# Formation of lipoxin B by the pure reticulocyte lipoxygenase

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The pure reticulocyte lipoxygenase converts 5,15-DiHETE via a lipoxygenase reaction to 5,14,15-trihydroxy-6,8,10,12-eicosatetraenoic acid (a lipoxin B isomer) as shown by GC/MS analysis of its trimethylsilyl ether. With arachidonic acid, 15-HETE and 15-HETE methyl ester this lipoxin B isomer was also formed. The results presented here indicate that pure mammalian lipoxygenases are able to form lipoxins via sequential multiple oxygenation of arachidonic acid or its hydroxy derivatives.

*Lipoxygenase* *Multiple oxygenation* *Lipoxin*

## 1. INTRODUCTION

In 1984 Serhan et al. [1,2] described the formation of trihydroxyeicosanoids containing a conjugated tetraene system, called lipoxins, by polymorphonuclear leukocytes. As concluded from their steric structure which was elucidated by co-chromatography with authentic standards [3–5] most of the lipoxin A isomers, in particular those containing an all-*trans* conjugated tetraene system, were formed via hydrolysis of an epoxy intermediate [3,6]. Conflicting results, however, were reported concerning the steric structure and consequently the biosynthetic pathway of lipoxin B isomers [7,8]. Recently Morris et al. [9] identified one-fifth of the lipoxin B isomers formed by leukocytes as 5*S*,14*R*,15*S* trihydroxy-6*E*,8*Z*,10*E*,12*E*-eicosatetraenoic acid. This product meets the structural requirements of a triple oxygenation product of arachidonic acid, but is biosynthesized via enzymatic epoxide hydrolysis [10].

Two years ago we reported that the lipoxygenases from reticulocytes and soybeans convert their reduced double oxygenation product, 5,15-DiHETE, to trihydroxyeicosanoids containing a conjugated tetraene system [11].

Here, it is shown that the main product formed during the 5,15-DiHETE oxygenation by reticulocyte lipoxygenase is 5,14,15-trihydroxy-6,8,10,12-eicosatetraenoic acid (a lipoxin B isomer). (See Note Added in Proof.)

*Abbreviations:* 15-H(P)ETE, 15-hydro(pero)xy-5,8,11,13(*Z,Z,Z,E*)-eicosatetraenoic acid; 5,15-DiHETE, 5,15 - dihydroxy - 6,8,11,13(*E,Z,Z,E*) - eicosatetraenoic acid; RP-HPLC, reversed-phase high-pressure liquid chromatography; SP-HPLC, straight-phase high-pressure liquid chromatography; GC/MS, gas chromatography/mass spectroscopy

## 2. MATERIALS AND METHODS

### 2.1. *Chemicals*

The chemicals used were obtained from the following sources: 5,8,11,14-eicosatetraenoic acid (90% pure), soybean lipoxygenase (type IV) and bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Serva (FRG); sodium borohydride from Merck (FRG).



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### 2.2. *Preparations*

Reticulocyte lipoxygenase was prepared to homogeneity as described in [12]. 15L$_S$-HETE was prepared from arachidonic acid with soybean lipoxygenase as described in [13] except that the reduction of 15-HPETE formed was performed with sodium borohydride prior to silica gel column chromatography. 5,15-DiHETE was prepared with a modified method according to [14] and subsequent SP-HPLC separation. 15L$_S$-HETE was used as substrate. The chemical nature of 15-HETE and 5,15-DiHETE prepared was confirmed by UV and IR spectroscopy and GC/MS analysis.

### 2.3. *Analytics*

RP-HPLC was performed with a Du Pont 8800 instrument equipped with a Zorbax-ODS RP-HPLC column (250 × 4.6 mm; 5 $\mu$m particle size). The methyl esters of the trihydroxyeicosanoids were eluted with a solvent system of methanol/water (60:40, v/v), flow rate 1 ml/min. The absorbance at 300 nm was recorded. GC/MS analysis was performed with a Hewlett Packard 5995 A GC/MS system equipped with an SE 54 column (25 m × 0.24 mm). Samples were applied with a home-made on-column injector. The trimethylsilyl ether of the trihydroxyeicosanoid methyl ester was eluted with a carrier gas (helium) flow of 1.8 ml/min. Initially the temperature was held for 1 min at 200°C and then increased at a rate of 5°C/min up to 260°C. The interface temperature was 270°C. The trihydroxyeicosanoid derivatives were eluted at 5.1 min. An electron energy of 70 eV was used.

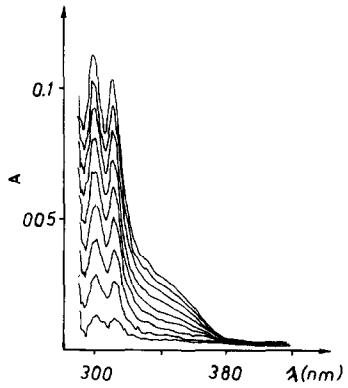


Fig.1. Formation of lipoxins during the oxygenation of 5,15-DiHETE. Reticulocyte lipoxygenase (8 nkat/ml linoleate oxygenase activity) was incubated with 5,15-DiHETE (15 $\mu$M) in 0.1 M phosphate buffer, pH 7.4 at 4°C. Repeated spectra of the reaction mixture were recorded in the range of 280–450 nm with a time interval of 5 min.

### 2.4. *Miscellaneous*

The molar absorption coefficients used were as follows: 15-HETE, $\epsilon_{234}$ = 28000 $(M \cdot cm)^{-1}$ [15]; 5,15-DiHETE, $\epsilon_{242}$ = 40000 $(M \cdot cm)^{-1}$ [14]; lipoxins, $\epsilon_{301}$ = 50000 $(M \cdot cm)^{-1}$ [2]. For RP-HPLC the free hydroxy fatty acids were methylated with diazomethane in ether. For GC/MS analysis the methylated hydroxy fatty acids were converted to their corresponding trimethylsilyl ethers by reaction with BSTFA (15 min at 60°C). UV spectrophotometric measurements were performed with a Pye Unicam SP 1750 spectrophotometer.

## 3. RESULTS

The pure reticulocyte lipoxygenase converted 5,15-DiHETE to product(s) possessing a con-

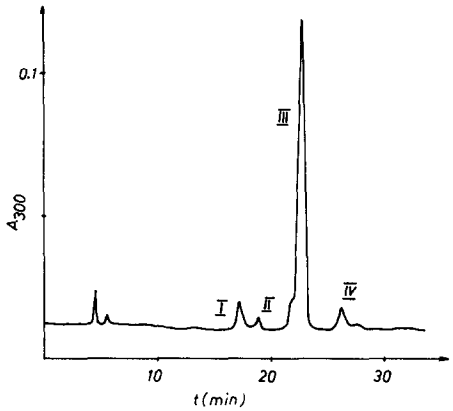


Fig.2. RP-HPLC of lipoxins formed by the reticulocyte lipoxygenase during 5,15-DiHETE oxygenation. Incubation conditions as in fig.1. Total volume of the preparation 1 l. After the reaction had ceased (no further spectral changes) the mixture was acidified to pH 3 and twice extracted with twice the volume of diethyl ether. The organic extracts were dried over anhydrous sodium sulfate, the solvent was evaporated under reduced pressure, the products were dissolved in 200 $\mu$l methanol and methylated with ethereal diazomethane. Unconverted 5,15-DiHETE was removed by SP-HPLC. The fractions containing conjugated tetraenes were pooled and an aliquot was subjected to RP-HPLC as described in section 2. Absorbance at 300 nm was recorded.

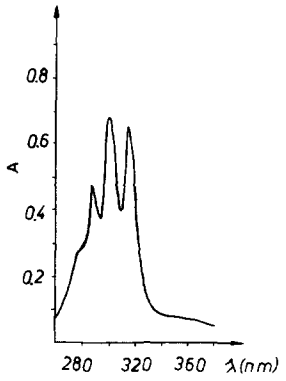


Fig.3. UV-spectrum of product III dissolved in methanol.

jugated tetraene system as indicated by the maxima of strong UV absorbance at 301 and 315 nm (fig.1). After the reaction had ceased (no further increase in absorbance at 315 nm) the products were extracted with diethyl ether and subjected to SP-HPLC to remove unconverted 5,15-DiHETE. Products with the spectral properties of conjugated tetraenes were collected, concentrated, methylated with diazomethane and subjected to RP-HPLC analysis. As seen from fig.2, four products absorbing at 300 nm were separated but only products III and IV show the spectral properties of conjugated tetraenes. The main product III was prepared by RP-HPLC and its UV-spectrum

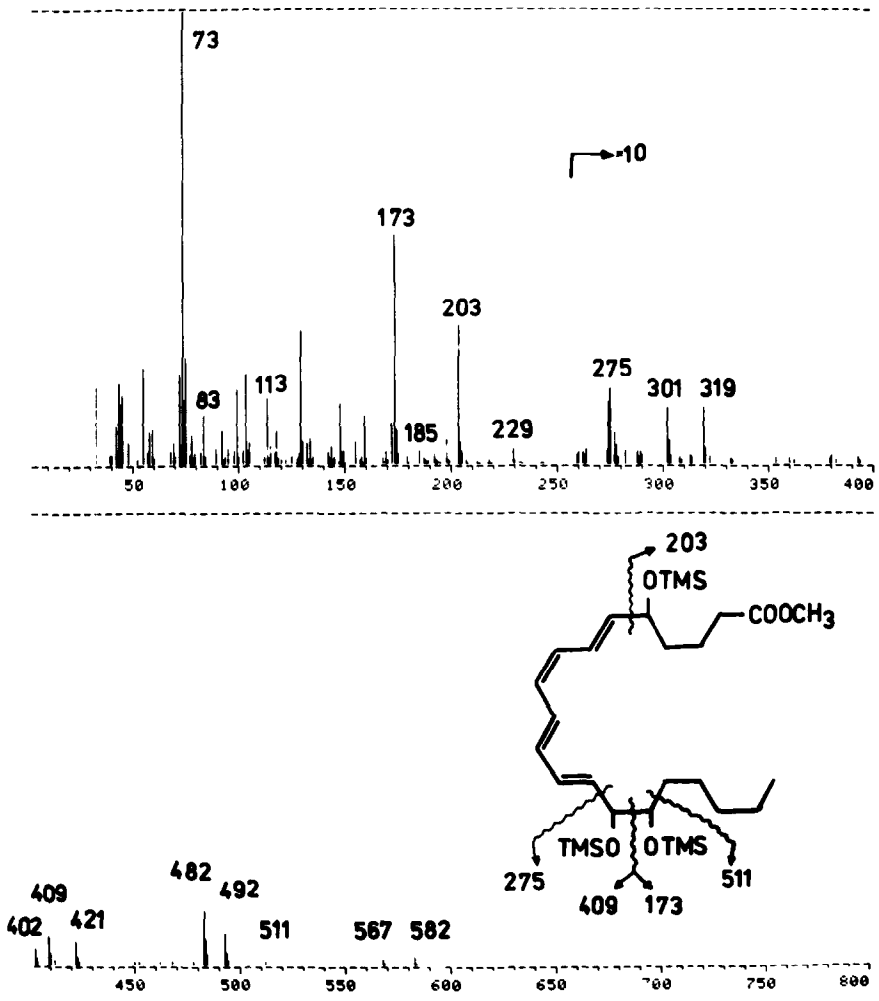


Fig.4. GC/MS analysis of product III. GC/MS analysis was performed as described in section 2. Inset: fragmentation pattern of product III indicating the positions of the OH groups.

was recorded (fig.3). The maxima of strong absorbance at 286, 301 and 315 nm point to the conjugated tetraene system [6]. Product III was silylated and subjected to GC/MS analysis. Fig.4 shows a representative mass spectrogram of it. It is evident that product III is a trihydroxyeicosanoid containing four double bonds indicated by the ions at $m/z$ 582 ($M^+$), 567 ($M^+ - 15$, loss of $CH_3$), 492 ($M^+ - 90$, loss of TMS-OH), 482 ($M^+ - 100$, rearrangement followed by a loss of $CH_3$-$(CH_2)_4HC=O$ [1,16]), and 402 ($M^+ - 180$, loss of 2 × TMS-OH). The positions of the hydroxy groups at $C_5$, $C_{14}$ and $C_{15}$ are indicated by the major fragment ions formed by cleavage of the carbon-carbon bonds on either side of the TMS-O-groups as shown in the inset in fig.4. In addition, secondary fragmentation of these ions (loss of TMS-OH, mass unit 90) were observed as indicated by the ions at $m/z$ 83, 113, 185, 319 and 421. Cleavage of the $C_7$–$C_8$ bond and the $C_{11}$–$C_{12}$ bond is proposed for the ions at $m/z$ 229 and 301, respectively. These data clearly indicate that product III is 5,14,15-trihydroxy-6,8,10,12-eicosatetraenoic acid, a lipoxin B isomer.

With arachidonic acid, 15-HETE and 15-HETE methyl ester as substrate this product was also formed as indicated by the spectral changes of the reaction mixture and RP-HPLC analysis of the products (not shown). For the formation of this product from 15-HETE methyl ester, it was shown that two atoms of atmospheric oxygen ($^{17}O$) were incorporated into the product. This result excludes the possible formation of the lipoxin B isomer via epoxide hydrolysis.

## 4. DISCUSSION

The demonstration of the formation of a lipoxin B isomer by the pure reticulocyte lipoxygenase underlines the universal capacity of this enzyme with respect to the arachidonic acid metabolism via the lipoxygenase pathway. Previously we have demonstrated the formation of 15-H(P)ETE, 12-H(P)ETE, 5,15-DiH(P)ETE, 8,15-DiH(P)ETE, 14,15-$LTA_4$ and 13-hydroxy-14,15-epoxyeicosatetraenoic acid [17–19]. Therefore the reticulocyte lipoxygenase is able to form compounds belonging to all important classes of lipoxygenase metabolites, such as hydroxyeicosanoids, epoxy-leukotrienes, lipoxins and hepoxiline isomers.

In the biosynthesis of the naturally occurring lipoxin B an enzymatic hydrolysis of an epoxide intermediate is involved [10]. In contrast, the results presented here indicate that pure lipoxygenases are able to form lipoxins via dioxygenation of 5,15-DiHETE, double dioxygenation of 15-HETE or sequential triple dioxygenation of arachidonic acid. Similar results were obtained with the purified 12-lipoxygenase from white cells [20]. Leukocytes convert 15-HPETE, 15-HETE and 5,15-DiHETE to lipoxin B [10]. However, in previous studies on the lipoxin formation mainly 15-HETE was used as substrate [2,3,6]. The use of 5,15-DiHETE is recommended for several reasons: (i) this product has been identified as one of the major double oxygenation products formed by leukocytes and by pure lipoxygenases; (ii) an intermediate formation of epoxy tetraenes is impossible, therefore no complex product pattern is expected; (iii) the last step of the oxygenation cascade can be studied separately.

Even though the absolute configuration of the chiral center $C_{14}$ and the geometry of the conjugated tetraene system were not elucidated by us (see Note Added in Proof), the steric structure of the product formed from 5S,15S-DiHETE can be predicted on the basis of the present knowledge on the stereochemistry of the lipoxygenase catalysis [21,22]. Considering the *cis-trans* isomerization following hydrogen removal, the direction of the radical rearrangement, the antarafacial character [22,23] and the specificity of the reticulocyte enzyme we propose the lipoxin B isomer formed by this lipoxygenase to be 5*S*,14*R*,15*S*-trihydroxy-6*E*,8*Z*,10*E*,12*E*-eicosatetraenoic acid. This product represents a sequential triple lipoxygenation product of arachidonic acid but seems to be identical to the most recently established structure for the naturally occurring lipoxin B which is formed via epoxide hydrolysis [10].

## NOTE ADDED IN PROOF

In recent experiments the complete steric structure of compound III has been elucidated by co-chromatography in RP-HPLC with the authentic standard of 5*S*,14*R*,15*S* trihydroxy-6*E*,8*Z*,10*E*, 12*E* eicosatetraenoic acid. Therefore the identity of compound III with the lipoxin B formed by leukocytes has been established. The various

authentic standards of trihydroxyeicosanoids were obtained from Dr Fitzsimmons from Merck Frost, Canada.

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