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12-Lipoxygenase from rat basophilic leukemia cells: separation from 5-lipoxygenase and temperature-dependent inactivation by hydroperoxy fatty acid

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12-Lipoxygenase and 5-lipoxygenase from rat basophilic leukemia cells were separated by protein-HPLC in a single step. Upon incubation in the presence of Ca^{2+}, 12-lipoxygenase converted arachidonic acid into 12(*S*)-hydroxyeicosatetraenoic acid and linoleic acid into 13(*S*)-hydro(pero)xyoctadecadienoic acid. The reaction products were analyzed by reversed-phase and chiral straight-phase HPLC with ultraviolet-detection. Using the cytosolic fraction of rat basophilic leukemia cells, optimal 12-lipoxygenase activity was observed at 10°C. At 37°C 12-lipoxygenase was very rapidly inactivated by its own product, hydroperoxy fatty acid, at low concentrations (10–100 nM).

Introduction

Rat Basophilic Leukemia (RBL-1) cells have been shown to possess 5-lipoxygenase activity. 5-Lipoxygenase has been purified to homogeneity [1,2]. However, there has been some uncertainty about the existence of a 12-lipoxygenase in RBL-1 cells. Jakschik et al. [3] observed that the $10\,000 \times g$ supernatant of RBL-1 cell homogenates produced small amounts of 12-HETE, upon incubation with arachidonic acid in the presence of Ca^{2+}. Later, the formation of 5(*S*),12(*S*)-diHETE, the product of double dioxygenation, was demonstrated, indicating the presence of a 12-lipoxygenase [4]. Hamasaki and Tai confirmed the production of 12-HETE by means of GC-MS [5]. They also suggested the presence of separate 12- and 5-lipoxygenases, based on the different behaviour of these enzymes towards inhibitors and heat treatment. However, lately other groups were unable to detect any substantial 12-lipoxygenase activity in RBL-1 cells [6–8].

This study provides further evidence for the existence of a 12-lipoxygenase in RBL-1 cells. 12- and 5-lipoxygenases were separated in a single chromatographic step. 12-Lipoxygenase was partially purified, the reaction products of the enzyme were identified, and some characteristics of the enzyme were established.

Materials and Methods

Materials

RBL-1 cells and foetal bovine serum were purchased from Flow Laboratories (Herts, U.K.). RPMI 1640 culture medium was obtained from Gibco (Grand Island, NY, U.S.A.). Arachidonic acid (> 99%) and linoleic acid (99%) were from Fluka AG (Buchs, Switzerland). Prostaglandin B_2, nordihydroguaiaretic acid, 12(*R*)-HETE, ATP and phosphatidylcholine (type III-E) were from Sigma Chemical Company (St. Louis, MO, U.S.A.). 1-Naphthoylchloride (> 98%) was obtained from Aldrich Chemical (Milwaukee, WI, U.S.A.). Anhydrous pyridine was kept over calcium chloride. Octadecyl solid-phase extraction columns (6 ml) were obtained from Baker (Deventer, The Netherlands). Sample filters (pore size 0.45 μm) were from Nikon Millipore Kogyo KK (Yonezawa, Japan). Methanol, water, hexane (Merck, Darmstadt, F.R.G.), and tetrahydrofuran (Baker) were of HPLC quality.

Abbreviations: H(P)ETE, hydro(pero)xyeicosatetraenoic acid; H(P)OD, hydro(pero)xyoctadecadienoic acid; RBL, rat basophilic leukemia.

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Cell culture and supernatant preparation

RBL-1 cells were grown in stationary tissue culture flasks in RPMI 1640 medium supplemented with 10% foetal bovine serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), L-glutamine (2 mM) and amphotericin (10 μg/ml). Cells were collected, centrifuged (10 min, 2000 × *g*, room temperature) and washed with phosphate-buffered saline (PBS, 0.9% (w/v) NaCl in 8.6 mM phosphate buffer, pH 7.4). Cell pellets were resuspended in PBS at $5 \cdot 10^7$ cells/ml and cells were disrupted by repeatedly freezing in liquid nitrogen and thawing. The cell lysate was centrifuged (20 min, 20000 × *g*, 4°C) and the resulting supernatant was brought to 5% ethylene glycol, frozen in liquid nitrogen and stored at −80°C until use.

Partial purification of 12-lipoxygenase from RBL-1 cells

Frozen RBL-1 cell supernatant was thawed and brought to 50% saturation with 100% saturated ammonium sulfate (pH 7.1). The mixture was stirred for 1 h at 0°C and centrifuged (10 min, 10000 × *g*, 0°C). The pellet was resuspended in buffer B (20 mM Tris-HCl (pH 7.1), 50 mM NaCl, 5% ethylene glycol and 100 μg/ml sonicated phosphatidylcholine) The resuspended pellet was dialyzed at 4°C against 200 ml of buffer B for 1 h. The dialyzed sample was centrifuged (10 min, 10000 × *g*, 0°C) and injected onto a TSK DEAE-5PW anion exchange HPLC column (7.5 × 75 mm) with a poly-ol Si 300 gel filtration HPLC guard column, pre-equilibrated at 0°C with buffer B, containing 20 μg/ml sonicated phosphatidylcholine at a flow rate of 0.5 ml/min. Elution was formed with a linear gradient of 50–400 mM NaCl in buffer B. For control experiments 5-lipoxygenase from RBL-1 cells was partially purified, based on the method of Hogaboom et al. [2], using a BioSil TSK-400 gel filtration HPLC column (7.5x300mm).

Protein concentrations were determined according to the method of Bradford [9].

Enzyme assay sample preparation and HPLC-analysis

The enzyme assay was carried out in PBS (supernatant) or buffer B (purified fractions). The amounts of hydro(pero)xy fatty acids formed upon incubation with substrate during 30 min in the enzyme assay were used as a measure of lipoxygenase activity. For 12-lipoxygenase the enzyme assay was performed at 10°C and pH 7.5 in a final volume of 500 μl containing 30 μM substrate, 1 mM Ca^{2+} and 5% ethylene glycol. For 5-lipoxygenase the enzyme assay was performed at 37°C and pH 7.5 in the presence of 30 μM substrate, 1 mM Ca^{2+}, 1 mM EDTA, 2 mM ATP and 5% ethylene glycol. In some cases other conditions were used, which will be indicated. Incubations were stopped by the addition of 500 μl methanol. Prostaglandin B_2 was added as an internal standard for HPLC analysis. The reaction products were extracted with octadecyl solid-phase extraction columns as described in [10] and analyzed with reversed-phase HPLC, using a CP microspher 3μ-C18 column (100 × 4.6 mm, Chrompack, Middelburg, The Netherlands). Elution was carried out with the solvent system tetrahydrofuran/methanol/water/acetic acid (25 : 30 : 45 : 0.1, v/v) which had been adjusted to pH 5.5 with ammonia [11]. The identity of the reaction products was confirmed by co-elution with reference compounds and by ultraviolet-detection. Reaction products were quantified by relating peak areas to that of the internal standard prostaglandin B_2. Molar absorption coefficients used were: 28650 $M^{-1} cm^{-1}$ at 280 nm for prostaglandin B_2, 40000 $M^{-1} cm^{-1}$ at 280 nm for the leukotrienes, 29500 $M^{-1} cm^{-1}$ at 237 nm for the hydro(pero)xides of arachidonic acid and 25000 $M^{-1} cm^{-1}$ at 237 nm for the hydro(pero)xides of linoleic acid [12,13].

Stereochemical analysis of 13-HOD and 12-HETE by chiral straight phase HPLC

Stereochemical analysis was performed according to [14]. 13-HOD and 12-HETE were converted into the corresponding methyl esters and further derivatized by reaction with 1-naphthoylchloride. The naphthoyl derivatives were purified by reversed-phase HPLC on a CP spher 8μ-C18 column (250 × 4.6 mm, Chrompack) using methanol/water (90 : 10, v/v), at a flow rate of 1 ml/min and the detection was at 237 nm. The purified naphtoyl derivatives were chromatographed on a DNBPG chiral stationary phase HPLC column (Bakerbond, covalently linked, 250 × 4.6 mm) using hexane/2-propanol (99.5 : 0.5, v/v) at a flow rate of 0.8 ml/min and the detection was at 237 nm. 13-HOD and 12-HETE were identified as *R*- or *S*-enantiomer by co-elution with reference compounds.

Preparation of 5-H(P)ETE, 12-HETE, 13-H(P)OD and 9-H(P)OD

5(*S*)-HPETE was biosynthesized from arachidonic acid by incubation with partially purified potato 5-lipoxygenase [15] and 12(*S*)-HETE by incubation with bovine platelets. 13(*S*)-HPOD was biosynthesized from linoleic acid by incubation with soybean lipoxygenase-1 at pH 9.0 [16] and 13(*R*,*S*)-HPOD by incubation with soybean lipoxygenase-2 at pH 9.0 [17]. 9(*R*,*S*)-HPOD was biosynthesized from linoleic acid by incubation with corn germ lipoxygenase at pH 6.6 [18]. The hydroperoxy fatty acids were reduced to the corresponding hydroxy compounds with $SnCl_2$. Purification of these compounds was performed with reversed-phase HPLC, as described under HPLC-analysis.

Results

12-Lipoxygenase from RBL-1 cell supernatant was purified 20-fold (Table I), using a TSK DEAE-5PW anion exchange HPLC column with a poly-ol Si 300 gel

TABLE I

Partial purification of 12-lipoxygenase from RBL-1 cells

Fractions were assayed for 12-lipoxygenase activity for 30 min at 20°C and pH 7.5 in the presence of 30 μM ETE, 1 mM Ca^{2+} and 5% ethylene glycol. The reaction mixtures were analyzed as described in Methods.

Sample	Total protein (mg)	Total activity (nmol)	Specific activity (nmol/mg)	-Fold purification	Yield (%)
20000 × *g* supernatant	94.0	6.0	0.06	1	100
0–50% precipitate	67.3	5.4	0.08	1.2	90
DEAE ion exchange pass-through					
fraction 10–16 min	0.840	0.58	0.69	10.9	10
fraction 12–14 min	0.173	0.23	1.31	20.5	4

filtration HPLC guard column (Fig. 1). Most of the 5-lipoxygenase was bound to the DEAE column and eluted at a NaCl concentration range of 330–360 mM. However, the 12-lipoxygenase was not bound to the column at pH 7 nor at pH 8, showing that the apparent pI of the enzyme must be at least > 7. The highest 12-lipoxygenase activity eluted in the pass-through fractions. The gel filtration guard column was necessary to obtain a slight retardation in the elution of this enzyme. Leaving out the gel filtration guard column led to co-elution of the enzyme with the bulk of protein in the pass-through fractions. For some control experiments 5-lipoxygenase from RBL-1 cells was partially purified, using a TSK-400 gel filtration HPLC column. The highest 5-lipoxygenase activity eluted in one peak after the total bed volume, showing a specific interaction between enzyme and column. This purification step provided an almost 250-fold enrichment of 5-lipoxygenase activity and separated it completely from 12-lipoxygenase acitivity as was determined by the absence of 12-HETE upon incubation with arachidonic acid (data not shown).

In the presence of 1 mM Ca^{2+} crude (20000 × *g* supernatant) as well as partially purified 12-lipoxygenase (DEAE pass-through fractions) converted arachidonic acid (10–300 μM) into 12-HETE. No conversion into 12-leukotrienes was observed. Stereochemical analysis of 12-HETE by means of chiral straight phase HPLC showed that the *S*-enantiomer was exclusively formed.

When the supernatant was incubated with linoleic acid (10–100 μM) the main products were 13-HOD and 13-HPOD. No conversion into 9-H(P)OD was observed. Preincubation with 10 mM indomethacin for 5 min did not influence the formation of 13-H(P)OD, whereas preincubation with 1 mM nordihydroguaiaretic acid for 5 min resulted in complete inhibition, indicating that the reaction is lipoxygenase-catalyzed and not cyclooxygenase-catalyzed. Incubation of partially purified 5- or 12-lipoxygenase with linoleic acid showed that 12-

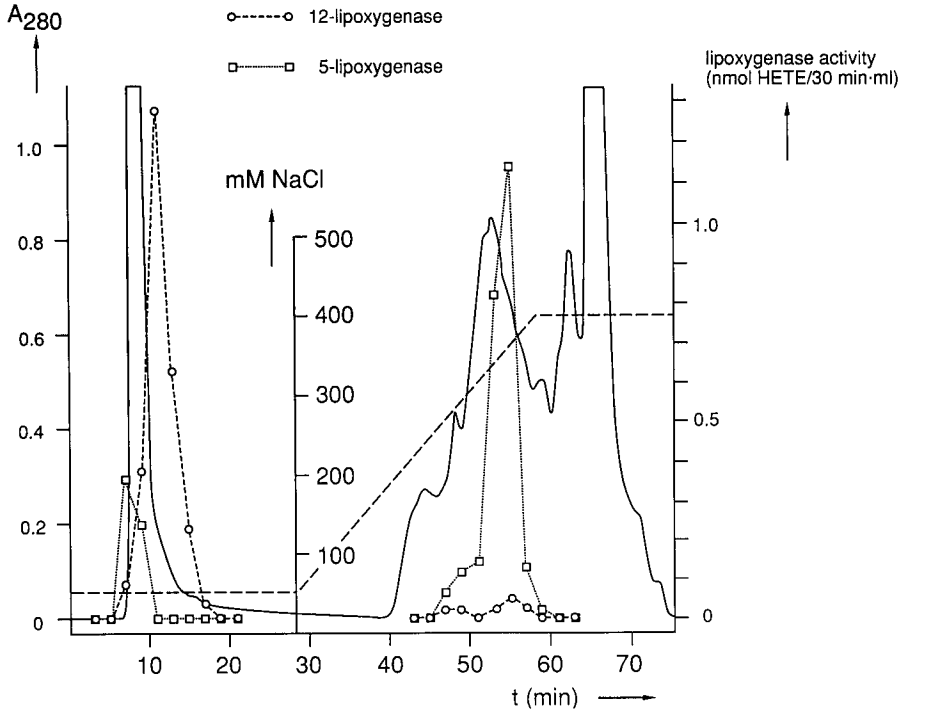

Fig. 1. Partial purification of 12-lipoxygenase from RBL-1 cells by a combination of gel filtration and weak anion exchange chromatography, as described in Methods. Fractions of 1 ml were collected and assayed for 12- and 5-lipoxygenase activity. The chromatogram is a typical example of 11 purifications.

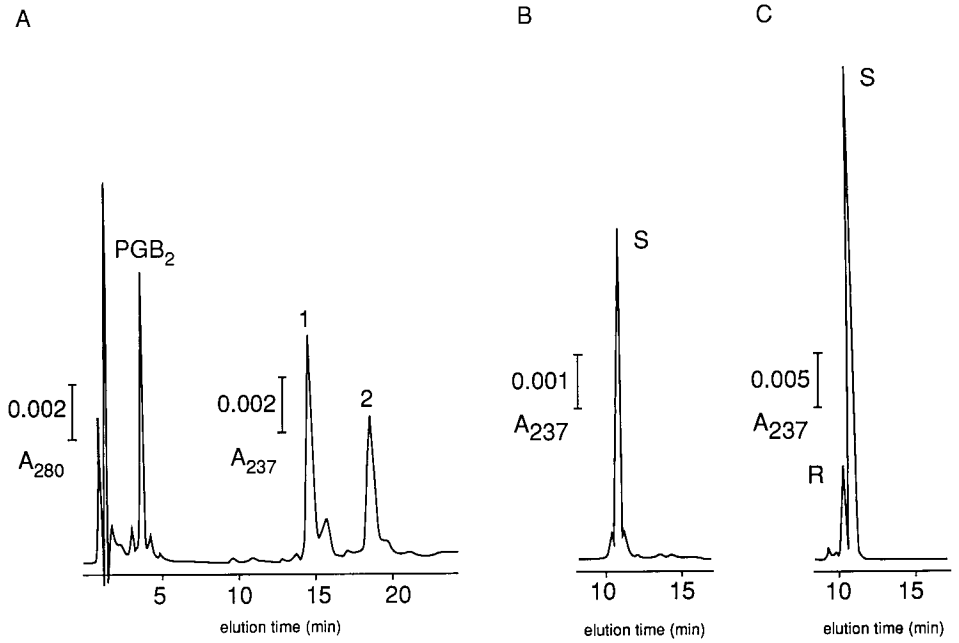

Fig. 2. (A) Reversed-phase HPLC chromatogram of reaction products formed from linoleic acid by partially purified 12-lipoxygenase from RBL-1 cells. The reaction products were extracted as described in Methods. 1. 13-HOD, 2. 13-HPOD. (B) Chiral straight-phase HPLC chromatogram of 13-HOD, formed from linoleic acid by 12-lipoxygenase from RBL-1 cells. (C) Chiral straight-phase HPLC chromatogram of reference 13(*R*)- and 13(*S*)-HOD.

lipoxygenase was capable of converting linoleic acid into 13-H(P)OD, whereas 5-lipoxygenase did not convert linoleic acid at all. Stereochemical analysis of 13-HOD showed that only the *S*-enantiomer was formed Fig. 2). Incubation of boiled supernatant or boiled purified fractions with substrate was always used as a control to exclude that autoxidation of fatty acids was involved.

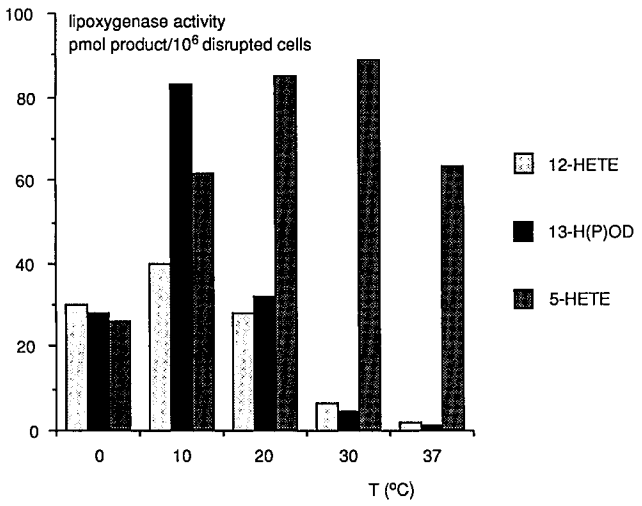

Fig.3. Temperature dependence of 12-lipoxygenase activity. 20000 × *g* Supernatant of RBL-1 cells was incubated with 60 μM linoleic acid or arachidonic acid for 30 min in the presence of 1 mM Ca^{2+}.

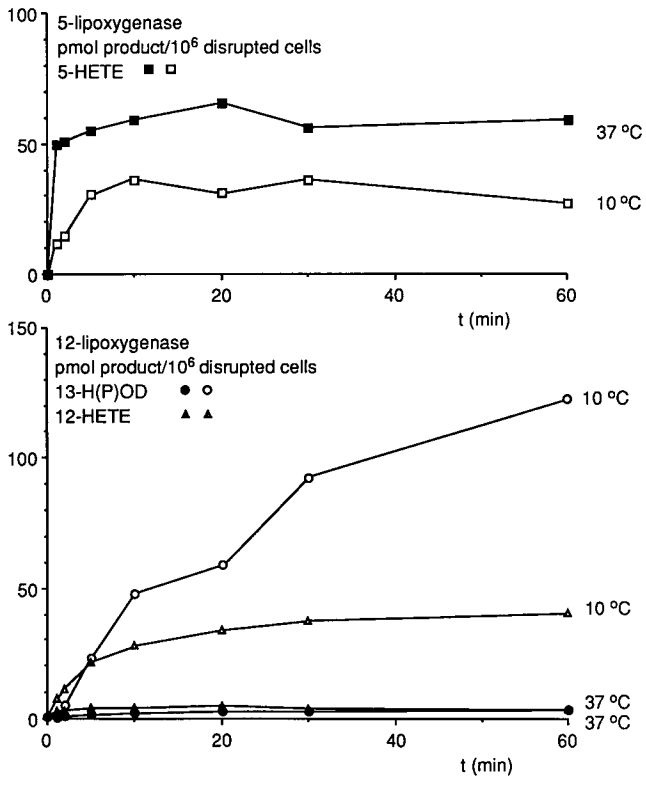

Fig. 4. Time-courses of 12-lipoxygenase- and 5-lipoxygenase-catalyzed reactions. 20000 × *g* Supernatant of RBL-1 cells was incubated with 60 μM linoleic acid or arachidonic acid at 10°C and 37°C in the presence of 1 mM Ca^{2+}.

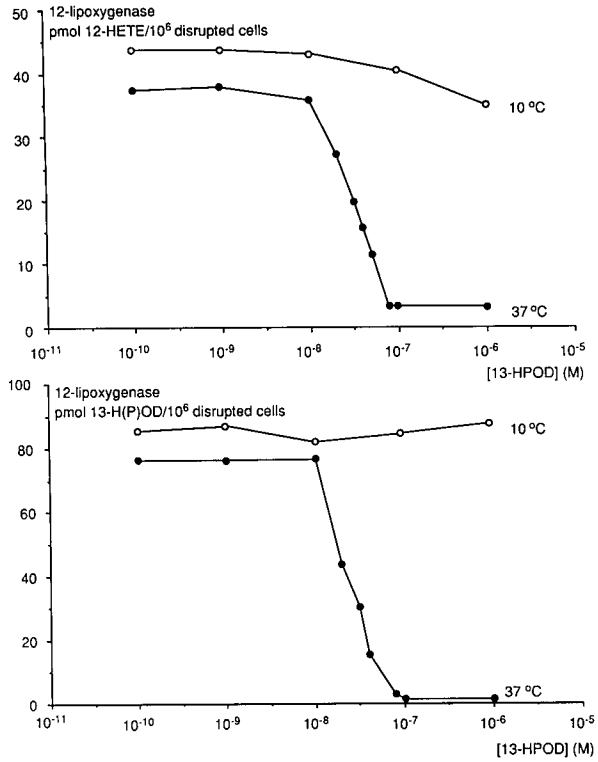

Fig. 5. Inhibition of 12-lipoxygenase by preincubation with its own reaction product. 20000 × *g* Supernatant of RBL-1 cells was preincubated for 2 min with 13(*S*)-HPOD at 37°C or 10°C and subsequently incubated with 60 μM linoleic acid or arachidonic acid for 30 min in the presence of 1 mM Ca^{2+}. The amounts of 13(*S*)-H(P)OD, produced during incubation, were corrected for added amounts of 13(*S*)-HPOD for preincubation.

Using the 20000 × *g* supernatant, 12-lipoxygenase was found to have maximal activity at 10°C, with linoleic acid as well as with arachidonic acid as substrate, while the enzyme became almost inactive at 30–37°C (Figs. 3 and 4). By contrast, 5-lipoxygenase activity was found to be maximal at 30°C. Time-courses showed that 5-lipoxygenase is inactive after 5 min at 37°C as well as at 10°C. However, 12-lipoxygenase was found to behave completely differently. At 10°C the enzyme was still active after 60 min of incubation, whereas at 37°C almost no 12-lipoxygenase-catalyzed reaction products were detectable. This observation could not be explained by thermolability of either enzyme or product, since both 12-lipoxygenase and 13(*S*)-HPOD were found to be stable when kept at 37°C for 10 min. However, when 12-lipoxygenase was preincubated for 2 min at 37°C with linoleic acid or arachidonic acid in the presence of 1 mM Ca^{2+}, cooled to 10°C and incubated for another 30 min, substrate conversion did not proceed at all. To further investigate this phenomenon 20000 × *g* supernatant was preincubated for 2 min at 37°C at various concentrations 13(*S*)-HPOD, cooled to 10°C and subsequently incubated with linoleic acid or arachidonic acid (Fig. 5). Upon preincubation with 13(*S*)-HPOD at 37°C inhibition of 12-lipoxygenase started off at hydroperoxy fatty acid concentrations above 10 nM and was almost complete at 100 nM. The latter concentration corresponds to the maximal product concentration observed upon incubation with substrate at 37°C. A similar experiment at 10°C showed no inhibition of 12-lipoxygenase. Since preincubation with 13(*S*)-HOD at 37°C did not cause inhibition of 12-lipoxygenase either, we conclude that 12-lipoxygenase is very rapidly inactivated at 37°C by formed hydroperoxy fatty acid.

Discussion

This study demonstrates that 12-lipoxygenase from RBL-1 cells can be separated from 5-lipoxygenase by means of HPLC. Our results show that linoleic acid is a substrate for 12-lipoxygenase but not for 5-lipoxygenase. 12-Lipoxygenase converts linoleic acid exclusively into 13(*S*)-H(P)OD. We observed maximal 12-lipoxygenase activity at 10°C. This could be a result of two different effects. Going from 0 to 10°C the enzyme seems to respond as expected. However, going from 10 to 37°C, the inhibition curves show that 12-lipoxygenase inactivates rapidly at elevated temperatures.

Preincubation with 13-HPOD at low levels (10–100 nM) immediately inactivates 12-lipoxygenase at 37 but not at 10°C. This explains why the product concentration after 30 min of incubation with 60 μM linoleic acid at 37°C hardly exceeded 100 nM, while at 10°C a product concentration of 5 μM was easily reached. Preincubation with 12-HPETE was not performed but since (pre)incubation with arachidonic acid at 37°C also yielded very low amounts of product, it is likely that inactivation of 12-lipoxygenase is caused here by very low concentrations of 12-HPETE. Whether simultaneously formed 5-HPETE by 5-lipoxygenase contributes to this inactivation or not, is under current investigation.

Inactivation of lipoxygenase by hydroperoxy fatty acid has been reported before, e.g., inactivation of potato lipoxygenase by 5-HPETE and 15-HPETE [19], inactivation of soybean lipoxygenase by hydroperoxy fatty acids [20,21], and inactivation of guinea pig neutrophil 5-lipoxygenase by 5-HPETE [22]. The same probably happens to the RBL-1 5-lipoxygenase [8]. The reticulocyte lipoxygenase is inactivated by nearly stoichiometric amounts of 13(*S*)-HPOD [23], probably by oxidation of a methionine residue in the active center of the enzyme [24,25]. This inactivation was observed at 37°C but hardly at low temperature (2°C), like we found for 12-lipoxygenase from RBL-1 cells. We found that 13(*S*)-HPOD inactivated the enzyme at low concentrations (10–100 nM). But since we could not determine the enzyme concentration in the supernatant, it remains unclear whether this inactivation is also at equimolar level or not. If inactivation of 12-lipoxy-

genase from RBL-1 cells happens in a way similar to that of the reticulocyte lipoxygenase, the temperature dependence of the inactivation may be explained by a conformational change around the active centre of the enzyme. A methionine residue, which could be hidden at 10°C, may become more easily accessible to a hydroperoxy fatty acid molecule at 37°C.

The rapid inactivation of 12-lipoxygenase from RBL-1 cells by hydroperoxy fatty acid at 37°C that we observed, may explain why some other groups, who studied the 5-lipoxygenase from RBL-1 cells at 37°C [3,6,8], were unable to detect any substantial 12-lipoxygenase activity and why Navé et al. [7] found only negligable 12-lipoxygenase activity at 25°C. Considering our results and those from other groups [3,6–8], we find it difficult to explain why Hamasaki and Tai find substantial 12-lipoxygenase activity at 37°C [5].

The biological significances of 12-HETE and 13-H(P)OD are manifold [26–28]. In vitro 12-lipoxygenase from RBL-1 cells produces these compounds at an apparent temperature optimum of 10°C. Such a low temperature optimum may have a (patho)physiological reason, or may be caused by removing the enzyme from its natural environment. Further studies on intact cells and purified enzyme will therefore be necessary.

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