Cloning and Characterization of a Bifunctional Leukotriene A₄ Hydrolase from Saccharomyces cerevisiae*

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In mammals, leukotriene A₄ hydrolase is a bifunctional zinc metalloenzyme that catalyzes hydrolysis of leukotriene A₄ into the proinflammatory leukotriene B₄ and also possesses an arginyl aminopeptidase activity. We have cloned, expressed, and characterized a protein from Saccharomyces cerevisiae that is 42% identical to human leukotriene A₄ hydrolase. The purified protein is an anion-activated leucyl aminopeptidase, as assessed by p-nitroanilide substrates, and does not hydrolyze leukotriene A₄ into detectable amounts of leukotriene B₄. However, the S. cerevisiae enzyme can utilize leukotriene A₄ as substrate to produce a compound identified as 5S,6S-dihydroxy-7,9-trans-11,14-cis-eicosatetraenoic acid. Both catalytic activities are inhibited by 3-(4-benzyloxyphenyl)-2-((R)-amino-1-propanethiol (thioamine), a competitive inhibitor of human leukotriene A₄ hydrolase. Furthermore, the peptide cleaving activity of the S. cerevisiae enzyme was stimulated approximately 10-fold by leukotriene A₄ with kinetics indicating the presence of a lipid binding site. Nonenzymatic hydrolysis products of leukotriene A₄, leukotriene B₄, arachidonic acid, or phosphatidylcholine were without effect. Moreover, leukotriene A₄ could displace the inhibitor thioamine and restore maximal aminopeptidase activity, indicating that the leukotriene A₄ binding site is located at the active center of the enzyme. Hence, the S. cerevisiae leukotriene A₄ hydrolase is a bifunctional enzyme and appears to be an early ancestor to mammalian leukotriene A₄ hydrolases.

Leukotriene A₄ hydrolase catalyzes the hydrolysis of 5S-trans-5,6-epoxy-7,9-trans-11,14-cis-eicosatetraenoic acid (LTA₄), into the proinflammatory mediator 5S,12R-dihydroxy-6,14-cis,8,10-trans-eicosatetraenoic acid (LTB₄), which is a potent chemotaxin and leukocyte activating agent (1, 2). LTA₄ hydrolase has a wide tissue distribution and has been purified from several mammalian sources as a soluble monomeric enzyme with a molecular mass of about 69 kDa. During catalysis, LTA₄ hydrolase is suicide inactivated through covalent binding of LTA₄ to the active site residue Tyr-378, a process that may be of importance for the overall regulation of LTB₄ biosynthesis (3–5).

The mammalian LTA₄ hydrolase is a metalloenzyme containing 1 mol of zinc per mol of protein. In addition to the epoxide hydrolase activity, i.e., the hydrolysis of LTA₄ into LTB₄, the enzyme also possesses an anion-dependent arginylaminopeptidase activity, the physiological role of which is presently unknown (6–9). The zinc atom is required for both catalytic activities and is bound to His-295, His-299, and Glu-318 (10). Because of its zinc binding motif and aminopeptidase activity, LTA₄ hydrolase is homologous to a multitude of other zinc peptidases present in a variety of species spanning from mammals to bacteria, in particular those belonging to the M1 family (11). On the other hand, the epoxide hydrolase activity, i.e., the production of LTB₄, has only been detected in vertebrates, including birds, fish, and frogs (12–15), and a nonmammalian form of LTA₄ hydrolase was recently purified from the African claw toad, Xenopus laevis. The toad enzyme contained zinc and exhibited both epoxide hydrolase and peptidase activity (16). In fact, formation of LTB₄ has never been convincingly demonstrated in any lower animal species, bacteria, or plants. Thus, an aminopeptidase-1 was recently cloned and characterized from Caenorhabditis elegans, that was 45% identical (63% similar) to the amino acid level to mammalian LTA₄ hydrolase (17) and exhibited an arginylaminopeptidase activity (Fig. 1). Despite this high level of sequence identity, the C. elegans enzyme failed to hydrolyze LTA₄ into LTB₄, and no other functional link to LTA₄ hydrolase was reported. Apparently, very little is known about the evolution of LTA₄ hydrolase and the phylogenetic relationship between its two catalytic activities.

In the course of sequencing the genome of Saccharomyces cerevisiae, an open reading frame was identified as an LTA₄ hydrolase homologue, with 42% identity (53% similarity) to human LTA₄ hydrolase (18). In the present study, we have cloned, expressed, and characterized the corresponding gene product (Fig. 1). We show that it is a bifunctional enzyme possessing an anion-activated leucyl aminopeptidase activity as well as an epoxide hydrolase activity toward LTA₄. Moreover, the aminopeptidase activity is strongly stimulated by LTA₄ in a fashion suggesting the presence of a lipid binding pocket located at the active center of the enzyme and presumably overlapping with the catalytic site(s). Hence, the homologue in S. cerevisiae appears to be an early ancestral gene to the vertebrate forms of LTA₄ hydrolase.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases, T4 DNA ligase, and T7 sequencing kit were purchased from Amersham Pharmacia Biotech.
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((2S,3R)-3-Amino-2-hydroxy-4-phenylbutanoyl)-1-leucine (bestatin), Ala-, Arg-, Glu-, Gly-, Leu-, Met-, Pro-, and Val-p-nitroanilide (p-NA) were from Sigma. Esterified LTA₄ was saponified in tetrahydrofuran with 1 M LiOH (6%, v/v) for 48 h at 4°C. The LTA₄ hydrolase inhibitors 3-(4-benzyloxyphenyl)-2-iodo-1-propanethiol (thioamine) and N-hydroxy-3-(4-benzyloxyphenyl)benzotriazolene (benzanilide) were synthesized as described (19, 20).

Isolation of RNA from S. cerevisiae—Cells from S. cerevisiae were grown in 10 ml of yeast medium (0.1 g of yeast extract, 0.1 g Bactopeptone, 0.2 g of glucose, and 0.2 mg of adenine) at 30°C to mid-exponential phase (A₅₅₀ = 1), pelleted by centrifugation for 3 min at 1,500 g. The cells were resuspended at 50 mg/mL in water for an additional 10 min. The centrifugation was repeated at 14,000 × g. Total RNA was isolated according to the hot acid phenol method (21). The RNA was precipitated with ethanol, dissolved in RNase-free water, and stored at −80°C until use.

Cloning of the S. cerevisiae LTA₄ Hydrolase—Reverse transcription of total yeast RNA (2 μg/20 μl of reaction mix) was performed using the Perkin-Elmer Genescript mRNA purification kit (Perkin-Elmer Cetus, Norwalk, CT). The expression vector pT3-yLTAh was kindly provided by Dr. W. H. F. Haas, Wageningen, The Netherlands. The cDNA was removed from pT3-yLTAh by cleavage with SaII and SpII restriction enzyme sites: FK22, covering the start codon (5’ GTG TTC GTC GAC GAT TTC GTC GCA ACG TAA ATC, SaII site underlined) and FK21 over the stop codon (5’ 9 GTG GCC TTT TCA AAG ACC TAA ATC, SpII site underlined). Using the phosphorylated PCR product, an enzyme was ligated into the pBluescript II KS(-) vector (Stratagene) as primers. A PCR was performed to amplify the S. cerevisiae LTA₄ hydrolase gene using the Expand™ high fidelity PCR system (Roche Molecular Biochemicals). A 30-μl reaction contained 1 × reaction buffer, 200 μM dNTP, 1 unit of Taq and Pwo DNA polymerase mix, and 2 μl cDNA. The sample was first denatured for 2 min at 95°C followed by 35 cycles composed of denaturation for 30 s at 94°C, annealing for 30 s at 50°C, and elongation for 2 min at 72°C. The PCR was terminated by an elongation at 72°C for 7 min. The PCR product, representing the entire coding sequence of the S. cerevisiae LTA₄ hydrolase, was digested with SaII and SpII, purified by low melting agarose (0.7%) electrophoresis, and cloned into an expression vector.

Expression of S. cerevisiae LTA₄ Hydrolase in Escherichia coli—For expression in E. coli, the plasmid pT3–12LO was used (22). The 12-lox/yoxyzen insert was removed by cleavage with SaII and SpII, and the opened vector was purified by agarose gel electrophoresis, and 2 μl cDNA. The sample was first denatured for 2 min at 95°C followed by 35 cycles composed of denaturation for 30 s at 94°C, annealing for 30 s at 50°C, and elongation for 2 min at 72°C. The PCR product was digested by an elongation at 72°C for 7 min. The PCR product, representing the entire coding sequence of the S. cerevisiae LTA₄ hydrolase, was digested with SaII and SpII, purified by low melting agarose (0.7%) electrophoresis, and cloned into an expression vector.

Expression of S. cerevisiae LTA₄ Hydrolase in Spodoptera frugiperda (Sf9) cells—Expression of the S. cerevisiae LTA₄ hydrolase gene was performed using the Bac-to-Bac® baculovirus expression system (Life Technologies, Inc.). The cDNA was removed from pT3–yLTAh by cleavage with SaII and SpII, purified by agarose gel electrophoresis, and ligated into the recombinant donor plasmid pFastBac1 opened with SphI and SalI and ligated into the Sf9 baculovirus insertion site using the Sf9/I and Sf9/SphI restriction enzyme sites. The preparation of the bacmid DNA, transfection of Sf9 cells, and selection of transfectants were performed according to the method of Briggs, using the P3-tet-LytA, which was transformed into competent DH10Bac cells (Life Technologies, Inc.) containing the bacmid plasmid pT3–12LO. The expression vector pT3–12LO was used (22). The 12-lox/yoxyzen insert was removed by cleavage with SaII and SpII, and the opened vector was purified by agarose gel electrophoresis, and 2 μl cDNA. The sample was first denatured for 2 min at 95°C followed by 35 cycles composed of denaturation for 30 s at 94°C, annealing for 30 s at 50°C, and elongation for 2 min at 72°C. The PCR product was digested by an elongation at 72°C for 7 min. The PCR product, representing the entire coding sequence of the S. cerevisiae LTA₄ hydrolase, was digested with SaII and SpII, purified by low melting agarose (0.7%) electrophoresis, and cloned into an expression vector.

RESULTS

Cloning, Expression, and Purification of S. cerevisiae LTA₄ Hydrolase—The LTA₄ hydrolase homologue was cloned by PCR from reverse transcribed total RNA isolated from S. cerevisiae (w303-1b), using two primers raised against the genomic sequence. The cDNA (2016 bp) was initially expressed in E. coli JM101 cells. The protein was purified by affinity chromatography using anion exchange, hydroxyapatite, hydrophobic interaction, and chromatofocusing resins. The level of expression was very low, and from 6 liters of medium, only 50 μg of protein was recovered. However, the identity of the isolated protein could be established by N-terminal amino acid sequencing, which...
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FIG. 1. A multiple alignment of amino acid sequences of the human LTA₄ hydrolase, the S. cerevisiae LTA₄ hydrolase, and the C. elegans API. The alignment was made using the GAP alignment program in the GCG package (30). The zinc binding ligands are indicated by shaded boxes. Amino acids that have been shown to be important for the peptidase activity of human LTA₄ hydrolase, i.e. Glu-296 and Tyr-383, are indicated by open boxes (31, 32). Tyr-378, the residue in human LTA₄ hydrolase to which LTA₄ binds covalently during suicide inactivation, is indicated by an asterisk and is replaced by a phenylalanine in both S. cerevisiae LTA₄ hydrolase (Phe-424) and C. elegans API (Phe-382). I and IV designate the N termini of variants I and IV, respectively. The E. coli protein has the same N terminus as variant IV.

To increase the yield of recombinant protein, the S. cerevisiae LTA₄ hydrolase was also expressed in Sf9 cells, using the baculovirus system, and was recovered in the cell pellet rather than in the medium. The protein was purified to homogeneity using the same procedure as described above, and the molecular mass was determined to 72 kDa by SDS-PAGE (Fig. 2). Typically, from 450 ml of infected Sf9 cell culture, 500 µg of purified enzyme was recovered, and unless otherwise stated, this preparation was used for the enzyme characterization.

Using Leu-p-NA as the substrate, the mean value of Vₘₐₓ for all three variants was calculated to 520 ± 110 nmol/mg/min (mean ± S.D.; n = 6), and the value for Kₘ was determined to 1.5 ± 0.4 mM (n = 6) (Table I). The values of Vₘₐₓ using Met- and Ala-p-NA as substrates, were calculated to 380 ± 110 (n = 3) and 240 ± 170 nmol/mg/min (n = 3), respectively, whereas the corresponding values of Kₘ were determined to be 1.8 ± 0.45 (n = 3) and 2.0 ± 1.0 mM (n = 3), respectively. As judged by values of kₐₚ/Kₘ, Leu-p-NA was the best substrate (420 ± 100 s⁻¹ M⁻¹; n = 6) followed by Met-p-NA (230 ± 10 s⁻¹ M⁻¹; n = 3) and Ala-p-NA (130 ± 30 s⁻¹ M⁻¹; n = 3). In all subsequent experiments, Leu-p-NA was used as the standard substrate. To allow a direct comparison, recombinant human LTA₄ hydrolase (0.7 µg) was assayed in a parallel experiment with Leu-p-NA, and the kinetic constants Vₘₐₓ and Kₘ were calculated to 310 nmol/mg/min and 0.15 mM, respectively (Table I).

FIG. 2. SDS-PAGE of purified yeast LTA₄ hydrolase. After the final step of purification, protein samples (0.3–0.9 µg) collected under each of the four peaks (I–IV) obtained in chromatofocusing chromatography were subjected to SDS-PAGE (Phast system, 10–15% gradient gel) and Coomassie staining. Molecular mass markers were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), a-lactalbumin (14.4 kDa). The pH optimum for the aminopeptidase activity of the S. cerevisiae LTA₄ hydrolase (Phe-424) and C. elegans API (Phe-382) was 7.0 (data not shown). Furthermore, the enzyme was dose-dependently stimulated by monovalent anions. As described previously for LTA₄ hydrolase, thiocyanate was a very potent activator of all three enzymes.

KINETIC CONSTANTS—Apparent kinetic constants were determined using Leu- and Ala-p-NA as substrates in 50 mM Tris-Cl and phosphate buffer containing 100 mM KCl, pH 7.5, containing 100 mM KCl and did not differ significantly between the three variants of the protein.

Effects of pH and Anions—The pH optimum for the aminopeptidase activity of the S. cerevisiae LTA₄ hydrolase (1 µg of enzyme, 1 mM Leu-p-NA) in both Tris-Cl and phosphate buffer containing 100 mM KCl was determined to 7.3 (data not shown). Furthermore, the enzyme was dose-dependently stimulated by monovalent anions. As described previously for LTA₄ hydrolase, thiocyanate was a very potent activator of all three enzymes.
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The enzymes (0.7 μg) were incubated with 0.125–8 μM Leu-p-NA in 250 μl of 50 mM phosphate buffer, pH 7.5, containing 100 mM KCl. For LTA₄ activation, the S. cerevisiae LTA₄ hydrolase was pretreated with LTA₄ (70 μM) for 20 min before adding the substrate. Incubations with recombinant human LTA₄ hydrolase were performed in parallel. Data are mean values of 2–6 samples.

| Species          | Kₘ (μM) | Vₘₐₓ (nmol/mg/min) | kₘₐₓ (s⁻¹) | kₗₐₜ/kₘₐₓ (s⁻¹ μmol⁻¹) |
|------------------|---------|--------------------|-------------|-------------------------|
| S. cerevisiae LTA₄ hydrolase | 1.5     | 520                | 0.63        | 430                     |
| S. cerevisiae LTA₄ hydrolase stimulated with LTA₄ | 2.1     | 4400               | 5.2         | 2400                    |
| Human LTA₄ hydrolase | 0.15    | 310                | 0.35        | 2400                    |

**Effects of Inhibitors**—Three competitive inhibitors of LTA₄ hydrolase, i.e. the general aminopeptidase inhibitor bestatin and two specific LTA₄ hydrolase inhibitors, a thioamine and a hydroxamic acid, were tested for their inhibitory effect on the S. cerevisiae LTA₄ hydrolase. The inhibitors were tested at concentrations ranging between 0.01 and 100 μM, using Leu-p-NA as substrate. For all three variants of the protein, the thioamine was shown to be the most potent inhibitor with an IC₅₀ of 0.06 ± 0.01 μM (mean ± S.D.; n = 4). The hydroxamic acid was the second best inhibitor, with an IC₅₀ of 0.24 ± 0.09 μM (n = 4), whereas bestatin was a relatively poor inhibitor of this protein, with an IC₅₀ of 3.2 ± 0.6 μM (n = 3). Parallel experiments were performed with recombinant human LTA₄ hydrolase, using Leu-p-NA as substrate, which showed a similar pattern of inhibition with IC₅₀ values for the three inhibitors of 0.02, 0.03, and 0.5 μM for the thioamine, the hydroxamic acid, and bestatin, respectively.

The mode of inhibition for bestatin and the thioamine was determined from Eadie-Hofstee plots of kinetic data obtained with untreated and inhibited enzyme. For both compounds, a mixed type of inhibition was observed, suggesting that the inhibitors bind at the active site but can not be fully displaced by the substrate (Fig. 3).

**Epoxyde Hydrolase Activity**—The S. cerevisiae LTA₄ hydrolase was assayed for its ability to convert LTA₄ into LTB₄. Aliquots of a pool of all three protein variants (2–10 μg) were incubated in 100 μl of 10 mM Tris·Cl, pH 8, with 30 μM LTA₄ for 60 s at room temperature. Under these experimental conditions, production of LTB₄ could not be detected in any of the incubations with the S. cerevisiae protein. Similar incubations were performed with the purified recombinant protein from E. coli, as well as crude yeast cell extracts, none of which generated detectable amounts of LTB₄.

Further analysis of the product profile revealed that the S. cerevisiae LTA₄ hydrolase converted LTA₄ into a less polar compound eluting late in the reverse phase HPLC chromatogram (Fig. 4). Comparison with synthetic standards showed that this peak coeluted with 5S,6S-dihydroxy-7,9-trans,11,14-cis-eicosatetraenoic acid (5S,6S-DHETE). Furthermore, the material under this peak exhibited a UV spectrum typical of a conjugated triene moiety, with λₘₐₓ at 274 nm, in agreement with previously published data for 5S,6S-DHETE (26). By peak area measurements, the specific activity of this activity was calculated to 20 ± 2.1 nmol/mg/min (mean ± S.D.; n = 6) using prostaglandin B₁ as the internal standard. Heating the S. cerevisiae LTA₄ hydrolase at 90 °C for 5 min completely abolished the formation of 5S,6S-DHETE demonstrating the enzymatic nature of its formation. Moreover, preincubation of the enzyme with the competitive inhibitor thioamine (30 μM) also abolished this enzyme activity (Fig. 4), suggesting that epoxide hydrolysis occurs at a site close to or identical to the aminopeptidase active site.

**Effects of LTA₄ on the Peptidase Activity**—To test whether the peptidase activity was inactivated by LTA₄, as is the case with all LTA₄ hydrolases described thus far (27), the S. cerevisiae protein (0.7 μg) was incubated with LTA₄ added three times at 10- or 20-min intervals, to a final concentration of 80 μM. Subsequent measurement of the enzyme activity, using 1 mM Leu-p-NA as substrate, revealed that LTA₄ stimulated the specific peptidase activity from 150 to 1800 nmol/mg/min, corresponding to an 11-fold stimulation. When Ala-, Arg-, Pro-, and Met-p-NA were tested, Ala-p-NA was the only other substrate that was hydrolyzed with increased efficiency. Thus, the specific activity was increased from 120 to 500 nmol/mg/min, corresponding to a 4-fold stimulation.

The stimulatory effect was not reversible, and 70% of the initial effect was still present in a sample stored for a month in the refrigerator. Furthermore, stimulated enzyme could be chromatographed on a Mono-Q column without significant loss of activity, indicating a tight binding between lipid and protein (data not shown).

Further kinetic experiments showed that the stimulation was both time- and dose-dependent (Fig. 5). Hence, addition of 30 μM LTA₄ to a standard incubation of enzyme and substrate (1 μg of protein, 1 mM Leu-p-NA) caused a rapid increase in reaction velocity over the first 5 min of incubation and then gradually leveled off during the following 15 min (Fig. 5A). When the S. cerevisiae enzyme was preincubated with increasing concentrations of LTA₄, the peptidase activity toward Leu-p-NA and Ala-p-NA was stimulated in a dose-dependent fash-
ion, reaching a maximum at about 80 and 40 μM, respectively (Fig. 5B). Moreover, the stimulatory effect appeared to obey saturation kinetics, and when the data were plotted according to the Eadie-Hofstee method, linear relationships (r² = 0.83 and 0.94) were observed. From the slope of the lines, apparent affinity constants (Kₐ) were calculated to 19 and 6 μM for Leu- and Ala-p-NA, respectively. From the same plots, maximal catalytic efficiencies (at 1 mM substrate) were determined for Leu-p-NA as substrate in 50 mM phosphate buffer, pH 7.5, containing 100 mM KCl. Thus, values of both Kₐ and Vₐₐₐₐmax had increased to 2.1 mM and 4400 nmol/min/mg, respectively. Consequently, the value of kcat/Kₐ increased from 430 to 2400 s⁻¹ M⁻¹ (Table I).

To study the specificity of the stimulatory effect, the S. cerevisiae enzyme (0.7 μg) was incubated with LTA₄ (70 μM), arachidonic acid (60 μM), or phosphatidylethanolamine (80 μg/ml) prior to addition of the substrate, Leu-p-NA. None of these lipids had any significant stimulatory effect on the aminopeptidase activity. Furthermore, incubations were performed in which LTA₄ was allowed to undergo spontaneous hydrolysis before addition of the enzyme. Under these conditions, no activation of the peptidolysis could be observed, indicating that the nonenzymatic hydrolysis products of LTA₄, primarily 6-trans-LTB₄ and 12-epi-6-trans-LTB₄, are without effect and that the intact epoxide moiety of LTA₄ is necessary for the stimulation. This result also shows that other minor components of the substrate mix, e.g. small amounts of LiOH, have no stimulatory effect. For comparison, similar experiments were performed in parallel with recombinant human LTA₄ hydrolase, which, as expected, was inhibited (65–70%) by 80 μM LTA₄.

Competition between LTA₄ and Inhibitors—The S. cerevisiae protein was preincubated with 0.06 μM thioamine or 4 μM bestatin, corresponding to 60% inhibition, and then stimulated with increasing doses of LTA₄. Again, the aminopeptidase activity was stimulated in a saturable manner, and from a linear

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**Fig. 4.** Reverse-phase HPLC profiles of products formed from LTA₄ by S. cerevisiae LTA₄ hydrolase in the absence or presence of the inhibitor thioamine. The top trace represents the products obtained when S. cerevisiae LTA₄ hydrolase (8 μg) was incubated with LTA₄ (30 μM) for 60 s at room temperature in 10 mM Tris-Cl, pH 7.5. Extractions and HPLC analysis was performed as described under “Experimental Procedures.” Arrows and roman numerals indicate the retention time of the internal standard prostaglandin B₁, 6-trans-LTB₄ (I), 12-epi-6-trans-LTB₄ (II), LTB₄ (III), 5S,6R-DHETE (IV), and 5S,6S-DHETE (V). The inset shows the UV spectrum of material eluting under peak V, which is typical for a conjugated triene and has a λmax at 274 nm. The bottom trace shows the profile of products formed by S. cerevisiae LTA₄ hydrolase (8 μg) incubated in the presence of 30 μM thioamine.

**Fig. 5.** A, time course for the stimulatory effect of LTA₄ on the aminopeptidase activity of the S. cerevisiae LTA₄ hydrolase. The enzyme (1 μg) was incubated with 1 mM Leu-p-NA in 250 μl of 25 mM Hepes buffer, pH 7.5, containing 100 mM KCl. The reaction velocity was monitored by measurements of A405 at the indicated time points. After 20 min, LTA₄ (30 μM) was added to the incubate, and A405 was assessed after 30 s, 1 min, 5 min, and 10 min. Filled circles indicate the reaction velocity of enzyme treated with LTA₄, and open circles indicate the velocity of the untreated control enzyme. Each data point represents the mean of duplicate samples. B, dose-response curve for LTA₄ activation of the aminopeptidase activity of S. cerevisiae LTA₄ hydrolase. S. cerevisiae LTA₄ hydrolase (0.7 μg) was pretreated with LTA₄ at different doses (10–160 μM) for 15 min on ice. The aminopeptidase activity was assayed using 1 mM Leu-p-NA (●) or Ala-p-NA (▲) dissolved in 50 mM Tris-Cl, pH 7.5, containing 100 mM KCl. Each data point represents the mean of duplicate samples.
LTA₄, the potency of the inhibitor was drastically reduced (approximately 500 times) with an IC₅₀ of 25 μM (results not shown), in agreement with a tight binding between LTA₄ and the protein.

DISCUSSION

LTA₄ hydrolase has been characterized from several mammals, but little, if anything, is known about its evolution and properties in lower animal species. The enzyme is distantly related to many bacterial and yeast proteases and aminopeptidases, e.g. aminopeptidase N and thermolysin, by virtue of its zinc binding site and aminopeptidase activity. Hence, LTA₄ hydrolase has been classified as a member of the M1 family of metalloproteases (11). However, the degree of identity or similarity at the amino acid level between LTA₄ hydrolase and other members of this family is usually low and confined to the zinc binding region. Using bioinformatics, several proteins with higher degree of homology have been identified. Thus, a protein with 45% identity (63% similarity) to LTA₄ hydrolase was found in C. elegans (17). However, expression and characterization of this gene revealed that it was yet another aminopeptidase without functional links to LTA₄ hydrolase.

In the present work, we have cloned and characterized another homologue of LTA₄ hydrolase present in S. cerevisiae, which was discovered serendipitously via the sequencing of the yeast genome (18). The S. cerevisiae enzyme was initially expressed in E. coli, but the recovery of recombinant protein was very poor. Edman degradation showed that it lacked the initial 40 amino acids at the N terminus, suggesting that translation is initiated at the second methionine (Met-40) of the open reading frame or that the protein undergoes N-terminal proteolytic processing. To get a higher yield of protein we turned to expression in a baculovirus insect cell (Sf9) system that generated four different products in the final step of chromatofocusing chromatography. The molecular masses of these four protein species were indistinguishable by SDS-PAGE (Fig. 2), and all exhibited similar catalytic properties. However, upon Edman degradation, two of the variants differed in their N termini, again suggesting different translational initiation sites or N-terminal proteolytic processing (Fig. 1). The S. cerevisiae protein is larger than the human and C. elegans proteins. It contains 672 amino acids and has an N-terminal extension of about 50 residues. In view of the sequencing data, it is tempting to speculate that a segment of the N terminus is not present in the mature S. cerevisiae protein.

The S. cerevisiae LTA₄ Hydrolase Is an Anion-stimulated Leucyl Aminopeptidase—The S. cerevisiae protein is an aminopeptidase, as assessed by enzyme activity determinations with synthetic chromogenic substrates of recombinant proteins from both E. coli and Sf9 cells. The best substrate was Leu-p-NA, a substrate specificity different from that of human LTA₄ hydrolase, which prefers Arg- or Ala-p-NA (28). As previously reported for LTA₄ hydrolase, the aminopeptidase activity of the S. cerevisiae homologue was greatly stimulated by thiocyanate and chloride in an allosteric fashion, with apparent affinity constants of approximately 28 and 120 mM, respectively. These data are in good agreement with the corresponding values for human LTA₄ hydrolase.

The S. cerevisiae LTA₄ Hydrolase Can Convert LTA₄ into 5S,6S-DHETE—The S. cerevisiae enzyme was tested for LTA₄ hydrolase activity. Under our experimental conditions (10 μg of enzyme, 30 μM LTA₄), we failed to observe any significant conversion of LTA₄ into LT₄. Interestingly, the S. cerevisiae enzyme was capable of hydrolyzing LTA₄ into another product, identified as 5S,6S-DHETE by its chromatographic mobility in reverse-phase HPLC and UV spectrometry (Fig. 4). Notably, enzymatic formation of this particular metabolite of LTA₄ has
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Previously only been described for a mutated form of LTA₄ hydrolase (26). Thus, a point mutation of Tyr-383 in human LTA₄ hydrolase generates a recombinant enzyme capable of converting LTA₄ into large amounts of 5S,6S-DHETE. In the S. cerevisiae protein, the corresponding amino acid is a tyrosine (Tyr-429) (Fig. 1). Nevertheless, this epoxide hydrolase activity and ability to turn over LTA₄ into 5S,6S-DHETE represents a functional link between the evolutionary distant S. cerevisiae enzyme and mammalian LTA₄ hydrolases.

LTA₄ Stimulates the Aminopeptidase Activity of S. cerevisiae LTA₄ Hydrolase—We also wanted to see whether the yeast homologue could be inhibited and covalently modified by LTA₄, as is the case with all LTA₄ hydrolases described thus far. To our surprise, we found that the aminopeptidase activity was time- and dose-dependently stimulated by LTA₄ in a fashion suggesting the presence of a lipid binding site. An apparent Kₐ for LTA₄ was calculated as 18 and 6 μM using Leu- and Ala-β-NA, respectively. The stimulatory effect was specific for LTA₄, because LTB₄, nonenzymatic hydrolysis products of LTA₄, arachidonic acid, or phosphatidylcholine had no effect. Several lines of evidence indicated that LTA₄ binds tightly to the S. cerevisiae protein, although we could not demonstrate covalent bond formation. In this context, it is interesting to note that during suicide inactivation, LTA₄ binds covalently to the phenolic hydroxyl group of Tyr-378 in the mammalian LTA₄ hydrolase (29), which in the S. cerevisiae homologue corresponds to Phe-424 (Fig. 1).

LTA₄ Stimulation Occurs via a Lipid Binding Pocket Located at the Active Center of the Enzyme—Further kinetic studies with active site-directed inhibitors allowed us to locate the LTA₄ binding site. Thus, stimulation with LTA₄ competed with bestatin and the thioamine (Fig. 6A). In the case of bestatin, the competition with LTA₄ was partial, whereas for the thioamine it was complete. This discrepancy between the two inhibitors agrees well with their proposed binding modes to the active site of LTA₄ hydrolase (Fig. 6B). Thus, bestatin is a derivative of leucine and is expected to occupy the peptide binding site, whereas the thioamine carries a bicyclic hydrophobic structure believed to mimic the fatty acid backbone of LTA₄. Furthermore, the K₅₅ values for LTA₄ were substrate-dependent and differed between Leu- and Ala-β-NA, suggesting some competition or interaction between the activator LTA₄ and the aminopeptidase substrates at the active site (Fig. 5B). In addition, the site for LTA₄ stimulation could be linked to the site for epoxide hydrolysis because the conversion of LTA₄ into 5S,6S-DHETE could be blocked with the thioamine inhibitor (Fig. 4), which in turn could be displaced by LTA₄. Hence, the binding site for LTA₄ appears to be located at the active center of the enzyme, presumably overlapping with the site(s) responsible for peptide and epoxide hydrolysis. A model for the active center of the S. cerevisiae LTA₄ hydrolase and its relation to the active center of the mammalian LTA₄ hydrolase is depicted in Fig. 7. To the best of our knowledge, leukotriene biosynthesis and formation of LTA₄ has never been described in yeast. Therefore, it appears likely that the LTA₄ binding site and/or the site for epoxide hydrolysis in the S. cerevisiae enzyme accommodates some other lipid substrate structurally related to LTA₄. Nevertheless, the S. cerevisiae homologue appears to be an early ancestral gene to LTA₄ hydrolase, thus sharing its unique ability to combine lipid and peptide metabolism in a single protein.

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