Leukotrienes A\textsubscript{4} Hydrolase/Aminopeptidase, the Gatekeeper of Chemotactic Leukotriene B\textsubscript{4} Biosynthesis*  

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The leukotrienes (LTs)\textsuperscript{1} are a family of lipid mediators that play important roles in a variety of allergic and inflammatory reactions (1, 2). These molecules are formed by leukocytes and are divided into two classes, the spasmodenic cysteinyl leukotrienes and LTB\textsubscript{4}, which is a classical chemoattractant that triggers adherence and aggregation of leukocytes to the endothelium at nanomolar concentrations. Recent data also indicate that LTB\textsubscript{4} is a chemoattractant for T-cells, creating a functional link between early innate and late adaptive immune responses to inflammation (3–5). In addition, LTB\textsubscript{4} participates in the host defense against infections (6) and is a key mediator of platelet-activating factor-induced lethal shock (7). Because of these powerful biological effects, LTB\textsubscript{4} is regarded as an important chemical mediator in a variety of acute and chronic inflammatory diseases, e.g. nephritis, arthritis, dermatitis, and chronic obstructive pulmonary disease (8). Moreover, only recently, several lines of pharmacological, morphological, biochemical, and genetic evidence have been gathered implicating LTs, in particular LTB\textsubscript{4}, as a mediator of vascular inflammation and arteriosclerosis (9). This article gives an overview of the biochemical, structural, and catalytic properties of LTA\textsubscript{4} hydrolase (LTA4H), which catalyzes the final and committed step in LTB\textsubscript{4} biosynthesis.  

LTA\textsubscript{4} Hydrolase is a Key Enzyme in the 5-Lipoxygenase Pathway  

In cellular biosynthesis of LTs, 5-lipoxygenase, assisted by 5-lipoxygenase-activating protein, converts arachidonic acid into the unstable epoxide LTA\textsubscript{4}, which in turn may be enzymatically conjugated with GSH to form LTC\textsubscript{4}, the parent compound of the cysteinyl leukotrienes, or hydrolyzed into LTB\textsubscript{4} by LTA4H (Fig. 1). Leukotrienes can also be formed via transcellular routes, where LTA\textsubscript{4} is donated from an activated leukocyte to a recipient cell for further metabolism by downstream enzymes, a process that was recently shown to occur in vivo (10). LTB\textsubscript{4} signals via a specific, high affinity, G-protein-coupled receptor (BLT\textsubscript{1}) (11). In addition, a second receptor for LTB\textsubscript{4} (BLT\textsubscript{2}) has been discovered, the functional role of which is presently not known (12). Interestingly, LTB\textsubscript{4} is also a natural ligand of the peroxisome proliferator-activated receptor \(\alpha\) class of nuclear receptors and has been suggested to play a role in lipid homeostasis (13).  

Leukotriene A\textsubscript{4} Hydrolase Is a Zinc-dependent Epoxide Hydrolase and Aminopeptidase  

LTA4H is a monomeric soluble protein that is widely distributed and has been purified from several mammalian sources (14). It resides in the cytosol, although nuclear localization has also been reported recently (15). The cDNAs encoding the human, mouse, rat, and guinea pig enzymes have been cloned and sequenced. The proteins contain 610 amino acids, excluding the first Met, and the calculated molecular mass of the human enzyme is 69,153 Da. The primary, secondary, and tertiary structures of LTA4H bear no resemblance to soluble xenobiotic epoxide hydrolase, although this enzyme also accepts LTA\textsubscript{4} as substrate (16). Human LTA4H exists as a single copy gene with a size of >35 kbp on chromosome 12q22 (17). The coding sequence is divided into 19 exons ranging in size from 24 to 312 bp, and the 5‘ upstream region (approximately 4 kbp) contains a phorbol ester response element (AP-2) and two xenobiotic response elements, the functional significance of which is presently unknown.  

LTA4H is highly substrate-specific. Besides LTA\textsubscript{4}, only the double bond isomers LTA\textsubscript{5} and LTA\textsubscript{3} are turned over by LTA4H, albeit at a low rate (18, 19). Typically, LTA4H is inactivated and covalently modified by its substrate LTA\textsubscript{4} during catalysis (18, 19). Using differential peptide mapping and site-directed mutagenesis, Tyr-378 has been identified as the site of attachment between lipid and protein, and the catalytic properties of the mutated protein [Y378F]LTA4H suggest that this residue may assist in the proper alignment of LTA\textsubscript{4} in the substrate binding pocket (20, 21). Although it has been reported that LTA4H may be phosphorylated at Ser-415 under certain conditions (22), no other specific post-translational modifications seem to occur.  

Sequence comparisons between LTA4H and several zinc hydrolases, e.g. aminopeptidase M and thermolysin, led to the discovery of a catalytic zinc site with the signature HEXXH\textsubscript{18}, and subsequent analysis by atomic absorption spectrometry revealed the presence of 1 mol of zinc/mol of protein (Fig. 2). As suspected from the homology to zinc proteases, LTA4H was found to possess a peptide cleaving activity. Unlike the epoxide hydrolase activity, i.e. the transformation of LTA\textsubscript{4} into LTB\textsubscript{4}, the aminopeptidase activity is activated by monovalent anions and albumin. It accepts a variety of substrates, and certain arginyl di- and tripeptides as well as \(\beta\)-nitroanilide derivatives of Ala and Arg are hydrolyzed with high efficiencies (23). Although it has never been experimentally verified, it is generally assumed that the aminopeptidase activity is involved in the processing of peptides related to inflammation and host defense.  

The Zinc Site and Catalytic Residues  

The three zinc binding ligands of the signature HEXXH\textsubscript{18} corresponds to His-295, His-299, and Glu-318 in LTA4H, and mutation of any of these residues leads to loss of the metal and catalytic activity (24). The conserved Glu-296 in the motif HEXXH was identified as the critical general base of the peptidase reaction without any apparent function in the ep-
oxide hydrolase reaction (25, 26). Furthermore, sequence comparisons with aminopeptidase N suggested that Tyr-383 might act as a proton donor in peptidolysis. Indeed, mutation of this residue resulted in selective abrogation of the aminopeptidase activity, thus supporting a catalytic role for Tyr-383 (27).

Several candidate catalytic residues were identified from the crystal structure of LTA4H (see below). Glu-271 is a component of a GXMEN motif, which is conserved among members of the M1 family of metallopeptidases and proposed to play a role in peptide substrate binding (28). We used mutagenesis and crystallography to detail the role of individual residues within the GXMEN motif and found that Glu-271 is indeed required for the peptidase activity and, unexpectedly, also for the epoxide hydrolase activity (29). With the same technique, it was demonstrated that Asp-375, located in a putative LTA4 binding pocket, is required for hydrolysis of LTA4 into LTB4 but not for the aminopeptidase activity (30). In addition, analysis of an Arg/Lys couple located at the entrance of the catalytic zinc site demonstrated that Arg-563 is a carboxylate recognition site that plays a key role in the epoxide hydrolase reaction and binds the C terminus of peptide substrates, assisted by Lys-565 (31).

Crystal Structure of LTA4 Hydrolase

The structure of LTA4H in complex with the competitive inhibitor bestatin has been determined at 1.95-Å resolution (32). The protein molecule is folded into three domains: N-terminal, catalytic, and C-terminal, that are packed in a flat triangular arrangement with approximate dimensions 85 × 110 × 50 Å3 creating a deep cleft in between (Fig. 3). The N-terminal domain has a large concave and hydrophobic surface area and is structurally similar to bacteriochlorophyll a (33). The fold of the catalytic domain is very similar to that of thermolysin although the sequence identity is only about 7% over the corresponding polypeptide chains. The C-terminal domain has structural features resembling a so-called armadillo repeat or HEAT region, which in turn suggests that it may take part in protein-protein interactions (34).

The zinc site is located at the bottom of the interdomain cleft. As predicted, the metal is bound to His-295, His-299, and Glu-318, with bestatin as the fourth ligand in a pentavalent coordination. In the vicinity of the prosthetic zinc, the catalytic residues Glu-271, Glu-296, and Tyr-383 are located. Behind the pocket occupied by the phenyl ring of bestatin there is an L-shaped hydrophobic cavity 6–7 Å wide, which stretches 15 Å deeper into the protein (Fig. 3). One patch of the cavity is hydrophilic, with Gln-134, Asp-375, and the hydroxyl of Tyr-267 clustering together. This cavity was probed by structural determination of complexes between LTA4H and specific, active site-directed inhibitors, some of which have been designed as LTA4 mimics (35). Indeed, the hydrophobic tail of the inhibitors, corresponding to the fatty acid backbone of LTA4, is buried into the narrow hydrophobic pocket, strongly indicating that it functions as a substrate binding cavity (Fig. 4).

Proposed Mechanism of the Epoxide Hydrolase Reaction

The stereochemistry at C-12 and double bond geometry are key structural determinants for the biological activity of LTB4. Consequently, the role of LTA4H during enzymatic hydrolysis of LTA4 into LTB4 is to generate the 12R epimer of the hydroxyl
LTA4. Arg-563 acts as a critical carboxylate recognition site. For further double bond geometry is controlled by the binding conformation of added at C-12 in a stereospecific manner, directed by Asp-375. The activation of the epoxide to form a carbocation intermediate. Water is polarized by the base, Glu-296, and attacks the substrate gets attached to Glu-271, acting as an N-terminal recognition group of the incoming tripeptide substrate. The lytic zinc is an activated water molecule that is displaced by a carbonyl is required for catalysis (19, 36). Furthermore, the catalytic zinc as well as Glu-271 will be proximal to the labile allylic group and to form the $\Delta^6$-cis-$\Delta^8$-trans-$\Delta^{10}$-trans configuration of the conjugated triene. This reaction is unique and requires control of the stereospecific introduction of a hydroxyl group at a site (C-12) distant from the reactive epoxide moiety (C-5—C-6). The crystal structure gives several important clues as to how LTA4H can execute its sophisticated chemistry.

If LTA4 is modeled into the hydrophobic pocket such that the 5,6-epoxide moiety is bound to Zn$^{2+}$, then C-7 to C-20 of the fatty acid backbone of LTA4 fits snugly into the deeper cavity, adopting a bent conformation (Fig. 4). Furthermore, the C-1 carboxylate can make direct electrostatic interactions with the positive charge of Arg-563. This crucial interaction will also ensure perfect substrate alignment required for catalysis (31), in agreement with the fact that the free carboxylic acid of LTA4 is required for catalysis (19, 36). Furthermore, the catalytic zinc as well as Glu-271 will be proximal to the labile allylic epoxide, suggesting that they will polarize a water molecule and promote an acid-induced activation and opening of the epoxide ring (Fig. 4). A carboxylation will be generated, according to an $S_{\text{n}}1$ reaction, whose charge is delocalized over the conjugated triene system (C-6 to C-12) leaving the planar sp$^2$ hybridized C-12 open for nucleophilic attack from either side of the molecule. In this model, Asp-375 would direct a water molecule for attack at C-12 and thus control the positional and stereospecific insertion of the 12R-hydroxy group in LTB$_4$, in agreement with mutational data (30).

Moreover, the shape and curvature of the LTA4 binding cavity also suggest the chemical strategy for creation of the 6-cis double bond in LTB$_4$. Because there is free rotation between C-6 and C-7 of LTA4, the enzyme may keep this bond in a "pro-cis" configuration in the transition state, which would promote the formation of a cis double bond from the carboxylation intermediate (Fig. 4). The entire modeled LTA4 molecule would then adopt a bent shape that fits very well with the architecture of the binding pocket. Hence, the critical double bond geometry at $\Delta^5$ in LTB$_4$ seems to be controlled by the exact binding conformation of LTA4 at the active site.

**Proposed Mechanism for the Aminopeptidase Activity**

In agreement with what has been discussed for thermolysin, the peptide cleaving activity of LTA4H most likely proceeds according to a general base mechanism (37). Thus, the catalytic zinc is complexed to its three amino acid ligands and an activated water molecule. The water is displaced from the zinc atom by the carbonyl oxygen of the substrate, which in turn gets anchored to the active site via its N-terminal $\alpha$-amino group binding to Glu-271. In this role, Glu-271 will stabilize the transition state and also contribute to the exopeptidase specificity of the enzyme (Fig. 4). The water molecule is simultaneously polarized by the carboxylate of Glu-296 to promote an attack on the carbonyl carbon of the scissile peptide bond. At the same time, a proton is transferred to the nitrogen of the peptide bond by Tyr-383.

**Two Catalytic Activities Exerted via Specific but Overlapping Active Sites**

Compilation of information from biochemical studies, mutational analysis, and x-ray crystallography leads to the conclusion that the two enzyme activities of LTA4H are exerted via distinct and yet overlapping active sites (Figs. 1 and 4). Thus, certain residues are specifically required for the aminopeptidase reaction, i.e. Glu-296 and Tyr-383, whereas Asp-375 is critical only for the epoxide hydrolase reaction. On the other hand, Glu-271, Arg-563, and the zinc atom are necessary for both catalyses. In fact, Glu-271 is a unique example of a residue that is shared between two catalytic machineries, yet carrying out a separate chemistry in each of the two enzyme reactions.

**Molecular Evolution of LTA4 Hydrolase**

Based on its zinc signature and aminopeptidase activity, LTA4H is classified as a member of the M1 family of zinc metallopeptidases, which includes enzymes such as aminopeptidase A, aminopeptidase B, and aminopeptidase N (28). However, the epoxide hydrolase activity appears to be unique for LTA4H and has not been detected with certainty in any human homologue, although conflicting data have been reported for aminopeptidase B (38, 39). LTA4H is present in several lower vertebrates including fish and frogs but not in lower species (40, 41). For instance, aminopeptidase 1 from *Caenorhabditis elegans*, which is 45% identical (63% similar) at the amino acid level to mammalian LTA4H, fails to hydrolyze LTA4 into LTB4 (42). On the other hand, an LTA4H that is 39% identical (53% similar) to the human enzyme has been cloned and characterized from yeast, *Saccharomyces cerevisiae* (43). The *S. cerevisiae* LTA4 is a zinc leucyl aminopeptidase with a primitive epoxide hydrolase activity against LTA4. Furthermore, binding of LTA4 to the active site leads to inactivation of the epoxide hydrolase activity and strong activation of the peptidase activity.

Together these data suggest that LTA4H has developed from an ancestral aminopeptidase, which initially possessed an al-
losteric lipid binding site. During evolution, the architecture was remodeled into an active site accommodating LTA₄. Subsequent structural optimizations further improved substrate alignment and finally allowed efficient catalysis and formation of LTB₄.

**LTA₄ Hydrolase, an Attractive Target for Structure-based Drug Design**

The specific roles of LTA₄ in acute and chronic inflammation have been mapped and corroborated by several animal models of perturbed biosynthesis or signaling, targeting all components of the biosynthetic pathway (7, 44–48). Together, this wealth of *in vivo* data points to LTA₄H as a potential target for development of anti-inflammatory drugs. Moreover, the pharmacological interest has been increased even further by the recent observations of increased protein expression in esophageal cancer and graft versus host disease following hematopoietic stem cell transplantation (49, 50).

Bestatin and captopril, inhibitors of aminopeptidases and angiotensin-converting enzyme, respectively, also inhibit LTA₄H (51). In addition, ω-(ω-arylalkyl)aryalkanoic acids and ketorolphan, a known inhibitor of enkephalin-degrading enzymes, are potent inhibitors (52, 53).

Several academic and industrial laboratories have developed more powerful and selective compounds. An α-keto-β-amino ester, a thioamine, and a hydroxamic acid were synthesized and found to be effective, tight binding inhibitors with IC₅₀ values in the low micromolar to nanomolar range (54, 55). Other series of potent inhibitors have also been developed by Searle, in particular SC-57461A, 3-[methyl[3-(4-phenylmeth-yl)phenoxylpropyl]amino]propanoic acid, which blocks inophore-induced LTB₄ production in human whole blood with an IC₅₀ of 49 nM, is orally active, and blocks arachidonic acid-induced ear edema in the mouse (56, 57). With the three-dimensional structure of LTA₄H at hand, it will now be possible to conduct rational structure-based drug design to tailor potent and selective inhibitors with desired pharmacological properties.

**REFERENCES**

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