The formation of products containing a conjugated tetraenoic system by pure reticulocyte lipoxygenase

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The pure lipoxygenase from reticulocytes converts 5,15-di-HETE at 2° C to product(s) showing a characteristic UV spectrum with maxima of strong absorbance at 300 and 316 nm and shoulders at 285 and 350 nm. Their formation was completely prevented by the lipoxygenase inhibitors, ETYA and NDGA, by heating of the enzyme and by anaerobiosis. At 35° C the products were formed only initially and in small amounts. The reaction products were purified by SP-HPLC and shown to migrate in the region of tri-HETEs in thin-layer chromatograms. The lipoxygenase from soybeans also converts 5,15-di-HETE to these product(s) with a comparable initial rate but different kinetics. These data suggest that 5,15-di-HETE is converted via a lipoxygenase reaction to 5,6,15-trihydroxy-7,9,11,13-(e,e,c,e)- and/or 5,14,15-trihydroxy-6,8,10,12-(e,c,e,e)-eicosatetraenoic acid, both of which contain a conjugated tetraene system.

Lipoxygenase product *Reticulocyte lipoxygenase* *Conjugated tetraene* *Reaction mechanism*

1. INTRODUCTION

A variety of lipoxygenase products, some of which are of great biological importance, have been isolated from various animal cells and tissues [1–3]. The purified lipoxygenases from soybeans [4] and reticulocytes [5] convert arachidonic acid to 15 L_s-HPETE and 15 L_s- and 12 L_s-HPETE, respectively. At higher lipoxygenase concentrations 5,15- and 8,15-di-HPETE were formed in addition to the mono-HPETEs [4,5]. It has been shown that these double dioxygenation products are formed by a secondary attack of the lipoxygenase on the primary dioxygenation product, 15 L_s-HPETE.

Recently, authors in [7] reported the formation of 5,6,15-trihydroxy-6,8,10,12-eicosatetraenoic acid by human leukocytes which showed a characteristic UV spectrum with three bands of intense absorbance at 287, 301 and 316 nm indicating a conjugated tetraene structure.

Here, it is shown that the pure lipoxygenases from reticulocytes and soybeans catalyze the formation of eicosanoids containing a conjugated tetraene system by a dioxygenation of 5,15-di-HETE or by a sequential triple dioxygenation of arachidonic acid.

Abbreviations: 15H(P)ETE, 15L_s-hydro(pero)xy-5,8,11,13-(c,c,c,e)-eicosatetetraenoic acid; 5,15-di-HETE, 5D_s,15L_s-hydroxy-6,8,11,13-(e,c,c,e)-eicosatetraenoic acid; ETYA, eicosatetraynoic acid; NDGA, nordihydroguaiaretic acid; tri-HETE, trihydroxyeicosatetraenoic acid; SP-HPLC, straight-phase high-pressure liquid chromatography; LTA_4, 5D_s-*trans*-5,6-oxido-7,9,11,14-(e,e,c,c)-eicosatetraenoic acid (leukotriene A_4)

2. MATERIALS AND METHODS

2.1. *Chemicals*

Arachidonic acid (90% pure) and soybean lipoxygenase (170000 units/mg) were obtained from Sigma. Nordihydroguaiaretic acid was purchased from Fluka (West-Berlin). Eicosatetraynoic acid was a kind gift from Dr Evstigneeva (Moscow).

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2.2. *Preparations*

15 L_S-HETE was prepared by the reaction of soybean lipoxygenase with arachidonic acid as in [8]. 5 D_S, 15 L_S-di-HETE was obtained by the reaction of soybean lipoxygenase with 15 L_S-HETE. Under our experimental conditions (1.6 mM 15 L_S-HETE, 250000 units soybeans lipoxygenase/ml, 2°C, 0.1 M sodium-borate buffer, pH 8.8) more than 90% of the di-HETE formed was 5,15-di-HETE as shown by SP-HPLC chromatography. Only a small amount of 8,15-di-HETE was found. Both 15 L_S- and 5 D_S, 15 L_S-di-HETE were purified by chromatography on a silica gel column and shown to be more than 90% pure by SP-HPLC analysis. Reticulocyte lipoxygenase was purified to homogeneity as in [9] and peak fractions of the isoelectric focussing were used for the experiments. The preparation of tri-HETE was performed as follows: 0.150 mg/ml (60 nkat/ml) purified reticulocyte lipoxygenase was incubated at 2°C in 0.1 M phosphate buffer (pH 7.4) with 280 μM 5 D_S, 15 L_S-di-HETE up to 4 h. The concentration of 5 D_S, 15 L_S-di-HETE was estimated using $\epsilon_{243} = 33\,500$ $(M \cdot cm)^{-1}$ in methanol [4].

To follow the reaction, spectra between 290 and 450 nm were recorded repeatedly by means of a Hitachi spectrophotometer 557. When the absorbance at 316 nm did not increase further, the reaction products were reduced by addition of sodium tetraborohydride. The mixture was acidified to pH 3 and twice extracted with diethylether. The organic layer was rewashed, dried over sodium sulfate, concentrated by evaporation and purified by SP-HPLC. Analytic and preparative SP-HPLC of the methylated products was performed on a silica gel column (Chromatronix Si-60, 250 × 4.6 mm; particle size 5 μm) with a Spectra Physics instrument; the solvent was hexane:isopropanol, 92:8 (v/v), flow 1 ml/min. Absorbance at 316 nm was monitored. Thin-layer chromatography was performed on precoated silica gel plates (Merck, FRG) with the solvent ethylacetate:2,2,4-trimethylpentane, 5:1 (v/v).

Methylation of the reaction products was performed with etheric diazomethane.

2.3. *Assay*

Arachidonate oxygenase activity was assayed as in [10].

3. RESULTS

As seen from fig.1, high concentrations of the pure reticulocyte lipoxygenase convert 5,15-di-HETE to a product mixture showing a characteristic UV spectrum with maxima of strong absorbance at 301 and 316 nm and shoulders at 285 (not shown) and 350 nm. With the denaturated enzyme the time-dependent changes of the spectral properties of the reaction mixture are completely prevented, parallel to the arachidonate oxygenase activity (table 1). Preincubation of the enzyme with NDGA, a powerful lipoxygenase inhibitor [11], and ETYA, a suicidal substrate for lipoxygenases [12], strongly inhibit the changes of spectral properties. As seen from table 1, the arachidonate oxygenase activity measured in aliquots of the preincubation samples was also inhibited. ETYA was shown to be somewhat less effective as an inhibitor under these conditions. However, at lower enzyme

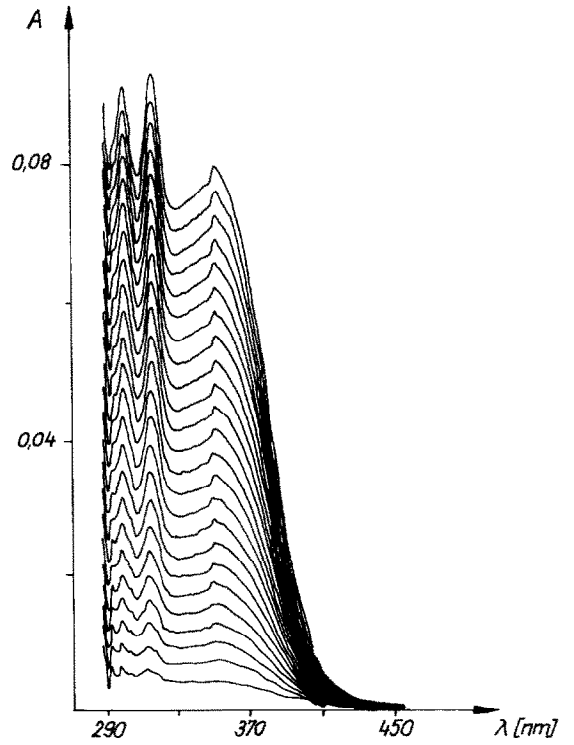


Fig.1. Formation of the tri-HETE by the pure reticulocyte lipoxygenase. Reticulocyte lipoxygenase (23 nkat/ml arachidonate oxygenase activity) was incubated as described in section 2. After every 2 min spectra were recorded repeatedly.

Table 1

Effects of heat denaturation, lipoxygenase inhibitors and anaerobiosis on the formation of tri-HETE

Conditions	Arachidonate oxygenase		Tri-HETE formation	
	$\Delta E_{234} \cdot min^{-1}$	Inhibition (%)	$\Delta E_{316} \cdot min^{-1}$	Inhibition (%)
Without inhibitor	0.510	–	0.003	–
Heating	0	100	0	100
NDGA	0	100	0	100
ETYA	0.100	81	0.0003	90
Anaerobiosis	not determined		0	100

Reticulocyte lipoxygenase was incubated with 5 D_s, 15 L_s-di-HETE as described in section 2. Absorbance at 316 nm was assayed. Heat denaturation of the enzyme was achieved by 5-min incubation at 80°C. The lipoxygenase was preincubated with the inhibitors (100 μM NDGA, 100 μM ETYA) for 15 min at 2°C. Afterwards, the reaction was started by addition of 5 D_s, 15 L_s-di-HETE. Aliquots of the incubation sample were taken off for the measurements of the arachidonate oxygenase activity. Anaerobiosis was achieved by long-lasting bubbling of all solutions with argon in ice

concentrations a complete inactivation of lipoxygenases was achieved [13]. Under anaerobic conditions no changes of the spectral properties of the reaction mixture were observed during an incubation period of 1 h. From fig.1 it is also evident that at 2°C the increase of the absorbance at 301 and 316 nm is linear over a long period (up to 3 h). In contrast, at 35°C only small amounts of the products possessing the characteristic UV spectrum are formed (not shown). Measurements of the arachidonate oxygenase activity in aliquots of this sample showed that the enzyme is completely inactivated during the first 8 min. Probably the hydroperoxy derivative of the 5,15-di-HETE formed by the lipoxygenase caused an inactivation of the enzyme similarly to hydroperoxy linoleic acid [14]. Indeed, the addition of glutathione–glutathione *S*-transferase, a system that reduces hydroperoxy lipids, decreased the inactivation of the arachidonate oxygenase activity and allowed the formation of the products with the characteristic spectral properties of the di-HETE conversion at 35°C.

Soybean lipoxygenase also converted 5,15-di-HETE at 2°C to product(s) showing a UV spectrum similar to the reticulocyte enzyme (not shown). However, as seen from fig.2 the spectral changes were not linear with time as shown for the absorbance at 316 nm (main peak). Since the arachidonate oxygenase activity remains active, an inactivation of the enzyme was excluded; on repeated addition of 5,15-di-HETE the reaction proceeded further.

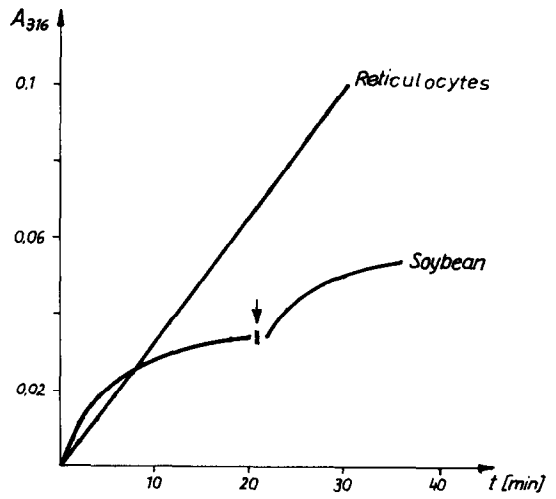


Fig.2. Comparison of tri-HETE formation by lipoxygenases from soybeans and reticulocytes. Comparable arachidonate oxygenase activities of both lipoxygenases (23 nkat/ml) were incubated with 280 μM 5 D_s, 15 L_s-di-HETE at 2°C in 0.1 M sodium phosphate buffer (pH 7.4), and the absorbance at 316 nm was monitored. The arrow indicates a second addition of 5 D_s, 15 L_s-di-HETE.

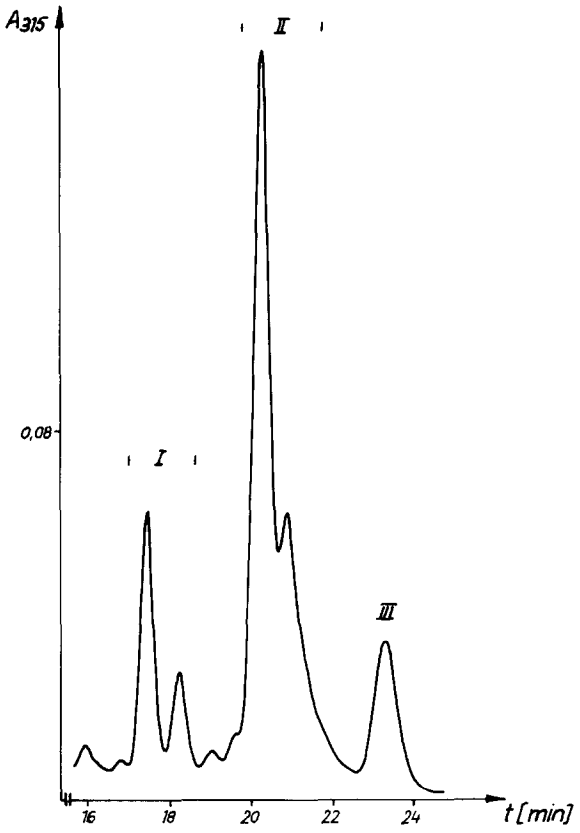


Fig.3. SP-HPLC analysis of the oxygenation products of 5,15-di-HETE. Product preparation and HPLC analysis as described in section 2. Before application to HPLC, the products were methylated with etheric diazomethane. Absorbance at 315 nm was monitored. Before retention time of 16 min no products with absorbance at 315 nm were eluted.

To obtain further information on the products formed during the reaction of reticulocyte lipoxygenase with 5,15 di-HETE, an SP-HPLC analysis was performed. As seen from fig.3, three main peaks showing an absorbance at 315 nm were eluted, but only the compound(s) II show the characteristic spectral properties of the incubation mixture.

The thin-layer chromatogram (fig.4) shows that compound(s) II (R_f = 0.54) is more polar than the substrate 5,15-di-HETE, moreover, it is co-chromatographed with a polar main product, probably tri-HETE, prepared by the haemoglobin-catalyzed decomposition of 15-HPETE [15] (not shown).

Using arachidonic acid as substrate, large

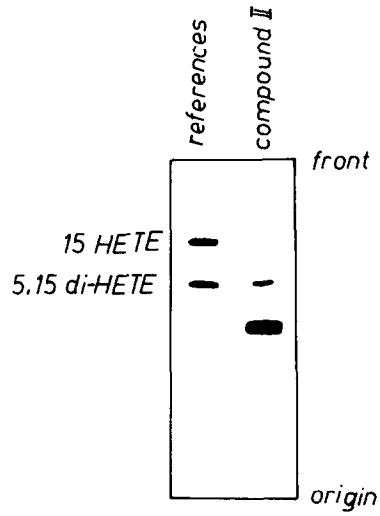
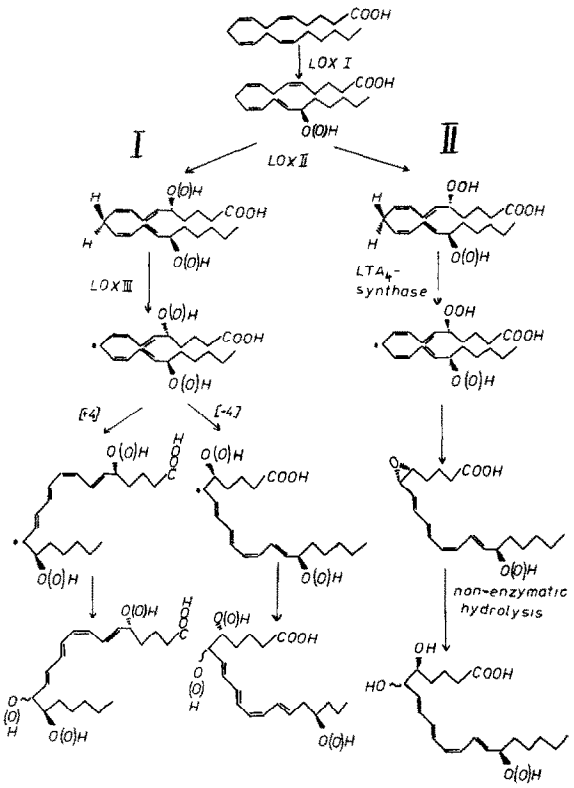


Fig.4. Thin-layer chromatography of compound(s) II. Fraction II of HPLC preparation was applied together with the standards on a precoated silica gel plate and the chromatogram was developed as described in section 2. The products are visualized with iodine.

amounts of 5,15-di-HETE and small, but detectable amounts of the compound(s) II were formed (not shown) as shown by SP-HPLC analysis.

4. DISCUSSION

The pure lipoxygenase from reticulocytes as well as the soybean lipoxygenase convert 5,15-di-HETE to product(s) showing in the UV region the spectral properties of a conjugated tetraene. Since this product formation is strongly inhibited by lipoxygenase inhibitors and by anaerobiosis, it may be concluded that 5,15-di-HETE is oxygenated via a lipoxygenase reaction to a hydroperoxy derivative of 5,15-di-HETE yielding, after borohydride reduction, a trihydroxyeicosanoid with a conjugated tetraene system. The probable reaction mechanism is shown in scheme 1. It is proposed that, starting from 5,15-di-HETE, the initial hydrogen removal takes place at the only double allylic carbon atom (C_{10}). As shown before, the reticulocyte lipoxygenase is able to remove hydrogen from C_{10} of arachidonic acid [5] and from 15-H(P)ETE [6]. Using arachidonic acid as substrate, the tri-HETE showing the spectral properties of a conjugated tetraene should be formed by a sequential triple dioxygenation of the fatty acid.



Scheme 1. Proposed mechanism of the formation of trihydroxyeicosanoids containing a conjugated tetraene system by attack of lipoxygenases on arachidonic acid (I) Lipoxygenase pathway. (II) LTA_4 synthase pathway.

The positional isomerism of the hydroperoxy group introduced by the lipoxygenase during the oxygenation of 5,15-di-HETE is not yet established. According to the mechanism of the lipoxygenase reaction the (−4)rearrangement of the radical would lead to the 5,6,15-tri-HETE and the (+4)rearrangement to the 5,14,15-tri-HETE. Work is in progress to obtain information on the stereochemistry of these lipoxygenase products.

Recently it has been shown [7] that a product with similar spectral properties, identified as 5,6, 15-tri-HETE, was formed during the conversion of 15-HPETE by human leukocytes. It has not yet been clarified if this product is formed by a sequential double dioxygenation of 15-HPETE by lipoxygenases or by a non-enzymatic hydrolysis of 15-hydroxy-5,6- LTA_4. In both cases the sequential action of two hydrogen-removing enzymes would be necessary. Therefore, both reaction mechanisms have great similarities.

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