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Spectroscopic studies on the interactions between lipoxygenase-2 and its product hydroperoxides

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The interactions of soybean lipoxygenase-2 (linoleate:oxygen oxidoreductase, EC 1.13.11.12) with three of its product hydroperoxides (9*R*-hydroperoxyoctadecadienoic acid (9*R*-HPOD), 9*S*- and 13*S*-HPOD) have been studied with optical and EPR spectroscopy as well as with stopped-flow kinetics. Upon addition of 1 molar equivalent hydroperoxide to the colourless resting enzyme at 4°C, chromophores in the near ultraviolet and at 580 nm arise, which are reminiscent of those observed in the interaction of soybean lipoxygenase-1 with 13*S*-HPOD. Stopped-flow analysis demonstrated that these chromophores are concomitantly formed. Upon addition of a molar excess of hydroperoxide at 4°C, the chromophore at 580 nm increases in intensity, but decreases immediately afterwards. The interactions with the 9*R*-, 9*S*- and 13*S*-HPODs are similar. The EPR spectra of the enzyme-HPOD samples are dominated by *g* 4.3 signals, which are ascribed to rhombic high-spin Fe(III). Also signals in the *g* 6 region are discernible, which arise from high-spin Fe(III)-species with ligand environments of distorted axial symmetry. From the occurrence of the same chromophores and EPR signals, it is concluded that the iron environment is similar to that in lipoxygenase-1. However, the following differences are noticed: 1. The chromophore at 580 nm and the *g* 4.3 signal arise more readily with lipoxygenase-2 than with lipoxygenase-1. 2. No enzyme species with only a *g* 6 signal can be obtained with lipoxygenase-2, which may be due to the unavoidable presence of denatured enzyme. Some of the spectroscopic features of the lipoxygenase-2–HPOD interactions resemble the effects of H_2O_2 on lipoxygenase-1. The effect of 0.1% ethanol, which alters the shape of the EPR signals around *g* 6 to give a more axial signal in lipoxygenase-1, also occurs with lipoxygenase-2. The intensities of the *g* 6 signals in the lipoxygenase-2 samples treated with one molar equivalent of HPOD decrease considerably upon anaerobic addition of linoleic acid. This is interpreted as a reduction of Fe(III) to Fe(II) and yields an EPR-silent enzyme species. This is considered as an indication that the function of iron and the mechanism of the catalysis are similar to those in lipoxygenase-1.

Introduction

Lipoxygenases (linoleate:oxygen oxidoreductase, EC 1.13.11.12) catalyse the dioxygenation of

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Abbreviation: HPOD, hydroperoxyoctadecadienoic acid.

fatty acids containing a 1*Z*,4*Z*-pentadiene system to form hydroperoxides. The dioxygenation can be very regio- and stereospecific, depending on the source of the enzyme and on the incubation conditions. Lipoxygenase-1 from soybeans catalyses the almost exclusive formation of 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid (13*S*-HPOD) from linoleic acid at pH 9.0 [1]. In order to observe an activity at this pH with soybean lipoxygenase-2, substrate inhibition has to be counteracted [2,3]. At low substrate concentration ($\leqq 100$ μM), this enzyme also displays its highest stereospecificity at pH 9.0 [3]. The hydroperoxide predominantly formed is 9*R*-hydroperoxy-10*E*,12*Z*-octadecadienoic acid (9*R*-HPOD, 51%) but 13*S*-HPOD (30%), 9*S*-HPOD (14%) and 13*R*-HPOD (5%) are also produced [3].

The existence of three [4] and even four [5] lipoxygenases in soybeans has been reported. We have recently demonstrated [6] that the enzymes which occur in addition to lipoxygenase-1 all give the same mixture of products, which justifies the classification of these enzymes as type-2 lipoxygenases and allows the use of the mixture of them for spectroscopic studies. The interaction of lipoxygenase-1 with its product hydroperoxide 13*S*-HPOD has been studied with various techniques (for a review, see Ref. 7). The resting lipoxygenase-1 contains high-spin Fe(II) [8], which upon addition of 13*S*-HPOD is oxidized to high-spin Fe(III). EPR studies [9] have provided information on the coordination of iron and the mechanism of the enzymic catalysis. From the presence of a *g* 6 signal, it is concluded that iron must have nearly axial ligand symmetry. With regard to the mechanism, it has been found that the iron in lipoxygenase-1 can shuttle between two valence states under the influence of substrate and products, i.e. reduction from Fe(III) to Fe(II) by linoleic acid and oxidation from Fe(II) to Fe(III) by 13*S*-HPOD [9]. Because of our interest in structures and mechanisms of lipoxygenases and the abundance of type-2 lipoxygenases, we decided to investigate the interaction of soybean lipoxygenase-2 with its three main product hydroperoxides to obtain information about the iron environment and the mechanism of the enzymic catalysis.

Materials and Methods

Enzyme purification. Lipoxygenase-2 was purified from soybeans (*Glycine max* (L.) Merr. var. Williams) by ion-exchange chromatography on CM-Sephadex C-50 and DEAE-Sephadex A-50 (Pharmacia) according to Slappendel [10]. For calculations, the values for $A^{0.1\%}_{280nm}$ and M_r were taken as 1.4 and 92000, respectively [6]. The enzyme was stored in 0.1 M imidazole-HCl buffer (pH 6.8), brought to 25% saturation with ammonium sulphate. The enzymic activity was 7.1 μmol $O_2 \cdot min^{-1} \cdot mg^{-1}$, as determined polarographically in a Gilson Oxygraph equipped with a Clark electrode (0.1 M sodium borate buffer, pH 9.0, 1.8 mM ammonium linoleate and 2 mM $CaCl_2$). Iron, manganese and copper contents of the preparation were determined with flameless atomic absorption spectrometry. The iron content was found to be 0.8 gatom per mol enzyme; the contents of the manganese and copper contaminants were 0.1 and 0.01 gatom per mol enzyme, respectively.

Hydroperoxides. 13*S*-HPOD, 9*S*-HPOD and 9*R*-HPOD were prepared by incubating linoleic acid (Lipid Supplies, St. Andrews, U.K.) with soybean lipoxygenase-1 at pH 9.0 [11], with corn germ lipoxygenase at pH 6.6 [11] or with soybean lipoxygenase-2 at 100 μM concentration at pH 9.0 [3], respectively. The products were separated from their positional isomers by high-performance liquid chromatography (HPLC) [12]. No attempt was made to separate optical isomers. For the optical purity of the preparations, see Ref. 3. All hydroperoxides were stored in ethanol at -20°C. Before addition to the enzyme, the ethanol was evaporated and the hydroperoxide dissolved in 1 M ammonia. Additions of 1 or 2.5 molar equivalents of HPOD to enzyme preparations were made on the basis of the iron content (see above).

Optical spectroscopy. Optical spectra were recorded on a Beckman Acta spectrophotometer equipped with a cooling bath to allow measurements at 4°C. The protein concentrations in this and the following experiment were 40.3 mg $\cdot$ ml^{-1}.

EPR spectroscopy. EPR spectra were recorded on a Varian E-9 spectrometer with 100 kHz modulation at 15 K using an Oxford Instruments cryostat. In order to quantify the signals, they

were integrated [13], and a Cu(II) solution in 2 M $NaClO_4$, pH 2, and a solution of human serum transferrin were used as references [14].

Stopped-flow kinetics. Stopped-flow experiments were carried out with a Durrum single-beam apparatus (light path 2 cm) in the absorbance mode, coupled to a Digital Equipment Minc computer for acquisition, storage and analysis of the data.

Results and Discussion

Optical spectra

The optical spectra of the resting lipoxygenase-2 and of the enzyme species formed upon addition of its main product hydroperoxides are shown in Fig. 1. The absorbance in the spectrum of the resting enzyme increases towards shorter wavelengths. The spectrum only shows typical protein features, apart from a small shoulder at 410 nm, the origin of which is as yet unknown. Such a shoulder has also been observed in some preparations of lipoxygenase-1 [15]. Upon addition of the various HPODs to the enzyme, chromophores arose around 350 nm and at 580 nm. The effects of 9*R*- and 9*S*-HPOD were identical. The effect of 1 molar equivalent 13*S*-HPOD (not shown) was different from that of the 9-HPODs, as it gave a lower intensity of the chromophore at 580 nm. Furthermore, less time was needed to attain the maximum intensity in this absorption band (5 and 20 min for the 13*S*-HPOD and the 9-HPOD under these conditions, respectively).

After the development of the chromophores

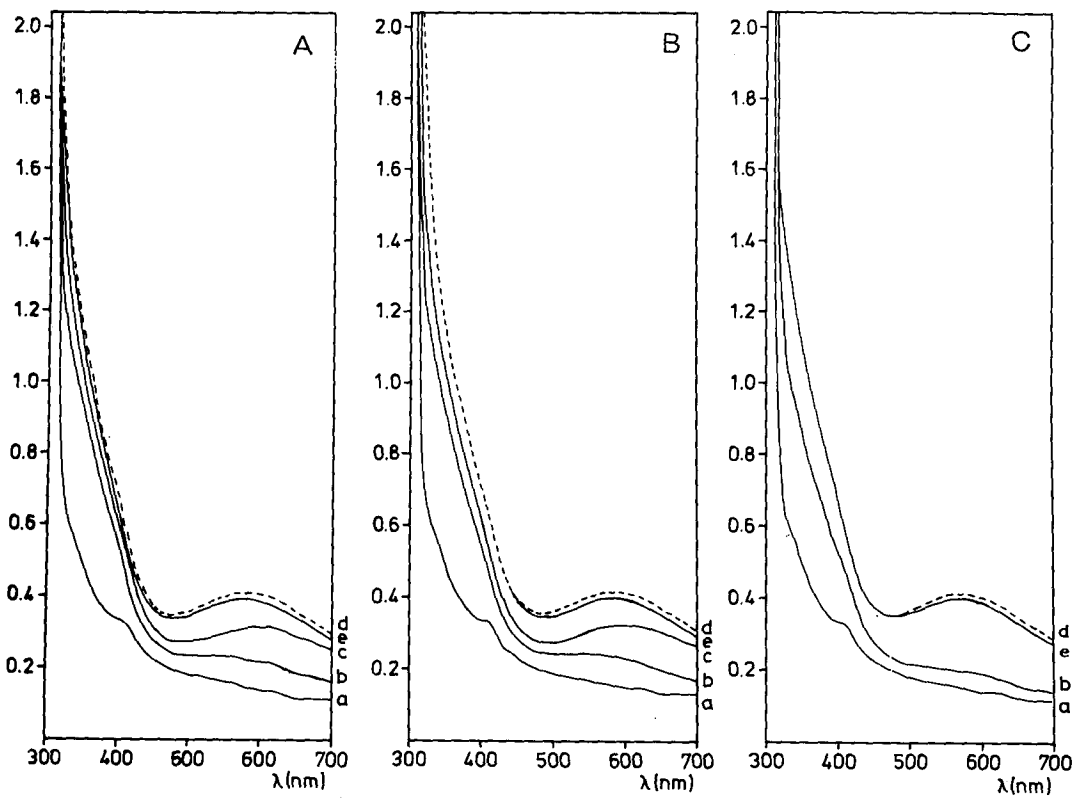

Fig. 1. Optical absorption spectra of lipoxygenase-2 species. A, 9*R*-HPOD; B, 9*S*-HPOD; C, 13*S*-HPOD. a, resting enzyme; b, 1 molar equivalent of HPOD (immediately after addition); c, 1 molar equivalent of HPOD (formation of chromophores completed); d, additional 1.5 molar equivalent of HPOD (immediately after addition); e, spectrum taken 4–6 min after the addition of a further 1.5 molar equivalent.

had come to completion and an aliquot for EPR studies had been taken, a further 1.5 molar equivalent of HPOD was added. With all HPODs there was an increase in the absorbance at 580 nm. The final absorbance at 580 nm was the same for all HPODs used.

Although the chromophores are reminiscent of those observed with lipoxygenase-1–13*S*-HPOD interactions [16], some differences are noteworthy. First, with lipoxygenase-2, 9*R*- and 9*S*-HPOD are both capable of forming the 580 nm chromophore [17]. With lipoxygenase-1 the 9*S*-HPOD can form the near-ultraviolet (350 nm) but not the 580 nm chromophore [16]. Secondly, with lipoxygenase-2, the chromophores in the near ultraviolet and at 580 nm start to arise concomitantly, and not sequentially, as is the case with lipoxygenase-1 [16]. This is concluded from the observation that after the addition of 1 molar equivalent HPOD to lipoxygenase-2 both the chromophores in the near ultraviolet and at 580 nm are observed, whereas with lipoxygenase-1 only the near-ultraviolet chromophore arises [16]. The 580 nm chromophores formed upon addition of excess hydroperoxide are unstable, as with lipoxygenase-1.

EPR spectra

Effect of one molar equivalent HPOD. The EPR spectra of lipoxygenase-2 samples of which optical spectra had been recorded are shown in Fig. 2. The spectrum of the resting enzyme (not shown) has a signal at *g* 4.3, which stems from high-spin Fe(III) in a rhombic ligand field [9]. In view of the instability of the enzyme (see below), it could well be that the signal originates from denatured enzyme.

The features observed upon addition of the various product hydroperoxides (Fig. 2A and B) are similar. The spectra are dominated by *g* 4.3 signals. It is difficult to quantify these signals accurately because of their broad wings and perturbing signals at *g* 6 and *g* 9. The reference sample, transferrin, has a *g* 4.3 signal with characteristics [18] similar to those of lipoxygenase, and integration between the same field limits should eliminate most of the errors. The final results of the quantification are given in Table I.

The signal at *g* 9 is associated with the *g* 4.3 signal [14]. The broad signals around *g* 6 stem from high-spin Fe(III) species in ligand fields with different degrees of axial symmetry. Compared to lipoxygenase-1, the axial ligand symmetry is considerably distorted. The rhombicity of the EPR signals of lipoxygenase-2 is even more pronounced than that observed with lipoxygenase-1 upon addition of SDS (sodium dodecyl sulphate) [19]. The signal arising at *g* 6 upon addition of 13*S*-HPOD to lipoxygenase-2 shows the most distinct features. It bears a remarkable resemblance to the EPR signal observed with a type-2 lipoxygenase from peas upon addition of 13*S*-HPOD [20]. However, in the latter case, the *g* 4.3 signal was relatively small in amplitude.

Integration of the *g* 6 signal of lipoxygenase-2 treated with HPOD in the absence of ethanol (see below) was possible in the case of the lipoxygenase-2–13*S*-HPOD interaction. The amount of EPR-visible iron was determined, assuming a *D* value equal to that of lipoxygenase-1 [21]. It proved impossible to integrate the *g* 6 signals in the other interactions, because of the proximity of the *g* 4.3 and *g* 9 signals. Therefore, the amount of iron represented by the *g* 6 signals in the interactions of lipoxygenase-2 with 9-HPODs was estimated by comparison of the amplitudes of rhombic and axial parts of the signals around *g* 6 with those of a lipoxygenase-1 sample of known concentration [14]. The results are presented in Table I.

The enzymic activities of the samples, which were thawed after the EPR measurements, are shown in Table II. There is a considerable loss of activity upon freezing, storage and thawing, which is only slightly higher in the case of the HPOD-treated samples than for a sample of resting enzyme that had only been frozen, stored and thawed. Only 9*R*-HPOD caused significant inactivation in addition to that caused by the procedures mentioned. The increase of the *g* 4.3 signal from 5% in the resting enzyme to 10% after addition of 13*S*-HPOD (Table I) may be due to oxidation of iron by 13*S*-HPOD.

In all interactions of lipoxygenase-2 and HPODs, about 25% of the iron is represented by the *g* 6 and *g* 4.3 signals taken together. This is considerably less than the *g* 6 signal alone in the lipoxygenase-1–13*S*-HPOD interaction (80%) [14]. The generally low amount of EPR-visible iron may be due to inactivation or denaturation of the

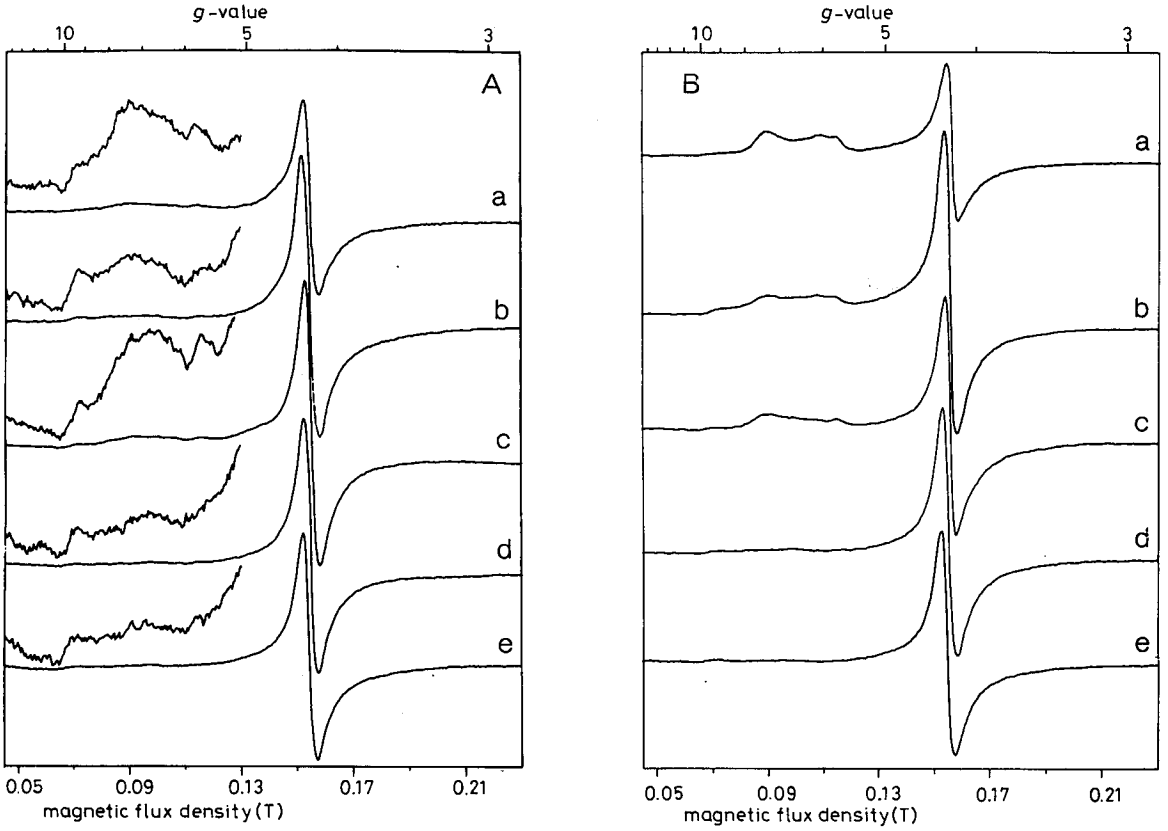

Fig. 2. EPR spectra of lipoxygenase-2 species. A, addition of 9*R*-HPOD; B, addition of 13 *S*-HPOD. The indices a–e correspond with the indices in Table I. EPR spectra a and b were taken of samples of which the optical spectra in Fig. 1 are indicated with c and e, respectively. The insets were recorded at a 10-times higher receiver gain.

TABLE I

QUANTIFICATION OF THE EPR SIGNALS OBSERVED IN THE INTERACTION OF SOYBEAN LIPOXYGENASE-2 WITH 9*R*-HPOD, 9*S*-HPOD AND 13*S*-HPOD

The numbers represent the amount of iron in the enzyme made EPR-visible, as percentage of the enzyme concentration. The figures in the resting enzyme column refer to enzyme that was not treated with any HPOD but otherwise handled like the other samples.

HPOD added:	9*R*		9*S*		13*S*		Resting enzyme:
g value:	6	4.3	6	4.3	6	4.3	4.3
a. 1 equivalent HPOD	7	16	9	14	15	10	5
b. Additional 1.5 equivalent of HPOD	2	19	3	25	13	19	
c. Sample a, 2 h at 4°C	7	11	6	9	10	10	6
d. Sample c, linoleic acid added under N_2	4	13	3	12	3	12	8
e. Sample d, flushed with O_2	2	9	2	10	2	10	
f. Sample a, 0.1% ethanol added	6	19	6	23	9	14	

TABLE II

ACTIVITY MEASUREMENTS ON THE LIPOXYGENASE-2 SAMPLES USED IN THE SPECTROSCOPIC STUDIES

The figures represent percentage specific activities related to the specific activity of freshly dialyzed lipoxygenase-2: 7.1 μmol O_2 $min^{-1} \cdot mg^{-1}$ protein. The figures in the resting enzyme column refer to enzyme that was not treated with any HPOD but otherwise handled like the other samples.

HPOD	9*R*	9*S*	13*S*	Resting enzyme
Untreated				100
After addition of 1 equivalent, freezing, storage, EPR measurement and thawing	41	50	54	55
After anaerobic addition of linoleic acid and admission of O_2	20	35	37	42

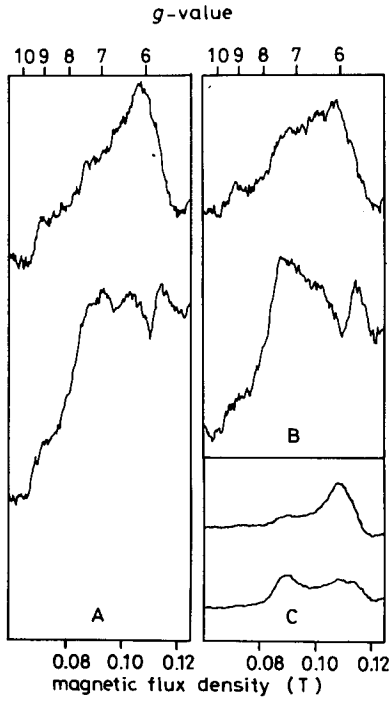

Fig. 3. Effect of 0.1% (v/v) ethanol on the EPR spectrum of lipoxygenase-2 to which 1 molar equivalent of HPOD has been added. A, 9*R*-HPOD; B, 9*S*-HPOD; C, 13*S*-HPOD. The lower spectra are comparable to those in Fig. 2A. In the upper spectra, ethanol has been added.

protein. It could well be that factors causing the ineffective conversion to EPR-visible iron at high concentrations of lipoxygenase-1 (L. Petersson et al., unpublished data) also occur with lipoxygenase-2 but at much lower concentrations. When lipoxygenase-2 is treated with 13*S*-HPOD more iron is EPR-visible at *g* 6 than upon treatment with 9-HPODs. Within experimental error the latter have identical effects as far as the amounts of EPR-visible iron are concerned.

Effect of ethanol. Ethanol has been reported to change the EPR spectrum of lipoxygenase-1 at as low a concentration as 0.05% (v/v) to give an almost exclusively axial signal [19]. It was considered worthwhile to determine whether a similar effect could be observed in the more rhombic EPR spectra of lipoxygenase-2. The results are shown in Fig. 3A–C. Upon addition of 0.1% (v/v) ethanol, a loss of rhombicity is indeed observed in the interaction of lipoxygenase-2 with all three product HPODs studied. As is shown in Fig. 3A–C, this effect is most pronounced in the interaction with 13*S*-HPOD. This observation suggests that the coordination spheres of iron are similar in lipoxygenase-1 and -2.

Effect of excess HPOD. Invariably, addition of 2.5 molar equivalents of HPOD to lipoxygenase-2 leads to a larger *g* 4.3 signal at the expense of the *g* 6 signal. The effect of 13*S*-HPOD is less pronounced than that of 9-HPODs (Table I). However, the values listed in Table I contain a varying amount of error due to both the shape of the EPR spectra and denaturation of the protein.

For lipoxygenase-1 it has been demonstrated that correlations exist between the chromophore in the near ultraviolet and the *g* 6 signal, and also between the chromophore at 580 nm and the *g* 4.3 signal [22]. In the latter study [22], the molar absorption coefficient of the 580 nm chromophore, which was originally found to be 1320 $M^{-1} \cdot cm^{-1}$ [15], was recalculated on the basis of an EPR-quantification study. It was found that the *g* 4.3 signal correlated to this chromophore represented only 10% of the iron [14], yielding a value of 10 000 $M^{-1} \cdot cm^{-1}$ for the molar absorption coefficient [22]. If the same correlations exist

in lipoxygenase-2 and the molar absorption coefficient is the same, one can calculate from the optical spectrum that, after the addition of 2.5 molar equivalents of either the 9*R*-, the 9*S*- or the 13*S*-HPOD, the 580 nm chromophore is present only in 6–7% of the enzyme molecules. This would be somewhat less than in the case of lipoxygenase-1. The quantification on the basis of the *g* 4.3 EPR signal of lipoxygenase-2 yields a significantly larger percentage. The difference indicates that there is a considerable contribution from denatured enzyme to the *g* 4.3 signal in the lipoxygenase-2–HPOD interactions. The contribution from denatured enzyme may be different for the resting enzyme and each of the HPOD-treated enzyme samples (Table I). The HPODs may in part and to different extents have the same effect on lipoxygenase-2 as H_2O_2 has on lipoxygenase-1, i.e. irreversible inactivation and concomitant formation of *g* 6 and *g* 4.3 signals upon titration with more than equivalent amounts [23].

Further EPR studies

In order to investigate whether a lipoxygenase-2 species with only a *g* 6 and no *g* 4.3 signal could be observed, the EPR tubes containing lipoxygenase-2 and HPOD in a molar ratio of 1 : 1 were thawed and kept in ice until the purple colour had disappeared, i.e. for 2 h, and then frozen again. As is evident from Fig. 2, there is still a *g* 4.3 signal, and the quantification (Table I) does not indicate a significant decrease in the amount of iron EPR-visible in this signal. This suggests that denaturation is an important factor in samples of this protein. This is different from the type-1 enzyme, which is much less prone to denaturation.

To test whether it was possible to make the enzyme EPR-silent by reducing the Fe(III) giving rise to the EPR signals (cf. Ref. 9), the samples were again thawed, flushed with nitrogen and mixed with a 5-fold excess of linoleic acid. The EPR spectra changed considerably and the amount of iron EPR-visible at *g* 6 could indeed be reduced (Table I), most clearly in the case of 13*S*-HPOD. However, the *g* 4.3 signal increased slightly in intensity.

To investigate whether the EPR signals would increase in intensity due to formation of HPODs from linoleic acid added in the preceding step, the thawed samples were flushed with oxygen. However, the total amount of EPR-visible iron did not increase significantly and the enzymic activity had diminished considerably (Table II). With lipoxygenase-1, shuttling between the two valence states of iron can readily and repeatedly be demonstrated with EPR spectroscopy using a single protein sample [9].

Stopped-flow studies

Lipoxygenase-2 was mixed at 40 or 50 μM concentration with amounts of 13*S*-HPOD varying from 0.1 to 1 molar equivalent. The development of the chromophores after mixing was monitored at 330 and 580 nm. With all hydroperoxides used in this study, the chromophores in the near ultraviolet and at 580 nm arise concomitantly. This is analogous to the observations for lipoxygenase-1 upon mixing with a molar excess 13*S*-HPOD [24]. The effect of mixing equimolar amounts of enzyme and HPOD is different for lipoxygenase-1 and -2. With lipoxygenase-1 only the near-ultraviolet chromophore is formed, whereas lipoxygenase-2 forms both the near-ultraviolet and the 580 nm chromophores.

Concluding remarks

From the results of this study, it is evident that in the interaction of lipoxygenase-2 with its three main product hydroperoxides the same chromophores are produced as in the interaction of lipoxygenase-1 with its main product hydroperoxide, 13*S*-HPOD [16]. The extent and the kinetics of the formation of the chromophore at 580 nm depend on the type of lipoxygenase and hydroperoxide. With lipoxygenase-2, no excess hydroperoxide is needed to start its formation.

The effect of ethanol on the EPR spectra and several additional data presented in this study suggest that the coordination spheres and functions of iron in lipoxygenases-1 and -2 are similar. Important differences between these two types of lipoxygenases have been found with respect to regio- and stereospecificities in the oxygenation of polyunsaturated fatty acids, amino acid compositions, IEPs [6] and immunological properties [25]. Despite the similarity of the coordination sphere of iron in both enzymes, the active site must have

differences with respect to the relative orientation of the substrates, i.e. the polyunsaturated fatty acid and dioxygen. On the basis of modification studies of lipoxygenase-1 [26] and -2 [6] we have recently suggested that a particular sulphydryl group which is present in lipoxygenase-1 but absent in lipoxygenase-2 plays a role in this respect. It has been suggested that the relatively high cooxidation activity of type-2 lipoxygenases can be explained in terms of a higher mobility of the fatty acid radical formed during the dioxygenation [27]. This effect may also account for the relatively low regio- and stereospecificity of the dioxygenation by lipoxygenase-2.

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