Leukotriene A₄ hydrolase: Insights to the molecular evolution by homology modeling and mutational analysis of enzyme from Saccharomyces cerevisiae

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Running title: Molecular evolution of LTA₄ hydrolase

Mammalian leukotriene A₄ (LTA₄) hydrolase is a bifunctional zinc metalloenzyme possessing an Arg/Ala aminopeptidase and an epoxide hydrolase activity, which converts LTA₄ into the chemoattractant LTB₄. We have previously cloned an LTA₄ hydrolase from S. cerevisiae with a primitive epoxide hydrolase activity and a Leu aminopeptidase activity, which is stimulated by LTA₄. Here we used a modeled structure of S. cerevisiae LTA₄ hydrolase, mutational analysis, and binding studies to show that Glu-316 and Arg-627 are critical for catalysis, allowing us to propose a mechanism for the epoxide hydrolase activity. Guided by the structure, we engineered S. cerevisiae LTA₄ hydrolase to attain catalytic properties resembling those of human LTA₄ hydrolase. Thus, six consecutive point mutations gradually introduced a novel Arg aminopeptidase activity and caused the specific Ala and Pro aminopeptidase activities to increase 24 and 63 times, respectively. In contrast to the wild type enzyme, the hexuple mutant was inhibited by LTA₄ for all tested substrates and to the same extent as for the human enzyme. In addition, these mutations improved binding of LTA₄ and increased the relative formation of LTB₄, whereas the turnover of this substrate was only weakly affected. Our results suggest that during evolution, the active site of an ancestral eukaryotic zinc aminopeptidase has been reshaped to accommodate lipid substrates, while using already existing catalytic residues for a novel, gradually evolving, epoxide hydrolase activity. Moreover, the unique ability to catalyze LTB₄ synthesis appears to be the result of multiple and subtle structural rearrangements at the catalytic center, rather than a limited set of specific amino acid substitutions.

Leukotriene (LT)₄ A₄ hydrolase (LTA₄H) catalyzes the hydrolysis of LTA₄ (5S-trans-5,6-oxido-7,9-trans-11,14-cis eicosatetraenoic acid) into LTB₄ (5S,12R-dihydroxy-6,14-cis-8,10-trans-eicosatetraenoic acid) a potent chemotactic agent and mediator of inflammation (1,2). The mammalian LTA₄H is a 70 kDa, bifunctional, zinc metalloenzyme, which, in addition to its epoxide hydrolase activity (hydrolysis of LTA₄ into LTB₄), possesses an anion dependent Arg and Ala aminopeptidase activity (3). The peptide and lipid substrates are hydrolyzed in the same active site and are thus mutually exclusive. LTA₄H is homologous to other aminopeptidases in a variety of species, ranging from mammals to bacteria, in particular those belonging to the M1 family (4). Members of this family share a common Zn binding signature, HEXXH, in which the His residues are the primary Zn binding ligands and Glu the general base catalyst required for peptidolysis. In LTA₄H, as well as most other M1 aminopeptidases, the third Zn binding ligand is a Glu located 18 residues downstream from the HEXXH motif, thus defining a HEXXH-(X)₁₈-E motif. However, the evolutionary relationship between the M1 aminopeptidases and LTA₄H is uncertain. Thus, aminopeptidase 1 from Caenorhabditis elegans is 45% identical (64% similar) at the amino acid level to mammalian LTA₄H and exhibits an Arg aminopeptidase activity (5). Despite this high level of sequence identity,
the C. elegans enzyme fails to hydrolyze LTA4 into LTB4 and no other functional link to LTA4H has been reported. In fact, enzymes carrying a distinct LTA4 hydrolase activity have only been detected among vertebrates, including birds, frogs and fish (6-8).

We have previously identified a putative ancestral LTA4H from Saccharomyces cerevisiae (scLTA4H) that carries a primitive epoxide hydrolase activity (9). The yeast enzyme is 39\% identical (58\% similar) to human LTA4H (humLTA4H). In contrast to humLTA4H, which only produces LTB4, scLTA4H hydrolyzes LTA4 into three products. The main product is 5S,6S-dihydroxy-7,9-trans-11,14-cis-eicosatetraenoic acid (5S,6S-DHETE), but also small amounts of LTB4 as well as 5S,12R-dihydroxy-6,10-trans-8,14-cis-eicosatetraenoic acid (ΔS-trans-Δ8-cis-LTB4) are produced (10). The yeast enzyme has a different preference for model peptide substrates. While humLTA4H exhibits the highest specific activity for the substrates Ala-p-nitroanilide (Ala-p-NA) followed by Arg-p-NA the yeast enzyme prefers Leu-p-NA followed by Met-p-NA. Interestingly, whereas the peptidase activity of humLTA4H is blocked by LTA4 the peptidase activity of scLTA4H is strongly activated by this lipid.

Recently, the crystal structure of humLTA4H was solved (11). The protein is folded into three domains with a deep cavity in between, which harbors the active center. The architecture of the active site was established and a model for binding of LTA4 was proposed, involving a narrow, L-shaped, hydrophobic side pocket. The structure clearly indicates that the conservation of active-site residues are not confined to residues of the HEXXH-(X)18-E motif but extends to residues lining the entire LTA4 binding pocket. Notably, the active site of LTA4H is composed of residues from all three domains.

Considering the sequence identity between human and yeast LTA4H as well as functional similarities and differences, we chose scLTA4H as a suitable target to study the molecular mechanisms underlying the evolution of mammalian LTA4Hs. To this end, a homology model of the scLTA4H enzyme, based on the crystal structure of humLTA4H, has been constructed and candidate catalytic residues have been subjected to mutational analysis. Using rational protein engineering, scLTA4H was converted to an enzyme with functional properties more similar to those of mammalian LTA4H. Together, our data provide novel insights to both the catalytic mechanisms and the molecular evolution of LTA4H.

MATERIALS AND METHODS

Materials – QuickChange Site-Directed Mutagenesis Kit (Stratagene), Ni-NTA (nickel-nitrioltriacetic acid; QIAGEN), and DYEnamic ET terminator cycle sequencing kit (Pharmacia Biotech) were used according to the manufacturer’s instructions. Standard chemicals were purchased from Sigma. LTA4 methyl or ethyl ester was obtained from Merck Frosst (Quebec, Canada). To obtain the free acid, the esterified LTA4 was saponified in aceton with 50 mM NaOH (20\% v/v) for 60 min at room temperature, or in tetrahydrofuran with 1 M LiOH (6\% v/v) for 48 h at 4°C.

Molecular modeling – Human LTA4H was aligned against homologous proteins, including scLTA4H, in the range between 33\% and 92\% sequence identity using ClustalW (12). From this multiple alignment a pair-wise alignment between scLTA4H and humLTA4H was derived (Fig. S1, Supplementary Material on line). The structure of humLTA4H (PDB ID 1HS6) was used as the template. The model was constructed using the WHATIF program, which was also used for initial geometry refinement (13). Manual adjustment of the model including rebuilding of loops with insertions and deletions was done with XtalView (14). Finally, the model was energy minimized using the YAMBER force field implementation of the Yasara software (15). After energy minimization the rmsd between the scLTA4H model and the template was 0.8 Å for 567 carbon-α atoms. Concerning model quality the final model fulfilled the requirements of the WHATCHECK program, where applicable (16).

Mutagenesis – Site-directed mutagenesis of scLTA4H cDNA in pT3_scLTA4H-40his, constructed as previously described (9), was carried out by PCR using the protocol for Stratagene QuickChange site-directed mutagenesis kit. The presence of mutations and the integrity of the sequences were verified by DNA sequencing using the dideoxy chain termination method. All mutants produced are described in Table S1, Supplementary Material on line. Specifically, mutants 3M, 4M, 5M and 6M are defined as Q412V/T418I/F424Y,
Expression and purification – Expression and purification of recombinant proteins were performed as previously described (10). Briefly, scLTA4H was expressed as a (His)6-tagged fusion protein and purified by affinity chromatography on a Ni-NTA column followed by chromatofocusing and anion exchange chromatography. Protein concentrations were determined according to the method of Bradford, using the Bio-Rad protein-assay reagent and bovine serum albumin as standard. To determine the purity of the protein samples, SDS-polyacrylamide gel electrophoresis (PAGE) was performed on a Phast system (Amersham Pharmacia Biotech) using 10-15% gradient gels. Bands of protein were visualized by staining with Coomassie Brilliant Blue.

Enzyme kinetic assays – For determination of apparent kinetic constants ($k_{cat}$ and $K_m$) for the aminopeptidase activity at least three sets of initial velocity measurements were performed. Aliquots (50 µl) of equilibrated solutions containing 1 µg protein, 500 mM KCl, 250 mM Tris-HCl, pH 7.5, were added to the wells of a microtiter plate. The reactions were initiated by the addition of 200 µl of aminoacyl-p-NA of various concentrations (12, 10, 5, 2.5, 1.25, 0.625, 0.3125, and 0.1525 mM) yielding a final reaction volume of 250 µl containing 1 µg protein, 100 mM KCl, 50 mM Tris-HCl, pH 7.5, and 9.6 - 0.125 mM Ala-p-NA. The formation of p-NA was subsequently monitored at ambient temperature for 15 min in a MCC/340 multiscan spectrophotometer as the increase in absorbance at 405 nm. Spontaneous hydrolysis of the various aminoacyl-p-NA was corrected for by subtracting the absorbance of incubations without enzyme. A molar response factor of 8065 M$^{-1}$ for p-NA was derived from a set of standard incubations of known concentration.

For determination of apparent kinetic constants ($k_{cat}$ and $K_m$) for the epoxide hydrolase activity at least three sets of initial velocity measurements were performed. Reactions were carried out in 100 µl aliquots of Tris-HCl, pH 7.5, containing 25 µg of protein. The reactions were initiated by the addition of various amounts of LTA4 (giving final concentrations ranging from 2 µM – 150 µM) and quenched after 30 s by the addition of 200 µl of methanol containing 1.5 µM prostaglandin (PG)B$_2$ as internal standard. The added volume of LTA4 never exceeded 2 µl. The samples were subsequently diluted with 1 ml water and acidified to pH $\approx 3.5$ with 10 µl of acetic acid (10 %) and metabolites extracted on solid phase Chromabond C$_{18}$ columns. Samples were loaded onto the columns, washed with 1 ml 25% methanol, and eluted with 250 µl 100% methanol. The samples were diluted with 250 µl water prior to HPLC analysis. Metabolites of LTA$_4$ were separated by isocratic reverse-phase HPLC on a Waters Nova-Pak C$_{18}$ column eluted with a mixture of methanol/acetonitrile/water/acetic acid (30:31:39:0.01 by vol.) at a flow rate of 1.2 ml/min. The UV detector was set at 270 nm and metabolites were quantified based on peak area measurements and the known extinction coefficients for the internal standard PGB$_2$ (30 000 M$^{-1}$ x cm$^{-1}$), LTB$_4$ (50 000 M$^{-1}$ x cm$^{-1}$), 5,6-DHETE (40 000 M$^{-1}$ x cm$^{-1}$) and Delta$^6$-trans-Delta$^8$-cis-LTB$_4$ (50 000 M$^{-1}$ x cm$^{-1}$).

For determination of the specific aminopeptidase and epoxide hydrolase activities the corresponding standard reactions were carried out with 1 mM aminoacyl-p-NA and 40 µM LTA$_4$, respectively.

Determination of the effects of LTA$_4$ on the peptidase activity of LTA4H – As a test for a stimulatory or inhibitory effect, LTA$_4$ was added at a single concentration (50 µM). To assess dose-response relationships, the specific peptidase activity was assayed in enzyme preparations pre-treated with various amounts of LTA$_4$ (typically 0.5 - 150 µM) for 20 to 30 min, prior to the activity measurements.

SPR Biosensor Analysis of bestatin binding – To assess the substrate affinity of the inactive mutants [E316Q] and [E316A]scLTA4H an interaction assay using an SPR biosensor, Biacore 3000 (Biacore, Uppsala, Sweden) was performed using the peptide analogue bestatin as the analyte. As a control experiment the same binding affinity of wild-type enzyme was determined. All binding experiments were performed at ambient temperature in a 10 mM Tris pH 7.4 running buffer supplemented with 0.005 % SP20.

Standard amine coupling (BIAapplication handbook;Biacore) was used for covalent protein immobilization onto a CM5 sensor chip. The proteins were kept at a concentration of 20 µg/ml in 10 mM sodium
acetate buffer pH 5.5 and injected onto the EDC/NHS (N-ethyl-N’-(3-dimethyl aminopropyl)-carbodiimide hydrochloride /N-hydroxysuccinimide) activated sensor surface at a flow-rate of 5 μl/min. To ensure that similar amounts of protein were immobilized in the three lanes the change in refractive index was monitored and immobilization stopped when the relative response reached approximately 11000 response units. To measure the real-time binding various amounts of bestatin (62.5, 125, 250 μM) in running buffer were injected, at a flow-rate of 20 μl/min for 2 minutes, onto the three surfaces containing immobilized protein. The SPR response caused by analyte binding and subsequent self-dissociation was monitored. To correct for background responses several injections containing buffer alone, were performed. For each concentration of bestatin the experiment was run in triplicate. To regenerate the chip surface bound bestatin was removed by washing with 20 μl of 1M ethanolamine.

**Competition assay for analysis of substrate binding** – To further assess the substrate affinity of the inactive mutant E316A 125μl of a solution containing 0.48 μM wild-type enzyme, 120 μM mutant enzyme, 200 mM KCl and 100 mM Tris pH 7.5 was added to a well of a micro-titre plate. To a reference well KCl and 100 mM Tris pH 7.5 was added to a well of a micro-titre plate. To a reference well the same amount of a mixture without mutant enzyme, but otherwise identical, was added. To initiate the reactions 125 μl of 120 μM Leu-p-NA was added. The resulting time-course of p-NA formation was subsequently monitored for 3 h. Obtained progress-curves were analyzed by the software package Dynafit (17) allowing the obtained data to be fitted to the following model: E + S ↔ ES, ES → E + P, M + S ↔ MP, were M denotes inactive mutant and MS denotes the mutant-substrate complex. Thus, the model states that both M and E bind the substrate but only E is capable of converting S into P. To evaluate the data the obtained progress-curves were compared to a simulated curve assuming a substrate affinity of the mutant equal to that of wild-type enzyme.

**Model fitting and statistical analysis** – Appropriate kinetic models were fitted against the experimental data from the enzyme kinetic experiments and the dose-response measurements, using non-linear regression. The models and the derived parameters were evaluated with the statistical tools provided by the Excel macro SolverStat as described by Comuzzi et al. (18). Reported standard errors are the ones given by the program. Activity measurements were analyzed using ANOVA and pair-wise comparisons performed according to the Tukey-Kramer procedure. All reported differences are significant at the 5% level or less, unless otherwise stated.

**RESULTS**

**Homology modeling** – A model of scLTA4H was constructed using humLTA4H as a structural template. Based on a multiple alignment generated with ClustalW all residues from 52 to 671 of the yeast enzyme were modeled using the WHATIF software. Inspection of the final model and the underlying alignment between human and scLTA4H, which served as the base for the modeling, indicates that all insertions and deletions occur in loop regions of humLTA4H (Figs. S1 and S2, Supplementary Material on line). This indicates a reliable alignment and is a prerequisite for homology modeling of good quality. The fact that the structural framework of the active site of humLTA4H is dictated by the fold and of residues of the catalytic domain, which exhibits higher sequence identity than the rest of the protein, further supports the predictive value of our model. Thus, the model indicates that scLTA4H possesses an active site similar to that of humLTA4H, which contains the established catalytic residues (presented as yeast residue/human residue) His-340/His-295, Glu-341/Glu-296, His-344/His-299, Glu-363/Glu-318, and Tyr-429/Tyr-383 as well as a hydrophobic channel possibly binding LTA4 (Fig. 1). In addition, three putative catalytic residues Glu-316/Glu-271, Asp-421/Asp-375, and Arg-627/Arg-563 were found at the active site and functionally analyzed (see below). Four residues directly lining the binding pocket of the human enzyme differ in yeast, namely: Glu-186/Gln-136, Gln-412/Val-367, Thr-418/Ile-372 and Phe-424/Tyr-378. The overall shape of the hydrophobic pocket appears slightly wider and shorter as compared to humLTA4H, in part due to the exchange of Val-367 -> Gln-412 (in yeast) at the bottom of the pocket (cf. Fig. 7). Furthermore, the model structure indicates that Asp-422 (Val-376 in humLTA4H) forms a novel interaction with polar residues of the C-terminal domain of the protein, which potentially could affect the...
shape of the active site. Also Asn-417, a loop residue close to the active site, which is absent in all mammalian LTA4H could have a potential effect on the chemistry of the active site. Glu-186, Gln-412, Asn-417, Thr-418, Asp-422, and Phe-424 were all subjected to mutational replacements, individually or in groups, in an effort to engineer scLTA4H into an enzyme with catalytic properties more similar to humLTA4H (see below).

Mutagenesis, expression and purification – In addition to wild-type scLTA4H, 17 mutants of scLTA4H were successfully constructed, expressed and purified to apparent homogeneity. The final yield was approximately 0.5-5 mg of protein per liter cell culture and the purity was at least 90% as judged from SDS-PAGE. The analyzed mutants are presented in Table S1 (Supplementary Material on-line) along with a short description of the rational behind the selection of mutations.

Effects of mutations on specific peptidase activities – The specific peptidase activity and effects of LTA4 on wild type and all 17 mutated proteins were analyzed using different derivatives of aminocaproic acid, i.e., Leu-, Ala-, Arg-, Lys-, Met-, Gly- and Pro-p-NA (Fig. 2). Mutations probing for catalytic residues identified Glu-316, conserved throughout the M1 family of aminopeptidases, and Arg-627, conserved in mammalian LTA4H:s, as critical for the peptidase activity of scLTA4H (Fig. 2). Thus, all enzyme variants of scLTA4H mutated at Glu-316 were virtually devoid of peptidase activity and mutants at position Arg-627 exhibited drastically reduced peptidase activities, even under stringent conditions with higher concentrations of enzyme and substrate (data not shown). The peptidase activity of neither of these mutants could be restored by LTA4 treatment. However, for E316D, a very small but significant residual activity could be detected. Thus, to restore the normal activity the enzyme concentration was increased approximately 300 times as compared to wild-type enzyme.

Mutations aimed at converting scLTA4H into an enzyme with properties similar to humLTA4H had variable effects on the peptidase activity. Thus, F424Y shows a strong increase in its Leu- and Met-p-NA hydrolyzing capacity, whereas the combined mutants 4M (E186Q/Q412V/T418I/F424Y), 5M (E186Q/Q412V/T418I/D422V/F424Y) and 6M (E186Q/Q412V/N417del/T418I/D422V/F424Y) just like humLTA4H, also hydrolyze Ala-p-NA efficiently. Of note, with increasing number of mutagenetic changes, the combined mutants gradually developed a strong Arg-p-NA hydrolyzing capacity, an enzyme activity that is prominent in humLTA4H but virtually absent in wild type scLTA4H. Also in terms of $k_{cat}/K_m$ the 6M mutant displayed highly increased values for all its activities and the preferred substrates were Arg-, Leu- and Ala-p-NA followed by Pro-, Lys- and Met-p-NA, just as for humLTA4H (Fig. 3).

Effects of mutations on the stimulatory effects of LTA4 on the peptidase activities of scLTA4H – Depending on the substrate, mutants were either stimulated, inhibited or unaffected by treatment with LTA4 (Fig. 2). Thus, with few exceptions, the Met-p-NA, Arg-p-NA or Ala-p-NA hydrolyzing activities were inactivated when treated with LTA4, whereas the Leu aminopeptidase activities were stimulated. Considering specific mutants, pre-treatment of 4M or 5M with LTA4 inhibited the peptidase activities for all substrates, except Leu-p-NA. However, for 5M the stimulatory effect of LTA4 on the specific Leu aminopeptidase activity was very low, corresponding to about 25%, as compared to approx. 600% in wild type scLTA4H. A similar reduction of the LTA4 stimulatory effect was also observed for the D422V mutant. Interestingly, for 6M, the mutant most closely resembling humLTA4H, the peptidase activities against all tested substrates were inhibited upon LTA4 treatment. In addition, the degree of inhibition for each substrate paralleled well the inhibitory profile of humLTA4H (Fig. 3).

Considering apparent kinetic constants for mutants exhibiting LTA4 activation or
inhibition of the peptidase activity, the LTA₄

treatment mainly affects the apparent $k_{cat}$

whereas the $K_m$ values are only affected to a

limited extent (Fig. S4, Supplementary

Material on line).

Dose-response relationships for the

stimulatory effect of LTA₄ on the peptidase

activity – Mutants exhibiting a strong

stimulatory effect of LTA₄ on the peptidase

activity for a particular substrate were

analyzed by dose-response measurements (Fig.

S5, Supplementary Material on line). Mutants

that mimic humLTA₄H, e.g., D422V,

N417del/D422V, 3M, and 4M are all activated
to a lesser degree than the wild-type enzyme,

and 6M is instead inhibited by LTA₄ (Table

1). Only D421N exhibited an increased

activation. The dose-response data for LTA₄

activation obeyed saturation kinetics

suggesting the presence of an allosteric LTA₄-

binding site. The concentration of LTA₄
giving half-maximal activation (apparent $K_A$

values) was estimated to 10 - 50 μM for all

mutants.

Determination of apparent $K_i$ for LTA₄

inhibition of the peptidase activity – The

inhibitory effect of LTA₄ on the Ala and Arg

aminopeptidase activities of 4M and 5M, as

well as on the Ala, Leu and Arg

aminopeptidase activities of 6M and

humLTA₄H were assayed and values of $K_i$
calculated (Table 1). The data indicate that the

mode of inhibition is substrate dependent and
gradually changes towards competitive as the

mutants become more similar to humLTA₄H.

Furthermore, for the most extensive mutant,

6M, the observed degree of inhibition is in

level with the inhibition of humLTA₄H.

Effects of mutations on the epoxide hydrolase

activities – The specific epoxide hydrolase

activities of all 17 mutants, as well as wild-
type enzyme, were assayed. Enzyme variants

of scLTA₄H mutated at Glu-316 or Arg-627

exhibited very low or non-detectable levels of

5S,6S-DHETE production and were classified

as inactive (data not shown). For mutants

displaying activity, the typical changes were a

moderate to significant decrease in both 5S,6S-

DHETE as well as LTB₄ production (Fig. 4).

An exception is the single mutant D421N,

which actually displayed an increased epoxide

hydrolase activity, due to increased formation

of 5S, 6S-DHETE under saturating conditions

(see further below). Notably, all combined

mutants displayed reduced rates of 5S,6S-

DHETE production. One mutant, viz. 6M,

exhibited a decrease in 5S, 6S-DHETE

formation with an intact, or even slightly

increased, ability to produce LTB₄. Similar

activities and product profiles were obtained

under stringent conditions with higher enzyme

and substrate concentrations (data not shown).

Determination of apparent turnover numbers

($k_{cat}$) for the epoxide hydrolase activity –

Mutants exhibiting a significantly changed

specific epoxide hydrolase activity were

analyzed under conditions with substrate

saturation. For the three enzymatic epoxide

hydrolase activities of scLTA₄H only the

activity generating 5S,6S-DHETE was

amenable to kinetic experiments; the other two

activities were too low. For assayed mutants,
significant changes were observed for Q412V,

D421N and D422V which all exhibited

between 3- and 4-fold increases in their

reaction rates at substrate saturation (data not

shown). For 4M, F424Y and N417del slight

decreases were observed (data not shown),
similarly to what was observed for their

specific activities (Fig. 4).

Effects of mutations on the relative LTB₄

formation – If the specific epoxide hydrolase

activities are assessed as the sum of 5S,6S-

DHETE and LTB₄ production, almost all

significant changes were reduced activities

(Fig. 4). However, if one considers the relative

amounts of the two products, significant

differences between mutants are observed. The

most pronounced effect was seen for the

hexuple mutant, 6M, which displayed an

LTB₄ to 5S,6S-DHETE ratio of 0.4. For

N417del/D422V and 5M, the corresponding

values are 0.1 and 0.2 and for wild-type

scLTA₄H the ratio is 0.08 (Fig. 4).

Effects of mutations of Glu-316 and Arg-627

on substrate affinity – To assess the role of

Glu-316 in substrate tethering of peptide α-

amino groups, an interaction assay using an

SPR sensor chip was performed with wild-
type enzyme along with the two inactive

mutants E316A and E316Q. The general

amino-peptidase inhibitor bestatin, possessing

a free amino group corresponding to the

α-amino group of peptides, was used to mimic

this specific enzyme-substrate interaction. The

time-course data for the binding responses did

not allow proper determination of dissociation

and association rate constants. However, in

qualitative terms the data show that binding of

bestatin is much weaker, or almost absent, in
E316Q and E316A compared to wild-type enzyme (Fig. 5).

To directly assess the change in aminopeptidase substrate affinity upon mutation of Glu-316, a progress curve analysis of the formation of p-NA was performed. In this experiment the inactive mutant E316A was present in high concentration in the reaction mixture together with wild-type enzyme and substrate. Thus, the inactive mutant competes for the substrate leading to effects on the observed progress of product formation, which is related to the substrate affinity and turnover of the mutant. Comparison of the experimentally observed progress curve with a simulated progress curve corresponding to a preserved substrate affinity of the mutant, demonstrates that mutation of Glu-316 leads to drastically reduced substrate binding (Fig. 5).

The role of Arg-627 as a carboxylate recognition site in substrate binding was evaluated by determining the effects of the mutations R627A and R627K on the $K_i$ of bestatin and a hydroxamic acid inhibitor, each containing carboxylate moieties resembling the carboxy groups of peptide substrates and LTA$_4$, respectively. Aliquots of mutated enzymes (1 µg in 250 µl 50 mM Tris-HCl, pH 7.5 containing 100 mM KCl) were incubated with 1 mM Ala-$p$-NA at increasing concentrations of bestatin and hydroxamic acid, typically 1, 2, 5, 10, 20 µM. For bestatin the IC$_{50}$ increased from 3.6±0.3 µM of wild-type enzyme to 24±3 and 79±5 µM of R627K and R627A, respectively. For the hydroxamic acid inhibitor the IC$_{50}$ changed from 1.5±0.1 µM of wild-type enzyme to 0.17±0.02 and 0.9±0.1 µM for R627A and R627K, respectively. We also modeled the tripeptide substrate Arg-Ser-Arg into the active site of scLTA4H and found that its C-terminal carboxyl group is well positioned for interaction with Arg-627 (Fig S6, Supplementary Material on line). Similarly, the carboxy moiety of LTA$_4$ also binds to this residue (Fig. 1).

**DISCUSSION**

Mammalian LTA4H is a bifunctional enzyme with an aminopeptidase and epoxide hydrolase activity, which generates the classical chemoattractant LTB$_4$. Interestingly, the two active sites share several catalytic residues and yet provide unique catalytic properties and substrate specificities. The bifunctional LTA4H from *S. cerevisiae* is the first example of an isoenzyme from an invertebrate organism and was chosen for studies of the molecular evolution of the enzyme’s two activities.

*S. cerevisiae* LTA4H possesses a more spacious LTA$_4$ binding pocket as compared to the human enzyme – The model structure of scLTA4H displays an active site and hydrophobic pocket that are slightly wider and shorter as compared to the LTA$_4$-binding pocket of humLTA4H (Fig. S2, Supplementary Material on line, Figs. 1 and 7). Several previously identified catalytic residues were found at or near the catalytic zinc site, i.e., His-340, His-344, and Glu-363, the canonical zinc binding ligands, as well as Glu-341 and Tyr-429, the general base and proton donor in the aminopeptidase reaction (10). In addition, Glu-316, Asp-421 and Arg-627 appeared to be potentially catalytic since they correspond to Glu-271, Asp-375 and Arg-563 in humLTA4H, all of which have defined roles in catalysis (3). Four residues directly lining the LTA$_4$-binding pocket of the human enzyme differ in yeast, namely: Glu-186/Gln-136, Gln-412/Val-367, Thr-418/Ile-372, and Phe-424/Tyr-378.

Glu-316 is essential for anchoring of the N-terminus of peptide substrates – Glu-316 of scLTA4H belongs to a GXMEN motif, which is conserved among M1 aminopeptidases and believed to function as an N-terminal recognition site for peptide substrates. Mutation of Glu-316 to either a Gln or an Ala residue rendered the enzyme virtually unable to turnover any peptide substrate. We assessed the binding capacity of the inactive mutants for the substrate mimetic bestatin by the SPR biosensor technique (Biacore), and for E316A we also performed a progress curve analysis for the hydrolysis of substrate (Fig. 5). Each method demonstrates that Glu-316 is indeed crucial for substrate binding. Hence, we propose that the role of Glu-316 is to anchor peptide substrates to the active site via binding to their free α-amino group (Fig. S6, Supplementary Material on-line).

Interestingly, catalysis appears to dependent on the precise positioning of the substrate. Thus, the mutant E316D, which has a preserved negative charge possesses some, however drastically reduced, aminopeptidase activity (data not shown). Even though the main role of this residue appears to be substrate anchoring an indirect role in catalysis cannot be excluded. On the other hand, the
generally strong effects on catalysis caused by mutations of this residue probably reflects its close proximity to other functional residues, e.g. residues of the Zn binding motif. Thus, mutation of this residue probably leads to perturbation of substrate positioning (with respect to other functional residues) resulting in a significant loss of enzyme function. These conclusions are also supported by structural and mutational data for humLTA4H as well as other mammalian members of the M1 family of aminopeptidases (19-22).

Glu-316 has a second essential role in LTA4 hydrolysis in which it acts as a general base and acid catalyst specifically coupled to 5,6S-DHETE production – All three mutants of Glu-316 were also unable to produce significant amounts of 5,6S-DHETE from LTA4 indicating that this residue is essential for the epoxide hydrolase activity. This agrees well with data for humLTA4H, which indicate that Glu-271 is critical for LTB4 formation by assisting in the initial opening of the epoxide ring of LTA4 (19).

Since scLTA4H mainly yields 5,6S-DHETE as product whereas humLTA4H produces LTB4, the reaction mechanisms of the two enzymes appear to be different. A possible role for Glu-316 in the epoxide hydrolase reaction, which also fits our structural model of scLTA4H, would be that of general base and acid catalyst. The binding mode of LTA4, as outlined in Fig. 6, is supported by the modeled structure of an LTA4/scLTA4H complex (Fig. 1), previous structure-activity relationships of hydroxamic acid inhibitors (23) as well as the structure of humLTA4H (11, 24). Furthermore, since the stereochemistry of the product, i.e., the vicinal diol 5,6S-DHETE, has been retained from the substrate, the allylic epoxide LTA4 (5S-trans-5,6-oxido-7,9-trans-11,14-cis-eicosatetraenoic acid), it could be inferred that the reaction proceeds according to an SN1 mechanism.

Mutation of Asp-421 in scLTA4H did not inhibit the enzyme’s epoxide hydrolase activity, as judged by the formation of 5,6S-DHETE, LTB4, and Δ6-trans-Δ8-cis-LTB4. Instead this mutation increases the formation of 5,6S-DHETE (Fig. 4). This was somewhat unexpected, at least with respect to the LTB4 isomers, since the human counterpart, Asp-375, seems to serve as a general base catalyst in LTB4 formation (25). However, in the model structure of scLTA4H, Asp-421 is too distant from the catalytic zine to be able to catalyze the introduction of water at C-6 of LTA4, which explains why it is not responsible for the formation of 5S,6S-DHETE.

Arg-627 is essential for both LTA4 hydrolysis as well as peptidolysis as a carboxylate recognition site – Arg-627 is a residue that is conserved among LTA4H isoenzymes but not in, e.g., aminopeptidase B where a Lys is found instead. Recent structural, mutational and binding data for humLTA4H have identified Arg-563 as a carboxylate recognition site common to both the epoxide hydrolase and peptidase activities (26). Mutation of Arg-627 in scLTA4H to either an Ala or Lys residue drastically reduced the ability of the enzyme to hydrolyze both peptides and LTA4. To assess the contribution of Arg-627 to substrate binding, we used inhibition assays with substrate mimics, as well as determinations of apparent kinetic constants. Thus, the IC50 value of bestatin, a peptide substrate mimic, against the Leu aminopeptidase activity increased 7-fold in the mutant R627K. Furthermore, removal of the positive charge by mutation of Arg-627 into an Ala residue, increased the IC50 of bestatin 22-fold and the Km values for Leu-p-NA and Ala-p-NA approximately 4-fold (data not shown). Although these data support a role for Arg-627 in peptide substrate binding, it can be argued that neither bestatin nor the nitroanilide substrates carry a carboxyl group that can be a reliable mimic of the C-terminus of a peptide substrate. In fact, the distance between Arg-627 and Glu-316 indicates that scLTA4H is a tripeptidase which was also supported in preliminary competition assays indicating that the tripeptide RSR is an excellent substrate for scLTA4H with a kcat of approx. 7 s−1 (data not shown). Thus, we modeled RSR into the active site of scLTA4H and found that Arg-627 can well bind the carboxylate of a tripeptide (Fig. S6, Supplementary Material on-line). Hence, together our data support the conclusion that Arg-627 functions as a carboxylate recognition site for peptide substrates.

Due to the chemical instability and reactivity of LTA4, it is not possible to assess binding of this molecule to an inactive mutant and thus the role of Arg-627 for carboxylate binding. Instead we used a hydroxamic acid inhibitor, originally developed as a stable mimic of LTA4, and studied the effects of mutations on inhibitor potency. In contrast to bestatin, the potency of the hydroxamic acid inhibitor exhibits a limited, if any, dependence on an intact Arg-627. In fact, the inhibitor was...
even more potent against the mutant as compared to wild type scLTA4H. Presumably, this reflects the flexibility of the fatty acid-like hydroxamic acid inhibitor (in contrast to bestatin) which allows it to find alternative, equally strong, binding conformations in the mutated enzyme. Furthermore, modeling of LTA4 into the active site of scLTA4H clearly indicates interactions between the C1 carboxyl group of the substrate and the positively charged guanido group of Arg-627 (Fig. 1). Together, these results suggest that Arg-627 acts as a carboxylate recognition site for LTA4. However, rather than providing binding strength per se, this residue appears to assure an accurate substrate positioning compatible with catalysis (Figs. 6 and 7), as previously demonstrated for humLTA4H (26).

*S. cerevisiae* LTA4H can be engineered to attain a peptide substrate specificity and sensitivity to LTA4-inhibition paralleling humLTA4H – With the exceptions of E316A and F424Y, most single point mutations did not generate major effects on the aminopeptidase activity of scLTA4H. The E186A mutation selectively increased the Pro-p-NA hydrolyzing activity (Fig. 2), which may be explained by the fact that an Ala residue at this position allows a more bulky N-terminal group, such as Pro, to fit into the S1 subsite. In contrast, the F424Y mutation led to strongly increased activities for all substrates normally accepted by wild type scLTA4H, i.e. Leu-, Met- and Ala-p-NA. Since this mutation mainly affects the *K* ~m~ of the enzyme this is probably caused by increased substrate affinity upon introduction of the Tyr hydroxyl group (10).

The combined mutants 4M, 5M and particularly 6M efficiently hydrolyze Arg-p-NA and exhibits increased rates of Ala-p-NA hydrolysis, both in terms of specific activities and *k* ~cat~/*K* ~m~ values (Figs. 2, 3 and Fig. S3, Supplementary Material on-line). The 6M mutant also exhibits increased rates of Pro-, Met- and Lys-p-NA hydrolysis (Fig. 3). For humLTA4H, Ala- and Arg-p-NA are turned over most efficiently, followed by Pro-, Leu-, Met- and Lys-p-NA (Fig. 3). In contrast, wild type scLTA4H is a Leu aminopeptidase, which hydrolyzes Ala-p-NA at significantly lower rates and Arg- and Pro-p-NA are barely accepted as substrates. Hence, mutants most closely mimicking the human enzyme, have attained aminopeptidase activities that are shared with humLTA4H. With the exception of Leu-p-NA, which is hydrolyzed at significantly higher rates in the hexuple yeast mutant than in humLTA4H, the substrate specificities are also very similar for these two enzymes (Fig. 3). Furthermore, the Ala and novel Arg aminopeptidase activities of mutants 4M and 5M were inhibited by treatment with LTA4 and for 6M all aminopeptidase activities were inhibited by LTA4 with a relative inhibitory profile similar to that observed for humLTA4H (Fig. 3). The gradual introduction of mutations in 4M, 5M and 6M also increase the enzyme’s sensitivity for LTA4 inhibition, as judged by decreased *K* _I_ ~i~ values, to levels comparable to those of humLTA4H (Table 1). Moreover, these mutations change the mode of inhibition to a competitive type, a feature typical of humLTA4H. Hence, not only the peptide substrate specificity of humLTA4H but also its sensitivity to inhibition by LTA4 can be engineered into scLTA4H.

LTA4 appears to bind in one productive and oneallostERIC conformation to scLTA4H – Several lines of evidence indicate that LTA4 binds in two conformations to scLTA4H, one productive that turns over LTA4, and one unproductive leading to stimulation of the peptidase activity (Fig. 7). The two activities of scLTA4H both require the catalytic zinc, Glu-316 and Arg-627, suggesting that the substrates of the two activities are mutually exclusive. Yet, LTA4 can both inhibit and stimulate the aminopeptidase activity of scLTA4H in a substrate-dependent manner. Thus, LTA4 treatment inhibits the Met peptidase activity of wild type and mutated enzymes as well as the Arg and Ala peptidase activity of certain mutants, whereas the Leu peptidase activity is generally stimulated (Fig. 2). Since the outcome of LTA4 treatment for a given enzyme depends on the chemistry of the peptide substrate it is reasonable to assume that LTA4, in its activating binding mode, is bound close to where the substrates differ, *viz.* the side-chain of the N-terminal residue. In humLTA4H this part of the active site is very narrow 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. Furthermore, LTA4 acts as a competitive inhibitor of the Ala-p-NA cleaving activity of 4M, 5M and 6M (Table 1), suggesting that this substrate, which mostly occupies the zinc binding site and wide portion of the active site, is only affected by LTA4 when bound in its elongated binding mode.
(Fig. 7). In contrast, LTA₄ gives a mixed type of inhibition against the substrate Arg-p-NA, presumably because its side-chain penetrates deeper into the hydrophobic S1 binding pocket, allowing interactions with LTA₄ bound in both conformations.

Mutations of scLTA₄H affect the rate of LTA₄ hydrolysis and product profile – When engineering scLTA₄H into an enzyme with properties resembling those of humLTA₄H, several different effects on the epoxide hydrolase activity could in principle occur. These include (i) increased turnover of LTA₄, (ii) reduced formation of 5S,6S-DHETE, and, in particular, (iii) increased formation of LTB₄. The structure of humLTA₄H shows that the putative LTA₄ binding cavity is formed at Q412V, do indeed increase the rate of LTA₄ turnover, as well as LTB₄. The hexuple mutant (6M), however, not only displayed a higher turnover (data not shown). Instead, we chose a strategy in which we exchanged a 108-residue segment of scLTA₄H for the corresponding part of humLTA₄H, which resulted in a protein that failed to express and fold properly (data not shown). Instead, we chose a strategy with a limited number of point mutations, selected from the modeled structure, possibly mimicking the molecular evolution of the enzyme.

Mutations assumed to cause significant changes to the active site of scLTA₄H, i.e. D422V, D422V/N417del and Q412V, do indeed increase the rate of LTA₄ turnover (data not shown). Thus, these mutations increase the 5S,6S-DHETE production approximately 3-fold as compared to wild-type enzyme while the LT₄ production remains the same. Since these effects were only observed under conditions with substrate saturation they probably reflect changes in both substrate affinity and turn over. Also the 3-fold increase observed for D421N (data not shown) suggests similar changes.

The combined mutants 3M, 4M and 5M exhibited reduced abilities to produce 5S,6S-DHETE as well as LTB₄. The hexuple mutant (6M), however, not only displayed a reduced ability to produce 5S,6S-DHETE but also retained (or slightly increased) its ability to convert LTA₄ into LTB₄. Thus, in relative terms this mutant exhibits increased LTB₄ over 5S,6S-DHETE production (Fig. 4), with a LTB₄:5S,6S-DHETE ratio of 0.4 compared to 0.08 for wild-type scLTA₄H. In addition, substrate binding appeared to be significantly improved since LTA₄ acted as a competitive inhibitor for the aminopeptidase activity of the hexuple mutant, across three tested substrates, with a potency profile similar to what is observed for humLTA₄H (Table 1).

Recent data show that the catalytic machinery of humLTA₄H is very sensitive to changes in the binding and precise alignment of LTA₄ along the active site (26). Even a minimal change, i.e. an Arg to Lys substitution, in the carboxylate recognition site (i.e. Arg-563 in humLTA₄H corresponding to Arg-627 in scLTA₄H) renders the enzyme inactive even though LTA₄ binding appears intact. Keeping this in mind it is perhaps not surprising that we were unable to engineer a mutant of scLTA₄H with a robust LTB₄ producing activity by a limited number of point mutations. Nonetheless, the catalytic and kinetic properties of the hexuple mutant indicate that structural changes have been introduced which indeed mimic steps in the evolution of the epoxide hydrolase activity thus leading to an LTA₄-binding cavity resembling the human counterpart.

Structural modifications of LTA₄H during evolution – LTA₄H is an unusual combination of an M1 zinc aminopeptidase and an epoxide hydrolase with a very strict substrate specificity, for which Glu-271, Asp-375, and Arg-563 are essential. Inspection of multiple sequence alignments, e.g. see the MEROPS peptidase database, merops.sanger.ac.uk (27), reveals that several M1 aminopeptidases of invertebrate organisms have the former two of these three residues conserved, i.e. those corresponding to Glu-316 and Asp-421 of scLTA₄H. The carboxylate recognition site (Arg-627 of scLTA₄H) is conserved within mammalian LTA₄H and also occurs in some related M1 aminopeptidases (26). Thus, one would expect, based on sequence similarity and our biochemical data, that the ability to hydrolyze LTA₄ into 5S,6S-DHETE and possibly into LTB₄, would exist among other members of this broad enzyme family. However, except for scLTA₄H such activities have never been demonstrated.

Given the evolutionary conservation of the peptide cleaving activity it seems likely that LTA₄H has been evolved from an ancestral aminopeptidase. Since only six point mutations
mutations were sufficient to convert scLTA4H into an enzyme with a peptidase activity resembling that of humLTA4H, it appears likely that scLTA4H represents a close ancestor among the lower eukaryotes. Indeed, according to a phylogenetic tree derived from an alignment of aminopeptidases of the M1 family, see the MEROPS peptidase database, merops.sanger.ac.uk (27), scLTA4H clusters together with mammalian LTA4H. Throughout the M1 family of aminopeptidases the catalytic domain is the most conserved domain. This suggests, and is indeed supported by our 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. Our data shows that the introduction of a limited number of new functionalities to an aminopeptidase active site is sufficient to improve LTA4 binding but additional changes are required to create the full machinery for efficient conversion of LTA4 into LTB4. How then has this specific ability been introduced? Inasmuch as the C-terminal domain of LTA4H appears to be most unique to LTA4Hs one may speculate that its intrinsic structural features and interactions with the N–terminal and catalytic domains, may be key to the process of fine-tuning the chemistry of the active site. Thus, in the course of evolution the binding pocket has been gradually reshaped in small steps to optimally fit LTA4 thereby favoring LTB4 production over other structural isomers. In this process, the substrate has been aligned with residues already serving in the peptidase activity, e.g. Glu-316 and Arg-627, thereby allowing them to perform novel epoxide hydrolase catalysis while at the same time maintaining the original peptidase activity.

SUPPLEMENTAL DATA

The pair-wise sequence alignment between scLTA4H and humLTA4H underlying the homology modeling (Fig. S1), the derived overall model of scLTA4H (Fig.S2), a table describing the produced mutants in condensed format (Table S1), a graph presenting $k_{cat}/K_m$ values for peptide hydrolysis by 6M, humLTA4H and scLTA4H (Fig. S3), two graphs presenting the $k_{cat}$ and $k_{cat}/K_m$ values for Leu-p-NA hydrolysis of LTA4 treated mutants (Fig. S4), a graph presenting the degree of LTA4-induced activation of the peptidase activity of various mutants (Fig. S5) and a figure depicting the modeled binding conformation of a Arg-Ser-Arg peptide to the active site of scLTA4H (Fig. S6) are available on line at the JBC Web site.

REFERENCES

1. Samuelsson, B. (1983) Science 220, 568-575
2. Funk, C. D. (2001) Science 294, 1871-1875
3. Haeggstrom, J. Z. (2004) J. Biol. Chem. 279, 50639-50642
4. Barret, A. J., Rawlings, N. D., and Woessner, J. F. (1998) in Handbook of proteolytic enzymes (Barret, A. J., Rawlings, N. D., and Woessner, J. F., eds), pp. 994-996, Academic Press, London, San Diego
5. Baset, H. A., Ford-Hutchinson, A. W., and O'Neill, G. P. (1998) J. Biol. Chem. 273, 27978-27987
6. Green, F. A., Herman, C. A., Herman, R. P., Claesson, H. E., and Hamberg, M. (1987) Biochem. Biophys. Res. Commun. 142, 309-314
7. Habenicht, A. J., Goerig, M., Rothe, D. E., Specht, E., Ziegler, R., Glomset, J. A., and Graf, T. (1989) Proc. Natl. Acad. Sci. USA 86, 921-924
8. Pettitt, T. R., Rowley, A. F., Barrow, S. E., Mallet, A. I., and Secombes, C. J. (1991) J. Biol. Chem. 266, 8720-8726
9. Kull, F., Ohlson, E., and Haeggström, J. Z. (1999) J. Biol. Chem 274, 34683-34690
10. Kull, F., Ohlson, E., Lind, B., and Haeggström, J. Z. (2001) Biochemistry 40, 12695-12703
11. Thunnissen, M. M. G. M., Nordlund, P., and Haeggström, J. Z. (2001) Nat. Struct. Biol. 8, 131-135
12. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) Nucleic Acids Res. 22, 4673-4680
13. Vriend, G. (1990) J. Mol. Graph. 8, 52-56
14. McRee, D. E. (1999) J. Strucr. Biol. 125, 156-165
15. Krieger, E., Darden, T., Nabuurs, S. B., Finkelstein, A., and Vriend, G. (2004) Proteins 57, 678-683
16. Hooft, R. W., Vriend, G., Sander, C., and Abola, E. E. (1996) *Nature* **381**, 272
17. Kuzmic, P. (1996) *Anal Biochem* **237**, 260-273
18. Comuzzi, C., Polese, P., Melchior, A., Portanova, R., and Tolazzi, M. (2003) *Talanta* **67**, 67-80
19. Rudberg, P. C., Tholander, F., Thunnissen, M. G. M., and Haeggström, J. Z. (2002) *J. Biol. Chem.* **277**, 1398-1404
20. Luciani, N., Marie-Claire, C., Ruffet, E., Beaufont, A., Roques, B. P., and Fournie-Zaluski, M.-C. (1998) *Biochemistry* **37**, 686-692
21. Vazeux, G., Iturrioz, X., Corvol, P., and Llorens-Cortes, C. (1998) *Biochem. J.* **337**, 260-273
22. Comuzzi, C., Polese, P., Melchior, A., Portanova, R., and Tolazzi, M. (2003) *Talanta* **67**, 67-80
23. Rudberg, P. C., Tholander, F., Andberg, M. B., Haeggström, J., Samuelsson, B., and Wong, C. H. (1998) *Chem. Eur. J.* **4**, 1698-1713
24. Hogg, J. H., Ollmann, I. R., Wetterholm, A., Andberg, M. B., Haeggström, J. Z., and Samuelsson, B. (1998) *Chem. Eur. J.* **4**, 1698-1713
25. Rudberg, P. C., Tholander, F., Andberg, M. B., Thunnissen, M. M. G. M., and Haeggström, J. Z. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 4215-4220
26. Thunnissen, M. M. G. M., Andersson, B., Samuelsson, B., Wong, C. H., and Haeggström, J. Z. (2002) *FASEB J.* **16**, 1648-1650
27. Rudberg, P. C., Tholander, F., Andberg, M., Thunnissen, M., and Haeggstrom, J. Z. (2004) *Proc. Natl. Acad. Sci. USA* **99**, 4215-4220
28. Zvelebil, M. J., Tolle, D. P., and Barrett, A. J. (2004) *Nucleic Acids Res.* **32**, D160-164
29. Barton, G. J. (1993) *Protein Engineering* **6**, 37-40
30. Guex, N., and Peitsch, M. C. (1997) *Electrophoresis* **18**, 2714-2723
31. Mueller, M. J., Blomster, M., Oppermann, U. C. T., Jörnvall, H., Samuelsson, B., and Haeggström, J. Z. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 5931-5935
32. Mueller, M. J., Andberg, M. B., Samuelsson, B., and Haeggström, J. Z. (1996) *J. Biol. Chem.* **271**, 24345-24348
33. Morris, G. M., Goodsell, D. S., Halliday, R. S., Huey, R., Hart, W. E., Belew, R. K., and Olson, A. J. (1998) *J. Computational Chemistry* **19**, 1639-1662
34. Junmei, W., Cieplak, P., and Kollman, P. A. (2000) *J Comput Chem* **21**, 1049-1074

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Footnotes

1 The abbreviations used are: LT, leukotriene; LTA4, leukotriene A4, 5S-trans-5,6-oxido-7,9-trans-11,14-cis-eicosatetraenoic acid; LTB4, leukotriene B4, 5S,12R-dihydroxy-6,14-cis-8,10-trans-eicosatetraenoic acid; Δ6-trans-Δ8-cis-LTB4, 5S,12R-dihydroxy-6,10-trans-8,14-cis-eicosatetraenoic acid; 5S,6S-DHETE, 5S,6S-dihydroxy-7,9-trans-11,14-cis-eicosatetraenoic acid; 5S,6S-DHETE, 5S,6S-dihydroxy-7,9-trans-11,14-cis-eicosatetraenoic acid; Δ6-trans-LTB4, 5S,12R-dihydroxy-6,8,10-trans-14-cis-eicosatetraenoic acid; 12-epi-Δ6-trans-LTB4, 5S,12S-dihydroxy-6,8,10-trans-14-cis-eicosatetraenoic acid, PGB1, prostaglandin B1; Ni-NTA, nickel-nitrilotriacetic acid; LTA4H, LTA4 hydrolase; scLTA4H, *Saccharomyces cerevisiae* LTA4 hydrolase; humLTA4H, human LTA4 hydrolase; aminoacyl-p-NA, aminoacyl-p-nitroanilide; p-NA, para-nitro aniline; SPR, surface plasmon resonance. 3M, 4M, 5M and 6M denotes the mutations Q412V/T418I/F424Y, E186Q/Q412V/T418I/F424Y, E186Q/Q412V/T418I/D422V/F424Y and E186Q/Q412V/N417del/T418I/D422V/F424Y, respectively.

Figure Legends

FIG. 1. Stereo diagram of the active sites of human and yeast LTA4H.

The stereo diagram shows active site structures of human and yeast LTA4H superimposed with the carbons of scLTA4H in light blue and of *hum*LTA4H in dark gray. Amino acid residues are labeled *hum*LTA4H/scLTA4H. A modeled binding conformation of LTA4 to *hum*LTA4H is shown in green. In this conformation, the ω-carbon of LTA4 clashes with the side chain of Gln-412 (located at the bottom of the hydrophobic pocket of scLTA4H) forcing the lipid tail of LTA4 to accommodate a
The loop segment shown is located between Gln-412/Val-367 and Asp-421/Asp-375 (yeast residue/human residue) and contains an insertion in the yeast enzyme; Asn-417. According to the modeled structure the residue next to Asp-375/Asp-421, Val-376/Asp-422 (omitted due to clarity), interacts with residues of the C-terminal domain in scLTA4H thereby possibly affecting the shape and chemistry of the active site (cf. Fig. 7 and Fig S2, Supplementary Material on line).

**FIG. 2.** - Effects of mutations on specific aminoacyl-p-NA hydrolyzing activities and modulation by LTA4. Panels A-E present the specific Leu-, Ala-, Met-, Arg-, and Pro-p-NA hydrolyzing activities of wild type and mutated enzymes that have been treated (filled bars) or not treated (open bars) with LTA4. The error bars indicate standard deviation and “nd” denotes “not determined”. Mutants with negligible specific activities, i.e. E316A, E316Q, R627K and R627A, are omitted. The activities of these mutants could not be restored by LTA4 treatment and were at the same low level with other substrates.

**FIG. 3.** - The relative specific aminopeptidase activities of humLTA4H, 6M and scLTA4H with and without LTA4 treatment. For each enzyme the specific Ala-, Leu-, Arg-, Met-, and Pro-p-NA hydrolyzing activities are normalized with respect to the substrate giving the highest activity for untreated enzyme (the reference bar is denoted with an ‘N’). ‘scWT’ and ‘hum’ denote wild-type scLTA4H and humLTA4H, respectively. Whereas wild-type scLTA4H exhibits strong activation of its Leu-, Ala-, Met- and Pro-p-NA hydrolyzing activities 6M exhibits, just as humLTA4H, inhibition of all its aminopeptidase activities. Note that the bars for the specific Ala-p-NA and Leu-p-NA hydrolytic activity of LTA4−treated scLTA4H are broken, the correct values are instead explicitly given on top of the bars.

**FIG. 4.** - Effects of mutations on specific epoxide hydrolase activities of scLTA4H. The graph presents the specific production of 5S,6S-DHETE (open part of each bar) and LTB4 (black part of each bar) for mutants aimed at mimicking humLTA4H. The bars denote mean values ± SD. The numbers given in the box below the graph corresponds to the LTB4:5S,6S-DHETE ratio.

**FIG. 5.** - Assessment of substrate binding to mutants of E316. (A), Time-course for the binding response of 125 μM bestatin (injected at a flow-rate of 20 ul/min) to wild-type (blue trace), E316Q (green trace) and E316A (pink trace) scLTA4H. For wild-type enzyme, the dissociation part of the experiment (from 180 seconds and onwards) clearly indicate a significant binding affinity between bestatin and the enzyme, as demonstrated by the slow decline of the curve towards basal levels. However, for both mutant enzymes the response immediately drops down to the basal level suggesting a drastically lowered, or virtually abolished, binding affinity. The response curves for binding of bestatin were corrected for the background response by subtraction of a previous injection with buffer alone. The experiment was performed at three different concentrations of bestatin and repeated three times for each concentration (data not shown). Similar results were obtained in each run. (B), The progress of of p-NA formation was monitored for 2 hours in the presence of wild-type enzyme alone (blue trace) as well as in the presence of wild-type and inactive mutant (E316A) enzyme (magenta trace). A simulated curve assuming preserved substrate affinity, equal to that of wild-type enzyme, of the inactive mutant is shown as a black trace. The addition of mutant enzyme (at a 250-fold higher concentration with respect to wild-type enzyme) to the reaction mixture has a very weak effect on the observed progress of product formation due to a drastically reduced substrate affinity of the mutant enzyme. Note that the axes are broken.

**FIG. 6.** - Model for the epoxide hydrolase reaction mechanism. The scheme shows a putative SN1 reaction mechanism for the conversion of LTA4 into the major product 5S,6S-DHETE by scLTA4H. Arg-627 holds the carboxy moiety of LTA4 whereas the epoxide oxygen binds to the Zn2+ ion, which functions as a Lewis acid, thus promoting activation and opening of the oxirane ring. In a second step, Glu-316 acts as a general base and polarizes a water molecule to facilitate its attack at C6 of the carbocation intermediate. In the final step, Glu-316 instead acts as an acid and delivers a proton to the oxyanion at C5, thus forming the second hydroxyl group of the vicinal diol.
Fig. 7. - Schematic model of the substrate-binding pocket of scLTA4H. Panel A shows a schematic representation of the LTA₄ binding pocket of humLTA4H along with several lining residues. It is composed of a rounded open section containing the zinc site and the carboxylate recognition site as well as a narrow hydrophobic pocket extending in an L-shaped fashion, which harbors the ω-end and fatty acid backbone of LTA₄. ‘3*’ indicate residues exchanged in the 3M mutant and ‘4*’, ‘5*’ and ‘6*’ indicate the additional residues exchanged in the mutants 4M, 5M and 6M, respectively. Panels B and C show the putative pocket of the yeast enzyme with LTA₄ in two binding modes. Residues around the catalytic Zn are highly conserved and also the basic residues binding the carboxy group of LTA₄ (as well as peptides). Residues lining the deeper part of the LTA₄ binding pocket differs between the two enzymes. As discussed in the text, the binding pocket of the yeast enzyme (solid line) appears to be more spacious than the human enzyme (dotted line). The double arrow at Asp-422 of scLTA4H denotes a putative interaction with residues of the C terminal domain that could affect the shape of the deeper part of the pocket. The additional space in scLTA4H could allow LTA₄ to bind in two possible conformations; one elongated (panel B) and one curled-up in the deeper part of the pocket (panel C), see text for further details. The latter binding mode would be the one compatible with activation of the peptidase activity. A peptide substrate would occupy the upper and middle part of the binding pocket. Depending on the side-chain of the peptide substrate it penetrates to a variable extent into the deeper part of the pocket thus interacting differently with LTA₄, as indicated by the shaded areas in dark and light gray representing the binding of Ala- and Arg-p-NA, respectively.

Table I

Inhibition of the Ala, Leu, Arg and Pro aminopeptidase activities of mutated scLTA4H by LTA₄. Aliquots of mutated enzymes (1 µg in 250 µl 50 mM Tris-HCl, pH 7.5 containing 100 mM KCl) were incubated with Ala-, Leu- or Arg-p-NA (9.6 - 0.125 mM) at varying concentrations of LTA₄, typically 1, 2, 5, 10, 20 µM. Values of $K_i$ were calculated as described in the methods section and are expressed as mean values ± SE. Note that the experiment is not applicable for the Leu-p-NA hydrolyzing activities of 4M and 5M since these activities are stimulated upon LTA₄-treatment.

| Mutant | $K_i$ (µM) | Substrate   | Type of inhibition |
|--------|------------|-------------|-------------------|
| 4M     | 12.6±4.8   | Ala-p-NA    | competitive       |
| 4M     | 0.57±0.04  | Arg-p-NA    | mixed*            |
| 5M     | 2.7±0.6    | Ala-p-NA    | competitive       |
| 5M     | 9.1±1.8    | Arg-p-NA    | mixed*            |
| 6M     | 0.58±0.07  | Ala-p-NA    | comp              |
| 6M     | 0.98±0.2   | Leu-p-NA    | comp              |
| 6M     | 1.0±0.1    | Arg-p-NA    | mixed             |
| humLTA4H | 1.1±0.1   | Ala-p-NA    | comp              |
| humLTA4H | 0.19±0.04 | Leu-p-NA    | comp              |
| humLTA4H | 0.27±0.04 | Arg-p-NA    | comp              |

* Discrimination between $K_{IIU}$ and $K_i$ not possible
Figure 1. Tholander et al.
Leu-p-NA

Activity (nmol/mg/min)

WT

F424Y

E186A

5M

4M

D422V

WT

F424Y

E186A

5M

4M

D422V

Activity (nmol/mg/min)

A

55.4±19

0.45±0.06

1.1±0.1

6.51±2.5

E316D

WT

F424Y

E186A

5M

4M

D422V

C

Pro-p-NA

Activity (nmol/mg/min)

WT

F424Y

E186A

5M

4M

D422V

WT

F424Y

E186A

5M

4M

D422V

Ala-p-NA

Activity (nmol/mg/min)

WT

F424Y

E186A

5M

4M

D422V

WT

F424Y

E186A

5M

4M

D422V

Met-p-NA

Activity (nmol/mg/min)

WT

F424Y

E186A

5M

4M

D422V

WT

F424Y

E186A

5M

4M

D422V

Arg-p-NA

Activity (nmol/mg/min)

WT

F424Y

E186A

5M

4M

D422V

WT

F424Y

E186A

5M

4M

D422V

Fig. 2 Tholander et al.
Figure 4. Tholander et al.
Figure 5. Tholander et al.
Figure 6. Tholander et al.
Figure 7. Tholander et al.
SUPPLEMENTAL DATA:
Table S1
Fig. S1 – Fig. S6
Table S1. Description of mutants of scLTA4H and their relation to humLTA4H

| Mutation(s) | Residue(s) in humLTA4H | Assigned role in humLTA4H | Type of mutation and rationale for mutant selection. | Residue conservation (1) |
|-------------|------------------------|--------------------------|---------------------------------------------------|--------------------------|
| Wild-type scLTA4H | - | - | - | About 40% overall identity to humLTA4H. |
| E316Q | E271 | Yes (19). | Test of catalytic function. Human residue involved in epoxide ring opening and binding of α-amino groups of peptides. In scLTA4H possibly general base in epoxide hydrolysis | Belongs to conserved GXMEN motif. |
| E316A | E316D | - | - | |
| R627A | R563 | Yes (26). | Test of catalytic function. Human residue involved in binding of substrate carboxy group. | Conserved within mammalian LTA4H:s and occurs in some lower organisms. |
| R827K | | | |
| E186Q | Q136 | No | Human mimic/test of catalytic function. Position suggesting involvement in binding of α-amino groups of peptides. Possible general base in epoxide hydrolysis. | Gln most common within protein family, yeast and rat LTA4H has a Glu. |
| E186A | | | |
| D421N | D375 | Yes (25). | Test of catalytic function. Human residue shown to be general base in LTb₄ formation without an identified role in peptidase activity. | Fully conserved within mammalian LTA4H:s, common in lower organisms. |
| F424Y | Y378 | Yes (see above). | Human mimic/test of catalytic function. Human residue involved in suicide inhibition and substrate alignment. Included in 4M. | All mammalian LTA4H:s has a Tyr and most lower organisms a Phe. |
| D422V | D376 | No | Human mimic/test of catalytic function. Possible domain interaction affecting shape of LTA₄ binding pocket. Located next to the general base required for LTb₄ production in human (D375). | All mammalian LTA4H:s have a Val residue whereas lower organisms have an Asp. |
| Q412V | V367 | No | Human mimic/test of catalytic function. Possibly affects nature and depth of LTA₄ binding pocket. Included in 4M. | Val residue in all mammalian LTA4H:s, most lower organisms has a Gln. |
| N417del (deletion of N417) | Not present | - | Human mimic. Loop residue located close to catalytic residue. Could possibly affect catalytic residues. | Loop insertion absent in all mammalian LTA4H. |
| N417del/D422V | Not present/D376 | No | D422V and N417del combined. Possibility to affect position of general base required for LTb₄ production in human (D375). | (Combination of mutants.) |
| 3M (= Q412V, T418I and F424Y) | V367, i372 and Y378 | Yes, for Y378 (see above). | Human mimic. Includes 3 residues that differ in human and yeast and directly face the putative binding pocket. | (Combination of mutants.) |
| 4M (= E186Q, Q412V, T418I and F424Y) | Q136, V367, i372 and Y378 | Yes, for Y378 (31, 32). | Human mimic. Includes all four residues that differ in human and yeast and directly face the putative binding pocket. | (Combination of mutants.) |
| 5M (as 4M plus D422V) | Q136, V367, i372, D376 and Y378 | Yes, for Y378 (see above). | Human mimic. Includes the 4M mutations and in addition D422V. | (Combination of mutants.) |
| 6M (as 5M plus N417del) | Q136, V367, not present, i372, D376 and Y378 | Yes, for Y378 (see above). | Human mimic. Includes the 4M mutations and in addition N417del and D422V. | (Combination of mutants.) |
The degree of conservation is indicated by colour code with values according to Zvelebil et al. (28). Blue represents the highest degree of conservation. Mutated residues are marked with an M. Letters 'a' and 'c' represent Glu-341 and Tyr-429, the general base and proton donor in the peptidase reaction, whereas 'b' represents Phe-429 and 'Z' the zinc binding ligands. Residues lining the deeper part of the putative binding pocket are indicated with open circles while filled circles indicate residues close to the upper part of the pocket. Domain borders are marked with open horizontal triangles. The secondary structure of humLTA4H is presented underneath the aligned sequences using cylinders and arrows to symbolize helices and β-strands, respectively. The figure was generated with Alscript (29).
Figure S2. Stereo diagram of the modeled structure of scLTA4H.

Stereo diagram of the modeled regions of scLTA4H (before energy minimization) superimposed on its template humLTA4H. The picture depicts the C-α trace of LTA4H and modeled regions of scLTA4H. The model is coloured according to the same scheme as in S1. Inserted and truncated segments of the model scLTA4H are shown in yellow. The position of the loop located close to the active site, which is discussed in the text, is indicated with an arrow. The corresponding loop is visible in the close-up in figure 1. The figure was generated with Swiss PDB viewer (30) and povray (www.povray.org).
Figure S3. $k_{\text{cat}}/K_m$ values of humLTA4H (white bars), 6M (gray bars) and scLTA4H (black bars) for hydrolysis of six different peptide substrates.
Figure S4. Effect of LTA$_4$ treatment on the kinetic parameters for peptide hydrolysis by wild-type and various mutants of scLTA4H. Apparent $k_{\text{cat}}$ (panel A) and $k_{\text{cat}}/K_m$ (panel B) values for Leu-p-NA hydrolysis by LTA$_4$-treated (black bars) and untreated (white bars) enzymes. The error bars indicate standard errors and “nd” denotes “not determined”.
Figure S5. *LTA*₄*-induced stimulation of the peptidase activity of wild type and mutated scLTA4H*. The specific peptidase activity of enzymes susceptible to LTA₄ stimulation was assayed with different peptide substrates and increasing amounts of LTA₄, until saturation was achieved. The maximum increase of the specific Leu-, Ala- and Pro-ₚ-NA aminopeptidase activities are presented. The bars denote mean value ± SE.
Figure S6. Modeled binding conformation of an Arg-Ser-Arg peptide. The figure presents certain key-interactions between the substrate (white carbons) and scLTA4H (turquoise carbons with the Zn-ion as an orange sphere). Only the functional groups of the Zn-binding ligands, i.e. His-340, His-344 and Glu-363, are presented. Arg-627 and Glu-316 together aligns, via binding of the C- and N-termini of the substrate, the scissile bond of the tripeptide substrate with the Zn ion and the general base catalyst Glu-341. In addition, Asp-421 and Glu-186 (both unlabeled in the figure) interacts with the guanido group and the free amine of the P1 residue, respectively. The binding conformation was obtained by docking using the Autodock3 software (33) followed by energy minimization of the lowest energy conformation using the Amber99 force field implementation of the YASARA software (34, 15). The figure was created with YASARA (www.yasara.org) and PovRay (www.povray.org).
Leukotriene A4 hydrolase: Insights to the molecular evolution by homology modeling and mutational analysis of enzyme from Saccharomyces cerevisiae
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