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FACTORS AFFECTING THE LINE-SHAPE OF THE EPR SIGNAL OF HIGH-SPIN Fe(III) IN SOYBEAN LIPOXYGENASE-1

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The yellow form of soybean lipoxygenase-1 (linoleate:oxygen oxidoreductase, EC 1.13.11.12), obtained upon addition of one molar equivalent of 13-L$_S$-hydroperoxy-9-*cis*,11-*trans*-octadecadienoic acid (13-L-HPOD) to the native enzyme, shows a complex EPR signal around *g* 6 which results from contributions of different high-spin Fe(III) species with rhombic or axial symmetry. The signal cannot be attributed to different enzyme-product complexes because removal of the products or variation of the concentration of the products in the enzyme solution does not lead to an EPR spectrum characteristic for one particular species. Upon varying the pH of the enzyme solution from 7 to 11 changes in the line-shape are observed, but no distinct spectrum of either a rhombic or an axial form could be observed. The relative amounts of the different species visible in the signal around *g* 6 are strongly affected by cyanide, primary alcohols or 13-L-hydroxyoctadecadienoic acid (reduced 13-L-HPOD). The presence of either of these substances causes a shift to an axial type of spectrum. *t*-Butanol and sodium dodecyl sulfate induce a shift towards a more rhombic line-shape. A shift to an axial type of spectrum is observed after storage of the enzyme in the yellow form at 4°C. Storage of the native form at 4°C also leads to changes which become apparent after oxidation in a similar axial type of spectrum. Reversion to the original, more rhombic, spectrum is possible by ammonium sulfate precipitation. It is concluded that the species giving rise to the EPR signal around *g* 6 are enzyme species differing only in the structure of the environment of the iron atom.

Introduction

13-L-HPOD is the main product of the incubation of soybean lipoxygenase-1 (linoleate:oxygen oxidoreductase, EC 1.13.11.12) with linoleic acid at pH 9.0 under aerobic conditions [1]. Addition of 1 molar equivalent of this product to the native, colourless and EPR-silent enzyme results in the formation of a yellow enzyme which shows a complex EPR spectrum around *g* 6 [2–4]. This spectrum has been attributed to high-spin Fe(III) species with different ligand symmetry. The number and relative amounts of species building up the *g* 6 signal vary [5,6]. This results in different line-shapes of the EPR spectrum of yellow lipoxygenase. It has been suggested [6] that pH, concentration of oxygen and conversion products of 13-L-HPOD have an influence on the spectrum.

In this paper an investigation of factors affecting the quantities of the different species is de-

Abbreviations: 13-L-HPOD, 13-L$_S$-hydroperoxy-9-*cis*,11-*trans*-octadecadienoic acid; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine.

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scribed and the nature of the species is further characterized.

Materials and Methods

Lipoxygenase-1 was isolated from soybeans according to the method described by Finazzi-Agrò et al. [7] with the modifications reported by Galpin et al. [8]. The specific activity of the enzyme was 3.92 μkat · mg^{-1}, corresponding to 235 μmol O_2 · min^{-1} · mg^{-1}. The enzyme activity was measured both polarographically in a Gilson oxygraph equipped with a Clark electrode (1.8 mM linoleic acid in 0.1 M borate buffer, pH 9.0) and photometrically in a Cary C118 spectrophotometer at 234 nm (molar absorption coefficient of 13-L-HPOD, 25 mM^{-1} · cm^{-1}).

The iron content was 0.97 mol per mol enzyme, determined by a colorimetric method and atomic absorption spectroscopy [6].

Linoleic acid was obtained from Lipid Supplies (St. Andrews University, St. Andrews, U.K.); [U-^{14}C]linoleic acid (948 Ci/mol) was purchased from New England Nuclear (Boston, MA, U.S.A.); 13-L-HPOD was prepared and purified according to the method of Verhagen et al. [9]. 12,13-Epoxy-11-hydroxyoctadecenoic acid, 13-oxotridecadienoic acid and 13-oxooctadecadienoic acid were obtained according to the method of Garssen et al. [10,11], and 13-hydroxyoctadecadienoic acid was prepared by reduction of 13-L-HPOD with $NaBH_4$. These compounds were purified as free acids by thin-layer chromatography on 0.50-mm precoated plates (Silicagel 60 F254, 20 × 20 cm, E. Merck, A.G., Darmstadt) in the solvent system hexane/diethyl ether/acetic acid (50:50:1, v/v). [^{13}C]Potassium cyanide (87.43 atom% ^{13}C) was supplied by Merck, Sharp and Dohme (Canada).

The ^{14}C-radioactivity of column eluate was determined using a Packard 2425 Liquid Scintillation Spectrometer. A Jasco J-500 spectrometer was used for recording CD spectra. EPR spectra at 9 GHz were recorded on a Varian E-9 spectrometer with 100 kHz field modulation (modulation amplitude 2 mT). Measurements were carried out at a temperature of 15 K using an Oxford Instruments liquid-helium cryostat [5].

EPR spectra presented in the same figure are corrected for small differences in the enzyme concentrations and the dimensions of the EPR tubes, thus allowing intensity comparison.

Results

The low-field part of the EPR spectrum of yellow lipoxygenase-1, obtained by addition of 1.0 molar equivalent of 13-L-HPOD to the native enzyme at pH 9.0, is shown in Fig. 1A. EPR lines are observed at g 7.4 and g 6.2–5.8 stemming from rhombic and nearly axial species, respectively [6]. The g_z parts of the resonances near 2.0 [6] are obscured by the presence of contaminating manganese (0.07 mol per mol enzyme [6]) and radical signals (insert, Fig. 1).

A. Interaction with products

Based mainly on simulation data, the complex signal around g 6 has been attributed to at least three species with different ligand symmetry [6]. It might be that these species represent different

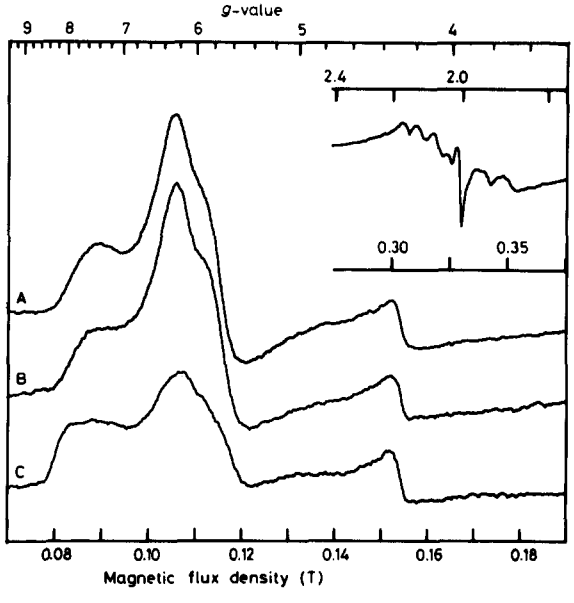

Fig. 1. EPR spectra of yellow lipoxygenase-1. A solution of native lipoxygenase (25.7 g/l) was incubated with a 13-L-HPOD solution (50.34 mM); final concentrations: 0.26 mM for both enzyme and 13-L-HPOD in 0.1 M borate buffer, pH 9.0. Samples have been taken before (A) and after (B) the removal of secondary products using a Sephadex G-25 column (see also Fig. 2). C. To a sample as described for B 13-oxooctadecadienoic acid has been added. Final concentration: 1.05 mM. Microwave frequency, 9.255 GHz; microwave power, 2 mW; temperature, 15 K.

enzyme-product complexes, because Verhagen et al. [12,13] have reported the formation of a variety of secondary products in the lipoxygenase-catalyzed conversion of 13-L-HPOD. Incubations were carried out by these authors both under aerobic [12] and anaerobic [13] conditions at enzyme/13-L-HPOD ratios of 1:19 and 1:38, respectively. For comparison with EPR conditions we have repeated these experiments with a molar ratio of 1:1.2 (enzyme/13-L-HPOD) and obtained similar results. In our case, the secondary products had to be extracted from the incubation medium according to the method of Bligh and Dyer [14], because of the relatively large amount of protein present in the incubation medium.

In order to study possible interaction between the enzyme and secondary products we have performed the following three types of experiment:

1. Removal of secondary products. After the addition of 13-L-HPOD to the native enzyme the reaction products were removed by gel chromatography [3]. The EPR spectrum of the yellow enzyme obtained after this purification step (Fig. 1B) differs only slightly from Fig. 1A.

The efficiency of the Sephadex G-25 column was checked using U-^{14}C-labelled 13-L-HPOD (Fig. 2). The amount of radioactive material present in the enzyme fractions was 6% of the total amount of radioactive material recovered. A qualitative analysis of the 6% fraction (performed in the same way as described above for the analysis of the secondary products) revealed a variety of

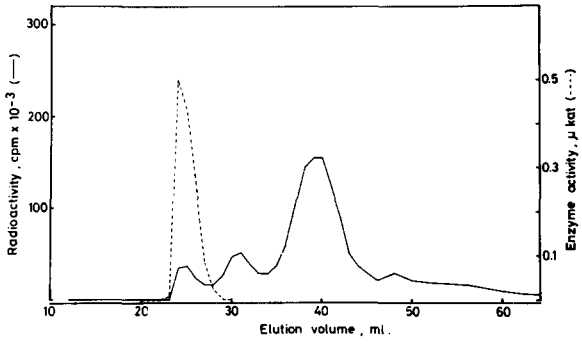

Fig. 2. Removal of secondary products from yellow lipoxygenase-1. A lipoxygenase-1 solution (52 g/l), incubated with U-^{14}C-labeled 13-L-HPOD (0.5 mM), final concentrations 0.225 mM and 0.250 mM, respectively, in 0.1 M borate buffer, was purified on a Sephadex G-25 column (75×1.0 cm).

products similar to the products of an incubation of native enzyme with 1 molar equiv. 13-L-HPOD. This points to a nonspecific binding of the radioactive material to the enzyme. Attribution of the 6% fraction to one of the three species building up the EPR signal around g 6 can also be excluded, because at least 25% of the total iron content is visible in each species [6].

2. Addition of secondary products. Up to 5 molar equiv. of three secondary products were added to the yellow enzyme which was purified by gel chromatography. No significant changes in the EPR spectrum of yellow lipoxygenase are observed upon addition of 13-oxotridecadienoic acid and 12,13-epoxy-11-hydroxyoctadecenoic acid. Interestingly, addition of 13-oxooctadecadienoic acid gives rise to a new signal at approx. g 8 at the expense of the axial species at g 6.2–5.8 (Fig. 1C).

3. Change of the relative amounts of secondary products by removal of oxygen before oxidation of the native enzyme. Yellow enzyme was prepared both under aerobic and anaerobic conditions because of observed differences in amount and nature of the reaction products formed in aerobic and anaerobic conversions of 13-L-HPOD [12,13]. Identical spectra around g 6 were obtained, thereby also indicating that oxygen has no direct influence on the shape of the signal.

B. pH influence on the signals around g 6

The relative intensities of the axial and rhombic signals have been studied as a function of pH. The NaOH/Bicine buffer used is believed to show only small changes in pH on freezing ($\Delta pH = \pm 0.1$) [15]. In order to eliminate the change in ionic strength of the buffer, NaCl (2.5 M) was added to the samples. The increase of the ionic strength of the solution results only in a small decrease of linewidth (see C). Fig. 3 gives EPR spectra of enzyme solutions dialyzed for 15 h at different pH. The spectra at pH 7.0 and 11.0 are similar to those at pH 8.0 and 9.4, respectively. The shape of the EPR spectrum shows a shift of the g 7.4 line to a higher g value with increasing pH. Quantification [6] of the amount of iron visible in the rhombic part of the spectrum (g 7.4–7.6) gives an increase of approx. 10% going from pH 8.0 to 9.4. Furthermore, simulations revealed that the apparent large change of the axial part of the spectrum (g 6.3–5.7)

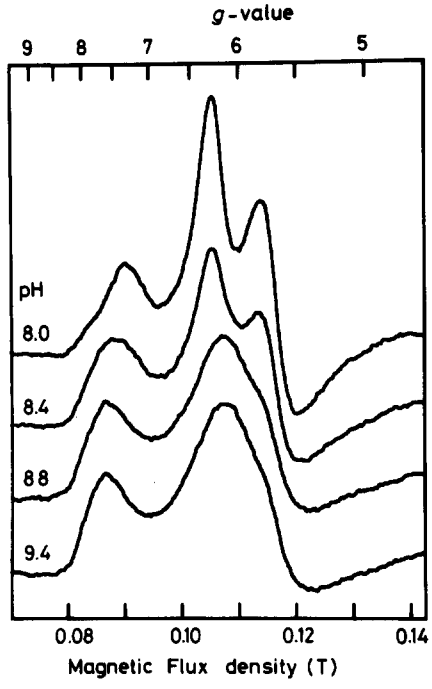

Fig. 3. EPR spectra of yellow lipoxygenase-1 solutions at different pH. Native lipoxygenase (26.3 g/l) was dialyzed for 15 h against 0.1 M Bicine/NaOH buffers. The pH of the enzyme solution was checked after addition of solid NaCl to native enzyme (NaCl 2.5 M). The solution was incubated with a 13-L-HPOD solution (21.6 mM), final concentration 0.27 mM for both enzyme and 13-L-HPOD. The EPR sample was frozen in liquid nitrogen 15 min after incubation, thus allowing complete formation of the yellow enzyme. Microwave frequency, 9.252 GHz; microwave power, 2 mW; temperature, 15 K.

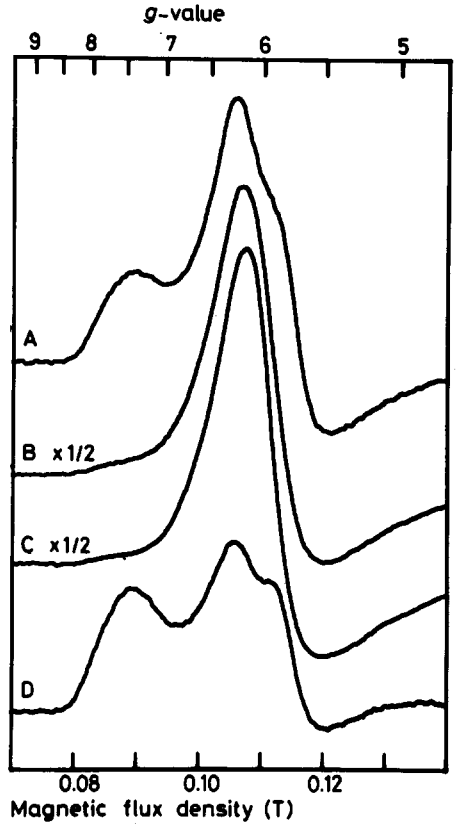

Fig. 4. EPR line-shape changes of yellow lipoxygenase-1. Yellow lipoxygenase (A) prepared as described for Fig. 1A was incubated with 10 molar equiv. KCN (B), 0.05% v/v ethanol (corresponding to 30 molar equiv.) (C) and 10 molar equiv. SDS (D). Microwave frequency, 9.256 GHz; microwave power, 2 mW; temperature, 15 K.

can be fully explained by change in linewidth of the axial species. The total amount of EPR-visible iron is constant over the pH range studied. The changes in line-shape upon titrating the enzyme solution were found to be reversible.

C. Effects of anions

In the study of the pH influence on the line-shape of the signal around g 6 2.5 M NaCl was used to keep the ionic strength constant at different pH values. A decrease of the linewidth of the signal around g 6 is observed. A similar effect is obtained upon addition of NaF, NaI, NaBr or sodium acetate (1.0 to 2.5 M) to a yellow enzyme solution (approx. 0.25 mM). No changes are observed at low concentrations (1–10 molar equiv.). Low concentrations (10 molar equiv.) of EDTA, azide and thiocyanate have no influence on the line-shape of the signal around g 6. At higher concentrations (approx. 2 M) changes of the signals also occur. However, under these circumstances an increase of signals around g 4.3 is observed, which is most probably due to partial denaturation of the enzyme. Upon incubation of yellow enzyme with azide the solution becomes slightly brown-coloured.

Addition of KCN to the yellow enzyme leads to a large change in the EPR spectrum (Fig. 4B). The axial part of the spectrum is increased at the expense of the rhombic part. No change in the total amount of EPR-visible iron nor any appearance of low-spin Fe(III) EPR signals is observed.

In order to study a possible binding of the cyanide anion to iron we have compared the EPR spectra of solutions of yellow lipoxygenase with $K^{13}CN$ and $K^{12}CN$. In the case of direct binding of the ^{13}C ($I = \frac{1}{2}$) to iron either a resolved hyperfine structure or at least a line-broadening results. A significant increase of the linewidth in the EPR spectrum of yellow enzyme preincubated with ^{13}CN could not be observed using computer analysis of the spectra ($\Delta B < 0.1$ mT), which indicates that the cyanide does not directly bind to the iron in the active site.

Sodium dodecyl sulfate (10 molar equiv.) gives a shift to a more rhombic EPR signal (Fig. 4D). Integration of the rhombic part of the spectrum [6] reveals that approx. 55% of the total iron is present in the rhombic species, whereas the rhombic component amounts to approx. 40% in the yellow enzyme, as shown in Fig. 4A [6].

D. Effect of alcohols

The effect of alcohols on the EPR line-shape has been studied in more detail because it turned out that very small amounts of ethanol in the 13-L-HPOD solution have caused the drastic changes of the EPR line-shape reported previously [5,6]. Fig. 4C shows the EPR spectrum of yellow lipoxygenase after the addition of 0.05% (v/v) ethanol, corresponding to 30 molar equiv. The signal is nearly axial and almost identical to that shown in Fig. 4B (KCN added to yellow enzyme). The effect of ethanol is reversible; after dialysis against 0.1 M borate buffer, pH 9.0, for 15 h a spectrum almost identical to that shown in Fig. 4A is obtained. The observed change in line-shape is not specific for ethanol. Methanol, 1-butanol, 2-methyl-1-propanol and 1-hexanol all have effects similar to those of ethanol. 13-L-Hydroxyoctadecadienoic acid, obtained by reduction of 13-L-HPOD, also induces a shift to an axial type of spectrum. However, *t*-butanol gives an increase of the rhombicity of the signal and an EPR spectrum similar to Fig. 4D is obtained after addition of 500 molar equiv.

No change in the amount of EPR-visible iron was observed after addition of ethanol, consistent with earlier observations [6]. Furthermore, no changes occur in the optical absorption spectrum (300–700 nm) [3,16] recorded at 77 K or room temperature and in the CD spectrum upon preincubation of yellow lipoxygenase-1 with 0.05% (v/v) ethanol. The enzyme activity is not diminished after preincubation with 0.05% (v/v) ethanol.

E. Storage of enzyme solutions

After isolation of lipoxygenase from soybeans the enzyme is stored at 4°C in a 0.1 M sodium acetate buffer, pH 5.0, brought to 25% saturation with solid ammonium sulfate. Before use the enzyme is precipitated by increasing the amount of ammonium sulfate to 60% saturation and then dialysed against the buffer desired for EPR experiments. An EPR spectrum of freshly dialyzed enzyme after conversion into the yellow Fe(III) form by 13-L-HPOD is given in Fig. 4A. The EPR spectrum obtained after storage of the native enzyme for approx. 4 weeks in a buffer solution at 4°C is similar to the spectra shown in Fig. 4B and C. Thus storage of the native enzyme in buffer leads after conversion into the yellow enzyme to a more axial type of spectrum. An EPR spectrum containing a rhombic component as shown in Fig. 4A can be obtained by ammonium sulfate precipitation of the stored native enzyme followed by dialysis against 0.1 M borate buffer, pH 9.0, and oxidation with 13-L-HPOD. Dialysis as such does not restore the EPR spectrum of the enzyme. No decrease of the activity of the native enzyme stored for 3 months at 4°C in 0.1 M borate buffer, pH 9.0, (enzyme concentration approx. 25 mg/ml) was observed.

Regarding the amount of EPR-visible iron, it does not make any difference whether the yellow enzyme is obtained from native enzyme directly or from native enzyme which has been stored in buffer at 4°C during 1–30 days. In contrast, storage of yellow enzyme at 4°C leads to a marked decrease of the amount of EPR-visible iron. The spectrum can be restored by addition of 13-L-HPOD, which indicates that during storage the iron is reduced by an as yet unknown mechanism.

Discussion

Previously, we have reported that the EPR spectrum around *g* 6 of yellow lipoxygenase consists of contributions of different species [5,6]. These species were thought [6] to be different enzyme-

product complexes because a variety of secondary products is formed upon addition of 13-L-HPOD to the native enzyme. However, variation of the concentration of the secondary products by (a) purification of the enzyme using Sephadex G-25, (b) addition of 1–5 molar equiv. of three secondary products and (c) aerobic and anaerobic incubation of the native enzyme with 13-L-HPOD, does not lead to an EPR spectrum characteristic for only one species (Fig. 1). Thus an assignment of the signals building up the spectrum around *g* 6 to distinct enzyme-product complexes can be excluded.

Upon pH-titration of the enzyme variation in the line-shape is observed (Fig. 3), but no separate spectrum of either a rhombic or an axial enzyme form could be obtained in the pH range 7–11. The change in line-shape in the axial part of the spectrum at approx. pH 8.5 might point to the presence of a titratable group in the vicinity of iron which only influences the axial part of the signal.

The absence of changes in the line-shape upon addition of small amounts of sodium halides or acetate indicates that there is no strong binding place for those anions in the environment of iron. The observed decrease of linewidth upon increasing the ionic strength might be caused by freezing effects leading to 'local' salt concentrations which affect the conformation of enzyme species [17].

The addition of 'iron-complexing' compounds results only for KCN in an alteration of the line-shape (Fig. 4B). Unlike most high-spin heme proteins no low-spin Fe(III) is obtained upon addition of cyanide. This might exclude H_2O as an iron ligand and indicate that cyanide is not directly bound to iron but to another place in the vicinity of iron. This is supported by the absence of any inhibition of the enzyme activity by cyanide [18] and also by the absence of any line-broadening of the EPR spectrum around *g* 6 after adding $K^{13}CN$.

SDS added to yellow enzyme gives an increase of the rhombicity of the signal around *g* 6 (Fig. 4D). This is also observed upon addition of 3 molar equiv. 13-oxooctadecadienoic acid (Fig. 1C). These substances possibly bind to the enzyme in such a way that it causes a distortion of the environment of iron.

The effect of alcohols on the line-shape of the signal around *g* 6 is very impressive. 0.1 μl ethanol in 200 μl enzyme solution, the usual sample volume, is sufficient for a shift to an axial type of spectrum as shown in Fig. 4C. Impurities of this order of magnitude readily arise because during storage the peroxides are dissolved in ethanol and ethanol is often used as a rinsing solvent for syringes. The optical and CD spectra show no changes upon addition of ethanol and this may indicate that large changes in the enzyme conformation do not occur. Therefore, we conclude that the species which give rise to the EPR signal around *g* 6 only differ in structure of the environment of iron. Thus, changes in the line-shape of the EPR signal correspond with changes in the relative amounts of species with different environments of iron. A small number of species with relatively low stabilization energy is present because the spectrum as shown in Fig. 1A can be simulated with three species [6]. The absence of a rigid porphyrin system for the coordination of iron [2] might account for the variations in symmetry in the environment of iron.

Acknowledgements

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