# *Saccharomyces cerevisiae* Leukotriene $A_4$ Hydrolase: Formation of Leukotriene $B_4$ and Identification of Catalytic Residues[†]

Filippa Kull,[‡] Eva Ohlson,[‡] Birger Lind,[§] and Jesper Z. Haeggström*,[‡]

*Department of Medical Biochemistry and Biophysics, Division of Chemistry II, and Institute of Environmental Medicine, Division of Metals and Health, Karolinska Institutet, S-171 77 Stockholm, Sweden*

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ABSTRACT: Leukotriene $A_4$ hydrolase in mammals is a bifunctional zinc metalloenzyme that catalyzes the hydrolysis of leukotriene $A_4$ into the proinflammatory mediator leukotriene $B_4$, and also possesses an aminopeptidase activity. Recently we cloned and characterized an leukotriene $A_4$ hydrolase from *Saccharomyces cerevisiae* as a leucyl aminopeptidase with an epoxide hydrolase activity. Here we show that *S. cerevisiae* leukotriene $A_4$ hydrolase is a metalloenzyme containing one zinc atom complexed to His-340, His-344, and Glu-363. Mutagenetic analysis indicates that the aminopeptidase activity follows a general base mechanism with Glu-341 and Tyr-429 as the base and proton donor, respectively. Furthermore, the yeast enzyme hydrolyzes leukotriene $A_4$ into three compounds, viz., 5*S*,6*S*-dihydroxy-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid, leukotriene $B_4$, and $\Delta^6$-*trans*-$\Delta^8$-*cis*-leukotriene $B_4$, with a relative formation of 1:0.2:0.1. In addition, exposure of *S. cerevisiae* leukotriene $A_4$ hydrolase to leukotriene $A_4$ selectively inactivates the epoxide hydrolase activity with a simultaneous stimulation of the aminopeptidase activity. Moreover, kinetic analyses of wild-type and mutated *S. cerevisiae* leukotriene $A_4$ hydrolase suggest that leukotriene $A_4$ binds in one catalytic mode and one tight-binding, regulatory mode. Exchange of a Phe-424 in *S. cerevisiae* leukotriene $A_4$ hydrolase for a Tyr, the corresponding residue in human leukotriene $A_4$ hydrolase, results in a protein that converts leukotriene $A_4$ into leukotriene $B_4$ with an improved efficiency and specificity. Hence, by a single point mutation, we could make the active site better suited to bind and turn over the substrate leukotriene $A_4$, thus mimicking a distinct step in the molecular evolution of *S. cerevisiae* leukotriene $A_4$ hydrolase toward its mammalian counterparts.

Leukotriene (LT)[1] $A_4$ hydrolase (LTA4H) catalyzes the hydrolysis of $LTA_4$ into the proinflammatory mediator $LTB_4$, which is a potent chemoattractant and leukocyte activating agent (*1, 2*). The mammalian LTA4H is a soluble monomeric metalloenzyme with a molecular mass of 69 kDa, containing one zinc atom per enzyme molecule (*3*−*5*). In addition to the epoxide hydrolase activity, i.e., the hydrolysis of $LTA_4$ into $LTB_4$, the enzyme possesses an anion-dependent peptidase activity, the physiological role of which is presently unknown (*4, 6*−*8*). The enzyme has a wide tissue distribution (*9*−*12*) and is found in cell types lacking the enzyme 5-lipoxygenase, which catalyzes the conversion of arachidonic acid into $LTA_4$, the substrate of mammalian LTA4H (*13, 14*). The zinc atom is required for both catalytic activities, and the metal is bound to His-295, His-299, and Glu-318, components of the zinc-binding motif in LTA4H (*15*). The peptide hydrolysis catalyzed by LTA4H has been proposed to follow a general base mechanism in which Glu-296 acts as the base and Tyr-383 as the proton donor. Mutation of any of these two residues abolishes the peptidase but not the epoxide hydrolase activity (*16, 17*). During catalysis, LTA4H is suicide-inactivated through covalent binding of $LTA_4$ to the active site residue Tyr-378 (*18*−*21*). This process blocks both enzyme activities and may be of importance for the overall regulation of $LTB_4$ biosynthesis.

Very little is known about the evolution of LTA4H. Although $LTB_4$ may be regarded as a component of the innate immune system, it is not known when this molecule, and the corresponding biosynthetic enzymes, appeared during evolution. Formation of $LTB_4$, i.e., the enzymatic product of LTA4H, has been described in birds, frogs, and fish (*22*−*26*) but seldomly, if at all, in nonvertebrate species. LTA4H has also been purified from *Xenopus laevis* oocytes, and this protein has a very high catalytic efficiency and the

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* Correspondence should be addressed to this author. Telephone: +46-8-728 7612. Fax: +46-8-736 0439. E-mail: Jesper.Haeggstrom@mbb.ki.se.

[‡] Department of Medical Biochemistry and Biophysics, Division of Chemistry II.

[§] Institute of Environmental Medicine, Division of Metal Toxicology.

[1] Abbreviations: LT, leukotriene; $LTA_4$, leukotriene $A_4$, 5*S*-*trans*-5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid; $LTB_4$, leukotriene $B_4$, 5*S*,12*R*-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid; $\Delta^6$-*trans*-$\Delta^8$-*cis*-$LTB_4$, 5*S*,12*R*-dihydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid; 5*S*,6*S*-DHETE, 5*S*,6*S*-dihydroxy-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid; $\Delta^6$-*trans*-$LTB_4$, 5*S*,12*R*-dihydroxy-6,8,10-*trans*-14-*cis*-eicosatetraenoic acid; 12-epi-$\Delta^6$-*trans*-$LTB_4$, 5*S*,12*S*-dihydroxy-6,8,10-*trans*-14-*cis*-eicosatetraenoic acid; $PGB_1$, prostaglandin $B_1$; PCR, polymerase chain reaction; FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis; RP-HPLC, reverse-phase high-performance liquid chromatography; Leu-*p*-NA, leucine-*p*-nitroanilide; thioamine, 3-(4-benzyloxyphenyl)-2-(*R*)-amino-1-propanethiol; Ni-NTA, nickel-nitrilotriacetic acid; Sf9, *Spodoptera frugiperda*; LTA4H, $LTA_4$ hydrolase; *sc*LTA4H, *Saccharomyces cerevisiae* $LTA_4$ hydrolase; humLTA4H, human $LTA_4$ hydrolase.

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Human          280 PTLLAGDKSLSNVIAHEISHSWTGNLVTNKTWDHFWLNEGHTVYLERHICGRLFGEKFRHFNALGGW
                   |||:| |:|  .|||||:.||||.|||||| .|.||||||| |||||| | | : ||  |||.||
S.cerevisiae   325 PTLIAHDRSNIDVIAHELAHSWSGNLVTNCSWNHFWLNEGWTVYLERRIIGAIHGEPTRHFSALIGW
                                    ↑
                                    1

Human          347 GELQNSVKTFGETHPFTKLVVDLTD.IDPDVAYSSVPYEKGFALLFYLEQLLGGPEIFLGFLKAYVE
                    :||||: .  :   |. || .| |   ||| |:|.||||||| |||:|| :|||   |  |:: | .
S.cerevisiae   392 SDLQNSIDSMKDPERFSTLVQNLNDNTDPDDAFSTVPYEKGFNLLFHLETILGGKAEFDPFIRHYFK
                                                    ↑    ↑
                                                    *    2
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FIGURE 1: Alignment of the amino acid sequences of human and *S. cerevisiae* LTA4H, over the proposed active sites. All functional amino acid residues are in boldface type. The putative zinc-binding ligands are indicated with boxes while residues implicated in catalysis are indicated with numbers. Residue 1 is a Glu in positions 296 and 341 of the human and yeast enzyme, respectively. Residue 2 is a Tyr in positions 383 and 429 in the human and yeast enzyme, respectively. An asterisk indicates a Tyr in position 378 of the human enzyme, involved in suicide inactivation, and a corresponding Phe in position 424 of *sc*LTA4H. The alignment was made using the GAP alignment program in the GCG package (*49*).

Table 1: Primers Used for PCR Mutagenesis of *S. cerevisiae* $LTA_4$ Hydrolase[a]

| mutation | primer | proposed function |
|---|---|---|
| His340 → Gln (H340Q) | 5′ TC GCC CAA GAA CTT GCT CAC 3′ | zinc ligand |
| His344 → Gln (H344Q) | 5′ T GCT CAA TCA TGG TCT GGT A 3′ | zinc ligand |
| Glu363 → Gln (E363Q) | 5′ G AAT CAA GGT TGG ACC GTT T 3′ | zinc ligand |
| Glu341 → Gln (E341Q) | 5′ CC CAT CAA CTT GCT CAC TCA 3′ | general base |
| Phe424 → Tyr (F424Y) | 5′ CCT GAC GAT GCC TAT TCC A 3′ | covalent binding of $LTA_4$ |
| Tyr429 → Phe (Y429F) | 5′ CC ACT GTT CCG TTC GAA AAG 3′ | proton donor, covalent binding of $LTA_4$ |

[a] Mutated bases are underlined.

ability to convert $LTA_4$ into two products, viz., $LTB_4$ and 5*S*,12*R*-dihydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid ($\Delta^6$-*trans*-$\Delta^8$-*cis*-$LTB_4$) (*27*). Amino acid sequencing of internal peptide fragments indicated that the *X. laevis* enzyme is about 65% identical to human $LTA_4$ hydrolase (humLTA4H).

Recently, we cloned a bifunctional LTA4H from *Saccharomyces cerevisiae* (*sc*LTA4H) possessing an anion-activated, leucyl aminopeptidase activity as well as an epoxide hydrolase activity in which $LTA_4$ is hydrolyzed into 5*S*,6*S*-dihydroxy-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (5*S*,6*S*-DHETE). The aminopeptidase activity of *sc*LTA4H is strongly stimulated by $LTA_4$ in a fashion suggesting the presence of a lipid-binding pocket (*28*). The *sc*LTA4H is about 42% identical (53% similar) to humLTA4H at the amino acid level, with the most highly conserved segments corresponding to the proposed active site (*29*) (Figure 1). In the present study we show that *sc*LTA4H is a zinc metalloenzyme capable of hydrolyzing $LTA_4$, not only into 5*S*,6*S*-DHETE, but also into $LTB_4$ and $\Delta^6$-*trans*-$\Delta^8$-*cis*-$LTB_4$. By mutagenetic replacements, we provide evidence that the aminopeptidase activity follows a general base mechanism. We also show that $LTA_4$ is a selective inhibitor of the epoxide hydrolase activity and, at the same time, an efficient stimulator of the aminopeptidase activity. Moreover, introduction of a Tyr residue at position 424 improves the ability of *sc*LTA4H to bind $LTA_4$ and catalyze the formation of $LTB_4$, thus mimicking a step in the molecular evolution of LTA4H.

## EXPERIMENTAL PROCEDURES

*Materials.* The ExpandTM high fidelity PCR system (Roche Molecular Biochemicals), Ni-NTA (nickel-nitrilotriacetic acid, QIAGEN), and the DYEnamic ET terminator cycle sequencing kit (Pharmacia Biotech) were used according to the manufacturer's instructions. Leucyl-*p*-nitroanilide (Leu-*p*-NA) was obtained from Sigma whereas restriction enzymes and T4 DNA ligase were purchased from Life Technologies, Inc. $LTA_4$ methyl or ethyl ester, from Merck Frosst, Quebec, Canada, or prepared as described in (*30*), were saponified in acetone 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.

*Expression of scLTA4H in Spodoptera frugiperda (Sf9) Cells.* The expression of *sc*LTA4H in Sf9 cells (Bac-to-Bac Baculovirus expression system, Life Technologies) and subsequent protein purification were performed essentially as described (*28*).

*PCR Mutagenesis of scLTA4H.* Six histidine codons were introduced into the plasmid pT3_*sc*LTA4H immediately upstream of the Met at position −40 by PCR mutagenesis using a synthetic oligonucleotide (5′ GGA TAA CGT CGA CAT GCA TCA CCA CCA TCA CCA TTT GCC TCT TTC AAT TGA GCA 3′). This primer contains a *Sal*I restriction site before the histidine codons, and the downstream primer covers a unique *Pst*I site. The amplified PCR fragment (digested with *Sal*I and *Pst*I) was ligated together with the remaining part of the pT3_*sc*LTA4H plasmid after cleavage with *Sal*I and *Pst*I to produce the expression plasmid pT3_*sc*LTA4H-40his. The resulting recombinant protein lacks 40 amino acids at the N-terminus and corresponds to 1 of the 4 protein variants (no. IV), previously identified in recombinant *sc*LTA4H expressed in a baculovirus/insect cell system (*28*).

Site-directed mutagenesis of *sc*LTA4H cDNA in pT3_*sc*LTA4H-40his was carried out by PCR according to the megaprimer method (*31*). In a first PCR reaction, a megaprimer is amplified, using one primer containing the mutation and a second primer covering a unique restriction site (Table 1). The megaprimer is then used in a second PCR reaction together with a fourth primer that covers another unique

restriction site. After cleavage, the resulting PCR fragment, containing the mutation, is ligated into the pT3_*sc*LTA4H-40his plasmid, previously cut with the same restriction enzymes. The mutations were verified by DNA sequencing using the dideoxy chain termination method.

*Expression of scLTA4H in Escherichia coli*. The pT3_*sc*-LTA4H-40his plasmid was transformed into competent *E. coli* (JM 101) cells. Expression and preparation of crude protein extracts were performed as described (*32*).

*Protein Purification.* The crude protein extract from the expression was filtered through a 0.45−0.80 μm filter and applied to a Ni-NTA column. The column was washed sequentially with 10 mM imidazole in 2 bed volumes each of 50 mM Tris-HCl, pH 8.0, followed by 1 M NaCl in 50 mM sodium phosphate, pH 6.8, and 50 mM Tris-HCl, pH 8.0. Finally, the protein was eluted with 2 bed volumes of 50 mM Tris-HCl, pH 8.0, containing 100 mM imidazole. The buffer was changed by gel filtration through a PD-10 column (Amersham Pharmacia Biotech). Further purification was performed by chromatofocusing using a Mono-P column (Amersham Pharmacia Biotech), preequilibrated with 25 mM Bis-Tris, pH adjusted to 7.0 with iminodiacetic acid. After sample application, adsorbed proteins were eluted with a pH gradient (7.0−4.5) by changing the buffer to Polybuffer 74 (Amersham Pharmacia Biotech), diluted 10 times in water and pH adjusted to 4.5 with iminodiacetic acid. The protein was eluted in fractions between pH 5.4 and 5.9. For final purification on anion exchange chromatography, a Mono-Q column (Amersham Pharmacia Biotech), equilibrated with 10 mM Tris-HCl, pH 8, was used. Adsorbed proteins were eluted with a linear gradient of KCl (0−500 mM), and active fractions were eluted at 150 mM KCl. For ultrafiltration, a microconcentrator (Centricon, Amicon) was used.

*Protein Determinations and SDS−PAGE.* Protein concentrations were determined according to the method of Bradford, using the Bio-Rad protein assay reagent and bovine serum albumin as standard. SDS−polyacrylamide gel electrophoresis (PAGE) was performed on a Phast system (Amersham Pharmacia Biotech) using 10−15% gradient gels. The protein was visualized by staining with Coomassie Brilliant Blue.

*Zinc Analysis*. Measurements of zinc were performed by graphite furnace atomic absorption spectrophotometry using a Perkin-Elmer 5000 Zeeman instrument equipped with an electrothermal atomization unit (HGA-500) and an automatic sample injector (AS-40). Zinc was analyzed at 213.9 nm using an EDL lamp. Zinc standards (10−200 ng/mL) were prepared in 0.03 M $HNO_3$ from stock standards of 1000 mg/L (British Drug House, U.K.) and diluted 1:1 in the sample cups with deionized water (Elgastat Spectrum R.O.1, ELGA, U.K.) prior to analysis. Samples of *sc*LTA4H were mixed with an equal volume of 0.03 or 0.1 M $HNO_3$. Standards and unknowns were analyzed in duplicates with blanks (0.015 or 0.05 M $HNO_3$) injected between each duplicate. Aliquots of 10 μL were injected onto the L'Vov platform in the HGA-500.

*Western Blot Analysis.* For Western blot, aliquots of protein (2 μg) in Laemmli sample buffer were heated to 95 °C for 5 min before being loaded onto an SDS−PAGE gel (stacking gel 5%; separating gel 10%) (*33*). The separated proteins were blotted onto a nitrocellulose membrane (Hybond-C, Amersham) at 100 V for 1 h. The membrane was blocked in 5% nonfat dried milk (w/v) in TBE buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl). Subsequently, the filter was incubated with a polyclonal rabbit antiserum raised against recombinant *sc*LTA4H, diluted 1:1000 in TBE with 5% FCS (fetal calf serum, w/v) and 0.02% $NaNO_3$. As second antibody, a donkey anti-rabbit antibody conjugated with horseradish peroxidase diluted 1:1000 in TBE with 5% FCS (w/v) was used. The binding of the primary antibody to its target protein was visualized by incubation of the membranes with 15 mg of 3,3′-diaminobenzidine (Sigma), 0.03% $H_2O_2$, and 0.03% $CoCl_2$ (w/v).

*Enzyme Activity Assay.* The peptidase activity was determined with a spectrophotometric assay in the wells of a microtiter plate, essentially as described (*34*). The enzyme was incubated with 1 mM Leu-*p*-NA in 50 mM Tris-HCl, pH 7.5, containing 100 mM KCl. Formation of the product (*p*-nitroaniline) was measured at room temperature as the increase in $A_{405}$ using a multiscan spectrophotometer, MCC/340 (LabSystems).

For determination of the epoxide hydrolase activity, incubations were performed with enzyme (20−100 μg) in 100 μL of 10 mM Tris-HCl, pH 7.5, with 6.25−80 μM $LTA_4$ for 60 s at room temperature. The reaction was stopped with 1−2 volumes of MeOH, and 400−700 pmol of prostaglandin $B_1$ ($PGB_1$) was added as an internal standard. The samples were subjected to solid-phase extraction (Chromabond $C_{18}$ EC, Macherey Nagel) and finally analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC). The column (Nova-Pak $C_{18}$, 3.9 × 150 mm, Waters) was eluted with a mixture of acetonitrile/methanol/water/acetic acid (30:30:40:0.01, v/v) at a flow rate of 1.0 mL/min. The absorbance of the eluate was monitored at 270 nm. The products were identified by their chromatographic mobility of relative standard compounds, as well as by UV spectrophotometry.

## RESULTS

*Expression and Purification of scLTA4H.* The *sc*LTA4H was initially expressed in Sf9 cells. The recombinant protein was recovered in the cell pellet rather than in the medium and was purified to homogeneity in five steps by FPLC, using anion exchange, hydroxyapatite, hydrophobic interaction, and chromatofocusing resins. Typically, from 450 mL of infected Sf9 cell culture, 500 μg of purified enzyme was recovered and was used for zinc analysis. From expression in Sf9 cells, four different translation variants of the protein have been described, I−IV (*28*). For further biochemical characterization and mutational analysis of *sc*LTA4H, variant IV was expressed in *E. coli* and purified. To this end, 40 codons were removed from the 5′-region of the original cDNA, which positions the Met at position −40 as the translation-initiation site. For rapid purification on nickel-affinity chromatography, a tag of six histidines was attached immediately after the start codon, and additional purification was achieved by chromatofocusing followed by a final step of ion exchange chromatography. The final yield was approximately 2−3 mg of protein per liter of cell culture, several log-orders of magnitude higher than what is obtained with the full-length protein (*28*). Unless otherwise stated, this preparation was used for characterization. Wild-type and mutated *sc*LTA4H were all recognized by an anti-*sc*LTA4H antibody (Figure 2).

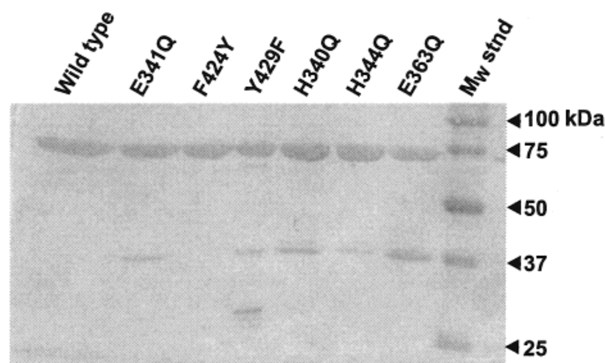


FIGURE 2: Western blot analysis of purified wild-type and mutated *sc*LTA4H. After the final step of purification, aliquots of all mutated proteins (2 $\mu$g) were subjected to SDS−PAGE (5% stacking gel, 10% separating gel). The gel was blotted onto a nitrocellulose membrane, incubated with an antiserum raised against *sc*LTA4H, and developed as described under Experimental Procedures. The molecular mass marker was a Precision Protein standard (Bio-Rad). Samples of wild-type and [F424Y]-, [H340Q]-, [H344Q]-, and [E363Q]*sc*LTA4H were from fresh enzyme preparations, whereas [E341Q]- and [Y429F]scLTA4H were from a 2 week old and a 3 month old preparation, respectively.

*Characterization of scLTA4H Expressed in E. coli.* The peptidase activity was determined from incubations of the enzyme (1 $\mu$g) with Leu-*p*-NA (1 mM) in 10 mM Tris-HCl, pH 7.5, containing 100 mM KCl at room temperature. The specific activity was calculated to 345 $\pm$ 56 nmol mg$^{-1}$ min$^{-1}$ (mean $\pm$ SD, $n$ = 17). The kinetic constants, $K_m$ and $V_{max}$, were determined to 1.97 $\pm$ 0.1 mM and 0.97 $\pm$ 0.1 $\mu$mol mg$^{-1}$ min$^{-1}$ (mean $\pm$ SD, $n$ = 3), respectively. The specificity constant, $k_{cat}/K_m$, was calculated to 593 $\pm$ 68 s$^{-1}$ M$^{-1}$ ($n$ = 3) (Table 2A). When the enzyme (1 $\mu$g) was treated with $LTA_4$ (40−60 $\mu$M) prior to incubation with Leu-*p*-NA, the specific peptidase activity was stimulated almost 10 times in a dose-dependent and saturable fashion, in agreement with previous observations (*28*). The affinity constant ($K_A$) for $LTA_4$ was calculated to 6.5 $\pm$ 0.8 $\mu$M (mean $\pm$ SD, $n$ = 5). Furthermore, the kinetic constants, $K_m$ and $V_{max}$, for hydrolysis of Leu-*p*-NA by the activated enzyme were calculated to 2.0 $\pm$ 0.1 mM and 9.3 $\pm$ 0.6 $\mu$mol mg$^{-1}$ min$^{-1}$ ($n$ = 3), respectively. Consequently, the value of $k_{cat}/K_m$ was calculated to be 5500 $\pm$ 440 s$^{-1}$ M$^{-1}$ ($n$ = 3). Thus, after $LTA_4$ treatment of the enzyme, the $V_{max}$ was raised from 0.97 $\pm$ 0.1 to 9.3 $\pm$ 0.6 $\mu$mol mg$^{-1}$ min$^{-1}$, i.e., a 9.6-fold increase (Table 2A).

The epoxide hydrolase activity, i.e., the conversion of $LTA_4$ into 5*S*,6*S*-DHETE, was determined from incubations of enzyme (20−100 $\mu$g) with $LTA_4$ (40 $\mu$M) in 10 mM Tris-HCl, pH 7.5, for 1 min at room temperature and analysis of products by RP-HPLC. The specific activity was calculated from peak area measurements to 22.5 $\pm$ 3.5 nmol mg$^{-1}$ min$^{-1}$ (mean $\pm$ SD, $n$ = 11), using $PGB_1$ as the internal standard. This activity is in good agreement with previous data for *sc*LTA4H expressed in Sf9 cells (*28*). In addition, we carried out a kinetic characterization of the conversion of $LTA_4$ into 5*S*,6*S*-DHETE by *sc*LTA4H. Thus, the enzyme was incubated with increasing concentrations of $LTA_4$, and values of $K_m$ and $V_{max}$ were calculated to 60 $\pm$ 26 $\mu$M and 37 $\pm$ 15 nmol mg$^{-1}$ min$^{-1}$ ($n$ = 3), respectively. The specificity constant, $k_{cat}/K_m$, was calculated to 742 $\pm$ 190 s$^{-1}$ M$^{-1}$ ($n$ = 3) (Table 2B).

*Enzymatic Hydrolysis of $LTA_4$ into $LTB_4$ and $\Delta^6$-trans-$\Delta^8$-cis-$LTB_4$.* When large amounts of enzyme (>30 $\mu$g) were used in incubations with $LTA_4$, two additional peaks could be observed in the RP-HPLC chromatogram, denoted III and IV (Figure 3). Taking into consideration the structure of the substrate $LTA_4$, the materials under peaks III and IV were tentatively identified as $LTB_4$ and $\Delta^6$-*trans*-$\Delta^8$-*cis*-$LTB_4$, respectively, as judged by their chromatographic retention, UV spectrum, and comparison with synthetic standards (Figures 3 and 4). The production of $LTB_4$ and $\Delta^6$-*trans*-$\Delta^8$-*cis*-$LTB_4$ was about 20% and 10%, respectively, of the production of 5*S*,6*S*-DHETE. Boiling the protein (10 min) prior to incubation with $LTA_4$ did not lead to any production of $LTB_4$, $\Delta^6$-*trans*-$\Delta^8$-*cis*-$LTB_4$, or 5*S*,6*S*-DHETE. The $K_m$ and $V_{max}$ values for $LTB_4$ production were determined to 20 $\pm$ 10 $\mu$M and 1.6 $\pm$ 1.2 nmol mg$^{-1}$ min$^{-1}$ (mean $\pm$ SD, $n$ = 3), respectively, and $k_{cat}/K_m$ was calculated to 0.16 $\pm$ 0.2 s$^{-1}$ M$^{-1}$ (Table 2B). The large standard deviations of these values are due to the relatively low accuracy in the kinetic measurements at low levels of $LTB_4$ formation. No determinations of apparent kinetic constants were performed for the formation of $\Delta^6$-*trans*-$\Delta^8$-*cis*-$LTB_4$.

*Selective Inactivation of the Epoxide Hydrolase Activity by $LTA_4$.* *sc*LTA4H was treated with $LTA_4$ (86−102 $\mu$M) for 10−20 min at room temperature. The $LTA_4$-treated protein was washed by ultrafiltration (30 kDa molecular mass cutoff), and aliquots of washed enzyme were analyzed by RP-HPLC to ensure that no $LTA_4$ metabolites remained in the samples. The epoxide hydrolase activity of $LTA_4$-treated and ultrafiltrated enzyme was then determined from incubations with a second dose of $LTA_4$ (43−51 $\mu$M) and analysis of products by RP-HPLC. Likewise, aliquots of the $LTA_4$-treated and ultrafiltrated enzyme were tested for remaining peptidase activity by incubations with 1 mM Leu-*p*-NA (Figure 5). Control samples of fresh enzyme were incubated with either $LTA_4$ (30 $\mu$g of protein, 43−51 $\mu$M $LTA_4$) or Leu-*p*-NA (1.5 $\mu$g of protein, 1 mM Leu-*p*-NA) to analyze the basal enzyme activities of untreated *sc*LTA4H. The epoxide hydrolase activity of *sc*LTA4H protein was inactivated by $LTA_4$. After ultrafiltration of the $LTA_4$-treated enzyme, only 26 $\pm$ 13% (mean $\pm$ SD, $n$ = 3) of the activity remained (Figure 5). At the same time, the peptidase activity was increased 808 $\pm$ 33% ($n$ = 3), in agreement with earlier results (*28*). $LTA_4$ methyl ester, which is a better inhibitor of humLTA4H as compared to the free acid of $LTA_4$ (*35*), neither inactivated the epoxide hydrolase activity nor stimulated the peptidase activity of *sc*LTA4H (results not shown).

*Zinc Analysis and Identification of Zinc-Binding Ligands.* *sc*LTA4H, expressed and purified from Sf9 cells, was subjected to atomic absorption spectrometry, which revealed the presence of 0.8 mol of zinc/mol of protein. Furthermore, each of the three putative zinc-binding ligands, His-340, His-344, and Glu-363, was exchanged for a Gln by site-directed mutagenesis (Figure 1). The overall expression of the three mutated proteins, [H340Q]-, [H344Q]-, and [E363Q]-*sc*LTA4H (in single-letter code for the amino acid change), was low, and the purified proteins were unstable, which suggests that they are sensitive to proteolytic degradation (Figure 2). The specific peptidase activities of freshly prepared [H340Q]-, [H344Q]-, and [E363Q]*sc*LTA4H were determined to 2.3 $\pm$ 0.2 (mean $\pm$ SD, $n$ = 4), 0.79 $\pm$ 0.5 ($n$ = 3), and 0.18 $\pm$ 0.03 nmol mg$^{-1}$ min$^{-1}$ ($n$ = 3),

Table 2: Apparent Kinetic Constants for Wild-Type and [F424Y]*sc*LTA4H[a]

(A) Peptidase Activity

| | untreated enzyme | | | activated enzyme (40 $\mu$M $LTA_4$) | | | $LTA_4$ |
|---|---|---|---|---|---|---|---|
| | $K_m$ (mM) | $V_{max}$ ($\mu$mol mg$^{-1}$ min$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$ M$^{-1}$) | $K_m$ (mM) | $V_{max}$ ($\mu$mol mg$^{-1}$ min$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$ M$^{-1}$) | $K_A$ ($\mu$M) |
| wild type | 1.97 ± 0.1 | 0.97 ± 0.1 | 593 ± 68 | 2.03 ± 0.1 | 9.3 ± 0.6 | 5500 ± 440 | 6.5 ± 0.8 |
| F424Y | 0.43 ± 0.1 | 0.77 ± 0.01 | 2100 ± 240 | 2.3 ± 0.4 | 20 ± 0.1 | 10500 ± 1600 | 1.2 ± 0.5 |

(B) Epoxide Hydrolase Activity, i.e., Hydrolysis of $LTA_4$ into 5*S*,6*S*-DHETE and $LTB_4$

| | 5*S*,6*S*-DHETE production | | | $LTB_4$ production | | |
|---|---|---|---|---|---|---|
| | $K_m$ ($\mu$M) | $V_{max}$ (nmol mg$^{-1}$ min$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$ M$^{-1}$) | $K_m$ ($\mu$M) | $V_{max}$ (nmol mg$^{-1}$ min$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$ M$^{-1}$) |
| wild type | 60 ± 26 | 37 ± 15 | 742 ± 190 | 20 ± 10 | 1.6 ± 1.2 | 147 ± 184 |
| F424Y | 15 ± 6.0 | 9.5 ± 1.0 | 840 ± 290 | 16 ± 7.5 | 2.7 ± 0.6 | 234 ± 94 |

[a] Data are presented as mean ± SD, $n = 3$.

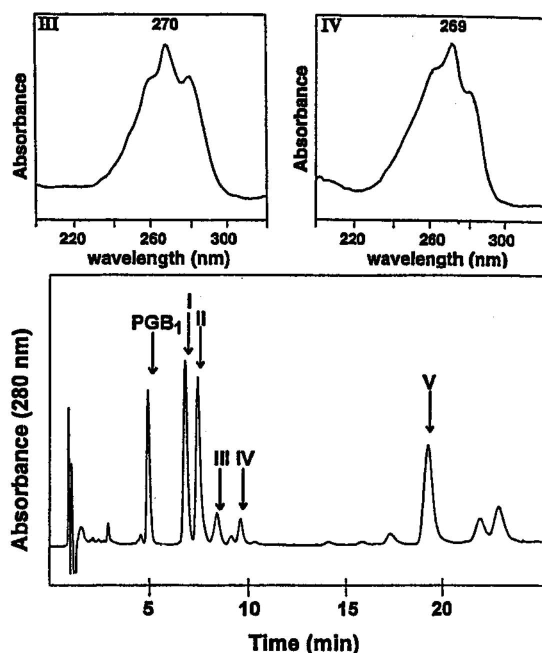


FIGURE 3: Reverse-phase HPLC profile of products formed from $LTA_4$ by *sc*LTA4H. *sc*LTA4H (30 $\mu$g) was incubated in 10 mM Tris-HCl, pH 7.5, with $LTA_4$ (40 $\mu$M) for 1 min at room temperature. Extraction and RP-HPLC analysis of products were performed as described under Experimental Procedures. $PGB_1$ was used as internal standard. The Roman numerals indicate the retention times of compounds tentatively identified as $\Delta^6$-*trans*-$LTB_4$ (I) and $\Delta^6$-*trans*-12-epi-$LTB_4$ (II), two nonenzymatic hydrolysis products of $LTA_4$ (*50*), $LTB_4$ (III), $\Delta^6$-*trans*-$\Delta^8$-*cis*-$LTB_4$ (IV), and 5*S*,6*S*-DHETE (V). The UV spectra of material eluting under peaks III and IV are shown in the top panels. Both spectra are typical of compounds containing a conjugated triene and have $\lambda_{max}$ at 270 and 269 nm, in agreement with published data for $LTB_4$ and $\Delta^6$-*trans*-$\Delta^8$-*cis*-$LTB_4$, respectively (*50, 51*).

respectively, which correspond to 0.7, 0.2, and 0.07% of the activity of the wild-type enzyme (Figure 6A). The epoxide hydrolase activities of [H340Q]-, [H344Q]-, and [E363Q]-*sc*LTA4H, i.e., their ability to convert $LTA_4$ into 5*S*,6*S*-DHETE, were calculated to 0.35 ± 0.2, 0.23 ± 0.1, and 0.48 ± 0.2 nmol mg$^{-1}$ min$^{-1}$ ($n = 3$), respectively, corresponding to 1.5, 1.0, and 2.1% of wild-type enzyme (Figure 6B). It should be noted that the sensitivity of the epoxide hydrolase assay is low and most likely overestimates the enzyme activity due to the simultaneous nonenzymatic formation of 5*S*,6*S*-DHETE. Thus, mutation of any of the three deduced

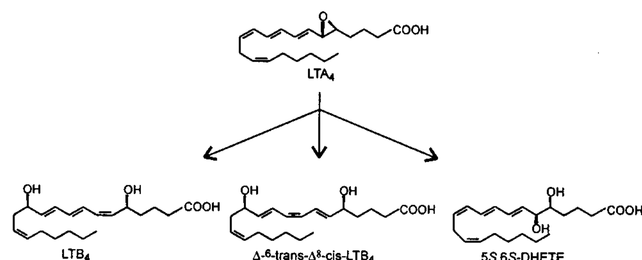


FIGURE 4: Enzymatic hydrolysis products formed from $LTA_4$ by *sc*LTA4H. The figure depicts the structure of the substrate $LTA_4$ (5*S*-*trans*-5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid) and the products $LTB_4$ (5*S*,12*R*-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid), $\Delta^6$-*trans*-$\Delta^8$-*cis*-$LTB_4$ (5*S*,12*R*-dihydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid), and 5*S*,6*S*-DHETE, (5*S*,6*S*-dihydroxy-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid).

zinc-binding ligands essentially abolished both the aminopeptidase and the epoxide hydrolase activity.

*Effects of Mutagenetic Replacements of Glu-341, Tyr-429, and Phe-424.* The potentially catalytic amino acids Glu-341 and Tyr-429 were exchanged for a Gln and Phe, respectively ([E341Q]- and [Y429F]*sc*LTA4H). In addition, a Phe in position 424 was exchanged for a Tyr ([F424Y]*sc*LTA4H), which is the corresponding residue in human LTA4H that is involved in suicide inactivation. The expression yields of these three mutants were approximately the same as for the wild-type enzyme.

The specific aminopeptidase activity of [E341Q]*sc*LTA4H was 0.23 ± 0.06 nmol mg$^{-1}$ min$^{-1}$ (mean ± SD, $n = 3$), corresponding to 0.07% of the wild-type enzyme (Figure 6A). For [Y429F]*sc*LTA4H, the specific peptidase activity was determined to 2.2 ± 0.2 nmol mg$^{-1}$ min$^{-1}$ ($n = 7$), which is about 0.6% of the activity of the wild-type enzyme. Due to the very low enzyme activities, no kinetic constants were determined for these two mutants. The peptidase activities of the mutants [E341Q]- and [Y429F]*sc*LTA4H were not significantly activated by $LTA_4$. The specific epoxide hydrolase activity, i.e., conversion of $LTA_4$ into 5*S*,6*S*-DHETE, of [E341Q]*sc*LTA4H was determined to 22.9 ± 3.5 nmol mg$^{-1}$ min$^{-1}$ (mean ± SD, $n = 5$), which is almost identical to the activity of the wild-type enzyme (Figure 6B). [Y429F]-*sc*LTA4H exhibited a specific activity of 3.7 ± 0.9 nmol mg$^{-1}$ min$^{-1}$ ($n = 8$), corresponding to about 16% of the activity of the wild-type enzyme. Apparently, mutation of Glu-341 and Tyr-429 selectively removes the aminopeptidase activity.

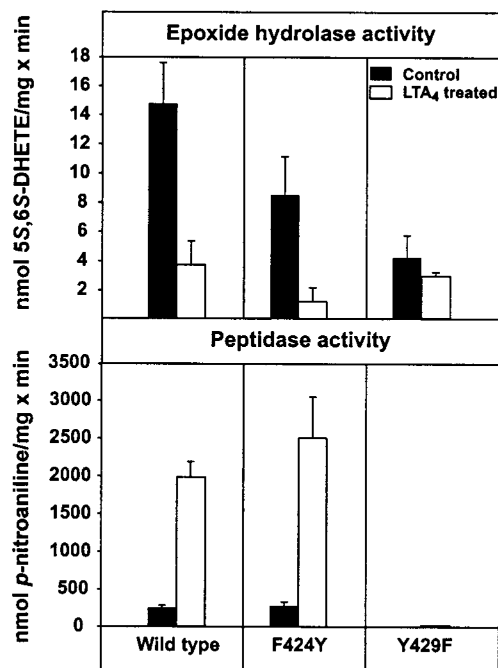


FIGURE 5: Effects of treatment with $LTA_4$ on the epoxide hydrolase and aminopeptidase activity of wild-type and [F424]- and [Y429F]*sc*LTA4H. *sc*LTA4H was treated with $LTA_4$ (86−102 μM), followed by removal of $LTA_4$ metabolites by ultrafiltration. Aliquots of this enzyme pool ($LTA_4$ treated) were incubated with $LTA_4$ (43−51 μM, 30 μg of enzyme) or with Leu-*p*-NA (1 mM, 1 μg of enzyme) to determine the remaining epoxide hydrolase and peptidase activities, as indicated by the open bars. As a control, the epoxide hydrolase and peptidase activities of untreated *sc*LTA4H, indicated by filled bars, were determined from similar incubation with $LTA_4$ or Leu-*p*-NA. The upper panel shows the effects of $LTA_4$ on the epoxide hydrolase activity whereas the lower panel shows the effects on the aminopeptidase activity.

In contrast to [E341Q]- and [Y429F]*sc*LTA4H, [F424Y]-*sc*LTA4H exhibited an unrestricted, or even increased, aminopeptidase activity mounting to 0.51 ± 0.07 μmol $mg^{-1}$ $min^{-1}$ ($n = 12$), corresponding to 147% of the activity of the wild-type enzyme (Figure 6A). The apparent kinetic constants, $K_m$ and $V_{max}$, were calculated to be 0.43 ± 0.1 mM and 0.77 ± 60 μmol $mg^{-1}$ $min^{-1}$ ($n = 4$), respectively, and the value of $k_{cat}/K_m$ was calculated to be 2140 ± 240 $s^{-1}$ $M^{-1}$ ($n = 4$) (Table 2A). Furthermore, mutation of Tyr-424 did not affect the stimulatory action of $LTA_4$ on the aminopeptidase activity. Thus, the mutant [F424Y]-*sc*LTA4H exhibited an 840 ± 350% ($n = 3$) increase of the specific activity when pretreated with $LTA_4$, as observed for the wild-type enzyme (Table 2A). The affinity constant ($K_A$) for activation of [F424Y]*sc*LTA4H by $LTA_4$ was calculated to be 1.2 ± 0.5 μM ($n = 4$). The Michaelis constant and $V_{max}$ for hydrolysis of Leu-*p*-NA by enzyme activated with $LTA_4$ were calculated as 2.3 ± 0.4 mM and 20 ± 0.1 μmol $mg^{-1}$ $min^{-1}$ ($n = 3$), respectively. In fact, $V_{max}$ was increased from 0.77 ± 0.06 to 20 ± 0.1 mmol $mg^{-1}$ $min^{-1}$, corresponding to a 19-fold increase. Consequently, $k_{cat}/K_m$ was calculated to be 10 500 ± 1600 $s^{-1}$ $M^{-1}$ ($n = 3$) (Table 2A).

Mutation of Tyr-424 did not significantly reduce the epoxide hydrolase activity, as judged by the 5*S*,6*S*-DHETE production. The [F424Y]*sc*LTA4H mutant exhibited a specific activity of 9.0 ± 1.8 nmol $mg^{-1}$ $min^{-1}$ ($n = 11$), which is about 40% of the activity of the wild-type protein (Figure 6B). This mutant also hydrolyzed $LTA_4$ into $LTB_4$ in the relative amount 0.3:1 ($LTB_4$/5*S*,6*S*-DHETE), whereas only minute amounts of $\Delta^6$-*trans*-$\Delta^8$-*cis*-$LTB_4$ were formed. The kinetic constants $K_m$ and $V_{max}$ for the conversion of $LTA_4$ into 5*S*,6*S*-DHETE were determined to be 15 ± 6.0 μM and 9.5 ± 1.0 nmol $mg^{-1}$ $min^{-1}$ ($n = 4$), respectively. For the production of $LTB_4$, $K_m$ and $V_{max}$ were determined to be 15.7 ± 7.5 μM and 2.7 ± 0.6 nmol $mg^{-1}$ $min^{-1}$ ($n = 3$). From these values, the specificity constants ($k_{cat}/K_m$) were calculated as 840 ± 290 and 234 ± 94 $s^{-1}$ $M^{-1}$ ($n = 3$) for 5*S*,6*S*-DHETE and $LTB_4$, respectively (Table 2B).

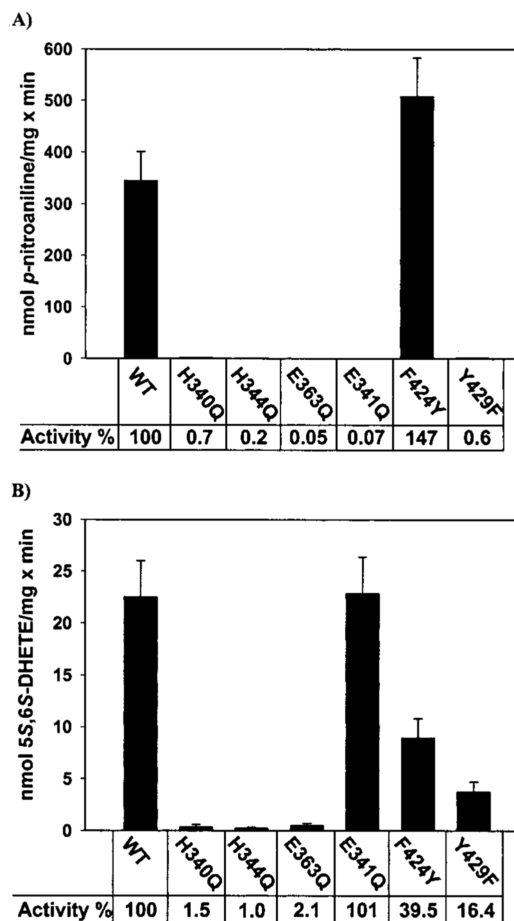


FIGURE 6: Effects of mutations on the epoxide hydrolase and aminopeptidase activity of *sc*LTA4H. Purified wild-type and mutated *sc*LTA4H (1 μg) were incubated with Leu-*p*-NA (1 mM) in 50 mM Tris-HCl, pH 7.5, containing 100 mM KCl at room temperature. The histogram in panel A depicts the resulting peptidase activity expressed in nmol of *p*-nitroaniline $mg^{-1}$ $min^{-1}$ (mean ± SD, $n = 3$). The enzyme activity is also indicated in relative numbers with the wild-type enzyme set to 100%. Purified wild-type and mutated *sc*LTA4H (30 μg) were also incubated in 10 mM Tris-HCl, pH 7.5, with $LTA_4$ (40 μM) for 1 min at room temperature. The histogram in panel B depicts the resulting epoxide hydrolase activity, measured as hydrolysis of $LTA_4$ into 5*S*,6*S*-DHETE and expressed as nmol $mg^{-1}$ $min^{-1}$ (mean ± SD, $n = 3$). The epoxide hydrolase activity is also indicated in relative numbers with the wild-type enzyme set to 100%.

Finally, [F424Y]- and [Y429F]*sc*LTA4H were tested for substrate-mediated inactivation of the epoxide hydrolase activity, as described above for the wild-type enzyme. After $LTA_4$ treatment (86−102 μM), only 13 ± 8% (mean ± SD, $n = 3$) of the epoxide hydrolase activity remained for

[F424Y]*sc*LTA4H while 76 ± 26% ($n = 3$) of the activity was still present in [Y429F]*sc*LTA4H (Figure 5).

## DISCUSSION

Mammalian LTA4H is a bifunctional zinc metalloenzyme with widespread occurrence in cells and tissues. The enzyme is unusual in that it utilizes distinct catalytic structures to integrate an aminopeptidase activity and a unique epoxide hydrolase activity in a common active center. Very little is known about the molecular evolution and properties of LTA4H from lower species. LTA4H is a member of the M1 family of metallopeptidases and is distantly related to many zinc proteases and aminopeptidases that are present in a variety of organisms from bacteria to mammals (*36*). For most of these proteins, the level of identity (similarity) with LTA4H is low and essentially confined to the zinc-binding site, whereas in some cases, e.g., an arginyl aminopeptidase in *C. elegans* (*37*), the degree of homology is higher and more evenly distributed along the primary structures. However, these proteins are solely proteases or aminopeptidases without an epoxide hydrolase activity.

As an exception which confirms the rule, we recently cloned and characterized *sc*LTA4H as a bifunctional enzyme with an anion-stimulated leucyl aminopeptidase activity and an epoxide hydrolase activity in which $LTA_4$ is hydrolyzed into 5*S*,6*S*-DHETE (*28*). We also showed that *sc*LTA4H possesses a lipid-binding pocket, which mediates stimulation of the peptidase activity when occupied by $LTA_4$.

*scLTA4H Can Hydrolyze $LTA_4$ into $LTB_4$ and $\Delta^6$-trans-$\Delta^8$-cis-$LTB_4$.* Recombinant *sc*LTA4H, expressed and purified from *E. coli*, exhibited properties in good agreement with data previously reported for *sc*LTA4H expressed in Sf9 cells (*28*). However, a detailed analysis of the enzymatic products generated from $LTA_4$ revealed that *sc*LTA4H has a broad catalytic repertoire. Thus, when larger amounts of enzyme were incubated with $LTA_4$, two additional enzymatic products were observed in the RP-HPLC chromatogram (Figure 3). These two products were tentatively identified as $LTB_4$ and $\Delta^6$-*trans*-$\Delta^8$-*cis*-$LTB_4$, and their formation was calculated to 18% and 10%, respectively, relative to the formation of 5*S*,6*S*-DHETE (Figure 4).

Formation of 5*S*,6*S*-DHETE as the major product from $LTA_4$ may be seen as a sign of a functional relationship between *sc*LTA4H and xenobiotic epoxide hydrolases, which invariably form vicinal diols from epoxides (*38*−*40*). In fact, soluble epoxide hydrolase accepts $LTA_4$ as substrate and converts it into 5*S*,6*R*-DHETE, i.e., an epimer of the product formed by *sc*LTA4H (*41, 42*). However, recent studies have demonstrated that in mammals soluble epoxide hydrolases have minimal, if any, sequence or structural similarity with LTA4H (*43*−*45*). Moreover, mutation of Tyr-383 in humLTA4H allows formation of 5*S*,6*S*-DHETE, indicating a phylogenetic relationship with *sc*LTA4H (*46*).

The $K_m$ values for the conversion of $LTA_4$ into 5*S*,6*S*-DHETE and $LTB_4$ were of the same order of magnitude and suggest that the substrate binds in a single productive conformation, yet allowing formation of several structurally distinct products. Of note, *sc*LTA4H is the first example of a nonvertebrate protein that can catalyze the hydrolysis of $LTA_4$ into $LTB_4$ and demonstrates that *sc*LTA4H has acquired some of the molecular determinants required for expression of this unusual enzyme activity. Moreover, the mere presence of this catalytic activity corroborates the notion that the yeast enzyme is an ancestral gene to LTA4H in vertebrates and mammals.

*scLTA4H Contains One Catalytic Zinc Complexed to His-340, His-344, and Glu-363.* *sc*LTA4H shares typical sequence motifs with other members of the M1 family of metallopeptidases (*36*), in particular the signature for a catalytic zinc site (Figure 1). Using atomic absorption spectrometry, we found that *sc*LTA4H is indeed a metalloenzyme containing one zinc per enzyme molecule (0.8 mol/mol). From sequence alignment with humLTA4H, the three putative zinc-binding ligands were identified as His-340, His-344, and Glu-363, each of which was mutated into a Gln (Figure 1). For all mutants, the expression yield was very low, and the purified proteins lacked significant enzyme activities. Furthermore, these proteins seemed to be unstable, as judged from repeated analysis with SDS−PAGE and Western blot, suggesting that the mutations had induced conformational changes that make the enzymes more sensitive to proteolytic degradation. Taken together, these data show that His-340, His-344, and Glu-363 are the zinc-binding ligands, and that the zinc atom is catalytic and also involved in the maintenance of the structural integrity of the protein.

*A General Base Mechanism for the Leucyl Aminopeptidase Activity of scLTA4H.* In the human enzyme, the peptidase activity has been suggested to follow a general base mechanism in which Glu-296 acts a base and Tyr-383 as a proton donor (*16, 17*). The corresponding amino acids in the yeast enzyme are Glu-341 and Tyr-429 (Figure 1). To investigate the importance of Glu-341 for the peptidase activity of the yeast enzyme, it was mutated into a Gln; i.e., the side chain carboxylate was removed. [E341Q]*sc*LTA4H lost the peptidase activity to near zero, and the activity could not be restored by $LTA_4$ (Figure 6A). In contrast, the epoxide hydrolase activity was unaffected as judged by the formation of 5*S*,6*S*-DHETE (Figure 6B). Thus, Glu-341 is specifically involved in the peptidase activity and could well serve as a base. Likewise, when Tyr-429 was mutated into a Phe, the peptidase activity was selectively removed, in agreement with an important role for this residue in the enzyme reaction. Together, these mutational data indicate that the aminopeptidase activity of *sc*LTA4H follows a general base mechanism, similar to the one discussed for the human enzyme (*16*). Hence, in this mechanism Glu-341 and Tyr-429 would be the base and proton donor, respectively (Figure 7).

*Selective Inactivation of the Epoxide Hydrolase Activity by $LTA_4$, Involvement of Tyr-429.* Typically, mammalian LTA4H undergoes suicide inactivation and covalent modification when exposed to $LTA_4$, and this process blocks both catalytic activities (*6, 21*). In our hands, the epoxide hydrolase activity of scLTA4H was also inactivated when pretreated with $LTA_4$. Thus, approximately 75% of the epoxide hydrolase activity, measured as the formation of 5*S*,6*S*-DHETE, was lost after $LTA_4$ treatment (86−102 $\mu$M). In sharp contrast, the peptidase activity was increased by > 800%, under the same experimental conditions (Figure 5). The mechanism(s) by which $LTA_4$ generates these opposite effects on the two enzyme activities is (are) presently not clear. Previous work has demonstrated that the stimulatory action of $LTA_4$ on the aminopeptidase activity is mediated

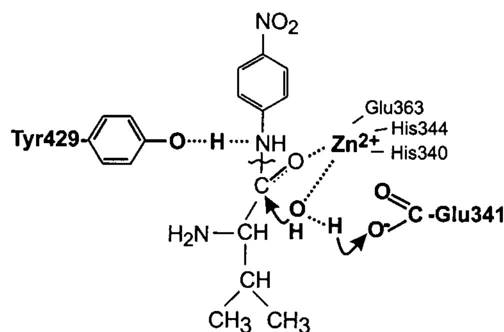


FIGURE 7: Model for the aminopeptidase reaction of *sc*LTA4H. In this scheme, a water molecule is displaced from the catalytic zinc by the incoming substrate Leu-*p*-NA. The water is further polarized by the base Glu-341 to promote its attack on the carbonyl carbon of the scissile peptide bond. In the final step of this reaction, a proton is donated from Tyr-429 to the peptide nitrogen.

via a lipid-binding pocket located at the active center of *sc*LTA4H (*28*). The inhibitory action on the epoxide hydrolase activity may be exerted via the same or a different binding site. On the other hand, the large difference in $K_m$ and $K_A$ for $LTA_4$ suggests that $LTA_4$ binds in one catalytic and one allosteric conformation, both of which may potentially lead to inactivation of the epoxide hydrolase activity. However, in analogy with the inactivation of humLTA4H, it is tempting to speculate that $LTA_4$ binds covalently to *sc*LTA4H. In the human enzyme, the site of attachment for $LTA_4$ is Tyr-378, and mutation of this residue into a Phe protects the enzyme from suicide inactivation (*21*). Since there is a Phe in the corresponding position (Phe-424) in *sc*LTA4H, it seems unlikely that this residue is involved in the inactivation process. Previous work by Mancini et al. has demonstrated that $LTA_3$, a double bond isomer of $LTA_4$, can bind covalently to Tyr-383 in humLTA4H (*47*). Interestingly, the corresponding amino acid in the yeast enzyme is also a Tyr (Tyr-429, cf. Figure 1), and when this residue was mutated into Phe, *sc*LTA4H was partially protected from $LTA_4$ inactivation (Figure 5). Hence, the phenolic hydroxyl group of Tyr-429 seems to be partially responsible for the substrate-mediated inactivation of *sc*LTA4H, perhaps as a site for covalent binding of $LTA_4$. Moreover, a covalent attachment of $LTA_4$ to the protein would exclude binding to the allosteric site since we have previously shown that $LTA_4$ can be displaced from this site by a specific tight-binding inhibitor (*28*). Determination of the structure of *sc*LTA4H will hopefully elucidate the spatial and functional relationships between the binding sites for $LTA_4$.

*Mutation of Tyr-424 Mimics a Step in the Molecular Evolution of scLTA4H.* As mentioned above, mutation of Tyr-378 in humLTA4H into a Phe protects the enzyme from suicide inactivation (*21*). Furthermore, [Y378F]humLTA4H can hydrolyze $LTA_4$ into both $LTB_4$ and $\Delta^6$-*trans*-$\Delta^8$-*cis*-$LTB_4$ (*48*), just like *sc*LTA4H (Figure 3). To investigate whether Phe-424 (equivalent to Tyr-378) is involved in the binding mode of $LTA_4$ and the production of $\Delta^6$-*trans*-$\Delta^8$-*cis*-$LTB_4$, we mutated this residue into a Tyr. The resulting mutant, [F424Y]*sc*LTA4H, hydrolyzed $LTA_4$ into very low to nondetectable amounts of $\Delta^6$-*trans*-$\Delta^8$-*cis*-$LTB_4$. Although the specific epoxide hydrolase activity (measured as the 5*S*,6*S*-DHETE production) was lower as compared to the wild-type protein, the formation of $LTB_4$ and thus the ratio $LTB_4$/5*S*,6*S*-DHETE were increased (Table 2B). In addition, the Michaelis constant was reduced, leading to a greater than 2-fold increase in the specificity constant ($k_{cat}/K_m$), which in turn indicates that the active site had become better adapted for binding and turnover of $LTA_4$ into $LTB_4$. Furthermore, the epoxide hydrolase activity was more sensitive to inactivation by $LTA_4$. Thus, after $LTA_4$ treatment, approximately 90% of the activity was lost. Taken together, these data indicate that during evolution, a Phe residue (Phe-424) at the active site has been exchanged for a Tyr, resulting in an enzyme that binds $LTA_4$ more tightly and converts this substrate into $LTB_4$ with an improved efficiency and specificity at the expense of 5*S*,6*S*-DHETE and $\Delta^6$-*trans*-$\Delta^8$-*cis*-$LTB_4$. At the same time, the enzyme is penalized by a catalytic restraint imposed by higher susceptibility to inactivation by $LTA_4$. Hence, mutation of Phe-424 into a Tyr residue appears to mimic a distinct step in the molecular evolution of *sc*LTA4H into its mammalian counterparts.

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