Reprinted from

Biochimica et Biophysica Acta, 488 (1977) 517—520
© Elsevier/North-Holland Biomedical Press

BBA Report

BBA 51211

ON THE PROPERTIES OF A PEA LIPOXYGENASE

LEOPOLD J.M. SPAAPEN, JOHANNES F.G. VLIEGENTHART and JAN BOLDINGH

Laboratory of Organic Chemistry, University of Utrecht, Croesestraat 79, Utrecht (The Netherlands)

(Received May 5th, 1977)

Summary

Pea lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12) contains one atom of iron per mol as was shown by atomic absorption analysis.

EPR experiments have shown that the iron in the enzyme interacts with substrate linoleic acid and product hydroperoxylinoleic acid. A parallel can be drawn between the role of iron in this enzyme and in soybean lipoxygenase 1 and between the course of the anaerobic conversion of hydroperoxylinoleic acid and linoleic acid by both enzymes.

Lipoxygenase (linoleate:oxygen oxidoreductase EC 1.13.11.12) catalyses the oxygenation by molecular oxygen of polyunsaturated fatty acids containing a 1,4-*cis,cis*-pentadiene system to conjugated hydroperoxy fatty acids [1]. It has been demonstrated that soybean lipoxygenase 1 contains one gatom of non-heme iron per mol of enzyme [2]. The apparent valence state of iron in the enzyme is influenced by substrate fatty acid and product hydroperoxide as was shown by EPR spectroscopy [3,4]. These observations confirmed the hypothesis that iron plays an essential role in the reactions catalysed by this enzyme. In the class of lipoxygenases, the soybean 1 enzyme holds an exceptional position with respect to its pH optimum of 9.0 in comparison to 6—7.5 for the other known lipoxygenases. Therefore it seemed appropriate to investigate if the latter group of enzymes also contain iron and to determine its function in the catalytic processes.

We have chosen pea lipoxygenase for this purpose. Its isolation and partial purification has already been described by several authors [5—8].

Lipoxygenase was isolated from green peas (*Pisum sativum* L. var. "Bliss Abundance") according to Eriksson and Svensson [5], but reduced glutathione was omitted throughout the procedure because this appeared to have no favourable effect on the yield. The enzyme turned out to be rather unstable,

particularly on column chromatography. However, it could be stored as a precipitate in a 50% ammonium sulphate solution for at least one month at 4°C without significant loss of activity. The specific activity of the enzyme was 4.3 μmol O_2/min per mg protein as was measured by a polarographic method using 1.8 mM ammonium linoleate as substrate in 0.1 M potassium phosphate buffer, pH 6.6. The purified enzyme gave a single band on polyacrylamide gel electrophoresis. The molecular weight was determined by sodium dodecyl sulphate electrophoresis according to Weber and Osborn [9] with the following proteins as standards: cytochrome *c* (horse heart) 12 400; myoglobin (sperm whale) 17 800; chymotrypsinogen A (beef pancreas) 25 000; ovalbumin 45 000, albumin (bovine) 67 000; soybean lipoxygenase 1 and 2, both 100 000. The obtained value of 98 000 daltons for the pea enzyme agrees satisfactorily with the value of 106 000 daltons reported by Haydar et al. [7]. However it should be mentioned that Arens et al. [8] and Eriksson and Svensson [5] have reported molecular weights of 78 000 and 72 000, respectively. Metal analysis by flameless atomic absorption spectrometry revealed the presence of 1.04 gatom Fe, 0.05 gatom Cu and 0.02 gatom Mn per mol of enzyme. The contents of Co and Mo in the enzyme solution were below the detection limit of 0.01 ppm (equivalent to 0.003 gatom Co and 0.002 gatom Mo per mol of enzyme).

To study the role of iron in the reactions catalysed by this enzyme, the influence of substrate fatty acid and product hydroperoxide on the apparent valence state of iron under various reaction conditions was investigated by EPR spectroscopy according to de Groot et al. [3]. EPR spectra were recorded on a Varian E-3 spectrometer with 100 kHz field modulation at a microwave power of 4 mW. All measurements were carried out at 15 K with a modulation amplitude of 10 G. In the EPR experiments, a solution of 0.19 mM of enzyme in 0.1 M Tris·HCl buffer, pH 7.0, was used. The EPR spectrum of the native enzyme shows a weak signal at $g = 4.23$ (Fig. 1A), which probably stems from contaminating iron and a sharp signal at $g = 1.97$, of unknown origin.

Addition of an equimolar amount of 13-L-hydroperoxylinoleic acid (13-L-ROOH) to the native enzyme turned the colour of the solution into yellow. The EPR spectrum of this solution contains signals at $g = 7.2$, 6.1, 5.7 and 4.2 (Fig. 1B) indicating that iron is now in a high spin ferric state with an axial ligand symmetry. Further addition of 13-L-ROOH to the yellow enzyme solution turned it into a purple coloured solution. This purple enzyme species is a rather unstable complex of yellow enzyme and product hydroperoxide. The hydroperoxide must be bound very close to iron since the ligand symmetry is changed from axial into rhombic, as is shown in the EPR spectrum (Fig. 1C); the intensities of the signals at $g = 7.2$, 6.1 and 5.7 are reduced, whereas the signal at $g = 4.2$ is strongly increased. Upon standing at room temperature, the purple species is reconverted into the yellow form. The EPR spectrum of the yellow enzyme derived from the purple one is almost identical to that of the yellow species obtained after treatment of the native enzyme with an equimolar amount of 13-L-ROOH (Fig. 1D). A new addition of 13-L-ROOH is necessary to regenerate the purple enzyme species.

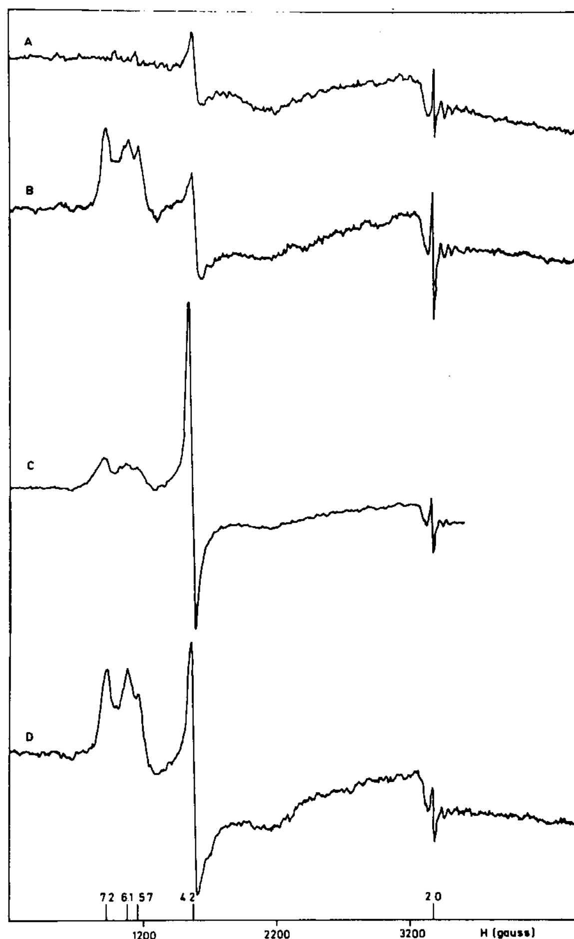

Fig. 1. Effect of 13-L-ROOH on the EPR spectrum of pea lipoxygenase. (A) 250 μl of the pea lipoxygenase solution (18.6 mg/ml); gain $4 \cdot 10^5$. (B) 4.3 μl of a 11.0 mM 13-L-ROOH solution was added to 250 μl of the pea lipoxygenase solution. Final concentrations: both 0.19 mM; gain $4 \cdot 10^5$. (C) another 8.5 μl of 11.0 mM 13-L-ROOH solution was added to the reaction mixture. Final concentrations: enzyme, 0.18 mM, 13-L-ROOH, 0.54 mM; gain $2 \cdot 10^5$. (D) the same reaction mixture as described under (C) after 45 min incubation at room temperature; gain $4 \cdot 10^5$. Microwave frequency, 9.325 GHz.

These experiments could be reproduced with a lipoxygenase preparation obtained from a wrinkled seeded pea variety. The results show a remarkable similarity with those described for soybean lipoxygenase 1 [10].

Under anaerobic conditions, the behaviour of pea lipoxygenase is also very similar. Addition of a molar excess of linoleic acid to the yellow enzyme at pH 7.0 under strictly anaerobic conditions leads to the formation of colourless ferrous enzyme. Moreover it was found that pea lipoxygenase is capable of catalysing the anaerobic conversion of 13-L-ROOH in the presence of linoleic acid both at pH 6.6 and 9.0. Under both conditions, 13-oxo-octa-9,11-dienoic acid and dimeric fatty acids are formed, but the chain cleavage reaction, leading to *n*-pentane and 13-oxo-trideca-9,11-dienoic acid, only occurs at higher pH (cf. refs. 11, 12). The same holds for the anaerobic reac-

tion of soybean lipoxygenase 1, which at low pH does not lead to chain cleavage products (unpublished results). Thus it seems that the course of the reaction is primarily determined by the pH of the reaction medium.

Moreover it has been observed that both the aerobic and the anaerobic reactions at pH 9.0 by the pea enzyme are stimulated by Ca^{2+} ions.

Acknowledgments

The authors are grateful to Mr. C.C. Saarloos (Unilever Research Laboratory, Vlaardingen) for performing the atomic absorption analysis.

References

1 Hamberg, M. and Samuelsson, B. (1965) Biochem. Biophys. Res. Commun. 21, 531—536
2 Chan, H.W. (1973) Biochim. Biophys. Acta, 327, 32—35
3 de Groot, J.J.M.C., Veldink, G.A., Vliegenthart, J.F.G., Boldingh, J., Wever, R. and van Gelder, B.F. (1975) Biochim. Biophys. Acta 377, 71—79
4 Pistorius, E.K., Axelrod, B. and Palmer, G. (1976) J. Biol. Chem. 251, 7144—7148
5 Eriksson, C.E. and Svensson, S.G. (1970) Biochim. Biophys. Acta 198, 449—459
6 Haydar, M. and Hadziyev, D. (1973) J. Sci. Fd. Agric. 24, 1039—1053
7 Haydar, M., Steele, L. and Hadziyev, D. (1975) J. Food Sci. 40, 808—814
8 Arens, D., Seilmeier, W., Weber, F., Kloos, G. and Grosch, W. (1973) Biochim. Biophys. Acta 327, 295—305
9 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
10 de Groot, J.J.M.C., Garssen, G.J., Veldink, G.A., Vliegenthart, J.F.G., Boldingh, J. and Egmond, M.R. (1975) FEBS Lett. 56, 50—54
11 Garssen, G.J., Vliegenthart, J.F.G. and Boldingh, J. (1971) Biochem. J. 122, 327—332
12 Garssen, G.J., Vliegenthart, J.F.G. and Boldingh, J. (1972) Biochem. J. 130, 435—442