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# LEUKOTRIENE $A_4$ HYDROLASE: AN EPOXIDE HYDROLASE WITH PEPTIDASE ACTIVITY

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Purified leukotriene $A_4$ hydrolase from human leukocytes is shown to exhibit peptidase activity towards the synthetic substrates alanine-4-nitroanilide and leucine-4-nitroanilide. The enzymatic activity is abolished after heat treatment (70 °C, 30 min). At 37 °C these substrates are hydrolyzed at a rate of 380 and 130 nmol/mg/min, respectively, and there is no enzyme inhibition during catalysis. Apo-leukotriene $A_4$ hydrolase, obtained by removal of the intrinsic zinc atom, exhibits only a low peptidase activity which can be restored by the addition of stoichiometric amounts of zinc. Reconstitution of the apoenzyme with cobalt results in a peptidase activity which exceeds that of enzyme reactivated with zinc. Preincubation of the native enzyme with leukotriene $A_4$ reduces the peptidase activity. Semipurified preparations of bovine intestinal aminopeptidase and porcine kidney aminopeptidase do not hydrolyze leukotriene $A_4$ into leukotriene $B_4$. 

Leukotriene (LT)$A_4$ hydrolase (EC 3.3.2.6) is a key enzyme in the transformation of arachidonic acid into the biologically active leukotrienes. It catalyzes the hydrolysis of the unstable epoxide intermediate $LTA_4$ into the dihydroxy fatty acid $LTB_4$ (1). Due to its powerful biological activities, $LTB_4$ is regarded as an important proinflammatory mediator (2). $LTA_4$ hydrolase is ubiquitous in mammalian tissues and the enzyme has without exception been purified as a soluble, monomeric protein with a $M_r$ of about 70 000 (for reviews see refs. 3 and 4). No cofactor requirement has been reported for the catalytic activity, but the substrate $LTA_4$ inactivates the enzyme and binds covalently to the protein (5,6). $LTA_4$, and to a lesser extent its isomers $LTA_5$ and $LTA_3$, are the only known substrates accepted by $LTA_4$ hydrolase (6-8).

Recently we identified leukotriene $A_4$ hydrolase as a zinc metalloenzyme (9) since the protein contains one zinc atom per enzyme molecule as determined by atomic absorption spectrometry. Furthermore, the metal atom was shown to have a catalytic function, since the apoenzyme of $LTA_4$ hydrolase was virtually inactive but could be reactivated by stoichiometric amounts of zinc (9). The predicted zinc binding site of $LTA_4$ hydrolase has a striking similarity to the corresponding primary structures of certain aminopeptidases and neutral proteases, typified by thermolysin (10,11). This structural feature of $LTA_4$ hydrolase prompted us to investigate its possible peptidase activity.

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## MATERIALS AND METHODS

Alanine-4-nitroanilide, leucine-4-nitroanilide and 1,10-phenanthroline were from Sigma and 4-nitroaniline was from Kebo, Stockholm. $LTA_4$ methyl ester (Upjohn) was saponified in tetrahydrofuran with 7% 1 M LiOH (v/v) at 4 °C. Metal free buffer was prepared from reagent grade Milli-Q water (Waters-Millipore). $ZnSO_4$ and $CoCl_2$ (Johnson-Matthey specpure) were dissolved in Milli-Q water.

**Enzyme preparations**

$LTA_4$ hydrolase was purified to apparent homogeneity from human leukocytes according to a procedure which will be presented elsewhere (Fig. 1). Purified enzyme was lyophilized and stored at -20 °C. The apoenzyme was obtained by treatment of purified $LTA_4$ hydrolase (700 μg; 1 mg/ml) with 3-10 mM 1,10-phenanthroline in 25 mM HEPES, pH 8, for 24 hrs at 4 °C. The chelator was removed by dialysis or ultrafiltration, as described (12). Reconstitution of the holoenzyme was performed as described (9). Bovine intestinal aminopeptidase and porcine kidney aminopeptidase (type IV-S) were purchased from Sigma and dissolved in 50 mM Tris-Cl, pH 8, prior to use. Protein concentrations were determined by the Bradford method (13).

**Enzyme assays**

Aminopeptidase activity was assayed spectrophotometrically, essentially as described (14). The substrates were dissolved in 50 mM Tris-Cl, pH 8, to a concentration of 1 mM, and incubated with various amounts of the respective enzymes. The product, 4-nitroaniline, was measured as an increase of absorbance at 410 nm, assuming an extinction coefficient of 8 850 $M^{-1}$ x $cm^{-1}$. In certain experiments, enzyme and substrate were incubated in the wells of a microtiterplate and the absorbance was measured with a Multiskan MCC/340 (Labsystems) at 405 nm. Amounts of product were calculated from a standard curve generated from measurements of known amounts of 4-nitroaniline in 50 mM Tris-Cl, pH 8. Spontaneous hydrolysis of the substrates was corrected for by subtraction of the background absorbance in samples without enzyme. $LTA_4$ hydrolase activity was measured by incubation with $LTA_4$ (25-90 μM) dissolved in tetrahydrofuran. Reactions were quenched with 2 volumes of methanol and a known amount of internal standard, prostaglandin (PG)$B_1$ (Upjohn) was added to the mixture. After acidification to pH ≈ 3, the samples were directly subjected to RP-HPLC analysis.

**Instrumental analysis**

For HPLC, an Ultrosphere ODS column (250 x 4.6 mm) was eluted with a mixture of acetonitrile/methanol/water/acetic acid (30:35:35:0.01, v/v) at a flow rate of 1 ml/min. Eluate absorbance was monitored at 270 nm. Quantitations of $LTB_4$ were performed by measurements of peak height ratios between $LTB_4$ and the internal standard $PGB_1$, as previously described (15). Absorbance measurements were carried out with a Hewlett-Packard 850A UV/VIS spectrophotometer.

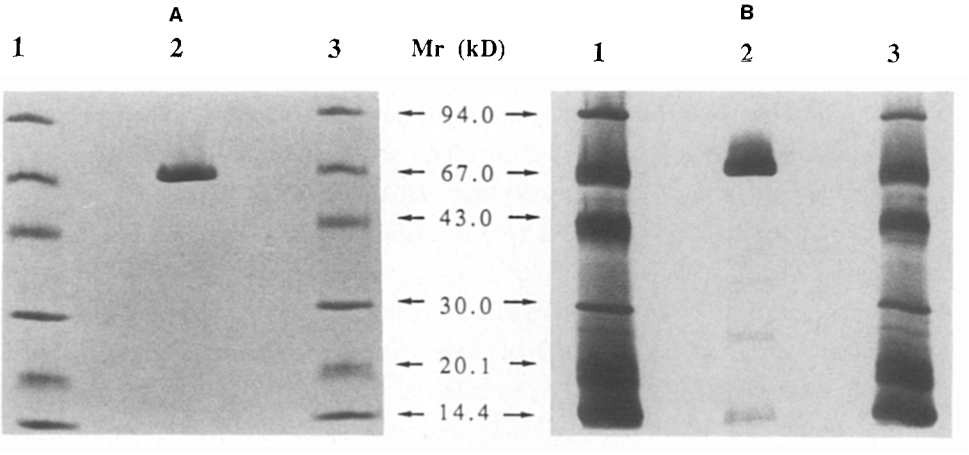



Fig.1. **SDS-PAGE of purified $LTA_4$ hydrolase.** Electrophoresis of purified enzyme (0.7 μg) was carried out on a Phast Gel (gradient 10-15) using a Pharmacia Phast System. The gel was stained with Coomassie brilliant blue (Panel A) or silver nitrate (panel B). Lanes 1 and 3 depict the molecular weight standards and lane 2 the enzyme sample.

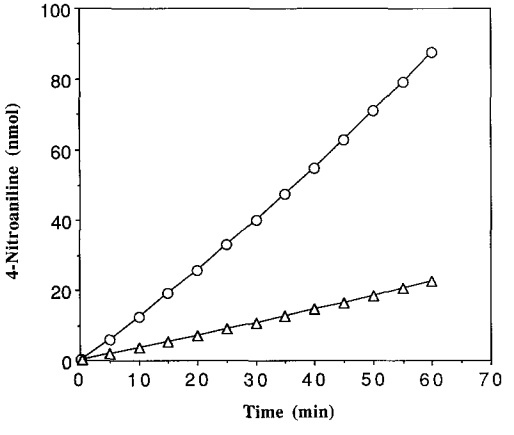
## RESULTS

### Peptidase activity of $LTA_4$ hydrolase

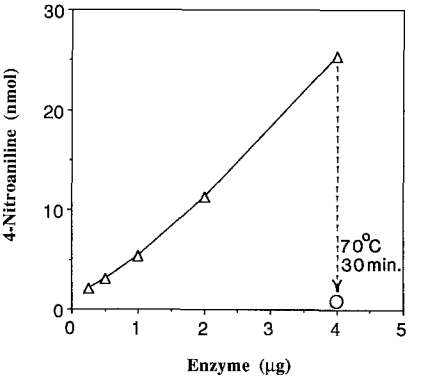
When $LTA_4$ hydrolase (0.14 μM) was incubated with alanine-4-nitroanilide (1 mM) a time-dependent increase in absorbance at 410 nm was observed, reflecting the formation of 4-nitroaniline and alanine (Fig.2). The reaction was dependent on the amounts of enzyme added to the incubate and was completely abolished after heat treatment at 70 °C for 30 min (Fig.3). The formation of product was nearly linear with time for more than an hour and thus there was no enzyme inhibition during catalysis (Fig.2). At a substrate concentration of 1 mM, the reaction rates at 25 and 37 °C, were calculated as approx. 240 and 380 nmol/mg/min, respectively (Table I). An additional substrate, viz. leucine-4-nitroanilide, was also tested and was found to be hydrolyzed less efficiently by $LTA_4$ hydrolase, corresponding to a rate approx. 25-35% of that obtained with the alanine conjugate (Fig.2; Table 1).

### Activity of apo-$LTA_4$ hydrolase

The apoenzyme of $LTA_4$ hydrolase was virtually inactive towards alanine-4-nitroanilide. However, addition of stoichiometric amounts of zinc resulted in a gradual increase in peptidase activity that became maximal at a value corresponding to a ratio, metal versus enzyme, of about 1:1, and then decreased upon further addition of zinc (Fig.4). Reconstitution of the apoenzyme with cobalt resulted in a similar pattern of reactivation, but the activity was not reduced at levels of metal exceeding a 1:1 molar ratio. In a separate experiment, the apoenzyme was reconstituted with 2 equivalents of cobalt or zinc, which resulted in enzymatic activities of about 300 and 160 nmol/mg/min (24 °C), respectively.




(2)




(3)

Fig.2. **Time course for the hydrolysis of synthetic amides by $LTA_4$ hydrolase.** The enzyme (0.14 μM) was incubated at 25 °C with 1 mM alanine- (o) or leucine-4-nitroanilide (Δ). The reactions were followed by measurements of the absorbance at 410 nm in the spectrophotometer cuvette.

Fig. 3. **Dose-response for the hydrolysis of alanine-4-nitroanilide by $LTA_4$ hydrolase.** Various amounts of $LTA_4$ hydrolase (0.5-4 μg in 250 μl) were incubated with 1 mM alanine-4-nitroanilide at 25 °C for 30 min. The product was measured spectrophotometrically at 405 nm as described in the methods section. The effect of heat treatment at 70 °C for 30 min on the enzyme activity is also shown (o).

Table I

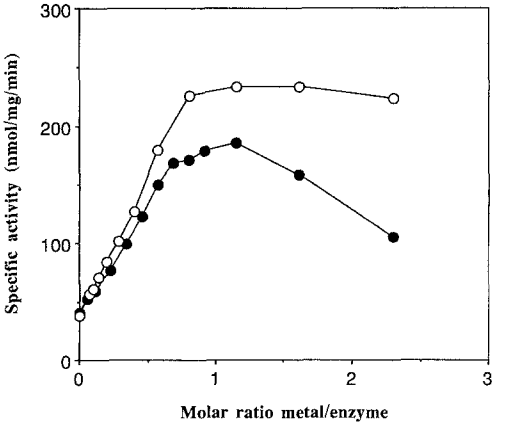
SPECIFIC ACTIVITY OF $LTA_4$ HYDROLASE TOWARDS SYNTHETIC AMIDES

| | Specific activity (nmol/mg/min) | |
|---|---|---|
| | alanine-4-nitroanilide | leucine-4-nitroanilide |
| 25 °C | 240 | 65 |
| 37 °C | 380 | 130 |

$LTA_4$ hydrolase was incubated with 1 mM alanine- or leucine-4-nitroanilide at 25 or 37 °C for 30 - 60 min. The specific activities were determined as described in the methods section and are expressed as nmol 4-nitroaniline or alanine/leucine formed per mg and min.

## Effects of $LTA_4$ on the peptidase activity of $LTA_4$ hydrolase

$LTA_4$ hydrolase (3 µg in 100 µl) was preincubated with either $LTA_4$ (90 µM) dissolved in tetrahydrofuran (1µl), or with tetrahydrofuran alone, and subsequently assayed for peptidase activity with alanine-4-nitroanilide. Exposure of enzyme to $LTA_4$ decreased peptidase activity, to a level corresponding to approx. 50% of that exhibited by untreated enzyme (Fig.5). When semipurified preparations of porcine kidney aminopeptidase (20 µg) and bovine intestinal aminopeptidase (25 µg) were incubated with $LTA_4$ (25 µM) for 5 min at room temperature, no formation of $LTB_4$ could be detected (data not shown).



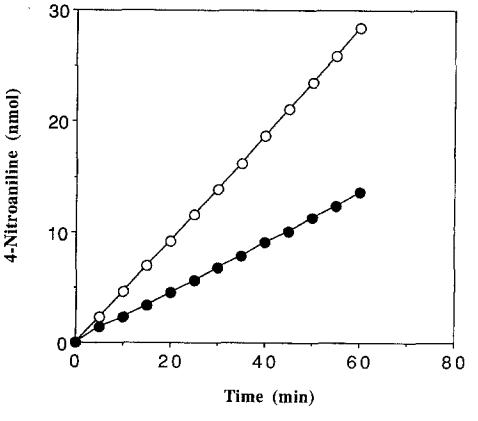




Fig.4. **Effects of zinc and cobalt on the peptidase activity of apo-$LTA_4$ hydrolase.** The apoenzyme (3 µg in 250 µl) was incubated with increasing amounts of $ZnSO_4$ (•) or $CoCl_2$ (o) and assayed for peptidase activity as described in the methods section. Enzymatic activity was determined from a 20 min incubation at 24 °C.

Fig. 5. **Effect of $LTA_4$ on the peptidase activity of $LTA_4$ hydrolase.** The enzyme (0.4 µM) was incubated with $LTA_4$ (90 µM) in tetrahydrofuran (•) or with solvent alone (o) for 30 min at 25 °C and subsequently assayed for peptidase activity as described in the methods section.

## DISCUSSION

In this paper we report that $LTA_4$ hydrolase, an epoxide hydrolase, the substrate of which is a fatty acid, also possesses a significant peptidase activity, as assessed by activity determinations towards two synthetic aromatic amides. The enzymatic nature of the reaction was evident from the dose dependence and sensitivity to heat treatment. The conclusion that the peptidase activity is attributable to the presence of $LTA_4$ hydrolase, and does not originate from some contaminating activity in the purified enzyme preparation (cf. Fig.1, panel B), is supported by experiments with apo-$LTA_4$ hydrolase. The apoenzyme is virtually inactive and is reactivated by the addition of zinc at a molar ratio of about 1:1 metal/$LTA_4$ hydrolase, a stoichiometry which would hardly be obtained with a contaminant. This pattern of reactivation of the apoenzyme upon addition of zinc is also in agreement with that which we have reported earlier for the hydrolysis of $LTA_4$ into $LTB_4$ (9). However, the two enzymatic activities exhibit some dissimilarities with respect to metal substitution of the apoenzyme. The peptidase activity, unlike the $LTA_4$ hydrolase activity (9), was reduced already when zinc was added at a molar ratio > 1 (Fig.4). On the other hand, neither of the two enzyme activities were decreased at similar levels of cobalt. Also, cobalt seemed more effective than zinc in restoring the peptidase activity, whereas the $LTA_4$ hydrolase activity was restored by both metals with similar efficacy (9).

At 37 °C, pH 8, the peptidase activity of $LTA_4$ hydrolase versus alanine-4-nitroanilide was calculated to 380 nmol/mg/min, which corresponds to about 2% of the activity reported for microsomal aminopeptidase purified from porcine intestine, at pH 7.3 (14). At the same temperature, the maximal initial reaction velocity ($V_{max}$) of the epoxide hydrolase activity, i.e. the conversion of $LTA_4$ into $LTB_4$, has been calculated to approx. 1700 nmol/mg/min (15). Without any attempts to optimize the reaction conditions with respect to e.g. substrate, pH, or buffer composition, the peptidase activity of $LTA_4$ hydrolase thus appears significant.

The predicted zinc binding sequence of $LTA_4$ hydrolase has much in common with the primary structures of a number of other known zinc containing hydrolases, all of which cleave peptide bonds (10,11,16-20). In conjunction with our previous data regarding the catalytic function of zinc in $LTA_4$ hydrolase (9), it seems reasonable to assume that the peptidase and epoxide hydrolase activities of $LTA_4$ hydrolase are exerted via the same active site, i.e. the catalytic zinc atom. This hypothesis is supported by the finding that apo-$LTA_4$ hydrolase was almost completely devoid of both peptidase and epoxide hydrolase activity which could be restored by zinc (9). Furthermore, it was recently demonstrated that the inactivation of $LTA_4$ hydrolase occuring when the enzyme is exposed to the epoxide $LTA_4$, is intimately related to enzymatic catalysis and thus presumably involves the active site (21). In contrast, the peptidase activity of $LTA_4$ hydrolase does not show any sign of substrate mediated inhibition during catalysis. However, preexposure of the enzyme to $LTA_4$ reduces the peptidase activity, in line with the concept of two enzymatic activities, one of which inactivates the enzyme during catalysis, at a single active site. The presence of two enzyme activities in $LTA_4$ hydrolase will probably assist the definition of the active site and the elucidation of the catalytic mechanism, as well as for the understanding of the substrate mediated inactivation/regulation of this enzyme.

A variety of functions have been established or proposed for aminopeptidases in mammals, e.g. final degradation and absorption of proteins from the intestine (22), inactivation of certain opioid peptides (enkephalins; 23), cleavage of angiotensin II into angiotensin III (24), and a dipeptidase converts leukotriene $D_4$ into $E_4$ (25). Thus, the physiological role of this novel enzymatic activity of $LTA_4$ hydrolase is not evident. We have previously reported that $LTA_4$ hydrolase is sometimes degraded upon storage, giving rise to a fragment with a Mr $\approx$ 50 000 - 55 000 (26). In light of the results from the present study, this phenomenon could be interpreted as an autocleavage by an endopeptidase activity of $LTA_4$ hydrolase itself, representing an additional mode of regulation for this enzyme.

For many years $LTA_4$ hydrolase was believed to reside exclusively in white blood cells, especially granulocytes. When enzymatic activity was found in mammalian plasma (27), and subsequently in almost all tissues and cells examined, this notion was revised (28, 29, 26, 30). Even some cells which do not express 5-lipoxygenase activity and therefore are unable to synthesize the substrate $LTA_4$, harbour $LTA_4$ hydrolase activity (31-33). Several studies *in vitro* have shown that $LTA_4$ can be formed in a donor cell and then transferred to a recipient cell for further conversions (34, 35). This process is referred to as transcellular metabolism and may be of importance under certain conditions. However, an additional enzymatic activity, and thus an additional biochemical function of the enzyme, offers the potential for a completely new interpretation. Consequently, it seems possible that $LTA_4$ hydrolase can exert different functions in different cells and tissues, and that the actual activity exhibited in a given system, is governed by the availability of substrate. However, until an endogenous substrate for the peptidase activity of $LTA_4$ hydrolase has been demonstrated, this discussion remains speculative.

## ACKNOWLEDGMENTS

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