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Use of Azidobestatin as a Photoaffinity Label To Identify the Active Site Peptide of Leucine Aminopeptidase†

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ABSTRACT: Aminopeptidases catalyze the hydrolysis of amino acid residues from the amino terminus of peptide substrates. They are found in most cells and tissues, and their activity has been implicated in myriad fundamental biochemical and physiological processes. Nevertheless, little is known about the structure of the aminopeptidase active sites. Beef lens leucine aminopeptidase (blLAP) can be considered prototypical of many enzymes in this family of peptidases. Bestatin, [(2*S*,3*R*)-(3-amino-2-hydroxy-4-phenylbutanoyl)-L-leucine] is a nonhydrolyzable substrate analogue of a peptide, PheLeu, which is rapidly cleaved by blLAP. Bestatin incorporates elements of the putative tetrahedral intermediate, and this results in a $>10^5$-fold enhancement of binding relative to analogous peptides. Bestatin is the most tightly bound inhibitor of many aminopeptidases. Bestatin was successively converted to nitrobestatin, *p*-aminobestatin, [^{3}H]-*p*-aminobestatin, and finally [^{3}H]-*p*-azidobestatin (pAB). Like bestatin, pAB is a slow binding inhibitor of LAP (K_i*, the dissociation constant for the final complex, = $\sim 4 \times 10^{-9}$; K_i, the dissociation constant for the initial collision complex, = $\sim 10^{-8}$). The $t_{1/2}$ for binding of 2×10^{-8} M and 8×10^{-8} M bestatin are ~60 min and ~38 min, respectively. pAB, nitrobestatin, bestatin, and physiological peptides appear to bind in the same site, the first three with similar avidity. In the dark, pAB and bestatin protect low concentrations of the enzyme against inactivation upon extensive dialysis. The $t_{1/2}$ for photoactivation of pAB is approximately 3 s. Irradiation of blLAP for such short periods of time resulted in insignificant change in activity. blLAP which was placed in 254-nm light in the presence of pAB was inactivated significantly. Treatment of photolabeled blLAP with trypsin produces only two peptides. Autoradiography and scintillation counting indicate that the active site is in the peptide which includes residues 138–487. Treatment of the same blLAP with hydroxylamine produces two different peptides, with the active site in the peptide 323–487. This indicates that the active site is in the carboxyl-terminal one-third of the protomer. It is likely that this photoaffinity label will be useful in identifying active sites in other aminopeptidases as well.

Aminopeptidases catalyze the hydrolysis of amino-terminal amino acid residues from peptide substrates. These enzymes are widely distributed throughout the plant and animal kingdoms, and most have broad specificity. Aminopeptidase catalysis occurs on cell surfaces, in cytoplasm, and within various cellular compartments, and several forms of these enzymes have been found in many tissues or cells (Ahmad & Ward, 1990; Chang & Smith, 1989; Ledeme et al., 1983; McDonald & Barrett, 1986; Oettgen & Taylor, 1985; Stirling et al., 1989; Taylor et al., 1982a, 1983, 1984a,b; Watt & Yip, 1989, and references cited within). Despite their ubiquitous distribution and myriad physiological roles (see Discussion), structural information regarding these enzymes is scanty. There has been no indication of the sites involved in substrate or inhibitor binding by covalent attachment of an active site label.

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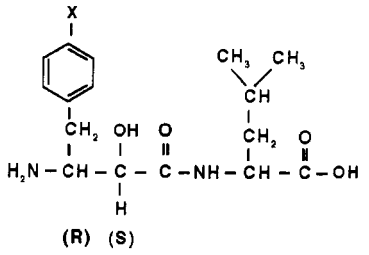
Scheme I



X = H bestatin; X = N_3 azidobestatin

Beef lens leucine aminopeptidase, blLAP,[1] (EC 3.4.11.1) is considered to be prototypical for a large class of aminopeptidases because it displays properties common to this group of enzymes: slow, relatively tight ($K_i = 10^{-9}$ M) binding of the transition state inhibitor, bestatin [(2*S*,3*R*)-(3-amino-2-hydroxy-4-phenylbutanoyl)-L-leucine] (Nishizawa et al., 1977; Ricci et al., 1982; Suda et al., 1976), and a requirement for metal ions for activity (Allen et al., 1983; Ocain & Rich, 1988; Patterson, 1989; Peltier & Taylor, 1986; Rich et al., 1984; Taylor et al., manuscript in preparation; Wilkes & Prescott, 1985). It is the interposition of the tetrahedral carbon and its functional groups (C2) (see Scheme I) which converts Phe to a statine, and it is this transformation which is responsible for the tighter binding of bestatin than the analogous peptide, PheLeu. Catalysis by LAP is most effective when peptides or peptide analogues have an N-terminal Leu (Delange & Smith, 1970; Hanson & Frohne, 1976; Smith & Hill, 1960), hence, the name. However, kinetic investigations indicate that different specificities may be indicated if longer peptide substrates are used (Taylor et al., 1981), and recent tests indicate that LAP and prolylaminopeptidase activities are due to the same enzyme (Turzynski & Mentlein, 1990).

blLAP was one of the first proteases discovered, and it is the best-studied AP with respect to sequence and composition (Cuypers et al., 1982; Hanson & Frohne, 1976; Carpenter & Vahl, 1973; Wallner and Taylor, submitted for publication), structure (Burley et al., 1990; Taylor et al., 1979, 1984b, 1989), and mechanism of action (Allen et al., 1983; Peltier & Taylor, 1986; Taylor et al., 1982b). Mature LAP is a hexamer comprised of six identical bilobal protomers (Burley et al., 1990; Carpenter & Harrington, 1972; Taylor et al., 1979), all of which are synthesized as identical precursors (Wallner and Taylor, submitted for publication). Each protomer contains 2 g-atoms of zinc, and one, or both of these, may be involved in substrate binding and/or catalysis (Allen et al., 1983; Burley et al., 1990; Taylor et al., 1982b). Immunological (Taylor et al., 1984a,b) and mechanistic (Hanson & Lasch, 1967) experiments indicate that within a species LAPs are indistinguishable; interspecies comparisons show a high level of conservation (Taylor et al., 1984a). Even where structural homology is less extensive, conservation of catalytic moieties appears to be strict (reviewed in Wallner and Taylor, submitted for publication).

In this work, we describe the preparation of a bestatin derivative, [^{3}H]-*p*-azidobestatin, which allowed us to identify that portion of LAP which is involved in binding substrate or transition-state analogues and which is presumably part of the active site. Relationships between active site occupancy by peptides, bestatin, nitrobestatin, and pAB are also documented, and use of the compound for physiological studies is discussed.

Materials and Methods

Materials. Leucine aminopeptidase was isolated from fresh, young beef eye lenses using an adaptation of the method of Allen et al. (Allen et al., 1983; Burley et al., 1990). All concentrations of LAP were determined by the Bio-Rad protein assay unless noted otherwise and are based on the protomer molecular weight of 54000 (Carpenter & Harrington, 1972; Wallner and Taylor, submitted for publication). Bestatin either was synthesized following the protocol of Suda et al. (1976) or was the kind gift of Dr. Umezawa. L-Leucylglycylglycine was purchased from Chemical Dynamics Corp. L-Amino acid oxidase (crude-dried venom from *Crotalus adamanteus*), *o*-dianisidine, horseradish peroxidase, and trypsin were purchased from Sigma Chemical Co. En^{3}hance was obtained from New England Nuclear. Trizma base, urea, and β-mercaptoethanol were obtained from Bio-Rad. EDTA, guanidine, and hydroxylamine were obtained from Sigma. Iodoacetamide and LiOH were obtained from Aldrich. NaOH was obtained from Baker, and formic acid was from Fisher. All other chemicals were purchased from either Bio-Rad, Baker, Fisher, Sigma, or Aldrich Chemical (St. Louis, MO) and were the finest purity available. Silica gel and cellulose plastic thin-layer chromatography plates were purchased from Kodak. HPLC was performed on an LKB 2150 LC pump with a 2138 UV detector, and UV spectroscopy was done on an LKB 4050 spectrophotometer. FTIR was done on an IBM ASPECT-3000 spectrophotometer, and NMR spectra were obtained on a Varian-Brucher 300 MHz multiprobe spectrometer.

Synthesis of p-Nitrobestatin. Nitration of bestatin was performed in a manner analogous to that of the nitration of phenylalanine (Bergel & Stock, 1954). To 1.0 mL of concentrated H_2SO_4 was slowly added 100 mg (0.3 mmol) of bestatin, and the resulting solution was stirred at 4 °C until the bestatin had dissolved. When solubilization of the bestatin was problematic, the mixture was gently heated to 25 °C or slightly higher, but this produced a minor amount of a side product. The solution was cooled in an ice bath, and 100 μL of the nitrating solution (1:1 v:v, H_2SO_4/fuming nitric acid) was added slowly in four 25-μL portions. Following the addition, the mixture was allowed to stir at 25 °C for an additional 30 min. The reaction mixture was then poured slowly over 30-mL of ice water, and the pH of the solution was adjusted to 5.5 with 2 N NaOH. The neutralized solution was lyophilized. The resulting semi-solid was triturated twice, each time with 15 mL of anhydrous methanol, and filtered. The remaining cake of inorganic salts was washed twice with 10 mL of anhydrous methanol, the organic phases were combined, and the methanol was removed by rotoevaporation, yielding 138 mg of an off-white solid. This was recrystallized from water/isopropanol, yielding 93 mg of the product, which ran as a single spot on silica gel and cellulose thin-layer chromatography. The material gave an intense UV absorption unlike the starting material, bestatin. The elution conditions were butanol, acetic, and water (25:4:10) in both cases, and the R_f's = 0.7 and 0.5 for silica gel and cellulose, respectively. This material exhibited a single peak (retention time 6.7 min) when analyzed by HPLC on a C_{18} column, eluted with 10 mM potassium phosphate (pH 6.8) and 5% methanol. The product had a characteristic UV absorption at λ_{max} = 276.0 nm (ϵ = 14.3 × 10^3 M^{-1} cm^{-1}), which is consistent with a nitroaromatic

[1] Abbreviations: AP, aminopeptidase; bl, beef lens; EDTA, ethylenediaminetetraacetic acid; FTIR, Fourier transform infrared spectroscopy; HA, hydroxylamine; HPLC, high-performance liquid chromatography; kDa kilodalton; LAP, leucine aminopeptidase; LeuGlyGly, leucylglycylglycine; pAB, *p*-azidobestatin; PAGE, polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate.

group but is different from bestatin (λ_{max} = 255.1 nm, ϵ = 1.9 × 10^2 M^{-1} cm^{-1}). The infrared spectrum exhibited characteristic aromatic absorptions between 3100 and 2990 cm^{-1} and strong bands corresponding to the aromatic nitro group at 1539, 1400, 856, and 702 cm^{-1}. In addition, there was a strong absorption at 3287 cm^{-1} that could be due to the hydrogen bonding of either the quaternary hydroxyl group or the amino function. The proton NMR (D_2O/DCl) had a doublet at δ = 1.9 (6 H), representing the methyl functions, a multiplet from δ = 1.4 to 1.6 (3 H) representing the aliphatic protons, a broad multiplet from δ = 3.0 to 3.9 (5 H) for the substituted aliphatic and benzylic protons, and two doublets at δ = 7.4 and 8.2 (2 H each) representing the aromatic protons.

Synthesis of [^3H]-*p-Aminobestatin.* To reduce the *p*-nitrobestatin, 125 mg (0.3 nmol) was dissolved in 10 mL of methanol at 0 °C containing 80 μL of concentrated HCl and mixed with 15 mg of 10% Pd/C. The suspension was stirred for 2.5 h under a slightly positive pressure of H_2 at 0 °C. The reaction was filtered through a pad of celite, and the pad was washed with 5 mL of methanol followed by 5 mL of ethanol. The organic solutions were combined and rotoevaporated yielding 120 mg of a solid. After recrystallization from methanol, 108 mg of the product was recovered. The product had a λ_{max} at 283 nm (ϵ = 1.37 × 10^3 M^{-1} cm^{-1}) and gave a single spot on TLC using silica gel and cellulose [R_f's = 0.3 and 0.4, respectively, when eluted with a solution of butanol, acetic acid, and water (25:4:10)] that was positive to ninhydrin and strongly UV absorbing.

HPLC on C_{18} showed one peak (retention time 3.4 min) when eluted with 10 mM potassium phosphate (pH 6.8) and 5% methanol, detected at 280 nm. The infrared spectrum had characteristic bands from 3400 to 2100 cm^{-1} for the C–H, N–H, and O–H stretching frequencies and other strong absorption bands at 1670, 1518, 1383, 1150, 966, 770, and 623 cm^{-1}. Much of the proton NMR (CD_3OD/DCl) showed essentially the same pattern as nitrobestatin. The methyl protons (6) appear at δ = 1.9, the aliphatic protons are in a broad peak from δ = 1.4 to 1.7 (3 H) and a broad multiplet from δ = 3.0 to 3.8 (5 H). The aromatic portion of the spectrum condensed to a singlet at δ = 7.6 (4 H) for the aminosubstituted ring. This same pattern is observed for *p*-aminophenylalanine.

p-Aminobestatin was exchange labeled by NEN–Du Pont. The *p*-aminobestatin (115 mg, 0.3 mmol) was dissolved in 5 mL of a suspension of DMF with 100 mg of 5% Rh on alumina. To this was added 25 Ci of ^3H_2O water, and the reaction was stirred at 50 °C overnight. The labiles were removed by a series of rotoevaporations with ethanol, and the final product was packed in ethanol. The final product contained 630 mCi, and the amount of the product, as calculated by using ϵ = 1.37 × 10^3, was found to be 100 mg. The product therefore was found to have a specific activity of 6.3 mCi/mg or 2.1 Ci/mmol. This material was used directly for the preparation of *p*-azidobestatin.

Synthesis of [^3H]-*p-Azidobestatin.* This synthesis was adapted from Schwyzer and Caviziel (1971). This procedure was repeated twice. The radiolabeled *p*-aminobestatin (100 mg, 0.28 mmol) was dissolved in 3.0 mL of 2 N H_2SO_4 and cooled in an ice bath. A solution of 37.5 mg of $NaNO_2$ (0.54 mmol) in 300 μL of water was added to the stirred mixture in six 50-μL portions over 30 min. The reaction was allowed to stir at 25 °C for an additional 20 min. A solution of 39 mg of NaN_3 (0.6 mmol) in 300 μL of water was added to this mixture in six aliquots. Each addition resulted in the release of nitrogen. The reaction mixture was cooled in an ice bath and stirred an additional 10 min, diluted with 6 mL of ice-cold water, and neutralized with 2 N NaOH (pH 6.8). The mixture was lyophilized, and the resulting tan solid was triturated 4 times with 2 mL of anhydrous ethanol. The organic phases were combined, and the solvent was removed on a rotary evaporator. This resulted in a yield of 84 mg (81%) of a material that showed one spot on TLC (silica gel R_f = 0.62 and cellulose R_f = 0.75) using a solution of butanol, acetic acid, and water (25:4:10) that was strongly positive to short-wavelength UV and positive to ninhydrin. For the *p*-azidobestatin, λ_{max} = 249.2 nm with an ϵ = 17.8 × 10^3 M^{-1} cm^{-1}. HPLC on a C_{18} column gave a single peak (retention time 7.3 min) when eluted with 10 mM potassium phosphate (pH 6.8) and 5% methanol and detected at 280 nm. The infrared spectra of the *p*-azidobestatin exhibited an extremely strong absorption at 2168 cm^{-1}, which is characteristic of the azido function. This was not observed in the precursors. Other strong and characteristic absorptions occurred at 3150 cm^{-1} for the C–H stretch, 2345 cm^{-1} for the azide, 1504 and 1350 cm^{-1} for the carboxylic acid, and 835 and 660 cm^{-1} for aromatic absorptions. The specific activities of the two preparations were 4.05 × 10^9 μCi/mol and 3.3 × 10^8 μCi/mol.

Enzyme Assays Using Pre-Steady-State and Steady-State Conditions. Assays were done under conditions which allow observation of pre steady state (Cha, 1976), steady state, or both (Morrison & Walsh, 1988). The data reported here were achieved using the former and the latter. Pre-steady-state data were obtained in three separate experiments. The data obtained from a combination of pre-steady-state and steady-state conditions were done twice. For each experiment, all assays were done in duplicate. The concentration of LAP was in the range of (4.6–26) × 10^{-10} M. In a typical experiment, the reaction was started by the addition of 0.8 mL of LAP (4.6 × 10^{-10} M final concentration) to 16.0 mL of 1.0, 2.0, 3.0, and 4.0 mM LeuGlyGly (final concentrations, each containing 2 × 10^{-8}, 4 × 10^{-8}, 5 × 10^{-8}, 6 × 10^{-8}, and 8 × 10^{-8} M bestatin. Duplicate samples (0.525 mL each) were taken every 10 min for 2–3 h. The extent of hydrolysis was determined by following the appearance of free leucine using L-amino acid oxidase (Nicholson & Kim, 1975). Under these conditions, LAP has been shown to hydrolyze just the leucyl–glycyl bond, resulting in free leucine and glycylglycine (Peltier & Taylor, 1986). A total of 1.0 mL L-amino acid oxidase reagent (LAOR) [2 mg of horseradish peroxidase, 10 mg of *o*-dianisidine, and 20 mg of L-amino acid oxidase, added to 100 mL of 0.05 M Tris-HCl (pH 8.0)] was added to each 0.525-mL aliquot after the reaction was quenched. Color was developed for 20 min at 37 °C; the color reaction was quenched by the addition of 0.74 mL of 50% sulfuric acid. Optical densities (530 nm) of the samples were read on a Perkin-Elmer Lambda I spectrophotometer equipped with a Super Sipper and MultiSampler Rack. The quantity of leucine produced, in nanomoles, was determined by comparison to a leucine standard curve. Rates of liberation of product were calculated by linear regression.

Progress curves (Figure 1) were traced using a quadratic fit, all of which had r^2 values greater than 0.98, and tangents were taken to obtain velocities.

Effect of Photoirradiation on LAP and Azidobestatin. The effects of photoirradiation on LAP and pAB were determined in separate experiments. All photoirradiation was performed at 254 nm in a Rayonet photochemical reactor consisting of 16 2537 A bulbs. To determine the susceptibility of LAP to UV-induced damage, 0.2 mL of LAP (9.3 × 10^{-5} M subunits) in a solution of 0.05 M Tris-HCl (pH 8.0) and 10^{-5} M $ZnCl_2$

was placed in capped 1-mL cuvetts. The sample was irradiated for 0–120 s. At designated times during the photoirradiation, 10-μL samples were removed and added to 240 μL of a solution of 0.05 M Tris-HCl (pH 8.0) and 10^{-5} M $ZnCl_2$. To avoid substrate depletion during the assay, the samples of photoirradiated LAP were further diluted (9.3×10^{-9} M subunits, final concentration) and were assayed using 5.0 mM LeuGlyGly (final concentration). Use of a nitrogen atmosphere provided no extra protection of the enzyme against UV-induced damage during the irradiation.

The effect of photoirradiation on the decomposition of azidobestatin was followed spectrophotometrically. A 0.2-mL sample of 37 mM azidobestatin was placed in a capped quartz tube and irradiated as above; 13.5-μL aliquots were taken at various times and were diluted (5×10^{-5} M pAB final concentration) in 10 mL of a solution of 0.05 M Tris-HCl (pH 8.0) and 10^{-5} M $ZnCl_2$. The diluted samples were scanned on a Beckman DU-7 Scanning Spectrophotometer, and the plots were overlaid.

Competition between Azidobestatin and Bestatin for the LAP Active Site. Fractional amounts of bestatin were added to samples containing LAP and azidobestatin before photolysis (Figure 2). The resultant percent of inhibition was plotted vs the number of bestatin equivalents to determine the ratio of binding of bestatin and azidobestatin to the LAP active site. A 3.0×10^{-8} M solution of LAP was incubated for 24 h at 4 °C with 1.2×10^{-6} M azidobestatin and 0, 0.6×10^{-6}, 1.2×10^{-6}, and 2.4×10^{-6} M bestatin. LAP was also incubated with 1.2×10^{-6} M bestatin without azidobestatin. All samples were photoirradiated for 20 s and then exhaustively dialyzed for 72 h vs a solution of 0.05 M Tris-HCl (pH 8.0) and 10^{-5} M $ZnCl_2$. Activity assays were carried out with 5 mM LeuGlyGly (final concentration).

Protection of LAP during Dialysis and Photolysis. A variety of experiments in this laboratory have shown that many small molecules offer protection to proteins against damage during storage and dialysis (Blondin & Taylor, 1987). To determine if the presence of bestatin protects LAP from photolytic damage, three samples of 3.0×10^{-8} M LAP were incubated overnight (4 °C, in the dark) with either 2.3×10^{-6} M pAB, 2.3×10^{-6} M bestatin, or no additions. Following the incubation, aliquots of each sample were assayed for activity using 5 mM LeuGlyGly as above, either directly, after dialysis (72 h, 4 °C, in the dark), or after photoirradiation (20 s) followed by dialysis vs a solution of 0.05 M Tris-HCl (pH 8.0) and 10^{-5} M $ZnCl_2$ (72 h, 4 °C, in the dark).

Photoaffinity Labeling of LAP with [^{3}H]Azidobestatin. Photoaffinity labeling was done with two preparations of [^{3}H]pAB. A 6.11×10^{-6} M solution of LAP (final concentration of subunits) was incubated for 48 h with 0.1–10 equiv of [^{3}H]pAB, followed by photoirradiation for 10 s. The photoirradiated sample was then exhaustively dialyzed vs a solution of 0.05 M Tris-HCl (pH 8.0) and 10^{-5} M $ZnCl_2$ for 72 h. The second preparation of [^{3}H]pAB had a specific radioactivity (3.29 mCi/mmol) which was about 10% of the first batch. Thus, for experiments which went beyond trypsinization, the ratio of label to enzyme was increased to 1:1.

Determination of the Labeled LAP Fragment. Tryptic digestion of the labeled LAP was performed according to Cuypers et al. (1982). To 0.18 mL of the labeled LAP mixture was added 0.02 mL of trypsin (2.25 mg/mL). Trypsinization was allowed to proceed for 3 h at 37 °C. It was stopped by the addition of 2× Laemmli (1970) sample buffer (1:1), and the solution was boiled for 2 min. Thirty-microliter samples were run on separate 10% SDS–PAGE at 150-V constant voltage. After electrophoresis, one gel was stained with Coomassie blue and then destained in 5:5:1 H_2O/MeOH/HOAc in a warm water bath for 6 h or until all of the background was removed. The destained gel was soaked in En^{3}hance for 1 h, rinsed, and soaked in cold water for 1 h before being dried and put in a cassette at −80 °C for 4 months for autoradiography. The second gel was cut into 1-cm pieces, each of which was incubated in a glass-counting vial with 1 mL of Soluene-350 and 9 mL of Hionic-Fluor (overnight, 25 °C) before being counted for tritium.

Reduction and Carboxamidomethylation. The methods were adapted from Melbye & Carpenter (1971). A total of 0.50 mL of 4.0 mg/mL [^{3}H]pAB-labeled blLAP in 0.1 M Tris (pH 8.5) was diluted to 1.4 mL with 0.1 M Tris (pH 8.5). To this was added 0.075 mL of 50 mg/mL EDTA (pH 10) and 0.90 g of urea. The solution was stirred until the urea was dissolved, and then the reaction vessel was blanketed with nitrogen and capped. A total of 0.025 mL of β-mercaptoethanol was injected into the solution, and the reaction was allowed to proceed with stirring for 3 h. After 3 h, 0.063 g of iodoacetamide was added to the reaction solution and the pH was monitored with pH strips. The pH was maintained at pH 8.5 with 1 M NaOH. The reaction proceeded at 25 °C for 30 min. It was then dialyzed against 0.025 M Tris (pH 8.0) and lyophilized.

Hydroxylamine Cleavage. This cleavage was done according to Balian and Bornstein (1977). This procedure was done twice, each time with the same result; 2.86 g of guanidine and 0.070 g of hydroxylamine were dissolved in 4.5 N LiOH at 0 °C. The pH was monitored and LiOH was added until pH 9.0 was reached, and the final volume was 5 mL. The mixture was heated to 45 °C in a water bath, and then 2.0 mL of this solution was added to 2.0 mg of lyophilized carboxamidomethylated LAP. The reaction vessel was shaken well to dissolve the protein and was then placed in a water bath at 45 °C. The pH was monitored with pH strips and maintained at 9.0 with 0.9 N LiOH. After 4 h, the reaction was quenched with 5 drops of 80% formic acid and the mixture dialyzed against 0.01 M Tris (pH 8.0) and then lyophilized overnight.

Approximately 400 μg of lyophilized hydroxylamine cleavage product was dissolved in 100 μL of 1× Laemmli buffer and boiled at 100 °C for 120 s. The sample was loaded into a 2.1-cm well on a 14 cm × 14 cm × 1.5 mm SDS–PAGE gel, which contained a 4% acrylamide stacking gel and 12% acrylamide separating gel. The gel ran at 30 mA constant amperage until the dye front reached 1 cm from the bottom of gel. The gels were stained and destained as noted above. The destained gel was soaked in destain containing 5% glycerol for 30 min, and then the lane corresponding to the HA-treated sample was cut into 5-mm pieces and placed into scintillation vials containing 1 mL of Soluene-350 and 9 mL of Hionic-Fluor. The gel slices were solubilized for 96 h, and the ^{3}H was counted in a Beckman LS5800 scintillation counter. These procedures were more difficult than the trypsin series since the specific radioactivity of the preparation of pAB used was only one-tenth of the level found in the preparation of pAB used for the trypsin experiments and because reaction conditions resulted in the loss of radiolabel.

Results

Synthesis of [^{3}H]-p-Azidobestatin and Slow, Competitive Binding of This Inhibitor to LAP. Successive conversion of bestatin to the *p*-nitro-, *p*-amino-, [^{3}H]-*p*-amino-, and finally to [^{3}H]-*p*-azidobestatin yielded a very photolabile (see below) inhibitor in reasonable overall yield. At each step of the

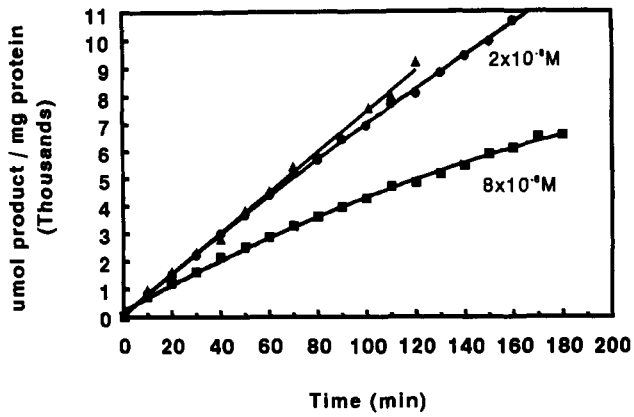


FIGURE 1: Slow binding of *p*-azidobestatin to LAP. LAP (4.26×10^{-10} M subunits) was added to 5.25 mM LeuGlyGly (final concentration 5 mM) containing 0, 2×10^{-8}, and 8×10^{-8} M azidobestatin. All reactions were carried out in a solution of 0.05 M Tris-HCl (pH 8.0) and 10^{-5} M $ZnCl_2$.

Scheme II

$$E + S \longrightarrow E + P$$
$$+$$
$$I \underset{k_4}{\overset{k_3}{<\!\!-\!\!-\!\!-\!\!-\!\!-\!\!-\!\!-\!\!-\!\!-\!\!>}} EI \underset{k_6}{\overset{k_5}{<\!\!-\!\!-\!\!-\!\!-\!\!-\!\!-\!\!-\!\!-\!\!-\!\!>}} EI^*$$

(fast: k_3, k_4; slow: k_5, k_6)

synthesis, the presence of critical functional groups, purity, and yield of the compound were confirmed by spectrophotometric and chromatographic techniques (see Materials and Methods). The wavelength of maximal absorbance, 249.2 nm, and the extinction coefficient, 17.8×10^3 M^{-1} cm^{-1}, are almost identical to the values for the pN_3Phe (Semeriva & Gratecos, 1982).

To try to simulate physiological conditions for the activity assay and to avoid potential confusion caused by using aminoacyl nitroanilides (Taylor et al., 1979), a tripeptide, LeuGlyGly, was used as substrate. During the time of these assays, thin-layer chromatography and amino acid analysis indicated that the GlyGly liberated upon the hydrolysis of LeuGlyGly is hydrolyzed to an insignificant extent (Peltier & Taylor, 1986). As compared with the linear appearance of product with increasing time in the absence of inhibitor (Figure 1, upper line), the rate of hydrolysis of LeuGlyGly prior to achieving the steady state is progressively attenuated with increasing time of contact of LAP with pAB (Figure 1, lower curves). The time of onset of inhibition and extent of inhibition was related to the concentration of inhibitor present. The linearity of the uninhibited assay indicates that, under the conditions used, there is no significant loss of zinc and no inactivation of the enzyme.

Use of pre-steady-state and steady-state data indicates that bestatin is a competitive inhibitor of LAP-catalyzed hydrolysis of LeuGlyGly. Similar kinetic tests indicate that pAB is also a competitive inhibitor of this reaction. Consequently, data was analyzed using data treatment appropriate for slow-binding competitive inhibitors (Cha, 1976; Morrison & Walsh, 1988).

Establishment of steady state for this competitive inhibitor has been described as follows: Dissociation constants and individual rate constants for the reaction described in Scheme II were obtained using a combination of pre-steady-state and steady-state reaction conditions. For reactions containing 2×10^{-8} M and 8×10^{-8} M pAB, $t_{1/2}$ was 60 min and 38 min, respectively. The dissociation constants are for the initial and final complexes, $K_i = 10^{-8}$ M, $K_i^* = 4 \times 10^{-9}$ M, respectively, and $k_5 = 3 \times 10^{-4}$ s^{-1} and $k_6 = 1.3 \times 10^{-4}$ s^{-1}. If the diffusion-controlled rate is assumed to be 10^7 M^{-1} s^{-1}, then k_4 is approximately 5×10^{-2} s^{-1}. It is clear that the transformation of EI to the tighter bound complex, EI*, and the dissociation of EI* is a relatively slow process. These rate constants were corroborated when pre-steady-state conditions (Cha, 1976) were used to obtain k_{on} ($=k_3k_5$) = 2.4 (±1.3) $\times 10^3$ M^{-1} s^{-1}.

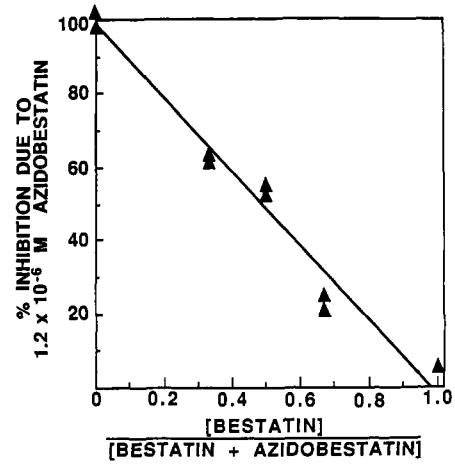


FIGURE 2: pAB and bestatin compete for the same binding site on LAP. LAP (3.0×10^{-8} M) was incubated overnight in the dark at 4 °C with 1.2×10^{-6} M azidobestatin and 0, 0.6×10^{-6}, 1.2×10^{-6}, or 2.4×10^{-6} M bestatin. The samples were all photolyzed for 20 s and then dialyzed in the dark (72 h, 4 °C) vs a solution of 0.05 M Tris-HCl and 10^{-5} M $ZnCl_2$, pH 8.0. Activity was determined on the basis of the hydrolysis of LeuGlyGly and is expressed as the percent of inhibition due to 1.2×10^{-6} M pAB. All assays gave linear plots for the evolution of product vs time.

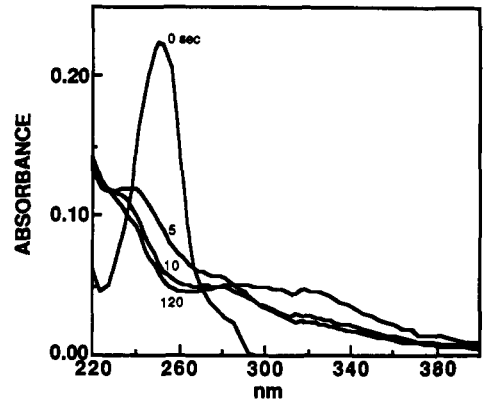


FIGURE 3: Absorption spectrum of azidobestatin before and after photolysis. A 1.0-mL sample of 1.30×10^{-5} M azidobestatin in a solution of 0.05 M Tris-HCl (pH 8.0) and 10^{-5} M $ZnCl_2$ was placed in a capped quartz tube and photoirradiated for 0, 5, 10, and 120 s in a Rayonet photochemical reactor consisting of 16 2537 A bulbs. After each time period of photoirradiation, an absorption spectrum of the sample was obtained. The concentration of azidobestatin was calculated on the basis of $\epsilon_{249.2nm} = 1.78 \times 10^4$ M^{-1} cm^{-1}.

The low dissociation constant of pAB suggested that labile derivatives of this transition state analogue would be a more effective label for the active site than peptide analogues, all of which have dissociation constants which are several orders of magnitude higher (Taylor et al., 1981; Escher et al., 1974).

Competition between pAB and Bestatin. When the extent of irreversible inhibition of LAP by pAB was tested in the absence or presence of various amounts of bestatin and the results were presented as the percent of inhibition due to pAB versus the fraction of [bestatin]/([bestatin] + [pAB]), a linear relationship with an intercept of unity was obtained (Figure 2).

Photoaffinity Labeling and Stability of LAP. Absorption spectra for azidobestatin before and following increasing times

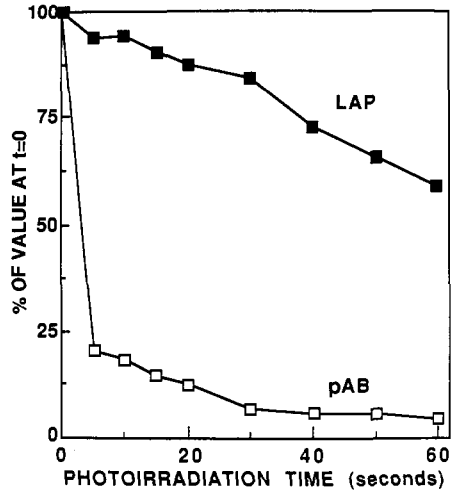


FIGURE 4: Effect of photolysis on LAP and azidobestatin. Two individual experiments were carried out, and the results are presented in one plot. A 6.11×10^{-7} M concentration of azidobestatin (25 °C, in a solution of 0.05 M Tris-HCl (pH 8.0) and 10^{-5} M $ZnCl_2$) was photoirradiated for 0–360 s. The optical densities at 249.2 nm after various times of photoirradiation were recorded and are presented as a percentage of the value at $t = 0$ (lower curve). A sample of LAP [9.3×10^{-6} M subunits, 25 °C, in a solution of 0.05 M Tris-HCl (pH 8.0) and 10^{-5} M $ZnCl_2$] was also photolyzed. Aliquots were removed, and activity (nanomoles per minute) was determined as indicated in the Materials and Methods section. Activity is presented as a percentage of the value at $t = 0$ (upper curve). The values at 4 min of photolysis for both LAP and azidobestatin were used as the baseline for the calculations of the percentage of the value at $t = 0$.

of photoirradiation are shown in Figure 3. A strong absorbance maximum is observed at 249.2 nm ($\epsilon = 1.78 \times 10^4$ M^{-1} cm^{-1}) for azidobestatin prior to photoirradiation (Figure 3, upper curve). Irradiation of azidobestatin resulted in a rapid and progressive decrease in absorbance at 249.2 nm and an increase in absorbance at 317 nm. Similar spectral changes were observed upon irradiation and decomposition of *p*-azidophenylalanine (Semeriva & Gratecos, 1982). Thus, the reduction in absorbance at 249.2 nm was considered to indicate the decomposition of the azido group in pAB, presumably with concomitant formation of a highly reactive nitrene (Semeriva & Gratecos, 1982). $t_{1/2}$ for this reaction was approximately 3 s. After 5 and 10 s of photoirradiation, 79% and 95.6% reductions in absorbance were observed, respectively. There was little further change in absorbance at 249.2 nm after 10 s and no change after 120 s of photoirradiation. Azidobestatin was similarly photolabile when it was photoirradiated in the presence of LAP. When bestatin was photoirradiated under these same conditions, no change in the absorption spectrum was observed (data not shown).

Photoirradiation of blLAP (Figure 4, upper curve) results in a steady decrease of measured activity, from 100% at $t =$ 0 to approximately 65% activity remaining at 60 s of photoirradiation. Less than 5% LAP activity remained after 4 min of photolysis. For comparison, data regarding the photoactivation of pAB are also plotted in this figure (Figure 4, lower curve). The $OD_{249.2}$ at 4 min was used in the calculation of the percent of photoactivation of azidobestatin (since after this time no significant change in $OD_{249.2}$ was observed). A 10-s period of photoirradiation was selected, since it maximized the effect of photolysis on azidobestatin in the presence of LAP (82% reduction at $OD_{249.2}$) yet only resulted in a 6% loss of LAP activity.

Prior work indicated that the presence of certain small molecules can enhance the stability of proteins, including LAP, under photooxidative stress (Blondin & Taylor, 1987; Gratecos et al., 1982). In order to determine the magnitude of the irreversible inhibition which resulted due to photoaffinity labeling, it was necessary to quantify the protective effect of inhibitor on LAP which was identically treated except for the photoirradiation. The specific activity of LAP that has been untreated is 26.0 mmol/(min·mg) (Table I). Incubation with 2.3×10^{-6} M bestatin or azidobestatin results in 97% inhibition. Activity is fully or almost fully restored upon removal of the bestatin [26.1 mmol/(min·mg)] or pAB [22 mmol/(min·mg)] by dialysis. In contrast, there was a 28% loss of activity upon dialysis of LAP which did not originally have the inhibitor present. It is plausible that the presence of the bestatin stabilizes LAP against adventitious removal or substitution of metal ions required for structural stability and/or catalytic function (Allen et al., 1983), or offers protection against oxidation and denaturation. When LAP was incubated with azidobestatin, photoirradiated, and then dialyzed, only about half of the activity was recovered.

Table I: Effect of Bestatin and Azidobestatin on Leucine Aminopeptidase Activity before and during Photoirradiation[a]

	activity [mmol/(min·mg)]		
	LAP	LAP + B	LAP + pAB
no treatment	26.0	0.9	1.1
dialysis	18.7	26.1	22.0
photoirradiation + dialysis	17.5	24.8	12.7

[a] LAP (3.0×10^{-8} M LAP subunits) was incubated overnight (4 °C, in the dark) with either 2.3×10^{-6} M azidobestatin (LAP + pAB), 2.3×10^{-6} M bestatin (LAP + B), or no additions (LAP) in a solution of 0.05 M Tris-HCl (pH 8.0) and 10^{-5} M $ZnCl_2$. These samples were subjected to three treatments and were assayed for activity using as substrate 5 mM LeuGlyGly in the same buffer. Treatments: no treatment, samples were assayed following the incubation; dialysis, samples were dialyzed vs a solution of 0.05 M Tris-HCl (pH 8.0) and 10^{-5} M $ZnCl_2$ (72 h, 4 °C, in the dark) before assaying for activity; photoirradiation + dialysis, samples were photolyzed for 20 s and then dialyzed as indicated above prior to assaying for activity. All protein concentrations were determined using the Bio-Rad assay.

Cleavage of LAP with Trypsin and Hydroxylamine before and after Reaction with [^{3}H]pAB. LAP appears on SDS–PAGE as a single band representing the monomer of 54 kDa (Figure 5A, lane a) (Carpenter & Harrington, 1972; Wallner and Taylor, submitted for publication. After trypsinization, little of the 54-kDa monomer band remains and two new bands with apparent masses of 36 and 18 kDa are observed (lane b). Trypsinization of LAP labeled with [^{3}H]pAB also results in two major bands when run on SDS–PAGE and stained with Coomassie blue dye (Figure 5B, lane a). These bands have molecular masses which are indistinguishable from those observed when unlabeled LAP is trypsinized (Figure 5A, lane b).

Autoradiography (Figure 5B, lane b) of the trypsinized [^{3}H]pAB-labeled LAP indicates that almost all of the radioactivity is in the large tryptic fragment, since only that band is obvious on the autoradiogram. This is corroborated by the recovery of 81% of the radioactivity applied to the gel in the gel slice corresponding to the large tryptic fragment (data not shown). Similar results were obtained in multiple experiments.

Cleavage of LAP with hydroxylamine was about 50% effective and resulted in two fragments. The larger fragment (~39 kDa) had a molecular mass slightly larger than the large tryptic fragment, and the smaller hydroxylamine fragment (~17 kDa) was slightly smaller than the small tryptic fragment (Figure 5A, lane c). Treatment with hydroxylamine of LAP which had been reacted with [^{3}H]pAB resulted in significant loss of radioactivity associated with the enzyme as determined by scintillation data from solubilized gel slices.

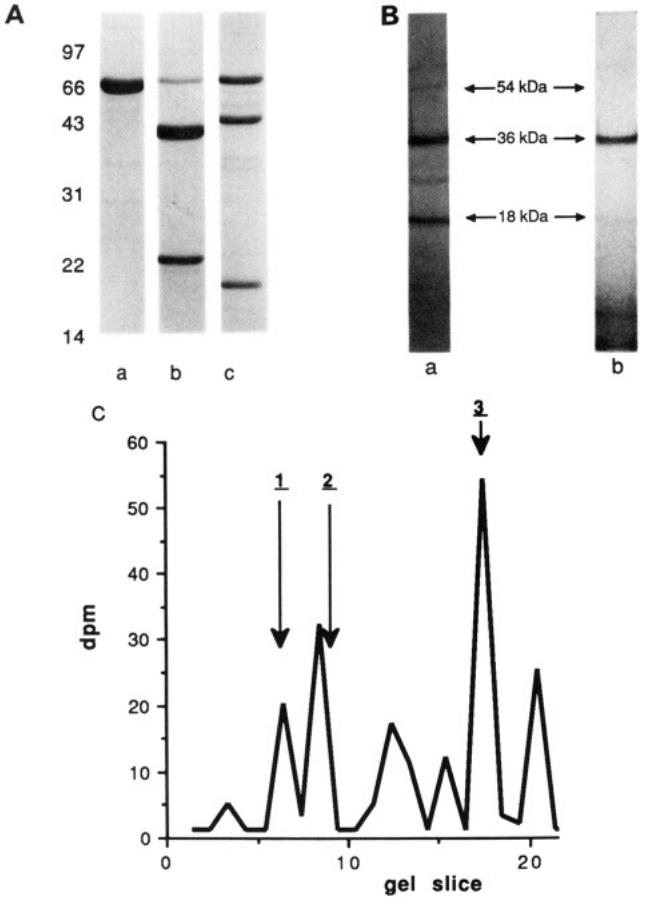


FIGURE 5: SDS–PAGE of LAP before and after reaction with trypsin, hydroxylamine, and [^{3}H]azidobestatin. (A) SDS–PAGE of LAP before and after reaction with trypsin and hydroxylamine. The sample was run on a 12% SDS–PAGE running gel with a 4% stacking at 150 V at constant current. Molecular mass standards from Bio-Rad: phosphorylase *b*, 97.2 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 42.7 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa. Lane a: LAP (10 μg). Lane b: LAP (20 μg) which had been treated with trypsin. Lane c: LAP (20 μg) which had been treated with hydroxylamine. (B) SDS–PAGE of LAP photolabeled with [^{3}H]pAB and then trypsinized. Lane a: Coomassie blue stained gel. Lane b: the gel was stained with Coomassie blue, destained completely, soaked in En^{3}Hance (1 h), rinsed and soaked in cold water (1 h), dried, and autoradiographed (−80 °C, 4 months). A total of approximately 11 000 counts was applied to the gel. (C) Plot of radioactivity in gel slices of [^{3}H]-*p*-azidobestatin-labeled LAP treated with hydroxylamine. LAP and ^{3}[H]-*p*-azidobestatin were combined and photoirradiated as indicated in the Materials and Methods section. The sample was dialyzed, lyopholized, and reconstituted with 1× Laemmli buffer prior to being applied to 12% SDS–PAGE. In order to diminish background radioactivity, the gel was stained and destained as usual prior to being cut into 5-mm pieces. Radioactivity in the gel slices was determined using scintillation counting (see Materials and Methods). The remaining average background radioactivity was 100 dpm/slice. This was subtracted from each slice value prior to preparation of the plot shown. The numbers 1, 2, and 3 refer to the position on duplicate gels where the uncleaved, large HA fragment, and small HA fragment are found, respectively.

Nevertheless, on a per milligram basis, the small HA fragment contained over 4 times the radioactivity associated with it as compared with the large HA fragment (Figure 5C). Similar results were obtained in two separate experiments.

Discussion

Early attempts to identify residues of LAP which comprise the active site by covalent attachment of a reporter group relied on an enzyme-bound nucleophile to displace nucleophilically-labile halides in substrate analogues. Aminoacyl chloromethyl ketones (Akhtar & Birch, 1972) and a variety of aminoacyl aminobenzenesulfonyl fluorides (Taylor et al., 1981) were effective reversible LAP inhibitors, but none of these compounds were covalently attached to specific portions of the enzyme. Reagents which react with specific amino acids (Carpenter & Vahl, 1973) also proved unrevealing. Alternate substrates were constructed to probe active sites, including the role of the metal ions (Taylor et al., 1982b) of aminopeptidases, but these did not indicate the desired active site information.

These data indicated that an acyl enzyme intermediate was probably not formed. This was corroborated by isotope exchange data using bestatin (Anderson et al., 1985). Thus, it appeared that other inhibitors which required an enzyme nucleophile to covalently bind to LAP would probably be without effect.

Prior inhibition tests indicated that there is considerable space in the hydrophobic binding site of blLAP (Taylor et al., 1981, 1982b; Hanson & Frohne, 1976). Azidophenylalanyl derivatives were employed as photoaffinity labels of hog kidney aminopeptidases, but labeling was nonspecific (Gratecos et al., 1982). This suggested, however, that a photolabile functional group could be incorporated into the phenylalanyl portion of a substrate or substrate analogue and that with judicious alteration of the substrate a selective and effective photoaffinity label for aminopeptidases might result.

Inhibitors with lower K_i offered the possibility of enhanced selectivity. Bestatin incorporates the structural and chemical elements of a putative transition-state analogue (Nishizawa et al., 1977), and it is not surprising that the K_i of this compound is approximately 10^5 lower than the K_m or K_i for peptides or peptide derivatives (Taylor et al., 1981; Hanson & Frohne, 1976). A survey of the current literature indicates that bestatin-type compounds are the most tightly bound substrate analogues to aminopeptidases. Incorporation of the statine moiety potentially offered significant enhancement in specificity.

The successful synthesis of a molecule which retains avid relatively slow binding of bestatin to blLAP (Figure 1) and which incorporates a photolabile azido functional group is described. The close resemblance of the photochemical properties of this compound with those of azidophenylalanyl derivatives (Semeriva & Gratecos, 1982) corroborates the success of incorporation into bestatin of the photolabile moiety.

The curvilinear downward shape of the line which traces liberation of product when pAB is included with substrate indicates that pAB is a slowly bound inhibitor of LAP (Figure 1). The $t_{1/2}$ is approximately 60 min in the presence of 2×10^{-8} M pAB and 38 min in the presence of 8×10^{-8} M pAB. Other kinetic and binding constants for pAB are also similar to the same measures regarding the interaction of bestatin or *p*-nitrobestatin and blLAP and for bestatin with hog kidney LAP (Taylor et al., manuscript in preparation). The ability to restore full activity when inhibitor is removed by dialysis indicates that the binding of pAB to LAP in the dark is completely reversible. The same is true for bestatin. The linear evolution of product in the absence of inhibitor indicates that substrate is not limiting under the conditions used for the assays (data not shown).

Kinetic and preliminary crystallographic data indicated that 1 bestatin is bound per subunit (Taylor et al., submitted for publication; Burley et al., 1992). The identity of the kinetic parameters for pAB and bestatin suggests that the same would hold for the photoaffinity label.

The linear relationship between the percent of inhibition due to azidobestatin and the intercept of 1 indicates that bestatin

and pAB compete for the same site (Figure 2). The clear competition between physiological peptide substrates, bestatin, and pAB suggests that each of these occupies the usual physiologial peptide binding site on the enzyme. Although hog kidney LAP is structurally very similar to blLAP (Taylor et al., 1984a,b), prior reports indicated that only one bestatin is bound per hog kidney LAP molecule (Wilkes & Prescott, 1985). Since the hog LAP is comprised of 6 identical protomers (Taylor et al., 1984b), the apparent discrepancy will be reexamined using X-ray diffraction.

Since the subunits of blLAP are found organized as hexamers under the conditions used for photoirradiation, the occupancy of each site by a pAB enhanced the chances of incorporation of label into the protein. The basis for the selection of an optimal photoirradiation time is to determine the time at which the labilization of azidobestatin is maximal yet the LAP inactivation due to photolysis is minimal. The data in Figure 4 and the calculation of $t_{1/2}$ of approximately 3 s for photoactivation of pAB indicated that at 10 s there would be almost total photoactivation of pAB with minimal photoinactivation of LAP. Loss of LAP activity under these conditions would be due to incorporation of the photoaffinity label. Prior to this evaluation, it was necessary to identify effects of the inhibitor during processing of the enzyme (in the dark).

Many small molecules confer some stabilization on proteins during photolysis, and it appeared that bestatin does the same (Table I). When the activities before and after photoirradiation of LAP after dialysis with and without bestatin are compared, it is evident that the presence of bestatin affords virtually full protection against the loss of activity usually encountered upon this prolonged dialysis. Significant inactivation after photolysis of LAP in the presence of pAB indicated that irreversible modification occurred. Enzymatic and chemical fragmentation was then used to determine which portions of the enzyme contained the label.

Although there are many theoretical trypsin cleavage sites on LAP, only one of these appears to be accessible (Cuypers et al., 1982). Trypsinization of LAP results in almost quantitative production of a large carboxyl-terminal fragment with an apparent mass of 37 kDa (residues 138–487). A smaller, amino-terminal fragment (residues 1–137) has a mass of 18 kDa (Figure 5A, lane b) as determined by SDS–PAGE. Hydroxylamine also results in a unique cleavage of LAP between residues 322 and 323 (Cuypers et al., 1982).

The location of radioactivity of azidobestatin in the large tryptic fragment indicates that LAP binds pAB in this region. The small hydroxylamine fragment (residues 323–487) is found within the large tryptic fragment. Subsequent identification of radioactivity in the small hydroxylamine fragment (Figure 5C) confirmed the trypsin experiments and showed that amino acids involved in binding the hydrophobic side chains of substrates are found in the carboxyl-terminal 15 kDa of the enzyme.

Recent X-ray diffraction studies of LAP with bestatin located the zinc-binding residues within the protomer (Burley et al., 1990) and proposed a substrate binding site (Burley, submitted for publication). The two Zn^{2+} are bound by Asp 255, Asp 273, Asp 332, and Glu 334, and the scissile peptide bond may interact with Lys 250 and Arg 336. Many of these are not found within the carboxyl-terminal hydroxylamine fragment of LAP. In contrast, LAP residues which appear to be most closely associated with the binding pockets for S1′ are Asn 330, Ala 333, and Ile 421 and for S1 are Met 270, Thr 359, Gly 362, Ala 451, and Met 454. All but Met 270 are found in the carboxyl-terminal hydroxylamine fragment. The location of Met 270 close to the ortho position of bestatin suggests that it might be less accessible to reaction with the nitrene. Thus, the data presented here are consistent with our proposed location of the active site as determined crystallographically.

Since its discovery in 1976 there have been over 200 publications which relate bestatin and physiological functions, usually via aminopeptidase activity. The following aminopeptidases are inhibited by bestatin: Arg (McDermott et al., 1988), M (Ward et al., 1990; Rich et al., 1984), B (Nishizawa et al., 1977; Harbeson & Rich, 1988), W (Gee & Kenny, 1987), cell surface AP (Aoyagi et al., 1976), AP from erythrocytes (Abramic & Vitale, 1989), AP from cornea (Sharma & Ortwerth, 1987), AP from *Aeromonas* (Wilkes & Prescott, 1985), and lens AP III (Sharma & Ortwerth, 1986), but not Met AP (Freitas et al., 1985) or *Xanthomonas* AP (Osada & Isono, 1986). Bestatin inhibits degradation of endogenous liver proteins (Botbol & Scornik, 1991), enkephalin [most recently treated in Suh and Tseng (1990) and Benter et al. (1990)], vasopressin (Johnson et al., 1984), melanotropin (Hui et al., 1983), bradykinin (Proud et al., 1987), angiotensin (Abhold et al., 1987), leukotriene (Hui et al., 1983), and α-bag cell peptide (Squire, et al., 1991). Dipeptide transport (Tomita et al., 1990), immune function and cancer (Umezawa, 1980), drinking response (Jensen et al., 1989), insulin- and EGF-induced DNA synthesis (Takahashi et al., 1985, 1989), DNA polymerase α and deoxynucleotide transferase activities (Leyhausen et al., 1984), and SAC- or PMA-stimulated B-cell proliferation (Morikawa et al., 1989) are all attenuated by bestatin.

Recent molecular genetic and biochemical experiments indicate that human, hog and beef lens LAP are similar structurally (Taylor et al., 1984a,b) and mechanistically (Oettgen & Taylor, 1985; Taylor & Nakamian, unpublished data), and that these enzymes and the *xerB* gene product, aminopeptidase I and aminopeptidase A, prolyl aminopeptidase, etc., form a mechanistically similar family of peptidases (Wallner and Taylor, submitted for publication). Furthermore, several aminopeptidases have binding constants for this inhibitor which are similar to that noted here. Since pAB can be expected to inhibit these enzymes in a similar fashion to blLAP, it is likely that pAB or related molecules can be used for further study of the mechanisms of action of the relevant peptidases. Given the diverse physiological functions which are affected by bestatin, it is also likely that pAB can be exploited for pharmacological purposes and elucidation of the physiological phenomena mentioned above.

Acknowledgments

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Structural Studies of the Acidic Transactivation Domain of the Vmw65 Protein of Herpes Simplex Virus Using ^{1}H NMR

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ABSTRACT: We have overproduced and purified the carboxy-terminal transactivation domain of Vmw65 (VP16) of herpes simplex virus, and studied potential folding of the domain by ^{1}H NMR. Two species of the acidic domain were obtained from the bacterial expression system, and we demonstrate that one of these represents read-through of the natural amber termination codon of the Vmw65 reading frame producing a larger polypeptide. Additional residues in the read-through product were identified by total amino acid analysis and by NMR. Study of the correctly terminated product by 1D NMR gave resonances which were clustered into groups around their random-coil chemical shift positions, and 2D NMR demonstrated that, even in mixed solvents containing up to 80% MeOH, there was very little evidence of secondary structure. Together these results indicate that the isolated acid domain has little if any α-helical content of any stable nature. We discuss these results with reference to the demonstrated activity of the acidic domain in a wide variety of polypeptide contexts.

Vmw65 (VP16) is a structural protein of herpes simplex virus (HSV) which is packaged into the tegument region of the virion at approximately 400-600 molecules per virion (Heine et al., 1974). In addition to its structural role, Vmw65 is a regulatory protein (Post et al., 1981; Campbell et al., 1984) which selectively induces the transcription of HSV immediate-early (IE) genes after virus infection by a complex interaction with at least two cellular factors including Oct-1 (McKnight et al., 1987; Kristie & Roizman, 1987; Gerster & Roeder, 1988; O'Hare & Goding, 1988; Stern et al., 1989; Preston et al., 1988) and CFF (VCAF-1, C1/C2) (Xiao & Capone, 1990; Kristie et al., 1989; Katan et al., 1990). A transcription complex containing these three proteins is selectively assembled on IE-specific, cis-acting regulatory elements (the TAATGARAT motif) present upstream of each IE gene (Mackem & Roizman, 1982a,b; Preston et al., 1984; Whitton et al., 1983; Treizenberg et al., 1988a; O'Hare & Hayward, 1987). Requirements within Vmw65 for transactivation are distinct and separable from those for complex formation (Treizenberg et al., 1988b; Greaves & O'Hare, 1989; Werstruck & Capone, 1989). Thus, removal of the acidic carboxy-terminal region of Vmw65 completely abrogates transactivation of IE genes but has no effect on complex formation. Moreover, this acidic region of Vmw65 will function in transcriptional activation when fused to a wide variety of different proteins, including, for example, the human estrogen receptor (Elliston et al., 1990), the chicken oncogene v-myb (Ibanez & Lipsick, 1990), and the yeast regulatory protein GAL4 (Sadowski et al., 1988; Cousens et al., 1989).

The carboxy-terminal region used in such "domain swap" experiments is highly acidic, containing 21 glutamic or aspartic residues and, from secondary structure predictions (Garnier et al., 1978), is proposed to form 2 helical regions separated by a poorly structured proline/glycine-rich loop. The prediction that this activation region of Vmw65 may adopt an α-helical conformation is consistent with the proposal of Ptashne and colleagues that acidic activation regions may have the general property of forming amphipathic helices having one hydrophilic face bearing the acidic residues, and one hydrophobic face (Ma & Ptashne, 1987; Giniger & Ptashne, 1987). However, although the activation regions of a number of other regulatory proteins are enriched in acidic residues (Hope et al., 1988; Hollenberg & Evans, 1988), there has been no direct examination of the physical structure of such regions and no analysis of the proposal for a requirement for α-helix formation.

Since the transcriptional function of the acidic region of Vmw65 is independent of the sequence of the DNA binding domain to which it is attached, we anticipated that any specification for folding would be entirely contained within the region itself. Here we report the absence of any major stable secondary structure of the domain and discuss this in light of current results from site-directed mutagenesis of the region and previous predictions on the requirement for α-helical formation for the function of acidic transactivation domains.

Materials and Methods

Plasmid Construction, Expression, and Purification. The coding region for the acidic carboxy-terminal 79 amino acids of Vmw65 protein of HSV-1 strain MP was fused in-frame

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