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SOYA-BEAN LIPOXYGENASE: AN IRON-CONTAINING DIOXYGENASE*

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SUMMARY

Metal analysis by atomic absorption indicated the presence of one atom of iron in soya-bean lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12). Preincubation of the enzyme at pH 9 with chelating agents resulted in substantial inhibition. The results may indicate a role for the iron atom in lipoxygenase action.

INTRODUCTION

Lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12) was first isolated by Theorell *et al.*[1] who obtained a crystalline enzyme from the soya-bean. More recent studies have shown the wider occurrence of this enzyme in the plant kingdom[2–4]. While they differ in their specificity with respect to the position of oxygenation, all these enzymes catalyse the formation of a hydroperoxide from linoleic acid. As they catalyse the incorporation of both atoms of a molecule of oxygen to the substrate, the enzymes may, therefore, be formally classified as dioxygenases. Unlike other enzymes in this class, however, no transition metal is believed to be associated with lipoxygenase. The requirement of a transition metal in dioxygenases may, at least, in part, originate from the high activation energy found in non-radical addition reactions between molecular oxygen and organic compounds. When conservation of spin angular momentum is considered, the allowed product between molecular oxygen in its ground state (a triplet) and an organic molecule (other than a radical) in its ground state (a singlet) is a triplet—usually of high energy. It is possibly this "spin-barrier" which accounts for the high activation energy in reactions catalysed by dioxygenases. The formation of a complex between oxygen and a transition metal can be an effective means of lowering the activation energy and hence provide a pathway for catalysis. As part of a programme of investigation into the mechanism of dioxygenation, the question of the presence of a transition metal in lipoxygenase has been reexamined.

* Results discussed herein were reported at the 11th World Congress of the International Society for Fat Research held on 18–22 June 1972, Göteborg, Sweden.

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MATERIALS AND METHODS

Enzyme purification

Soya-bean lipoxygenase (Type I) was purchased from Sigma (London), Chemical Co. Ltd, S.W.6, Great Britain. The enzyme (2 g) was dissolved in 0.02 M phosphate buffer (20 ml, pH 6.2). After removal of insoluble material by centrifugation, it was applied onto a column (7 cm × 55 cm) of Sephadex G-200 and eluted with the same buffer. Fractions containing the enzyme were pooled, concentrated by ultra-filtration and chromatographed on a column (2.5 cm × 7 cm) of DEAE-cellulose in 0.02 M phosphate buffer, pH 6.2, made up in de-ionised water. The enzyme was eluted as a single peak by a KCl gradient (0–50 mM). Fractions containing the enzyme were pooled and concentrated by ultra-filtration. Disc-gel electrophoresis (7.5% polyacrylamide) showed a single protein band in gels (4 mm diameter) containing 10 μg and 50 μg of protein*.

Atomic absorption

After the above purification, the lipoxygenase was prepared for metal analysis by atomic absorption as follows: a solution (1 mg/ml) of the enzyme was dialysed against de-ionized water in an EDTA-treated dialysis bag for 60 h with five changes of water in a polyethylene container (500 ml). The dialysis residue was passed through a column (0.8 cm × 7 cm) of Sephadex G-25 in de-ionized water and lyophilised. The solid enzyme (10 mg) was dissolved in de-ionized water (5 ml) to make up the solution for analysis. A fraction of the same volume as that containing the enzyme but from a region in the DEAE-cellulose chromatography that did not contain any ultraviolet absorbing material, was concentrated and treated as above and used as a control.

Atomic absorption was measured on a Pye-Unicam SP 1900 double beam spectrophotometer. Standard solutions (0.5 ppm and 1.0 ppm) of each metal to be determined were used to calibrate the instrument.

Enzyme assay and inhibition

Lipoxygenase activity was measured by the increase in absorbance at 234 nm of a 0.2 mM solution of linoleic acid in 0.2 M Tris buffer (pH 9.0). 1 unit of enzyme causes an absorbance increase of 0.001 per min at 25 °C with a final reaction volume of 3 ml and a light-path of 1 cm.

The purified enzyme had an activity of 280 000 units/mg.

For the inhibition studies, the enzyme ($5 \cdot 10^{-7}$ M) was incubated at 25 °C in 0.1 M Tris buffer (pH 9.0) containing the stated (Table II) molarity on inhibitor. Apart from KCN and EDTA which were added as aqueous solutions, the inhibitors were dispersed by addition as solutions in acetone, containing Tween 80 such that the final concentrations of acetone and Tween were 2% and 0.001%, respectively. Two controls were used both containing the same concentration of the enzyme. In one, acetone and Tween were added to the above final concentrations. The activities of the enzyme were identical in the two controls.

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* The enzyme isolated has a pH maximum of 8.5 for linoleate oxygenation and corresponds to lipoxygenase I (Christopher *et al.*[5]).

RESULTS AND DISCUSSION

In the account of their original study, Theorell *et al.*[1] reported the presence of a small amount of iron (≈0.3 mole per mole of enzyme) in lipoxygenase. Following the observation that chelating agents failed to inhibit the enzyme, the iron was regarded as an impurity. Care has been taken in the present investigation to ensure the production of a purified sample of the enzyme as free from metal contaminants as possible. Apart from being homogeneous in disc-gel electrophoresis, the enzyme used for analysis had a specific activity comparable to that obtained previously by isoelectric focussing[6]. Steps were taken to remove possible metal contaminants prior to metal analysis and a protein-free fraction from the final step in the purification of the enzyme was treated in parallel with the enzyme containing fraction and used as a control. Results obtained (Table I) show that iron is present in lipoxygenase to the

TABLE I

ANALYSIS OF METALS IN SOYA-BEAN LIPOXYGENASE

Metal	*Metal content in ppm*		
	Enzyme (2 mg/ml)	*Control*	*Atoms per mole of enzyme*
Cu	0.0	0.0	0.0
Mn	0.1	0.0	0.1
Fe	1.0	0.0	0.9
Zn	0.0	0.0	0.0
Mg	0.0	0.0	0.0

extent of one atom per mole of enzyme. Moreover, of five metals analysed, only iron is present to an appreciable degree. Confirmation of the presence of iron in lipoxygenase was obtained by the inhibition of the enzyme by a variety of chelating agents. Like Theorell *et al.*[1] we were unable to observe any inhibition of the enzyme by these reagents even at high concentrations if they are added at the time of the enzyme assay. However, when the enzyme is preincubated at pH 9.0 with the chelating agents, considerable inhibition is observed even at low concentrations of the inhibitors (Table II). Powerful chelating agents such as diphenylthiocarbazone, 1,10-phenanthroline and 2,2'-dipyridyl are particularly effective*. Sodium diethyldithiocarbonate does not inhibit as it is rapidly decomposed at this pH.

The results discussed here support but do not prove a role for the iron atom in catalysis in lipoxygenase. However, in view of the general involvement of transition metals in catalysis in dioxygenases and the absence of other reasonable means of lowering the activation energies in dioxygenation reactions, a role for the iron atom is strongly implicated.

* These results appear to be at variance with those reported by Roza and Francke (see preceding article: *Biochim. Biophys. Acta*, 327, 24–31). This could be due to the use of much lower concentrations of the enzyme ($5 \cdot 10^{-7}$ M) and dispersal agents for the solubilisation of the inhibitors in this study. The author thanks Ir Francke for communicating their results before publication.

TABLE II

EFFECT OF INHIBITORS ON LIPOXYGENASE ACTIVITY AFTER PREINCUBATION

Inhibitor	*Concentration (M)*	*Inhibition (%)*
Diphenylthiocarbazone	10^{-5}	100
	10^{-6}	32
1,10-Phenanthroline	10^{-5}	35
2,2'-Dipyridyl	10^{-5}	53
8-Hydroxyquinoline	10^{-4}	44
KCN	10^{-4}	71
EDTA	10^{-4}	60

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