Reticulocyte lipoxygenase exhibits both *n*-6 and *n*-9 activities

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Purified reticulocyte lipoxygenase converts arachidonic acid to both 15- and 12-hydroxyperoxyeicosatetraenoic acids. The proportion of the two reaction products does not change during the purification procedure as shown by HPLC analysis. By means of isoelectric focusing it was not possible to separate the *n*-6 and *n*-9 activities. Reticulocyte lipoxygenase was completely inactivated by both 5,8,11-eicosatriynoic and 5,8,11,14-eicosatetraynoic acids in contrast to soybean lipoxygenase-1 which was inactivated only by 5,8,11,14-eicosatetraynoic acid. These results indicate that reticulocyte lipoxygenase exhibits both *n*-6 and *n*-9 activities. A contamination of the enzyme preparation with other lipoxygenases, e.g., the *n*-9 lipoxygenase from thrombocytes appears to be excluded.

Lipoxygenase *Positional specificity* *Reticulocytes* *15-Hydroperoxyeicosatetraenic acid* *12-Hydroperoxyeicosatetranoic acid* *Arachidonic acid*

1. INTRODUCTION

Lipoxygenases with different positional specificities have been identified in various animal cells [1–3]. The positional specificity determines the final products of the lipoxygenase pathway and, therefore, its biological function [4,5]. In [6] it was reported that the lipoxygenase of rabbit reticulocytes possesses both *n*-6 and *n*-9 activities as judged from the analysis of the reaction products. Here, experimental evidence is presented which indicates that the *n*-6 and *n*-9 activities are caused by one enzyme.

2. MATERIALS AND METHODS

Reticulocyte lipoxygenase was purified to homogeneity in electrophoresis and immunoprecipitation as in [7]. 5,8,11,14-All-*cis*-eicosatetraenoic acid (arachidonic acid) and 9,12-all-*cis*-octadecadienoic acid (linoleic acid) were obtained from Sigma (Switzerland). 11,14,17-All-*cis*- and 5,8,11-all-*cis*-eicosatrienoic acids were gifts from Dr R. Bryant (Washington DC). Samples of 5,8,11,14-eicosatetraynoic and 5,8,11-eicosatriynoic acids were gifts from both Dr D.H. Nugteren (Vlaardingen) and Dr S. Hammarström (Stockholm). Soybean lipoxygenase (grade IV) was purchased from Sigma (St Louis MO). Lipoxygenase activities were measured spectrophotometrically as in [8]; the assay media used were 0.1 M potassium phosphate (pH 7.4) containing 0.2% sodium cholate and 5% ethanol for reticulocyte lipoxygenase and 0.1 M sodium borate (pH 9.0) for soyban lipoxygenase unless stated otherwise.

3. RESULTS AND DISCUSSION

From the results of the high-pressure liquid chromatography (HPLC) analysis of the reaction products from arachidonic acid (table 1) it may be seen that the *n*-6 and *n*-9 activities were co-purified during the enzyme preparation. The data in fig.1 demonstrate that during isoelectric focusing no separation of the two activities could be achieved. It may be seen, that the *n*-9 activity measured as the oxygenation rate with 5,8,11-eicosatrienoic acid as substrate parallels the *n*-6 activity with linoleic acid as substrate.

Strong evidence for the conclusion that the two

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Table 1

High-pressure liquid chromatography of the reaction products of reticulocyte lipoxygenase with arachidonic acid during enzyme preparation

Purification step	15-Hydroperoxy-5,8,11,13-eicosatetraenoic acid (%)	12-Hydroperoxy-5,8,10,14-eicosatetraenoic acid (%)
$(NH_4)_2SO_4$ precipitation (0.55 saturation)	87	13
Ion exchange chromatography	92	8
Isoelectric focusing	80	20

Amounts of reticulocyte lipoxygenases with comparable lipoxygenase activity (2.3–2.7 nkat) were incubated with 100 μM arachidonic acid in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.2% sodium cholate and 5% ethanol at 2°C for 8 min. The reaction was stopped by acidification to pH 3. The mixture was extracted twice with diethylether. The extract was dried over sodium sulfate and concentrated by evaporation and was then subjected to silica gel thin-layer chromatography (solvent = hexane : diethylether : acetic acid, 60 : 40 : 1). Zones of hydroperoxyeicosatetraenoic acid were scraped off, eluted with ethanol and aliquots were subjected together with authentic standards to high-pressure liquid chromatography. HPLC was performed on a Zorbax–SIL column (4.6 × 250 mm) with a DuPont–HPLC system at 25°C. The samples were eluted isocratically with the solvent (hexane : 2-propanol : acetic acid, 986 : 13 : 1, by vol.) at 1.0 ml/min. The UV absorbance at 234 nm was monitored. Authentic standards of hydroperoxyeicosatetraenoic acid were prepared as in [9].

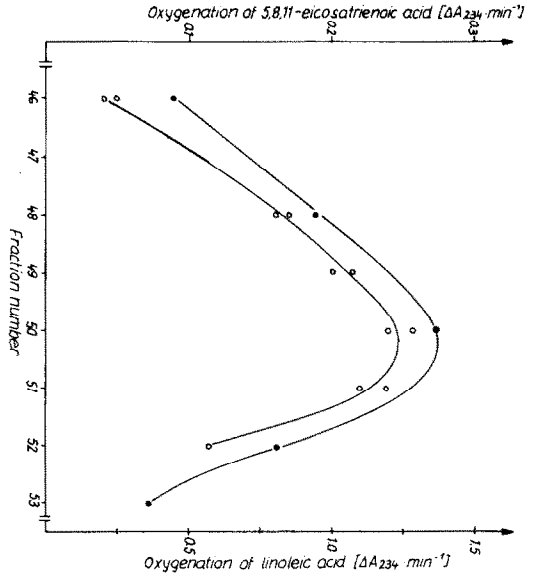

Fig.1. Isoelectric focusing of reticulocyte lipoxygenase. Isoelectric focusing was performed as in [7]. Fractions of 2.5 ml were obtained and checked for *n*-6 and *n*-9 activity. The *n*-9 activity was estimated with 100 μM, 5,8,11-all-*cis*-eicosatrienoic acid (○—○, left ordinate) in the assay system as in section 2. The *n*-6 activity was estimated with 0.53 mM linoleic acid as substrates (●—●, right ordinate). Protein fractions lacking lipoxygenase activity were omitted. Equal volumes of the fractions were added.

activities reside in one enzyme was obtained by experiments with polyacetylenic fatty acids. According to the Downing hypothesis [10] these substances act as suicidal substrates*. Whereas 5,8,11,14-eicosatetraynoic acid inactivates both *n*-6 and *n*-9 lipoxygenases, 5,8,11-eicosatriynoic acid is a strong inactivator only for *n*-9 lipoxygenases, since it lacks the allylic hydrogen at the *n*-8th carbon atom which is necessary for hydrogen abstraction by *n*-6 lipoxygenases. For the same reason 5,8,11-eicosatrienoic acid is a substrate only for *n*-9 lipoxygenases, whereas 11,14,17-eicosatriynoic acid is oxygenated only by *n*-6 lipoxygenases. Table 2 shows that reticulocyte lipoxygenase oxygenates both substrates with preference for the *n*-6 substrate, whereas soybean lipoxygenase, known to be a *n*-6 lipoxygenase [11], oxygenates 5,8,11-eicosatriynoic acid only to a minor extent. From table 3 it can be seen that 5,8,11,14-eicosatetraynoic acid inactivates the lipoxygenases from both reticulocytes and soybeans in contrast to 5,8,11-eicosatriynoic acid, which inactivates only the reticulocyte lipoxygenase. Even though the

* We have experimental evidence which indicates that the conversion of polyacetylenic fatty acid is the rate-limiting step in the inactivation of lipoxygenases (unpublished)

Table 2

Oxygenation of 5,8,11- and 11,14,17-eicosatrienoic acid by the lipoxygenases from reticulocytes and soybeans

Lipoxygenase from	Activity ($\Delta A_{234} \cdot min^{-1}$)	
	5,8,11-eicosa-trienoic acid	11,14,17-eicosa-trienoic acid
Reticulocytes (8 μg/ml)	0.35	2.0
Soybeans (0.35 μg/ml)	0.03	1.6

Activities were measured in the standard system with a substrate concentration of 100 μM

Table 3

Inactivation of lipoxygenases by polyacetylenic fatty acids

Inactivator	μM	Enzyme inactivation from:	
		Reticulo-cytes (%)	Soybeans (%)
5,8,11-Eicosa-triynoic acid	1.3	55	0
	6.6	90	0
	6.6	100	5
5,8,11,14-Eicosa-tetraynoic acid	0.12	51	6
	12	100	60

Reticulocyte lipoxygenase (11.5 μg/ml peak fraction of isoelectric focusing) and soybean lipoxygenase (1.75 μg/ml) were preincubated with acetylenic fatty acids in the assay medium (see section 2) at 22°C for 30 s. Afterwards lipoxygenase reaction was started by addition of 0.27 mM linoleic acid and residual activities were measured polarographically (oxygen electrode)

concentration of 5,8,11-eicosatriynoic acid necessary for half-inactivation is one order of magnitude higher than that of 5,8,11,14-eicosatetraynoic acid, complete inactivation of reticulocyte lipoxygenase can be achieved at low concentrations of 5,8,11-eicosatriynoic acid. Since the remaining activities were measured with linoleic acid as substrate, which is specific for *n*-6 lipoxygenases, it appears to be established that 5,8,11-eicosatriynoic acid is able to inactivate the *n*-6 lipoxygenase activity. This result can be understood only, if in the case of reticulocyte lipoxygenase one enzyme molecule is able to attack polyunsaturated fatty acids for initial hydrogen abstraction at different neighbouring allylic hydrogen atoms of the fatty acid chain. The subsequent step of the reticulocyte lipoxygenase reaction (i.e., the oxygen insertion) must also be directed enzymatically for both products, 12- and 15-hydroperoxyeicosatetraenoic acids, since only the *S*-enantiomers were found [6]. A non-enzymatic oxygenation or a dissociation of the arachidonic acid radical from the active center of the enzyme with subsequent isomerization would have led to the formation of a racemic mixture. This has been demonstrated to be the case for the 9-hydroperoxy-10,12-octadecadienoic acid, which is a side product of the soybean lipoxygenase-1 reaction with linoleic acid [12].

A contamination of the enzyme preparation by another *n*-9 lipoxygenase such as that from platelets can be excluded for several reasons:

(1) The homogeneity of the enzyme in sodium dodecylsulfate polyacrylamide electrophoresis;
(2) The lack of any lipoxygenase activity in enzyme preparations from blood with low reticulocyte percentages. If thrombocyte lipoxygenase were responsible for the *n*-9 activity it should have been noticeable in preparations from reticulocyte-poor blood;
(3) The lack of separation of *n*-6 and *n*-9 activities during the purification procedure including the parallel behaviour of the two activities during isoelectric focusing;
(4) The total inactivation of reticulocyte lipoxygenase by 5,8,11-eicosatriynoic acid. If a contamination of the enzyme preparation by another lipoxygenase possessing *n*-9 specificity were responsible for the formation of 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid, 5,8,11-eicosatriynoic acid should have only inactivated this part of the enzyme mixture leaving the *n*-6 activity of reticulocyte lipoxygenase unaffected.

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