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# Leukotriene $A_4$ hydrolase, a bifunctional enzyme

## Distinction of leukotriene $A_4$ hydrolase and aminopeptidase activities by site-directed mutagenesis at Glu-297

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We previously obtained evidence for intrinsic aminopeptidase activity for leukotriene (LT)$A_4$ hydrolase, an enzyme characterized to specifically catalyse the hydrolysis of $LTA_4$ to $LTB_4$, a chemotactic compound. From a sequence homology search between $LTA_4$ hydrolase and several aminopeptidases, it became clear that they share a putative active site for known aminopeptidases and a zinc binding domain. Thus, Glu-297 of $LTA_4$ hydrolase is a candidate for the active site of its aminopeptidase activity, while His-296, His-300 and Glu-319 appear to constitute a zinc binding site. To determine whether or not this putative active site is also essential to $LTA_4$ hydrolase activity, site-directed mutagenesis experiments were carried out. Glu-297 was mutated into 4 different amino acids. The mutant E297Q (Glu changed to Gln) conserved $LTA_4$ hydrolase activity but showed little aminopeptidase activity. Other mutants at Glu-297 (E297A, E297D and E297K) showed markedly reduced amounts of both activities. It is thus proposed that either a glutamic or glutamine moiety at 297 is required for full $LTA_4$ hydrolase activity, while the free carboxylic acid of glutamic acid is essential for aminopeptidase.

Leukotriene; Leukotriene $A_4$ hydrolase; Aminopeptidase; Zinc-metalloprotease; Site-directed mutagenesis

## 1. INTRODUCTION

Leukotrienes (LTs) constitute a class of lipid mediators which are involved in inflammatory/allergic processes, and have various physiological and pathological functions [1–5]. Arachidonic acid is converted to $LTA_4$ by 5-lipoxygenase [6–10], and is subsequently hydrolysed to $LTB_4$ by cytosolic $LTA_4$ hydrolase (EC 3.3.2.6) [11–14]. Biochemical and immunohistochemical studies of $LTA_4$ hydrolase have shown that this enzyme is ubiquitously distributed in various cells and tissues of the guinea pig [15,16]. The most dense immunohistochemical staining was observed in epithelial cells of the guinea pig small intestine [16] which is deficient in 5-lipoxygenase [17]. The different distribution of the two enzyme activities was also shown in human erythrocytes, vascular endothelial cells and human B-lymphocytes [18–22] and had been explained by an intercellular transfer mechanism of $LTA_4$; i.e. from neutrophils to erythrocytes or from neutrophils to endothelial cells. Another possible explanation is that this enzyme might possess an alternative enzyme activity towards different substrates. Several groups of investigators including ours, have recently found a sequence domain of $LTA_4$ hydrolase which is homologous to the active site and zinc-binding motif of several aminopeptidases (Fig. 1a) [23–26]. In the aminopeptidase N, a highly conserved region, VXXHEXXH, is considered to constitute a functional protease domain [27], which is also conserved in other zinc-metalloproteases such as thermolysin, endopeptidase, and collagenase. Two conserved His residues in the motif and another Glu putatively act as ligands for zinc ion binding, and an invariant Glu residue adjacent to the first. His is one of the active sites for hydrolysis of peptides [28]. These observations led us to study the zinc content and putative peptidase activity of $LTA_4$ hydrolase. We found that both native and recombinant $LTA_4$ hydrolases intrinsically contain an equimolar zinc ion and have the aminopeptidase activity [29]. Other workers also reported similar observations [30–33], thus, $LTA_4$ hydrolase was shown to be a bifunctional enzyme, possibly acting towards $LTA_4$ and various peptides, two physicochemically different components in the cell.

To clarify whether or not the putative active site (Glu-297) is also essential for $LTA_4$ hydrolase activity, we carried out site-directed mutagenesis experiments. $LTA_4$ hydrolase and aminopeptidase activities were

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thus separated: a mutant E297Q possesed $LTA_4$ hydrolase and reduced aminopeptidase activity, while in other mutants (E297A, E297K), both activities were substantially diminished. Possible mechanisms of the enzyme reaction are briefly discussed.

## 2. MATERIALS AND METHODS

### 2.1. *Mutagenesis of human $LTA_4$ hydrolase cDNA*

We carried out a site-directed mutagenesis by the polymerase chain reaction method [34] using partial subclones of the EX85 expression vector constructed for the expression of a full-length human $LTA_4$ hydrolase cDNA [35,36] in *E. coli*. The recombinant plasmid LTA858 (used for Y384F) and LTA851 (used for other mutants), contained C-terminal and N-terminal halves of EX85, respectively. The regions contiguous to desired mutation sites were amplified in two separate PCR reactions using four different primers. Briefly, a common set of primer 1 and 4 including a *Bgl*II and *Eco*RI site, respectively, and specifically designed primers 2 and 3 containing the mutated sites were synthesized using an ABI 381B DNA synthesizer. Primers 1 + 3 and 2 + 4 were used as separate pairs to obtain the 'upstream' and 'downstream' PCR fragment, respectively. Both fragments were mixed and amplified using primers 1 + 4 to obtain a PCR fragment of 324 bp containing the mutated site. This fragment was digested with *Eco*RI abd *Bgl*II and inserted into an *Eco*RI-*Bgl*II fragment of EX851 (E297A, E297D, E297Q, E297K, and E319K) or pUC 18 (Y384F). Mutated EX851 plasmids were digested with *Eco*RI and ligated to the remaining half of $LTA_4$ hydrolase cDNA (LTA858). For Y384F, the pUC18 insert was excised with *Eco*RI and inserted into the *Eco*RI site of EX851. Each mutated site was confirmed by double-stranded DNA sequencing with Sequenase Version 2.0 (USB). The sequences of the 4 primers used are as follows:
Primer 1: 5'-GCAGAAGATCTGGGAGGACC-3';
Primer 2: 5'-ATTGCACATXXXATATCTCA-3' in which XXX represents: GCA for E297A, AAA for E297K, CAA for E297Q, GAC for E297D; or 5'-GGTTAAATAAGGGACATACT-3' for E319K;
Primer 3: antistrand sequence to each primer 2.
Primer 4: 5'-GGCCAGTGAATTCTGTAGTT-3'
In Y384F, primer 1 as above was combined with primer 2 (5'-CAGTTCCCTTTGAGAAGGG-3'), primer 3 (antistrand to primer 2), primer 4 (5'-GGCCAGTGAATTCCATTTAAAAA-3') for PCR.

### 2.2. *Overproduction and purification of wild-type and mutant $LTA_4$ hydrolase proteins*

Human recombinant wild-type and mutant $LTA_4$ hydrolases were overexpressed in *E. coli* YA21 according to Minami et al. [37]. The wild-type and two mutants (E297Q, E319K) were purified as described [37].

### 2.3. *Assay of enzyme activities and protein concentration*

The $LTA_4$ hydrolase activity was determined as described [13]. The peptidase activity was measured as described [29]. Protein concentrations were determined according to Bradford [38], using bovine serum albumin as a standard.

### 2.4. *Immunoblot analysis*

The wild-type and mutant proteins were electrophoresed on a 10% polyacrylamide gel/0.1% SDS, and electrotransferred to a nitrocellulose membrane. These proteins were immunostained using an affinity-purified anti-$LTA_4$ hydrolase antibody [16].

### 2.5. *Quantitation of $LTA_4$ hydrolase proteins*

The quantitation of the expressed $LTA_4$ hydrolase proteins was carried out by enzyme-linked immunosorbent assay (ELISA). The microtiter plates were coated with diluted lysates containing either mutant or wild-type enzyme. Anti-$LTA_4$ hydrolase rabbit antiserum and peroxidase-labeled anti-mouse IgG antibody were used to generate a color reaction product of the substrate, o-phenylendiamine. The color reaction was evaluated from the absorbance at 492 nm. Background absorbance was measured using the lysate of *E. coli* transformed with pUC9 (vector) only.

### 2.6. *Atomic absorption spectrometry and circular dichroism*

The mutant E319K was purified as described [29]. The zinc content was measured using a Hitachi Atomic Absorption Spectrometer model Z-6100. Circular dichroism was analyzed with a JASCO CD spectrometer model J-720 using the enzyme solution (0.3 mg of enzyme/ml of 10 mM potassium phosphate buffer, pH 7.5).

## 3. RESULTS AND DISCUSSION

We obtained evidence that $LTA_4$ hydrolase possesses an equimolar zinc ion and has intrinsic aminopeptidase activity [29]. Thus, $LTA_4$ hydrolase is now considered to represent a unique bifunctional enzyme case in which the water molecule is incorporated into $LTA_4$ and peptides.

To examine the molecular mechanisms involved in the bifunctional activity of $LTA_4$ hydrolase, we carried out site-directed mutagenesis of $LTA_4$ hydrolase cDNA. Single amino acid mutations of Glu-297, the putative active site of aminopeptidase, to 4 different amino acids were carried out (E297A, E297K, E297D and E297Q in Fig. 1b). Crude extracts were used for assays of enzyme activities, because the degradation of some mutants proteins were constantly observed in spite of addition of a cocktail of protease inhibitors. The expressed enzyme protein amount of mutant and wild-type $LTA_4$ hydrolases was checked by ELISA and immunoblot analysis (Fig. 3). After substracting the basal activity of the vector control, the measured activity was divided by the estimated enzyme amount and described as the specific activity. The relative activity for $LTA_4$

(A)

| | | | | | | |
|---|---|---|---|---|---|---|
| human $LTA_4$ hydrolase | [29] | VIAHEIAHSW | (14) | LNEQH | (59) | SVPYER |
| human aminopeptidase N | | VIAHELAHQW | (14) | LNEQP | (60) | AISYER |
| rabbit aminopeptidase N | | VVAHELAHQW | (14) | LNEQP | (58) | SITYSR |
| rat aminopeptidase N | | VIAHELAHQW | (14) | LNEQP | (59) | SITYSR |
| *E.coli* aminopeptidase A | | VIGHEYFHNW | (14) | LKEGL | (55) | LTVYER |
| *B.subtilis* thermolysin | | VTAHEHTHGV | (15) | LNESP | (65) | GGVHIN |

(B)

| | | | | | | |
|---|---|---|---|---|---|---|
| wild type | [29] | VIAHEIAHSW | (14) | LNEQH | (59) | SVPYER |
| E297A | | ----A----- | | ----- | | ----- |
| E297D | | ----D----- | | ----- | | ----- |
| E297K | | ----K----- | | ----- | | ----- |
| E297Q | | ----Q----- | | ----- | | ----- |
| E319K | | ---------- | | --K-- | | ----- |
| Y384F | | ---------- | | ----- | | ---F-- |

Fig. 1. (A) The homologous region of $LTA_4$ hydrolase, aminopeptidases and thermolysin ([24,25]). ●, putative active site; △, putative zinc-binding site; □, proton donor residue. (B) Structure of the constructed mutants. The figures in parentheses denote the number of amino acid residues between each motif.

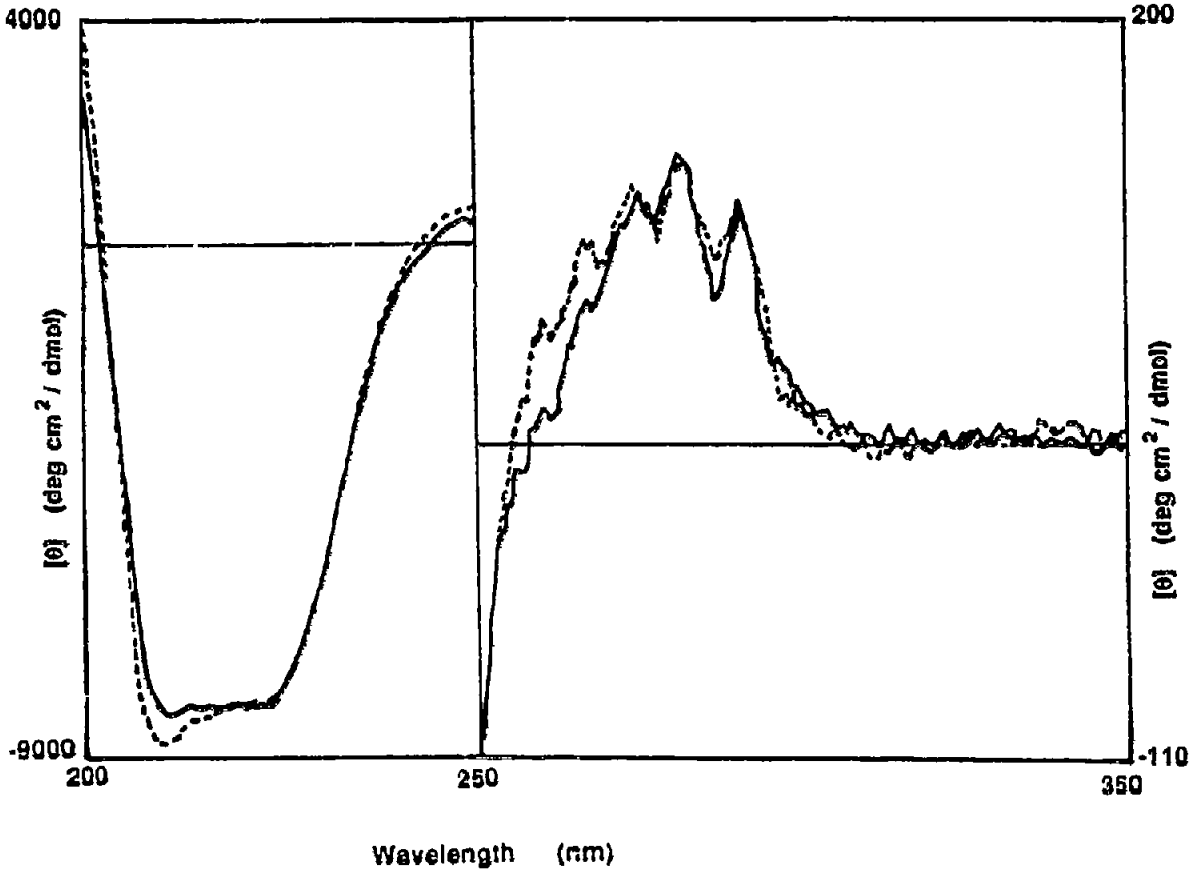


Fig. 2. Peptide region (200-250 nm) and aromatic region (250-350 nm) circular dichroism spectra of the wild-type and mutant E297Q $LTA_4$ hydrolase in 10 mM phosphate buffer (pH 7.5) at 20°C. (Solid line) E297Q; (dashed line) wild-type enzyme.

hydrolase and aminopeptidase of wild-type and mutant enzymes are shown in Table I. E297Q exhibited a full $LTA_4$ hydrolase activity but only reduced peptidase activity. In other mutants (E297D, E297A and E297K), both activities were markedly reduced. Although the aminopeptidase activity of wild-type $LTA_4$ hydrolase was inhibited by $LTA_4$ [29], aminopeptidase activities of the 6 mutants were apparently unaffected by addition of $LTA_4$. The result suggests that aminopeptidase activities observed in the mutant lysates may be mainly derived from increased intrinsic aminopeptidase activities of *E. coli* due to transformation. To support this view, the purified preparation of the wild-type enzyme and E297Q and E297K mutants displayed significantly lower aminopeptidase activity.

Table I

Relative activities of $LTA_4$ hydrolase and aminopeptidase of the mutant human $LTA_4$ hydrolase

| | $LTA_4$ hydrolase activity (%) | Aminopeptidase activity (%) |
|---|---|---|
| Wild-type | 100 | 100 |
| E297Q | 104 | 14 |
| E297A | 30 | 25 |
| E297K | 13 | 16 |
| E297D | 17 | 38 |
| E319K | n.d. | 12 |
| Y384F | n.d. | 13 |

Quantitation of the activity and the expressed enzyme protein (ELISA) were carried out using the lysates prepared from mutant or wild-type $LTA_4$ hydrolases. The relative activity is defined by the ratio (%) between the mutant and the wild-type-specific enzymic activities, with wild-type-specific enzymic activities taken as 100%. n.d., not detectable.

Peptide and aromatic region of the circuluar dichroism (CD) profile of the mutant E297Q exhibited no significant changes compared with the wild-type enzyme (Fig. 2), thereby suggesting that tertiary structures were not heavily affected by this mutation. Thus, the loss of peptidase activity in all mutants at Glu-297 confirms that Glu-297 constitutes the active site for aminopeptidase activity of $LTA_4$ hydrolase.

The structural requirement of $LTA_4$ hydrolase activity constitutes a more equivocal issue. While three mutants at Glu-297 (E297A, E297D and E297K) showed reduced enzyme activity, the mutant E297Q exhibited an intact $LTA_4$ hydrolase activity (1.92 $\mu$mol $LTB_4$/min·mg protein) compared with the wild-type enzyme


Fig. 3. Immunoblot analysis of the mutants and wild-type enzyme. Lane 1, E297A; lane 2, E297K; lane 3, E297D; lane 4, Y397F; lane 5, E297Q; lane 6, E319K; and lane 7, wild-type.

(1.93 $\mu$ $LTB_4$/min·mg protein). Thus, the side chain of the glutamic moiety [$-CH_2-CH_2-CO-R$ where R = OH (Glu) or R = $NH_2$ (Gln)] seems to be the required structure for $LTA_4$ hydrolase rather than the glutamic acid itself. It is also possible that the tertiary structure surrounding the Glu-297 may highly affect the active site pocket, and have a different effect on each mutant, though a precise mechanism would need to be determined. The entire catalytic mechanism for $LTA_4$ hydrolase seems intact in E297Q, since $LTA_4$ hydrolase activity showed characteristic suicide-type inactivation by its substrate $LTA_4$ and was also inhibited by *L*-leu thiol in a similar manner as for the wild-type enzyme [13, 39].

His-296, His-300 and Glu-319 probably constitute the zinc binding ligands [24,25]. Based on this proposal, Medina et al. constructed three mutants (H296Y, H300Y and E319Q) for mouse recombinant $LTA_4$ hydrolase, and found a parallel loss of enzyme activities and zinc content [40]. In the present study, the mutant E319K (Fig. 1b) showed a loss of two enzyme activities with a loss of zinc content (2% as compared to the wild type). These data clearly support our previous notion [29] that the presence of the zinc ion in the protein is a requisite for both enzyme activities.

We found that a putative proton donor motif for various aminopeptidases and carboxypeptidases was also conserved in $LTA_4$ hydrolase (Fig. 1a). In this alignment, Tyr-384 is a possible candidate for a proton donor site required in aminopeptidase reaction [41]. In the mutant Y384F, however, the two enzyme activities were below detection limit, because this mutant protein was poorly expressed or degradated fast (Fig. 3).

Studies are continuing to closely examine the physiological events linked to the aminopeptidase activity in $LTA_4$ hydrolase.

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