations in 0.1 M phosphate buffer pH 7.0: lipoxygenase 0.49 mM; 13-HPOD 0.45 mM. B. Simulated spectrum obtained by addition of two components. The g values, linewidths and relative weights of the components are: (6.20, 5.80, 2.00), (7, 8, 25) mT, 2.0; and (6.55, 5.45, 1.98), (10, 12, 25) mT, 1.0, respectively.

Fig. 6. Experimental and simulated EPR spectra of purple lipoxygenase. A. Experimental spectrum. 8.4 μl of a 13-HPOD solution (44.3 mM) was added to the sample described in Fig. 2B. Final concentrations in 0.1 M borate buffer, pH 9.0: lipoxygenase 0.52 mM; 13-HPOD 1.90 mM. B. Simulated spectrum obtained by addition of three 'g 6' components and three 'g 4' components. The g values, linewidths are relative weights of the 'g 6' components are (7.55, 4.25, 1.83), (12, 35, 25) mT, 1.0; (6.20, 5.80, 2.00), (8, 20, 25) mT, 0.25; and (6.55, 5.45, 1.98), (12, 20, 25) mT, 0.1, respectively. The 'g 4' components are (4.24), (12) mT, 0.055; (4.29), (6) mT, 0.027; and (4.32), (3.5) mT, 0.022, respectively. The latter three components are taken as isotropic and therefore only one figure for the g value and linewidth is used.

trum without any g 7.4 feature has also been observed for yellow lipoxygenase at pH 9 prepared in the same way as described in Fig. 2B. The origin of the large variation in shape of the signal is not yet clear, but pH, concentration of oxygen [8] and conversion products of 13-HPOD might have an influence. A study of the factors determining the shape of the spectrum is in progress. The simulation of the 'axial' type of spectrum as shown in Fig. 5B is a sum spectrum of only two components having g values identical with those of the components C and D in Fig. 4 and having almost the same linewidths.

The EPR spectrum of purple lipoxygenase obtained by addition of 3 mol-equiv. of 13-HPOD to the yellow enzyme shows a decreased axial part of the signal around g 6 and a strongly increased signal at g 4.3 compared to the spectrum of yellow lipoxygenase (cf. Figs. 2B and 6A). The g 4.3 signal stems from the middle Kramers' doublet of high-spin Fe(III) in a so-called rhombic environment [13]. Contributions to the EPR spectrum of transitions in the other doublets as observed for protocatechuate 3,4-dioxygenase, another non-heme dioxygenase [13,23], are visible at approx. g 9.6 if a high gain is used (Fig. 3B). A simulated spectrum of the purple enzyme is presented in Fig. 6B. For the g 6 part essentially the same three species as in the simulation of the spectrum of the yellow enzyme (Fig. 4) are used but with different relative weights. The g 4.3 signal is also simulated in order to determine the amount of Fe(III) visible in that signal (see below). The number of species used for the simulations is kept limited, although indications are present for more species, for instance the larger amplitude above the baseline at about 0.12 T in spectrum A compared to spectrum B (Fig. 6).

Determination of EPR-visible iron

For the calculation of the amount of EPR-visible iron the signal intensity and the population of the doublet which gives rise to the EPR signal must be known. The most straightforward method for the determination of the signal intensity in an EPR derivative spectrum should be double integration of the total spectrum. However, only for the g 4.3 signal in purple lipoxygenase double integration is possible because this signal is sufficiently large and also

TABLE II

EPR-VISIBLE IRON IN LIPOXYGENASE-1

The amount of iron visible in the EPR spectra is given as a percentage of the total iron content. The percentages are representative for at least two samples.

Enzyme form	Spectrum	Signal around g 6		Signal at g 4.3 *	
		Simulation	Integration	Simulation	Integration
Native	Fig. 2A	<1		0.1	
Yellow	Fig. 2B			0.6	
	Fig. 4E	81			
	Fig. 4F	80			
Yellow 'axial' type	Fig. 5	68 **	73 **	0.5	
Purple	Fig. 6	76		8	13

* Values are corrected for the signal of an impurity in the EPR cavity and quartz dewar (90% of the signal shown in Fig. 2A). Simulations for the native and yellow enzyme forms are not shown.

** Calculated using D = 2.3 K, mean D value for axial species (Table I in Ref. 14).

separated from other resonances (Fig. 6A). The signal intensity can also be obtained by simulation of the spectrum (Fig. 6B). The population of the doublet can be calculated from the zero-field splitting constants. For this reason we have recently determined the zero-field splitting constants of lipoxygenase-1 using the temperature dependence of the signal intensity and the microwave frequency dependent shift of g values [14]. Concerning the g 4.3 signal, an upper limit of 3 K of the zero-field splitting (Z) has been found [14]. This value implies that the population of the middle doublet is practically equal to 1/3 of the total population at the temperature used. Cu(II) can be employed as a reference, but an Fe(III) solution giving a signal around g 4.3 is probably a better choice. Therefore we have used Fe-transferrin which has a small zero-field splitting [20] and in which all iron is EPR detectable [19]. The results of the determination of the amount of EPR-visible iron using both integration and simulation are presented in Table II. In both the native and yellow enzyme (Figs. 2 and 5) the amount of EPR-visible iron at g 4.3 is small (less than 1% of the total iron content), probably arising from contaminating iron in the enzyme solution or denaturated enzyme. The g 4.3 signal of the purple enzyme (Fig. 6) also represents only a relatively small amount of the total iron content (8—13%), despite the impressive amplitude of the signal.

The complexity and anisotropy of the high-spin Fe(III) signals of lipoxygenase around g 6 and the presence of traces of manganese and copper limit the application of a double integration procedure for the total spectrum and make other methods necessary. The amount of iron contributing to the signal around g 6 has been determined by three methods:

(1) For signals having an axial character like that shown in Fig. 5, the signal around g 6 was integrated by a semi-empirical method [19].

(2) In case the g 7.4 peak of the least axial species is present (Figs. 2B, 4 and 6) an integration measuring the area under a separated peak [19] was carried out thus yielding an estimate of the amount of iron visible in that part of the signal around g 6 (i.e. the component shown in Fig. 4B).

(3) The calculated intensity of a simulated spectrum was used for the determination of the amount of EPR-visible iron.

The population of the lowest doublet ($N_{M_S=\pm\frac{1}{2}}$) responsible for the signals around g 6 can be calculated from Eqn. 2

$$N_{M_S=\pm\frac{1}{2}} = N_{\text{total}} \cdot [1 + \exp(-2D/kT) + \exp(-6D/kT)]^{-1} \qquad (2)$$

This equation is based on a Boltzman distribution assuming that the middle and the highest doublets are $2D$ and $6D$ higher in energy, respectively, where D is the zero-field splitting constant recently reported [14].

The intensities of the high-spin Fe(III) signals around g 6 are summarized in Table II. In all cases a considerable amount of iron is EPR detectable (68—89%). This indicates that earlier described qualitative EPR work [8,9,11] dealt with major enzyme forms. Interestingly, there is no significant difference in the total amount of EPR-visible iron in the yellow and purple enzyme forms. The results obtained from the integration and simulation procedures are consistent within experimental error (Table II and Fig. 5). Comparing the amounts of EPR-visible iron for Figs. 4E and 4F in Table II shows that the applied refine-

ment of the simulation does not lead to a significant change in the amount of EPR-visible iron. Despite the large variation in the shape of the g 6 signal in the EPR spectrum of the yellow form (cf. Figs. 4 and 5) there is no large difference in the amount of EPR-visible iron. The amounts of iron visible in the EPR signal at approx. g 7.4 were determined for both yellow and purple lipoxygenase (Figs. 4 and 6) by integration and simulation as described above. The two methods agreed and consistently gave a larger amount for the g 7.4 signal in the purple enzyme (53% of the total iron content compared to 40% in the yellow form, using D = 3.1 K, mean D value for rhombic species [14]). The larger amount of iron visible in the rhombic signal in the purple enzyme must correspond to a decrease in the axial part because the sum of the rhombic and axial part is almost the same for both enzyme forms (Table II).

The errors in the determinations of the EPR-visible iron are considered to be rather large (at least 10%). One reason is that the complexity of the EPR spectra of lipoxygenase makes it difficult to achieve a perfect fit of experimental and simulated spectra if the number of different components is kept reasonably small. Also the uncertainty in the D value (unless stated otherwise a mean D value of 2.7 K is used [14]) leads to errors in the determination of the amount of EPR-visible iron. By using the extreme values of D, i.e. axial species 1.5—3.0 K; rhombic species 1.8—4.4 K [14], a deviation of about 10% in the amount of EPR-visible iron is obtained. The figures for the total amount of EPR-visible iron seems consistently to fall slightly below 100%. A possible reason might be that radicals form diamagnetic complexes with Fe(III), making part of the iron EPR invisible. The occurrence of radicals in the conversion of linoleic acid and 13-HPOD catalyzed by lipoxygenase has been suggested [3,8, 24] and under anaerobic conditions the presence of linoleic acid radicals has been reported [25]. A radical type of signal at g 2 is always observed in the EPR spectra of yellow and purple lipoxygenase (Fig. 2B and Refs. 8 and 11). We have determined the concentration of the radical by double integration of a more expanded EPR spectrum and found it to be less than 3% of the protein concentration. However, from this figure no definite conclusion about any iron-radical interaction can be drawn.

In conclusion, the findings on the total amount of EPR-visible iron as derived from integration and simulation procedures are consistent with the hypothesis of De Groot et al. [8] concerning the functional role of iron in the enzyme catalysis.

Acknowledgements

We would like to thank Professor Tore Vänngård for many useful discussions and Mr. F.J. Slikkerveer (Unilever Research Laboratory, Vlaardingen, The Netherlands) and Dr. L.-G. Danielsson (Chalmers Institute of Technology, Department of Analytical Chemistry) for performing the atomic absorption analysis and Håkan Mattsson for the chemical iron-determination. This work was supported by funds from the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO), from the Swedish Natural Science Research Council and by a short-term EMBO fellowship to S.S.

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