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Magnetic susceptibility studies on yellow and anaerobically substrate-treated yellow soybean lipoxygenase-1

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The magnetic susceptibilities of yellow soybean lipoxygenase-1 before and after anaerobic reduction with the substrate linoleic acid have been studied over the temperature range 10–170 K. Theoretical temperature dependencies of the magnetic susceptibility, calculated by means of a spin Hamiltonian, have been fitted to the experimental data. The conversion of native into yellow enzyme (involving iron oxidation) by (13*S*)-hydroperoxy-9*Z*,11*E*-octadecadienoic acid ((13*S*)-HPOD) was found by EPR and light-absorption studies to be progressively less effective at increasing enzyme concentrations. A theoretical model could be fitted to the experimental susceptibility data of a yellow sample by assuming the EPR-invisible iron fraction to contain high-spin Fe(II) with its zero-field splitting parameter *D* in either of the ranges 8 ± 3 cm^{-1} or -6.5 ± 1.5 cm^{-1}. Our results indicate a ligand environment of axial symmetry, probably with a rhombic distorsion, and suggest that the EPR-invisible iron fraction has remained Fe(II), just like in the native enzyme. There is no evidence that the EPR-invisible iron is due to high-spin Fe(III) antiferromagnetically coupled to a radical. Our results for the anaerobically substrate-treated yellow lipoxygenase are consistent with the presence of high-spin Fe(II) with *D* in either of the ranges $12.5^{+1.5}_{-2}$ cm^{-1} or -12^{+3}_{-2} cm^{-1}. It is concluded that linoleic acid can reduce the iron of yellow lipoxygenase. The present results are definite evidence for a valence change during the anaerobic reaction. Reduction of iron by linoleic acid is possibly also a feature of the dioxygenation reaction.

Introduction

Lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12) is a non-heme iron dioxygenase which catalyzes the dioxygenation of polyunsaturated fatty acids containing a 1*Z*,4*Z*-pentadiene system. Lipoxygenase-1 from soybeans catalyzes the almost exclusive formation of (13*S*)-hydroperoxy-9*Z*,11*E*-octadecadienoic acid ((13*S*)-HPOD) from 9*Z*,12*Z*-octadecadienoic acid (linoleic acid) [1]. Under anaerobic conditions, and in the presence of both linoleic acid and its primary reaction product, lipoxygenase-1 catalyzes the formation of 13-oxo-9*Z*,11*E*-fatty acids, fatty acid dimers, with or without epoxy groups, and *n*-pentane [2,3]. During this reaction, linoleyl radicals can be trapped [4].

Native lipoxygenase-1 is colourless and virtually EPR-silent. Addition of an equivalent amount of (13*S*)-HPOD yields an active yellow enzyme

Abbreviations: EPR, electron paramagnetic resonance; (13*S*)-HPOD, (13*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid.

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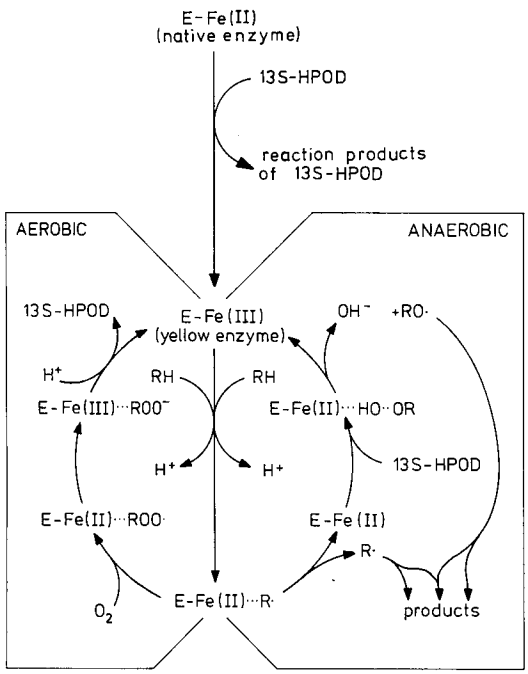
species with a light-absorption shoulder around 330 nm, and EPR signals around $g = 6$, stemming from high-spin Fe(III) [5,6]. The intensities of these spectroscopic features were found to be linearly correlated with the amount of (13*S*)-HPOD added, for additions up to 1 molar equivalent and enzyme concentrations below 0.4 mM [7]. The yellow species is in turn converted to a colourless EPR-silent form upon anaerobic addition of linoleic acid [5,8].

On the basis of the experimental evidence, reaction mechanisms were proposed for both the aerobic and the anaerobic reaction, involving a shuttling of the valence state of iron in lipoxygenase between Fe(II) and Fe(III) (see Scheme I) [5]. The reaction of the yellow enzyme species with linoleic acid plays a key role in both reaction mechanisms. It was proposed to involve the formation of a linoleyl-radical by hydrogen abstraction at *C*-11 of linoleic acid and a concomitant reduction of iron. Hydrogen abstraction has previously been shown to be the rate-limiting step of the overall reaction [9].



Scheme I. Proposed mechanism for the aerobic and anaerobic reactions catalyzed by soybean lipoxygenase-1, adapted from Ref. 5, suggesting a key role for the reduction step of yellow enzyme with linoleic acid in the catalytic cycle. RH = linoleic acid; R˙ = linoleyl radical; RO˙ = (13*S*)-HPOD alkoxy radical; ROO˙ = (13*S*)-HPOD peroxy radical; ROO^- = (13*S*)-HPOD peroxy anion.

In the presence of dioxygen the catalytic cycle is completed with the oxygenation of the linoleyl radical, and the regeneration of the Fe(III)-containing yellow form. Under anaerobic conditions the reaction proceeds to a stage, in which the iron is present either as Fe(II) or Fe(III), depending on the relative amounts of linoleic acid and (13*S*)-HPOD originally present. If linoleic acid prevails a reduced Fe(II)-containing enzyme form results.

In recent years magnetic studies have clarified the nature of some lipoxygenase-1 species. EPR-spectroscopic studies show that the high-spin Fe(III) population of the yellow enzyme species is heterogeneous with respect to the symmetry of the ligand environment. The values of the zero-field splitting parameter, *D*, describing axial distortion, were estimated to be in the range 1.0–3.1 cm^{-1} [10]. In all quantitative EPR studies, the amount of EPR-visible iron was found to be lower than 100% [7,11]. It was suggested that formation of diamagnetic complexes between radicals and Fe(III) could explain the presence of an EPR-invisible iron fraction.

Using a highly sensitive magnetic balance of the Faraday type [12,13] and measuring the temperature dependence of the magnetic susceptibility, Slappendel et al. [14] showed that the native enzyme contains high-spin Fe(II). This finding was corroborated by ^{1}H-NMR studies on the proton relaxation enhancement resulting from the interaction of the iron in native lipoxygenase-1 with the protons of *n*-butanol [14], and confirmed by Cheesbrough and Axelrod [15], who studied the shift induced in the NMR signal from the methyl protons in methyl α-D-glucoside. The latter authors also found that yellow lipoxygenase-1 contains high-spin Fe(III), which is in agreement with results from EPR spectroscopy [5,11]. Petersson et al. [16] found the temperature dependence of the susceptibility between 10 and 170 K to be compatible with a *D* value in either of the ranges 8.5–11.9 cm^{-1} or -6.9– -5.5 cm^{-1} for the high-spin Fe(II) in native lipoxygenase-1. A comparison of results from oxygenated and deoxygenated native enzyme indicated that dioxygen does not significantly af-

fect the electronic state of the iron. Feiters et al. [17] showed that the deoxygenated enzyme does not produce the EPR signal typical of the reaction with (13*S*)-HPOD, when reacted with 1 molar equivalent of linoleic acid, and thus ruled out the possibility that dioxygen is coordinated very strongly.

In this paper, a magnetic susceptibility study on both yellow and anaerobically substrate-treated yellow lipoxygenase is presented. We report a partial conversion of the native enzyme into the yellow enzyme by (13*S*)-HPOD at high enzyme concentrations (0.5–2.0 mM). The degree of conversion is found to be dependent on the enzyme concentration as concluded from light absorption and EPR studies. Our results clarify the valence and spin states of iron in the different enzyme species. The anaerobically substrate-treated yellow lipoxygenase is found to contain high-spin Fe(II), verifying that linoleic acid can efficiently reduce the iron. Information on the symmetry of the iron environment is obtained through determination of the zero-filed splitting parameters. Implications for the reaction mechanism of lipoxygenase are discussed.

Materials and Methods

Soybean lipoxygenase-1 was isolated according to Slappendel [18], dialyzed against 0.1 M sodium borate buffer (pH 9.0), and concentrated in a collodion bag SM 13200 (Sartorius-Membranfilter, Göttingen, F.R.G.) to a concentration of 1.55 mM for the susceptibility measurements. The enzyme concentration was determined from the absorbance at 280 nm, using $A_{280}^{0.1\%} = 1.6$, with an estimated accuracy of $\pm 5\%$. Dilution effects in sample preparation due to addition of (13*S*)-HPOD or linoleic acid were taken into account. The native enzyme contained 0.97 mol iron and 0.07 mol manganese per mol enzyme [11]. The EPR-detectable high-spin Fe(III) in the native enzyme, 0.01 mol per mol enzyme [11], as well as the manganese were conceived as contaminants.

The specific activity, measured after the concentration procedure, was 240 μmol O_2/min per mg protein, as determined polarographically with a Clark electrode mounted on a Gilson 5/6 Oxygraph maintained at 25 °C. Ammonium linoleate in air-saturated 0.1 M sodium borate buffer (pH 9.0) was used as the substrate.

(13*S*)-HPOD was prepared by incubation of lipoxygenase-1 with linoleic acid (Lipid Supplies, St. Andrews University, St. Andrews, U.K.) and purified by high-performance liquid chromatography (HPLC) [19]. Before addition to lipoxygenase-1, (13*S*)-HPOD and linoleic acid were dissolved in 1.0 M ammonia. This solvent was preferred to ethanol, because the latter had been reported to influence the shape of the EPR spectrum of yellow lipoxygenase [20]. Mixing of borate buffer (pH 9.0) and 1.0 M ammonia in the same ratio as in our experiment typically causes the pH to rise 0.6 unit. In this pH range, the shape of the EPR spectrum and the specific activity do not change [19,20]. Furthermore, the protein is expected to have an additional buffering effect.

The yellow enzyme sample for the susceptibility measurements was prepared aerobically by initially adding 1.1 molar equivalents of (13*S*)-HPOD to the concentrated native enzyme, and subsequently small amounts until the absorbance at 330 nm no longer increased. The total amount of (13*S*)-HPOD added was 1.65 molar equivalents. After magnetic susceptibility measurements and thawing, the same sample was deoxygenated at 4 °C. The deoxygenation was performed directly in the sample holder by flowing humidified pure argon over the sample surface for about 40 min, while stirring [12,13]. Deoxygenated twice-distilled water was used in the gas wash bottles. The substrate-treated yellow enzyme form was prepared by adding 1.20 molar equivalents of linoleic acid under anaerobic conditions.

The yellow lipoxygenase samples used in the spectroscopic studies were prepared by adding 1.04 ± 0.06 molar equivalents of (13*S*)-HPOD to solutions of the native enzyme at different concentrations. Parallel samples were used for the EPR and light-absorption measurements.

Magnetic susceptibility measurements were carried out with a sensitive magnetic balance of the Faraday type [12,13]. EPR spectra at 15 K were recorded on a Varian E-9 X-band spectrometer equipped with an Oxford Instruments helium-flow cryostat. A 100 kHz field modulation was used. The amount of EPR-visible iron was determined by integration and simulation of the spectrum as

described previously [11,21]. The light absorbance at 330 nm was measured with a HP 8450A spectrophotometer.

Theory

Calculation of magnetic susceptibilities

An interpretation of the experimental susceptibility data in terms of ligand field symmetry requires theoretical temperature dependences for the susceptibilities of both high-spin Fe(II) and high-spin Fe(III). In such calculations expressions for the electronic energy levels of the iron are needed. The spin Hamiltonian formalism offers a convenient method to describe the electronic ground state multiplets by means of a small number of parameters. It was adopted in the present case on similar grounds as previously given for the iron of the native enzyme [16]. Thus the ground-state energy levels were obtained for both iron forms by means of the spin Hamiltonian

$$\mathcal{H} = D\left[S_z^2 - \tfrac{1}{3}S(S+1)\right] + E\left[S_x^2 - S_y^2\right] + g\beta\vec{H}\cdot\vec{S}$$

where D and E are the axial and rhombic zero-field splitting parameters, respectively, g the electronic g value, β the Bohr magneton, H the applied magnetic field and S the fictitious spin quantum number. The basis set $|M_s\rangle$ was used in the calculations with $M_s = 2,\ 1,\ 0,\ -1,\ -2$ for Fe(II) and $M_s = 5/2,\ 3/2,\ 1/2,\ -1/2,\ -3/2,\ -5/2$ for Fe(III). The energies in either case were first calculated with the applied magnetic field parallel to the z-axis, using Cardano's method [22,23] to obtain exact solutions of the cubic determinant equations involved. The energies with H parallel to the x- and y-axes were determined by rotation of the coordinate system as described by Orton [24].

The molar susceptibility for a transition metal ion in an environment of low symmetry is dependent on its orientation in the applied field. With the field along any of the principal axes the susceptibility is obtained from the electronic energy levels, E_{ij}, through

$$\chi^j_{\text{mole}} = \frac{N}{H}\frac{\sum_{i=1}^{n} -\frac{\partial E_{ij}}{\partial H} e^{-E_{ij}/kT}}{\sum_{i=1}^{n} e^{-E_{ij}/kT}}$$

where $j = x,\ y,\ z$. $\boldsymbol{k}$ is the Boltzmann constant, T the absolute temperature and N Avogadro's number. The summation should be done for all levels of the ground state multiplet. Thus $n = 5$ for high-spin Fe(II) and $n = 6$ for high-spin Fe(III).

For a frozen solution with randomly oriented molecules an averaged susceptibility value is required. For each species this was approximated as

$$\chi^{\text{ave}}_{\text{mole}} = \tfrac{1}{3}\left[\chi^x_{\text{mole}} + \chi^y_{\text{mole}} + \chi^z_{\text{mole}}\right]$$

which is strictly valid only when $\beta H/kT \ll 1$. The approximation has been analyzed in the specific case of a d^5 high-spin system in D_{4h} or D_{3d} symmetry and was found to be satisfactory when $\beta H/kT < 0.1$ [25], a condition which is met in the entire temperature range of the present study.

The theoretical temperature dependency of the force in the Faraday balance was calculated as described previously [16] with contributions from either or both of the two high-spin iron populations and also paramagnetic contaminants.

Results and Discussion

Yellow enzyme sample

The temperature dependent contribution to the molar magnetic susceptibility of the yellow enzyme sample was obtained as described previously [13,16]. It is displayed in Fig. 1 as a function of inverse temperature. The dotted theoretical curve was fitted to the experimental points as described below, and the solid straight line represents the best fit to the data in the temperature range where the susceptibility is proportional to $1/T$ (Curie behaviour). The slope of the line was used to calculate the value of the effective Bohr magneton number, n_{eff}, in the high temperature limit by means of the relation

$$n_{eff} = \left[\frac{3k}{N\beta^2}\cdot\frac{\mathrm{d}(\chi_{\text{mole}})}{\mathrm{d}(1/T)}\right]^{1/2}$$

After correction, as described previously [14], for the contributions from contaminating manganese (0.07 mol per mol enzyme and probably high-spin Mn(II)) and iron (0.01 mol per mol enzyme as high-spin Fe(III)), a value for n_{eff} of 5.6 ± 0.3 was obtained.

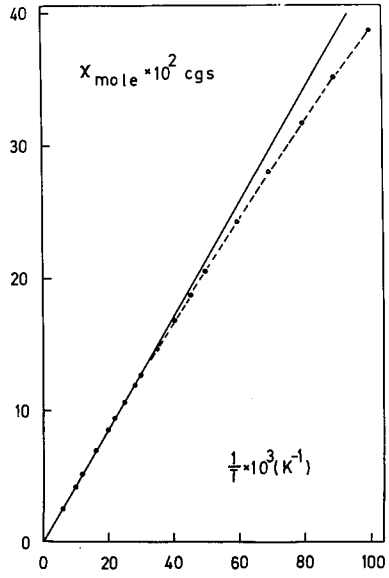


Fig. 1. The temperature dependent contribution to the molar magnetic susceptibility for soybean lipoxygenase-1 with 1.65 equivalents of the product (13*S*)-HPOD added. 31 μl of 33 mM (13*S*)-HPOD in 1 M NH_3 was added to 400 μl of 1.55 ± 0.08 mM lipoxygenase in 0.1 M borate buffer (pH 9.0). The final concentrations of lipoxygenase and (13*S*)-HPOD were 1.44 mM and 2.37 mM, respectively. An aliquot of 101 μl was transferred into the sample holder. The dotted curve was fitted to the experimental data assuming 1.49 mM iron, of which 50% was supposed to be high-spin Fe(III) and 50% high-spin Fe(II). High-spin Fe(III) was characterized by $D = 1.9$ cm^{-1}, $|E/D| = 0.01$ and $g = 2.0$, and high-spin Fe(II) by $D = 8.5$ cm^{-1}, $|E/D| = 0$ and $g = 2.1$. The root mean square difference between a calculated and an experimental susceptibility value was $\Delta\chi = 4 \cdot 10^{-4}$ cgs in this fit. Contaminating iron and manganese were supposed to give spin only contributions merely. An averaged value of 16.3 kOe was used for the applied magnetic field. The solid straight line represents the best fit to the experimental points at temperatures above 50 K.

This value is lower than expected for a sample containing only high-spin Fe(III), for which n_{eff} values in the range 5.8–6.0 have been reported at ambient temperatures [26]. It is within the range 5.1–5.7 observed for high-spin Fe(II), but significantly higher than the value 5.2 ± 0.3, which was reported for native lipoxygenase-1 [14]. EPR quantification showed that only $45 \pm 10\%$ of the iron in the yellow sample was detectable as high-spin Fe(III). The observed Bohr magneton number then indicates that the EPR-invisible fraction may be present as high-spin Fe(II) or as high-spin Fe(III) antiferromagnetically coupled to a radical produced by the interaction with (13*S*)-HPOD. The first of these two possibilities was tested by using a microcomputer in a grid search to fit a theoretical model (as worked out in the Theory section) with contributions from both high-spin forms to the experimental data. The fraction of high-spin Fe(III) was allowed to be within the range $45 \pm 10\%$ of the total, with its zero-field splitting parameters restricted to $D = 1.9 \pm 0.5$ cm^{-1} and $|E/D| < 0.04$. The range for D was chosen to be centered around the mean D value estimated from EPR spectroscopic studies for the Fe(III) population of the yellow-enzyme form [10]. Contaminating iron and manganese were assumed to be within the range 0.08 ± 0.02 mol per mol enzyme (See Materials and Methods). The electronic g values were approximated to be isotropic with $g = 2$ for high-spin Fe(II). For high-spin Fe(II) g values in a range slightly above $g = 2$ were also allowed.

Satisfactory computer fits could then be found with D values in either of the ranges 8 ± 3 cm^{-1} or -6.5 ± 1.5 cm^{-1} and g values in the range 2.04–2.20 for the EPR-invisible high-spin Fe(II) fraction. Though the best fits (Fig. 1) were obtained with $|E/D| = 0$ it was not possible to discriminate between $|E/D|$ values in the range 0–0.33. These results indicate an axial symmetry for the ligand environment of the iron, possibly with a rhombic distortion.

Apparently, it is not possible in this case to uniquely determine the sign of D from the temperature dependency of the average susceptibility. Such difficulties have been pointed out previously by Mitra [27].

In our previous evaluation of the zero-field splitting parameters for the high-spin Fe(II) of the native-enzyme perturbation calculation was used to derive the energy levels [16]. With the present more accurate calculation we find the same experimental data to be compatible with D values in either of the ranges 9 ± 2 cm^{-1} or -8 ± 1.5 cm^{-1} and g values in the range 2.0–2.08. Also in this case, our experimental data do not allow a discrimination between $|E/D|$ values in the range 0–0.33. The results are in fair agreement with the ranges 8.5–11.9 cm^{-1} or -6.9– -5.5 cm^{-1} obtained for D using the perturbation calculation with $g = 2$ [16]. A comparison of results indicates

that the EPR-invisible iron of the yellow sample has remained Fe(II) just like in the native enzyme. The small differences compared to the iron of the native form could be caused by (13*S*)-HPOD, but could also be due to products formed during the conversion of native into yellow enzyme by (13*S*)-HPOD, in line with the effect of 13-oxo-9,11-octadecadienoic acid on high-spin Fe(III) in yellow lipoxygenase [20].

The degree of conversion of native into yellow form upon addition of (13*S*)-HPOD has previously been reported to be around 80% [11] and 95% [7] at 0.5 mM and 0.37 mM enzyme concentration, respectively, with stoichiometric amounts of product. In the present magnetic susceptibility experiment, with an enzyme concentration of 1.55 mM, the degree of conversion, as determined by EPR quantitation, is no more than roughly 45%, even with a molar excess of (13*S*)-HPOD added. In order to examine a possible dependency of the degree of conversion on the enzyme concentration, stoichiometric amounts of (13*S*)-HPOD were added at different enzyme concentrations. Subsequently, the amounts of high-spin Fe(III) at $g = 6$ and 7.4 were determined from the EPR spectra. The light absorbance at 330 nm, which is correlated to the amount of high-spin Fe(III) [7], was also monitored. In the present experiments, a correlation factor of 0.99 was found between the total EPR intensity and the increase in A_{330}. As shown in Fig. 2, the degree of conversion decreases progressively with increasing enzyme concentration, giving only about 35% conversion with 1.5 mM enzyme. The molar extinction coefficient that can be derived from the present data is 3000 $M^{-1} \cdot cm^{-1}$, which is considerably higher than the value of 1800 $M^{-1} \cdot cm^{-1}$ previously reported by Spaapen et al. [28]. This difference could reflect an overestimation of the degree of conversion in their work. Spaapen et al. corrected their absorbances for residual absorption at 330 nm by 20% of the (13*S*)-HPOD products formed. A corresponding correction was not done in the present work, since the amount of such products was not determined. However, even if an unrealistically high yield of 100% were assumed for products with residual absorption at 330 nm, the corresponding correction would not bring the two extinction coefficients into agreement, since in this case a value of 2300 $M^{-1} \cdot cm^{-1}$ would be obtained.

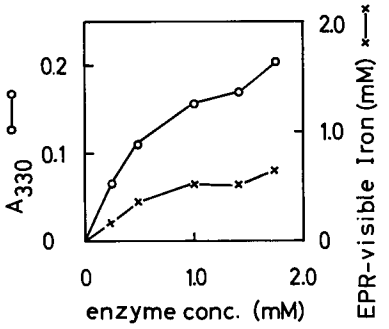


Fig. 2. Conversion of native into yellow lipoxygenase at different enzyme concentrations as monitored by EPR and light absorption measurements. Samples were prepared by adding 1.04 ± 0.06 molar equivalents of (13*S*)-HPOD (40 mM in 1.0 M NH_3) to 600 μl native lipoxygenase in 0.1 M borate buffer (pH 9.0). After mixing and incubating for 3 min at 5 °C, 2× 200 μl were used for EPR samples and 100 μl was diluted with 900 μl borate buffer for absorbance measurements at 330 nm. Corrections were made for protein absorbances by using native lipoxygenase as a reference solution.

Several alternative explanations for the observed low degree of conversion of native into yellow lipoxygenase at high enzyme concentrations can be considered, and will be discussed individually: (1) the low ratio of the dioxygen and enzyme concentrations, (2) mixing problems due to high viscosity, (3) acceleration of the previously reported spontaneous reduction of yellow lipoxygenase and (4) antiferromagnetic interaction between high-spin Fe(III) and radicals in a fraction of the yellow lipoxygenase sample.

(1) Under anaerobic conditions, hydroperoxide conversions by the enzyme proceed relatively quickly [29,30]. If these hydroperoxide conversions are catalyzed by the Fe(III) enzyme when some of it has been formed, addition of more (13*S*)-HPOD does not necessarily lead to an oxidation of the iron in the remaining Fe(II) enzyme. In an air-saturated solution at 4 °C (dioxygen concentration 0.4 mM) of 1.5 mM lipoxygenase concentration, one might suppose that part of the enzyme molecules in fact experience anaerobiosis and that addition of hydroperoxide in excess of the dioxygen concentration is not effective in the oxidation of the iron. However, this would not be in agreement with the results of experiments at relatively low

enzyme concentration (0.2–0.4 mM), showing that the reaction of native enzyme with (13*S*)-HPOD leads to qualitatively and quantitatively identical EPR spectra under aerobic and anaerobic conditions [20]. Also, if the dioxygen concentration would be the only factor, one would expect to be able to circumvent the problem by carrying out the reaction at relatively low enzyme concentration (0.5 mM) and then concentrating the enzyme solution. However, also in this case the percentage of EPR-visible iron is lower after concentration.

(2) It could be argued that, at high enzyme concentrations, the viscosity could cause mixing problems, which would allow a significant consumption of (13*S*)-HPOD in reactions catalyzed by the Fe(III) enzyme already formed (cf. (1)). Only a fraction of the (13*S*)-HPOD added would then be available for iron oxidation. Polymer formation causing steric hindrance for (13*S*)-HPOD to reach the iron could also be a problem. However, we have no indications for irreversible polymerization, since concentrated enzyme was always fully active under assay conditions.

(3) A marked decrease of the amount of EPR-visible iron in yellow lipoxygenase upon storage at 4° C has been reported [20]. This was explained as a spontaneous reduction of iron, proceeding by an as yet unknown mechanism. If it would involve collisions between molecules the reduction would be more effective at higher enzyme concentrations. We have no indications for iron–iron interactions, as dilution of relatively concentrated samples (0.8–1.0 mM) to 0.15 mM did not change the total amount of EPR-visible iron nor the shape of the EPR signal.

(4) The possibility that Fe(III) is actually formed but to a large extent antiferromagnetically coupled to radicals of some kind must be kept in mind. The formation of radicals by electron transfer to or from iron has been proposed in the current reaction mechanism [5], but there is no evidence for an antiferromagnetic interaction in any lipoxygenase species, except for the NO complex of the native enzyme [31]. If there would be antiferromagnetic interaction in the yellow lipoxygenase sample, then, the energy separation to the first excited state of the coupled unit would be determined by the exchange integral, J. At temperatures where the thermal energy becomes comparable to this energy separation the susceptibility would be expected to deviate from Curie behaviour (cf. Ref. 26, pp. 170–203). No such deviation is observed. Interestingly enough, however, when the yellow enzyme sample was stored in the cryostat of the instrument at −100° C, and measured again four days after the first session, a small Curie dependent contribution had arisen. This contribution presumably originates from a radical which would then amount to about 7 to 8% of the protein concentration. Upon storage at 77 K, yellow enzyme samples have been found to give radical signals in EPR, which probably originate from the protein chain [18,32].

We conclude that the low yield of yellow enzyme at high enzyme concentrations is mainly due to an accelerated rate of spontaneous reduction of the yellow form. However, we cannot exclude that mixing problems in combination with the low ratio of dioxygen and enzyme concentrations as well as polymer formation could affect the conversion of native into yellow enzyme.

Anaerobically substrate-treated yellow enzyme

In Fig. 3, the temperature dependent part of the magnetic susceptibility is shown for the yellow sample (Fig. 1) after addition of substrate under anaerobic conditions. A Bohr magneton number of 5.2 ± 0.3 was evaluated in the high temperature range, where the susceptibility follows Curie behaviour. The EPR spectrum for this sample lacked features in the $g = 6$ region and was almost identical to that of the native enzyme. No light absorption peaks were found in the visible range. In combination, our data indicate that this enzyme species contains high-spin Fe(II) only, and that linoleic acid has reduced the high-spin Fe(III) fraction of the yellow sample. Thus, the fraction of high-spin Fe(II) in our yellow enzyme sample did not become diamagnetic upon anaerobic addition of linoleic acid. This is not in agreement with results of Cheesbrough and Axelrod [15], who reported that native enzyme becomes diamagnetic upon such an addition. Hindrance by linoleic acid of the interaction between the reference compound and the metal centre in their experiment may account for this discrepancy.

Acceptable computer fits were obtained for a uniform population of high-spin Fe(II) with D

values in either of the ranges $12.5^{+1.5}_{-2}$ cm^{-1} or -12^{+3}_{-2} cm^{-1}, and g values in the range 2.08–2.16. For negative D values it was not possible to discriminate between $|E/D|$ values in the range 0–0.33, while for positive D values $|E/D|$ was found to be in the range 0.1–0.33. In both cases, the best fits were obtained with $|E/D|$ values close to 0.33 (cf. Fig. 3).

The observed values of the zero-field splitting parameters are close to those of the high-spin Fe(II) of the native enzyme, but indicate that there may be small differences in the symmetry of the iron environment. These differences may be explained by the presence of possibly unreacted linoleic acid as well as reaction products of both linoleic acid and (13*S*)-HPOD in the anaerobic sample.

It may be argued that the hydrogen abstraction step [5,9] can affect the iron environment. In this step the electron is accommodated on the iron [5], but it is not known where the remaining proton resides. It is unlikely that it is on water, since the hydrogen abstraction takes place in the hydrophobic cleft of the protein [33], close to iron, and water is not a ligand of iron [20]. Therefore, it is possible that an amino-acid residue in the environment of the iron is protonated. This could then affect the contribution to the susceptibility from the iron fraction which was reduced by linoleic acid.

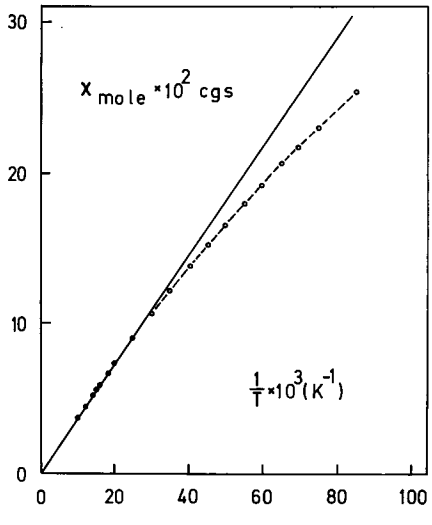


Fig. 3. The temperature-dependent contribution to the molar magnetic susceptibility for the sample of Fig. 1 with 1.20 equivalents of the substrate linoleic acid anaerobically added. 3.3 μl of 52.2 mM linoleic acid in 1.0 M NH_3 was added to 101 μl of the yellow sample. The final concentrations of lipoxygenase and linoleic acid were 1.39 mM and 1.67 mM, respectively. The dotted curve was fitted to the data assuming the iron to be in the high-spin Fe(II) state with $D = 12.5$ cm^{-1}, $|E/D| = 0.3$ and $g = 2.12$. The root mean square difference between a calculated and an experimental susceptibility value was $\Delta\chi = 2 \cdot 10^{-4}$ cgs in this fit. Curie behaviour was supposed also in this case for the contaminating iron and manganese. The solid straight line represents the best fit to the experimental points at temperatures above 50 K.

From the present data it is not possible to discriminate between a homogenous and a slightly heterogeneous high-spin Fe(II) population in the anaerobically substrate-reduced sample and we cannot decide whether the relatively high D value is caused by linoleic acid, reaction products or amino-acid protonation.

Our data do not exclude that free radicals created upon the reduction of iron could be present. As a consequence of the hydrogen abstraction an unpaired electron could be localized on linoleic acid, but the electron could also be transferred to an amino-acid residue of the protein. The effect of allowing radicals in the fitting procedure, to an extent corresponding to the amount of yellow enzyme reduced, is mainly to increase the absolute value of D by about 2 cm^{-1}. A significant rhombic distortion in the iron environment is also indicated, since $|E/D|$ values above 0.15 are required for acceptable fits. To account for the absence of an EPR signal from a radical this model would require a magnetic interaction between the reduced iron and the radical. This interaction should be weak, say of dipolar or exchange origin, since an interaction term is not required to account for the susceptibility data. It should be noticed that both models, with and without radical present, are in agreement with the reaction scheme proposed by de Groot et al. [5] (Scheme I).

Concluding remarks

It is now well established that the Fe(III) in yellow lipoxygenase can be reduced by the substrate linoleic acid, and that iron shuttles between the Fe(II) and Fe(III) states during the anaerobic reaction. It is reasonable to assume that the reduction of the yellow enzyme by linoleic acid also

occurs under aerobic conditions [5]. Since dioxygen is apparently not coordinated to the iron of the native enzyme [16,17], the present results indicate that lipoxygenase acts by a mechanism of fatty-acid substrate activation involving a valence change of iron, rather than by dioxygen activation.

The feature of substrate activation is an analogy to protocatechuate-3,4-dioxygenase [34]. However, in the catalytic cycle proposed for this enzyme [34,35] iron remains Fe(III). On the other hand, Fe(II) occurs in the native enzyme and the enzyme-substrate complex of protocatechuate-4,5-dioxygenase [36]. Thus, among the non-heme iron dioxygenases, lipoxygenase, in changing its valence state, appears to take an unique position.

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