A LIPOXYGENASE IN RABBIT RETICULOCYTES WHICH ATTACKS PHOSPHOLIPIDS AND INTACT MITOCHONDRIA

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1. Introduction

Soluble lipoxygenases are known to occur in a variety of plants [1]. For a long time attempts to detect genuine soluble lipoxygenases in animal tissues were unsuccessful [1,2]. Only recently Nugteren has described a lipoxygenase in blood platelets but not in other tissues which preferentially attacks arachidonic acid [3]. In this report we describe a soluble lipoxygenase in rabbit reticulocytes. A remarkable feature of the lipoxygenase from reticulocytes is the ability to cause lysis of intact mitochondria by peroxidation of their lipids. Pure phospholipids are also attacked. It is proposed that the action of the lipoxygenase on mitochondria is involved in the degradation of mitochondria during the maturation process of the reticulocytes.

2. Materials and methods

The lipoxygenase was partly purified by the following way. The osmotic haemolysate from washed rabbit reticulocytes (bleeding anaemia) was cautiously adjusted to pH 6.0 and the stroma was removed by centrifugation at 20 000 *g* for 20 min. The lipoxygenase in the stroma-free supernatant fluid was stabilized by addition of mercaptoethanol (final concentration 5 mM). Then an ammonium sulphate precipitation at 0.55 saturation was carried out. Further purification was accomplished by chromatography on DEAE-Sephadex A 50 in 0.01 M Tris–HCl pH 7.4 and elution by a NaCl gradient from 0.01 M up to 0.4 M. The active fraction was eluted at about 0.1 M NaCl. The fraction revealed only few distinct protein peaks in the diselectrophoresis, but no haemoproteins as judged by the absence of an absorbancy maximum in the 410 nm region. This fraction was used for most of the experiments. The mol. wt was estimated by gel chromatography on Sephadex G-200. The isoelectric point was determined by isoelectric focussing on Ampholine pH 5–8.

Isolated mitochondria from rat liver [4] were suspended in the following medium: 125 mM mannitol, 60 mM KCl, 60 mM Tris, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 0.5 mM EDTA, pH 7.4.

Phospholipids were extracted from rat liver mitochondria and fractionated by chromatography on silicic acid. The phospholipid classes obtained were identified by thin-layer chromatography. Lecithin from eggs was prepared according to Rhodes and Lea [6]. A methanolic solution of the lipids was added to ten volumes of 0.1 M potassium phosphate buffer pH 7.4. The methanol was removed by a stream of nitrogen before the addition of lipoxygenase.

The rat liver mitochondria were incubated with the lipoxygenase preparation for 60 min unless noted otherwise. The released malate dehydrogenase (MDH) was measured in the supernatant of the incubation mixture according to Siegel and Englard [7]. A sample treated with 0.2% Triton X-100 final concentration served as control for 100% release of MDH. The determination of malonyl dialdehyde (MDA) was performed by the thiobarbituric acid method.

3. Results

Lipoxygenase from reticulocytes produces drastic lysis of both inner and outer membranes of rat liver

Table 1
Release of MDH and formation of MDA from rat liver mitochondria by the lipoxygenase from reticulocytes

Expt. No.	Incubation (min)	Amount of enzyme (μl)	MDH release (%)	MDA formation (E_{532})
I	0	40	4	0.032
	15	40	15	0.312
	30	40	28	0.524
	60	40	47	1.032
	120	0	16	0.032
II	60	0	21	0.040
	60	3	24	0.062
	60	10	35	0.138
	60	30	86	0.432

mitochondria [8]. This lysis was not accompanied either by a release of free fatty acids or by the formation of free amino groups. Hence, an involvement of a phospholipase A or a proteolytic enzyme was excluded. In contrast, the release of MDH as a measure of the rupture of both mitochondrial membranes was paralleled by a formation of MDA which reflects a peroxidation of lipids (table 1). Lysis and formation of MDA coincided practically with regard to their dependence on the amount of lipoxygenase, time, temperature and pH. There was only little variation with pH between 6.5 and 8.0, but a strong increase with temperature up to 37°C. Above 40°C the enzyme was inactivated. A Q_{10} value of about 1.8 is consistent with the enzymatic nature of the rate-limiting step of the attack on mitochondria. Both lysis and MDA formation were strongly influenced by substrates and effectors of the mitochondrial metabolism. Succinate plus ADP prevented completely the action of the lipoxygenase. Cyanide, tiron and α-naphthol inhibit the action of the lipoxygenase on mitochondria, whereas α-tocopherol and propyl gallate were without effect (table 2). EDTA, *o*-phenanthroline in the presence and absence of mercaptoethanol and 8-oxyquinoline at 1 mM as well as 0.1 mM *p*-chloromercuribenzoate were also without effect.

The lipoxygenase also caused a formation of MDA from pure phospholipid suspensions. All main

Table 2
Effect of inhibitors on the activity of the lipoxygenase on mitochondria

Addition	Release of MDH (%)	Effect (%)	Formation of MDA[a] (E_{532})	Effect (%)
None	89	–	0.182	
2 mM KCN	36	−59	0.040	−78
1 mM α-naphthol	8	−92	0.027	−85
1 mM α-tocopherol	67	−25	0.210	+12
None			0.700	
1 mM tiron, 22 h			0.570	−19
None			0.642	
1 mM tiron, 41 h			0.400	−38

[a] Corrected for the controls.

Table 3
MDA formation from mitochondrial phospholipid classes by the lipoxygenase from reticulocytes

Phospholipid class	Amount of P-Lipid[a] (mg/sample)	Formation of MDA[b] (E_{532})
Cardiolipin	0.50	0.029
Phosphatidyl ethanolamine	0.51	0.046
Phosphatidyl choline	0.54	0.045
Complete mixture	0.51	0.096

The turbidity in the samples for the colorimetric determination caused by phospholipids was abolished by addition of 1% Triton X-100.
[a] Dry weight.
[b] Corrected for the controls.

phospholipid classes present in mitochondrial membranes (phosphatidyl ethanolamine, lecithin, cardiolipin) were attacked, cardiolipin albeit only weakly. The extent of MDA formation was highest with a complete mixture of mitochondrial lipids (table 3). The MDA formation was dependent on the amount of phospholipids and that of the enzyme. It was completely abolished by heat inactivation. In contrast, the nonenzymatic peroxidation of phospholipids induced by haeme compounds was heat-stable. The MDA formation from phospholipid suspensions was increased in the presence of 1% cholate. Under these conditions it was linearly dependent on the amount of lipoxygenase (fig.1). There are some differences between the actions of the lipoxygenase on mitochondria and on phospholipid suspensions. Whereas cyanide inhibited partly both actions (the peroxidation of egg yolk lecithin by 5 mM cyanide was inhibited by 45%); propyl gallate inhibited only the action on phospholipids (table 4). Furhtermore, there was an anomalous increase of action with decrease of temperature from 37 to 15°C. From these differences it is evident that there are differences in the actions of lipoxygenase dependent on substrates.

Some properties of the lipoxygenase from reticulocytes are summarized in table 5.

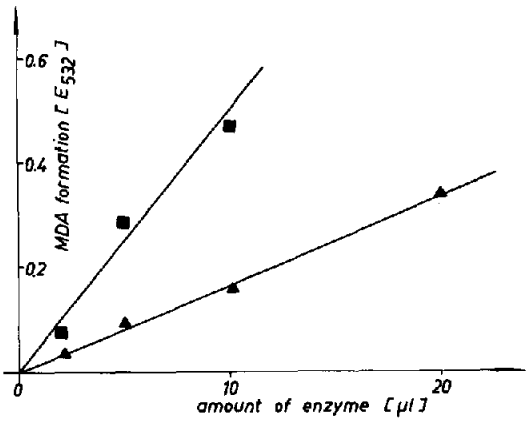

Fig.1. Formation of MDA from egg lecithin by the lipoxygenase from reticulocytes in the presence of 1% sodium cholate. (▲) 20 min incubation. (■) 60 min incubation. Values corrected for the controls. The enzyme solution contained 2.2 mg protein per ml (absorbancy at 280 nm).

Table 4
Effect of propyl gallate on the action of the lipoxygenase on mitochondria and egg yolk lecithin

Sample	Formation of MDA (E_{532})	
	Mitochondria	Lecithin
Control	0.033	0.054
Lipoxygenase	0.683	0.267
Lipoxygenase + 1 mM propyl gallate	0.628	0.049

4. Discussion

From the coincidence of the extent of MDA formation and that of the release of MDH as well as the ability to catalyse the peroxidation of pure phospholipids in the absence of any additional factor it is evident that we are dealing with a lipoxygenase. Its action differs from the nonenzymatic catalysis by haeme compounds in the ability to attack intact mitochondria and in the heat-lability of the enzyme. The linear increase of the action with the amount and the Q_{10} value argue strongly for a genuine enzymatic nature of the actions described.

The lipoxygenase from reticulocytes corresponds to that from soybeans in the following properties: mol. wt [9], similar behaviour on DEAE exchange resins [9], slow inactivation by the Fe-III chelating agent tiron [10] and unsensitivity against a variety of other chelators [11] as well as resistance against *p*-chloromercuribenzoate. In contrast, only the enzyme from reticulocytes is inhibited by cyanide, but not that from

Table 5
Properties of the lipoxygenase from reticulocytes

Occurrence:	reticulocytes, but not erythrocytes
Molecular weight:	120 000 (gel chromatography)
Isoelectric point	5.5 (isoelectric focussing)
Inhibitors:	CN^-, tiron, α-naphthol;
Without effect:	EDTA; 8-Oxyquinoline, *p*-Chloromercuribenzoate
Prosthetic group:	Fe^{III} (?)
Membrane binding affinity:	Binding to submitochondrial particles

soybeans. Futhermore, we failed to detect any inactivation of the reticulocyte enzyme by *o*-phenanthroline plus mercaptoethanol [10]. There are insufficient data to compare in details the enzymes from blood platelets and reticulocytes; one difference is the insensitivity to cyanide of the lipoxygenase from blood platelets [3].

The lipoxygenase of reticulocytes may be involved in the degradation of mitochondria which is one of the main features of the metabolism of these cells. The action of this enzyme may proceed in a complex interplay with respiratory inhibitors which are present in reticulocytes as well. A detailed discussion of the possible biological importance of the reticulocyte lipoxygenase will be subject of a further publication.

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