An EPR Study on the Binding of Alcohols to Soybean Lipoxygenase-1 *

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Lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12) is a dioxygenase catalyzing the reaction between molecular oxygen and polyunsaturated fatty acids with a 1,4-*cis*,*cis*-pentadiene system.[1] Soybean lipoxygenase-1 contains per molecule one non-heme iron atom which switches between the ferric and ferrous states during catalysis as demonstrated by EPR.[2] In the oxidized form the so-called yellow enzyme shows a complex EPR signal around g 6 stemming from high-spin Fe(III) species. The line shape of the EPR spectrum changes substantially upon addition of small amounts of ethanol and other alcohols.[3] This change in line shape corresponds to a change in the relative amounts of species having different environments of iron.[3]

The purpose of this investigation was to obtain more information on the effects of alcohols on the EPR spectrum of yellow lipoxygenase to gain a better understanding of the binding of the alcohols and the natural substrates.

Experimental. Lipoxygenase-1 was isolated from soybeans according to Finazzi Agrò *et al.*[4] The specific activity was 235 μmol O_2 min^{-1} mg^{-1}. The iron content was 0.97 mol per mol enzyme and the amount of contaminating Mn was found to be 0.07 mol per mol enzyme. 13-L- and 9-D-hydroperoxy-octadecadienoic acid (HPOD) were prepared by aerobic incubation of linoleic acid with soybean lipoxygenase-1 at pH 9.0[5] and corn-germ lipoxygenase at pH 6.6,[6] respectively, and purified by HPLC. 13-L- and 9-D-hydroxy-octadecadienoic acid (HOD) were prepared by reduction with $NaBH_4$ of 13-L- and 9-D-HPOD, respectively. These compounds were purified by TLC on 0.50 mm precoated plates in the solvent system hexane – diethylether – acetic acid (50:50:1, v/v/v). The other chemicals were of reagent grade. EPR spectra at 9 GHz were recorded on a Varian E-9 spectrometer with a 100 kHz field modulation (modulation amplitude 2 mT). Spectra presented in the same figure are corrected for small differences in enzyme concentrations and in dimensions of the EPR tubes thus allowing a direct comparison of intensities.

Results. Yellow lipoxygenase-1, obtained by oxidation of the native, colourless and EPR-silent enzyme with one molar equivalent 13-L-HPOD, shows a complex EPR spectrum around g 6 (Fig. 1A). This signal is built up by at least three different high-spin Fe(III) species with different symmetry.[7] The most axial and the most rhombic species have g_x equal to 6.2 and 7.5, respectively. The small signal at g 4.3 has been attributed to contaminating high-spin Fe(III).[2] The presence of contaminating manganese and radical signals near g 2 obscures the g_z-parts of the high-spin Fe(III) resonances (insert Fig. 1). Fig. 1B gives the spectrum of yellow lipoxygenase to which 30 molar equivalents of ethanol (corresponding to 8.5 mM) were added. Similar spectra are obtained upon addition of butanol-1 and hexanol-1. Titration curves of yellow lipoxygenase with ethanol, butanol-1 and hexanol-1 are presented in Fig. 2. The changes in the EPR spectra are measured by the ratio (R) of the amplitudes of the rhombic (at g 7.5) and axial (at g 6.0 – 6.2) parts.

In order to mimic the situation with substrate fatty acid and product hydroperoxide, hydroxy-octadecadienoic acids were also used. EPR spectra obtained upon addition of 3 molar equivalents 9-D- or 13-L-HOD to yellow lipoxygenase are shown in Fig. 3. Titration curves are given in the insert. In contrast to the other alcohols a decrease of the total signal intensity is observed upon titration of yellow enzyme with 9-D- or 13-L-HOD.

Discussion. In a study on the nature and relative amounts of the high-spin Fe(III) species that build up the complex g 6 signal of yellow lipoxygenase, a large effect of alcohols on the relative amounts of high-spin Fe(III) species has been reported.[3] The low concentration of alcohols (*e.g.* ethanol) which is required for a shift to an axial type of spectrum as shown in Fig. 1B for ethanol suggests that alcohols have a specific affinity for binding to the enzyme, probably in the environment of iron. The titration curves of various alcohols (Fig. 2) clearly show a more pronounced effect on the EPR spectrum with increasing carbon chain length. Thus, the affinity of the alcohols for binding on the enzyme increases with chain length. This result is in line with observations of Mitsuda *et al.*[8] who have reported that the degree of inhibition of lipoxygenase by saturated monovalent alcohols increases with an increase of the chain length of the alcohols. A binding of the alcohols at a hydrophobic region

*Communication at the Meeting of the Swedish Biochemical Society in Gothenburg, 7 – 8th June, 1982.

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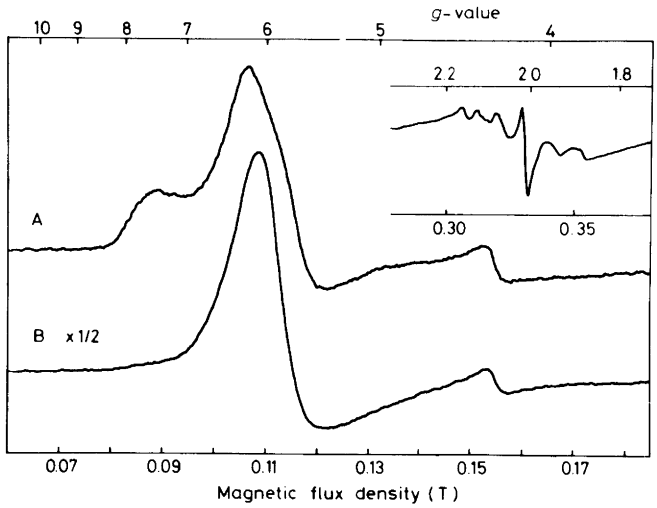

Fig. 1. Effect of ethanol on the line shape of the g 6 signal of yellow lipoxygenase-1. A. A solution of native enzyme (26 g/l) was incubated with a 13-L-HPOD solution (43.3 mM); final concentrations: 0.26 mM for both enzyme and 13-L-HPOD in 0.1 M borate buffer pH 9.0. Insert: High field part of the spectrum. B. 1 μl of an ethanol solution (1.7 M) was added to the sample described for A. Final concentrations: Enzyme 0.26 mM and ethanol 8.5 mM. Microwave frequency 9.256 GHz; microwave power 2 mW; temperature 15 K.

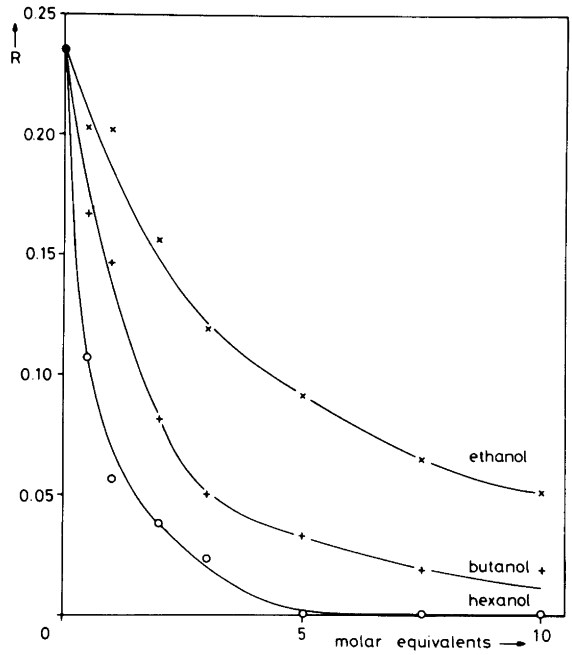

Fig. 2. Titration of yellow lipoxygenase-1 with ethanol, butanol-1 and hexanol-1. On the ordinate the ratio of the rhombic and axial components (R) is given. To yellow enzyme samples prepared as described for Fig. 1A small amounts of alcohol solutions (0.2 % v/v) were added. The amplitude of the rhombic part measured at g 7.5 is corrected for the contribution of the axial part at this g-value.

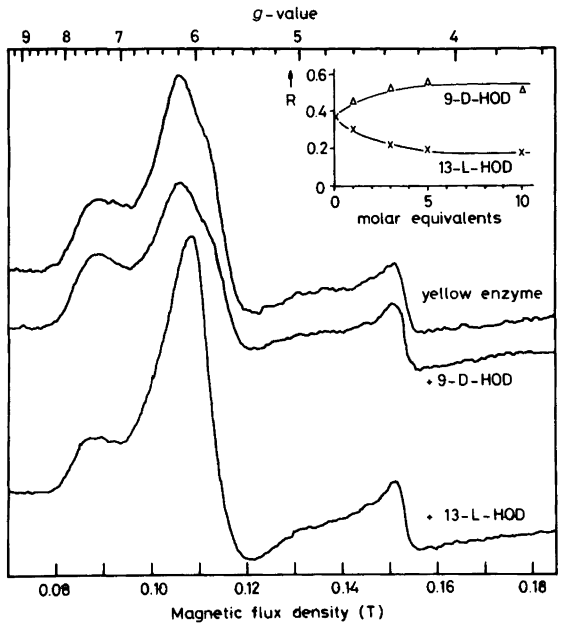

Fig. 3. Effect of hydroxy-octadecadienoic acids on the line shape of the signal around *g* 6 of yellow lipoxygenase-1. A yellow enzyme solution prepared as described for Fig. 1A was incubated with 3 molar equivalents of 9-D-HOD or 13-L-HOD. Final concentrations: Enzyme 0.22 mM and HOD 0.66 mM in 0.1 M borate buffer pH 9.0. Microwave frequency 9.179 GHz; microwave power 2 mW; temperature 15 K. Insert: Titration of yellow lipoxygenase-1 with 9-D- HOD and 13-L-HOD. On the ordinate the ratios of the amplitudes of the rhombic and axial components (R) are given.

which also serves as a binding site for the substrate has been suggested.[8] The effects of hydroxy-octadecadienoic acids, which have a hydrophobic part similar to the product hydroperoxy-octadecadienoic acid, have been investigated. The results are also relevant to the binding of the substrate octadecadienoic acid because the affinity constants of substrate and product are of the same order of magnitude.[9,10] For the two hydroxy-octadecadienoic acids different effects on the line shape are observed (Fig. 3). 13-L-HOD gives a shift to an axial type of spectrum whereas 9-D-HOD induces a rhombic shift. Unlike 13-keto-octadecadienoic acid [3] 9-D-HOD does not cause the appearance of a new rhombic species. This experiment makes clear that besides the chain length other structural elements (*i.e.* positions and configurations of the double bonds and the presence of substituents) are important for binding. Although different effects of 9-D-HOD and 13-L-HOD on the EPR spectrum of yellow lipoxygenase are observed, the titration curves of both compounds (insert Fig. 3) indicate that the changes are almost complete after addition of approx. 5 molar equivalents. This amount is comparable to that for hexanol-1 which has also a saturated chain of five carbon atoms and has the highest affinity for binding to lipoxygenase (Fig. 2). This is consistent with studies on the substrate specificity,[11] showing that the best substrates are polyunsaturated fatty acids with the pentadiene system at the n-6 position, *i.e.* a saturated chain of five carbon atoms.

This EPR study on the binding of monovalent alcohols and hydroxy-octadecadienoic acids to lipoxygenase-1 supports the concept that the hydrophobic part of the substrate is very important for binding to the enzyme.

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Received June 14, 1982.