Leukotriene A4 hydrolase/aminopeptidase

GLUTAMATE 271 IS A CATALYTIC RESIDUE WITH SPECIFIC ROLES IN TWO DISTINCT ENZYME MECHANISMS

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Leukotriene A4 hydrolase/aminopeptidase is a bifunctional zinc metalloenzyme that converts the fatty acid epoxide leukotriene A4 into leukotriene B4, a potent chemoattractant and immune-modulating lipid mediator. Recently, the structure of leukotriene A4 hydrolase revealed that Glu-271, which belongs to a conserved GXMEN motif in the M1 family of zinc peptidases, and Gln-136 are located at the active site. Here we report that mutagenic replacements of Glu-271, but not Gln-136, abrogate both catalytic activities of leukotriene A4 hydrolase. Furthermore, the 2.1 Å crystal structure of [E271Q]leukotriene A4 hydrolase revealed minimal conformational changes that could not explain the loss of enzyme function. We propose that the carboxylate of Glu-271 participates in an acid-induced opening of the epoxide moiety of leukotriene A4, and formation of a carbocation intermediate. Moreover, Glu-271 appears to act as an N-terminal recognition site and may potentially stabilize the transition-state during turnover of peptides, a property that most likely pertains to all members of the M1 family of zinc aminopeptidases. Hence, Glu-271 is a unique example of an amino acid, which has dual and separate functions in two different catalytic reactions, involving lipid and peptide substrates, respectively.

The leukotrienes (LTs) are potent chemical mediators in a variety of allergic and inflammatory reactions. The biosynthesis of LTs, 5-lipoxygenase converts arachidonic acid into the unstable epoxide LTA4. This intermediate may in turn be conjugated with GSH to form the spasmogenic LTC4 or hydrolyzed into the proinflammatory lipid mediator LTB4, in a reaction catalyzed by LTA4 hydrolase (LTA4H). The enzyme product LTB4 is a classical chemoattractant and triggers adherence and aggregation of leukocytes to the endothelium at nanomolar concentrations. In addition, LTB4 modulates immune responses, participates in the host-defense against infections, and is a key mediator of PAF-induced lethal shock. These effects are signaled via a specific, high-affinity, G protein-coupled receptor for LTB4 (BLT1) (9). In addition, a second receptor for LTB4 (BLT2) was recently discovered, the functional role of which is presently not known (10).

LTA4 hydrolase (EC 3.3.2.6) is a bifunctional zinc metalloenzyme, which integrates a sophisticated epoxide hydrolase activity, specific for the fatty acid derivative LTA4, with an anion-dependent aminopeptidase activity in a common active center; for a review see Ref. 11. The enzyme belongs to the M1 family of zinc metallopeptidases (12), which include enzymes such as aminopeptidase A (EC 3.4.11.7, APA), aminopeptidase B (EC 3.4.11.6, APB) and aminopeptidase N (EC 3.4.11.2, APN) (see Fig. 1). A common structural feature in this group of enzymes is the zinc binding motif HEXXH(X8)HE (13), and in LTA4H the zinc is coordinated by His-295, His-299, and Glu-318 (14). Both enzyme activities of LTA4H require the catalytic zinc.

The aminopeptidase activity accepts a variety of substrates and certain arginyl di- and tripeptides as well as p-nitroanilide derivatives of Ala and Arg are hydrolyzed with high efficiencies (15). Although it has never been proven, it is generally assumed that the aminopeptidase activity is involved in the processing of peptides related to inflammation and host-defense. Furthermore, this enzyme activity can be selectively abolished by mutation of either of the conserved Glu-296 or Tyr-383, presumably acting as a general base and proton donor, respectively (16, 17). The members of the M1 family of metallopeptidases also share a conserved motif, GXMEN (Fig. 1), that is located N-terminally to the zinc site, and for APA and APN recent data have suggested that it participates in the exopeptidase function of these enzymes (18, 19).

The epoxide hydrolase reaction, i.e. the conversion of LTA4 into LTB4, is unique in that the stereospecific introduction of a hydroxy group occurs at a site distant from the epoxide moiety, and it has been suggested that it proceeds via a delocalized carbocation intermediate. Unlike the aminopeptidase activity, the epoxide hydrolase activity of LTA4H is restrained by suicide inactivation, which involves binding of LTA4 to Tyr-378 (20, 21). However, besides the zinc binding ligands, no single amino acid residue critical for the epoxide hydrolase activity has yet been identified.
the observed and calculated factor amplitudes, respectively. Components of $h$ are omitted during the refinement process. Both participates in the initial activation of the epoxide moiety mechanisms. We propose mechanistic models in which Glu-271 might be involved in the aminopeptidase activity of LTA4H. Yet, Glu-271, which is located within the G-men motif, and Gln-136 are positioned at the active site near the structure, Glu-271, makes interactions with bestatin suggesting that they residues at the catalytic zinc site.

Recently, we determined the x-ray crystal structure of LTA4H at 1.95 Å resolution in complex with bestatin (22). In the structure, Glu-271, which is located within the GXMEN motif, and Gln-136 are positioned at the active site near the catalytic residue Glu-296, as well as the catalytic zinc and its three amino acid ligands (Fig. 2). Furthermore, Glu-271 and Gln-136 make interactions with bestatin suggesting that they might be involved in the aminopeptidase activity of LTA4H.

In the present report, we show that Glu-271, but not Gln-136, is a catalytic residue that is shared between the epoxide hydrolase and aminopeptidase activities of LTA4H. Yet, Glu-271 carries out a separate chemistry in each of the two reaction mechanisms. We propose mechanistic models in which Glu-271 both participates in the initial activation of the epoxide moiety of the substrate LTA4 and contributes to the binding of the N-terminal amine and the exopeptidase nature of the peptidaceavleaving activity. Moreover, it is likely that the conclusions regarding the aminopeptidase activity are valid for all members of the M1 family of metallopeptidases and that the GXMEN sequence qualifies as a consenus motif for an N-terminal recognition site.

**EXPERIMENTAL PROCEDURES**

*Mutagenesis of Human LTA4H cDNA—Site-directed mutagenesis was carried out by PCR on the recombinant plasmid pT3MB4 for expression of His$_6$-tagged LTA4H in Escherichia coli. Briefly, it involves two consecutive steps of PCR comprising four different primers, according to the megaprimer method (23). The first reaction includes a modified primer (A) carrying the mutation and a second primer (B) comprising a restriction site within the LTA4H cDNA. Together, primers A and B amplify a DNA fragment denominated megaprimer. In the next PCR, the megaprimer is used together with a fourth primer (C) containing another restriction site to amplify a final fragment carrying the mutation and different restriction sites at its ends. Polymerase chain reactions were carried out in a total volume of 50 µl, using 1× Pfu polymerase buffer, 125–150 ng each of primers A and B, 1 unit Pfu polymerase, 10 nmol each of dNTPs and 100 ng of template. In the second reaction, 15–20 µl of the first PCR mix was used to supply with the megaprimer, which was used together with primer C (125–150 ng). The amplification program included an initial round of denaturation at 94 °C (60 s), annealing at 55–66 °C (60 s) and elongation at 72 °C (90 s) followed by 30 cycles of denaturation (45 s), annealing (30 s), and elongation (60 s) on a PE GeneAmp PCR System 2400.

For generation of [Q136L], [Q136N] and [Q136A]LTA4H, a SalI and BglIII site were used whereas a BglII and the BfrI site were used for [G268A], [G269A], [M270L], [E271Q], [E271D], [E271A] and [N272A]LTA4H. DNA fragments were cleaved with SalI/BglII or BglII/BfrI and purified by agarose gel electrophoresis (1.5%) followed by extraction (QIAEX II Gel Extraction Kit). Mutated fragments were ligated into pT3MB4 (T4 DNA Ligase Protocol), opened with the respective pair of restriction enzymes. Competent *E. coli* cells (JM101) were transformed with mutated recombinant plasmid and grown in LB medium containing ampicillin (100 µg/ml). Stock cultures were kept at 70 °C in a 1:1 mixture of culture medium and 40% (v/v) glycerol/0.75% (w/v) NaCl, respectively. Recombinant plasmids were purified using Wizard Miniprep Plus, and the entire mutated inserts were all sequenced using Dynamic ET terminator cycle sequencing kit (Amerham Biosciences, Inc.) to confirm that no nucleotide alterations had occurred in addition to the desired mutation.

*Protein Expression and Purification—*Mutated enzymes were expressed as N-terminal His$_6$-tagged fusion proteins in *E. coli* (JM101) cells grown at 37 °C in M9 medium (50 mM Na$_2$HPO$_4$, 22 mM KH$_2$PO$_4$, 200 mM NaCl, 5 mM MgSO$_4$, and 0.4% glucose). Enzymes were purified as described previously (22).

**FIG. 1. Conserved sequence motifs within the M1 family of metallopeptidases.** The three zinc-binding ligands are denoted $L_1$, $L_2$, and $L_3$. Catalytic residues are indicated for the epoxide hydrolase activity (■) and/or the aminopeptidase activity (○), respectively. Numbering of residues refers to the sequence of LTA4H.

**TABLE I**

| Data collection and refinement statistics |
|-----------------------------------------|
| **Data collection**                      |
| Diffraction limit (Å)                   | 2.05 |
| Wavelength (Å)                          | 0.9831 |
| Completeness (%)                        | 99.6 |
| Mean I/σ(I)                             | 10.3 |
| Multiplicity of observation             | 4.5 |
| $R_{merge}^{*}$ (%)                     | 8 |

| **Refinement statistics**               |
|-----------------------------------------|
| $R$-factor (%)                          | 18.2 |
| $R_{free}^{*}$ (%)                      | 23.3 |
| r.m.s.d. in bond distance (Å)           | 0.01 |
| r.m.s.d. in bond angle (°)              | 1.6 |

$R_{merge} = \frac{\Sigma_h \Sigma_i |I_i(h) - \bar{I}(h)|}{\Sigma_h \Sigma_i I_i(h)}$, where $I_i(h)$ is the ith measurement of reflection $h$ and $\bar{I}(h)$ is the weighted mean of all measurements of $h$.

$R = \frac{\Sigma_h |F_{calc}(h) - F_{obs}(h)|}{\Sigma_h F_{calc}(h)}$, where $F_{calc}$ and $F_{obs}$ are the observed and calculated factor amplitudes, respectively.

$R_{free}$ is the $R$-factor calculated for the test set of reflections, which are omitted during the refinement process.

And B amplify a DNA fragment denominated megaprimer. In the next PCR, the megaprimer is used together with a fourth primer (C) containing another restriction site to amplify a final fragment carrying the mutation and two different restriction sites at its ends. Polymerase chain reactions were carried out in a total volume of 50 µl, using 1× Pfu polymerase buffer, 125–150 ng each of primers A and B, 1 unit Pfu polymerase, 10 nmol each of dNTPs and 100 ng of template. In the second reaction, 15–20 µl of the first PCR mix was used to supply with the megaprimer, which was used together with primer C (125–150 ng). The amplification program included an initial round of denaturation at 94 °C (60 s), annealing at 55–66 °C (60 s), and elongation at 72 °C (90 s) followed by 30 cycles of denaturation (45 s), annealing (30 s), and elongation (60 s) on a PE GeneAmp PCR System 2400.

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**Protein Expression and Purification—** Mutated enzymes were expressed as N-terminal His$_6$-tagged fusion proteins in *E. coli* (JM101) cells grown at 37 °C in M9 medium (50 mM Na$_2$HPO$_4$, 22 mM KH$_2$PO$_4$, 200 mM NaCl, 5 mM MgSO$_4$, and 0.4% glucose). Enzymes were purified as described previously (22).

**FIG. 2. Structure of LTA4H, spatial relationships between residues at the catalytic zinc site.** The figure depicts the juxtaposition of Glu-271 and Gln-136 relative the known catalytic residues Glu-296 and Tyr-383 as well as the prosthetic zinc. The inhibitor bestatin is depicted in light gray. Hydrogen bonds are indicated with green dotted lines, and the metal coordination with black dotted lines.
20 mM NH4Cl, 8.5 mM NaCl), pH 7.4, containing 0.4% glucose (w/v), 0.2% (w/v) casamino acids, 2 mM MgSO4 and 0.1 mM CaCl2. At A260 0.2, isopropyl-p-b-thiogalactopyranoside was added to a final concentration of 100 μM. Cells were harvested at A600 1.8, pelleted at 1000 × g and resuspended in 30 ml of homogenization buffer (50 mM Tris-HCl, pH 8.0, containing soybean trypsin inhibitor) supplemented with phenylmethylsulfonyl fluoride (1 mM). Nucleic acids were removed by streptomycin sulfate precipitation. After centrifugation (10,000 × g for 15 min), the supernatant was filtered (0.22 μm) and applied to a nickel-NTA (nitriilosacetic acid) resin. The column was washed with 1 bed volume of 50 mM Tris-HCl, pH 8.0, 50 mM sodium phosphate buffer, pH 6.8, and 0.5 M NaCl, and 50 mM Tris-HCl, pH 8.0, with each solution supplemented with 10 mM imidazole. The final wash consisted of 0.8 bed volumes of 50 mM Tris-HCl, pH 8.0, containing 100 mM imidazole. The His-tagged protein was eluted with 0.6 bed volumes of 50 mM Tris-HCl, pH 8.0, containing 100 mM imidazole. If required, a final step process, programs included in the AutoDock 3.0 program package were used for molecular modeling. The aminopeptidase activity was determined in a spectrophotometric assay at 405 nm, using a MCC/340 spectrophotometer and bovine serum albumin as standard (24).

Aminopeptidase Activity Assays—The aminopeptidase activity was determined in a spectrophotometric assay at 405 nm, using a MCC/340 multispectrophotometer, essentially as described (25). Briefly, the enzyme (1–20 μg) was incubated at room temperature in 96-well microplates with alanine-p-nitroanilide ( Ala-p-NA) as substrate in 50 mM Tris-HCl, pH 8.0, containing 100 mM KCl. Additional aminopeptidase assays were performed similarly with Leu-p-NA and Lys-p-NA as substrates in 50 mM Tris-HCl, pH 8.0, containing 100 mM KCl. The aminopeptidase activity was determined in a spectrophotometric assay at 405 nm, using a MCC/340 spectrophotometer and bovine serum albumin as standard (24).

Epoxide Hydrolyase Activity Assays and Reverse-phase HPLC—The epoxide hydrolyase activity was determined from incubations of enzyme (1–20 μg) in 100 μl of 10 mM Tris-HCl, pH 8.0, with LTA4 (2.5–125 μM) for 30 s on ice. Reactions were quenched with 200 μl of MeOH, followed by the addition of 0.4 nmol of prostaglandin B1 (PGB1) or PGB2 as internal standard. Samples were acidified with 5 μl of acetic acid (10%), and metabolites were extracted on solid phase Chromabond C18 columns. Enzymatic metabolites of LTA4 were identified and quantified by reverse-phase HPLC. Metabolites of LTA4 were separated by isocratic reverse-phase HPLC on a Waters Nova-Pak C18 column eluted with a mixture of methanol/acetonitrile/water/acidic acid (30:30:40:0.01 by volume) at a flow rate of 1.2 ml/min. The UV detector was set at 270 nm, and Chromatography Station for Windows version 1.7 computer software. Calculations were based on peak area measurements and the known extinction coefficients for the internal standards PGB1 and PGB2 (30 000 M⁻¹ × cm⁻¹) as well as LTB4 (50 000 M⁻¹ × cm⁻¹).

Crystallization—Plate-like crystals of [E271Q]LTA4H were obtained by liquid-liquid diffusion in capillaries, as described (25). Briefly, 5 μl of

| Mutant | Epoxide hydrolyase activity | Aminopeptidase activity |
|--------|-----------------------------|-------------------------|
|        | kcat | Km | kcat/Km | kcat | Km | kcat/Km |
| Q136A  | 34 ± 2 | 67 ± 16 | 51 | 103 ± 10 | 108 ± 24 | 95 |
| Q136L  | 57 ± 5 | 179 ± 59 | 32 | 0.1 ± 0.004 | 24 ± 4 | 0.004 |
| Q136N  | 20 ± 2 | 15 ± 10 | 113 | 218 ± 6 | 73 ± 5 | 301 |
| Q269A  | 113 ± 8 | 97 ± 19 | 117 | 21 ± 1 | 66 ± 6 | 31 |
| Q269N  | 135 ± 9 | 202 ± 43 | 67 | 56 ± 2 | 189 ± 13 | 30 |
| M270L  | 19 ± 1 | 21 ± 6 | 88 | 0.6 ± 0.03 | 44 ± 5 | 1 |
| E271A  | ND | ND | ND | 18 ± 1 | 221 ± 20 | 8 |
| E271D  | ND | ND | ND | 6 ± 0.63 | 44 ± 5 | 1 |
| E271Q  | ND | ND | ND | 18 ± 1 | 221 ± 20 | 8 |
| N272A  | 31 ± 2 | 30 ± 7 | 102 | 9 ± 1 | 111 | 3 |

* The mutant Q136L was catalytically active but did not obey saturation kinetics, using Ala-p-NA as substrate. However, at [S] = 8 mM, the relative V reached 4 ± 0.2% of the Vmax for wild type enzyme.

Molecular Modeling—To model the binding of Ala-p-NA, the program AutoDock 3.0 (30) was used. The substrate, assumed to be protonated with a positively charged amino group, was initially modeled manually into the active site of LTA4H using the program XtalView (29). During the refinement 582 water molecules, one Zn²⁺ ion, one acetate molecule, and one Yb³⁺ ion were added. After a final round of refinement, including all data between 25–21 Å and all 610 amino acids, the best factor was 18.2% and the Rfree factor was 23.3%. Most of the model of [E271Q]LTA4H is in good density except for the His tag and the first four N-terminal residues. The model exhibits a highly restrained but acceptable stereochemistry with 99.6% of the residues in the most favored or additionally allowed regions of the Ramachandran plot. Root mean square deviations for bond lengths and angles are 0.01 Å and 1.6°, respectively.

Molecular Docking—The binding of Ala-p-NA, the program AutoDock 3.0 (30) was used. The substrate, assumed to be protonated with a positively charged amino group, was initially modeled manually into the active site of LTA4H using the program XtalView. The binding conformation was then optimized using the local search algorithm of AutoDock 3.0. Prior to the docking, addition of polar hydrogens and assessment of atomic charges to the model of LTA4H was done using the program QUANTA (Molecular Simulations, Inc) and XtalView (29). During the refinement 582 water molecules, one Zn²⁺ ion, one acetate molecule, and one Yb³⁺ ion were added. After a final round of refinement, including all data between 25–21 Å and all 610 amino acids, the best factor was 18.2% and the Rfree factor was 23.3%. Most of the model of [E271Q]LTA4H is in good density except for the His tag and the first four N-terminal residues. The model exhibits a highly restrained but acceptable stereochemistry with 99.6% of the residues in the most favored or additionally allowed regions of the Ramachandran plot. Root mean square deviations for bond lengths and angles are 0.01 Å and 1.6°, respectively.

**RESULTS**

**Mutagenetic Replacements, Expression, and Purification of Recombinant Proteins**—To detail their function we exchanged Glu-271 for a Gln, Asp, or Ala residue, whereas Glu-136 was replaced with an Asn, Leu, and Ala by site-directed mutagenesis. In addition, the remaining residues in the GXXEN signature, viz. Gly-268, Gly-269, Met-270, and Asn-272 were mutated into Ala, Ala, Leu, and Ala, respectively. The resulting 10 mutants were all expressed as His₆-tagged fusion proteins in E. coli to allow rapid and convenient purification on nickel-NTA resins. The level of expression was similar for wild type enzyme.
and all mutants, with a final yield of about 2–5 mg of purified protein per liter of cell culture.

Effects of Mutation of Glu-271—Exchange of Glu-271 for a Gln, Asp, or Ala residue resulted in a complete loss of the epoxide hydrolase activity (Table II), even when the amounts of enzyme were increased 40 times (100 μg) in the incubation mixture. In contrast, mutants at the other positions within the GXMEN motif, i.e. [G268A], [G269A], [M270L], and [N272A]LTA4H, displayed variable, albeit significant, epoxide hydrolase activities. Thus, values of $V_{\text{max}}$ and $k_{\text{cat}}$ ranged between 19–135% relative to the wild type enzyme, whereas the corresponding values for $K_m$ ranged between 21–202%. As a result, the specificity constant $k_{\text{cat}}/K_m$ varied between 67 and 117%.

The aminopeptidase activity of [E271Q], [E271D], and [E271A]LTA4H against Ala-p-NA was essentially abolished (Table II) and similar results were obtained with Lys-p-NA and Leu-p-NA (data not shown). In contrast, the aminopeptidase activity of [G268A], [G269A], [M270L], and [N272A]LTA4H were moderately, if at all, affected with values of $V_{\text{max}}$ ($k_{\text{cat}}$) and $K_m$ ranging between 18–218% and 66–221%, relative to the wild type enzyme. The corresponding specificity constants were between 8 and 301% that of the wild type enzyme.

Effects of Mutation of Glu-271—Exchange of Glu-271 for a Gln, Asp, or Ala residue did not significantly affect the epoxide hydrolase activity of LTA4H (Table II). Values of $V_{\text{max}}$ ($k_{\text{cat}}$) and $K_m$ for [Q136L], [Q136N], and [Q136A]LTA4H reached 20–57% and 18–179% of the wild type control, respectively. On the other hand, the mutations led to highly variable effects on both the turnover, and the Michaelis constant for the peptide substrate Ala-p-NA (Table II). Thus, [Q136L]LTA4H did not obey saturation kinetics and displayed a linear increase in reaction velocity in the substrate interval 0.125–8.0 mM Ala-p-NA. In contrast, [Q136N]LTA4H lacked almost completely the ability to hydrolyze Ala-p-NA, whereas the peptidase activity of [Q136A]LTA4H did not differ from that of wild type enzyme, with values of $V_{\text{max}}$ ($k_{\text{cat}}$) and $K_m$ mounting to 103 and 108%, respectively. This large variation in the effects of mutation of Gln-136 makes it difficult to draw any conclusions regarding its functional role. However, since the mutant in which the side chain was extensively truncated retained all of its peptide-cleaving ability, the most likely interpretation is that Gln-136 does not play any significant role in this catalytic reaction.

Structure of [E271Q]LTA4H—The mutant and wild type enzymes crystallize non-isomorphously and also bind a different number of Yb$^{3+}$ ions. Thus, the wild type enzyme crystallizes in space-group $P2_12_12_1$ with three Yb$^{3+}$ ligands per asymmetric unit, whereas the mutant enzyme crystallizes in space-group $P2_12_12_1$ with only one Yb$^{3+}$ ligand per asymmetric unit.

In the wild type structure, the Zn$^{2+}$ ion is coordinated to His-295, His-299, one carboxylic oxygens of Glu-318, and the hydroxyl and carboxyl oxygens of bestatin in a pentavalent coordination sphere, forming a square-based pyramid (Fig. 2). In the structure of [E271Q]LTA4H, the Zn$^{2+}$ is bound to its three canonical amino acid ligands and one carboxylic oxygen of an acetate molecule. These four ligands form an almost ideal tetrahedral coordination geometry. The acetate molecule is also hydrogen-bonded to Tyr-383 and Gln-271.

Exchange of Glu-271 for a Gln does not cause any significant conformational change of the active site (Fig. 3). Superpositioning of the mutant and wild type models gives an overall r.m.s. deviation for 610 α-carbons of 0.45 Å. Inspection of the superimposed models shows that the differences are evenly spread over the molecules. The only exceptions are two surface loops, which exhibit higher r.m.s. deviations; these differences in α-carbon positioning are probably due to crystal packing differences. Of note, residues lining the active site, including the deeper part of the hydrophobic pocket, show a positional shift in relation to the wild type structure, which is comparable with the rest of the molecule. Thus, the major outline of the active site is well conserved in the mutant structure. However, when comparing the structures of the wild type and mutated enzyme in detail, a small rearrangement of spatially neighboring residues can be observed, which may be explained by the mutation and the absence of inhibitor in the crystal of [E271Q]LTA4H. Directly affected residues, besides Glu-271, are Gln-296 and Gln-136. The changes are subtle and mainly affect the hydrogen-bonding network.

The orientation of the amide side-chain of Gln-271 cannot be judged from electron density, and it was instead determined based on changes in hydrogen-bonding network. Glu-271 in the wild type structure is hydrogen-bonded to its own backbone amide and to the free amine of the bound inhibitor bestatin. In [E271Q]LTA4H, the orientation of the amide side group does not allow direct interaction with its backbone amide. Instead, new direct hydrogen bonds are observed to the acetate bound in the active site and to a water molecule now occupying the position held by the free amine of bestatin in the wild type structure. The absence of inhibitor and the hydrogen bonding to the amide group of Glu-271 pull Glu-296 toward the backbone amide of Gln-271.

Furthermore, in the mutant structure, Glu-136 is flipped about 90 degrees, which creates a hydrogen bond to Glu-271 and to a new water molecule replacing the free amine of bestatin. Another water molecule, at a position corresponding to the phenyl ring of bestatin, also makes a hydrogen bond to Gln-136. Thus, both the mutation and the absence of inhibitor could explain the positional change of Gln-136. Similarly, the torsional flip observed for Met-270 and the positional shift of Gln-134 are most likely effects caused by the absence of inhibitor.

Modeling of Ala-p-NA into the Active Site of LTA4H—Ala-p-NA is a good aminopeptidase substrate for LTA4H. The binding conformation with the lowest energy of a docked Ala-p-NA at the active site of LTA4H agreed very well with biochemical data and is presented in Fig. 4. Thus, the free amine of Ala-p-NA donates one hydrogen bond to each of the carboxyl groups of Glu-271 and Glu-318 and one to the amine carbonyl oxygen of Gln-136. Furthermore, the amide carbonyl oxygen of Ala-p-NA is bound to the Zn$^{2+}$ ion, and the carbonyl carbon is at a
suitable position for attack from a water molecule activated by Glu-296. In addition, Tyr-383, which is believed to act as a proton donor in the final step of the peptidase reaction, is appropriately hydrogen-bonded to the amide nitrogen of the substrate. This alignment of Ala-p-NA indicates that it is a chemically good mimic of tripeptides, the preferred amine- and nitro-groups of peptidase substrates of LTA4H (15). Moreover, this conclusion agrees well with the fact that the amino- and nitro-groups of Ala-p-NA are separated by the same number of covalent bonds as the N and C termini in tripeptide substrates.

DISCUSSION

Recently, we solved the x-ray crystal structure of LTA4H in complex with bestatin at 1.95 Å resolution (22). At the zinc site, in juxtaposition to the previously identified catalytic residues, Glu-296 and Tyr-383, a Glu and Gln residue were located in seemingly strategic positions, and their interactions with the inhibitor bestatin indicated that they could well be involved in the aminepeptidase reaction (Fig. 2). The carboxylic acid, Glu-271, is conserved among members of the M1 family of metallopeptidases in a GXXMEN motif (Fig. 1), whereas the amine, Gln-136, is not well conserved within this enzyme family. To detail the functional role of Glu-271 and Gln-136 for the two catalytic activities of LTA4H, they were subjected to mutational analysis.

Glu-271 Is a Common Catalytic Residue in Both the Epoxide Hydrolase and Peptidase Reactions—Exchange of Glu-271 for a Gln, Ala, or Leu residue completely abolished the ability of LTA4H to convert LTA4 into LTB4 (Table II) as well as its amide cleaving activity against Ala-p-NA (Table II). In contrast, mutation of any of the remaining residues within the GXXMEN motif preserved a significant epoxide hydrolase and peptidase activity. On the other hand, mutagenetic replacements of Gln-136 did not allow us to ascribe any specific functional role for this residue (Table II).

The finding that mutation of Glu-271 abolishes both catalytic activities of LTA4H was unexpected and warranted further studies to rule out that the recombinant proteins had lost the prosthetic zinc or had undergone major conformational changes at the active site. To this end, we determined the x-ray crystal structure of the mutant with the most conservative replacement, i.e. [E271Q]LTA4H. This structure revealed the presence of the catalytic metal and an almost completely conserved overall architecture, particularly at the active site, which demonstrates that the loss of enzyme function was site-specific and could be attributed to the removal of the carboxylate from the side chain of residue 271. Hence, we conclude that Glu-271 is involved in key steps of the epoxide hydrolase and peptidase reactions. In fact, Glu-271 is the first amino acid, besides the three zinc-binding ligands, that has been shown to be required for the epoxide hydrolase mechanism. Moreover, to our knowledge, this is the first example of a single amino acid residue that is critical and common for two distinct catalytic reactions, which turn over lipid and peptide substrates, respectively.

Role of Glu-271 in the Epoxide Hydrolase Mechanism, Assistance in an Acid-induced Activation, and Opening of the Epoxide—Based on the structure of LTA4H as well as data obtained by studies of structure activity relationships of tight-binding hydroxamic acid inhibitors, we recently proposed a mechanistic model for enzymatic hydrolysis of LTA4 into LTB4 (22, 32). It postulates that LTA4 is bound to the active site with the oxirane ring near the zinc ion and the C7-C20 olefinic tail buried in a narrow, L-shaped, hydrophobic pocket in the protein. In this model, which apparently is incomplete, the zinc acts as a weak Lewis acid to activate and open the epoxide ring. In light of the present mutational data and the fact that Glu-271 is located in the immediate vicinity of the zinc, it seems likely that Glu-271 is required in the initial epoxide activation. Thus, we propose that Glu-271 polarizes a water molecule bound to the zinc and that the resulting proton will catalyze an acid-induced opening of the oxirane ring (Fig. 5A). This SN1 reaction will generate a carbocation intermediate whose positive charge will be delocalized over the conjugated triene system. In the last step of the reaction, a nucleophilic attack will occur at C12 to introduce the 12R-hydroxyl group of LTB4 (Fig. 5A).

Even if several lines of evidence support this model, we cannot rule out an alternative mechanism, which includes the formation of an ester intermediate. In this scheme, the zinc alone activates and opens the epoxide, and the carboxylate of Glu-271 attacks LTA4 at C6 to form an ester intermediate. In a concerted SN2′ reaction, this ester can then be attacked by an
hydroxyl group or a carboxylate at C12, and the negative charge can move along the conjugated triene system, eventually leading to an alkyl-oxygen cleavage instead of a normal ester cleavage (Fig. 5B). This model is attractive since formation of an alkylenzyme ester intermediate is a key feature in the mechanisms of other epoxide hydrolases, in particular soluble and microsomal epoxide hydrolase (33–36). In this context it is interesting to note that LTA4 is an excellent substrate of soluble epoxide hydrolase, which converts the allylic 5S,6R-epoxide into a 5S,6R dihydroxy acid (37). On the other hand, the crystal structure of soluble epoxide hydrolase was recently solved and revealed no structural similarity with LTA4H (35).

In addition, alkyl-oxygen cleavages of esters are rare reactions although they have been described for certain allylic or benzylic esters (38).

**Role of Glu-271 in the Aminopeptidase Reaction, an N-terminal Recognition Site for Peptide Substrates, and a Determinant for the Exopeptidase Specificity of LTA4H**—The total loss of aminopeptidase activity in [E271Q]LTA4H points to a critical role of Glu-271 in this catalytic mechanism. Several lines of evidence indicate that this residue acts as an anionic binding site for peptide substrates. Thus, in the structure of LTA4H in complex with bestatin, Glu-271 makes hydrogen bonds to the free amine of the inhibitor, which chemically resembles a peptide substrate (22). Furthermore, Glu-271 is ideally positioned in the structure to bind the N terminus of Ala-p-NA or an arginyl tripeptide, the preferred peptidase substrate of LTA4H. In addition, this residue, or any equivalent functionality, is absent in the structure of thermolysin, a classical zinc endopeptidase that accommodates peptide substrates of any length (39). In agreement with the crystallographic data, modeling of Ala-p-NA, a chemically good mimic of a tripeptide substrate into the active site of LTA4H, positions Glu-271 within hydrogen bonding distance to the terminal amino group of the substrate (Fig. 4). Together, these data imply that Glu-271 acts as an N-terminal recognition site for peptide substrates, and in this capacity Glu-271 will play a critical role for the exopeptidase nature of the peptide-cleaving activity of LTA4H. The drastic effects of mutations on \( k_{cat} \) with abolishment of catalytic activity suggest that Glu-271 may also be involved in the stabilization of the transition-state (Table II). For APN and APA, it has been suggested that such a transition-state involves an interaction between the nitrogen of the N-terminal

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**FIG. 5.** Mechanistic models for the conversion of LTA4 into LTB4 by LTA4H. A, Glu-271 activates a water molecule bound to the zinc to promote an acid-induced opening of the epoxide and the generation of a carboxylation intermediate. For details see “Discussion.” B, Glu-271 forms an ester intermediate at C6 of LTA4 followed by a concerted nucleophilic attack at C12 and migration of negative charge over the conjugated triene system. In the final step of the reaction sequence, the carboxylate is regenerated via an alkyl-oxygen bond cleavage. For further details, see “Discussion.”

**FIG. 6.** Model for the aminopeptidase activity of LTA4H. Glu-296 acts a general base and polarizes a water molecule for nucleophilic attack at the carbonyl carbon of the scissile peptide bond. Tyr-383 donates a proton to the peptide nitrogen, and Glu-271 binds the terminal amino group. For details, see “Discussion.”
Glutaminase is a bifunctional residue in LTA4 hydrolase

amino group of the peptide substrate and the catalytic zinc (18, 19). However, in our model for the binding of Ala-p-NA, the distance between these two atoms is too long to allow such an interaction (Fig. 4). Instead, the model indicates that the amino group of the substrate is positioned within hydrogen-bonding distance to Glu-318.

The model for binding of Ala-p-NA is in excellent agreement with previous biochemical and mutational data and implies that Glu-271, as well as Glu-296 and Tyr-383, can be fitted into a general base mechanism for the aminopeptidase activity (Fig. 1). Further-

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REFERENCES

1. Samuelsson, B. (1983) Science 2 20, 568-575
2. Samuelsson, B., Dahlén, S.-E., Lindgren, J.-Å., Rouzer, C. A., and Serhan, C. N. (1987) Science 2 37, 1171-1176
3. Ford-Hutchinson, A. W., Bray, M. A., Doig, M. V., Shipley, M. E., and Smith, M. J. H. (1988) Nature 3 260, 364-365
4. Griffiths, R. J., Smith, M. A., Boachie, M. L., Stock, J. L., Slam, E. J., Milici, A. J., Scampoli, D. N., Esksa, J. D., Byrum, R. S., Koller, B. H., and McNeil, J. D. (1997) J. Exp. Med. 1 85, 1123-1129
5. Bailie, M. B., Standiford, T. J., Laichalk, L. L., Coffey, M. J., Strieter, R., and Peters-Golden, M. (1996) J. Immunol. 1 57, 5221-5224
6. Mancuso, P., Nana-Sinkam, P., and Peters-Golden, M. (2001) Infect. Immun. 6 9, 2011-2016
7. Chen, X. S., Shelier, J. R., Johnson, E. N., and Funk, C. D. (1994) Nature 3 72, 179-182
8. Byrum, R. S., Goulet, J. L., Snowvaert, J. N., Griffiths, R. J., and Koller, B. H. (1999) J. Immunol. 1 63, 6810-6819
9. Yokomizo, T., Izumi, T., Chang, K., Takowa, Y., and Shimizu, T. (1997) Nature 3 87, 620-624
10. Yokomizo, T., Kato, K., Terawaki, K., Izumi, T., and Shimizu, T. (2000) J. Exp. Med. 1 92, 421-431
11. Haeggstrom, J. Z. (2000) Am. J. Resp. Crit. Care Med. 1 61, S25-S31
12. Barret, A. J., Rawlings, N. D., and Woessner, J. F. (1998) in Handbook of Proteolytic Enzymes (Barret, A. J., Rawlings, N. D., and Woessner, J. F., eds), pp. 994-996, Academic Press, London
13. Vallee, B. L., and Audl, D. S. (1990) Biochemistry 2 9, 5647-5659
14. Medina, J. F., Wetterholm, A., Rådmark, O., Shapiro, R., Haeggstrom, J. Z., Vallee, B. L., and Samuelsson, B. (1991) Proc. Natl. Acad. Sci. U. S. A. 8 8, 7620-7624
15. Oening, L., Grenier, J. K., and Fitzpatrick, F. A. (1994) J. Biol. Chem. 2 69, 11209-11237
16. Wetterholm, A., Medina, J. F., Rådmark, O., Shapiro, R., Haeggstrom, J. Z., Vallee, B. L., and Samuelsson, B. (1992) Proc. Natl. Acad. Sci. U. S. A. 9 9, 9141-9145
17. Blomster, M., Wetterholm, A., Mueller, M. J., and Haeggstrom, J. Z. (1995) Eur. J. Biochem. 2 31, 528-534
18. Vazeux, G., Iurriaz, C., Corvol, P., and Lorenz-Cortez, C. (1998) Biochem. J. 3 34, 407-413
19. Luciani, N., Marie-Claire, C., Ruffet, E., Beaumont, A., Roques, B. P., and Fournié-Zaluski, M.-C. (1998) Biochemistry 3 37, 686-692
20. Mueller, M. J., Wetterholm, A., Blomster, M., Jornvall, H., Samuelsson, B., and Haeggstrom, J. Z. (1995) Proc. Natl. Acad. Sci. U. S. A. 9 2, 8383-8387
21. Mueller, M. J., Blomster, M., Oppermann, U. C. T., Jornvall, H., Samuelsson, B., and Haeggstrom, J. Z. (1996) Proc. Natl. Acad. Sci. U. S. A. 9 3, 5931-5935
22. Thunnissen, M. G. M., Nordlund, P., and Haeggstrom, J. Z. (1991) Nat. Struct. Biol. 8, 131-135
23. Sarkar, G., and Sommer, S. S. (1999) BioTechniques 8, 404-407
24. Bradford, M. M. (1976) Anal. Biochem. 7 2, 248-254
25. Wetterholm, A., Haeggstrom, J. Z., Samuelsson, B., Yuan, W., Munoz, B., and Wong, C. H. (1996) J. Pharmacol. Exp. Ther. 2 75, 31-37
26. Owinowski, Z. (1995) Data Collection and Processing. Proceedings of the CCP 4 Study Weekend, pp. 56-62, SERC Daresbury Laboratory, Warrington, UK
27. Navaza, J. (1994) Acta Crystallogr. Sect. A 5 0, 157-163
28. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J.-S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. Sect. D Biol. Crystallogr. 5 4, 905-921
29. McRee, D. E. (1999) J. Struct. Biol. 1 25, 156-165
30. Morris, G. M., Goodsell, D. S., Halliday, R. S., Huey, R., Hart, W. E., Belew, R. K., and Olson, A. J. (1998) J. Comput. Chem. 1 9, 1639-1662
31. Stewart, J. J. P. (1990) J. Comput. Aided Mol. Des. 4, 1–45
32. Hogg, J. H., Ollmann, I. R., Wetterholm, A., Blomster Andberg, M., Haeggstrom, J. Z., Samuelsson, B., and Wong, C.-H. (1998) Chem. Eur. J. 4, 1697-1713
33. Arand, M., Grant, D. F., Beetham, J. K., Friedberg, T., Oesch, F., and Hammock, B. D. (1994) FEBS Lett. 3 38, 251-256
34. Arand, M., Wagner, H., and Oesch, F. (1996) J. Biol. Chem. 2 71, 4223-4229
35. Argiriadi, M. A., Morisseau, C., Hammock, B. D., and Christianson, D. W. (1999) Proc. Natl. Acad. Sci. U. S. A. 9 6, 10637-10642
36. Zeu, J., Hallberg, B. M., Bergfors, T., Oesch, F., Arand, M., Mowbray, S. L., and Jones, T. A. (2000) Structure 8, 111-122
37. Haeggstrom, J. Z., Meijer, J., and Rådmark, O. (1996) J. Biol. Chem. 2 61, 6332-6337
38. March, J. (1985) Advanced Organic Chemistry, John Wiley & Sons, New York
39. Holmes, M. A., and Matthews, B. W. (1982) J. Mol. Biol. 1 60, 623-639
40. Laustsen, P. G., Vang, S., and Kristensen, T. (2001) Eur. J. Biochem. 2 68, 98–104
41. Gues, N., and Peitsch, M. C. (1997) Electrophoresis 1 8, 2714–2723
Leukotriene A₄ Hydrolase/Aminopeptidase: GLUTAMATE 271 IS A CATALYTIC RESIDUE WITH SPECIFIC ROLES IN TWO DISTINCT ENZYME MECHANISMS

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