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12-Lipoxygenase from rat basophilic leukemia cells, an oxygenase with Leukotriene A_4-synthase activity

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Rat basophilic leukemia cells exhibit 12-lipoxygenase activity only upon cell disruption. 12-Lipoxygenase may also possess 15-lipoxygenase activity, as is indicated by the formation of low amounts of 15(*S*)-HETE, in addition to the predominant product 12(*S*)-HETE, upon incubation of partially purified 12-lipoxygenase with arachidonic acid. With 5(*S*)-HPETE as substrate not only 5(*S*), 12(*S*)-diHETE and 5(*S*), 15(*S*)-diHETE are formed, but also LTA_4, as was indicated by the presence of LTA_4-derived LTB_4-isomers. 12-Lipoxygenase from rat basophilic leukemia cells has many features in common with 12-lipoxygenase from bovine leukocytes. As was suggested for the latter enzyme, 12-lipoxygenase from rat basophilic leukemia cells may represent the remaining LTA_4-synthase activity of 5-lipoxygenase, of which the 5-dioxygenase activity has disappeared upon cell disruption. Such a possible shift from 5-lipoxygenase activity to 12-lipoxygenase activity could not simply be induced by interaction of cytosolic 5-lipoxygenase with a membrane fraction after cell disruption, but may involve release of membrane-associated 5-lipoxygenase upon disruption of activated rat basophilic leukemia cells.

Introduction

The leukotrienes are a well characterized group of arachidonic acid metabolites with very pronounced biological activities. They play an important role in inflammatory and allergic responses [1,2]. The first step in the biosynthesis of the 5-series leukotrienes involves the dioxygenation of arachidonic acid to produce 5(*S*)-HPETE, catalyzed by 5-lipoxygenase. 5-Lipoxygenases also catalyze the subsequent synthesis of leukotriene A_4 (LTA_4) from 5(*S*)-HPETE [3–5]. Beside 5-lipoxygenases, also 12- and 15-lipoxygenases are found in mammalian systems. Lipoxygenases have been purified from various sources [4–8] and genes coding for lipoxygenases have been cloned and sequenced [9–14].

Bovine leukocytes have been demonstrated to possess both 5- and 12-lipoxygenase activity [15,16]. It was suggested that both 5- and 12-lipoxygenase activity are associated with the same protein [17]. The 12-lipoxygenase, which became apparent upon disruption of the cells, was shown to possess also LTA_4-synthase activity. Rat basophilic leukemia (RBL-1) cells have been shown to possess both 5- and 12-lipoxygenase activity as well [5,18–21]. 5-Lipoxygenases show a preference for hydrogen abstraction at a position, determined by the distance to the carboxylic end of the molecule [22,23]. 12-Lipoxygenase from RBL-1 cells may behave similarly, since both arachidonic acid and linoleic acid were demonstrated to be good substrates, giving rise to the formation of 12(*S*)-H(P)ETE and 13(*S*)-H(P)OD, respectively [21]. Both the formation of 12(*S*)-H(P)ETE from arachidonic acid by 12-lipoxygenase and that of LTA_4 from 5(*S*)-HPETE by 5-lipoxygenase [24,25] require the abstraction of hydrogen at C10. Therefore, we investigated the possible LTA_4-synthase activity of 12-lipoxygenase from RBL-1 cells.

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Abbreviations: H(P)ETE, hydro(pero)xyicosatetraenoic acid; 5(*S*)H, 12(*S*)-HPETE, 5(*S*)-hydroxy-12(*S*)-hydroperoxyicosa-6*E*,10*E*,8*Z*,14*Z*-tetraenoic acid; H(P)OD, hydro(pero)xyoctadecadienoic acid; RBL, rat basophilic leukemia.

Materials and Methods

Materials

RBL-1 cells and foetal bovine serum were purchased from Flow Laboratories (Herts, UK). RPMI 1640 culture medium was obtained from Gibco (Grand

Island, NY, USA). Arachidonic acid (> 99%) and linoleic acid (99%) were from Fluka (Buchs, Switzerland). Prostaglandin B_2, ATP, 12(*R*)-HETE, reduced glutathione and phosphatidylcholine (type III-E) were from Sigma (St. Louis, MO, USA). 5(*S*)-, 12(*S*)- and 15(*S*)-HPETEs and 5(*S*)-, 12(*S*)- and 15(*S*)-HETEs were obtained from Cascade Biochem (Reading, UK). Octadecyl solid-phase extraction columns (6 ml) were obtained from Baker (Deventer, Netherlands). Sample filters (pore size 0.45 μm) were from Nikon Millipore Kogyo KK (Yonezawa, Japan). Methanol and water (Merck, Darmstadt, Germany), and tetrahydrofuran (Baker) were of HPLC quality.

Cell culture and preparation of cytosolic and membrane fractions

RBL-1 cells were grown and harvested as described before [21] and resuspended in phosphate-buffered saline (PBS, 0.9% (w/v) NaCl in 8.6 mM phosphate buffer, pH 7.4) at $5 \cdot 10^7$ cells/ml. For experiments concerning 5-lipoxygenase, EDTA was added to PBS (final concentration 1 mM). 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 pellet and supernatant are described as the membrane fraction and 20000 × *g* cell supernatant, respectively. The membrane fraction was washed and resuspended in a volume of PBS, equal to that of the initial cell suspension. For some experiments, the 20000 × *g* cell supernatant was recentrifuged (1 h, 100000 × *g*, 4°C) and the resulting pellet and supernatant are described as the microsomal enriched and cytosolic fraction, respectively. The microsomal enriched fraction was washed and resuspended in a volume of PBS, equal to that of the initial cell suspension.

Partial purification of 12-lipoxygenase and 5-lipoxygenase from RBL-1 cells

12-Lipoxygenase was purified about 300-fold from the 20000 × *g* cell supernatant, using a TSK DEAE-5PW anion-exchange HPLC column (7.5 × 75 mm) with a polyol Si 300 gel-filtration HPLC guard column, as described previously [26]. For some experiments 12-lipoxygenase was partially purified using a BioSil TSK-400 gel filtration column (7.5 × 300 mm). Cell supernatant was brought to 50% saturation with 100% saturated ammonium sulfate (final pH 7.1) and the protein precipitate was resuspended in buffer A (50 mM Tris-HCl (pH 7.1), 50 mM NaCl and 5% ethylene glycol), centrifuged and applied to the column, preequilibrated at 0°C with buffer A at a flow rate of 1 ml/min. The highest 12-lipoxygenase activity eluted as a single peak after 10–15 min. In this way, 12-lipoxygenase was purified about 40-fold from the 20000 × *g* cell supernatant (data not shown). The enzyme preparation obtained by using either method did not display any 5-lipoxygenase activity as was determined by the absence of 5-lipoxygenase-catalyzed reaction products upon incubation with arachidonic acid at 10 or 37°C in the presence of 1 mM Ca^{2+}, 1 mM EDTA, and 2 mM ATP.

5-Lipoxygenase was partially purified from the 20000 × *g* cell supernatant as described before [21]. The enzyme preparation did not display any 12-lipoxygenase activity as was determined by the absence of 12-HETE upon incubation with arachidonic acid.

Protein concentrations were determined according to Bradford [26].

Enzyme assay, sample preparation and HPLC-analysis

The enzyme assay was carried out in PBS (cell supernatant) or elution buffer (purified fractions). The amount of product formed upon incubation with substrate during 30 min was used as a measure of lipoxygenase activity. Unless otherwise indicated, enzyme assays were performed for 30 min 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 12-lipoxygenase or at 37°C in the presence of 30 μM substrate, 1 mM Ca^{2+}, 1 mM EDTA, 2 mM ATP and 5% ethylene glycol for 5-lipoxygenase. Incubations were stopped by the addition of 500 μl methanol and formed hydroperoxy fatty acids were reduced to the corresponding hydroxy fatty acids by the addition of reduced glutathione (final concentration 5 mM). Prostaglandin B_2 (PGB_2) was added as an internal standard for HPLC-analysis. The reaction products were extracted and subsequently analyzed with reversed-phase HPLC as described before [21,28]. The identity of the reaction products was confirmed by co-elution with reference compounds and by UV-spectrum. Molar absorption coefficients used were: 28650 M^{-1} cm^{-1} at 280 nm for prostaglanding B_2, 25000 M^{-1} cm^{-1} at 237 nm for 13(*S*)-HOD, 29500 M^{-1} cm^{-1} at 237 nm for H(P)ETEs, and 40000 M^{-1} cm^{-1} at 280 nm for leukotrienes and diH(P)ETEs except for 5(*S*), 15(*S*)-diHETE: 33500 M^{-1} cm^{-1} at 243 nm and for 8,15-diHETEs: 30000 M^{-1} cm^{-1} at 280 nm [29,30].

Preparation of 5,12-diHETE, 5,15-diHETE, 8,15-diHETE, 14,15-diHETE and 13-HOD

5(*S*),12(*S*)-diHETE was biosynthesized from 12(*S*)-HETE and 5(*S*),12(*R*)-diHETE from 12(*R*)-HETE by incubation with soybean lipoxygenase-1 at pH 8.75 (J. Verhagen, unpublished data). 5(*S*),15(*S*)-diHETE and 8(*S*),15(*S*)-diHETE were prepared from 15(*S*)-HETE by incubation with soybean lipoxygenase-1 at pH 8.75 [29]. 14(*R*),15(*S*)-diHETE was produced from arachidonic acid by incubation with porcine leukocytes. 13(*S*)-HPOD was formed from linoleic acid by incubation with soybean lipoxygenase-1 at pH 9.0 [31]. Hydroperoxy fatty acids were reduced to the correspond-

ing hydroxy compounds with $SnCl_2$. Purification of these compounds was performed with reversed-phase HPLC, as described under HPLC-analysis.

Cell viability

Trypan blue exclusion was used to monitor cell viability. Cell viability was generally over 90% and was not altered when cells were treated for 30 min at 37 or 10°C with 10 μM A23187. When also 60 μM arachidonic acid or linoleic acid was included in the incubation mixture, cell viability decreased till approx. 80% at 37°C, but hardly decreased at 10°C.

Results

Incubation of partially purified 12-lipoxygenase with arachidonic acid

When 20 000 × *g* cell supernatant was incubated with arachidonic acid at 10°C, the formation of 5(*S*)- and 12(*S*)-HETE was observed [21]. When incubation was performed with partially purified 12-lipoxygenase, only formation of 12(*S*)-HETE was observed [21]. However, in both incubation mixtures also a small amount of 15-HETE was found (data not shown). Initially this was ascribed to autoxidation of the arachidonic acid stock solution used in those studies. In the current study, we used arachidonic acid, which had been purified extensively on a silica-based column under argon and contained no autoxidation products at all. After incubation with 12-lipoxygenase, partially purified by using an anion-exchange HPLC column as described in Materials and Methods, again the presence of a low amount of 15-HETE was observed (peak 1, Fig. 1), which was found to be the *S*-enantiomer (data not shown). Similar results were obtained with 12-lipoxygenase, partially purified using a gel-filtration HPLC column as described in Materials and Methods. Neither 12(*S*)-HETE nor 15(*S*)-HETE was produced upon incubation of arachidonic acid with a boiled enzyme preparation. 15(*S*)-HETE was not produced either upon incubation of arachidonic acid with 5-lipoxygenase, partially purified as described in Materials and Methods.

Incubation of partially purified 12-lipoxygenase with 5(S)-HETE or 5(S)-HPETE

Upon incubation of partially purified 12-lipoxygenase (TSK DEAE anion-exchange column) at 10°C with 5(*S*)-HETE, two products of double dioxygenation were formed, being 5,12-diHETE and 5,15-diHETE (Fig. 2). When the peak of 5,12-diHETE was rechromatographed using methanol/ water/ acetic acid, 65 : 35 : 0.1 (by vol.) (pH 5.5) as an eluent, it was found to co-elute with standard 5(*S*),12(*S*)-diHETE, but not with 5(*S*), 12(*R*)-diHETE. The peak of 5, 15-diHETE was found to co-elute with standard 5(*S*), 15(*S*)-diHETE. The same products were formed when

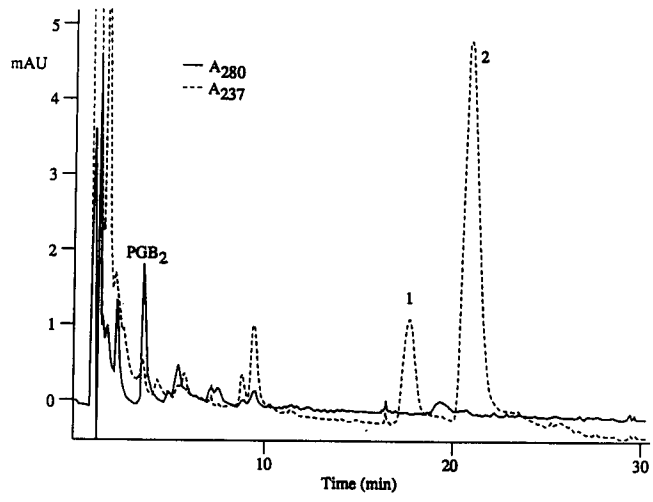

Fig. 1. Reversed-phase HPLC chromatogram of reaction products formed from arachidonic acid by partially purified 12-lipoxygenase from RBL-1 cells. Incubation was performed for 30 min at 10°C and pH 7.5 in the presence of 30 μM arachidonic acid, 1 mM Ca^{2+}, and 5% ethylene glycol. The reaction products were extracted as described in Materials and Methods. 1. 15(*S*)-HETE, 2. 12(*S*)-HETE.

12-lipoxygenase was incubated with 5(*S*)-HPETE but now also LTA_4 was formed, indicated by the presence of LTA_4-derived LTB_4-isomers (Fig. 3A). When this LTB_4-fraction was rechromatographed using the system mentioned above, separation into two peaks in a 1 : 1 ratio was obtained, co-eluting with the two LTB_4-isomers, 12(*S*), 6*E*-LTB_4 and 6*E*-LTB_4. Formation of LTB_4 was not observed, indicating that LTA_4-hydrolase activity was absent in the enzyme preparation. Upon incubation of 12-lipoxygenase with 5(*S*)-HPETE at 37°C instead of 10°C, only slightly smaller amounts of LTA_4-derived LTB_4-isomers were observed (Fig. 3B). However, 5(*S*),12(*S*)-diHETE was not formed at 37°C. Incubation of boiled purified fractions with substrate was always used as a control to exclude that autoxidation of fatty acids was involved. Although the 12-lipoxygenase preparation exhibited LTA_4-synthase activity, no 5-lipoxygenase activity was observed upon incubation with arachidonic acid at 10 or 37°C. Similar results were obtained with 12-lipoxygenase, partially purified using a gel-filtration HPLC column.

Incubation of partially purified 12-lipoxygenase with 15(S)-HETE or 15(S)-HPETE

Incubation of 12-lipoxygenase with 15(*S*)-HETE at 10°C led to the formation of two compounds, co-eluting with the products of double dioxygenation, 8(*S*), 15(*S*)-diHETE and 14(*R*),15(*S*)-diHETE, respectively (Fig. 4A). The same products were formed, though to a lesser extent, when 12-lipoxygenase was incubated with 15(*S*)-HPETE. However, in the latter case also 14, 15-LTA_4 was formed, indicated by the presence of two 8, 15-dihydroxy products formed non-enzymatically by

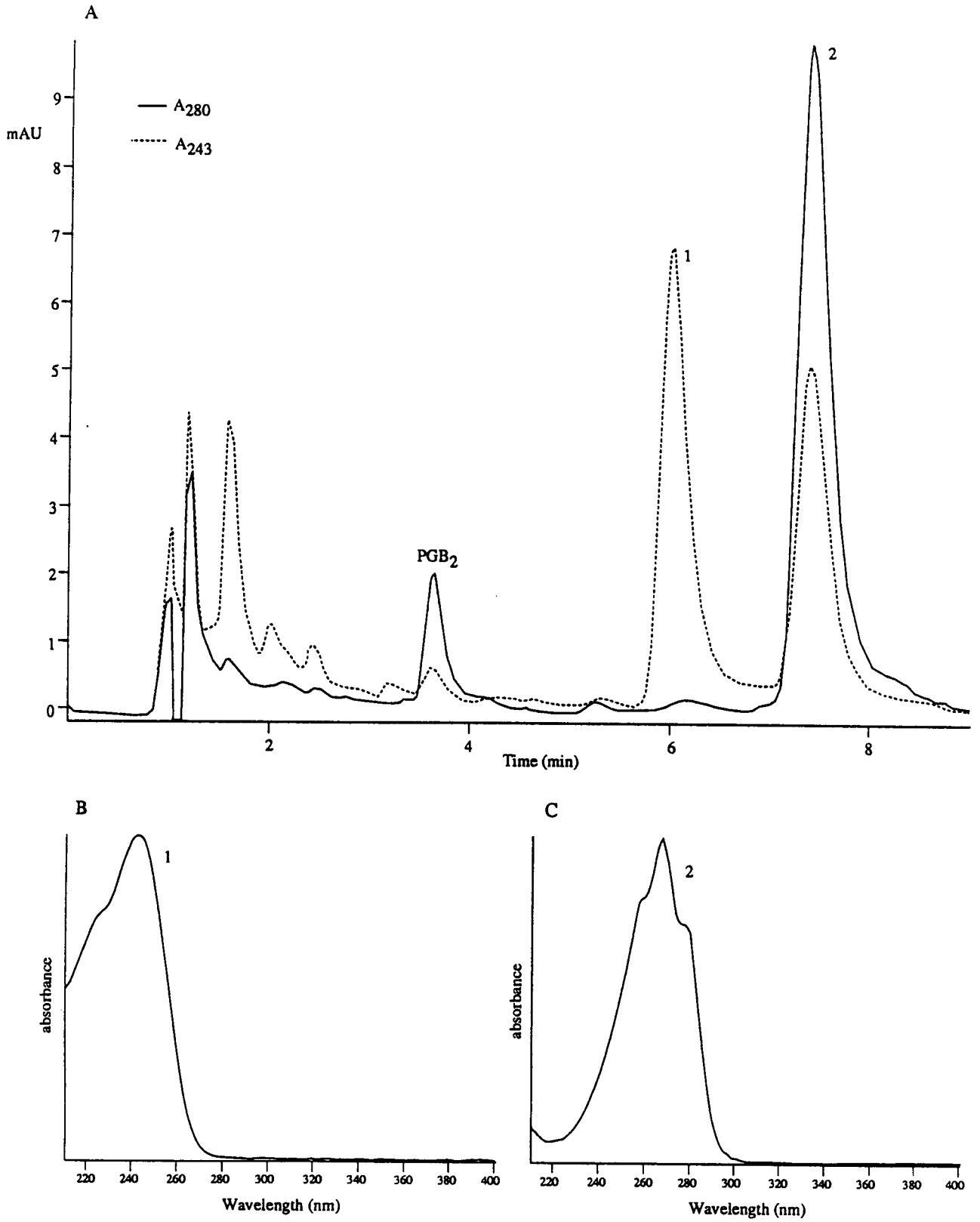

Fig. 2. (A) Reversed-phase HPLC chromatogram of reaction products formed from 5(*S*)-HETE by partially purified 12-lipoxygenase from RBL-1 cells. Incubation was performed for 30 min at 10°C and pH 7.5 in the presence of 30 μM 5(*S*)-HETE, 1 mM Ca^{2+} and 5% ethylene glycol. The reaction products were extracted as described in Materials and Methods. 1. 5(*S*),15(*S*)-diHETE, 2. 5(*S*),12(*S*)-diHETE; (B) UV-spectrum of 5(*S*),15(*S*)-diHETE, showing an absorption maximum at 243 nm; (C) UV-spectrum of 5(*S*),12(*S*)-diHETE, showing absorption maxima at 259, 269, and 279 nm.

hydrolysis of 14, 15-LTA_4 (Fig. 4B).

When partially purified 12-lipoxygenase was incubated with 12(*S*)-HPETE, no conversion into 12-leukotrienes was observed.

Experiments with intact RBL-1 cells

Walstra et al. reported that for bovine leukocytes, 12-lipoxygenase activity became apparent only upon cell disruption [16]. They further suggested that this 12-lipoxygenase activity could represent the remaining LTA_4-synthase activity of the 5-lipoxygenase after the dioxygenase activity had vanished upon cell disruption [17]. Therefore, we investigated whether RBL-1 cells show a similar phenomenon.

When intact RBL-1 cells were incubated at 37°C with arachidonic acid in the presence of A23187 and Ca^{2+}, the formation of 5-lipoxygenase-catalyzed reaction products (LTB_4, LTB_4-isomers, LTC_4, and 5(*S*)-HETE) was observed (data not shown). However, upon incubation for 30 min at 10 or 37°C with arachidonic acid or linoleic acid in the presence of A23187 and Ca^{2+}, only very small amounts of 12-lipoxygenase-catalyzed reaction products were formed, which can be ascribed to 12-lipoxygenase released into the medium by lysis of a few cells during incubation (Table I) (see also under Cell viability, Materials and Methods). Subsequent disruption of intact cells after incubation for 30 min at 37°C with substrate, A23187, and Ca^{2+}, by freezing the incubation mixtures in liquid nitrogen and thawing, yielded considerable 12-lipoxygenase activity at 10°C (Table I). The 20000 × *g* supernatant of disrupted RBL-1 cells exhibited an even higher 12-lip-

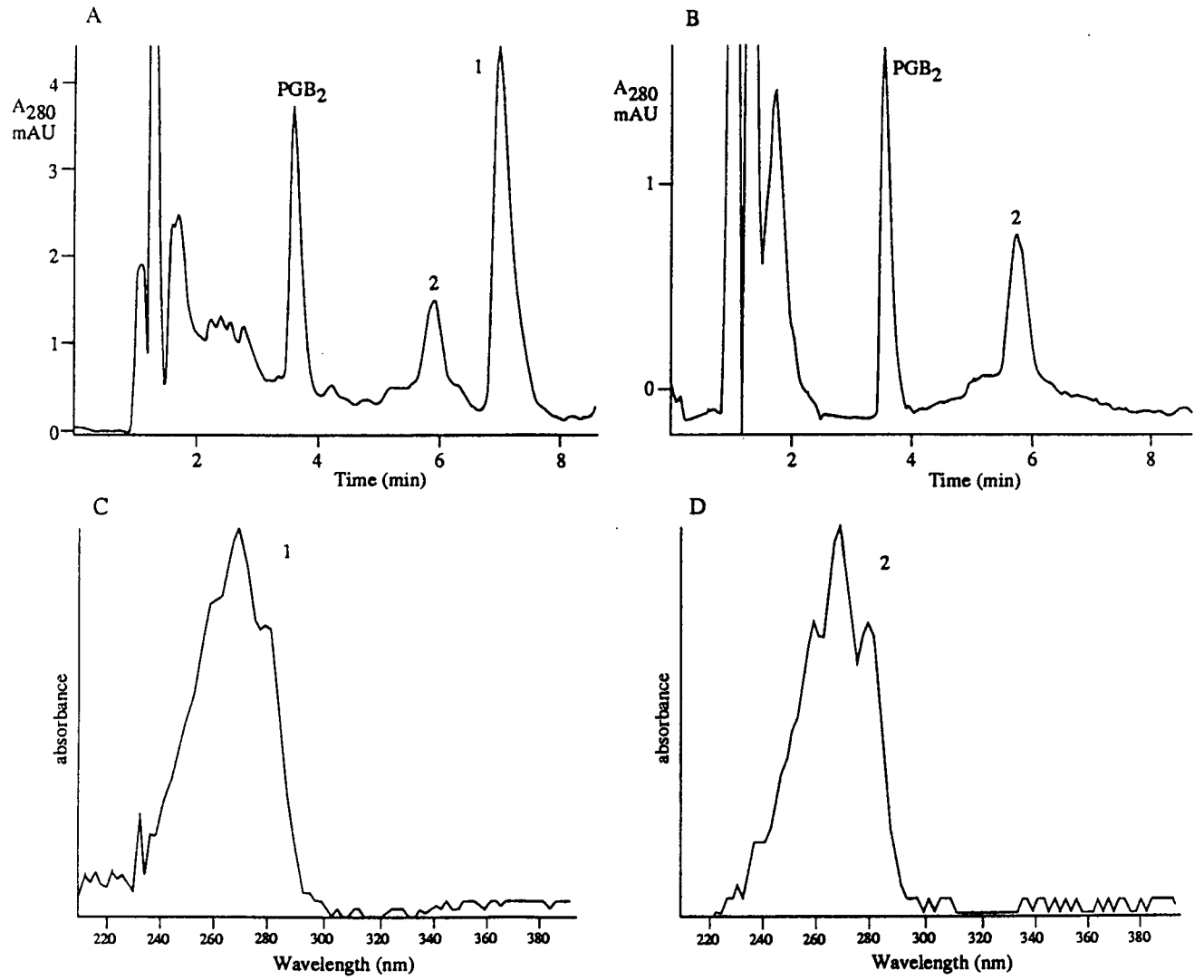

Fig. 3. Reversed-phase HPLC chromatograms and UV-spectra of reaction products formed from 5(*S*)-HPETE by partially purified 12-lipoxygenase from RBL-1 cells. The reaction products were extracted as described in Materials and Methods; (A) Incubation with 5(*S*)-HPETE at 10°C, 100 ng PGB_2 added. 1. 5(*S*), 12(*S*)-diHETE, 2. 12(*S*), 6*E*-LTB_4 + 6*E*-LTB_4; (B) incubation with 5(*S*)-HPETE at 37°C, 50 ng PGB_2 added. 2. 12(*S*), 6*E*-LTB_4 + 6*E*-LTB_4; (C) UV-spectrum of 5(*S*), 12(*S*)-diHETE, showing absorption maxima at 259, 269, and 279 nm; (D) UV-spectrum of 12(*S*), 6*E*-LTB_4 + 6*E*-LTB_4, showing absorption maxima at 259, 269, and 279 nm.

oxygenase activity. Apparently, like 12-lipoxygenase from bovine leukocytes, 12-lipoxygenase from RBL-1 cells exhibits activity only upon cell disruption. However, this was not accompanied by a total disappearance of 5-lipoxygenase activity, as was observed for bovine leukocytes [17]. Even a higher amount of 5(*S*)-HETE was found upon incubation of cell supernatant with arachidonic acid (Table I). This may be explained by a higher substrate turnover in cell supernatant than in intact RBL-1 cells, since upon activation of intact RBL-1 cells by A23187 and Ca^{2+}, a part of 5-lipoxygenase is translocated to the membrane, thereby losing its activity [32]. Also incorporation of 5(*S*)-HETE into phospholipids of the cell membrane may reduce the amount of 5(*S*)-HETE detected [33]. Furthermore, a portion of 5(*S*)-HETE may be metabolized in intact cells by reductase activity, as was reported for porcine leukocytes [34].

Nevertheless, a relatively small amount of 12-lipoxygenase could account for the amounts of product formed at 10°C, since inactivation of the enzyme at 10°C is slow [21]. If a similar phenomenon as was reported for bovine leukocytes, also occurs with RBL-1 cells, this 12-lipoxygenase activity may originate from the 5-lipoxygenase. To investigate whether such a shift from 5-lipoxygenase to 12-lipoxygenase activity could possibly be induced, further experiments with 5-lipoxygenase were performed.

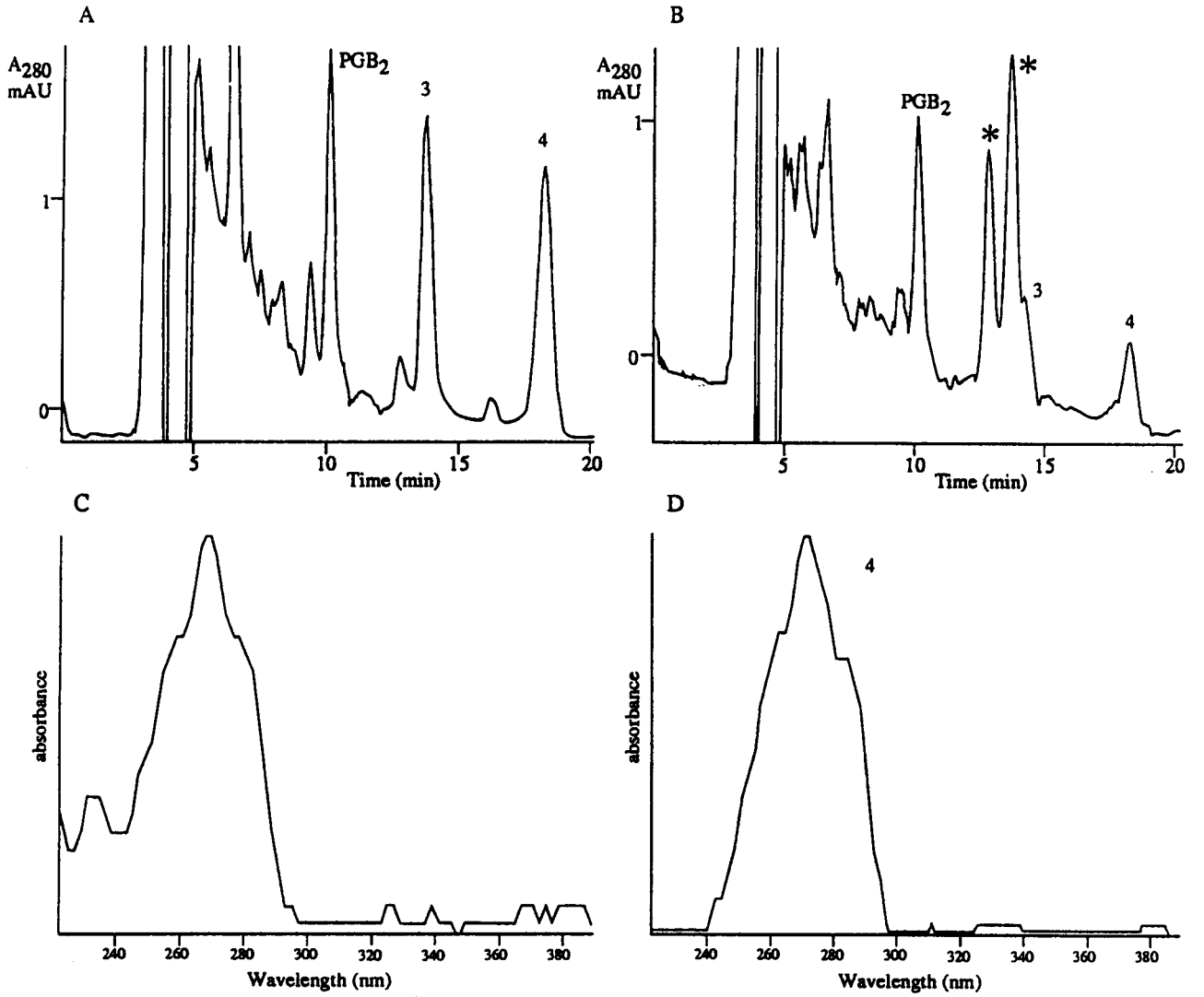

Fig. 4. Reversed-phase HPLC chromatograms and UV-spectra of reaction products formed from 15(*S*)-HETE or 15(*S*)-HPETE by partially purified 12-lipoxygenase from RBL-1 cells. The reaction products were extracted as described in Materials and Methods; (A) Incubation with 15(*S*)-HETE at 10°C. 3. 8(*S*), 15(*S*)-diHETE, 4. 14(*R*), 15(*S*)-diHETE; (B) incubation with 15(*S*)-HPETE at 10°C. 3. 8(*S*), 15(*S*)-diHETE, 4. 14(*R*), 15(*S*)-diHETE. *. 8, 15-dihydroxy products; (C) UV-spectrum of 8(*S*), 15(*S*)-diHETE and of 8, 15-dihydroxy products, showing absorption maxima at 259, 269, and 279 nm; (D) UV-spectrum of 14(*R*), 15(*S*)-diHETE, showing absorption maxima at 263, 273, and 283 nm.

TABLE I

5-Lipoxygenase and 12-lipoxygenase activity of intact and disrupted RBL-1 cells

RBL-1 cells were collected as described in Materials and Methods and resuspended in PBS at a final concentration of $2 \cdot 10^7$ cells/ml. Cells were incubated for 30 min at 10 or 37°C (pH 7.4) in the presence of 10 μM calcium ionophore A23187, 60 μM substrate, and 1 mM Ca^{2+} (12-lipoxygenase); or in the presence of 10 μM A23187, 60 μM substrate, 1 mM EDTA, 2 mM ATP, and 1 mM Ca^{2+} (5-lipoxygenase). After 30 min, the incubation mixture was centrifuged (10 min, 5000× *g*, 4°C). PGB_2 was added to the supernatant and reaction products were extracted and analyzed. 20000× *g* Supernatant of RBL-1 cells was incubated with 60 μM arachidonic acid or linoleic acid at 10 or 37°C. Values represent the mean of duplicate incubations with one cell preparation. Experiments were performed in duplicate.

Incubation	Activity (pmol product/10^6 (disrupted) cells)		
	5-Lipoxygenase	12-Lipoxygenase	
	5-HETE	12-HETE	13-HOD
Intact cells			
+ substrate, 30 min, 37°C	21.8	0.2	0.3
+ substrate, 30 min, 10°C	8.1	0.6	1.0
Intact cells + substrate, 30 min, 37°C → disruption →			
30 min, 37°C		3.6	7.1
30 min, 10°C		43.7	84.0
Intact cells → disruption → 20000× *g* supernatant			
+ substrate, 30 min, 37°C	70.7	1.9	2.4
+ substrate, 30 min, 10°C	54.6	60.5	109.2

Interaction of 5-lipoxygenase with the membrane in intact cells stimulated with A23187

In nonstimulated RBL-1 cells 5-lipoxygenase is cytosolic [5]. Activation of RBL-1 cells by 10 μM A23187 in the presence of Ca^{2+} leads to release of LTC_4. This process is associated with a partial translocation of 5-lipoxygenase to a membrane site, as was demonstrated in Ref. 32 by examining the relative distribution of 5-lipoxygenase by immunoblotting. Similar results have been described for human leukocyte 5-lipoxygenase [35]. This enzyme was even shown to bind to an 18 kDa integral membrane protein, 'Five-Lipoxygenase-Activating-Protein (FLA)', which may serve as a membrane anchor for activated 5-lipoxygenase [36,37]. This membrane-associated 5-lipoxygenase was suggested to serve for leukotriene synthesis and as a consequence, the enzyme is thought to be inactivated. No activity could be detected for the membrane-bound RBL-1 5-lipoxygenase, either [32]. In order to investigate whether this membrane-bound 5-lipoxygenase could be dissociated upon cell disruption and exhibit LTA_4-synthase and 12-lipoxygenase activity, RBL-1 cells were treated for 30 min at 37°C with or without A23187 and Ca^{2+}. After stimulation with A23187 and Ca^{2+}, 5-lipoxygenase activity in the 20000 × *g* cell supernant had decreased, while 12-lipoxygenase activity had increased (Table II). No 5-lipoxygenase or 12-lipoxygenase activity could be detected in the membrane fraction.

Interaction of partially purified 5-lipoxygenase with membrane fractions

It was reported that upon disruption of RBL-1 cells in the presence of 0.05–10 μM Ca^{2+}, cytosolic 5-lipoxygenase becomes associated with the membrane fraction, thereby losing its activity [32]. Moreover, in the presence of Ca^{2+}, purified 5-lipoxygenase was

TABLE II

Effect of stimulation of RBL-1 cells with A23187 on 5-lipoxygenase and 12-lipoxygenase activity

RBL-1 cells were resuspended in PBS at a final concentration of $2 \cdot 10^7$ cells/ml and stimulated for 30 min at 37°C (pH 7.4) with 10 μM calcium ionophore A23187 and 1 mM Ca^{2+}. Then, cells were centrifuged (10 min, 2000× *g*, room temperature), washed with PBS, and resuspended in PBS, containing 2 mM EDTA. Cells were immediately disrupted by freezing in liquid nitrogen and thawing. The 20000× *g* cell supernatant and the membrane fraction (20000× *g* cell pellet) were obtained as described in Materials and Methods. Aliquots of 500 μl were assayed for 5-lipoxygenase and 12-lipoxygenase activity by incubation with 60 μM arachidonic acid or linoleic acid. Values represent the mean of duplicate incubations with one cell preparation. Experiments were performed in duplicate.

		Stimulation	
		without A23187	with A23187
5-Lipoxygenase activity with arachidonic acid	(%)	100	83
12-Lipoxygenase activity with arachidonic acid	(%)	100	128
with linoleic acid		100	131

TABLE III

Effect of interaction of 5-lipoxygenase with membrane fractions on enzyme activity

(B) Cytosolic 5-lipoxygenase from $0.5 \cdot 10^8$ cells was incubated with the microsomal membrane-enriched fraction of the same cells at 4°C for 1 h in the presence of 1 mM Ca^{2+} in a final volume of 2 ml. At the end of the incubation, the mixture was centrifuged (20 min, 100000× *g*, 4°C) to separate the microsomal membrane-enriched fraction from the cytosolic fraction. The microsomal membrane-enriched fraction was washed and resuspended in PBS. Aliquots of 1 ml were assayed for 5-lipoxygenase and 12-lipoxygenase activity by incubation with 60 μM arachidonic acid or linoleic acid, as described in Materials and Methods; (C) partially purified (p.p.) 5-lipoxygenase from $2.5 \cdot 10^8$ cells was incubated with the membrane fraction of the same cells at 4°C for 1 h in the presence of 1 mM EDTA and 2 mM Ca^{2+}, in a final volume of 10 ml. At the end of the incubation, the mixture was centrifuged (20 min, 20000× *g*, 4°C) to separate membranes from the soluble fraction. The membrane pellet was washed and resuspended in PBS containing 1 mM EDTA and 2 mM Ca^{2+}. Aliquots of 1 ml were assayed for 5-lipoxygenase and 12-lipoxygenase activity by incubation with 60 μM arachidonic acid or linoleic acid. Values represent the mean of duplicate incubations with one enzyme preparation. Experiments were performed in duplicate. n.d., not detectable.

Incubation	12-Lipoxygenase activity (nmol 13-HOD/30 min per ml)	5-Lipoxygenase activity (nmol 5-HETE/30 min per ml)
A 20000× *g* supernatant	3.0	1.9
20000× *g* pellet	n.d.	0.1
B 100000× *g* supernatant	3.3	
100000× *g* pellet	n.d.	
100000× *g* supernatant + pellet + Ca^{2+}, 1 h →		
100000× *g* supernatant	3.0	
100000× *g* pellet	n.d.	
C p.p. 5-lipoxygenase	n.d.	1.2
p.p. 5-lipoxygenase + Ca^{2+} + 20000× *g* pellet, 1 h →		
20000× *g* supernatant	n.d.	0.02
20000× *g* pellet	n.d.	0.01

shown to bind to a purified membrane fraction. The enzyme was also shown to associate with the microsomal membrane fraction, though to a lesser extent. The membrane-association could not simply be reversed by the addition of EDTA, as was reported for the membrane-association of human leukocyte 5-lipoxygenase [38]. The possible role of membrane fractions prompted us to investigate whether an interaction of 5-lipoxygenase with these fractions during cell disruption could be accompanied by a shift from 5- to 12-lipoxygenase activity.

Upon incubation with linoleic acid the microsomal membrane-enriched fraction did not exhibit detectable 12-lipoxygenase activity (Table IIIA-B). All 12-lipoxygenase activity present in the 20000 × *g* cell supernatant was found in the 100000 × *g* cytosolic fraction. After incubation with cytosolic 5-lipoxygenase in the presence of Ca^{2+} for 1 h at 4°C still no microsomal membrane-associated 12-lipoxygenase activity could be observed (Table IIIB).

After incubation of partially purified 5-lipoxygenase with the membrane fraction in the presence of Ca^{2+}, 5-lipoxygenase activity could hardly be detected in the soluble fraction (Table IIIC). No activity was found for the membrane-bound 5-lipoxygenase either. However, this was not accompanied by appearance of 12-lipoxygenase activity in either soluble or membrane fraction.

Interaction of 5-lipoxygenase with membrane fractions upon cell disruption in Ca^{2+}-containing media

Cells were disrupted in the presence or absence of 1 mM EDTA. Recovery of 5-lipoxygenase had decreased when EDTA was left out (Table IV), as was reported before [18,19]. When cells were disrupted in the presence of 1 mM Ca^{2+}, recovery of 5-lipoxygenase activity was even lower. However, no simultaneous increase in

TABLE IV

Cell disruption in EDTA or Ca^{2+}-containing media

RBL-1 cells were resuspended in PBS and disrupted in the presence of Ca^{2+} or EDTA, while temperature did not exceed 4°C. The 20000× *g* cell supernatant and the membrane fraction were obtained and aliquots of 500 μl were assayed for 5-lipoxygenase and 12-lipoxygenase activity by incubation with 60 μM arachidonic acid or linoleic acid, as described in Materials and Methods. Values represent the mean of duplicate incubations with one cell preparation. Experiments were performed in duplicate.

	Cell disruption		
	−EDTA	+EDTA	+1 mM Ca^{2+}
5-Lipoxygenase activity (%) with arachidonic acid	100	130	85
12-Lipoxygenase activity (%)			
with arachidonic acid	100	87	91
with linoleic acid	100	97	92

12-lipoxygenase activity was observed. It should be noted that preincubation of cell supernatant at 1 mM Ca^{2+} at room temperature was reported to result in a decrease in 5-lipoxygenase activity of 90% within 1 min [32]. However, in spite of the presence of 1 mM Ca^{2+} during cell disruption, we now observed a decrease in 5-lipoxygenase activity in the subsequently prepared cell supernatant of only 35%. The presence of a membrane fraction in the mixture, directly after cell disruption, may have reduced the inactivation of 5-lipoxygenase by Ca^{2+}, as was reported before [32]. Still, we observed considerable 5-lipoxygenase activity when this membrane fraction was removed and temperature was raised from 0 to 37°C prior to incubation with substrate (for 5-lipoxygenase assay), thereby creating the conditions for a rapid inactivation of 5-lipoxygenase, as reported in Ref. 32. This may be explained by binding of part of Ca^{2+} to the membrane fraction, thereby reducing the actual Ca^{2+}-concentration to a level, insufficient for an almost total inactivation of 5-lipoxygenase.

Discussion

This study shows that RBL-1 cells exhibit 12-lipoxygenase activity only upon cell disruption. 12-Lipoxygenase may also possess 15-lipoxygenase activity, as is indicated by the formation of low amounts of 15(*S*)-HETE, next to the predominant product 12(*S*)-HETE, upon incubation of partially purified 12-lipoxygenase with arachidonic acid. Analogous to this, the formation of both 5(*S*),12(*S*)-diHETE and 5(*S*),15(*S*)-diHETE is observed with 5(*S*)-HETE as substrate. Similar results were obtained with 12-lipoxygenase obtained by using a different purification method. Therefore, it seems unlikely that the formation of 15(*S*)-HETE and 5(*S*),15(*S*)-diHETE is due to a contaminating co-eluting 15-lipoxygenase. Instead, we suggest that these products are formed by the 12-lipoxygenase itself, as was also observed for 12-lipoxygenases from porcine leukocytes [7,39], bovine leukocytes and platelets [40] and human platelets [41].

Partially purified 12-lipoxygenase, which contained no contaminating 5-lipoxygenase activity, converted 5(*S*)-HPETE not only into 5(*S*),12(*S*)-diHETE, but also into LTA_4. Similar results were found with 12-lipoxygenase obtained by using a different purification method. Therefore, it seems unlikely that the LTA_4-synthase activity is due to a contaminating co-eluting enzyme. Instead, we suggest that 12-lipoxygenase from RBL-1 cells possesses LTA_4-synthase activity, as was also reported for 12-lipoxygenase from bovine leukocytes [17]. Also in this case, 12-lipoxygenase activity was found only upon sonication of these cells [16]. In cell supernatant, bovine leukocyte 12-lipoxygenase converted 5(*S*)-HPETE into LTA_4 [17] and the purified enzyme also formed 5(*S*),12(*S*)-diHETE [40]. It was suggested that the bovine leukocyte 12-lipoxygenase represents the remaining LTA_4-synthase activity of the 5-lipoxygenase, of which the 5-dioxygenase activity was destroyed upon cell disruption [17]. Both the formation

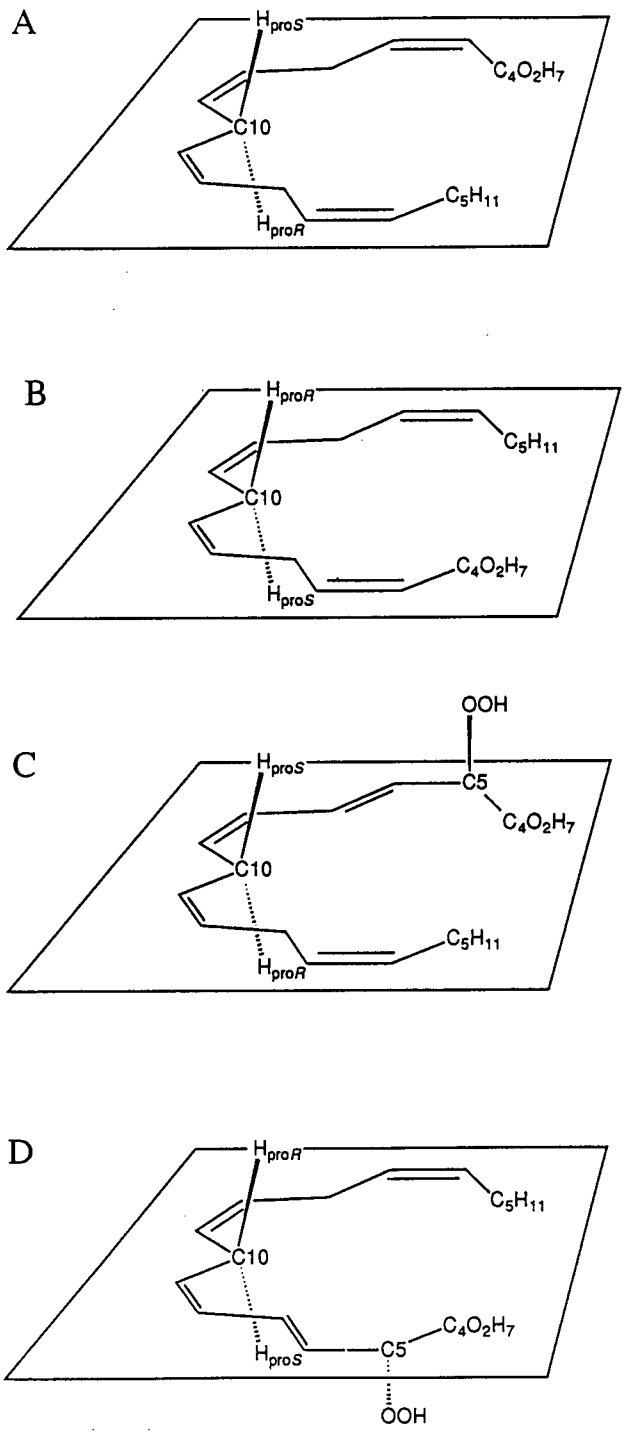

Fig. 5. Three-dimensional depiction of arachidonic acid on the enzyme; (A) Arachidonic acid in the normal position; (B) arachidonic acid in the inverted position, (C) 5(*S*)-HPETE in the normal position; (D) 5(*S*)-HPETE in the inverted position.

of 12(*S*)-HPETE from arachidonic acid and the formation of LTA_4 from 5(*S*)-HPETE are initiated by the abstraction of hydrogen at C10. However, formation of LTA_4 from 5(*S*)-HPETE requires the abstraction of $H_{pro R}$ (Fig. 5C), followed by a radical shift towards the carboxylic end, whereas formation of 12(*S*)-HPETE from arachidonic acid requires the abstraction of $H_{pro S}$, followed by a radical shift towards the methyl end (Fig. 5A) [17]. For definition of pro*R* and pro*S* see Ref. 42. Therefore, upon abstraction of $H_{pro R}$ at C10 from arachidonic acid, the formation of 12(*R*)-HPETE or 8(*R*)-HPETE would be expected. However, upon incubation of 12-lipoxygenases from bovine leukocytes [16] or RBL-I cells [21] with arachidonic acid, 12(*S*)-HPETE is exclusively formed. If we assume an inverted position of arachidonic acid on the active centre (Fig. 5B), as was suggested by Walstra et al. for 12-lipoxygenase from bovine leukocytes [17], $H_{pro S}$ will be abstracted and the radical shift will still proceed towards the methyl end (in the same direction as for the formation of LTA_4), resulting now in the formation of 12(*S*)-HPETE. Similarly, the formation of 5(*S*),12(*S*)-diHETE and 13(*S*)-HPOD can be explained by assuming an inverted position of 5(*S*)-H(P)ETE (Fig. 5D) and linoleic acid, respectively. An inverted position of the substrate on the enzyme has also been suggested for the formation of 8(*S*), 15(*S*)-diHETE by double dioxygenation of arachidonic acid [17,43].

Upon incubation with arachidonic acid or linoleic acid, product formation by 12-lipoxygenase was observed at 10°C, while at 37°C the enzyme was rapidly inactivated by formed hydroperoxy fatty acid [21]. Also with 5(*S*)-HETE as substrate, product formation was observed at 10°C, but not at 37°C, probably by a rapid inactivation of 12-lipoxygenase at 37°C by formed 5(*S*)H, 12(*S*)-HPETE. With 5(*S*)-HPETE as substrate, formation of 5(*S*), 12(*S*)-diHETE is observed only at 10°C, while formation of LTA_4 is observed at 10°C and to a somewhat lesser extent also at 37°C. Therefore, we suggest that when 5(*S*)-HPETE is bound in inverted position, 12-lipoxygenase is rapidly inactivated at 37°C, by direct action of 5(*S*)-HPETE or by possibly formed 5(*S*), 12(*S*)-diHPETE. The observed formation of LTA_4 at 37°C implies that normal binding of 5(*S*)-HPETE does not cause direct inactivation of 12-lipoxygenase at 37°C. This is in accordance with the observation that the intact 5-lipoxygenase/LTA_4-synthase complex forms LTA_4 at both 10 and 37°C. A survey of products formed from various substrates by partially purified 12-lipoxygenase from RBL-1 cells is given in Table V.

12-Lipoxygenase from RBL-1 cells converted 15(*S*)-HPETE into two products of double dioxygenation, 8(*S*), 15(*S*)-diHETE and 14(*R*), 15(*S*)-diHETE, and into 14, 15-LTA_4. Hence, 12-lipoxygenase from RBL-1 cells may also possess 14, 15-LTA_4-synthase activity. The same products were formed upon incubation of 15(*S*)-HPETE with 12-lipoxygenases from porcine leukocytes [7], bovine Leukocytes and platelets [40], and human platelets [41].

12-Lipoxygenases from RBL-1 cells and bovine leukocytes have many features in common, like LTA_4-synthase activity, enzyme activity in the absence of Ca^{2+}, (Refs. 26 and 16, respectively), reactivity towards linoleic acid, 5(*S*)-H(P)ETE, and 15(*S*)-HPETE, and appearance of enzyme activity, only upon cell disruption. So far, the reason for the latter property is not clear. 12-Lipoxygenase could represent the remaining LTA_4-synthase activity of 5-lipoxygenase, of which the 5-dioxygenase activity has disappeared upon cell disruption. Our experiments with intact RBL-1 cells show that such a possible shift from 5-lipoxygenase activity to 12-lipoxygenase activity can not simply be influenced by disrupting cells in the presence of Ca^{2+} or by incubation of partially purified 5-lipoxygenase with a membrane fraction in the presence of Ca^{2+}, both reported to cause 5-lipoxygenase to associate with membrane fractions [32]. Besides, it was demonstrated for intact cells that part of the 5-lipoxygenase is translocated to the membrane, induced by increased intracel-

TABLE V

Product formation from various substrates by 12-lipoxygenase from RBL-1 cells

Reaction products were extracted and analyzed as described in Materials and Methods.

Substrate	Substrate binding	Reaction profile
Arachidonic acid, 10°C	inverted	formation of 12(*S*)-HETE
Arachidonic acid, 37°C	inverted	rapid inactivation of 12-lipoxygenase
Linoleic acid, 10°C	inverted	formation of 13(*S*)-HOD
Linoleic acid, 37°C	inverted	rapid inactivation of 12-lipoxygenase
5(*S*)-HETE, 10°C	inverted	formation of 5(*S*), 12(*S*)-diHETE
5(*S*)-HETE, 37°C	inverted	rapid inactivation of 12-lipoxygenase
5(*S*)-HPETE, 10°C	inverted	formation of 5(*S*), 12(*S*)-diHETE
	normal	formation of LTA_4
5(*S*)-HPETE, 37°C	inverted	rapid inactivation of 12-lipoxygenase
	normal	formation of LTA_4

lular Ca^{2+}-concentrations [32]. Such an increase in intracellular Ca^{2+}-concentration was obtained by treatment with A23187 and Ca^{2+}. However, Ca^{2+} can also be released from intracellular stores upon activation of RBL cells [44–46]. The intracellular Ca^{2+}-concentration in non-stimulated RBL cells is approx. 100 nM. Activation of the cells by antigenic stimulation, monitored by the release of histamine, was accompanied by an increase in intracellular Ca^{2+}-concentration to approx. 500 nM, which appeared to be due to release of intracellularly stored Ca^{2+} and an influx of extracellular Ca^{2+} provided that the extracellular Ca^{2+}-concentration was at least 50 μM. The culture medium that we used, RPMI 1640, contained 425 μM Ca^{2+}. In addition to antigenic stimulation, activation of RBL-1 cells may also occur upon mechanical stress, e.g., when we harvested attached cells by scraping with a rubber policeman. Subsequent cell disruption may release the membrane-associated 5-lipoxygenase, which, as a consequence of the membrane-association, exhibits different product formation properties and behaviour on protein-HPLC columns. This may explain our observation that after treatment of intact cells with A23187 and Ca^{2+}, 5-lipoxygenase activity in the $20\,000 \times g$ cell supernatant had decreased, whereas 12-lipoxygenase activity had increased.

Alternatively, upon cell disruption some 5-lipoxygenase may be degraded proteolytically in such a way that the 5-dioxygenase activity is destroyed. With arachidonic acid as substrate, the remaining LTA_4-synthase then produces 12(*S*)-HPETE, thus behaving apparently as a 12-lipoxygenase.

Another explanation may be that an authentic 12-lipoxygenase, like the platelet 12-lipoxygenase, is present in the intact RBL-1 cell, but inaccessible for substrate, similar to the suggestion in Ref. 47 for the 15-lipoxygenase in human leukocytes. Cell disruption could then liberate the enzyme in a way that substrate conversion can occur. However, authentic 12-lipoxygenases do not possess LTA_4-synthase activity. Therefore, the presence of an authentic 12-lipoxygenase in RBL-1 cells is unlikely.

It seems more likely that, similar to 12-lipoxygenase from bovine leukocytes, also 12-lipoxygenase from RBL-1 cells represents the remaining LTA_4-synthase part of 5-lipoxygenase after the 5-dioxygenase activity has disappeared. Recently, it was reported that mutations at certain amino acids of human reticulocyte 15-lipoxygenase resulted in a shift from 15-lipoxygenation to 12-lipoxygenation [48]. Further studies, for instance using monoclonal antibodies or molecular cloning of the enzymes, will be necessary to elucidate the identity of 12-lipoxygenase in RBL-1 cells. Another approach may be the development of a quantitative polymerase chain reaction assay to analyze the mRNAs for these enzymes [49].

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References

1 Samuelsson, B., Dahlén, S.-E., Lindgren, J.Å., Rouzer, C.A. and Serhan, C.N. (1987) Science 237, 1171–1176.
2 Lewis, R.A., Austen, K.F. and Soberman, R.J. (1990) N. Engl. J. Med. 323, 645–655.
3 Shimizu, T., Rådmark, O. and Samuelsson, B. (1984) Proc. Natl. Acad. Sci. USA 81 689–693.
4 Ueda, N., Kaneko, S., Yoshimoto, T. and Yamamoto, S. (1986) J. Biol. Chem. 261, 7982–7988.
5 Hogaboom, G.K., Cook, M., Newton, J.F., Varrichio, A., Shorr, R.G.L., Sarau, H.M. and Crooke, S.T. (1986) Mol. Pharmacol. 30, 510–519.
6 Rouzer, C.A. and Samuelsson, B. (1985) Proc. Natl. Acad. Sci. USA 82, 6040–6044.
7 Yokoyama, C., Shinjo, F., Yoshimoto, T., Yamamoto, S., Oates, J.A. and Brash, A.R. (1986) J. Biol. Chem. 261 (16714–16721.
8 Sigal, E., Grunberger, D., Craik, C.S., Caughey, G.H. and Nadel, J.A. (1988) J. Biol. Chem. 263, 5328–5332.
9 Balcarek, J.M., Theisen, T.W., Cook, M.N., Varrichio, A., Hwang. S.-M., Strohsacker, M.W. and Cook, S.T. (1988) J. Biol. Chem. 263, 13937–13941.
10 Rouzer, C.A., Rands, E., Kargman, S., Jones, R.E., Register, R.B. and Dixon, R.A.F. (1988) J. Biol. Chem. 263, 10135–10140.
11 Matsumoto, T., Funk, C.D., Rådmark, O., Höög, J.-O., Jörnvall, H. and Samuelsson, B. (1988) Proc. Natl. Acad. Sci. USA 85, 26–30.
12 Funk, C.D., Furci, L. and FitzGerald, G.A. (1990) Proc. Natl. Acad. Sci. USA 87, 5638–5642.
13 Yoshimoto, T., Suzuki, H., Yamamoto, S., Takai, T., Yokoyama, C. and Tanabe, T. (1990) Proc. Natl. Acad. Sci. USA 87, 2142–2146.
14 Sigal, E., Craik, C.S., Highland, E., Grunberger, D., Costello, L.L., Dixon, R.A.F. and Nadel, J.A. (1988) Biochem. Biophys. Res. Commun. 157, 457–464.
15 Walstra, P., Verhagen, J., Veldink, G.A. and Vliegenthart, J.F.G. (1984) Biochim. Biophys. Acta 795, 499–503.
16 Walstra, P., Verhagen, J., Vermeer, M.A., Veldink, G.A. and Vliegenthart, J.F.G. (1987) Biochim. Biophys. Acta 921, 312–319.
17 Walstra, P., Verhagen, J., Veldink, G.A. and Vliegenthart, J.F.G. (1987) Biochem. Biophys. Res. Commun. 149, 258–265.
18 Jakschik, B.A., Sun, F.F., Lee, L-H. and Steinhoff, M.M. (1980) Biochem. Biophys. Res. Commun. 95, 103–110.
19 Goetze, A.M., Fayer, L., Bouska, J., Bornemeier, D. and Carter, G.W. (1985) Prostaglandins 29, 689–701.
20 Hamasaki, Y. and Tai, H-H. (1984) Biochim. Biophys. Acta 793, 393–398.
21 Van der Donk, E.M.M., Verhagen, J., Veldink, G.A. and Vliegenthart, J.F.G. (1991) Biochim. Biophys. Acta 1081, 135–140.
22 Jakshik, B.A., Sams, R., Sprecher, H. and Needleman, P. (1980) Prostaglandins 20, 401–410.
23 Kühn, H., Heydeck, D., Wiesner, R. and Schewe, T. (1985) Biochim. Biophys. Acta 830, 25–29.
24 Maas, R.L., Ingram, C.D., Taber, D.F., Oates, J.A. and Brash, A.R. (1982) J. Biol. Chem. 257, 13515–13519.
25 Panossian, A., Hamberg, M. and Samuelsson, B. (1982) FEBS Lett. 150, 511–513.
26 Van der Donk, E.M.M., Dubois, G.R., Verhagen, J., Veldink,

G.A. and Vliegenthart, J.F.G. (1991) Biochim. Biophys. Acta 1074, 443–447.
27 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
28 Verhagen, J., Wassink, G.A., Kijne, G.M., Viëtor, R.J. and Bruijnzeel, P.L.B. (1986) J. Chromatogr. 378, 208–214.
29 Van Os, C.P.A., Rijke-Schilder, G.P.M., Van Halbeek, H., Verhagen, J. and Vliegenthart, J.F.G. (1981) Biochim. Biophys. Acta 663, 177–193.
30 Verhagen, J., Veldink, G.A., Egmond, M.R., Vliegenthart, J.F.G., Boldingh, J. and Van der Star, J. (1978) Biochim. Biophys. Acta 529, 369–379.
31 Van Os, C.P.A., Vente, M. and Vliegenthart, J.F.G. (1979) Biochim. Biophys. Acta 574, 103–111.
32 Wong, A., Hwang, S.M., Cook, M.N., Hogaboom, G.K. and Crooke, S.T. (1988) Biochemistry 27, 6763–6769.
33 Costello, P.B., Baer, A.N. and Green, F.A. (1991) Inflammation 15, 269–279.
34 Wainwright, S., Falck, J.R., Yadagiri, P. and Powell, W.S. (1990) Biochemistry 29, 10126–10135.
35 Rouzer, C.A. and Kargman, S. (1988) J. Biol. Chem. 263, 10980–10988.
36 Miller, D.K., Gillard, J.W., Vickers, P.J., Sadowski, S., Leveille, C., Mancini, J.A., Charleson, P., Dixon, R.A.F., Ford-Hutchinson, A.W., Fortin, R., Gauthier, J.Y., Rodkey, J., Rosen, R., Rouzer, C., Sigal, S., Strader, C.D. and Evans, J.F. (1990) Nature (Lond.) 343, 278–281.
37 Dixon, R.A.F., Diehl, R.E., Opas, E., Rands, E., Vickers, P.J., Evans, J.F., Gillard, J.W. and Miller, D.K. (1990) Nature (Lond.) 343, 282–284.
38 Rouzer, C.A. and Samuelsson, B. (1987) Proc. Natl. Acad. Sci. USA 84, 7393–7397.
39 Yoshimoto, T., Miyamoto, Y., Ochi, K. and Yamamoto, S. (1982) Biochim. Biophys. Acta 713, 638–646.
40 Takahashi, Y., Ueda, N. and Yamamoto, S. (1988) Arch. Biochem. Biophys. 266, 613–621.
41 Hada, T., Ueda, N., Takahashi, Y. and Yamamoto, S. (1991) Biochim. Biophys. Acta 1083, 89–93.
42 Hanson, K.R. (1966) J. Am. Chem. Soc. 88, 2731–2742.
43 Kühn, H., Schewe, T. and Rapoport, S.M. (1986) Adv. Enzymol. 58, 272–311.
44 Beaven, M.A., Rogers, J., Moore, J.P., Hesketh, T.R., Smith, G.A. and Metcalfe, J.C. (1984) J. Biol. Chem. 259, 7129–7136.
45 Marcotte, G.V., Millard, P.J. and Fewtrell, C. (1990) J. Cell. Physiol. 142, 78–88.
46 Hide, M. and Beaven, M.A. (1991) J. Biol. Chem. 266, 15221–15229.
47 McGuire, J., McGee, J., Crittenden, N. and Fitzpatrick, F. (1985) J. Biol. Chem. 260, 8316–8319.
48 Sloane, D.L., Leung, R., Craik, C.S. and Sigal, E. (1991) Nature 354, 149–152.
49 Funk, C.D. and FitzGerald, G.A. (1991) J. Biol. Chem. 266, 12508–12513.