Leukotriene A₄ hydrolase: a critical role of glutamic acid-296 for the binding of bestatin

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Leukotriene A₄ hydrolase is a bifunctional Zn²⁺-containing enzyme catalysing the formation of the potent chemotaxin leukotriene B₄. From an analysis of three mutants of Glu-296 we have found that this catalytic residue is critical for the binding of bestatin, a classical aminopeptidase inhibitor. For bestatin, but not for three other tight-binding inhibitors, the IC₅₀ values for inhibition of the epoxide hydrolase activity decreased in the

mutants to 0.7–0.003 % of the control. Hence Glu-296 is an important structural determinant for binding of bestatin to leukotriene A₄ hydrolase; this conclusion might also apply to other members of the M1 family of metallopeptidases.

Key words: aminopeptidase, inflammation, leukotriene B₄, mutagenesis, zinc.

INTRODUCTION

Leukotriene A₄ (LTA₄) hydrolase (EC 3.3.2.6) catalyses the final step in the biosynthesis of the potent chemotaxin leukotriene B₄ (LTB₄). The substrate of this reaction, i.e. the labile epoxide intermediate LTA₄, is formed from arachidonic acid via the enzyme 5-lipoxygenase [1]. LTA₄ hydrolase is a soluble (molecular mass approx. 69 kDa) epoxide hydrolase, ubiquitous in mammalian tissues. Enzyme catalysis is typically accompanied by the inactivation and covalent coupling of LTA₄ to the protein, apparently at Tyr-378 [2,3].

LTA₄ hydrolase contains a zinc-binding motif structurally similar to those present in certain zinc proteases, e.g. aminopeptidase N and thermolysin (Figure 1). Consequently, LTA₄ hydrolase contains one catalytic Zn²⁺ ion complexed to His-295, His-299 and Glu-318 [4–6]. In addition, the enzyme possesses a peptide-cleaving activity with specificity for arginyl dipeptides and tripeptides [6–8]. However, the physiological substrate for the peptidase is not yet known. On the basis of the zinc signature (Figure 1), the peptidase activity and the overall structural similarity, LTA₄ hydrolase has been classified as a member of the M1 family of metallopeptidases [9].

Some of the zinc proteases and aminopeptidases also share a conserved Glu residue, in juxtaposition to the first Zn²⁺-binding

ligand (L₁ in Figure 1) [10]. In a previous study we showed that mutation of Glu-296, the corresponding residue in LTA₄ hydrolase, abrogated the peptidase activity with only minor effects on the epoxide hydrolase activity [11]. Hence Glu-296 is critical for the peptidase activity, in which it probably acts as a general base. Because both enzyme activities are closely related, as judged by substrate competition, susceptibility to competitive inhibitors, and requirement for Zn²⁺, the two active sites are overlapping but not identical.

Bestatin, a general aminopeptidase inhibitor, and captopril inhibit both catalytic activities of LTA₄ hydrolase [12]. Several other potent and specific inhibitors have also been synthesized, including an α -oxo- β -amino ester, a thioamine and a hydroxamic acid [13,14].

In the present study we have used the mutants [E296Q]-, [E296D]- and [E296N]-LTA₄ hydrolase, to show that Glu-296 is a structural determinant for the tight-binding properties of bestatin, a classical prototype for an aminopeptidase inhibitor. These conclusions might also be valid for bestatin inhibition of other metallopeptidases homologous with LTA₄ hydrolase, particularly those belonging to the M1 family.

EXPERIMENTAL

Materials

LTA₄ methyl ester (Merck Frosst, Quebec, Canada) was saponified in tetrahydrofuran with 1 M LiOH (6 %, v/v) for 48 h at 4 °C. Bestatin and alanine-4-nitroanilide were purchased from Sigma. 4-Nitroaniline was from Kebo (Stockholm, Sweden). T7 Sequencing kit, restriction endonucleases and nucleic-acid-modifying enzymes were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Vent DNA polymerase was from New England Biolabs (Beverly, MA, U.S.A.). Oligonucleotides were synthesized by Scandinavian Gene Synthesis AB (Köping, Sweden).

Site-directed mutagenesis of LTA₄ hydrolase cDNA and expression in *Escherichia coli*

Mutations of recombinant mouse LTA₄ hydrolase were performed by PCR mutagenesis as described [15]. Primers A and B

	L ₁	L ₂	L ₃
LTA ₄ hydrolase	V I A H E I S H S W T 295	F W L N E G H T V 318	
Aminopeptidase N	V I A H E L A H Q W T 388	L W L N E G F A S 411	
Aminopeptidase B	V I I H E I S H S W F 323	F W L N E G F T M 346	

Figure 1 Sequence comparison between the Zn²⁺-binding motifs of LTA₄ hydrolase, aminopeptidase N and aminopeptidase B

All three enzymes are members of the M1 family of metallopeptidases.

Abbreviations used: LTA₄, leukotriene A₄; LTB₄, leukotriene B₄; PGB₁, prostaglandin B₁.

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were JF21 and JF27 [15]. Primer C was the mutagenetic primer in each case (site mutation underlined): JF37 (5'-GACATATCTCATAGCTGGACAGG-3') for Asp-296; JF38 (5'-AACATATCTCATAGCTGGACAGG-3') for Asn-296, both phosphorylated at the 5' end. Primer D was JF13 (5'-AATCAGCAACAATCAGTTCCT-3'), a reverse primer matching nt 1856–1836 (following the cDNA numbering in [16]). Primers used for the construction of [E296Q] and [S298A]LTA₄ hydrolase were as described in [11]. Mutated proteins were expressed in *E. coli* (JM 101) transformed with the corresponding mutated plasmid, as described [15]. Sequence analysis of the entire cDNA inserts confirmed that no alterations of the protein primary structure, other than the desired mutation, had occurred.

Purification of recombinant LTA₄ hydrolase

Both mutated and unmutated proteins were purified essentially as described [17]. The procedure involved precipitations, anion exchange, hydrophobic interaction and chromatofocusing chromatographies and resulted in apparently homogeneous proteins. The yield was 0.5–1 mg of protein per litre of cell culture. After the final purification step, the buffer was changed to 50 mM Tris/HCl, pH 8, by repeated centrifugation on a Centricon-30 microconcentrator (Amicon, Danvers, MA, U.S.A.) and stored at 4 °C. SDS/PAGE was performed on a Phast system (Pharmacia) with 10–15% (w/v) gradient gels. Bands of protein were detected by staining with Coomassie Brilliant Blue. Protein concentrations were determined by the Bradford method [18], with BSA as standard.

Determinations of enzyme activities and kinetic constants

The kinetic constants (K_m , k_{cat} and k_{cat}/K_m), for the epoxide hydrolase activity (i.e. the hydrolysis of LTA₄ into LTB₄) of wild-type and [E296Q]LTA₄ hydrolase were determined by incubations of the respective enzyme (2 µg in 100 µl of 50 mM Hepes, pH 7.9) at room temperature for 15 s with the substrate LTA₄ (5–60 µM), added in 1 µl of tetrahydrofuran. The reaction was quenched with 200 µl of methanol, and prostaglandin B₁ was added as an internal standard. Product formation was analysed by reverse-phase HPLC, as described [17]. Quantifications of LTB₄ were made by area integration with Baseline 810 computer software (Waters) based on a standard curve obtained from an analysis of known amounts of prostaglandin B₁ and LTB₄. Kinetic data were calculated and plotted by the method of Eadie and Hofstee.

For determinations of IC₅₀ values for bestatin, the enzyme (2 µg in 100 µl of 50 mM Hepes, pH 7.9) was preincubated for 40 min at room temperature with various quantities of inhibitor. LTA₄ (20 µM) was added and the reaction was allowed to continue for 15 s before being quenched with methanol. Similar conditions were used for determinations of IC₅₀ values for α -oxo- β -amino ester, thioamine and hydroxamic acid. For determinations of K_i values for bestatin against wild-type and [E296Q]LTA₄ hydrolase, 2 µg of enzyme was incubated with LTA₄ (8–54 µM) under otherwise similar conditions. The K_i values were obtained from Dixon plots [19].

The peptidase activity was determined spectrophotometrically in 50 mM Tris/HCl, pH 7.5, containing 100 mM NaCl. The assays were performed by incubating the enzyme (1–26 µg) with alanine-4-nitroanilide (1 mM) in 250 µl of buffer at room temperature, as described previously [17]. Reaction rates were calculated from the increase in A_{405} over 30 min (wild-type) or 4–5 h (mutants) of incubation.

RESULTS

Properties of the mutated enzymes

In the present study we used wild-type enzyme, the previously constructed mutants [E296Q]- and [S298A]-LTA₄ hydrolase [11], and two additional mutants at position 296, namely [E296D]- and [E296N]-LTA₄ hydrolase. The cDNA species were expressed in *E. coli* and the recombinant enzymes were purified to apparent homogeneity and assayed for enzyme activities. The catalytic properties of [E296Q]LTA₄ hydrolase, the most extensively used mutant in the present study, were as described in [11] and listed in Table 1. The mutants [E296D]- and [E296N]-LTA₄ hydrolase, isolated from two or three separate expressions, were practically devoid of peptidase activity (0.25% and 0.1% of the wild-type enzyme's) and converted LTA₄ into LTB₄ with specific activities of 490 ± 120 nmol/min per mg (mean $\pm$ S.D., $n = 3$) and 310 nmol/min per mg ($n = 2$), corresponding to 70% and 45% respectively of the unmutated enzyme. The properties of [E296D]- and [E296N]LTA₄ hydrolase corroborate our previous conclusions on the role of Glu-296 for the peptidase activity of LTA₄ hydrolase. In addition, they show that a Glu $\rightarrow$ Asp mutation, which preserves the carboxy moiety but shortens the side chain by one methylene group, also results in a loss of the peptidase activity. The peptidase activity thus depends on the presence of a carboxylate group at a correct distance from the peptide backbone of the substrate.

Effects of mutations on the potency of bestatin

Purified mutated enzymes were incubated with various concentrations of bestatin and assayed for remaining epoxide hydrolase activity. The IC₅₀ values for the wild-type enzyme and the mutants [E296Q]-, [E296D]- and [E296N]-LTA₄ hydrolase, were calculated as being 0.14 ± 0.09 µM (mean $\pm$ S.D., $n = 5$), 19 µM ($n = 2$), 5.1 mM ($n = 1$) and 24 µM ($n = 2$) respectively (Table 2). Thus the mutation of Glu-296 into Gln, Asp or Asn led to a decrease in inhibitor potency to 0.7%, 0.003% and 0.6% respectively. These effects were specific for mutations at this position because a positive control, [S298A]LTA₄ hydrolase (see Figure 1), exhibited an IC₅₀ of 0.17 µM, in good agreement with that of the wild-type enzyme.

Three structurally different, tight-binding inhibitors, namely an α -oxo- β -amino ester, a thioamine and a hydroxamic acid (Figure 2), were tested against the wild-type enzyme and [E296Q]- and [E296D]-LTA₄ hydrolase. The inhibitor potencies did not differ between the mutated and unmutated enzymes, indicating that Glu-296 has an insignificant role in the binding of these drugs to the active site. Results for the hydroxamic acid are shown in Figure 3.

Determination of K_m , k_{cat} , $K_i^{bestatin}$ and activation energy (E_a) for wild-type and [E296Q]LTA₄ hydrolase

The catalytic properties and binding of bestatin were investigated further for wild-type and [E296Q]LTA₄ hydrolase. Thus from Eadie–Hofstee plots of kinetic data obtained from incubations of enzyme at different concentrations of substrate, the apparent K_m and k_{cat} for the wild-type enzyme were calculated as being 4.9 µM and 0.9 s⁻¹ respectively (Table 1). In the same experiment, [E296Q]LTA₄ hydrolase exhibited a K_m of 11 µM and a k_{cat} of 1.4 s⁻¹. To assess the binding between enzyme and bestatin without interference by changes in substrate affinity, the K_i for bestatin was determined from Dixon plots [19]. For wild-type and [E296Q]LTA₄ hydrolase, K_i values were calculated as 0.7

Table 1 Kinetic constants for the formation of LTB₄ and the binding of bestatin by the wild-type enzyme and the mutant [E296Q]LTA₄ hydrolase

Kinetic data were obtained from incubations of 2 µg of enzyme with LTA₄ (5–60 µM) for 15 s at room temperature. The specific activities were calculated and plotted by the method of Eadie and Hofstee. For determination of K_i , the enzyme was preincubated for 40 min with various concentrations of bestatin before the addition of LTA₄. The K_i values were obtained from Dixon plots [19].

Kinetic constant	Wild-type	E296Q mutant
K_m (µM)	4.9	11
k_{cat} (s ⁻¹)	0.9	1.4
$10^{-5}k_{cat}/K_m$ (s ⁻¹ · M ⁻¹)	1.9	1.2
K_i (µM)	0.7	21

Table 2 Effects of mutations of Glu-296 on the inhibitor potency of bestatin against the epoxide hydrolase activity of LTA₄ hydrolase

Incubations were performed with 2 µg of enzyme in 100 µl of 50 mM HEPES, pH 7.9, at room temperature with the substrate LTA₄, as described in the Experimental section. The enzyme was preincubated for 40 min with bestatin before the addition of LTA₄. Abbreviation: n.d., not determined. For comparison, estimated values of K_m are also included.

Variable	Wild-type	Mutant ...	E296Q	E296D	E296N	S298A
IC ₅₀ (µM)	0.14		19	5100	24	0.17
K_m (µM)	4.9		11	1–5*	5	n.d.

* Two determinations were carried out.

and 21 µM respectively (Table 1). For the mutants [E296D]- and [E296N]-LTA₄ hydrolase, no detailed graphical determinations of apparent kinetic constants were made. However, from incubations of these mutants with LTA₄ at 5, 15 and 60 µM, K_m

values for [E296D]- and [E296N]-LTA₄ hydrolase were estimated at $1 \mu\text{M} < K_m < 5 \mu\text{M}$ and $5 \mu\text{M}$ respectively (results not shown). Apparently, none of the mutations caused any significant change in the K_m for LTA₄.

The specific epoxide hydrolase activity of [E296Q]LTA₄ hydrolase was more sensitive to temperature changes than that of the unmutated enzyme. From kinetic data obtained at different temperatures (0, 7, 15 and 30 °C), the relative increase in specific activity per 10 °C (Q_{10}) for wild-type and [E296Q]LTA₄ hydrolase were calculated as 17 % and 49 % respectively (five experiments). The change in specific activity was not always linear over the whole temperature range, which might reflect sudden, temperature-dependent, transitions between different protein conformations. The E_a values for wild-type and [E296Q]LTA₄ hydrolase were calculated from Arrhenius plots ($\log V$ against $1/T$) as 3.3 ± 0.4 kJ/mol (mean $\pm$ S.D., $n = 3$) and 17.5 ± 5 kJ/mol ($n = 4$) respectively.

DISCUSSION

Certain metalloproteases share a conserved Glu in their Zn²⁺-binding motifs (Figure 1). This signature is also present in LTA₄ hydrolase and we have previously shown that the corresponding Glu-296 is a catalytic residue, which presumably activates the water molecule during peptidolysis [11]. In the present study we took advantage of the epoxide hydrolase activity, i.e. the conversion of LTA₄ into LTB₄, of the mutants [E296Q]-, [E296D]- and [E296N]-LTA₄ hydrolase to explore the role of Glu-296 for the tight-binding properties of bestatin and three other specific LTA₄ hydrolase inhibitors.

Bestatin is a classical inhibitor of aminopeptidases, efficient against, for example, the dizinc peptidase leucyl aminopeptidase as well as the monozinc peptidases aminopeptidase B and LTA₄ hydrolase [12,20,21]. Furthermore, it has been suggested that a Zn²⁺ ion and an acidic residue are involved in the binding of the drug to the active site [20,22]. Because bestatin is a prototype for an aminopeptidase inhibitor and many newly synthesized com-

Bestatin

α-Keto-β-amino ester

Thioamine

Amino hydroxamic acid

Figure 2 Structures of four tight-binding inhibitors of LTA₄ hydrolase

Figure 3 Inhibition of the epoxide hydrolase activity of wild-type (●) and [E296Q]LTA₄ hydrolase (■) by bestatin (A) and amino hydroxamic acid (B)

Enzyme (2 μ g) in 100 μ l of 50 mM Hepes, pH 7.9, was preincubated with various quantities of inhibitor for 40–60 min at room temperature. LTA₄ (20 μ M) was added and the reaction was stopped after 15 s by being quenched with 2 vol. of methanol. The internal standard prostaglandin B₁ was added and the samples were subjected to solid-phase extraction and reverse-phase HPLC for the analysis of LTB₄ formation.

pounds have been developed with bestatin as a template [23–25], we investigated the potential involvement of Glu-296 in the binding of bestatin and three other more specific and tight-binding inhibitors of LTA₄ hydrolase. Interestingly, for all mutants of Glu-296, i.e. [E296Q]-, [E296D]- and [E296N]-LTA₄ hydrolase, we found that the inhibitor potency of bestatin decreased markedly to 0.7–0.003 % of the potency towards the wild-type enzyme. This effect is specific for Glu-296 because a control mutant, [S298A]LTA₄ hydrolase, exhibited an IC₅₀ (0.17 μ M) similar to that of wild-type enzyme (0.14 μ M). A more detailed kinetic examination of wild-type and [E296Q]LTA₄ hydrolase revealed similar K_m values for LTA₄ and the values of K_i for bestatin were in good agreement with the IC₅₀ values

Figure 4 Model for the binding of bestatin to the active site of LTA₄ hydrolase

A proposed mechanism for inhibition with bestatin is shown, in which the free amine and the carbonyl oxygen are co-ordinated to the Zn²⁺ ion and the intervening hydroxy group is co-ordinated to a carboxylate group of the enzyme.

(Tables 1 and 2). Estimation of the K_m for [E296D]- and [E296N]-LTA₄ hydrolase also showed that a change in substrate affinity could hardly account for the large decrease in inhibitor potency. However, these estimations were performed in a rather crude fashion and therefore we cannot rule out the possibility that minor alterations in K_m contribute to the effect. In any event, Glu-296 seems to be an important structural determinant for the binding of bestatin, probably owing to its charged carboxylate. A putative model for the inhibitor binding is depicted in Figure 4. Because the equivalent of Glu-296 is conserved within the Zn²⁺-binding motifs of certain metalloproteases, our conclusions might prove applicable to the inhibition of other members of this enzyme class, particularly those belonging to the M1 family of zinc peptidases, by bestatin [9].

Considering the differences between mutants with regard to loss of inhibitor potency, it seems likely that structural features of the active site, other than Glu-296, also influence the binding of inhibitor. Thus each of the mutations might induce minor conformational changes at the active site that can improve or weaken the binding. Experimental evidence in support of such structural changes was the increased activation energy of [E296Q]LTA₄ hydrolase. Nevertheless, for all mutants tested, bestatin was a very poor inhibitor.

The loss of inhibitor potency was specific for bestatin. Thus, of four potent tight-binding inhibitors, bestatin was the only one that became essentially inactive against the mutants in position 296 (compare Figures 2 and 3). Glu-296 has been suggested to participate in the binding of all these inhibitors [14,23–25] but the IC₅₀ values for each of the α -oxo- β -amino ester, the thioamide and the hydroxamic acid were almost identical for the wild-type, [E296Q]- and [E296D]-LTA₄ hydrolases respectively. Apparently, Glu-296 does not have a major role in the binding of these inhibitors to the active site of LTA₄ hydrolase. A comparison of the four inhibitor structures (Figure 2) rather suggests that the bicyclic hydrophobic portions of the α -oxo- β -amino ester, the thioamide and the hydroxamic acid, which mimic the fatty acid backbone of LTA₄, are more important for the tight binding properties of these compounds.

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