# Determination of the Kinetic Parameters of *Escherichia coli* Leader Peptidase Activity Using a Continuous Assay: The pH Dependence and Time-Dependent Inhibition by β-Lactams Are Consistent with a Novel Serine Protease Mechanism

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ABSTRACT: Bacterial leader peptidase (LPase) is a potential target for the development of novel anti-infective agents, but to data only peptides based upon natural macromolecular substrates have been reported as inhibitors. In this work is described a continuous assay for *Escherichia coli* LPase activity, based upon Ac–WSASALAKI–AMC (**I**) as the substrate, that can be monitored either spectrophotometrically or spectrofluorometrically. The LPase reaction is coupled to the liberation of AMC (aminomethylcoumarin) via a nonspecific leucine aminopeptidase. LPase and a short form of the enzyme (LPase-sf) lacking the membrane spanning domains displayed saturable kinetics toward **I**. The second-order rate constants were approximately $2 \times 10^5$ $M^{-1}$ $h^{-1}$ at pH 7.5 and were comparable to those reported in the literature for peptide substrates based upon natural cleavage sites in preproteins. LPase was inhibited by β-lactams. [*S*-(*R**,*S**)]-4-[(1-(((1-(5-toluoyl)butyl)amino)carbonyl)-3,3-dimethyl-4-oxo-2-azetidinyl)oxy]benzoic acid (L-684,-248, 588 μM) inhibited the LPase-catalyzed hydrolysis of 50 μM **I** and 125 μM Ac–WLVP–Nleu–LSFAAEGDDPA–$NH_2$ by 30% and 88% over 1 and 4 h, respectively. The inhibition of LPase by L-684,248 and its C-4 diasteromer was time dependent and yielded second-order rate constants ($k_{inact}/K_i$) of 12 and 7.7 $M^{-1}$ $min^{-1}$, respectively. The process was structurally specific as the C-3 diethyl substituted β-lactam (C-4 *S*-isomer) was inactive. The latter data correlate with the LPase preference for alanine at the $P_1$ position of peptide substrates [Kuo et al. (1993) *Arch. Biochem. Biophys. 303,* 274–280]. The reaction of LPase with **I** was essentially pH independent from pH 5 to 9. This observation suggests that the enzyme does not require a catalytic histidine or lysine to act as a general base. These data and the previous reports that β-lactams are serine protease inhibitors suggest that bacterial LPase is a member of a new family of serine proteases that lacks a complete catalytic triad.

*Escherichia coli* leader peptidase (LPase),[1] an integral membrane protein, catalyzes the removal of the signal or leader sequence as one of the last steps of translocation of proteins across membranes [for reviews of this process see Randall et al. (1987) and Saier et al. (1989)]. The transport process is similar in both prokaryotes and eukaryotes, although in the latter the signal peptide is removed on the endoplasmic reticulum during translocation into the ER lumen (Blobel & Doberstein, 1975; Jackson et al., 1977; Sabatini et al., 1982; Lively & Walsh, 1983; Mollay et al., 1982; Fujimoto et al., 1984). While much of the work on LPases has been conducted with the *E. coli* enzyme, similar enzymes are found in both Gram-negative and Gram-positive bacteria as well as yeast. Date (1983) demonstrated that LPase is an essential enzyme in *E. coli*. Therefore, LPase has potential as a novel target for therapeutic intervention in infectious diseases. The only inhibitor of LPase activity reported to date was the 23 amino acid residue signal sequence of M13 procoat (MKKSLV-LKASVAVATLVPM–LSFA),[2] which inhibited the cleavage of both procoat and preMBP[3] *in vitro* (Wickner et al., 1987). Recently, Kuo et al. (1993) confirmed this observation with peptide substrates *in vitro*. The *Salmonella typhimurium* LPase (van Dijl et al., 1990) displays 94% homology to the

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[1] In this work LPase refers to wild-type leader peptidase as isolated and, therefore, contains fragments generated by autocatalytic breakdown (Kuo et al., 1993). LPase-sf refers to the Δ-2-75 cloned short form reported by Kuo et al. (1993).

[2] Peptide-based substrates and inhibitors are abbreviated using the standard one-letter representation of the amino acids. Additional functional groups are abbreviated as follows: Ac-, *N*-acetyl; AMC, 7-amido-4-methylcoumarin; BOC, *tert*-butyloxycarbonyl; FMOC, 9-fluoroenylmethyloxycarbonyl; $NH_2$, C-terminal amide; Nleu, norleucine; tBu, *tert*-butyl.

[3] Abbreviations: APMSF, aminophenylmethanesulfonyl fluoride; DCC, dicyclohexylcarbodiimide; DMAP, (dimethylamino)pyridine; EDTA, ethylenediaminetetraacetic acid; ESI-MS, liquid chromatography electrospray ionization mass spectrometry; HPLC, high-pressure liquid chromatography; HOBT, 1-hydroxybenzotriazole; LAP, leucine aminopeptidase; L-680,831, [*S*-(*R**,*S**)]-4-[(1-(((1-(4-methylphenyl)butyl)-amino)carbonyl)-3,3-diethyl-4-oxo-2-azetidinyl)oxy]benzoic acid; L-680,-833, [*S*-(*R**,*S**)]-4-[(1-(((1-(4-methylphenyl)butyl)amino)carbonyl)-3,3-diethyl-4-oxo-2-azetidinyl)oxy]benzeneacetic acid; L-684,248, [*S*-(*R**,*S**)]-4-[(1-(((1-(4-methylphenyl)butyl)amino)carbonyl)-3,3-dimethyl-4-oxo-2-azetidinyl)oxy]benzoic acid; L-684,249, [*R*-(*R**,*S**)]-4-[(1-(((1-(4-methylphenyl)butyl)amino)carbonyl)-3,3-dimethyl-4-oxo-2-azetidinyl)oxy]benzoic acid; MES, 2-[*N*-morpholino]ethanesulfonic acid; MBP, maltose binding protein; OMPA, outer membrane protein A; PAGE, polyacrylamide gel electrophoresis; PB-74, Pharmacia chromatofocusing polybuffer 74; PIPES, piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]; PMSF, phenylmethanesulfonyl fluoride; PPE, porcine pancreatic elastase; proOMPA, outer membrane protein A precusor; preMBP, maltose binding protein precursor; preRBP, ribose binding protein precurser; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TAPS, *N*-tris-[hydroxymethyl]methyl-3-aminopropanesulfonic acid; TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; TFA, trifluoroacetic acid; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; ZPCK, *N*-carbobenzoxy-L-phenylalanine chloromethyl ketone.

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*E. coli* enzyme, and even *Pseudomonas fluorescens* LPase (Black et al., 1992) retains 50% homology. These data suggest that the enzyme from *E. coli* is an appropriate model for the LPases derived from more therapeutically relevant infectious agents. While the bacterial LPases appear to be monomeric, single-gene products, the mammalian enzymes appear to be multimeric with nonidentical subunits (Baker & Lively, 1987; Shelness et al., 1988; Greenburg et al., 1989; YaDeau et al., 1991). In addition, there appear to be differences in the signal sequences recognized by eukaryotic and prokaryotic enzymes (von Heijne, 1984a; von Heijne & Abrahmsen, 1989). While signal sequences from the two sources are somewhat interchangeable, there are decreases in the processing efficiency of signal sequences in foreign hosts (Ngsee & Smith, 1990; Smith et al., 1985; Chang et al., 1987). These observations suggest differences in the specificity and structure of eukaryotic and bacterial LPases that could be exploited in the development of specific anti-infective agents.

The mechanism of LPase remains undefined. It does not appear to fit into any of the four classes of proteases based upon the lack of inhibition by inhibitors of serine enzymes, thioenzymes, metalloenzymes, and aspartyl enzymes (Zwizinski et al., 1981; Kuo et al., 1993). Arg-77 appears to be critical for activity versus macromolecular substrate (Bilgin et al., 1990). Sung and Dalbey (1992) have recently reported from site-directed mutagenesis studies that Ser-90 and Asp-143 were critical for catalysis. These authors suggested that this demonstrated that LPase was a novel serine protease. Tschantz et al. (1993) provided support for this proposal when they reported that replacement of Ser-90 with a cysteine resulted in a catalytically competent protein that was inhibited by *N*-ethylmaleimide. It was unclear from this work what the relative specific activities of the wild type and cysteine mutant were. These workers as well as Black (1993) further suggested that a lysine residue was critical. These data support the original proposal by Black that leader peptidase is analogous to the class A $\beta$-lactamases that utilize a serine and lysine residue to carry out catalysis (Herzberg & Moult, 1987; Strynadka et al., 1992; Adachi et al., 1991). The studies discussed above, although elegant, suffer from the usual uncertainties associated with mutagenesis in the absence of structure.

LPase does not appear to recognize a specific amino acid sequence in native protein substrates (von Heijne, 1983), although alanine is the most common site of cleavage (von Heijne, 1984b). Ala-X-Ala is the most common sequence found in $P_3P_2P_1$ subsites[4] of macromolecular substrates (von Heijne, 1983, 1986). Several studies indicate that proline in the $P_1'$ position of macromolecular substrates eliminates processing by the enzyme (Nilsson & von Heijne, 1992; Barkocy-Gallagher & Bassford, 1992). Kuo et al. (1993) reported that LPase prefers alanine in the $P_1$ position of peptide substrates. Even with glycine in this position, which does not violate von Heijne's rules (small aliphatic residues at $P_1$; von Heijne, 1983) for protein processing by LPase, an otherwise identical peptide was not processed. These data suggest that recognition of small substrates or inhibitors may be more subsite specific than the general rules that govern precursor protein recognition. The interactions with other subsites within the LPase active site remain undefined. A detailed understanding of the catalytic mechanism and substrate subsite interactions would facilitate the design of specific inhibitors for this enzyme.

[4] The enzyme subsites and the amino acid residues of substrates are numbered according to the nomenclature of Schecter and Berger (1967).

The development of inhibitors and mechanistic studies have been hampered by the lack of quantitative continuous assays for LPase activity. Until recently, the enzyme was assayed with PAGE assays with preproteins such as proOMPA (Dalbey & Wickner, 1985) and bacteriophage M13 procoat (Zwizinski & Wickner, 1980). Kuo et al. (1993) and Dev et al. (1990) have reported peptide substrates based upon the cleavage sites of procoat and preMBP, respectively. In both of these studies LPase activity was monitored with fixed-time assays by HPLC. While these assays represented significant improvements and allowed the development of screening assays to search for LPase inhibitors, they were somewhat tedious and insensitive due to the relatively low specific activity of LPase. The incorporation of radiolabel into a peptide substrate improved the sensitivity but still required HPLC separation (Dev et al., 1990). Kuo et al. (1993) reported that LPase catalyzed the hydrolysis of Ac–WSASALAKI between the alanine and lysine residues. In this work we report the development of a continuous assay for LPase activity based upon this peptide substrate that can be monitored either spectrophotometrically or spectrofluorometrically. This development should greatly facilitate mechanistic studies and the development of potential therapeutic agents. We demonstrate infidelity in LPase cleavage sites and provide data that suggest that LPase does not utilize a catalytic histidine residue. In addition, we report the first nonpeptide inhibitors of LPase. The molecular class of these compounds further supports the placement of LPase into a new class of serine proteases.

## MATERIALS AND METHODS

Native LPase was isolated according to published procedures from *E. coli* MC1061/pRD8, which was a gift from W. Wickner at UCLA (Dalbey & Wickner, 1985; Wolfe et al., 1982, 1983a,b). Δ-2-75 LPase (LPase-sf) was prepared according to the method of Kuo et al. (1993). Porcine kidney leucine aminopeptidase M (LAP) was purchased from Sigma Chemical Co. and dialyzed versus 40 mM $KPO_4$ at pH 7.7 prior to use. Peptides were purchased from either Bachem Biosciences (Philadelphia, PA), Bachem California, or Multiple Peptide Systems and dissolved in DMSO prior to use. The blocked amino acids and I–AMC were purchased from Bachem Biosciences. L-680,833, L-680,831, L-684,248, and L-684,249 were synthesized according to published procedures (Shah et al., 1992)[5] and dissolved in DMSO prior to use. All other reagents were of analytical grade and purchased from commercial sources. Buffers were titrated to the appropriate pH with either HCl or KOH. Protein concentrations were determined with the BCA protein reagent (Pierce Chemical Co.) with bovine serum albumin (Sigma) as a standard. High-pressure liquid chromatography electrospray ionization mass spectrometry (ESI-MS) was conducted on a Finnigan TSQ-700 spectrometer interfaced with an Applied Biosystems 140B syringe pump and a 10 cm × 150 $\mu$m $C_4$ column and eluted with a 5–90% acetonitrile gradient containing 0.05% TFA. UV–visible spectroscopy was conducted on a Molecular Devices ThermoMax plate reader or a Varian 2200 spectrophotometer equipped with an IBM-PS2-55SX and Galactic Industries Spectracalc software for data collection and analysis. Fluorescence spectroscopy was conducted on a Perkin-Elmer 650-40 spectrofluorometer equipped with an IBM-PC for data collection and analysis or an SLM Aminco 8000 spectro-

[5] Also see Merck and Co. (1989) Eur. Patent 0 337,549. These are highly substituted lactams and completely stable at pH 7.5 and 37 °C for periods of 30 days (Knight et al., 1992a).

fluorometer. HPLC was conducted on a Waters system equipped with Maxima 820 software for control of two Model 510 pumps and for integration of either the fluorescence detected by a Model 470 detector or UV–visible absorbance detected by a Model 490E detector. A Zymark robot prepared the reaction mixtures and removed aliquots for injection into a Valco injector. Samples were typically eluted from a 150 × 4.6 mm DuPont Zorbax $C_{18}$ column with a 2–85% acetonitrile gradient containing 0.05% TFA except where noted.

Ac–WSASALAKI–AMC was initially synthesized according to the following procedure. *N*-α-FMOC-tBOC-lysine was attached to [(4-dimethoxyphenyl)phenoxy]polystyrene resin using DCC and DMAP. FMOC was removed with piperidine. Conventional solid phase peptide synthesis methodology was then used to couple the amino acid derivatives in the following order: FMOC-A-OH, FMOC-L-OH, FMOC-A-OH, FMOC-S(tBu)-OH, FMOC-A-OH, FMOC-S(tBu)-OH, FMOC-W-OH, to produce FMOC-WS(tBu)-AS(tBu)-AK(BOC)-resin. Removal of FMOC with piperidine was followed by acetylation of the amine with acetic anhydride. The peptide was removed from the resin with 1% TFA in $CH_2Cl_2$ to yield the acid. The protected peptide was coupled to I–AMC using DCC and HOBT according to the procedure of Konig and Geiger (1970). The tBu protecting groups were removed with TFA to yield Ac–WSASALAKI–AMC. The FAB mass spectrum of the product displayed a parent ion of 1145 amu ($M + H^+$). Bachem Biosciences synthesized later lots of the peptide, after the substrate activity was demonstrated.

*LPase activity* was initially determined by HPLC according to the method of Kuo et al. (1993) with 125 μM Ac–WLVP–Nleu–LSFAAEGDDPA–$NH_2$ as substrate. Native LPase (0.2–4 μM) was assayed at 37 °C in buffer A [10% DMSO, 0.5% *n*-octyl glucoside, 20-fold dilution of PB-74 (polybuffer 74, Pharmacia), and 40 mM $PO_4$ at pH 7.7]. LPase-sf (0.2–4 μM) was assayed in either buffer A or B (10% DMSO and 40 mM $PO_4$ at pH 7.7) which lacked detergent and PB-74. The activity of Ac–WASALAKI–AMC versus both LPase and LPase-sf was monitored initially by HPLC to assess the cleavage site. The AMC-containing products were monitored by both UV–visible (215 nm) and fluorescence detectors (excitation at 320 nm and emission at 460 nm). The products were collected from the column and analyzed by ESI-MS. In the presence of LAP, any C-terminal products produced by LPase were hydrolyzed to liberate AMC. This product was monitored by either fluorescence (excitation at 380 nm and emission at 460 nm) or by UV–visible spectroscopy at 356 nm ($\epsilon$ = 17 300 $M^{-1}$ $cm^{-1}$). The fluorescence assays were conducted in 3-mL volumes with 5 units of LAP, while the individual UV–visible assays were conducted in 1 mL with 2–5 units of LAP. In the 96-well plate assay the final volume was 0.2 mL (path length = 0.55 cm and $\epsilon$ = 9500 $M^{-1}$ 0.55 $cm^{-1}$) of buffer A or B containing 2.5 units of LAP and 5% DMSO. (In control experiments up to 15% DMSO did not affect the reaction over 4 h.) LPase reactions were monitored for 1–4 h. In preliminary experiments both LPase and LAP were stable over this time period under the various conditions used in this work.

The pH dependence of the native LPase (1 μM) catalyzed hydrolysis of 5–320 μM Ac–WSASALKI–AMCA (above 320 μM the substrate was insoluble) was examined at 37 °C in 0.2 mL of 10% DMSO, 0.5% *n*-octyl glucoside, 20-fold dilution of PB-74, and 40 mM concentrations of the following buffers: MES (pH 5.5, 6.0, and 6.4), PIPES (pH 6.4, 7.0, and 7.3), TES (pH 7.3, 7.5, and 7.9), and TAPS (pH 7.9, 8.5, and 8.8). In preliminary experiments 2.5 units of LAP was stable and was sufficient to couple the LPase hydrolytic reaction to the production of AMC over this pH range. In addition, the reaction was examined with buffers at overlapping pH values to demonstrate that there were no artifacts due to the buffer composition. The rate at each substrate concentration (5, 10, 20, 40, 80, 160, and 320 μM) at each pH was determined in duplicate. The data were fit to eq 2 to obtain the kinetic parameters. Below pH 7.3, $V_m/K_m$ was determined from the linear slope of the velocity obtained between 20 and 80 μM substrate.

*LPase Inhibition.* The inhibition of LPase and LPase-sf by β-lactams was examined using the continuous assay. The reactions were initiated with enzyme. In initial experiments the amount of inhibition was determined in a 1-h incubation and the percent inhibition determined at this time from the change in absorbance in the presence and absence of inhibitor. In addition, L-684,248 was preincubated with LPase-sf for 1 h at 25 °C and the reaction initiated with substrate. Since the reaction appeared to be time dependent, full progress curves of the LPase-sf reaction were conducted at higher concentrations of the inhibitors. Detailed conditions are given in Table 3 and Figure 3. In control reactions the compounds did not inhibit the hydrolysis of KA–AMC by leucine aminopeptidase. The inhibition of LPase was also examined with the HPLC-based assay (125 μM Ac–WLVP–Nleu–LSFAAEGDDPA–$NH_2$).[6]

*Data Analysis.* The slopes of linear progress curves were determined by a linear least-squares fit of the data to eq 1

$$Y = v_i X + B \quad (1)$$

$$Y = V_m[S]/(K_m + [S]) \quad (2)$$

$$Y = v_0 t + ((v_0 - v_s)(1 - e^{(-k_o t)})/k_o) + A_0 \quad (3)$$

$$k_o/[I] = k_{inact}/(K_i(1 + [S]/K_m)) \quad (4)$$

using GRAFIT (Erithacus Software; Leatherbarrow, 1992). The kinetic constants for the reaction of the LPases with substrate were determined by nonlinear regression which fit the initial velocity as a function of substrate concentration to eq 2 using GRAFIT or the Fortran programs of Cleland (1979).[7] The rates determined from reactions monitored by HPLC were calculated from the concentration of substrate converted over time based upon the integrated areas of the substrate peak before and after reaction. The percent inhibition in the presence of inhibitors was calculated from the rates obtained in the presence and absence of inhibitors. The second-order rate constants for the inhibition of LPase-sf by time-dependent inhibitors were calculated by calculating the first-order rate constant for the inhibition according to eq 3 and then correcting for the ratio of $[S]/K_m$ using eq 4 (Knight et al., 1992).

## RESULTS

The initial HPLC chromatograms of the reaction of native LPase and LPase-sf with Ac–WSASALAKI–AMC yielded

[6] The incubation time is greater in this assay, which should result in increased inhibition by a time-dependent inhibitor. In addition, the ratio $[S]/K_m$ of the assay with this substrate [$K_m > 250$ μM (Kuo et al., 1992)] differs from that with 50 μM Ac–WSASALAKI–AMC. Finally, the stability of the E–I complexes formed is unknown and may effect the percent inhibition observed.

[7] Direct comparison of the results obtained from this program with the programs of Cleland (1979) yielded essentially identical results.

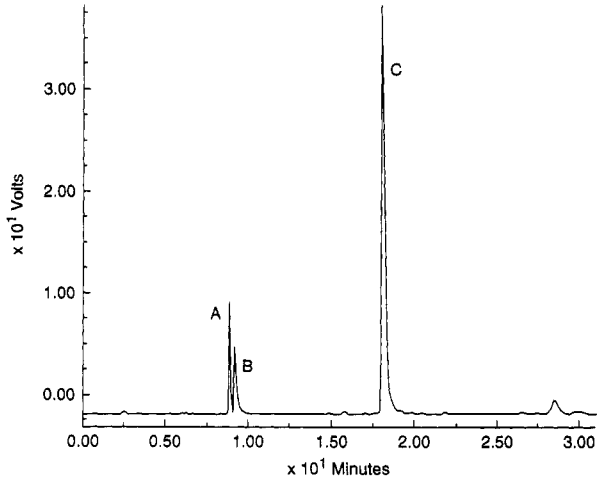



FIGURE 1: HPLC chromatogram of the reaction of LPase with 250 μM Ac–WSASALAKI–AMC (C) after 4 h obtained with an acetonitrile step gradient (0.05% TFA) from a $C_{18}$ column. The steps were 15–25% from 0 to 3 min, 25–55% from 3 to 22 min, and 55–85% from 22 to 26 min. The eluant was monitored by fluorescence (excitation 320 nm and emission 460 nm). Two AMC-containing products were observed (**A** and **B**). On the basis of the order of elution from the column, **A** and **B** are likely KI–AMC and LAKI–AMC, respectively. Ac–WSASALA, which elutes in the injection front with this gradient, was also detected by fluorescence (excitation 280 nm and emission 348) but is not observed at the wavelengths used in this chromatogram.

Table 1: Integrated Area of the Two AMC-Containing Peptides Produced during the Hydrolysis of Ac–WSASALAKI–AMC by LPases[a]

| enzyme | product 1 | product 2 | substrate remaining |
|---|---|---|---|
| LPase | $1.18 \times 10^6$ | $1.61 \times 10^6$ | $1.67 \times 10^7$ |
| LPase-sf | 320 000 | 480 000 | $7.8 \times 10^6$ |

[a] These data were obtained by integration of the fluorescence (excitation 320 nm and emission 460 nm) of the two AMC-containing products observed in HPLC chromatograms such as Figure 2. The concentrations of Ac–WSASALAKI–AMC were 250 and 125 μM for the reactions with LPase and LPase-sf, respectively. The units are counts.

two apparent products (data not shown). The first species that eluted from the columns coeluted with synthetic Ac–WSASALA during reversed-phase HPLC. The ESI-MS spectrum of this species displayed a parent ion of 747.2 amu (calculated 746.9). This was the same product observed by Kuo et al. (1993) during the LPase-catalyzed hydrolysis of Ac–WSASALAKI. The second species eluting from the column actually contained three different components when analyzed by ESI-MS. The mass spectrum of this mixture indicated the presence of Ac–WSASA (536.6 amu, calculated 562.6), KI–AMC (417.4 amu, calculated 416.6), and LAKI–AMC (601.7 amu, calculated 600.9). These data indicate that LPase has catalyzed the hydrolysis at both the A–L and A–K bonds.[8] The two AMC-containing products were resolved, and a typical chromatogram is presented in Figure 1. The amount of cleavage at both sites was estimated from the areas of the two AMC-containing products to be ~50% for both LPase and LPase-sf (see Table 1).

The strategy for the continuous assay of LPase is outlined in Scheme 1.[9] The progress curves for hydrolysis of Ac–WSASALAKI–AMC by LPase were linear to at least 10%

[8] Leucine aminopeptidase is specific for L-amino acids (Pfleiderer, 1970). Therefore, the alternate cleavage site could not be the result of racemization of the lysine residue during synthesis, as suggested in review, as this product, LA–(D)KI–AMC, would not be hydrolyzed to free AMC by LAP.

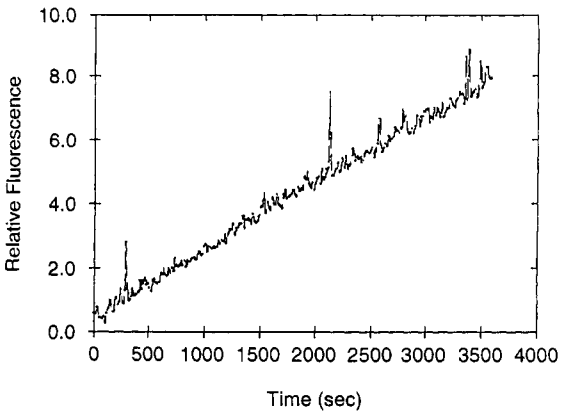



FIGURE 2: Progress curve of the hydrolysis of 20 μM Ac–WSASALAKI–AMC by 0.75 μM LPase-sf in 3 mL of buffer B as detected by fluorescence. The total conversion was less than 6%.

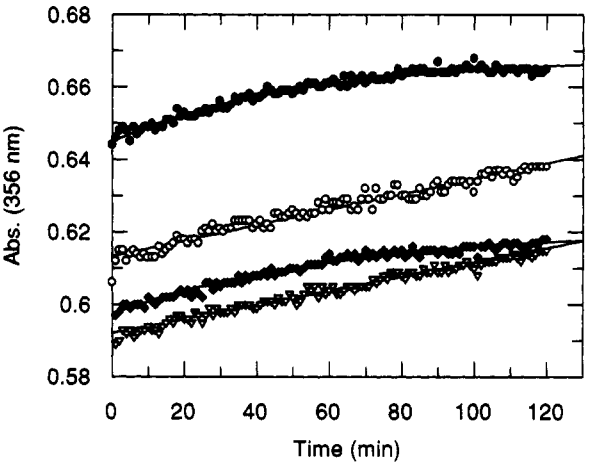
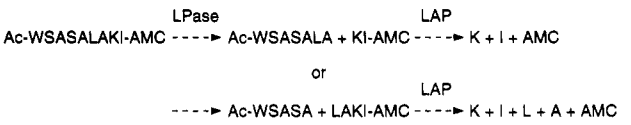



FIGURE 3: Linear progress curves (▽, ○) for the hydrolysis of 50 μM Ac–WSASALAKI–AMC by 1 μM LPase-sf in 0.20 mL of buffer B as detected using a 96-well UV–visible plate reader. The curves are the calculated fit to the linear equation. The nonlinear progress curves were obtained from the same reaction in the presence of 1.88 mM L-684,248 (●) and L-684,249 (◆). The curves are the theoretical fit to eq 3.

Scheme 1




conversion when monitored by UV–visible or fluorescence spectroscopy (data not shown). Examples of the progress curves observed by fluorescence and UV–visible spectroscopy using a 96-well plate reader are shown in Figures 2 and 3. The reaction was not limited by the concentration of LAP, although at much lower LAP concentrations there is an obvious lag in the progress curve before the steady-state rate is attained (data not shown). The reaction was linear with LPase concentration. For example, the ratio of the slopes observed by fluorescence detection from 0.5, 1, and 2 μm LPase was 0.53:1:2.1. The dependence of the rate of hydrolysis by LPase-sf on the concentration of Ac–WSASALAKI–AMC at pH 7.5 is presented in Figure 4. Similar saturation kinetics were observed for LPase-sf. From these data the individual kinetic constants were calculated and are compared in Table 2.

The dependence of $V_m$ and $V_m/K_m$ for the hydrolysis of Ac–WSASALAKI–AMC on pH is shown in Figure 5. At

[9] The original strategy, based on only a single cleavage site to produce KI–AMC, was modified after dual cleavage sites were observed.

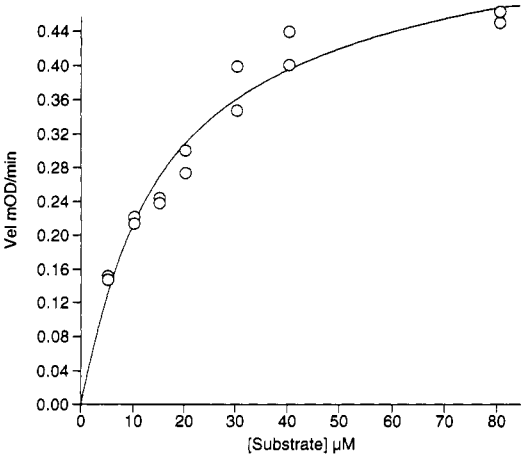



FIGURE 4: Dependence of the velocity of the LPase-sf (1 $\mu$M) catalyzed hydrolysis of Ac–WSASALAKI–AMC on the concentration of substrate in buffer B without detergent.

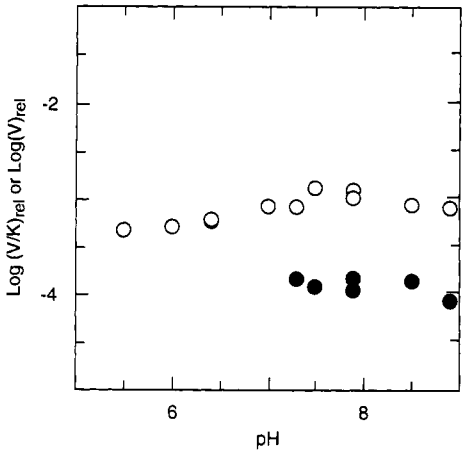


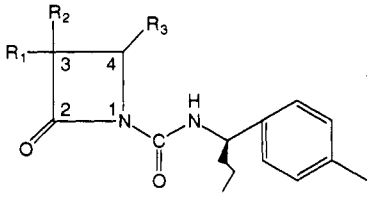

FIGURE 5: Dependence of $V_m/K_m$ (○) and $V_m$ (●) for the hydrolysis of Ac–WSASALAKI–AMC by LPase on pH.

Table 2: Comparison of the Kinetic Parameters for the Hydrolysis of Ac–WSASALAKI–AMC by LPase and LPase-sf

| enzyme | $k_{cat}$ ($h^{-1}$) | $k_{cat}/K_m$ ($M^{-1}$ $h^{-1}$) | $K_m$ ($\mu$M) |
|---|---|---|---|
| LPase | 16.5 ± 1.2 | (1.66 ± 0.29) × $10^5$ | 78 ± 18 |
| LPase-sf (no detergent) | 3.6 ± 0.2 | (2.1 ± 0.19) × $10^5$ | 16.7 ± 2.2 |
| LPase-sf (plus detergent) | 2.42 ± 0.15 | (1.5 ± 0.18) × $10^5$ | 16.1 ± 2.8 |

acidic pH values only $V_m/K_m$ could be determined since 320 $\mu$M Ac–WSASALAKI–AMC was insufficient to obtain complete saturation of LPase (data not shown). There was only a 3-fold decrease in $V_m/K_m$ upon going from pH 7.3 to 5.0, and there was approximately a 2-fold decrease in $V_m$ between pH 7.3 and 8.9. The rates obtained at 320 $\mu$M substrate at pH 5.5, 6.0, 6.4, and 7.0 were 70, 80, 93, and 104%, respectively, of the $V_m$ obtained at pH 7.3.

The two C-2 dimethyl substituted $\beta$-lactams, L-684,248 and L-684,249, inhibited LPase-sf and LPase, but *S*-stereochemistry at C-4 was preferred. Preincubation of LPase-sf with L-684,248 yielded increased inhibition. The time-




| L-number | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| 680,831 | Ethyl | Ethyl | (S)-P-Hydroxybenzoic Acid |
| 680,833 | Ethyl | Ethyl | (S)-P-Hydroxyphenylacetic Acid |
| 684,248 | Methyl | Methyl | (S)-P-Hydroxybenzoic Acid |
| 684,249 | Methyl | Methyl | (R)-P-Hydroxybenzoic Acid |

dependent nature of the inhibition of LPase-sf by both L-684,-248 and L-684,249 was evident from progress curves of the reactions (see Figure 3). The second-order rate constants for the inhibition are reported in Table 3. The C-2 diethyl substituted $\beta$-lactams were not inhibitory.

Table 3: Inhibition of LPase and LPase-sf by $\beta$-Lactams

| compd | % inhibition LPase-sf[a] | % inhibition LPase[a] | % inhibition LPase[b] | $k_o/[I]$[c] LPase-sf | $k_{inact}/K_i$[d] ($M^{-1}$ $min^{-1}$) LPase-sf |
|---|---|---|---|---|---|
| L-680,831 | nd[e] | nd | 0 | | |
| L-680,833 | 4 ± 2 | 2 ± 2 | 0 | | |
| L-684,248 | 30 ± 5 | 24 ± 4 | 88 ± 2 | | |
| 1 h preinc | 51 ± 7 | nd | nd | | |
| 0.942 mM | | | | 12.3 ± 0.4 | 49.1 ± 1.6 |
| 1.88 mM | | | | 11.7 ± 0.5 | 46.7 ± 2.0 |
| L-684,249 | 11 ± 1 | nd | 30 ± 2 | 7.7 ± 0.7 | 30.8 ± 2.8 |

[a] These values were determined in duplicate or triplicate at pH 7.5 at 25 °C with 50 $\mu$M Ac–WSASALAKI–AMC with 0.51 mM L-680,833, 0.588 mM L-684,248, and L-684,249 with 1 $\mu$M enzyme. The percent inhibition is based upon the difference in $A^{356}$ of the reactions after 1 h. In addition, L-684,248 was preincubated with LPase-sf for 1 h at 25 °C, and the reaction was initiated with the substrate. [b] These values were determined with the HPLC assay with 125 $\mu$M Ac–WLVP–Nleu–LSFAAEGDDPA–$NH_2$, pH 7.5. The percent inhibition is that obtained over a 4-h reaction time at 37 °C. The concentrations of L-684,248, L-684,269, and L-680,831 were 0.588, 0.588, and 1 mM, respectively. [c] $k_o$ was determined from the nonlinear progress curves over 120 min for the hydrolysis of 50 $\mu$M Ac–WSASALAKI–AMC at pH 7.5 and 1 $\mu$M LPase-sf and $k_o/[I]$ calculated. The concentrations of L-684,248 and L-684,249 used were 0.942 and 1.88 mM in triplicate. The progress curves obtained for the inhibition by 0.942 mM L-684,249, while nonlinear, did not reach sufficient inhibition over the time alloted, and therefore only the data at 1.88 mM were used to calculate the second-order rate constant. [d] This parameter was calculated from the measured $k_o/[I]$ according to eq 4. The value of $1 + [S]/K_m$ was 3.99. [e] Not determined.

## DISCUSSION

LPase catalyzed the hydrolysis of Ac–WSASALAKI–AMC at two sites, A–L and A–K, with approximately equal efficiency. The A–K site is that predicted by the preMBP sequence (Emr et al., 1980a; Perlman & Halverson, 1983) and was observed by Kuo et al. (1993) when Ac–WSASALAKI was tested as a substrate. The addition of the C-terminal AMC affects the cleavage site. This could be explained by recognition of hydrophobic residues in either $P_5'$, $P_6'$, or $P_7'$. For example, these residues are Gly-Ile-Tyr in the *Bacillus* outer wall protein (Tsubio et al., 1986), which suggests that hydrophobic residues are tolerated in these positions of bacterial LPase substrates. LPase displays amazing fidelity in the correct processing of precursor proteins, although there have been recent reports of infidelity in processing of preMBP at the analogous A–L bond in mutants that have the Ala normally at $P_1$ replaced with Asp, His, or Pro residues (Fikes et al., 1990; Barkocy-Gallagher & Bassford, 1992). These substitutions resulted in decreased processing efficiency. Sasamoto et al. (1989) have reported infidelity during the processing of precursor of $\alpha$-amylase by *Bacillus subtilis* signal peptidase *in vivo*. Furthermore, Barkocy-Gallagher and Bassford report that replacement of the $P_1'$ residue with proline in a preMBP chimer containing the preRBP signal sequence results in cleavage at an A–M in the $P_4$–$P_3$ of the sequence ...–A–M–A–K–... rather than the predicted A–K, but once

again with decreased efficiency. We observed no evidence of cleavage at the third alanine (A–S) site. LPase is known to require a hydrophobic region beginning approximately at –6 ($P_6$). Ac–WSA is likely insufficient to occupy this binding pocket which precludes cleavage of Ac–WSASALAKI–AMC at the A–S bond. The cleavage fidelity observed with macromolecular substrates may be due to the presentation of the cleavage site to the LPase active site by interactions with the membrane *in vivo* or detergent micelles *in vitro*. In fact, a number of groups have suggested that these interactions are primary determinants of the cleavage site of preproteins [for example, see Ohno-Iwashita and Wickner (1983)]. This fidelity may not be conserved with small peptide substrates where these interactions are lacking.

The kinetic parameters displayed by LPase and LPase-sf toward Ac–WSASALAKI–AMC are similar, but both $k_{cat}$ and $K_m$ are lower with the short form of the enzyme. This could have been the result of differences in the ratio of cleavage at the two sites, but this was not observed. At this time we do not have an explanation for this difference, but the second-order rate constants which reflect the true specificity of substrates is essentially the same versus both proteins. It is highly unlikely that the $K_m$ values reflect true dissociation constants ($K_s$) from a Michaelis complex. First, Kuo et al. (1993) report that the reaction of LPase with two peptides, Ac–WLVP–Nleu–LSFAAEGDDPA–$NH_2$ and Ac–WSA-SALAKI, that represent the spanning cleavage sites of procoat and preMBP was linear up to 250 $\mu$M, suggesting the $K_s$ for these two peptides was greater than this concentration. Second, Dev et al. (1990) report that the $K_m$ values for the peptides in their studies are $\geq$1 mM. In addition, if LPase is indeed a serine protease, then the $K_m$ will reflect the relative ratio of the acylation and deacylation rate constants according to eqs 3 and 4 and will likely be $\leq K_s$ [for example, see Stein et al. (1985) and Brocklehurst and Topham (1990)].

$$K_m = K_s(k_{deacyl}/(k_{acyl} + k_{deacyl})) \quad (5)$$

$$K_s = (k_{off} + k_{acyl})/k_{on} \quad (6)$$

In addition, nonproductive binding could lower both $k_{cat}$ and $K_m$ by the factor $1 + K_s/K_s'$, where $K_s'$ is the dissociation constant in the nonproductive binding mode. This will not affect the second-order rate constant $k_{cat}/K_m$ (Fersht, 1977). Therefore, the latter constant should be used for comparison of both specificity of substrates and different enzyme forms. The difference in kinetic parameters between the two LPases could be explained if nonproductive binding was more prominent with the short form of the enzyme. This is not a far-fetched proposal as the interaction of LPase and signal peptides with the detergent micelles in these studies could have an effect on substrate presentation to the active site.

The activities of the substrates reported in Table 2 versus LPase are comparable to those reported in the literature when $k_{cat}/K_m$ is compared. The second-order rate constants reported by Kuo et al. (1993) for two peptide substrates were approximately $8 \times 10^4$ $M^{-1}$ $h^{-1}$. The best peptide substrate reported by Dev et al. (1990) displayed a $k_{cat}/K_m$ of $1.2 \times 10^5$ $h^{-1}$ $M^{-1}$.

The pH dependence of $V/K$ of the LPase reaction with Ac–WSASALAKI–AMC did not demonostrate any ionizable groups between pH 5.5 and 8.9.[10] While there was a slight decrease in $V/K$ at lower pH, the magnitude precludes attributing this to the ionization of a catalytic group. This is consistent with the proposal that LPase is a serine protease that lacks the active site histidine (Sung & Dalbey, 1992). In classical serine proteases one observes the ionization of the histidine with a p$K_a$ of approximately 7.0 with a decrease in this parameter below pH 7.0 [for example, see Chabin et al. (1993)]. Interestingly enough, both $V_m$ and $K_m$ appear to increase below pH 7.0 as we did not observe saturation at the highest soluble concentration of substrate at low pH, yet the velocities at this substrate concentration were comparable to the maximum velocity at pH 7.3. These data would suggest that the protonation of a group in the ES complex at low pH might favor a catalytic step(s) which is reminiscent of aspartyl proteases [for a discussion of the aspartyl protease mechanism, see Fruton (1976) and Rich (1985)]. In these enzymes two aspartates function as both general acid and base catalysts. In the case of LPase, if the rate of deacylation of an acyl-enzyme intermediate were rate determining and this step were enhanced at lower pH, then the $K_m$ would be expected to increase at lower pH according to eq 3. Deacylation (and acylation) at lower pH could be facilitated by a protonated aspartate acting as a general acid to neutralize the incipient negative charge that develops on the carbonyl oxygen upon formation of a tetrahedral intermediate. An additional aspartate residue could function as a general base to enhance the nucleophilicity of both the active site serine during acylation and a water molecule during deacylation, although the p$K_a$ of such a group would have to be lower than that of a typical acid as is seen in the aspartyl proteases. At neutral pH this residue would be the only functioning acid–base catalytic residue. Sung and Dalbey (1992) have suggested from mutagenesis studies that at least one aspartate is required for catalysis. In addition, these workers proposed that several aspartates were required in detergent extracts, although one was suggested to play a structural role. The ionization state of the acids was not addressed in these studies. This would suggest that LPase might be classified as an acidic serine protease, but additional work is obviously needed to establish the pH dependence of this reaction at low pH.

There was also a slight decrease in LPase activity (both $V$ and $V/K$) at higher pH. Black (1993) has suggested from site-directed mutagenesis studies that Lys-145 is necessary for LPase activity *in vivo*. From these data and sequence alignments, he suggested that the LPase mechanism is analogous to class A $\beta$-lactamases which utilize an active site lysine(s). Strynadka et al. (1992) have proposed that neutral Lys-73 functions as the general base to activate the serine for nucleophilic attack in these lactamases. This is analogous to the role played by a histidine in classical serine proteases. This proposal requires a neutral Lys residue. For LPase to utilize this mechanism would require an active site lysine possessing a p$K_a$ below 5 as there are no ionizations observed over the pH range studied in this work. In fact, Knap and Pratt (1991) have argued that the group which displays a p$K_a$ of 7.5 in the class A $\beta$-lactamase reactions is actually Lys-234 and not Lys-73. Lys-234 is thought to interact with the

[10] During review it was suggested that interpretation of the pH profiles is complicated by lack of information concerning, the product ratio as a function of pH. If the product ratio changed as a function of pH, then a pH dependence of the reaction would be expected, unless the selectivity of cleavage at one site was increased, but offset by a decrease at the other site as a function of pH. While this cannot be ruled out with the available data, the ionization of an essential histidine residue in a "classical" serine protease mechanism residue would not be expected to show this characteristic. The slight decrease in $V_m/K_m$ at high pH is due to an increase in $V_m$ and not $K_m$, which would be predicted if there was a change in the selectivity toward the cleavage sites. Once again, we cannot rule out changes in the product ratio, but the absence of a dramatic pH dependence on either the acid or base side argues against a critical acid–base catalyst possessing a p$K_a$ in the pH range examined.

carboxylate of β-lactam substrates (Herzberg & Moult, 1987; Moews et al., 1990). Herzberg and Moult (1987) have also argued that the $pK_a$ of the catalytic Lys-73 in class A β-lactamases is too high to support the role proposed by Strynadka et al. Instead they suggest that the major role of the *positively* charged Lys-73 is to stabilize the anionic tetrahedral intermediate through an electrostatic interaction. In this model the proximity of the positive charge would also be expected to decrease the $pK_a$ of the nucleophilic serine, but due to electrostatics not general base catalysis. If LPase has a catalytic Lys residue as suggested by Black (1993), then the pH dependence of the reaction suggests that this residue has a normal $pK_a$ (>9). This residue could play an electrostatic role in the catalytic mechanism similar to that proposed by Herzberg and Moult for the class A β-lactamases or function as a general acid catalyst, but the results from the LPase pH studies suggest that the enzyme does not utilize a catalytic Lys as a general base catalyst.

β-Lactams inhibit LPase activity. While the potency of the compounds used in this study is not dramatic, these are the first reported nonpeptide inhibitors of LPase. There is a dependence on both β-lactam structure and stereochemistry which suggests that the inhibition is specific. The enzyme prefers smaller groups in the C-2 position of the β-lactam ring and *S*-stereochemistry at the C-4 position. The activity results of dimethyl versus diethyl analogs parallel those reported by Knight et al. (1992a) for the inhibition of porcine pancreatic elastase by β-lactams. From those observations and substrate specificity studies they concluded that the orientation of the lactam in the active site placed the dialkyl substitution (C-2) in the $P_1$ specificity pocket of elastases. It is interesting to note that the substrate specificity for alanine over valine reported by Kuo et al. (1993) for LPase was analogous to that reported for PPE (Zimmerman & Ashe, 1977). There was little or no stereochemical discrimination for the C-4 position of β-lactams by PPE. HLE, on the other hand, displayed a 23-fold preference for the *S*-C-4-stereoisomer. LPase appears to be intermediate between these two extremes in the C-4 stereochemical preference. In addition, the inhibition of LPase by L-684,248 and L-684,249 was time dependent. This is analogous to the inhibition of elastases and cathepsin G by β-lactams (Knight et al., 1992a,b). These enzymes are inhibited by β-lactams via formation of stable acyl-enzymes (Knight et al., 1992a; Chabin et al., 1993). Furthermore, β-lactamases also utilize an acyl-enzyme intermediate during the hydrolysis of β-lactams. The inhibition data reported in this work suggest that LPase utilizes nucleophilic catalysis. These observations coupled with the site-directed mutagenesis studies reported by several groups (Sung & Dalbey, 1992; Tshantz et al., 1993; Black, 1993) and pH dependence reported in this work suggest that LPase is a serine protease that lacks a catalytic histidine. The mechanism of both substrate hydrolysis and inhibition of LPase by β-lactams likely involves acylation of an active site serine enzyme. We do not as yet have information on the stability of the acyl-enzymes produced from the β-lactams.

## CONCLUSIONS

A continuous spectrophotometric and spectrofluorometric assay for signal peptidase activity was developed. The assay is amenable to automation in a 96-well plate format. The pH dependence of $V_m/K_m$ for this substrate supports the proposal that an active site histidine is not required for catalysis. The inhibition of LPase by β-lactams suggests that the enzyme belongs to a new class of serine proteases. Furthermore, β-lactams as well as other classes of acylating agents could be developed as inhibitors of LPase to test the therapeutic potential of LPase inhibition.

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