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CATALYTIC MECHANISMS AND EVOLUTION OF LEUKOTRIENE A₄ HYDROLASE

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ABSTRACT

Inflammation is the first response of the body to infection or physical irritation. This response can potentially trigger the whole immune system but in the initial stage mainly involves leukocytes of the innate immune system and a complex cascade of chemical mediators, which by different means control, maintain and resolve the process. One group of such mediators is the leukotrienes, among which LTB₄ is found. LTB₄ has several immunomodulating properties and mainly acts by recruiting leukocytes to the site of injury or infection. LTB₄ is mainly formed by leukocytes of the innate immune system, but recruits cells of both the innate as well as the adaptive immune systems. Thus, it constitutes an important link between the two systems. Moreover, LTB₄ is known to be involved in several pathological inflammatory conditions.

Leukotriene A₄ hydrolase (LTA₄H) is a bifunctional zinc metalloenzyme that catalyzes the last step in the formation of LTB₄. In addition, LTA₄H catalyzes hydrolysis of oligo-peptides. Hence, the enzyme is bifunctional and the two activities of LTA₄H actually share a common active site. While LTB₄ has a characterized biological functions in the inflammatory response, the physiological relevance of the peptidase activity of LTA₄H is yet unknown.

According to sequence homology, LTA₄H sorts as a member of the M1 family of aminopeptidases, a vast enzyme family found in most organisms and with a variety of biological functions. Among these peptidases, a subset present in vertebrates has the intrinsic capacity to catalyze LTA₄→LTB₄ formation.

The present investigations deal with the catalytic mechanism of both activities of LTA₄H, as well as the divergent evolution of LTA₄H from ancestral aminopeptidases. For the first part of the work, the recently determined crystal structure of LTA₄H served as a guideline for the design of experiments. In these, an approach mainly including mutagenesis, enzyme kinetics, molecular modeling and crystallography led to determination of the basic requirements for catalysis by LTA₄H as well as an insight into the molecular events underlying its evolution from ancestral aminopeptidases. To refine these findings, a novel assay for enzyme kinetics was developed, which allowed a faster and more adequate analysis of the peptidase activity. Finally, the novel assay in combination with crystal structure determinations of LTA₄H in complex with different ligands, allowed a yet deeper understanding of the reaction mechanism for peptide hydrolysis catalyzed by LTA₄H.

For the peptidase activity it was specifically shown that the peptide substrate is anchored between Arg-563 and Glu-271 with its C- and N-terminal, respectively. Together, these two residues function as an effective filter which selectively favors peptide substrates consisting of three residues, *i.e.* tri-peptides. The specific preference of the enzyme for arginyl tri-peptides is achieved by interaction between basic N-terminal groups of the substrate and Asp-375. During the course of peptide hydrolysis, Tyr-383 together with the zinc ion function as a site for oxyanion stabilization of the reaction intermediate. The general base Glu-296, not only facilitates the nucleophilic attack by the hydrolytic water, but also functions as a proton shuffle, which in the last step of peptide hydrolysis protonates the leaving amine. Considering sequence homology, these findings to a large extent holds true also for other metallopeptidases of the MA clan and specifically for aminopeptidases of the M1 family.

For the epoxide hydrolase activity, it was shown that the carboxylate group of LTA₄ binds to Arg-563 to achieve proper positioning, with respect to catalytic residues, of the reacting moieties of the substrate. During LTA₄ hydrolysis, Glu-271 and Asp-375 are required for epoxide ring opening and for catalyzing the stereospecific attack by the hydrolytic water at carbon 12 of LTA₄.

Notably, Arg-563 and Glu-271 are essential for both reaction mechanisms. While Arg-563 serves the same purpose in both reaction mechanisms, *i.e.* binding of substrate carboxylates, Glu-271 has distinct roles in each reaction. The latter observation, with a single residue serving in two different catalytic mechanisms, is unique to LTA₄H.

Often, assaying peptidase activity either involves complex assays, when utilizing natural substrates, or relies on chromogenic model substrates of limited physiological relevance. The novel assay developed circumvents these problems and allows simple and fast screening of natural peptide substrates and determination of kinetic constants for their hydrolysis.

For the evolutionary studies, the yeast homologue of human LTA₄H, an aminopeptidase with substrate specificities distinct from human LTA₄H, was used. This enzyme is activated by LTA₄ but also to some extent hydrolyzes it. For peptide hydrolysis, it was shown that the yeast enzyme uses the corresponding residues as human LTA₄H. Additionally, it was shown that the active site pocket of the yeast enzyme allows LTA₄ to bind in two conformations: one peptidase-activating and one compatible with LTA₄ hydrolysis. A few point mutations of the yeast enzyme, which made it more similar to human LTA₄H, sufficed to reengineer the pocket to more resemble the corresponding human one with LTA₄-inhibition replacing the LTA₄-activating effect. Thus, it appears as LTA₄H through evolution has fine-tuned an existing lipid binding site to optimize it for LTA₄-binding and turnover.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

- I. Leukotriene A₄ Hydrolase/Aminopeptidase – Glutamate 271 is a catalytic residue with specific roles in two distinct enzyme mechanisms.
Peter C. Rudberg, Fredrik Tholander, Marjolein M.G.M. Thunnissen & Jesper Z. Haeggström (2002)
J. Biol. Chem. **277**, 1398-1404
- II. Leukotriene A₄ hydrolase: Selective abrogation of leukotriene B₄ formation by mutation of aspartic acid 375.
Peter C. Rudberg, Fredrik Tholander, Marjolein M.G.M. Thunnissen, Bengt Samuelsson & Jesper Z. Haeggström (2002)
Proc. Natl. Acad. Sci. U.S.A **99**, 4215-4220
- III. Leukotriene A₄ Hydrolase – Identification of a common carboxylate recognition site for the epoxide hydrolase and aminopeptidase substrates.
Peter C. Rudberg, Fredrik Tholander, Martina Andberg, Marjolein M.G.M. Thunnissen & Jesper Z. Haeggström (2004)
J. Biol. Chem. **279**, 27376-27382
- IV. Leukotriene A₄ hydrolase, insights into the molecular evolution by homology modeling and mutational analysis of enzyme from *Saccharomyces cerevisiae*.
Fredrik Tholander, Filippa Kull, Eva Ohlson, Jawed Shafqat, Marjolein M.G.M Thunnissen & Jesper Z. Haeggström (2005)
J. Biol. Chem. **280**, 33477-33486
- V. Assay for rapid analysis of the tri-peptidase activity of LTA₄ hydrolase.
Fredrik Tholander & Jesper Z. Haeggström (2006)
Manuscript
- VI. Structural basis for peptide hydrolysis by M1 aminopeptidases.
Fredrik Tholander, Marjolein M.G.M Thunnissen & Jesper Z. Haeggström
Manuscript



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LIST OF ABBREVIATIONS

12HD/PGR	12-hydroxyl-LTB ₄ dehydrogenase/prostaglandin reductase
5-LO	5-lipoxygenase
5S,6S-DHETE	5S,6S-dihydroxy-7,9- <i>trans</i> -11,14- <i>cis</i> -eicosatetraenoic acid
AA	arachidonic acid, 5,8,11,14- <i>cis</i> -eicosatetraenoic acid
ACE	angiotensin converting enzyme
AP	aminopeptidase
C5a	complement factor 5a
COX	cyclooxygenase
cPLA ₂	cytosolic phospholipase A ₂
CYP	cytochrome P450
cysLT	cysteinyl leukotriene
C- α	α -carbon
DHETE	Dihydroxy-eicosa-tetraenoic acid
ER	endoplasmatic reticulum
FLAP	5-lipoxygenase activating protein
HETE	Hydroxy-eicosa-tetraenoic acid
HPLC	high performance liquid chromatography
HSA	human serum albumin
iNOS	inducible nitric oxide synthase
LT	leukotriene
LTA ₃	leukotriene A ₃ , 5S- <i>trans</i> -5,6-oxido-7,9- <i>trans</i> -11- <i>cis</i> -eicosatetraenoic acid
LTA ₄	leukotriene A ₄ , 5S- <i>trans</i> -5,6-oxido-7,9- <i>trans</i> -11,14- <i>cis</i> -eicosatetraenoic acid
LTA ₄ H	LTA ₄ hydrolase
LTA ₅	leukotriene A ₅ , 5S- <i>trans</i> -5,6-oxido-7,9- <i>trans</i> -11,14,17- <i>cis</i> -eicosatetraenoic acid
LTB ₄	leukotriene B ₄ , 5S,12R-dihydroxy-6,14- <i>cis</i> -8,10- <i>trans</i> -eicosatetraenoic acid
LTC ₄	leukotriene C ₄ , 5S-hydroxy-6R-S-glutathionyl-7,9- <i>trans</i> -11,14- <i>cis</i> -eicosatetraenoic acid
LTD ₄	leukotriene D ₄ , 5S-hydroxy-6R-S-cysteinylglycyl-7,9- <i>trans</i> -11,14- <i>cis</i> -eicosatetraenoic acid
LTE ₄	leukotriene E ₄ , 5S-hydroxy-6R-S-cysteinyl-7,9- <i>trans</i> -11,14- <i>cis</i> -eicosatetraenoic acid
LTC ₄ S	Leukotriene C ₄ synthase
MHC	major histocompatibility complex
NADP	nicotinamide adenine dinucleotide phosphate
NOS	nitric oxide synthase
PAF	platelet activating factor
PMN	polymorphonuclear neutrophil
<i>p</i> -NA	<i>para</i> -nitroanilide
PPAR	peroxisome proliferator-activated receptor
r.m.s.d	root mean square deviation
<i>sc</i> LTA ₄ H	<i>Saccharomyces cerevisiae</i> LTA ₄ hydrolase
sEH	soluble epoxide hydrolase
siRNA	short interfering RNA
SPR	surface plasmon resonance
TIFF3	tricorn interacting factor F3

1 INTRODUCTION

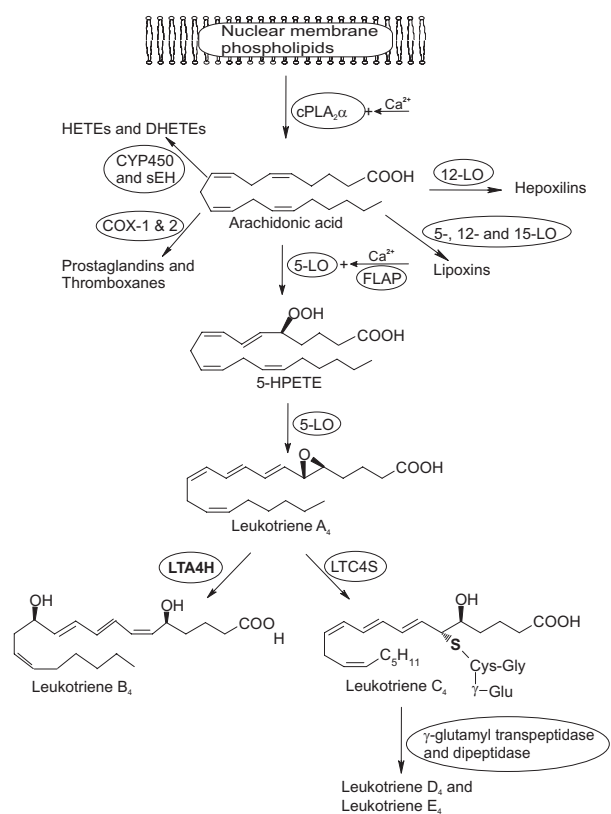
Inflammation is a defensive non-specific reaction by tissues subjected to injury, infection or chemical agents that involves a complex array of chemical mediators serving a multitude of functions. Some of these substances aim at the recruitment of leukocytes to the affected tissue thus constituting the initial signal that triggers the first line defense of the host and subsequently the whole immune system. While tissues locally affected by inflammation exhibit characteristic signs, *e.g.* local pain, edema and hyperemia, the whole organism may also be affected by the severe effects of a systemic inflammation.

One group of substances responsible for such signaling events are the eicosanoids, all oxygenated fatty acid derivatives of arachidonic acid (AA). This family of structurally related substances acts locally as paracrine hormones and includes subgroups such as leukotrienes (LT), prostaglandins, thromboxanes, prostacyclin and the lipoxins.

The enzyme leukotriene A₄ hydrolase (LTA₄H) is involved in leukotriene biosynthesis by catalyzing the formation of leukotriene B₄ (LTB₄), a potent chemoattractant and immunomodulating agent. As such, LTB₄ is pivotal to the inflammatory response and renders LTA₄H particularly interesting for medical and detailed biochemical studies.

2 LEUKOTRIENE BIOSYNTHESIS

This thesis deals with LTA₄H, an enzyme involved in LT metabolism, therefore focus will naturally be on this branch of the AA metabolic tree (Fig. 1). Biosynthesis of LTs occurs in response to increased levels of the second messenger calcium, triggered by various cellular stimuli. The increased level of calcium activates cytosolic phospholipase A₂ (cPLA₂) leading to its translocation to the nuclear membrane and assembly, by as yet not fully understood mechanisms, with other proteins involved in downstream metabolism of LTs, *i.e.* 5-lipoxygenase (5-LO), 5-lipoxygenase activating protein (FLAP), LTA₄H and leukotriene C₄ synthase (LTC₄S) [1, 2]. At the nuclear membrane cPLA₂ specifically catalyzes hydrolysis of the sn-2 ester bound arachidonic acid of phospholipids (phospholipases constitutes a large group of enzymes but the subtype cPLA₂ α of the GIV subgroup is of greatest importance in the context of LT biosynthesis). Via crucial interplay with the nuclear membrane protein FLAP, the liberated AA is transferred to the cytosolic enzyme 5-LO, which is also recruited to the nuclear membrane upon cell stimulation and activated by, for instance, the



Figur 1. The leukotriene pathway. Schematic representation of leukotriene metabolism and enzymes acting therein. Other pathways for arachidonic acid metabolism and the related key enzymes are also indicated. Increased levels of the second messenger Ca²⁺ activate cPLA₂, which liberates arachidonic acid from phospholipids of the nuclear membrane. The increased Ca²⁺ levels also activate the downstream enzyme 5-LO. It is generally believed that enzymes involved in LT biosynthesis upon activation gather at the nuclear membrane. LTA₄H, the subject of this thesis is given in bold type, catalyzes the stereospecific hydrolysis of LTA₄ into LTB₄. See text for further details. All enzymes are circled. Given enzyme acronyms are: cPLA₂, cytosolic phospholipase A₂; LO, lipoxygenase; LTA₄H, leukotriene A₄ hydrolase; LTC₄S, leukotriene C₄ synthase; CYP450, cytochrome P450; sEH, soluble epoxide hydrolase; COX, cyclooxygenase. Metabolite acronyms: HETE, hydroxy-eicosa-tetraenoic-acid; DHETE, dihydroxy-eicosa-tetraenoic-acid.

increased levels of calcium and membrane proximity [3, 4]. In two consecutive steps, 5-LO then oxidizes AA by the incorporation of molecular oxygen [5, 6]. The result of this reaction is Leukotriene A₄ (LTA₄), a highly unstable compound containing an epoxide allylic to a conjugated triene system. LTA₄ defines a branching point in LT metabolism and is the starting metabolite for the two groups of biologically active LTs. Thus, one alternative for further metabolism of LTA₄ includes conjugation with glutathione to yield the first cysteinyl containing LT (cysLT) LTC₄, the precursor of the smooth muscle constrictors LTD₄ and LTE₄, which mediate their effects via so-called CysLT receptors [7]. Membrane bound peptidases are responsible for trimming of the peptide moieties of the cysLTs, thereby converting LTC₄ into LTD₄ and LTE₄ [8-10]. Alternatively, LTA₄ can be stereo specifically hydrolyzed by LTA4H (the subject of this thesis) to give LTB₄.

2.1 OTHER AA METABOLIC PATHWAYS

Besides the 5-LO initiated pathway AA can also meet other metabolic destinies via the action of various oxidative enzymes (Fig. 1). For instance, while different catalytic actions of 5-, 12- or 15-lipoxygenase on AA can generate lipoxins, substances believed to possess anti-inflammatory actions [11, 12], 12-lipoxygenase is also able to initiate the formation of hepoxilins, substances first described as regulators of insulin secretion [13, 14]. A type of cyclic oxidation of AA, which generates ring-structured molecules, is carried out by the cyclooxygenase enzymes COX-1 and -2 and initiates the formation of the prostaglandins and thromboxanes, a vast family of potent mediators with a multitude of different effects [15]. In addition, certain members of the cytochrome P450 (CYP) family of enzymes can oxidize AA to produce yet other biologically active compounds, which in turn can be further metabolized by, for instance soluble epoxide hydrolase [16, 17].

The broad tissue distribution of LTA4H in contrast to the limited occurrence of cells that express 5-LO, and thereby have the ability to synthesize LTA₄, has led to the exploration of possible transcellular routes for metabolism of LTA₄. Indeed, such processes have been described in erythrocytes and platelets [24, 25].

The sub-cellular localization of LTA4H is believed to be mainly cytosolic [26] but nuclear co-localization with 5-LO in rat alveolar macrophages and basophilic leukemia cells has been described to occur under certain circumstances [27]. Also certain lung cells lacking 5-LO, and thereby the ability to autonomously produce LTB₄, have LTA4H localized to the nucleus in a manner correlating with cell differentiation [28]. These reports suggest that the sub-cellular localization of LTA4H might be dynamic and variable with respect to cell type and physiological state.

3 LTA4H TISSUE DISTRIBUTION AND LTB₄ BIOSYNTHESIS

LTA4H is expressed in most cells of the body but its cellular presence is not sufficient for LTB₄ biosynthesis, since this obviously requires the presence of its precursor LTA₄, which in turn requires the presence of other AA metabolizing enzymes, see section 2 above. Thus, production of LTA₄ is restricted to cells expressing 5-LO, *i.e.* certain leukocytes, which consequently are the only cells that have the full machinery for LTB₄ production. Particularly phagocytic leukocytes produce LTB₄, *e.g.* neutrophils [18, 19], monocytes and macrophages [20-23].

4 LTB₄ – A LEUKOCYTE ATTRACTANT ACTING THROUGH SPECIFIC RECEPTORS

LTB₄ is an extremely potent chemotaxin for leukocytes, originally described to act towards polymorphonuclear neutrophils (PMN). At nanomolar concentrations LTB₄ triggers adherence and aggregation of leukocytes to the blood vessel endothelium subsequently followed by diapedesis and migration of leukocytes into the extracellular space [29]. At higher concentration LTB₄ stimulates neutrophil degranulation leading to increased release of lysosomal enzymes, calcium mobilization and superoxide anion generation [30].

Most of these effects of LTB₄ are probably mediated by an LTB₄-specific G-protein coupled receptor, BLT1 [31]. However, a second receptor has been discovered, BLT2, whose functionality is less characterized [32]. The BLT1 has a higher affinity for LTB₄ (K_D of 1.1 nM), as compared to BLT2 (K_D of 23 nM). Quite obviously, after synthesis LTB₄ needs to cross the cell membrane to reach its receptors, some reports suggest that this process is carrier mediated and energy dependent [33, 34].

The two BLT encoding genes are clustered and actually overlap to some extent and the overall sequence identity is about 45% at the amino acid level. The genomic organization of these genes, as well as their degree of similarity, suggests that they arose by duplication of an ancient single gene. While BLT2 is more ubiquitously expressed in the body BLT1 is more specific for leukocytes, however, the cell distribution pattern and expression appears to be variable for both types [35].

The existence of BLT1 on cells of both the adaptive as well as the innate immune system suggests that not only PMNs are recruited by LTB₄. Indeed, recent reports show that LTB₄ mediates recruitment of certain types of T-cells to inflamed tissues [36-38]. Consequently, since LTB₄ is particularly secreted by leukocytes of the innate immune system (*e.g.* macrophages) and attracts cells of both the adapted (*e.g.* cytotoxic T effector cells) as well as the innate immune system (*e.g.* neutrophils), it constitutes a link between the two. Thus, in addition to the classical view of LTB₄ as a pure neutrophil chemoattractant, LTB₄ also serves to attract cells of the adaptive immune system thus facilitating their search for target cells.

4.1 ALTERNATIVE LTB₄ SIGNALING

Besides the BLT receptors it should also be noted that LTB₄ has been shown to be a natural ligand of the peroxisome proliferator-activated receptor, PPAR α . This is a nuclear transcription factor regulating genes involved in fatty acid β - and ω -oxidation. Interestingly, PPAR α deficient mice

display prolonged response to inflammatory stimuli. Thus, LTB₄ has the ability to directly modulate the gene expression of certain genes involved in lipid catabolism and thereby to modulate lipid homeostasis [39].

In addition, LTB₄ has been shown to affect the levels of nitrite (a spontaneously formed product of the reaction between NO, water and oxygen) in macrophage culture media thus suggesting a coupling between LTB₄ and enzymes of the nitric oxide pathway [40].

Another alternative signaling pathway is calcium mobilization via the Ryanodine receptor (RyR), an intracellular calcium release channel. The RyR is present in muscle cells and neurons and is modulated in response to a variety of endogenous substances, as well as the plant alkaloid Ryanodine, hence the name. Normally, approximately 50 μ M Ca²⁺ alone fully activates the channel while co-ligands significantly lower this level. The receptor can be fully activated by 100 nM LTB₄ in a Ca²⁺ dependent manner, similar to the effect of other endogenous substances [41, 42]. This activating effect by LTB₄ is counterbalanced by AA mediated inactivation of the IP₃ receptor, thus putatively defining a regulatory circuit of LT biosynthesis.

LTB₄ also has the ability to activate the capsaicin (an ingredient of hot pepper) receptor VR1 of sensory neurons, suggesting a novel and as yet unexplored mechanism of action for LTB₄ in the central and peripheral nervous system [43].

5 ROLE OF LTB₄ - LESSONS FROM ANIMAL MODELS

The *in vivo* role of LTB₄ has been examined in several animal models, some examples from the literature will here be briefly summarized. For instance, knockout studies of LTA4H and the BLT1 receptor have been performed. The function of BLT1 has also been explored by overexpression of the human receptor in transgenic mice. In addition, the LT pathway has been completely disrupted in mice by deletion of the genes encoding 5-LO, FLAP and cPLA₂α.

5.1 LTA4H, 5-LO AND FLAP DEFICIENT MICE

Mice deficient in LTA4H, and thus lacking the ability to convert LTA₄ into LTB₄, have been generated by targeted gene disruption [44]. These mice develop normally and are healthy. Analysis of their reactivity against various proinflammatory stimuli revealed that LTA4H is required for the formation of LTB₄ during an *in vivo* inflammatory reaction. Comparing the phenotype of these mice with that of 5-LO deficient mice, allowed an analysis of the relative contribution of LTB₄ and cysteinyl-LTs, to a specific inflammatory response. Thus, LTB₄ is responsible for the characteristic influx of neutrophils, which follows topical application of AA, and contributes to the vascular changes observed in this inflammatory model. In zymosan-A induced peritonitis, LTB₄ modulates only the cellular component of the response whereas LTC₄ appears to be responsible for the plasma protein extravasation. Furthermore, both 5-LO and LTA4H deficient mice survive the lethal effects of PAF (Platelet activating factor)-induced systemic shock, thus identifying LTB₄ as a key mediator of this reaction [44, 45].

Moreover, LTB₄ is possibly involved in the modulation of chronic and acute immune responses as demonstrated by the reduced severity of induced arthritis in FLAP deficient mice, thus completely lacking all LTs [46]. LTB₄ also participates in the host-defense against infections, as demonstrated by the possibility to partly reverse the increased sensitivity to *Klebsiella pneumoniae* infection of 5-LO deficient mice by the addition of exogenous LTB₄ [47]. Furthermore, impaired neutrophil phagocytosis, caused by the complete loss of LT production in 5-LO deficient mice or by blocking LTB₄ signaling by a BLT1 receptor antagonist, can be partly restored by exogenously added LTB₄ [48].

5.2 BLT1 OVEREXPRESSING MICE

Transgenic mice designed to over express the human BLT1 receptor have been constructed [49]. These mice appear healthy and do not exhibit any major pathology. PMN trafficking to skin

microabscesses, and to lungs after ischemia-reperfusion, is dramatically increased in these animals. In contrast, 5-LO deficient mice show diminished PMN accumulation in reperfused lungs. Furthermore, in the BLT1 transgenic animals increased 5-LO expression and product formation occurs, suggesting the presence of a positive feedback loop in LT biosynthesis. These data provide further evidence for the importance of BLT1 mediated LTB₄ signaling in PMN recruitment and in the regulation of the 5-LO pathway. In addition, it offers a model for acute skin inflammation and reperfusion-induced second-organ injury in mice.

5.3 BLT1 DEFICIENT MICE

Mice deficient in BLT1 have been constructed by targeted gene disruption [50, 51]. BLT1 (-/-) mice develop normally and appear to exhibit a normal phenotype. Whereas peritoneal neutrophils of these mice do not respond to LTB₄ for calcium mobilization or chemotaxis, they respond normally to the inflammatory mediators PAF and complement fragment C5a. Interestingly, female BLT1 (-/-) mice exhibit a distinct increase in survival from PAF-induced anaphylaxis as compared to male BLT1 (-/-) mice. Furthermore, in these mice neutrophil influx following zymosan-induced peritonitis and AA-triggered acute ear inflammation is markedly reduced as compared to normal mice.

5.4 cPLA₂ DEFICIENT MICE

cPLA₂, the enzyme releasing AA from membrane lipids, thus initiating the entire eicosanoid cascade, has also been deleted in mice [52]. The relative effects caused by the absence of each specific eicosanoid in these animals are of course difficult to assess. However, examples of interesting effects exhibited by such animals include, resistance to a model of human multiple sclerosis, decrease lung tumorigenesis, decreased susceptibility to cerebral ischemia/reperfusion and resistance to chemically induced neurotoxicity [53-57].

6 LTB₄ CATABOLISM

No route selectively dedicated for LTB₄ degradation has been described. However, LTB₄ can be catabolized, and its inherent signaling capacity thereby attenuated, by several pathways.

6.1 ω-OXIDATION

ω-oxidation catalyzed by enzymes of the cytochrome P450 (CYP) family, in particular CYP3F, occurs in neutrophils and macrophages. In this process the ultimate carbon of the lipid tail is oxidized, via hydroxylation, to a carboxylate group. The enzymes performing these actions exhibit rather broad substrate specificity and also oxidize, for instance, other eicosanoid metabolites and fatty acids [58-60].

6.2 12-KETO OXIDATION

Another type of oxidation of LTB₄ is NADP dependent and catalyzed by 12-hydroxyl-LTB₄ dehydrogenase, or prostaglandin reductase, (12HD/PGR) yielding 12-keto LTB₄ [61, 62]. As the name suggests, this enzyme also catalyses oxidation of other eicosanoids. The structure of this enzyme from both human and cavia porcellus have been determined by x-ray crystallography [63] (for the human enzyme there is no published paper, but the structure has been solved and the coordinates deposited at the protein data bank by Turnbull *et al.* at the Structural Genomics Consortium (SGC), see pdb entry 1ZSV).

6.3 β-OXIDATION

Also β-oxidation of LTB₄, as well as ω-oxidized LTB₄ (*i.e.* 20-carboxy LTB₄), has been detected to occur in human liver preparations [64].

6.4 CONJUGATION

Additionally, LTB₄ degradation could follow common detoxification routes in the liver, as suggested by the detection of LTB₄ conjugated with glucuronate in liver preparations [64, 65]. Also conjugation of 12-oxo-LTB₄ (the product of 12HD/PGR) with glutathione has been detected in cultured human keratinocytes, thus suggesting the presence of unknown metabolic pathways [66].

7 PATHOPHYSIOLOGY AND DISEASES

Even though LTB₄ has not yet been identified as the sole factor of any pathological condition it is most certainly involved in a variety of inflammatory disorders. The direct role of LTB₄ is, as outlined above, cellular recruitment, which is a central part of the normal inflammatory response. If this delicate act becomes unbalanced a pathological condition will most likely arise. Some examples of

inflammatory conditions that have been reported to involve LTB₄ will be presented below.

Airway inflammation. For instance, it has been found that exhaled breath condensate of patients suffering from chronic obstructive pulmonary disease, cystic fibrosis and asthma contains elevated levels of LTB₄ [67, 68]. In addition, sputum from patients with chronic bronchitis and bronchiectasis has also been shown to contain significant levels of LTB₄ [69].

Other chronic inflammatory disorders. Besides the role of LTB₄ in airway inflammation, increased levels of LTB₄ have been observed in chronic inflammatory disorders such as rheumatoid arthritis [70], psoriasis [71], gout [72] and inflammatory bowel disease [73, 74].

Heart disease. There are also findings indicating that LTs play a role in the pathophysiology of cardiovascular disease [75, 76]. For instance, perfusion of isolated rat hearts with low doses of bacterial toxins leads to cardiac dysfunction partly attributable to the observed LTB₄ synthesis [77, 78]. Moreover, LTA4H has been shown to be upregulated in the hearts of angiotensin II-induced hypertensive rats, thus providing further evidence for a role of LTA4H in inflammatory reactions *in vivo* [79]. (Since angiotensin-II is a peptide, albeit not a substrate for LTA4H, this suggests a possible involvement of the aminopeptidase activity of LTA4H in peptide degradation.)

Indirectly, studies of 5-LO deficient mice link LTB₄ to heart related diseases. For instance, mice lacking 5-LO exhibit decreased progression of aortic lesion formation implying involvement of LTs in atherosclerosis [80]. Genetic studies also emphasize the importance of genes involved in LT biosynthesis. Thus, polymorphism in the promoter region of the 5-LO gene is associated with variability in vessel wall thickness. It has also been shown that dietary AA increases inflammatory mediators in persons with a certain 5-LO promoter variant [81]. In another study, one haplotype of the FLAP encoding gene was identified as a marker for susceptibility to myocardial infarction and that males with myocardial infarction that carry this haplotype exhibit increased production of LTB₄ in stimulated neutrophils [82]. For the LTA4H gene, a certain haplotype has been shown to be associated with a moderate risk of myocardial infarction for people of certain ethnicity [83]. Furthermore, Qiu *et al.* recently found that LTA4H and 5-LO, but not LTC4S, are upregulated in atherosclerotic plaques of humans thus providing further support for a role of LTB₄ in cardiovascular disease [84].

Cancer. Tumor progression quite often involves an element of inflammation [85, 86]. The association of LTA4H overexpression in esophageal

adenocarcinomas in rats [87] and the finding that the nuclear LTB₄ receptor, PPAR α , is linked to hepatocellular carcinoma, exemplifies this statement [88]. Consequently, LTs could play important roles in cancer, thus defining enzymes involved in LT metabolism as novel targets for cancer therapy.

8 A NOTE ON DRUGS TARGETING LT METABOLIZING ENZYMES

Currently, there are no drugs in clinical use directly targeted against LTA₄H. However, a drug indirectly affecting LTB₄ biosynthesis is Zileuton, which inhibits the enzyme 5-LO and thereby blocks further metabolism of AA destined for LT biosynthesis. Moreover, the glucocorticoids inhibit the activity of 5-LO via upregulation of lipocortin, a membrane binding protein blocking the substrate access of 5-LO, and thereby also affecting LT metabolism.

9 LTA₄H – BASIC BIOCHEMICAL PROPERTIES

Native LTA₄H from human neutrophils was the first LTA₄H to be purified and characterized [89]. Subsequently, purification and characterization of native and recombinant enzyme from various sources led to a detailed exploration of the biochemical properties of the enzyme [90]. LTA₄H is a 69 kDa enzyme composed of 610 amino acids and is expressed in most cells of the human body.

The LTA₄H gene is a 35 kbp single copy gene divided into 19 exons and mapped to chromosome 12q22 [91]. A putative phorbol-ester response element (AP-2) and two xenobiotic-response elements (XRE) have been identified in the promoter region of the gene but the relevance of these *cis*-acting elements has not been determined.

LTA₄H catalyses the hydrolysis of the epoxide moiety of LTA₄ whereupon the product LTB₄ is formed. In the common type of hydrolytic reactions the scissile bond and point of water introduction involve the same carbon. In contrast, in LTA₄H the reaction occurs at two sites simultaneously; the cleaved bond and the point of water introduction are separated by a conjugated triene system.

Besides the LTB₄ forming activity LTA₄H also possesses a peptide hydrolyzing activity of unknown physiological relevance. Thus, the enzyme is a bi-functional metalloenzyme exhibiting two zinc ion dependent activities, *i.e.* the LTB₄ forming activity and a peptide hydrolyzing activity. For the nomenclaturist, it should be pointed out that the Enzyme Commission classifies LTA₄H as an ether hydrolase with EC number 3.3.2.6 and not as an aminopeptidase (*i.e.* EC number starting with 3.4.11.--).

9.1 THE PEPTIDASE ACTIVITY OF LTA₄H

According to sequence similarity, LTA₄H sorts as a member of the MA clan of metallopeptidases, all sharing a common Zn-binding motif (see further in section 13 below) [92, 93]. Originally, the similarity of LTA₄H with other well-characterized peptidases, such as thermolysin, led to the discovery of the catalytic Zn-binding site and the bifunctional nature of the enzyme, *i.e.* the additional peptidase activity of the enzyme [94-97].

Both activities of LTA₄H are zinc dependent, *i.e.* they depend on the integrity of the Zn-binding site. The different substrate types, LTA₄ and peptides, are known to be competitive with respect to each other and are also inhibited by the same set of synthetic inhibitors. These facts originally led to the mapping of the active sites of the two activities as partly overlapping. Furthermore, the zinc binding ligands have been mapped to His-295, His-299 and Glu-318 by site directed mutagenesis followed by zinc analysis and activity determinations of the mutated enzymes [98].

Due to homology with related peptidases, residues putatively involved in the peptidase activity could be identified. Thus, sequence alignments were utilized to identify Glu-296 and Tyr-383 as the general base and acid catalyst, respectively [99-101]. By the use of site directed mutagenesis these residues were found to be essential for the peptidase activity, thereby supporting the suggested catalytic roles in the reaction mechanism [100, 102, 103]. (However, in the present work the previously assigned role of Tyr-383 is challenged.)

A variety of peptide substrates including certain arginyl di- and tri-peptides as well as a number of chromogenic *para*-nitroanilide (pNA) derivatives of various amino acids are efficiently hydrolyzed by LTA₄H [94, 96, 104]. In terms of catalytic efficiency the di- and tri-peptides are as good substrates as LTA₄ suggesting that this activity is of physiological relevance. Other accepted peptide substrates of LTA₄H are certain opioid peptides, *e.g.* met5-enkephalin and dynorphin, however these are hydrolyzed with very low efficiencies [105, 106]. Thus, a variety of peptides are hydrolyzed by LTA₄H, however, it should be emphasized that an endogenous peptide substrate has not yet been identified.

The specific peptidase activity of LTA₄H is activated by albumin (approximately 10-fold) and by several monovalent anions (approximately 20-fold), *e.g.*, thiocyanate, chloride and bromide ions [107, 108]. The stimulatory effect of chloride obeys saturation kinetics suggesting the presence of an anion-binding site with an apparent affinity constant for chloride ions of 100 mM. Conversely, chloride ions do not affect the epoxide hydrolase activity.

The fact that the Cl⁻ concentration is considerably higher in the extra cellular compartment as compared to the intracellular milieu points at the possibility that the aminopeptidase activity of LTA4H might act in the extracellular space. However, at none of these location an obvious role or physiological substrate is readily identified.

9.2 THE EPOXIDE HYDROLASE ACTIVITY

Prior to structural determination of LTA4H, the only feature that was known to be absolutely essential for epoxide hydrolase catalysis was the presence of an intact Zn-binding site. However, some other interesting catalytic features regarding the epoxide hydrolase activity were well explored, *i.e.* suicide inactivation and determinants governing product stereochemistry.

Mass spectrometry, peptide mapping, site directed mutagenesis and enzyme kinetic studies unveiled the intriguing feature of suicide inactivation. In this process the substrate, LTA₄, becomes covalently attached to Tyr-378 of the active site during catalysis resulting in a complete block of both enzymatic activities [94, 109-114]. Mechanistically, various types of suicide inhibition exists but it commonly occurs when an enzyme deals with a highly reactive molecular species, *e.g.* in reactions catalyzed by lipoxygenases, cyclooxygenase, prostaglandin I₂ synthase and cytochrome P450 [115-119].

Tyr-378 was not only found to be critical for suicide inhibition but also to affect the stereo specificity of LTA₄ hydrolysis since mutation of this residue led to the formation of a novel product, in addition to LTB₄. It was identified as 5*S*,12*R*-dihydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid, *i.e.* a double bond isomer of LTB₄ [120].

Also Tyr-383 was found to affect the stereochemistry of the product, since mutation of this residue led to the formation of a novel side-product identified as 5*S*,6*S*-DHETE. Formation of a product with this stereochemical configuration suggested that the reaction proceeded via a carbocation intermediate [121]. Thus, Tyr-378 and Tyr-383 were shown to play important but not essential roles in the LTA₄ hydrolase activity.

10 INHIBITORS OF LTA4H

Before the discovery of the aminopeptidase nature of LTA4H, inhibitors of the enzyme were restricted to LTA₄ (as an irreversible suicide inhibitor) and the closely related substrate analogues LTA₃ and LTA₅ [109, 122]. Later investigations have mainly discovered an array of peptidomimetica, including captopril and bestatin, known to be efficient inhibitors of homologous aminopeptidases [123, 124]. In addition, some inhibitors to a larger extent

mimicking the LTA₄ molecule have been developed. Thus far, none of the inhibitors are specific for only one of the activities. Even though some of the compounds are powerful inhibitors of the enzyme and also show good activities in whole cell assays, none has proved to be a promising drug candidate. Some published inhibitor structures along with *K_I* and/or *IC*₅₀ values are presented in Table I.

Table I. LTA4H inhibitors

Inhibitor	Inhibition of peptidase activity <i>in vitro</i>	Inhibition of LTB ₄ synthesis in whole cells	Structure and mode of binding with respect to cavity, if determined.
Captopril [125, 126]	100 nM ^a	360 μM ^b	
Thioamine [125]	15 nM ^a	100 nM ^b	
Bestatin [125, 126]	200 nM ^a	40 μM ^b	
Amino hydroxamic acid [127]	2 nM ^a	300 nM ^b	
Kelatorphan [128]	7 nM ^b	>8 μM ^b	
SC-57461 [129-131]	27 nM ^b	49 nM ^b	
α-keto-β-amino ester [125]	20 nM ^b	200 nM ^b	

^aReported number is *K_I* value.

^bReported number is *IC*₅₀ value.

Structures and reported inhibition constants of various inhibitors of LTA4H. The crystal structure of the upper five inhibitors in complex with LTA4H has been determined (for kelatorphan, unpublished data) and their modes of binding, with respect to the hydrophobic cavity and the zinc site, are schematically given in the table. Relevant references for the determination of inhibition parameters are given in the table.

11 CRYSTAL STRUCTURE OF LTA4H

11.1 GROSS STRUCTURAL FEATURES

The crystal structure of LTA4H in complex with the competitive inhibitor bestatin has been solved at 1.95 Å resolution [132]. The protein is folded in three domains: an N-terminal domain surprisingly similar in structure to bacteriochlorophyll α [133], a catalytic domain which despite low sequence similarity (only 7%) is structurally very homologous to thermolysin [134] and a C-terminal domain organized in a superhelical fashion resembling a so called HEAT motif region [135]. The three domains are packed together with approximate dimensions 85 Å x 65 Å x 50 Å. The packing arrangement between the three domains forms a deep cavity at the interface. The cavity is limited by walls mostly formed by residues of the catalytic- and C-terminal domains with only minor contributions from the N-terminal domain. The cavity consists of a spacious and hydrophilic part separated by the zinc-binding site. At the zinc site the cleft extends into a narrow and bent tunnel that penetrates into the catalytic domain. The tunnel gradually becomes more hydrophobic the deeper it penetrates the protein interior. From the general outline of this cavity the basic binding mode of LTA₄ becomes evident (Fig. 2).

11.2 THE ACTIVE SITE AS PROBED BY BESTATIN

In the crystal structure of LTA4H the deep cavity harbors the Zn-binding site, the bound inhibitor bestatin and residues previously identified as being of catalytic relevance, *i.e.* Glu-296 and Tyr-383, and is hence identified as the active site, for details of binding see Fig. 3. The proposed roles of these residues are readily confirmed by the structure. Thus, previously reported reaction mechanism for the peptidase activity, *i.e.* with Tyr-383 acting as a proton donor and Glu-296 acting as a general base, is roughly compatible with structural data [102, 103]. In addition, candidate residues for substrate binding and specificity can be identified from the structure. For the epoxide hydrolase reaction mechanism, putative catalytic residues can be easily identified (Fig. 2).

11.3 INHIBITOR-LTA4H COMPLEXES

In addition to the first structure of LTA4H, which was a complex between LTA4H and bestatin, crystal structures of LTA4H complexed with captopril, a thioamine and an amino hydroxamic acid inhibitor have been determined [136]. Captopril, a widely used antihypertensive drug targeted against the angiotensin converting enzyme (ACE, EC 3.4.15.1), inhibits the aminopeptidase activity of LTA4H with a reported K_I of 0.1 μ M

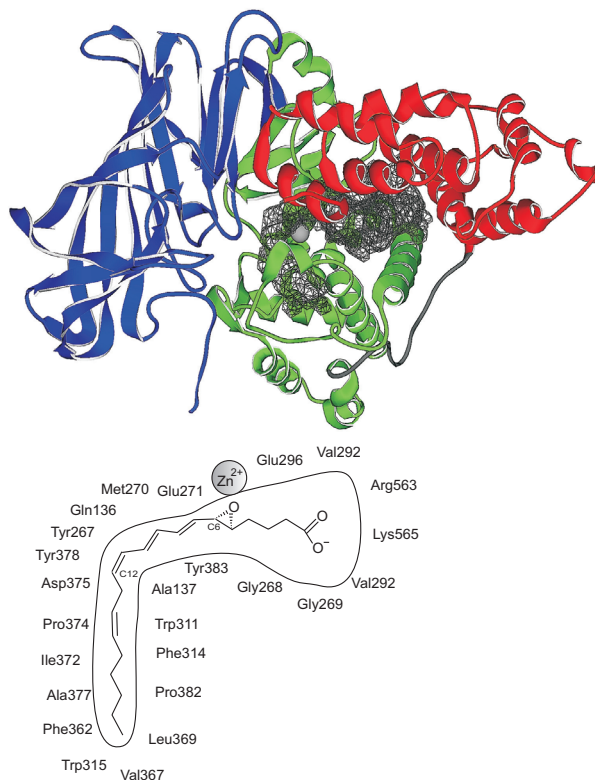


Figure 2 Structure of LTA4H. Upper panel. Overall crystal structure of LTA4H. The N-terminal domain is depicted in blue, the catalytic domain in green and the α -helical C-terminal domain in red. The deep cavity formed between the domains is depicted as a black mesh. Lower panel. Schematic binding of LTA₄ to LTA4H. In this simplistic 2-dimensional model, several key catalytic or substrate binding residues can be identified. Asp-375 is the most obvious candidate residue to act as a base catalyst in the introduction of water at C-12 of LTA₄. The epoxide oxygen is suitably positioned to interact with the zinc ion and the carboxylate moiety appears to interact with the basic residues Arg-563 and Lys-565. The C-7 to C-20 fatty acid backbone binds to the deepest and most hydrophobic part of the pocket and the conjugated triene system aligns with residues of a slightly more polar part of the binding pocket, *e.g.* Glu-271, Tyr-378 and Tyr-383.

while the latter two are powerful inhibitors acting in the low nM range [125, 127]. Interestingly, oral captopril reduces the LTB₄ synthesizing capacity of human neutrophils *in vivo*, a fact that might explain some of the anti-inflammatory properties of this drug [137-139].

11.3.1 The captopril-LTA4H complex

In the structure, LTA4H does not form extensive contacts with captopril. The molecule is located in the more spacious part of the binding cavity. The main interaction is between the thiol group of the inhibitor and the zinc ion. An additional strong interaction is formed between the carboxy group and the basic residue Arg-563. A schematic representation of enzyme-inhibitor interactions is given in Figure 4.

Despite the fact that captopril is a rather modest inhibitor of LTA₄H, the LTA₄H-captopril crystal structure provides valuable information of how captopril might bind to ACE, as well as to other aminopeptidases. In fact, the crystal complex between LTA₄H and captopril is the first example of a structure of this drug in complex with a metallopeptidase of the MA clan. Even though structural data for ACE in complex with captopril is absent it is generally assumed that the molecular mode of action of captopril involves chelation of the catalytic zinc ion(s) by its thiol group [140]. The crystal structure of the complex between LTA₄H and captopril emphasize this assumption and further suggests that similar interactions might occur between captopril and other metallopeptidases of the MA clan.

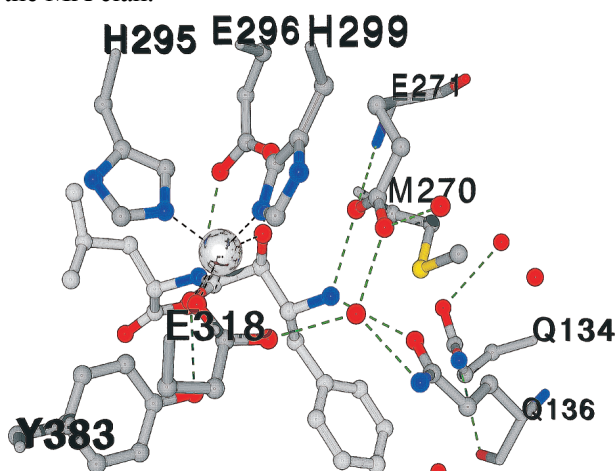


Figure 3. Close-up view of the active site with the bound inhibitor bestatin. Carbon atoms of protein residues are shown in gray, the bound inhibitor in white and water molecule in red. The coordination of the zinc ion (shown as a metallic sphere) is indicated by dotted black lines. Hydrogen bonds are shown as green dotted lines. Note the hydrogen bond between the free amino-group of the inhibitor and Glu-271, which is further discussed in sections 18.2.1, 18.2.2 and 18.3.1.

The hydroxyl and carbonyl oxygens of bestatin together chelate the zinc ion with binding distances of 2.1 and 2.4 Å, respectively. Together the metal ligands (*i.e.* the hydroxyl and carbonyl oxygens of bestatin, His-295, His-299 and Glu-318) form a metal coordination geometry described by a square based pyramid. The free amino group of bestatin H-bonds to Gln-136 and Glu-271 with binding distances of 2.9 and 2.6 Å, respectively. One of the carboxylate oxygens of Glu-296 is H-bonded to the hydroxyl group of bestatin, the other to its amide nitrogen, with respective binding distances of 2.5 and 3.0 Å. The carbonyl oxygen of the inhibitor is also H-bonded to the hydroxyl group of Tyr-383 with a binding distance of 2.4 Å. The phenyl moiety of the inhibitor packs with the enzyme in the region where the conjugated triene system of LTA₄ is believed to bind. The carboxylate moiety of bestatin interacts with Arg-563 and Lys-565 via a bridging water molecule (not shown due to clarity).

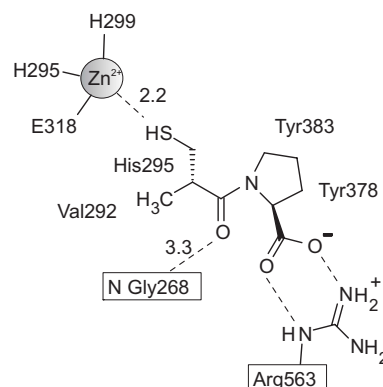


Figure 4. Schematic representation of the binding of captopril to the active site of LTA₄H. Important hydrogen bonds and other non-bonding interactions are depicted by dotted lines. Note the interaction between the zinc ion and the thiol group of captopril and the interactions between Arg-563 and the carboxy group of captopril. Bond distances are given in Å.

11.3.2 The thioamine–LTA₄H complex

The thioamine inhibitor belongs to a group of inhibitors developed to mimic the transition state of peptide cleavage and to provide a hydrophobic moiety resembling the lipid tail of the substrate LTA₄ [141, 142]. Similarly to captopril the thiol group of the thioamine inhibitor binds to the zinc ion and together with the three amino acid ligands a tetrahedral coordination of the zinc ion is formed. In contrast to captopril the thioamine forms extensive interactions with the enzyme, *e.g.* the amine group is firmly anchored to the carboxy group of Glu-271 and the benzyloxyphenyl moiety fills up most of the hydrophobic pocket where LTA₄ is proposed to bind (Fig. 5). Binding within this pocket is unspecific and mainly consists of hydrophobic interactions and aromatic stacking. Nevertheless, the extensive packing with the enzyme in this part of the binding pocket accounts for the increased potency of this inhibitor as compared to captopril.

11.3.3 The hydroxamate–LTA₄H complex

Improved inhibitor potency was achieved by the addition of the metal-chelating hydroxamates to the benzyloxyphenyl moiety of the thioamine inhibitor. Further coupling of a butanoic or pentanoic acid moiety to the chelating group improved inhibitor potency even more [127]. The structure of LTA₄H complexed with such an inhibitor exhibits basically the same binding pattern, with respect to the benzyloxyphenyl part of the molecule, as the thioamine inhibitor. The additional carboxylic acid tail of the molecule forms two highly polar hydrogen bonds with the positively charged side chain of Arg-563 and the intervening aliphatic chain is positioned similarly to captopril. Interestingly, the expected pentavalent coordination of the zinc ion as observed in similar complexes with thermolysin

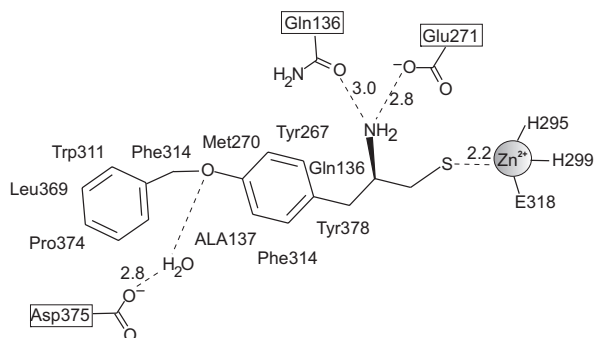
complexed to other hydroxamates [143] or between LTA₄H and bestatin (*cf.* Fig. 3) [132], is not observed. Instead, only one of two potentially metal chelating oxygens forms a direct coordinate bond with the zinc ion (the other is located outside coordinate bond distance and interacts weaker with the metal ion) thus forming a tetrahedral metal coordination geometry. A schematic representation of enzyme-inhibitor interactions is given in Figure 6.

12 LTB₄ BIOSYNTHESIS IS SPECIFIC FOR VERTEBRATES BUT NOT LTA₄ HYDROLYSIS

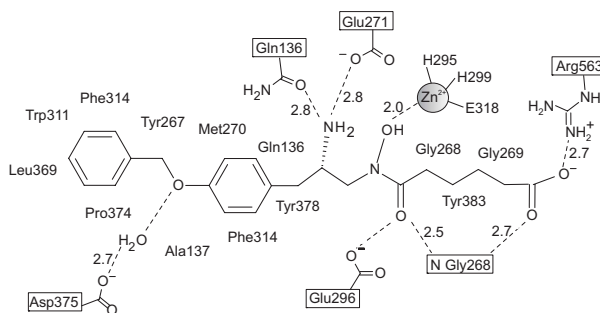
Most organisms, ranging from mammals to plants and bacteria, possess an aminopeptidase of the M1 family homologous to LTA₄H. Several of these possess a relatively high sequence identity of about 40% with respect to human LTA₄H. The mammalian subgroup of “true” LTA₄H enzymes (*i.e.* with the specific capacity to convert LTA₄ to LTB₄) exhibits a high degree of sequence identity with a maximal sequence divergence of 10%. Lower vertebrates exhibit around 60% sequence identity, as exemplified by LTA₄H from *Xenopus leavis*, which has been cloned and purified (unpublished data). Recombinant *X. leavis* LTA₄H has a peptidase activity and a strong LTB₄ forming epoxide hydrolase activity and additionally forms the product Δ^6 -*trans*- Δ^8 -*cis*-LTB₄ (unpublished data), thus confirming the studies on native *X. leavis* LTA₄H [144]. LTB₄ synthesis, suggesting the presence of an LTA₄H, has also been demonstrated in other lower vertebrates including fish and frogs [145-148]. In contrast, no non-vertebrate species has been shown to be able to synthesize LTB₄ in substantial amounts (see below).

Despite a high degree of sequence identity to human LTA₄H, particularly for residues lining the active site, the LTB₄ forming activity appears to be unique for vertebrates and not widely spread among non-vertebrate LTA₄H homologues. For instance, the activity has neither been detected in mammalian aminopeptidase B enzymes nor in homologous aminopeptidases of lower organisms (both groups exhibiting about 40% overall sequence identity and even higher in the catalytic domain), although conflicting data exist in the literature [149, 150].

An LTA₄H from *Saccharomyces cerevisiae* that is 39% identical (53% similar) to the human enzyme has been cloned and characterized [151, 152]. The *sc*LTA₄H is a zinc leucyl aminopeptidase with an epoxide hydrolase activity that mostly converts LTA₄ into 5*S*,6*S*-dihydroxy-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (5*S*,6*S*-DHETE) but also small amounts of LTB₄ and 5*S*,12*R*-dihydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid (Δ^6 -*trans*-



Figur 5. Schematic representation of the binding of the thioamine inhibitor to the active site of LTA₄H. Important hydrogen bonds and other non-bonding interactions are depicted by dotted lines. Note the interactions between the zinc ion and the thiol group of the inhibitor and between Glu-271 plus Gln-136 and the amino group of thioamine. Asp-375 is bridged to the ether oxygen of thioamine via an intervening water molecule. This oxygen occupies a position close to the proposed position of carbon 12 of LTA₄ (*cf.* Fig. 2). In addition, residues involved in the extensive hydrophobic interactions between inhibitor and protein are shown. Bond distances are given in Å.



Figur 6. Schematic representation of the binding of the hydroxamic acid inhibitor to the active site of LTA₄H. Important hydrogen bonds and other non-bonding interactions are depicted by dotted lines. Note the interaction between the zinc ion and the hydroxy group of the inhibitor, between the free amino group of the inhibitor and Glu-271 plus Gln-136 and between the carboxyl group of the inhibitor and Arg-563. Asp-375 is bridged to the ether oxygen via an intervening water molecule. This oxygen occupies a position close to the proposed position of carbon 12 of LTA₄ (*cf.* Fig. 2). In addition, residues involved in the extensive hydrophobic interactions between inhibitor and protein are shown. Bond distances are given in Å.

Δ^8 -*cis*-LTB₄) are formed. Furthermore, LTA₄ can bind tightly to the *S. cerevisiae* enzyme, which leads to inhibition of the epoxide hydrolase activity and strong activation of the peptidase activity [153]. Hence, the *S. cerevisiae* enzyme is the first example of a non-vertebrate LTA₄H that possesses an epoxide hydrolase activity. The yeast enzyme is only little, if at all, susceptible to suicide inactivation, and carries a Phe residue at the position corresponding to Tyr-378 of the human protein (mutating Tyr-378 of the human enzyme to a Phe

residue protects the enzyme from suicide inactivation, see section 9.2).

An aminopeptidase from *Caenorhabditis elegans* has also been cloned and characterized. It is 45% identical (63% similar) at the amino acid level to human LTA4H and exhibits an arginyl aminopeptidase activity [154]. Notably, despite this high level of sequence identity the *C. elegans* enzyme fails to hydrolyze LTA₄ into LTB₄ and no other functional links to LTA4H have been reported.

13 THE MA CLAN OF AMINOPEPTIDASES

Peptidases are one of the most diverse classes of enzymes that exist and peptidase-encoding genes actually constitute more than 4% of the human genome. To date, six distinct catalytic types of peptidases have been described. Based on sequence similarity, the known peptidases are divided into 48 clans, which are further subdivided into 184 families. One clan of peptidases is the so-called MA clan of zinc metallopeptidases, the metzincins, all sharing some resemblance to thermolysin (EC 3.4.24.27), and all possessing a HEXXH sequence signature. This motif defines the catalytic Zn-binding motif including the two Zn-coordinating His residues and the catalytic base, *i.e.* the Glu of the motif, which is required for peptidolysis. The zinc ion is also coordinated by a third protein ligand, a Glu, Asp or His residue, located on an α -helix at some distance (typically 18-20 residues) from the other zinc ligands. This third residue defines a division of the MA clan into two additional subclans: the MA(E) subclan in which it is a Glu residue and the MA(M) subclan in which it is an Asp or a His residue. The MA(E) clan is divided into 24 families among which the M1 family of aminopeptidases, which includes LTA4H, is found. M1 aminopeptidases cleave off the N-terminal residues of peptides thereby releasing a single amino acid.

13.1 THE M1 FAMILY OF AMINOPEPTIDASES

M1 aminopeptidases are found throughout all kingdoms of life and have various biological functions. For instance, they are known to be involved in the MHC class I antigen processing pathway (ERAP), in the protein degradation pathway of *thermoplasma acidophilum* (the tricorn interacting factor F3), in the regulation of Thyrotropin Releasing Hormone (pyroglutamyl-peptidase II), in the degradation of the neuropeptide enkephalin in brain (ApPS and APN) in metastasis-prone cancers (APN) and in cytokine degradation (APN). Humans possess 12 aminopeptidases of the M1 family as defined by sequence similarity, 11 of

these are briefly summarized in Table II (the 12:th is LTA4H). With respect to LTA4H, other M1 aminopeptidases are not only interesting because they are homologs, but also because their biological functions may reflect a role for the peptidase activity of LTA4H. In the context of possible functions for the aminopeptidase activity of LTA4H, which are not covered by other human M1 aminopeptidases, degradation of dietary peptides might be considered. The fact that the type of peptide which is the preferred substrates of LTA4H, *i.e.* di- and tri-peptides, and not only free amino acids, are efficiently taken up by cells in the gastrointestinal tract via the actions of oligo peptide transporters, further supports this notion [155]. Interestingly, there are several reports suggesting that dietary short peptides have a variety of biological effects, *e.g.* immunomodulating properties [156].

Since LTA4H prefers arginyl tri-peptides, another noteworthy and possible role for the aminopeptidase activity of LTA4H could be to supply inducible nitric oxide synthase (iNOS), in for instance macrophages, with its substrate under inflammatory conditions. Furthermore, besides its role as a substrate for NOS, arginine has a variety of direct biological effects, *e.g.* it can modulate vascular reactivity by direct binding to potassium channels, it plays a role in glucose metabolism via stimulation of insulin and glucagon secretion and inhibits leukocyte adhesion to the non-endothelial matrix [157-159].

Table II. Enzymes of the M1 aminopeptidase family in human.

Enzyme	Id.	Biological Roles
Aminopeptidase N (EC 3.4.11.2) (CD13) Refs.: [160-162]	25%	Ectoenzyme involved in tumor angiogenesis and tumor metastasis possibly through regulation of cell migration. Marker for certain type of leukemia. Inhibition leads to strong immunosuppression. Expressed in most T-cells. Dogs with naturally diminished expression exhibit normal phenotype.
Aminopeptidase A (EC 3.4.11.7) (Angiotensinase A) Refs.: [163-169]	24%	Ectoenzyme degrading angiotensin II thus forming angiotensin III. Role in brain to control blood pressure. Regulator of blood vessel formation. APA deficient mice develop normally but fail to mount proper angiogenic response to hypoxia and growth factors.
Pyroglutamyl-peptidase II (EC 3.4.19.6) (TRH-Degrading AP, Thyrotropin-releasing hormone-degrading ectoenzyme) Ref.: [170]	25%	Ectoenzyme that degrades and inactivates the neuropeptide thyrotropin-releasing hormone. Thereby a potential regulator of thyrotropin and prolactin suggesting a role in e.g. control of body temperature and heart rate, stimulator of locomotor activity, effects on arousal and pain perception.
Puromycin sensitive aminopeptidase (EC 3.4.11.14) (ApPS, Cytosol alanyl aminopeptidase, Enkephalin-degrading AP) Refs.: [161, 171]	24%	Ectoenzyme inactivating enkephalins and other bioactive peptides. Involved in e.g. cell cycle regulation, apoptosis, protein turnover and antigen presentation. Broad tissue distribution. Knockout mice exhibit dwarfism, increased anxiety, and extended latency of pain reaction. Infertile male and female due to impaired spermatogenesis and inability to form corpus luteum, respectively.
Placental leucine aminopeptidase (EC 3.4.11.3) (Cystinyl AP, Oxytocinase, Insuline-responsive AP, IRAP) Ref.: [172]	25%	Ectoenzyme degrading oxytocin and vasopressin, thereby a putative regulator of labor and uterine contraction. Translocates to cell membrane in response to insulin in adipocytes and muscle cells. Receptor of angiotensin IV in brain and thereby possibly involved in learning and memory. Knockout mice appear normal, particularly no effect on labor.
Aminopeptidase B (EC 3.4.11.6) (Arginyl AP, RNPEP) Ref.: [173]	37%	Unknown biological function. Strictly removes basic residues. Broad tissue distribution.
Aminopeptidase PILS (Puromycin Insensitive Leucyl specific AP, Endoplasmatic Reticulum AP-1, ERAP-1) Ref.: [174]	22%	Processing of peptides for MHC Class I antigen presentation. Induced by interferon- γ . Broad tissue distribution with highest in e.g. spleen, thymus, liver, heart and placenta. AP PILS siRNA treatment of HeLa cells leads to increase of MHC Class I on cell surface.
RNPEP-like protein (Mernam-AA050 peptidase) Ref.: [175]	33%	Differently expressed in immortalized human esophageal epithelial cell lines and malignant transformed esophageal carcinoma cell line.
Leukocyte-derived arginine aminopeptidase (L-RAP, ERAP-2, Mernam-AA066 peptidase, AP MAMS) Ref.: [176]	23%	Membrane associated peptidase found on the luminal side of ER. Induced by interferon- γ . Preference for arginyl-peptides. Expressed in most tissues with high levels in spleen and leukocytes.
Laeverin (Mernam-AA009 protein, Mernam-AA120 peptidase) Refs.: [177, 178]	25%	Overexpression in lymphoblastoid B-cell lines from patients with rheumatoid arthritis. Expressed in human extravillous trophoblasts, which invade the maternal decidua. Possesses a transmembrane domain.
Aminopeptidase O (Mernam-AA139 putative peptidase) Ref.: [179]	22%	Preference for synthetic arginyl-peptides. Broad tissue distribution but highest expression in pancreas, placenta, liver, testis and heart.

The first column gives the enzyme name and EC number, if assigned, along with some common alternative names followed by selected references. Column two indicates sequence identity (%) with LTA4H according to pair-wise blast alignments. In column three, the given biological roles are only examples and not meant to be exhaustive.

14 STRUCTURES OF OTHER METALLOPEPTIDASES

14.1 THE TRICORN INTERACTING FACTOR

Within the M1 family of aminopeptidases human LTA4H constitutes the only mammalian enzyme with an experimentally solved 3-dimensional structure. However, an M1 aminopeptidase from the archaea *thermoplasma acidophilum*, the tricorn interacting factor F3, has been structurally determined [180]. This protein is about 25% identical, 41% similar, to human LTA4H. While the N-terminal and catalytic domains of the two enzymes are folded very similar, with an r.m.s.d for the superimposed structures of 1.42 Å for 311 equivalent α -carbons, the C-terminal domains are significantly different (Fig. 7). The tricorn interacting factor is considerably longer than human LTA4H, 780 residues compared to 610 for human LTA4H. When superimposing LTA4H with the tricorn interacting factor F3 the backbones of the two proteins do not align after the catalytic domain. The whole C-terminal of the tricorn interacting factor F3 differs in size, as well as in orientation with respect to the other domains, as compared to human LTA4H. This leaves the active site of the tricorn protease considerably more accessible. In contrast to LTA4H, which possesses a large extended active site pocket, the archaean enzyme only exhibits a small pocket, putatively assigned as a S1¹ specificity pocket. The fold of the C-terminal domain of the archaean enzyme exhibits both similarities and differences compared to human LTA4H. While the last part has a similar superhelical fold as human LTA4H, the intervening part, which connects the catalytic and the C-terminal domains, does not resemble human LTA4H. Compared to LTA4H, the tricorn interacting factor F3, exhibits an insertion between the catalytic and c-terminal domains.

14.2 THERMOLYSIN

Thermolysin is an endoprotease from *Bacillus thermoproteolyticus* and constitutes the classical example of a Zn-metalloprotease. Hence, it defines the fold of proteins of the MA clan of peptidases. The enzyme itself belongs to the M4 family of peptidases. Despite low similarity at the sequence level (only 7%) LTA4H and thermolysin share a

¹ As defined by Schechter and Berger peptide substrates are numbered with start at the scissile bond counting towards the termini, residues at the N-terminal side are numbered P1, P2, ... and residues at the C-terminal side P1', P2', ... The corresponding enzyme specificity pockets are numbered accordingly but with the letter S [181].

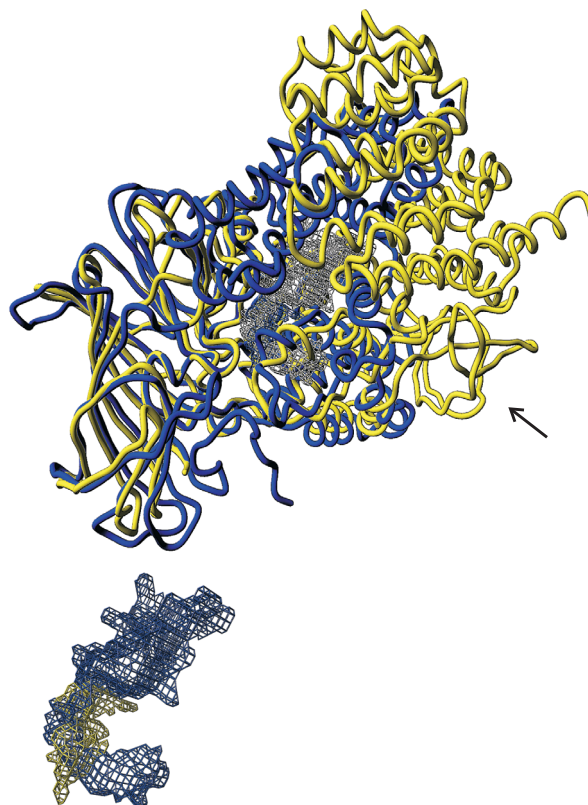


Figure 7. The tricorn interacting factor F3 (TIF3) and LTA4H superimposed. LTA4H is shown in blue and TIF3 in yellow. The hydrophobic cavity of LTA4H is depicted as a gray mesh. Whereas the N-terminal and catalytic domains of the two enzymes have similar folds the C-terminal domains differ. With respect to LTA4H, TIF3 possesses an additional domain in between the catalytic and the C-terminal domains (indicated with an arrow). The C-terminal domain of LTA4H, upper right hand side of the structure, packs tightly with the rest of the enzyme and in a sense defines a lid for the cavity. In contrast, the C-terminal domain of TIF3 has an orientation that leaves the active site considerably more accessible. **Lower panel:** In contrast to LTA4H, TIF3 only possesses a small active site cavity, presumably corresponding to the S1 specificity pocket. The cavity of LTA4H is shown in blue and TIF3 in yellow.

surprising structural similarity. Superimposing LTA4H onto thermolysin yields a fit with an r.m.s.d of 1.94 Å for 146 equivalent C- α positions (Fig. 8). Thermolysin has a considerably shorter sequence length than LTA4H and the structural similarities between the enzymes are confined to regions of the catalytic domain of LTA4H.

14.3 ANGIOTENSIN CONVERTING ENZYME

Angiotensin-converting enzyme is an ectoenzyme active in the conversion of angiotensin I to the biologically active angiotensin II, and in the degradation of bradykinin and other bioactive peptides. Hence, the enzyme constitutes a common target for the treatment of high blood pressure. Interestingly, a drug used for the treatment of such conditions, captopril, also inhibits LTA4H, see

above. However, apart from the HEXXH motif, the two enzymes do not share any significant sequence or structural similarities. Angiotensin-converting enzyme is a **carboxypeptidase** of the M2 family which releases two residues at the c-terminus of its substrates.

14.4 ANTHRAX LETHAL FACTOR

The peptidase anthrax lethal factor is a metallopeptidase of the M34 family and a virulence factor of *Bacillus anthracis*, the causative bacteria of anthrax. As a very unique enzyme it defines the single member of the M34 “family” of peptidases. It does not exhibit any similarities, neither at the sequence level nor at the structural level (besides the HEXXH motif), to any other member of the MA clan of metallopeptidases, including LTA4H.

15 OTHER PROTEINS FUNCTIONALLY RELATED TO LTA4H

Other proteins, which are not peptidases, are interesting with respect to LTA4H due to their ability to bind fatty acids, particularly arachidonic acid and LTB₄. For instance, human serum albumin (HSA), rabbit 15-lipoxygenase, 12-hydroxy-LTB₄-dehydrogenase and COX-1 constitute such proteins for which the structures have been determined. Of particular interest are COX-1 and HSA, since the crystal structures of these proteins in complex with AA have been determined. Regarding general features for lipid binding they both exhibit similar patterns as LTA4H, with a hydrophobic pocket capped with basic groups suitable for binding of the carboxylate moiety of the lipid [182-184].

16 METHODOLOGY

To provide a detailed description of the principles of each specific experimental technique that was applied throughout the present work is out of the scope of this thesis. However, relevant methodological details are given in the methods section of each paper.

17 AIMS OF THE PRESENT INVESTIGATION

As always in research, aims change during the process and new aims and new questions are constantly formed. For instance, a putatively simple problem originally tackled, quite often turns out to be very complex once proper knowledge about it has been gained. So, to simply state a number of aims that were originally defined is an oversimplification and will never be completely true. However, the

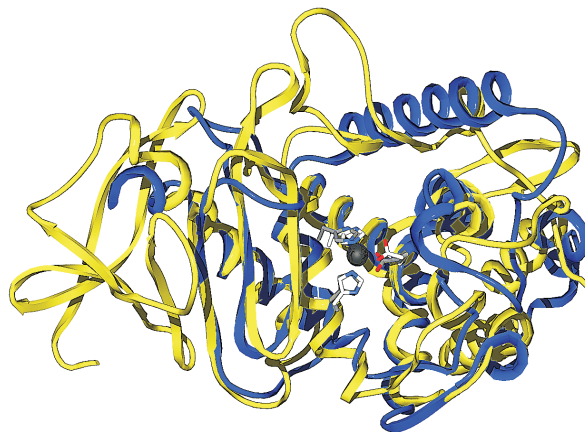


Figure 8. The catalytic domain of LTA4H superimposed onto thermolysin. LTA4H is shown in blue and thermolysin in yellow. The Zn coordinating ligands of both enzymes are shown with carbon atoms shown in white and gray for thermolysin and LTA4H, respectively. Due to clarity, the Zn ion is only shown for LTA4H. Note how the structural elements that build up the active site of the two enzymes are folded very similar and particularly the nearly identical positioning of the Zn coordinating ligands.

initial intention of the present study was not far from what is suggested by the thesis title, *i.e.* to gain insights into the *Catalytic mechanisms and evolution of LTA4H*. Initial aims and strategies will be further addressed in sections 18.1-18.2, but can be summarized as two key points:

- To understand the structural changes underlying the evolution of LTA4H by studies of homologous LTA4H from *Saccharomyces cerevisia* using approaches including mutagenesis, molecular modeling and enzyme kinetics.
- To understand the molecular details required for LTA4H-catalyzed hydrolysis of peptides and LTA₄ using approaches including mutagenesis, enzyme kinetics and crystallography.

18 RESULTS AND DISCUSSION

18.1 OUTLINE OF RESULT

Each of the papers I-III deals with very detailed chemistry of the two reaction mechanisms of human LTA₄H. Quite obviously, the picture became clearer the more data that were produced. In order to make the presentation here as simple as possible, the findings would probably benefit most if summarized as two reaction mechanisms only, one for LTA₄ hydrolysis and one for peptide hydrolysis. However, in order to separate between the specific findings, each paper also deserves to be reviewed separately. Therefore, the findings from paper I-III will first be presented in condensed format, in the following sections (sections 18.2.1 and 18.2.2). Thereafter, each of the paper I-III will be discussed in separate sections (sections 18.3.1, 18.3.2 and 18.3.3, respectively).

Paper IV deals with the molecular evolution of the reaction mechanisms of LTA₄H and will be reviewed in a separate section (section 18.3.4). The subject of this study was the yeast homologue of human LTA₄H. The relationship between these enzymes renders the findings concerning the yeast enzyme in parts valid also for the human counterpart. Hence, the data of this paper will in part provide additional support for the findings of paper I-III.

Paper V presents the development of a new assay for simple spectrophotometric analysis of the tri-peptidase activity of LTA₄H. Since previously applied assays for the peptidase activity was either limited to the analysis of chromogenic model substrates, or experimentally complex for the natural peptides, the progress made in this work was crucial for improving future studies. This paper will be briefly addressed in a separate section (section 18.3.5).

Paper VI, which deals with the peptidase reaction mechanism of LTA₄H, will also be discussed in a separate section (section 18.3.6). It confirms and refines findings from paper I-IV as well as earlier work on this subject.

18.2 RATIONALE FOR PAPERS I-III AND RESULTS IN BRIEF

18.2.1 Clues from structural data

The crystal structures of LTA₄H complexed with different inhibitors were the pivot around which new questions regarding the detailed functionality of the enzyme was defined. The peptide-like inhibitor bestatin indicated potential residues involved in peptidolysis whereas the more LTA₄-like inhibitors (the hydroxamic acid inhibitor and the thioamine inhibitor) pinpointed residues

possibly involved in hydrolysis of LTA₄. For instance, whereas the LTA₄H-bestatin structure indicate that Glu-271 and Gln-136 form hydrogen bonds to the free amino group of bestatin, the carboxylate moieties of the LTA₄-like inhibitors interact with Arg-563 and Lys-565 (Figs. 3, 5-6). The former interaction suggests a role in the recognition of the α -amino group of peptide substrates the latter suggests a role in carboxylate recognition for both substrates. In addition, the very shape of the extended cavity in the enzyme helped to define the general outline of LTA₄ binding, which indicates that the carboxylate of LTA₄ interacts with Arg-563 or Lys-565, that the epoxide of LTA₄ is positioned close to the zinc ion and that carbon 12 of LTA₄ is close to Asp-375 (Fig. 2). The latter in turn, suggests a role for Asp-375 as a base catalyst in the introduction of water at carbon 12 of LTA₄. This is also inferred by the water-mediated interactions seen between the ether oxygen (which occupies a similar position as carbon 12 of LTA₄ in the binding model) of the hydroxamic acid or the thioamine inhibitors and Asp-375 (Figs. 5-6). Thus, these structural observations helped to identify novel catalytic residues: four residues potentially critical for peptidase hydrolysis and three for LTA₄ hydrolysis.

18.2.2 Paper I-III. Basic requirements for the reaction mechanisms of LTA₄H

To verify stipulated hypotheses, an approach including site-directed mutagenesis and various kinds of enzyme kinetic experiments were undertaken. Data for the activities of mutant enzymes along with kinetics for substrate turn-over by mutant enzymes were used to define the functions of the specific residues. Also, to get an improved understanding of substrate binding, molecular modeling was utilized. Finally, to exclude any unexpected effects of the mutations, such as major structural changes, crystal structures of selected mutants were determined.

As proposed, Glu-271 was identified as the key determinant for the exopeptidase activity of the enzyme via its binding of the α -amino group (Paper I). Considering sequence conservation of related enzymes, this feature probably holds true for the whole M1 family of aminopeptidases. Unexpectedly, Glu-271 was also found to have an essential role also in epoxide hydrolysis. This kind of dual functionality of a single residue is an enzymatic feature unique to LTA₄H. In contrast, Gln-136 had a very limited effect on catalysis.

Arg-563 was identified as a carboxylate binding site for both peptide substrates as well as LTA₄ (Paper III). Lys-565, on the other hand, appeared to affect only the binding of peptide

substrates (and to a lesser extent as compared to Arg-563) and did not affect LTA₄ binding.

The role of Asp-375 was verified as the general base responsible for proton abstraction from the water molecule attacking LTA₄ (Paper II). This role also placed certain restraints on the binding positioning of LTA₄, thereby verifying the general features of the proposed binding mode (Fig. 2). The overall results from paper I-III is summarized in Figures 9 as two very schematic reaction mechanisms.

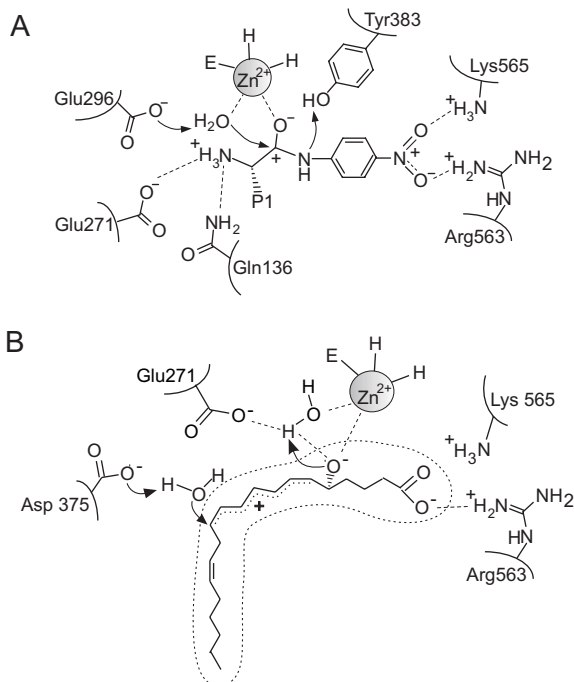


Figure 9. Reaction mechanisms of LTA₄H based on results in Papers I-III. **A.** Putative mechanism for the peptidase reaction. The carbonyl oxygen of the amide bond is coordinated to the Zn ion. Together with the zinc ion, Glu-296 polarizes a water molecule, thus facilitating its attack on the carbonyl carbon. Simultaneously, a proton is donated by Tyr-383. (Note that the role of Tyr-383 is redefined in paper VI.) Glu-271 anchors the free amino group of the peptide substrate. Arg-563 and Lys-565 anchor the nitro moiety of ala-*p*-NA, which to some extent corresponds to the C-terminal carboxyl moiety of the substrate. **B.** Putative mechanism for the epoxide hydrolase reaction. Initially the Zn ion, assisted by the carboxylate of Glu-271, facilitates the opening of the oxirane ring whereupon a carbocation intermediate is formed. The correct double-bond conformation of the product LTB₄ (Δ^6 -*cis*- Δ^8 -*trans*- Δ^{10} -*trans*) is simply controlled by the shape of the binding pocket, see further in sections 19.3, 19.4 and 19.5. Asp-375 assists in the stereospecific nucleophilic attack of water at C-12 of LTA₄, which leads to the formation of LTB₄. The basic residues Arg-563 and Lys-565 bind LTA₄ in a position compatible with catalysis. The dotted black line describes a schematic outline of the binding cavity.

18.3 SPECIFIC RESULTS OF EACH PAPER

18.3.1 Paper I. Leukotriene A₄ Hydrolase/ Aminopeptidase - Glutamate 271 is a catalytic residue with specific roles in two distinct enzyme mechanisms

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 [185]. As outlined above, the glutamate of this motif, Glu-271 in LTA₄H, binds the free amino group of bestatin, providing further support for this notion. In addition, this amino group also forms a hydrogen bond to Gln-136. To investigate the importance of residues involved in N-terminal recognition of peptide substrates, all residues within the GXMEN motif, along with some spatially neighboring residues including Gln-136 were mutated. Interestingly, all site-specific mutants retained their catalytic function with the exception of mutants of Glu-271, which had lost not only the peptidase activity but also the epoxide hydrolase activity. Furthermore, the crystal structure of the most conservative of the inactive mutants, *i.e.* [E271Q]LTA₄H, revealed that the overall structure was intact and particularly that the integrity of the catalytic zinc site was preserved. Hence, these combined mutational and structural data strongly indicate that Glu-271 is required for both enzyme reactions.

Considering the spatial position of Glu-271 (close to the catalytic zinc), it seems likely that its side-chain carboxylate is close to the epoxide moiety of LTA₄ and actually participates in the opening of the oxirane ring (Fig. 9). For the aminopeptidase activity, on the other hand, the function appears to be of another character. Thus, considering the interactions of Glu-271 with alanine-*p*-nitroanilide, which was observed upon modeling of this substrate into the active site of LTA₄H, it seems likely that the carboxylate of Glu-271 functions as an anchor for the N-terminal α -amino group of peptide substrates (Fig. 9). As such, this residue will be a determinant for the exopeptidase activity of the enzyme and thereby contribute to substrate alignment, which in turn will influence transition-state stabilization and substrate turnover. This conclusion also agrees well with site-directed mutagenesis studies of other zinc aminopeptidases and possibly holds true for all aminopeptidases of the M1 family [186, 187].

18.3.2 Paper II. Leukotriene A₄ hydrolase: Selective abrogation of leukotriene B₄ formation by mutation of aspartic acid 375

Two chemical features are critical for the biological activity of LTB₄: the chirality of the 12(R)-hydroxyl group and the Δ^6 -*cis*- Δ^8 -*trans*- Δ^{10} -*trans* conformation of the triene structure. As outlined in section 18.2.1, the crystal structure of LTA₄H identifies Asp-375 as an evident candidate residue to act as a general base in the epoxide hydrolase reaction and hence as the residue dictating the chirality of carbon 12 of LTB₄. To detail the function of Asp-375, this residue along with some spatially neighboring residues was mutated and their enzymatic activities assayed. In line with the proposed role as a general base in the epoxide hydrolase reaction all mutations of Asp-375 leads to the selective loss of the LTB₄ producing activity of the enzyme whereas the aminopeptidase activity is preserved. (Note that the latter statement will be refined in paper V and VI.) The crystal structure of [D375N]LTA₄H was determined and apart from the mutation *per se* no gross structural changes were observed. The changes actually observed were very subtle and only affected the hydrogen bonding network. Hence, secondary effects explaining the loss of activity was ruled out and the role of Asp-375 as a general base in the epoxide hydrolase reaction mechanism was further supported by the mutant structure.

According to the schematic binding model presented in Figures 2 and 9, the allylic epoxide of LTA₄ is presented to the zinc ion and the C7-C20 aliphatic tail fits into the extended binding cavity. This orientation of the substrate suitably positions Asp-375, along with putative hydrolytic water molecules, close to carbon 12 of LTA₄. This binding model along with biochemical data clearly demonstrates that Asp-375 could act as a general base in the epoxide hydrolase reaction and, as such, would be a critical determinant for the biological activity of LTB₄.

18.3.3 Paper III. Leukotriene A₄ Hydrolase - Identification of a common carboxylate recognition site for the epoxide hydrolase and aminopeptidase substrates

As outlined in sections 18.2.1 and 18.2.2, the crystal structure suggests a role for Arg-563 and Lys-565 in carboxylate binding of substrates. Also earlier work, utilizing chemical modification, indicated the presence of essential Arg residues [188]. To investigate the function of these two basic residues in carboxylate recognition they were subjected to mutagenesis and the enzymatic behavior of each mutant was assayed. The experiments indicated that mutation of Arg-563, but not Lys-565, led to

complete loss of the epoxide hydrolase activity. For the peptidase activity, mutants of both positions affected the activity, however to a variable extent. The effects caused by mutating Arg-563 were considerably stronger than the effects caused by mutants of position Lys-565. In contrast to the epoxide hydrolase activity, several mutants at position Arg-563 retained small but significant peptidase activities. This observation helped to further examine the LTA₄-binding properties of mutated enzyme. Thus, determination of the inhibitory potency of LTA₄, as well as the LTA₄-like hydroxamic acid inhibitor, against the peptidase activity was possible. Surprisingly, LTA₄ binding was not significantly changed whereas the binding constant for the hydroxamic acid inhibitor increased between five to ten times. Finally, any unexpected effects, such as large structural changes, apart from the mutation itself, were ruled out by determining the crystal structure of the mutant [R563A]LTA₄H.

Taken together, the results suggest that Arg-563 acts as a carboxylate recognition site for LTA₄ and peptide substrates. For the latter, additional binding strength is provided by Lys-565. For the epoxide hydrolase reaction however, the role of Arg-563 appears to be accurate substrate positioning compatible with catalysis, rather than providing binding strength *per se*. Presumably, the preserved binding constant for LTA₄ inhibition reflects the flexibility of the fatty acid LTA₄, which allows it to find alternative, equally strong, binding conformations in the mutated enzyme. However, these alternative conformations are not compatible with catalysis. Thus, the lipid tail could probably slide to some extent along the hydrophobic tunnel without any significant loss of binding strength and the carboxy moiety have a great flexibility to find alternative sites for strong polar interactions.

18.3.4 Paper IV. Leukotriene A₄ hydrolase, insights into the molecular evolution by homology modeling and mutational analysis of enzyme from *S. cerevisiae*

Saccharomyces cerevisiae LTA₄H (*sc*LTA₄H) is the first example of an LTA₄ hydrolyzing enzyme in an invertebrate organism. In yeast, LTA₄ hydrolysis yields three different products: mostly 5*S*,6*S*-DHETE but also small amounts of LTB₄ and Δ^6 -*trans*- Δ^8 -*cis*-LTB₄ are produced. The peptidase activity of the yeast enzyme exhibits a different preference for peptide substrates compared to human LTA₄H and the activity is stimulated by LTA₄, contrary to the human enzyme which is inhibited. Functional similarities and differences, along with a sequence similarity of about 40%, renders *sc*LTA₄H an interesting target for studies of the molecular evolution of LTA₄H. To this end, we

undertook a thorough study including homology modeling and extensive mutagenesis in combination with various enzyme kinetic approaches. By using the modeled structure of *scLTA4H* we were able to rationally choose residues for mutagenesis.

Mutagenesis of residues important for LTA₄ hydrolysis. The modeled structure of *scLTA4H* along with the underlying sequence alignment indicated that only four residues directly lining the active site differed in the two enzymes. In addition, two residues close to the active site were interpreted as being of relevance for the chemistry of the enzyme: a loop residue, which is deleted in all mammalian LTA₄Hs, and an Asp residue, which is replaced by a Val residue in mammalian LTA₄H. While the former may potentially affect the position of other active site residues the latter may form strong domain interaction in the yeast enzyme only. This set of residues was mutated, both separately and in groups. Thus, a series of mutants with increased similarity to human LTA₄H were created. Analysis of these mutants were expected to mainly enlighten the mechanisms underlying the evolution of LTA₄ binding and turnover.

Mutagenesis of conserved residues. A second group of residues, which are conserved within this enzyme family, was also analyzed. These residues already had defined catalytic roles in human LTA₄H. Therefore, these mutations served the purpose to verify, and possibly refine, their roles in *scLTA4H*.

Catalysis of peptide hydrolysis is identical in yeast and human LTA₄H. For the latter group of residues we found that the functionality for terminal recognition of peptide substrates is preserved (*cf.* papers I and III). Thus, the functions of residues Glu-316 and Arg-627 of *scLTA4H* parallel those of Glu-271 and Arg-563 in human LTA₄H for anchoring of N- and C-termini, respectively. The kinetic analysis of the function of Glu-316 was partly more extensive than for the corresponding residue (Glu-271) in human LTA₄H including both surface plasmon resonance (SPR) and enzyme kinetics with progress curve analysis. This additional information probably holds true also for related aminopeptidases possessing the GXMEN motif, including human LTA₄H. Thus, the main role of this residue appears to be substrate anchoring but an indirect role in catalysis also seems likely. In general, mutating this residue leads to an inactive enzyme, however, in some instances residual activity remains. This effect is not solely explained by the role of the residue as an N-terminal anchor. Probably, it also reflects its close proximity to other functional residues, *e.g.* residues of the Zn-binding motif. Thus, mutation of this residue probably leads to a perturbation of substrate positioning (with

respect to other catalytic residues) resulting in a significant loss of enzyme function.

LTA₄ hydrolysis in yeast and human LTA₄H is partly different. Concerning epoxide hydrolysis the functions of Arg-627 and Glu-316 also parallel the corresponding human residues (Arg-563 and Glu-271). Thus, Arg-627 binds the carboxy moiety of LTA₄ and Glu-316 is essential for the stereo specific opening and hydrolysis of the epoxide ring. Since *scLTA4H* mainly yields 5*S*,6*S*-DHETE, the involvement of Asp-421 (corresponding to Asp-375 in human LTA₄H) in this specific reaction is excluded due to the architecture of the active site; this residue is simply too far away from Glu-316 and the epoxide of the substrate. Also, mutating Asp-421 into an Asn actually increased the 5*S*,6*S*-DHETE producing activity to some extent, which for the same stereochemical reasons exclude direct catalytic involvement but suggests a possible involvement in LTA₄ binding. For Glu-316, an obvious role would be that of a general acid-base catalyst responsible for both water introduction at carbon 6 and protonation of the adjacent oxyanion thus yielding the vicinal diol 5*S*,6*S*-DHETE.

Formation of other products does not exhibit any major dependence on the mutated residues, if any significant effects were observed they actually showed increased activities. Since the rate of formation of these compounds is very low one may speculate that, for the formation of these products, *scLTA4H* simply serves as a (catalytic) binding surface allowing certain spontaneous hydrolysis products to form while preventing others.

scLTA4H can be mutated to attain catalytic properties similar to human LTA₄H. For mutants of the yeast enzyme that mimic human LTA₄H several conclusions were drawn. Guided by the structure, *scLTA4H* (*scLTA4H*) was engineered to attain catalytic properties resembling those of human LTA₄H. Thus, in the process of gradually increasing the similarity between *scLTA4H* and human LTA₄H the substrate specificity of the yeast enzyme was successively changed to more resemble the human enzyme. In particular, the disfavored substrates Arg-*p*-NA and Pro-*p*-NA of wild type *scLTA4H* were actually the preferred substrates for the most extensive mutant, just as for human LTA₄H. Furthermore, the observed LTA₄-stimulation of the peptidase activity of wild-type *scLTA4H* was gradually transformed into LTA₄-inhibition. In the most extensive mutants, LTA₄ inhibits the peptidase activity for all tested substrates, just as for human LTA₄H.

Interestingly, for some mutants the effect of LTA₄ was found to depend upon the nature of the peptide substrate: some substrates were activated and some were inhibited upon LTA₄ treatment. This

was also the case for wild-type scLTA4H for which the very weak Arg- and Met-p-NA activities were inhibited by LTA₄ whereas the Ala- and Leu-p-NA activities were stimulated more than 10-fold. Since the outcome of LTA₄ treatment depends on the chemistry of the peptide substrate it is reasonable to assume that LTA₄ is bound close to where the substrates differ, *viz.* the side-chain of the N-terminal residue. In human LTA4H this part of the active site is very narrow therefore forcing LTA₄ to bind in an extended conformation. This in turn, suggests that the LTA₄ binding pocket of scLTA4H has to be considerably more spacious to allow LTA₄ to bind differently. Hence, LTA₄ appears to bind in two distinct conformations to scLTA4H: one productive compatible with substrate turnover and one allosteric compatible with activation of the peptidase activity. Concerning LTA₄ hydrolysis, the most extensive mutants exhibited a lowered 5S,6S-DHETE production whereas LTB₄ production remained intact. In relative amounts however, this group of mutants exhibited increased LTB₄ producing activities.

The epoxide hydrolase activity of LTA4H has evolved by re-utilizing residues with distinct functions in the aminopeptidase reaction for novel tasks. Inspecting multiple sequence alignments between proteins of the M1 family shows that several of the active site residues are conserved. Thus, it appears reasonable to assume that the ability to hydrolyze LTA₄ would exist in several other members of this enzyme family. However, with the exception of scLTA4H, and possibly aminopeptidase B [149] this has not been observed. Interestingly, a phylogenetic tree derived from such an alignment indicates that scLTA4H clusters together with mammalian LTA4Hs. Throughout the M1 family of aminopeptidases the catalytic domain is the most conserved portion of the protein. This suggests, and is indeed supported by the present data, that changes of the catalytic domain mainly affects the peptidase activity, but also, to some extent, creates the structural basis required for LTA₄ binding. Even though a limited number of mutations were sufficient to create an enzyme that exhibited an LTA₄ binding pattern similar to human LTA4H, additional structural changes, obscured to us, are required to create an enzyme capable of efficient and specific LTB₄ production. Possibly such changes may involve the C-terminal domain of the protein, in particular since this is the domain most unique to mammalian LTA4Hs.

Thus, in the course of evolution the binding pocket has been gradually reshaped in small steps to fine-tune LTA₄ binding. In this process, the substrate has been aligned with residues already serving in the peptidase activity, thereby allowing

them to perform novel chemistry compatible with optimal LTB₄ production. Notably, this process has occurred while at the same time maintaining the original peptidase activity.

18.3.5 Paper V. Assay for rapid analysis of the tri-peptidase activity of LTA₄ hydrolase

A limitation in the analysis of the peptidase activity of LTA4H has been the difficulty by which real peptide substrate could be utilized. Instead, chromogenic model substrates of limited physiological relevance have been used. Alternatively, one had to rely on complex analysis involving derivatization and HPLC analysis of the cleaved peptides for analysis of natural substrates, a method associated with considerable data noise. In this work a new assay is presented that circumvents these problems. The assay relies on the theory of competing substrates and is fast and simple to perform. The basic idea is that two competing substrates are present in the reaction mixture: one chromogenic for which the enzyme has a low specificity constant and one spectrophotometrically invisible substrate with a high constant. The result is that the high-affinity substrate will compete the low-affinity substrate out leading to very low turnover of the latter. Successively, as the high-affinity substrate is consumed, the rate of turnover of the low-affinity substrate will increase. Spectrophotometric monitoring of the reaction for the chromogenic substrate over time gives a curve with a distinct lag phase followed by a sudden burst. Analysis of the obtained progress curves allows determination of kinetic parameters.

In terms of kinetic parameters the peptidase activity of LTA4H is at least as efficient as its LTA₄ hydrolyzing activity. However, the physiological relevance of this activity has not been established, nor has an endogenous peptide substrate been identified. Our new assay will greatly facilitate the search for such substances, it will be valuable when performing enzyme kinetic studies and it constitutes a tool for structure-activity studies utilizing various peptide substrates. Since LTA4H belongs to the M1 family of aminopeptidases the assay should be directly applicable also for other aminopeptidases. Furthermore, the principles of this assay should be applicable for any system where two competing substrates, among which one is chromogenic, with sufficiently different affinities are available.

18.3.6 Paper VI. Structural basis for peptide hydrolysis by M1 aminopeptidases

In papers I-III several details of the peptidase reaction mechanism were elucidated. However, when analyzing the peptidase activity in those studies a chromogenic model substrate was utilized. They also lacked a binding model for a real peptide substrate based on experimental data, *i.e.* a crystal structure. To complement these issues we determined the structure of the peptidolytically inactive [E296Q]LTA4H mutant in complex with two different tri-peptides and of wild type LTA4H in complex with a tri-peptide analogous inhibitor. Glu-296 is part of the metallopeptidase consensus motif, HEXXH, and constitutes the essential general base catalyst.

The investigated inhibitor, denoted RB3040, is a mimic of the tetrahedral intermediate of peptide hydrolysis in which a PO₂-CH₂ link replaces the P1-P1' peptide bond. Moreover, we also analyzed the kinetics for tri-peptide hydrolysis by wild type and the D375A mutant of LTA4H, using the novel assay presented in Paper V, and determined inhibition properties of RB3040. Together, the data made it possible to describe binding of peptide substrates by LTA4H in great detail, to define sub-pocket specificity and also to propose a very detailed reaction mechanism.

Tri-peptide substrates bind along the GXMEN motif with their termini anchored to Arg-563 and Lys-565. Specifically, the peptide substrate backbone was shown to bind along the conserved GXMEN motif, as an anti-parallel β-strand, with its termini interacting with Arg-563 and Glu-271. Thus, the roles of these two residues, as proposed in Papers I and III, were confirmed. From the structures it was also evident that these two residues together dictate the optimal length of the substrate to three residues.

During peptidolysis a positional shift of the substrate leads to exchange of Zn-coordinating groups without changing the overall coordination pattern. Surprisingly, there was a slight discrepancy in the binding of the tri-peptides as compared to the inhibitor. Whereas the tri-peptides directly coordinated the catalytic zinc ion with their N-terminal amino groups the corresponding group of the inhibitor instead exhibited a strengthened interaction with Glu-271. The interpretation was that the substrate exhibits a small but significant movement during the course of the reaction, which leads to exchange of Zn-coordinating groups without disruption of the overall coordination pattern. Such a movement assures an optimal binding of the transition state and is achieved by certain groups, particularly exemplified by Asp-375 and Glu-271 that basically pull the substrate apart.

Despite the fact that Asp-375 does not form a direct H-bond with the substrate, enzyme kinetic data indicate that this residue is crucial for turnover of substrates with N-terminal arginine residues. This further supports a scheme in which the substrate slightly moves during the reaction.

The S1 sub-pocket is the major site of substrate specificity. Considering sub-pocket specificity, it was concluded that the S1 pocket is the major specificity site. In contrast to the S1'-S2' pockets, which are loosely defined, the S1 pocket is distinctly narrow with a hydrophobic entrance and a more hydrophilic bottom, thus being ideal for binding arginine residues. Since Asp-375 is located at the bottom of the pocket it is a major contributor to this functionality. These findings are in good agreement with a previous report by Örning *et al.* [104].

Tyr-383 and the zinc ion together stabilize the oxyanion of the reaction intermediate. Tyr-383 has been previously proposed to act as a proton donor to the leaving amine in the very last step of the peptidase reaction mechanism. However, such a role is not compatible with the findings of paper VI, which instead indicate that Tyr-383, together with the zinc ion, stabilizes the oxyanion of the tetrahedral reaction intermediate. Thus, Tyr-383 and the catalytic Zn together function as an oxyanion stabilizing site. The role as an acid catalyst in the last step of the reaction is instead assigned to Glu-296, protonated as a consequence of previous catalytic steps, where it acted as a general base during the nucleophilic attack of water. Consequently, the basic steps of the reaction mechanism parallel those of thermolysin with Tyr-383 corresponding to His-231 of thermolysin.

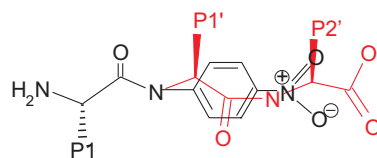
LTA4H as a model system for peptide hydrolysis by M1 aminopeptidases. This work presents the first example of an M1 metallopeptidase in complex with a natural substrate. It should be noted that our high-quality structural data involves the whole sequence of a very good substrate complexed to an MA aminopeptidase. In addition, the utilized reaction intermediate analogue corresponds to the exact length of the substrate. For the purpose of evaluating the catalytic properties of the enzyme these are very important factors. Hence, we believe that LTA4H could serve as a model system for peptide hydrolysis by MA metallopeptidases in general and for M1 aminopeptidases in particular.

19 CONCLUDING REMARKS AND LOOSE ENDS

Papers I-III basically use the same approach to elucidate the reaction mechanisms of LTA₄H. Mutagenesis and enzyme kinetics in combination with crystallography and modeling made it possible to draw detailed and reliable conclusions. However, a limitation of these studies is that they employed a chromogenic model substrate for analysis of the peptidase activity and that they lacked experimentally determined models for substrate binding. This was partially overcome by molecular modeling, which in principle can give very realistic answers but suffers from the fact that one never knows how close to the real answer the modeled result is. For the peptidase activity, these concerns were overcome by the results presented in paper V-VI, which involved the development of a new and simple assay that allowed direct assessment of the tripeptidase activity of LTA₄H and the determination of the crystal structure of LTA₄H in complex with tri-peptides. These findings neatly complemented the results from paper I-III and made it possible to create a very detailed view of the reaction mechanism for peptide hydrolysis by LTA₄H. Since LTA₄H belongs to the M1 family of aminopeptidases, which defines structurally very similar enzymes, particularly for the catalytic domain, this mechanism will be representative for this whole enzyme family. For the epoxide hydrolase activity however, some concerns still remain, these will be discussed in sections 19.3 - 19.5.

19.1 IMPORTANCE OF ASSAYING THE PROPER SUBSTRATE

Paper V shows that considerably different results could be obtained when assessing the peptidase activity with a model substrate as compared to a natural peptide. As shown, the first approach made a mutant of LTA₄H appear unaffected when assayed with a model substrate whereas it was virtually inactive when assayed with the natural substrate. Furthermore, LTA₄H exhibits a different substrate specificity profile for the various chromogenic model substrates, as compared to natural peptides, a possibly trivial observation considering the chemical differences of the substrates (Fig. 10). Whereas the preferred natural substrate of LTA₄H is arginyl-tripeptides the favored model substrate is *ala-p-NA*. This discrepancy in substrate specificities between real tri-peptide substrates and the model substrates can be explained by the rigidity of the *para*-nitroanilide moiety of the model substrate. This rigidity prevents the model substrate P1 residue from binding in a conformation perfectly mirroring the binding of a tri-peptide.



Figur 10. Comparison of model and natural peptide substrates. The model substrate *ala-p-NA* layered on top of a tri-peptide. The deviating part of the tri-peptide is shown in red. The two molecules are identical in the N-terminal part. There is no resemblance of the middle and C-terminal residues of the tri-peptide in the model substrate. Even though the N-terminal residues are identical the remaining part of the model substrate, and especially its rigidity, perturbs the binding of the model substrate with respect to a natural peptide. However, to some extent the nitro group of the model substrate roughly corresponds to the C-terminal carboxy group of a peptide substrate. These two functionalities are also located at a similar distance from the N-terminus.

19.2 IMPLICATIONS FOR INHIBITOR DESIGN

In addition to the conclusions regarding substrate binding and reaction mechanisms, outlined throughout this thesis, the crystal structures of LTA₄H in complex with various ligands are of great value for inhibitor design, and thereby for the process of rational drug design. Available inhibitors are detailed probes of the active site. In addition to the Zn-binding site and nearby residues, *e.g.* Gln-136, Glu-271 and members of the GXMEN motif, the deeper part of the hydrophobic binding pocket appears to be of importance for overall inhibitor binding strength. In this region of the binding pocket, properly designed inhibitors can make, not only, extensive hydrophobic interactions, but also form specific hydrogen bonds with certain residues within a polar patch of the cavity, *e.g.* Asp-375. None of the inhibitors developed thus far have any groups presenting hydrogen bond donors to Asp-375. Exploring such an interaction would possibly add new inhibitor binding strength and specificity. Moreover, inhibitors selective for the LTA₄ hydrolyzing activity only, should preferably be designed to target the deeper part of the pocket. As judged by the structure, this should in principle be possible.

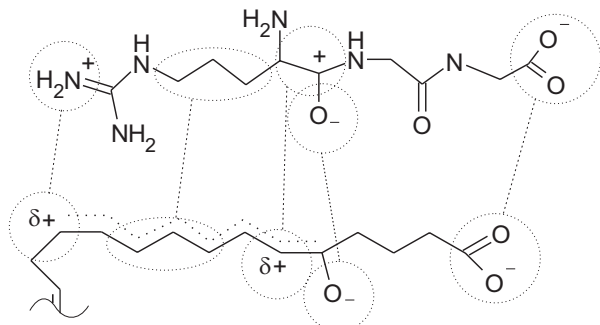
In addition, the more spacious part of the large cavity, which is formed in between the three domains of LTA₄H, is very polar in nature and offers many sites for specific interactions. With the notable exception of Arg-563 and Lys-565 these sites are basically not utilized by the present inhibitors. These binding sites define in part the S1' and S2' subsite of the enzyme and, if utilized, would most certainly add strength to the binding of inhibitors.

19.3 THE ROLES OF TYR-378 AND TYR-383 IN THE EPOXIDE HYDROLASE REACTION

According to previous findings, Tyr-378 is not only involved in suicide inhibition but also, along with Tyr-383, affects the formation of proper stereochemistry of the product. This is a factor critical for the biological activity of LTB₄. Tyr-383 and Tyr-378 are hydrogen-bonded to each other and in the model for LTA₄ bonding they are close to the conjugated triene system. In fact, their positions likely affect substrate alignment and thereby have the ability to influence the double bond conformation of the product.

In the model for LTA₄ binding, Tyr-378 is close to carbon 12 of LTA₄, suggesting that LTA₄ could be covalently attached to the enzyme via an ether link to this residue, thus explaining its involvement in suicide inhibition. Furthermore, the slight perturbation of the shape of the active site caused by mutation of Tyr-378 could allow LTA₄ to bind in alternative conformations. This could explain the formation of an additional product with a double bond conformation different from LTB₄, *i.e.* Δ^6 -*trans*- Δ^8 -*cis*- Δ^{10} -*trans*-LTB₄, by mutants of this residue.

Mutation of Tyr-383 to a Phe would create additional space around the zinc ion. Possibly, this could allow an additional water molecule to bind near carbon 6 of the bound LTA₄, which in turn could be the reason for the formation of 5*S*,6*S*-DHETE. However, a more intriguing explanation for the behavior of this mutant involves a more



Figur 11. Similarities between LTA₄ and tri-peptides. From a chemical perspective, a tri-peptide and LTA₄ actually have some clear similarities. This is especially evident if one considers the carbocation reaction intermediates. As indicated by the lines, both intermediates have similar charge distributions and a similar aliphatic moiety. Therefore, they might interact with the same catalytic enzyme residues in similar manners. Also the carboxyl moieties of both substrates are at similar positions. Notably, the greater flexibility of the C1-C5 chain of LTA₄, as compared to the corresponding part of the tri-peptide, makes it possible for the LTA₄ carboxylate to end up at the same position as the tri-peptide counterpart. This in turn, would allow it to interact with the same enzyme residues.

direct role for Tyr-383 in LTA₄ hydrolysis. Since the intermediates of LTA₄ hydrolysis and peptide hydrolysis have several properties in common (Fig. 11) it might be possible that also the binding and stabilization of these species are achieved through the same residues. Furthermore, according to our findings several residues actually function in both reactions and in some cases they even perform the same task. Thus, it is then tempting to hypothesize that the enzyme achieves stabilization of reaction intermediates of the two reactions by very similar means. If so, the oxyanion stabilization of the tetrahedral reaction intermediate of peptide hydrolysis, performed by the zinc ion together with Tyr-383, may parallel the stabilization of the intermediate of LTA₄ hydrolysis. Thus, Tyr-383 together with the zinc ion may bind the oxyanion formed at the 5-*S* oxygen when LTA₄ is hydrolyzed (for further details, see section 19.5). A mutation of Tyr-383 that disrupts this functionality would then lead to a less precise binding of LTA₄ and, specifically, the reaction intermediate. Such a shift in substrate binding could allow the hydrolytic water molecule intended for peptide hydrolysis to attack carbon 6 of LTA₄ whereupon 5*S*,6*S*-DHETE would be formed.

19.4 MISSING PIECES OF THE REACTION MECHANISM FOR LTA₄ HYDROLYSIS

For LTA₄ hydrolysis some issues still remain unclear: Firstly, the exact role of Glu-271 is not clear and two possible roles for this residue were proposed in paper I. Secondly, even though Asp-375 was identified as a general base facilitating introduction of water at carbon 12 of LTA₄ (Paper II), it is unclear how protonation of the 5-*S* oxygen and re-protonation of the enzyme are achieved?

Since the crystal structure of the LTA₄H-tripeptide complex was extremely valuable for the detailed understanding of the peptidase reaction, maybe the most straightforward way for resolving the remaining concerns about LTA₄ hydrolysis would be to determine the structure of LTA₄H, or mutant LTA₄H, complexed with LTA₄ or LTB₄. This would not immediately straighten out all question marks, but definitely some since it would reveal the exact position of reacting groups with respect to the substrate. Experimentally, this would not be a completely easy task, particularly since LTA₄ is a highly unstable molecule.

The problem regarding protonation is especially complex considering that the point of the nucleophilic attack by water and the point of protonation of the substrate are separated by a considerable distance. This is in sharp contrast to the common type of hydrolytic reaction in which this occurs at the same site. In LTA₄H, the acidic

proton resulting from the nucleophilic attack on carbon 12 presumably resides on Asp-375. From this position it has to travel a considerable distance in order to protonate the 5-S oxyanion. The hydrogen bonded residues Tyr-378 and Tyr-383 may provide (part of) such a path since Tyr-378 is involved in suicide inhibition, and thus resides close to carbon 12 of LTA₄, and because Tyr-383 may interact with the oxyanion of the reaction intermediate, as described in the following section 19.5.

However, these events may as well occur via bulk solvent. Especially if a 5-S alkoxide LTB₄ species is sufficiently stable to leave the enzyme, without reversing the reaction, it would be protonated by bulk solvent. Then in turn, the greater access to the active site would aid solvent assisted re-protonation of the enzyme. It should be emphasized that issues concerning protonation pathways are rather speculative, especially since they are more or less impossible to verify experimentally. Nevertheless, acid-base catalysis is of key importance to enzyme function and therefore of great interest.

19.5 A NEW MODEL FOR LTA₄ BINDING?

Transferring the binding mode outlined for tripeptides, as outlined in section 18.3.6 and Paper VI, to LTA gives a model for binding of the carbocation intermediate of LTA₄ hydrolysis. In such a model the 5-S oxyanion of the intermediate is bound between the zinc ion and Tyr-383. In the context of LTA₄ hydrolysis, it should be noted that LTA₄ is a very labile molecule, which is spontaneously hydrolyzed with a half-life of about four seconds in neutral solution at room temperature, and even faster under acidic conditions [189]. (The high reactivity of LTA₄ is accounted for by the allylic position of the epoxide, itself a reactive functionality.) The fact that acid increases the rate of spontaneous hydrolysis indicates that means to achieve stabilization of the oxyanion of the intermediate are crucial for the reaction rate. Furthermore, since the products of spontaneous hydrolysis of LTA₄ are rather similar to LTB₄ (mostly 12-hydroxyl-epimers with an all-*trans* conformation of the double bonds), it seems as if the main task of the enzyme is to control the stereochemistry of the reaction.

An LTA₄-binding model must account for the differences in double bond conformation of the conjugated trienes of LTA₄ (Δ^7 -*trans*- Δ^9 -*trans*- Δ^{11} -*cis*) and LTB₄ (Δ^6 -*cis*- Δ^8 -*trans*- Δ^{10} -*trans*). Through the reaction, the position of the conjugated triene-system is transferred by resonance from the position between C7-C12 in LTA₄ to C6-C11 in LTB₄. Thus, the enzyme must keep the C6-C7 bond

of LTA₄, which will form the novel *cis*-bond of LTB₄, in a *pro-cis* conformation. This is a single bond, to some extent rotationally restrained by its vicinity to the epoxide moiety, which upon formation of the carbocation intermediate acquires double bond character. Thus, if LTA₄ is properly bound by the enzyme the correct C6-C7 double bond conformation, *i.e.* *cis*, of LTB₄ could be formed without breaking any double bonds of the substrate.

Similarly, in order to obtain *R* configuration of the 12-hydroxyl group of LTB₄ the planar carbon 12 of the carbocation intermediate must face Asp-375, as well as hydrolytic water molecules, correctly.

Interactions and conformational requirements such as those described above, place distinct restraints on the overall LTA₄ binding and thus make it easier to create an accurate binding model for both the substrate as well as the carbocation intermediate. In future studies, a binding model accounting for these restraints could possibly help to refine the reaction mechanism for LTA₄ hydrolysis to a higher level of detail.

19.6 A NOTE ON EXPERIMENTAL LIMITATIONS

It should be kept in mind that all studies have their limitations and therefore future studies will always refine or even challenge present findings. In our studies some chemical phenomena, *e.g.* reaction energetics, molecular dynamics and protonation states of individual residues, were not fully addressed by the applied methodology. These might be of particular importance to enzyme function. To some extent however, enzyme kinetics reflects the energetics of the overall chemical reaction and one could of course extend our studies to include more mutants, more structures and more extensive and advanced kinetic studies to reach an even higher level of experimental detail. However, to reach this goal alternative approaches and technologies should preferably be utilized. For instance, this could involve theoretical or experimental techniques such as quantum mechanical calculations to study energetics, fluorescence techniques to study enzyme dynamics, nuclear magnetic resonance to study enzyme dynamics and protonation states. Yet other approaches for the specific studies of protonation states include neutron diffraction crystallography, mass spectrometry and theoretical pK_a predictions. (Actually, the last strategy was applied on LTA₄H without revealing any abnormal pK_a shifts in the active site.)

19.7 EVOLUTIONARY ASPECTS

Biosynthesis of LTB₄ has been shown to occur in a variety of vertebrates but not in lower animals. Most likely, this correlates with the presence of enzymes specific for the LT pathway, *i.e.* 5-LO and LTA₄H. As discussed in paper IV, the overall sequence similarity with human LTA₄H of a given homologous enzyme, is not sufficient to judge

whether it can catalyze the formation of LTB₄ or not. However, this might be achieved if the sub-sequence similarity with each specific domain of LTA₄H is considered, especially if one also takes into account whether or not a specific organism possesses a 5-LO enzyme. Since several organisms have their genomes fully sequenced this is now a trivial task. This can be exemplified by an identity

Identity (%) matrix for whole sequence.

	Human	Orangutan	Guinea pig	Pig	Mouse	Chinchilla	Rat	Chicken	X. tropicalis	X. leavis	Zebra fish	C. intestinalis	C. elegans	Mosquito	Fruit fly	S. pombe	S. cerevisiae	Rat APB	Human APB	Arabidopsis	
Human	ID	99	93	95	93	94	93	76	68	67	64	55	44	45	43	38	37	34	33	32	
Orangutan	ID	ID	93	95	93	93	76	68	68	64	55	44	44	44	43	39	37	34	34	32	
Guinea pig	ID	ID	ID	92	90	94	91	75	66	66	63	54	43	44	43	38	36	33	33	32	
Pig	ID	ID	ID	ID	93	92	93	77	67	67	64	56	44	45	44	39	37	35	34	33	
Mouse	ID	ID	ID	ID	ID	91	97	76	67	66	64	54	44	45	42	38	36	34	33	32	
Chinchilla	ID	ID	ID	ID	ID	ID	92	76	67	66	63	54	44	44	43	38	37	34	33	32	
Rat	ID	ID	ID	ID	ID	ID	ID	76	66	65	64	54	44	44	42	38	36	33	33	32	
Chicken	ID	ID	ID	ID	ID	ID	ID	ID	70	71	68	53	44	44	43	39	37	35	35	33	
X. tropicalis	ID	ID	ID	ID	ID	ID	ID	ID	ID	93	68	54	45	43	42	39	37	33	32	33	
X. leavis	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	68	54	46	43	42	39	37	34	33	33	
Zebra fish	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	52	44	43	42	39	36	33	32	33	
C. intestinalis	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	44	46	46	41	36	33	32	32	
C. elegans	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	39	41	38	34	29	27	30	
Mosquito	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	58	38	35	33	33	32	
Fruit fly	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	36	34	31	31	32	
S. pombe	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	45	29	28	30	
S. cerevisiae	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	29	29	29	
Rat APB	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	88	29	
Human APB	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	29
Arabidopsis	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID

Identity (%) matrix for N-terminal domain.

	Human	Orangutan	Guinea pig	Pig	Mouse	Chinchilla	Rat	Chicken	X. tropicalis	X. leavis	Zebra fish	C. intestinalis	C. elegans	Mosquito	Fruit fly	S. pombe	S. cerevisiae	Rat APB	Human APB	Arabidopsis	
Human	ID	99	94	94	93	92	91	69	62	61	61	45	39	43	35	29	32	26	25	30	
Orangutan	ID	ID	94	94	93	91	90	68	62	61	61	45	39	43	36	30	32	26	25	30	
Guinea pig	ID	ID	ID	92	91	93	90	68	62	61	62	46	38	42	36	30	32	26	25	29	
Pig	ID	ID	ID	ID	93	89	91	70	61	61	63	45	39	43	36	29	31	27	26	30	
Mouse	ID	ID	ID	ID	ID	96	68	61	59	62	45	39	43	35	29	31	26	26	30		
Chinchilla	ID	ID	ID	ID	ID	ID	ID	89	67	60	59	60	44	37	41	35	29	32	25	29	
Rat	ID	ID	ID	ID	ID	ID	ID	ID	67	60	58	61	43	38	41	34	27	32	25	24	29
Chicken	ID	ID	ID	ID	ID	ID	ID	ID	ID	63	64	64	41	39	44	36	29	30	29	29	28
X. tropicalis	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	91	64	48	43	42	38	31	30	25	24	30
X. leavis	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	63	46	43	42	38	31	31	26	25	31
Zebra fish	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	50	40	42	38	33	32	25	23	29
C. intestinalis	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	38	44	41	31	28	25	23	29
C. elegans	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	40	38	33	33	23	22	28
Mosquito	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	48	32	35	26	26	32
Fruit fly	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	29	30	26	26	33
S. pombe	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	39	20	19	24
S. cerevisiae	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	25	25	24
Rat APB	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	81	23
Human APB	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	22
Arabidopsis	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID

Identity (%) matrix for catalytic domain.

	Human	Orangutan	Guinea pig	Pig	Mouse	Chinchilla	Rat	Chicken	X. tropicalis	X. leavis	Zebra fish	C. intestinalis	C. elegans	Mosquito	Fruit fly	S. pombe	S. cerevisiae	Rat APB	Human APB	Arabidopsis	
Human	ID	99	93	96	93	95	94	83	76	75	69	70	58	55	59	51	51	49	49	43	
Orangutan	ID	ID	93	97	94	96	94	82	76	76	70	70	58	55	59	51	51	49	49	43	
Guinea pig	ID	ID	ID	93	91	95	91	81	72	72	69	67	56	55	58	50	50	46	47	41	
Pig	ID	ID	ID	ID	94	96	95	81	75	74	69	70	58	56	59	51	50	49	49	43	
Mouse	ID	ID	ID	ID	ID	95	99	82	73	73	68	68	58	56	58	50	50	49	48	41	
Chinchilla	ID	ID	ID	ID	ID	ID	95	84	75	74	70	69	58	56	58	50	51	49	49	43	
Rat	ID	ID	ID	ID	ID	ID	ID	82	73	73	69	68	58	56	57	50	49	49	48	42	
Chicken	ID	ID	ID	ID	ID	ID	ID	ID	77	78	73	67	60	55	58	52	53	49	51	44	
X. tropicalis	ID	ID	ID	ID	ID	ID	ID	ID	ID	96	77	66	59	50	53	51	50	47	46	43	
X. leavis	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	77	67	60	50	52	52	49	47	47	44	
Zebra fish	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	64	58	51	54	50	46	46	46	43	
C. intestinalis	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	58	57	58	55	50	47	48	44	
C. elegans	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	50	54	53	45	41	40	39	
Mosquito	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	70	49	45	46	47	43	
Fruit fly	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	47	44	43	44	41	
S. pombe	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	55	40	40	40	
S. cerevisiae	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	38	39	40	
Rat APB	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	94	42	
Human APB	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	43
Arabidopsis	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID

Identity (%) matrix for C-terminal domain.

	Human	Orangutan	Guinea pig	Pig	Mouse	Chinchilla	Rat	Chicken	X. tropicalis	X. leavis	Zebra fish	C. intestinalis	C. elegans	Mosquito	Fruit fly	S. pombe	S. cerevisiae	Rat APB	Human APB	Arabidopsis	
Human	ID	99	92	92	91	92	94	77	65	63	59	45	26	32	31	32	25	23	22	20	
Orangutan	ID	ID	91	92	91	92	93	77	65	63	59	45	26	32	31	32	25	23	22	20	
Guinea pig	ID	ID	ID	89	88	94	90	75	62	62	57	44	28	32	30	32	24	24	23	21	
Pig	ID	ID	ID	ID	92	90	92	79	63	62	59	47	27	34	31	34	25	24	23	21	
Mouse	ID	ID	ID	ID	ID	89	96	78	64	63	61	45	27	32	29	33	25	24	23	21	
Chinchilla	ID	ID	ID	ID	ID	ID	ID	91	75	63	62	57	44	28	33	31	33	24	25	24	20
Rat	ID	ID	ID	ID	ID	ID	ID	ID	78	64	63	60	45	27	33	31	34	25	23	22	21
Chicken	ID	ID	ID	ID	ID	ID	ID	ID	ID	70	70	64	46	26	31	31	34	25	24	23	23
X. tropicalis	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	91	61	46	27	34	33	30	27	25	24	21
X. leavis	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	62	45	29	35	34	31	26	25	24	21
Zebra fish	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	37	26	32	27	29	26	27	26	23
C. intestinalis	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	31	34	34	34	25	23	22	20
C. elegans	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	24	28	27	20	22	20	21
Mosquito	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	51	30	24	24	24	15
Fruit fly	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	32	26	24	23	18
S. pombe	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	38	24	22	23
S. cerevisiae	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	22	21	22
Rat APB	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	90	19
Human APB	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	19
Arabidopsis	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID

Figure 12. Sequence identity matrices for LTA₄H homologues of different species, ranging from plant to primates. As indicated, the left top panel shows identities with respect to the whole sequence of LTA₄H and the lower left panel for the N-terminal. Top right and lower right panels show identities for the catalytic and C-terminal domains, respectively. Identities greater than 70% are shaded dark blue, from 50-70% light blue and 35-50% light yellow. Species with names given in red are those that possess a putative 5-LO enzyme. Species with names underlined belong to a group of animals (e.g. fish or primates) among which LTB₄ biosynthesis has been experimentally verified. Note that the similarities are not evenly distributed over the whole sequence. Whereas conservation of the N-terminal domain exhibits a pattern similar to the

matrix, in which the pair-wise sequence identities of LTA₄H homologous of selected animals are compared. As shown in Figure 12, this clearly indicates that “real” LTA₄Hs (*i.e.* with the ability to catalyze LTA₄→LTB₄ formation) are specific for vertebrates. However, it also suggests that the ascidian *Ciona intestinalis* may have the enzymatic machinery for LTB₄ synthesis. *C. intestinalis*, or Sea squirt, is a chordate (*i.e.* a group of animals including the vertebrates and some closely related invertebrates. *Chorde* is Greek for cord, thus suggesting the presence of a spinal cord like structure.), which develops through extensive metamorphosis from larva (or tadpole) to adult animal. While the adult animal bears no signs of a spinal chord the larva possesses a notochord (*i.e.* a cellular rod-shaped body that runs the length of a vertebrate embryo and supports the nerve cord, it develops into the vertebrae of the spinal column). Interestingly, during metamorphosis ascidians have been shown to upregulate certain genes which are homologous to genes involved in the inflammatory response of humans [190]. Thus, *c. intestinalis* might provide a suitable model system for further evolutionary studies of LTA₄H.

In paper IV some structural changes underlying the evolution of LTA₄H were investigated. The data indicate that LTA₄H has evolved from an ancestral aminopeptidase, which initially possessed an allosteric lipid-binding site. During evolution, the architecture of this site was gradually changed to become an active site accommodating LTA₄. Subsequent optimizations of the structure further improved substrate alignment, and finally allowed efficient catalysis of LTA₄→LTB₄ formation. Interestingly, these structural changes occurred without disrupting the peptidase activity of the enzyme. Thus, it appears as if there has been an evolutionary pressure to keep the peptidase activity of the enzyme and that this activity is of physiological relevance: a futile enzymatic activity would most likely be deleted during the course of evolution. Indeed it is possible to selectively remove the peptidase activity by simple point mutations, as shown by mutagenesis studies [100, 103]. Alternatively, the peptidase activity is just an evolutionary remnant made possible because of the chemical similarities of the two substrates or reaction intermediates (Fig. 11). Whichever alternative is correct remains to be elucidated. However, it is always more tempting to believe in conspiracy rather than mere chance!

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