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A QUANTITATIVE OPTICAL AND EPR STUDY ON THE INTERACTION BETWEEN SOYBEAN LIPOXYGENASE-1 AND 13-L-HYDROPEROXYLINOLEIC ACID

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, NL-3522 AD Utrecht (The Netherlands) and* [b] *Chalmers Institute of Technology and University of Göteborg, Department of Biochemistry and Biophysics, S-412 96 Göteborg (Sweden)*

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The optical absorbance changes at 370 nm observed upon addition of 13-L$_S$-hydroperoxy-9-*cis*,11-*trans*-octadecadienoic acid (13-L-HPOD) to native lipoxygenase-1 are linearly correlated to the amount of Fe(III) iron visible in the EPR signal around *g* 6. The yellow enzyme form represents lipoxygenase with iron in the Fe(III) state. Addition of a molar excess of 13-L-HPOD to either native or yellow lipoxygenase results in the formation of a purple enzyme form with an additional EPR signal at *g* 4.3 stemming from Fe(III) in a ligand field of rhombic symmetry, representing 10% of the total iron content. The absorbance at 570 nm is linearly correlated with the intensity of the *g* 4.3 signal. The purple enzyme form is a lipoxygenase-13-L-HPOD complex with a conformation of the iron environment that allows charge transfer from amino acids ($\varepsilon_{570} = 1 \cdot 10^4\ M^{-1} \cdot cm^{-1}$).

Introduction

Addition of 13-L-HPOD to native, colourless and EPR-silent Fe(II)-lipoxygenase results in the formation of a yellow form which can turn into a purple-coloured Fe(III)-enzyme form, depending on the amount of 13-L-HPOD supplied [1]. Optical absorption spectra of the various enzyme forms have been reported [1,2]. Upon titrating the native enzyme with 13-L-HPOD [3] or with linoleic acid in an aerobic system [4] the maximum absorbance at 370 nm of the yellow enzyme form is obtained when about 1 molar equivalent is added. EPR spectra of the yellow and purple enzyme forms show a complex signal around *g* 6 [1,3] which has been attributed to at least three high-spin Fe(III) species with largely axial ligand symmetry [5,6]. The purple enzyme shows an additional signal at *g* 4.3 arising from high-spin Fe(III) in a ligand field of rhombic symmetry [7]. The spectrum around *g* 6 of the purple enzyme has a larger contribution from the most rhombic component. The purple enzyme form represents an enzyme-13-L-HPOD complex [1,8] which is rather unstable and, on standing, gradually reverts to the yellow form [1]. In the EPR spectrum the *g* 4.3 signal diminishes and eventually a spectrum is obtained which is indistinguishable from that of yellow lipoxygenase-1 [1]. The rate of the decomposition of the complex depends on temperature and dioxygen concentration [1,9,10]. The amounts of iron visible in the EPR spectra of the various enzyme forms have been determined by simulation and integration of the spectra, showing that the total amounts of EPR-visible iron around *g* 6 in yellow and purple lipoxygenase are at least 75% of the total iron content [5]. The *g* 4.3 signal of the purple enzyme represents about 10% of the total iron content [5].

Abbreviation: 13-L-HPOD, 13-L$_S$-hydroperoxy-9-*cis*,11-*trans*-octadecadienoic acid.

Studies describing changes in the optical absorbance [3,4] and fluorescence [4] upon titrating the native enzyme with either 13-L-HPOD or linoleic acid in an aerobic system suggest a correlation between the optical spectroscopic data and the oxidation state of iron in lipoxygenase. Information on the oxidation state of iron in lipoxygenase has been obtained from EPR-spectroscopic experiments [1,3,7] and magnetic susceptibility measurements [11]. In this paper the quantitative correlations between the yellow colour and the intensity of the EPR signal around g 6 and between the purple colour and the g 4.3 signal intensity are described. Furthermore, results of a quantitative study on the decomposition of the purple enzyme are presented.

Materials and Methods

Yellow and purple lipoxygenase-1 were obtained by incubation of native lipoxygenase-1 with 13-L-HPOD as described earlier [5].

EPR spectra were recorded with a Varian E-9 spectrometer with 100 kHz field modulation at 15 K using an Oxford Instruments cryostat [12]. Optical absorption spectra were recorded at 293 K with small-volume cells with 1 cm pathlength on a Beckman Acta spectrophotometer, and at 77 K in EPR tubes using a dual-wavelength spectrophotometer (Johnson Research Foundation DBS-2) with provisions for storing the spectrum of a reference substance in digital form [13]. Rapid mixing of the enzyme and 13-L-HPOD solutions was performed with the apparatus described by Wilson et al. [14]. The amount of EPR-visible iron was determined by simulation and integration methods described earlier [5].

Results and Discussion

A. Correlation between the yellow colour and the intensity of the EPR signal around g 6

Upon titrating native lipoxygenase with 13-L-HPOD a high-spin Fe(III) signal becomes visible in the EPR spectrum around g 6. Using simulation procedures [5] the amount of high-spin Fe(III) visible around g 6 is determined as a function of the amount of 13-L-HPOD added (Fig. 1). The maximum amount of EPR-visible iron is obtained when 1 molar equivalent of 13-L-HPOD is added. The iron in lipoxygenase can thus be oxidized from Fe(II) to Fe(III) by 13-L-HPOD in a stoichiometric reaction. The formation of the purple enzyme upon further addition of 13-L-HPOD beyond a 1 : 1 stoichiometric ratio (Fig. 1) is accompanied by a slight decrease of the amount of iron visible around g 6 and the formation of a new signal at g 4.3. The correlation between the optical absorbance and the intensity of the EPR signal around g 6 could be studied directly by using a recently developed method for the measurement of optical absorbance in the EPR tubes [13]. A linear correlation is observed upon titrating native en-

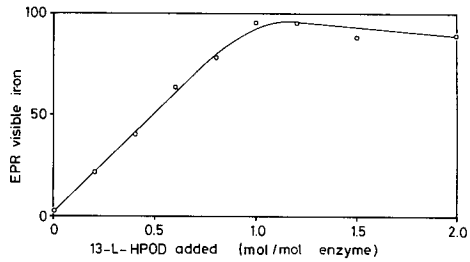


Fig. 1. Titration of native lipoxygenase with 13-L-HPOD. Small amounts of 13-L-HPOD (64.3 mM) were added to 250 μl native enzyme (0.33 mM in 0.1 M borate buffer, pH 9.0). On the ordinate the amount of iron visible in the EPR signal around g 6 is given as a percentage of the total iron content.

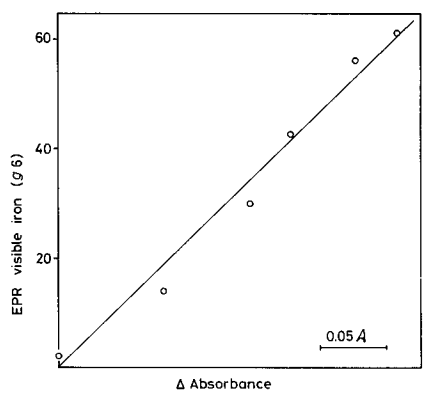


Fig. 2. Correlation between the amount of iron visible in the EPR signal around g 6 and the yellow colour of lipoxygenase-1. Small amounts of 13-L-HPOD (38.9 mM) were added to 200 μl native enzyme (0.37 mM in 0.1 M borate buffer, pH 9.0). Absorbance ($A_{370}-A_{460}$) was measured on the sample in the EPR tube at 77 K. On the ordinate the amount of iron visible in the EPR spectrum around g 6 is given as a percentage of the total iron content.

zyme with 13-L-HPOD (Fig. 2) which holds up to equimolar amounts. This implies that earlier studies [1,4,8], in which optical absorbance changes were reported, dealt in fact with changes in the valence state of iron as has been proposed by Egmond et al. [4] for the chromophore formed upon treatment of native lipoxygenase with 13-L-HPOD or H_2O_2. The absorption coefficient derived from the difference spectrum of yellow – native lipoxygenase at 370 nm is $1.8 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ [2]. This is considerably lower than that observed for other proteins giving g 6 signals, which are generally heme proteins. It is, on the other hand, considerably higher than that observed in simple high-spin Fe(III) complexes, indicating an unusual coordination sphere of iron in lipoxygenase.

B. Correlation between the purple colour and the intensity of the EPR signal at g 4.3

Purple-coloured lipoxygenase can be obtained upon addition of a molar excess of 13-L-HPOD to the native and yellow enzyme form [1]. The EPR spectrum of the purple enzyme shows an increase of the signal at g 4.3 and a change of the signal around g 6 to a more rhombic type when compared to the yellow enzyme form [1]. The amount of EPR-visible iron at g 4.3 is approx. 10% of the total iron content [5], balancing the decrease of the amount of EPR-visible iron around g 6. A titration with 13-L-HPOD as carried out for the yellow enzyme is difficult to perform accurately because the purple enzyme form is labile and gradually reverts to the yellow enzyme form [1]. For this reason the correlation between the purple colour and the intensity of the EPR signal around g 4.3 (Fig. 3) is studied by monitoring the decomposition of the purple complex obtained by addition of 4 molar equivalents of 13-L-HPOD to native lipoxygenase. The molar excess of 13-L-HPOD is sufficient for the formation (> 99%) of the enzyme-

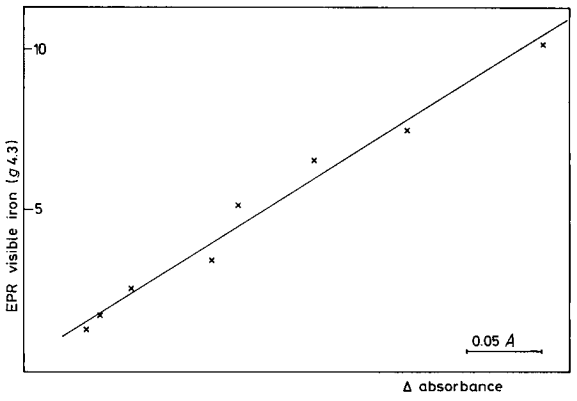


Fig. 3. Correlation between the intensity of the EPR signal at g 4.3 and the purple colour of lipoxygenase-1. Native enzyme (0.25 mM in 0.1 M borate buffer, pH 9.0) was incubated with 4 molar equivalents of 13-L-HPOD (37.0 mM). The sample (250 µl) was frozen in liquid nitrogen directly after mixing. In order to allow a decomposition of the enzyme-13-L-HPOD complex the sample was brought to room temperature during periods of approx. 1 min. The absorbance was measured as difference of A_{570} and A_{450}. On the ordinate the amount of iron visible in the EPR signal at g 4.3 is given as a percentage of the total iron content.

13-L-HPOD complex as calculated from the affinity constant of the yellow enzyme for 13-L-HPOD [8]. A linear correlation is observed between the purple colour and the g 4.3 signal intensity. This observation leads to the suggestion that only the enzyme molecules which give rise to the g 4.3 signal (approx. 10% of the enzyme concentration [5]) form a type of complex with 13-L-HPOD that absorbs at 570 nm (Scheme 1). In principle, the purple complex need not necessarily have a 1 : 1 stoichiometry, but kinetic studies [15] suggest a single binding site. In view of this, the molar absorption coefficient ε_{570} of the purple enzyme-13-L-HPOD complex is $1 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$, being roughly 10-times as large as the value which was earlier calculated on the basis of the assumption

E-Fe(III)	+	13-L-HPOD	⇆	E-Fe(III) ··· 13-L-HPOD	↔	E-Fe(III) – – – 13-L-HPOD
yellow				yellow		purple
EPR around g 6				EPR around g 6 (less axial than E-Fe(III))		EPR at g 4.3

Scheme I. Interaction of lipoxygenase-1 and 13-L-HPOD.

that all enzyme molecules form an enzyme -13-L-HPOD complex which absorbs at 570 nm [1,2]. This new value of ε_{570} for purple lipoxygenase is much higher than the molar absorption coefficient of the purple coloured Fe(III)-EDTA-peroxo complex which is formed during the superoxide dismutation in a model system ($\varepsilon_{520} = 530\ M^{-1} \cdot cm^{-1}$) [16,17]. However, human transferrin, an iron-containing protein which gives rise to an EPR signal round g 4.3 stemming from approx. 100% of the iron content [18], has a molar absorption coefficient $\varepsilon_{465} = 5 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ [19], which means $\varepsilon_{465} = 2.5 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ per iron. For transferrin the absorption band at 465 nm is ascribed to ligand-to-metal charge-transfer transitions [20]. Such a charge-transfer transition could also be responsible for the absorbance at 570 nm of purple lipoxygenase, indicating that aromatic or sulfur-containing amino acids may contribute to the liganding of iron in lipoxygenase-1. During the formation of the purple enzyme form the amount of iron visible in the rhombic part of the EPR signal around g 6 increases from 44 to 56% of the total iron content in yellow and purple lipoxygenase, respectively [5]. This indicates that the axial symmetry of the environment of iron is slightly disturbed upon addition of an excess of 13-L-HPOD to the yellow enzyme. Probably, approx. 10% of the enzyme-13-L-HPOD complex has obtained a conformation which allows charge-transfer between amino acids and iron.

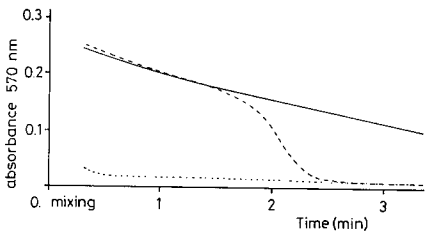
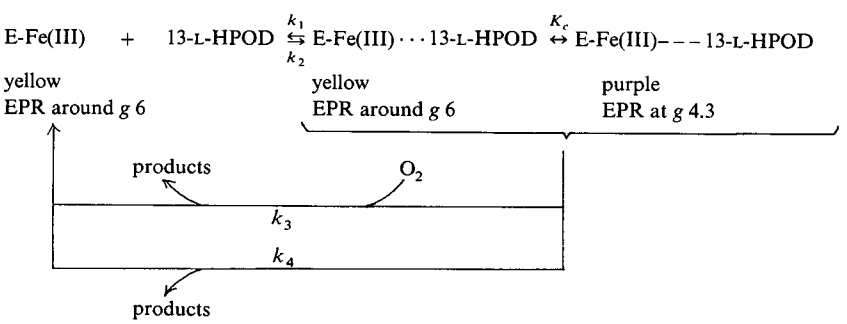


Fig. 4. Decomposition of purple lipoxygenase-1. Native enzyme (0.37 mM in 0.1 M borate buffer, pH 9.0) was incubated with 4 molar equivalents of 13-L-HPOD (70.9 mM) in a 1-cm cell at 293 K. The dioxygen concentration of the enzyme solution was approx. 1.25 (———), 0.25 (------) and less than 0.02 mM (· · · · · ·). The time course of the reaction was recorded at 570 nm approx. 20 s after the incubation.

C. Decomposition of the enzyme-13-L-HPOD complex

Owing to the conversion of 13-L-HPOD by the enzyme, the enzyme-13-L-HPOD complex decomposes [1,8]. The hydroperoxidase activity of lipoxygenase-1 has been described for anaerobic [9] and aerobic [6,10] systems (Scheme II).

The decomposition of the enzyme-13-L-HPOD complex can be monitored at 570 nm. The time course of the decomposition starting with different dioxygen concentrations in the reaction mixture is presented in Fig. 4. At high dioxygen concentration (1.25 mM) the decomposition takes place at a relatively slow rate, whereas at low dioxygen concentration (< 0.02 mM) the decomposition is almost complete after 20 s. At a dioxygen concentra-



Scheme II. Formation and decomposition of the lipoxygenase-13-L-HPOD complex. Products include epoxy-hydroxyoctadecenoic acid, 13-oxotridecadienoic acid and 13-oxo-octadecadienoic acid [9,10].

tion of approx. 0.25 mM a biphasic decomposition reaction is observed because of dioxygen depletion. This is consistent with the observations of Verhagen et al. [10] that a dioxygen consumption of 0.7 mol per mol 13-L-HPOD takes place and that the hydroperoxidase activity is 4-times higher under anaerobic conditions. The maximum concentration of purple enzyme which can be obtained depends on the relative magnitudes of the reaction rate constants, the dioxygen concentration and the equilibrium constant (K_c) of the conformations of the enzyme-13-L-HPOD complex (Scheme II). At 4°C the formation of the enzyme-13-L-HPOD complex proceeds within a few milliseconds [8] and k_3 and k_4 are of the order of 0.02 min^{-1} [8] and 0.08 min^{-1} [8,10], respectively. The amount of iron visible in the g 4.3 signal of the purple lipoxygenase does not become significantly higher than 10% of the total iron content when a rapid mixing and freezing system [14] is used. This indicates that the amount of enzyme-13-L-HPOD complex which has an environment of iron allowing charge-transfer is not readily affected by variation of the experimental conditions.

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