Purification and characterization of leukotriene A_4 hydrolase from *Xenopus laevis* oocytes

Filippa Strömberg-Kull, Jesper Z. Haeggström*

Department of Medical Biochemistry and Biophysics, Division of Chemistry II, Karolinska Institutet, S-171 77 Stockholm, Sweden

Received 6 June 1998; revised version received 8 July 1998

Abstract In mammals, leukotriene A_4 hydrolase converts leukotriene A_4 into the proinflammatory mediator leukotriene B_4. We have purified and characterized a non-mammalian leukotriene A_4 hydrolase from *Xenopus laevis* oocytes. This enzyme contains one zinc atom and catalyzes an anion-dependent peptidase activity, two key features of the mammalian enzymes. The amino acid sequence of an internal segment is 60% identical with human leukotriene A_4 hydrolase but only 27% identical with rat aminopeptidase B. The *Xenopus laevis* enzyme is catalytically very efficient and, unlike the human enzyme, converts leukotriene A_4 into two enzymatic metabolites, viz. leukotriene B_4 and Δ^6-*trans*-Δ^8-*cis*-leukotriene B_4.

Key words: Leukotriene A_4 hydrolase; Leukotriene B_4; Aminopeptidase; Inflammation; *Xenopus laevis*

1. Introduction

Leukotriene (LT) A_4 hydrolase catalyzes the final step in the biosynthesis of LTB_4, a potent chemotaxin and leukocyte activating agent [1]. The mammalian LTA_4 hydrolase is a soluble monomeric metalloenzyme with a molecular mass of about 69 kDa containing one catalytic zinc [2–4]. In addition to the epoxide hydrolase activity, i.e. the hydrolysis of LTA_4 into LTB_4, the enzyme also possesses an anion-dependent peptidase activity, the physiological role of which is still unknown [4–7]. The primary structure of LTA_4 hydrolase is 33% identical to that of aminopeptidase B, which has also been reported to possess LTA_4 hydrolase activity [8].

In a recent study we found that organ extracts of the African claw toad *Xenopus laevis* converted LTA_4, not only into LTB_4, but also into a novel biologically active metabolite, structurally identified as 5*S*,12*R*-dihydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid (Δ^6-*trans*-Δ^8-*cis*-LTB_4) [9]. We also discovered that the reproduction organs, i.e. oocytes and testes, are very rich in LTA_4 hydrolase activity, a circumstance which has now enabled us to purify and characterize the enzyme from a non-mammalian source. In the present report we show that the *Xenopus laevis* protein is a bifunctional zinc metalloenzyme which is structurally distinct from aminopeptidase B and exhibits high catalytic efficiencies as well as the ability to convert LTA_4 into Δ^6-*trans*-Δ^8-*cis*-LTB_4.

*Corresponding author. Fax: (46) (8) 736 0439.
E-mail: jesper.haeggstrom@mbb.ki.se

Abbreviations: LTA_4, leukotriene A_4, 5*S*-*trans*-5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid; LTB_4, leukotriene B_4, 5*S*, 12*R*-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid; Δ^6-*trans*-Δ^8-*cis*-LTB_4, 5*S*,12*R*-diHETE, 5*S*, 12*R*-dihydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid; FPLC, fast protein liquid chromatography

2. Materials and methods

2.1. Preparation of oocytes

Female *Xenopus laevis* toads, 60–90 g (Blades Biological, UK), were decapitated and the oocytes were removed and kept on ice prior to homogenization in one part (mass/vol.) 50 mM Tris-HCl buffer, pH 8, and 2 mM EDTA, utilizing a Potter-Elvehjem homogenizer. The $10\,000 \times g$ supernatants of the homogenates are referred to as cytosol.

2.2. Protein purification

Nucleic acids were removed from cytosol by streptomycin sulfate precipitation followed by centrifugation ($10\,000 \times g$; 15 min; 4°C). The supernatant was subjected to ammonium sulfate precipitation on ice. After removal of the 0–35% precipitate, the solution was saturated to 80%. Proteins were pelleted by centrifugation and dissolved in 10 mM Tris-HCl, pH 8.

For anion-exchange chromatography on FPLC (Pharmacia), a column packed with Q Sepharose Fast Flow (Pharmacia) was equilibrated with 10 mM Tris-HCl, pH 8. The 35–80% ammonium sulfate fraction was dialyzed against the equilibration buffer, treated with dithiothreitol (2 mM, 30 min), and applied to the column. Adsorbed proteins were eluted with a gradient of KCl (0–500 mM) and the enzyme activity was recovered between 140 and 190 mM. Active fractions were supplemented with 20% (mass/vol.) ammonium sulfate and applied to a phenyl Superose, HR 5/5 (Pharmacia) column preequilibrated in 20 mM Tris-HCl buffer, pH 8, containing 150 mM ammonium sulfate. A linear gradient of ammonium sulfate (150–0 mM) was applied and the enzyme activity was eluted between 67 and 45 mM. For hydroxyapatite chromatography, a TSKgel HA-1000 column (Tosohaas) was equilibrated in 10 mM potassium phosphate buffer, pH 7.1, supplemented with 0.2 mM $CaCl_2$. Pooled fractions from the hydrophobic interaction chromatography were applied in the equilibration buffer. The column was eluted with a gradient of potassium phosphate (10–400 mM) and active fractions were eluted between 150 and 190 mM. The final purification was achieved by chromatofocusing on a MonoP column HR 5/20 (Pharmacia) preequilibrated with 25 mM Bis-Tris, pH adjusted to 7.1 with iminodiacetic acid. Active fractions from the hydroxyapatite column, in 10 mM Tris-HCl, pH 8.0, were applied to the column which was then eluted with a pH gradient (7.1–4.5). LTA_4 hydrolase eluted at a pH = p*I* of about 6.1. For buffer exchange, a final chromatographic step was performed on a MonoQ HR 5/5 column (Pharmacia). LTA_4 hydrolase was also purified from isolated human leukocytes, essentially as described [10].

2.3. Determinations of enzyme activities and protein concentrations

The epoxide hydrolase activity was determined as described [9]. Briefly, aliquots of the enzyme pools (1% by vol.) in 100 μl, 10 mM Tris-HCl, pH 8, were incubated with 25 μM LTA_4 for 10 s at room temperature. Formation of LTB_4 was analyzed by reverse-phase HPLC at 270 nm, using prostaglandin B_1 as the internal standard. The column (Nova-Pak C_{18}, 4 μm Radial-Pak cartridge, 5 × 100 mm, Waters) was eluted with a mixture of acetonitrile/methanol/water/acetic acid (30:35:35:0.01, v/v) at 1.0 ml/min.

The peptidase activity was determined, essentially as described [10]. Alanine-4-nitroanilide was dissolved to a concentration of 1 mM in 50 mM Tris-HCl, pH 7.5, containing 100 mM KCl or, in some experiments, KSCN, NaBr, NaCl, NaF, NaI, $NaNO_3$ or NaSCN between

PII: S0014-5793(98)00918-1

12.5 and 800 mM. The product (4-nitroaniline) was measured spectrophotometrically at 405 nm.

Protein concentrations were determined according to Bradford [11], using the Bio-Rad protein assay reagent and bovine serum albumin as standard.

2.4. SDS-PAGE, Western blot, and isoelectric focusing

Aliquots of purified protein (2–4 μg) were subjected to SDS-PAGE (stacking gel 5%; separating gel 10%) on a Mini-Protean II (Bio-Rad) apparatus [12] or on a PhastSystem (Pharmacia) using PhastGels, gradient 10–15%. The gels were stained with Coomassie brilliant blue or $AgNO_3$. Western blot analysis was carried out with an affinity-purified polyclonal antiserum against human LTA_4 hydrolase, as described [9]. For isoelectric focusing, PhastGels IEF 3-9 were used and stained with Coomassie brilliant blue.

2.5. Amino acid sequence analysis

LTA_4 hydrolase (40 μg; 570 pmol) in 10 mM Tris-HCl, pH 8, was digested with Lys-C protease (4 μg, at 37°C, 18 h). Peptides were separated by HPLC on a Vydac C_8 column (2.1×150 mm), eluted with a gradient of acetonitrile (5–80%) in 0.1% trifluoroacetic acid. Peptides were sequenced on an Applied Biosystems instrument model 477A or 494.

2.6. Metal analyses

Zinc was measured by graphite furnace atomic absorption spectrophotometry using a Perkin-Elmer 5000 Zeeman instrument with an electrothermal atomization unit (HGA-500). Zinc was analyzed at 213.9 nm using an EDL lamp. Mixed zinc standards (British Drug House, UK) were prepared in 0.03 M HNO_3 (10–200 ng/ml) and diluted 1:1 in the sample cups with deionized water (Elgastat Spectrum R.O.1, ELGA, UK) prior to analysis. Samples of LTA_4 hydrolase were mixed with an equal volume of 0.03 or 0.1 M HNO_3. Standards and unknowns were analyzed in duplicate.

3. Results

3.1. Purification and physicochemical properties of Xenopus laevis LTA_4 hydrolase

LTA_4 hydrolase was isolated from *Xenopus laevis* oocytes. Typically, the overall purification was about 8000-fold with a recovery of 30% (Table 1), resulting in an apparently homogeneous preparation (SDS-PAGE with silver staining). From SDS-PAGE, the M_r of the *Xenopus laevis* LTA_4 hydrolase was estimated to 69 000 and the isoelectric point was found to be 6.1, as judged by isoelectric focusing (PhastGel) and chromatofocusing (MonoP column). Atomic absorption spectrometry revealed the presence of 0.8 mol zinc per mol enzyme. The amphibian enzyme was not recognized by an antiserum against human LTA_4 hydrolase in Western blot analysis and attempts to determine the N-terminal amino acid sequence did not yield any result, indicating the presence of a blocked N-terminus. To make possible an evaluation of the structure, the *Xenopus laevis* enzyme was therefore digested with Lys-C protease and seven internal peptides, varying in length between 5 and 15 amino acid residues, were sequenced. Altogether, these peptides contained 62 residues and were 60–100% (mean value 74%) identical to human LTA_4 hydrolase and 17–50% (mean value 32%) identical to rat aminopeptidase B. The longest 15 residue peptide (ELLEPSVYEFAETEK) exhibited 60% identity to the corresponding segment of human LTA_4 hydrolase (residues 225–239) but only 27% identity to rat aminopeptidase B (residues 252–267).

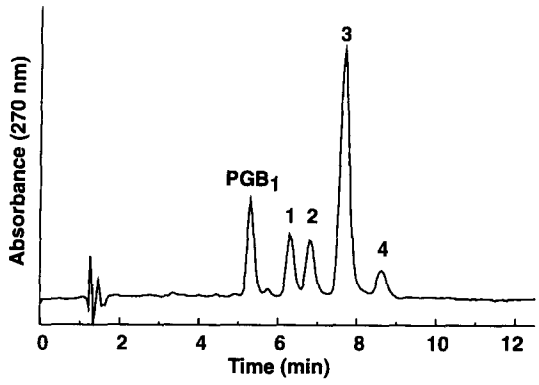

Fig. 1. Reverse-phase HPLC profile of the products formed in incubations of purified LTA_4 hydrolase from *Xenopus laevis* oocytes with LTA_4. The reverse-phase HPLC analysis was performed as described in Section 2. PGB_1 denotes the internal standard prostaglandin B_1. Peaks 1 and 2 elute with two non-enzymatic hydrolysis products of LTA_4. Peaks 3 and 4 have the same retention times as LTB_4 and Δ^6-*trans*-Δ^8-*cis*-LTB_4, respectively.

3.2. Catalytic properties

The purified *Xenopus laevis* LTA_4 hydrolase (0.2 μg) efficiently converted LTA_4 into LTB_4 at a pH optimum of 8.5 (Tris-HCl or phosphate buffer). The specific epoxide hydrolase activity was 6.7 ± 1.6 μmol/mg/min (mean ± S.D., $n = 8$) as compared to only 0.95 ± 0.3 μmol/mg/min (mean ± S.D., $n = 4$) for the human LTA_4 hydrolase (1 μg). Multiple exposures (4 times) to LTA_4 (25 μM, 30 min) led to a partial inactivation of the *Xenopus laevis* enzyme, suggesting that it was susceptible to suicide inactivation.

The *Xenopus laevis* enzyme also produced a second enzymatic metabolite of LTA_4. Thus, reverse-phase HPLC analysis of enzyme samples incubated with LTA_4 revealed four peaks (Fig. 1). Peaks 1 and 2 cochromatographed with the two non-enzymatic hydrolysis products of LTA_4. The third peak (3) eluted with synthetic LTB_4 whereas the fourth peak (4) had the same HPLC retention time and UV spectrum (in MeOH) as a recently identified enzymatic product of LTA_4, viz. 5*S*,12*R*-dihydroxy-8,14-*cis*-6,10-*trans* eicosatetraenoic acid, also termed Δ^6-*trans*-Δ^8-*cis*-LTB_4 or 5*S*-12*R*-di-HETE [9]. The formation of Δ^6-*trans*-Δ^8-*cis*-LTB_4 appeared at all stages of purification and always in the ratio 1:10,

Table 1
Purification of LTA_4 hydrolase from *Xenopus laevis* oocytes

Step	Total protein (mg)	Total activity (nmol/mg)	Specific activity (nmol/mg/min)	Yield (%)	Purification (-fold)
Dialysis	1650	2018	1.2	100	1
Q Sepharose	9.5	1900	202	95	168
Phenyl Sepharose	1.5	1627	1085	80	904
Hydroxyapatite	0.3	882	3095	44	2580
MonoP	0.07	486	6480	24	5400
MonoQ	0.071	704	9850	30	8200

relative to LTB_4. Incubations of the human leukocyte LTA_4 hydrolase with LTA_4 generated no other product than LTB_4, as judged by reverse-phase HPLC.

When the *Xenopus laevis* LTA_4 hydrolase (0.2 μg) was incubated in 50 mM Tris-HCl (pH 7.5), with 100 mM KCl, hydrolysis of the synthetic peptidase substrate alanine-4-nitroanilide (1 mM) into alanine and 4-nitroaniline was observed. The pH optimum for this peptidase activity (Tris-HCl or phosphate buffer) was determined to 7.5. The specific activity was calculated to 581 ± 213 nmol/mg/min (mean ± S.D., $n = 5$). Under the same conditions, the human leukocyte enzyme (0.2 μg) exhibited a specific activity of 209 ± 99 nmol/mg/min (mean ± S.D., $n = 4$).

The peptidase activity of the *Xenopus laevis* LTA_4 hydrolase towards alanine-4-nitroanilide was dose dependently stimulated by a number of monovalent anions added as potassium and/or sodium salts. The choice of cation did not influence the effect of the anion. Among the anions tested, thiocyanate was found to be the most effective activator followed by bromide and chloride ($SCN^- \gg Br^- > Cl^- \approx I^- \approx NO_3^- \gg F^-$). For chloride ions, the pattern of stimulation seemed to obey saturation kinetics and from Eadie-Hofstee plots of the kinetic data, an apparent affinity constant (K_A) for chloride was calculated to 80 mM. Bromide or thiocyanate caused a similar activation, but concentrations exceeding 200 mM suppressed the enzyme activity. A value of K_A for thiocyanate (0–100 mM) was estimated to 30 mM. Chloride (150–600 mM KCl) did not stimulate the epoxide hydrolase activity.

3.3. Apparent kinetic constants

Apparent kinetic constants for the two activities were determined for both the *Xenopus laevis* and the human LTA_4 hydrolase. Values of K_m and V_{max} were calculated from Eadie-Hofstee plots of the kinetic data.

For the epoxide hydrolase activity, 0.3 μg of the amphibian enzyme or 1 μg of the human enzyme were incubated with varying amounts of LTA_4 (5–90 μM). In a typical experiment with the *Xenopus laevis* enzyme, the K_m and V_{max} for LTA_4 were calculated to 20 μM and 12.0 μmol/mg/min, respectively (mean of duplicates). Corresponding values for the human enzyme were 2.7 μM and 1.3 μmol/mg/min (mean of duplicates), respectively (Table 2).

For the peptidase activity, 0.25 μg each of *Xenopus laevis* and human LTA_4 hydrolase were incubated with varying amounts of alanine-4-nitroanilide (0.125–16 mM). The values of K_m and V_{max} were determined to 2.4 mM and 1800 nmol/mg/min (mean of triplicates), respectively, for the *Xenopus laevis* enzyme. For the human enzyme the values were 0.7 mM and 470 nmol/mg/min (mean of triplicates), respectively (Table 2).

4. Discussion

4.1. General properties of Xenopus laevis LTA_4 hydrolase

In the present study we purified LTA_4 hydrolase to apparent homogeneity from a non-mammalian species, i.e. the African claw toad *Xenopus laevis* (Table 1), making possible metal analysis and assay for a peptidase activity. The amphibian enzyme contained one zinc atom per enzyme molecule and displayed a peptide cleaving activity against the synthetic substrate alanine-4-nitroanilide. Hence, *Xenopus laevis* LTA_4 hydrolase is a bifunctional zinc metalloenzyme, key properties of the mammalian counterparts [4,5].

The peptidase activity of the *Xenopus laevis* LTA_4 hydrolase was greatly stimulated by monovalent anions. For chloride, the physiologically most relevant anion, an apparent affinity constant (K_A) was calculated to be 80 mM, in good agreement with the value of 100 mM previously obtained with the human enzyme [6]. The profound and selective effects of chloride suggests that the peptidase activity may be regulated by the steady-state levels or fluxes of this electrolyte. Moreover, further studies are warranted to clarify the function of this peptide cleaving activity and explain the presence of the enzyme in the reproduction organs of *Xenopus laevis*.

In contrast to mammalian forms of LTA_4 hydrolase, the N-terminus of the *Xenopus laevis* enzyme appeared to be blocked. Furthermore, the amphibian protein was not detected in Western blots, using a probe for the human protein. To get an estimate of the structural conservation of the *Xenopus laevis* enzyme, several internal peptides were isolated and sequenced. Thus, seven peptides, encompassing 62 amino acid residues, were ~70% identical to the corresponding segments of human LTA_4 hydrolase and their sequences clearly distinguished the toad enzyme from aminopeptidase B (~30% identity), an enzyme which has also been shown to possess LTA_4 hydrolase activity [8].

4.2. The Xenopus laevis LTA_4 hydrolase displays a high catalytic efficiency

The apparent kinetic constants for the epoxide hydrolase and peptidase activities differed considerably between the *Xenopus laevis* and human LTA_4 hydrolase (Table 2). Thus, the value of V_{max} for the epoxide hydrolase activity of the *Xenopus laevis* enzyme was approximately 9-fold higher as compared to the human enzyme. On the other hand, it displayed a 7-fold higher K_m for LTA_4 and therefore, the specificity constants (k_{cat}/K_m) for LTA_4 were almost the same for the two species, suggesting that the two active sites are equally well adapted to process the substrate LTA_4. Of note, due to the instability of LTA_4 we consider the differences in substrate turnover (k_{cat}) more significant than those of the Michaelis

Table 2
Apparent kinetic constants for the epoxide hydrolase and peptidase activity of LTA_4 hydrolase from *Xenopus laevis* oocytes and human leukocytes

	Epoxide hydrolase activity		Peptidase activity	
	Xenopus laevis	Human	*Xenopus laevis*	Human
K_m (μM)	20	2.7	2400	700
V_{max} (μmol/mg/min)	12	1.3	1.8	0.5
k_{cat} (s^{-1})	14	1.5	2.1	0.5
k_{cat}/K_m (s^{-1} M^{-1})	6.9×10^5	5.5×10^5	860	770

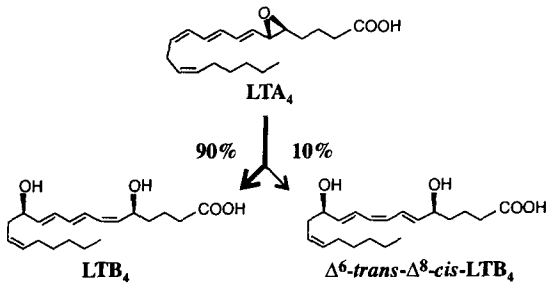

Fig. 2. Structure and formation of the enzymatic metabolites of LTA_4 generated by *Xenopus laevis* LTA_4 hydrolase.

constant. When comparing the peptidase activities, the values of both V_{max} and K_m were again higher (approx. 3.5-fold for both parameters) for the *Xenopus laevis* enzyme. As for the epoxide hydrolase activity, the specificity constants (k_{cat}/K_m) were almost the same for the two species.

4.3. *The Xenopus laevis LTA_4 hydrolase can form a second enzymatic metabolite of LTA_4*

In a recent study, we found that organs of *Xenopus laevis* contained an enzyme activity which converted LTA_4 into a novel, biologically active, product identified as Δ^6-*trans*-Δ^8-*cis*-LTB_4 [9]. The relative formation of Δ^6-*trans*-Δ^8-*cis*-LTB_4 versus LTB_4 was always about 1:10. The *Xenopus laevis* LTA_4 hydrolase that was purified in the present study has the ability to convert LTA_4 into both Δ^6-*trans*-Δ^8-*cis*-LTB_4 and LTB_4 in a ratio of about 1:10 (Fig. 1) and can account for both of the enzyme activities observed in the organ extracts (Fig. 2).

Typically, LTA_4 hydrolase becomes suicide inactivated and covalently modified by its lipid substrate LTA_4 [13]. In this process, Tyr-378 is a primary site for covalent binding of LTA_4 since exchange of this residue for a phenylalanine resulted in a recombinant enzyme that was protected from suicide inactivation [14]. Interestingly, this mutant, [Y378F]LTA_4 hydrolase, exhibited an increased turnover of LTA_4 together with an increased K_m and converted LTA_4 into both LTB_4 and Δ^6-*trans*-Δ^8-*cis*-LTB_4 and thus shares several catalytic properties with the *Xenopus laevis* enzyme. However, the latter enzyme is catalytically even more efficient and, in contrast to [Y378F]LTA_4 hydrolase, appears to be suicide inactivated by the substrate LTA_4. Nevertheless, it is tempting to speculate that Tyr-378 is located in a segment of LTA_4 hydrolase which may determine some of the catalytic differences between the human and of the *Xenopus laevis* enzyme.

Acknowledgements: The authors are grateful to Anders Wetterholm for discussions and advice. We also thank Birger Lind, the Institute of Environmental Medicine, Karolinska Institutet, for help with the metal analyses. This work was financially supported by The Swedish Medical Research Council (O3X-10350), The European Union (BMH4-CT960229), and Konung Gustav V:s 80-årsfond.

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