Inhibition of Bovine Lens Leucine Aminopeptidase by Bestatin: Number of Binding Sites and Slow Binding of This Inhibitor†

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ABSTRACT: Aminopeptidases catalyze the hydrolysis of amino acid residues from the amino terminus of peptide substrates. Their activity has been implicated in myriad fundamental biochemical and physiological processes, and alterations in aminopeptidase activity have been correlated with a variety of pathologies. Nevertheless, information about this group of proteases is less well developed. Bovine lens leucine aminopeptidase (blLAP) can be considered prototypical of many enzymes in this family of peptidases. It shows common features of (1) requiring divalent metal ions for activity, (2) having a relatively large size, and (3) having slow, relatively tight binding of bestatin, a transition-state analog of the substrate PheLeu. Bovine lens LAP is the only bestatin-inhibitable aminopeptidase for which structural and mechanistic data are available. However, full exploitation of these data required knowledge of the number of inhibitor molecules bound per subunit. Independent direct binding experiments and kinetic determinations indicate that one bestatin is bound per subunit in blLAP. K_i and K_i^* for formation of the initial and final complexes are approximately 1.1×10^{-7} and 1.3×10^{-9} M, respectively. The mode of binding is slow and competitive. The $t_{1/2}$ for formation and deformation of the final enzyme–inhibitor complex is approximately 30 and 22 min, respectively, with 10^{-8} M bestatin. To perform these measures, a new assay using physiological peptides (LeuGlyGly) as substrate was adapted. Taken together with prior NMR, photoaffinity labeling, and crystallographic data, these binding data allow us to propose a mechanism of the blLAP-catalyzed hydrolysis of peptides.

Aminopeptidases catalyze the hydrolysis of amino-terminal amino acids from the amino terminus of protein and peptide substrates. Many aminopeptidases are inhibited in a slow binding process (Cha, 1976; Morrison & Walsh, 1988) by bestatin [(2*S*,3*R*)-(3-amino-2-hydroxy-4-phenylbutanoyl)-L-leucine], require zinc ions for activity, and are oligomeric, and several appear to have common residues used for metal ion binding and substrate recognition (Wallner et al., submitted for publication; Stirling et al., 1989; Burley et al., 1992; Taylor, 1993). Since bovine lens leucine aminopeptidase (blLAP)[1] shares these characteristics and because there are more structural data regarding this aminopeptidase, it can be considered prototypical of the class of bestatin-inhibitable aminopeptidases. Despite the more advanced state of knowledge regarding LAP from bovine lens, there has been no independent determination of the stoichiometry of binding of substrates or inhibitors. This prohibited full exploitation of knowledge regarding the structure and composition of the active site to describe the binding process and to predict a mechanism of peptide hydrolysis. The situation is further complicated since other proposals regarding the stoichiometry of bestatin binding to homologous aminopeptidases (Wilkes & Prescott, 1985) were in contrast to information available regarding blLAP.

Catalysis by LAP is most effective when peptides and peptide analogs have an N-terminal Leu (Delange & Smith, 1971; Hanson & Frohne, 1976; Smith & Hill, 1960). However, LAP shows very broad specificity. Kinetic investigations indicate that different specificities may be observed when longer peptides are used (Taylor et al., 1981), and even prolyl aminopeptidase activity appears to be due to LAP (Turzynski & Mentlein, 1990). Thus, it was not surprising that LAP catalyzes the complete degradation of lens proteins to yield free amino acids (Taylor et al., 1982a). Activity of LAP has been found in most tissues, fluids, and organelles in a wide variety of species throughout the plant and animal kingdoms (Hanson & Frohne, 1976; Taylor et al., 1984a; Taylor, 1993a,b). The essentiality of aminopeptidase activity in vivo is indicated since inhibition by bestatin results in rapid intracellular accumulation of peptides (Botbol & Scornik, 1991). Similar experiments indicate that aminopeptidases are required for clearance of many types of peptides and hormones [reviewed in Taylor et al. (1992)]. Altered LAP activity has been associated with many physiological functions and conditions [see references in Taylor et al. (1992, 1993) and Wallner et al. (submitted for publication)]. The enzyme is comprised of six identical bilobated subunits; in its native state, each subunit contains two zinc ions (Hanson & Frohne, 1976; Taylor et al., 1979; Burley et al., 1992; Carpenter & Vahl, 1973; Melbye & Carpenter, 1971). The two zinc ions have been described as less and more readily exchangeable, and the exchange of each zinc with other metal ions has been demonstrated (Allen et al., 1983; Carpenter & Vahl, 1973; Thomson & Carpenter, 1976a,b). NMR measurements

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[1] Abbreviations: bl, bovine lens; hk, hog kidney; LAP, leucine aminopeptidase, LAOR, L-amino acid oxidase reagent; LeuGlyGly, leucylglycylglycine; LβNA, leucyl-β-naphthylamide; LpNA, leucyl-*p*-nitroanilide; SEM, standard error of mean; Tris, tris(hydroxymethyl)-aminomethane.

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indicate that substrates bind within a hydration sphere of the readily exchanged metal ion (Taylor et al., 1982b), but affinity-labeling experiments imply that an acyl enzyme is not formed (Taylor et al., 1981).

Bestatin, a dipeptide analog of PheLeu, is one of the most potent inhibitors of many aminopeptidases, including leucine aminopeptidase (Umezawa et al., 1976). It has been suggested that bestatin mimics the tetrahedral intermediate of hydrolysis, using the C-2 OH to form a complex with the active-site Zn^{2+} (Nishizawa et al., 1977) of these aminopeptidases.

In this work, kinetic constants for the slow binding of bestatin to LAP are described, and the number of bestatin molecules bound per LAP molecule, resulting in 100% inhibition, are indicated by two methods. To monitor activities, a new assay which employs physiological peptides as substrates was adapted. On the basis of this stoichiometry and available kinetic and structural data, a mode of binding of substrates and a mechanism of peptide hydrolysis are presented.

MATERIALS AND METHODS

Materials. L-Leucylglycylglycine was purchased from Chemical Dynamics Corp. L-amino acid oxidase (crude dried venom from *Crotalus adamateus*), *o*-dianisidine, and horseradish peroxidase were purchased from Sigma Chemical Co. All other chemicals were of analytical grade. Bestatin was either synthesized following the protocol of Suda et al. (1976a,b) or obtained as a kind gift from Dr. Umezawa. Reagents for the Coomassie blue dye binding assay were purchased from Bio-Rad.

Enzyme Preparation. Bovine lens LAP was isolated from fresh, young bovine lenses, which were removed from eyes within 2 h of sacrifice (Hanson & Frohne, 1976; Burley et al., 1990). The purified blLAP was stored at 4 °C, in 0.05 M Tris-HCl (pH 8.0) containing 10^{-5} M $ZnCl_2$.

Protein concentrations were determined by using either OD_{280}, the Bradford dye binding assay (1976) using γ-globulin as standard, or amino acid analysis. Prior to amino acid analysis, blLAP was hydrolyzed with gas-phase HCl in sealed evacuated tubes. Amino acids were separated on a 15-cm C_{18} PicoTag HPLC column, using a Waters PicoTag HPLC system, and quantified using the PicoTag method (precolumn derivatization with phenyl isothiocyanate). Calculations of concentrations were based on amounts of alanine and methionine, and were corroborated by levels of many other amino acids. The level of specific amino acids in blLAP was assumed to be the average of the two published amino acid compositions of the enzyme (Hanson & Frohne, 1976). This average was in good agreement with the composition deduced from the gene sequence (Wallner et al., 1987).

The dye binding assay gave values for blLAP which were 3-fold higher than concentrations indicated by amino acid analysis. Concentrations of blLAP obtained by OD_{280} readings and concentrations indicated by amino acid analysis were indistinguishable.

Amino acid analysis was also used to determine the concentration of hog kidney cytosolic LAP because chromatographic experiments indicated that commercial preparations of the hk enzyme have a chromophore that absorbs at 280 nm, which is unlikely to be protein. Since the amino acid compositions of hk- and blLAP are very similar (Taylor et al., 1984a; Wallner et al., submitted for publication), the amino acid analysis published for the bl enzyme was used to estimate the composition of the hk enzyme. Concentrations of hkLAP determined by dye binding and OD_{280} as opposed to amino acid analysis were 3.1-fold and 9.6-fold higher than indicated by amino acid analysis, and these ratios varied considerably with the lot of hk enzyme. Concentrations of blLAP are given based on the monomer molecular mass of 54 000 g/mol (Carpenter & Harrington, 1972) unless otherwise indicated.

Thin-Layer Chromatography. In order to confirm that only the LeuGly bond was cleaved during the assay, thin-layer chromatography was employed using a solvent system of 2-propanol/NH_4OH/H_2O (8:2:1). Twelve micromoles each of leucine, leucylglycine, glycylglycine, and glycine and 10 μmol of LeuGlyGly were applied to a silica-coated glass plate (Baker). Twenty microliters of a mixture of blLAP and 5 mM LeuGlyGly (incubated overnight at 37 °C) was also applied. After completion of the chromatography, the silica plate was sprayed with a ninhydrin solution (50 mL of 0.2% ninhydrin in absolute ethanol + 10 mL of glacial acetic acid), and the color was developed upon drying in a drying oven.

Enzyme Assays. Bovine lens LAP was assayed at 37 °C using 0.05 M Tris-HCl buffer (pH 8.0) containing 10^{-5} M $ZnCl_2$. This was done in an attempt to maintain sufficient zinc in the active site (Carpenter & Vahl, 1973). Appropriately diluted enzyme was added to substrate at a ratio of 1:20 (diluted enzyme to substrate, v/v), and the reaction was quenched at specific time points by taking aliquots (0.525 mL) and boiling for 8 min. Hydrolysis of 5–10 mM LeuGlyGly was determined by following the appearance of free leucine using the LAOR assay of Nicholson and Kim (1975). LeuGlyGly concentrations above 10 mM showed substrate inhibition with blLAP and hkLAP. One milliter of LAOR (2 mg of horseradish peroxidase, 10 mg of *o*-dianisidine, and 20 mg of L-amino acid oxidase in 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. Then the color reaction was quenched by the addition of 0.74 mL of 50% sulfuric acid. Optical densities at 530 nm were determined on a Perkin-Elmer Lambda I spectrophotometer equipped with a Super Sipper and MultiSampler Rack. The quantity of leucine produced (nanomoles) was determined by comparison to a leucine standard curve.

Time Course of Inhibition and Determination of Kinetic Constants Using Pre-Steady-State and Steady-State Conditions. Two experiments were carried out to determine the time course of the inhibition of LAP by bestatin. The first progress curve (Figure 2, upper curve) was generated by the addition of 1 mL of blLAP (3.6×10^{-8} M subunits) to 20 mL of 10.0 mM substrate containing 1.1×10^{-8} M bestatin. The lower curve resulted from the addition of 1 mL of enzyme (3.6×10^{-8} M subunits blLAP preincubated at 37 °C for 30 min with 2.1×10^{-7} M bestatin) to 20 mL of 10.0 mM LeuGlyGly. The final conditions attained in both cases were 9.52 mM LeuGlyGly, 1.7×10^{-9} M blLAP subunits, and 10^{-8} M bestatin. Duplicate aliquots were taken from each reaction mixture every 10 min for 150 min, and the data were plotted as leucine produced versus time.

Other assays with lower concentrations of enzyme and higher concentrations of bestatin were used in order to approach steady state in less than 120 min and were used to corroborate the data shown in Figure 1. Assays were performed by (a) the addition of 2 mL of blLAP (1.7×10^{-10} M blLAP subunits final concentration) to 40 mL of 10.5 mM LeuGlyGly containing 1×10^{-8} M bestatin (final concentration) or (b) the addition of 2 mL of LAP (2.6×10^{-10} M blLAP subunits final concentration) to 40 mL of 10.5 mM LeuGlyGly containing 5×10^{-7} M bestatin (final concentration). The reactions proceeded by 120 min. Duplicate aliquots were taken every 10 min, and curves of nanomoles of product vs time were plotted. Reaction rates for the steady-state portions of the curves were determined by linear regression. Correlation coefficients of the lines calculated during different time

intervals were compared to determine the beginning of the steady-state portion for each curve generated. Kinetic constants were calculated for duplicate runs at both conditions and are presented as an average value.

Determination of Kinetic Constants under Pre-Steady-State Conditions. Pre-steady-state data were used to determine the type of inhibition and to corroborate the kinetic constants obtained by the methods noted above. Reactions were started by the addition of 0.8 mL of blLAP (final concentration 2.77×10^{-9} M LAP subunits) 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 h. Progress curves were plotted as leucine produced versus time.

Stoichiometry of Binding of Bestatin per Hexamer. Two methods were used to determine the ratio of bestatin bound per LAP hexamer. Each was done in duplicate and the experiment repeated at least twice. First, blLAP (1.3×10^{-8} M hexamers, 7.8×10^{-8} monomers) was titrated with inhibitor (0×10^{-8}, 2×10^{-8}, 4×10^{-8}, and 6×10^{-8} M) and the activity plotted as percent activity vs equivalents of bestatin (Wilkes & Prescott, 1985).

Next, a direct method to assess the ratio of bestatin to blLAP was employed. One-milliliter plastic tuberculin syringes were packed with a 1-mL bed of Sephadex G-50 superfine in 0.05 M Tris-HCl (pH 8.5)/10^{-5} M $ZnCl_2$. The columns were spun at 100*g* for 5 min at 4 °C, and the eluent (100 μL) was collected in a 1.5-mL microfuge tube. To ascertain the utility of the column, it was necessary to demonstrate that all free bestatin could be separated from blLAP and the blLAP–bestatin complex. Samples (100 μL) containing enzyme alone, bestatin alone, or enzyme saturated with bestatin were applied to the column after 100 μL of the buffer was removed. Over 98% of the protein was recovered in the void volume. Unbound bestatin was retained on the column until the seventh 100-μL fraction was eluted.

In order to quantify the number of sites occupied by bestatin on 100%-inhibited blLAP, 7.2×10^{-6} M blLAP subunits were incubated alone or with 5 mM bestatin in 0.05 M Tris-HCl/10^{-5} M $ZnCl_2$, pH 8.5. One hundred microliter samples were applied to columns containing 1 mL of Sephadex G-50, equilibrated in the same buffer without bestatin. One hundred microliter fractions of blLAP alone or blLAP–bestatin complex were obtained in the void. From the 100-μL fractions, four 10-μL samples were taken for protein determination (Bradford, 1976). Fifty microliters was diluted 100-fold in 0.05 M Tris-HCl/10^{-5} $ZnCl_2$, pH 8.5, incubated at 4 °C for 6 h, and then assayed in 5 mM LeuGlyGly as above. LAP activity was determined as initial velocities. The percent activity of the previously totally inhibited blLAP was calculated as the ratio of the activity of the blLAP-diluted bestatin solution to the activity of free blLAP (see below). This is then compared to a standard curve constructed from samples for which activities were determined for known amounts of bestatin and known amounts of blLAP.

A standard curve was prepared by incubating 9.3×10^{-8} M LAP (subunits) with 0, 1.54×10^{-8}, 3.08×10^{-8}, 4.62×10^{-8}, 6.17×10^{-8}, 7.7×10^{-8}, and 9.25×10^{-8} M bestatin for 6 h at 4 °C. These are concentrations of bestatin that would be expected after blLAP/bestatin mixtures were eluted from the spin column and diluted, indicating the occupation of zero through six sites on the LAP molecules. Activity was measured by adding 0.02 mL of 205 mM LeuGlyGly to 0.8 mL of diluted blLAP/bestatin mixtures. Aliquots (0.05 mL) were taken at 0, 5, 10, 15, and 20 min, and the amount of Leu liberated was determined as described above. The standard curve was constructed by plotting percent activity as a function of bestatin concentration. The slight difference in the amount of blLAP in the standards (9.3×10^{-8} M subunits) as opposed to the experimental samples 7.2×10^{-8} M (subunits) was not associated with differences in inhibition at fixed levels of bestatin.

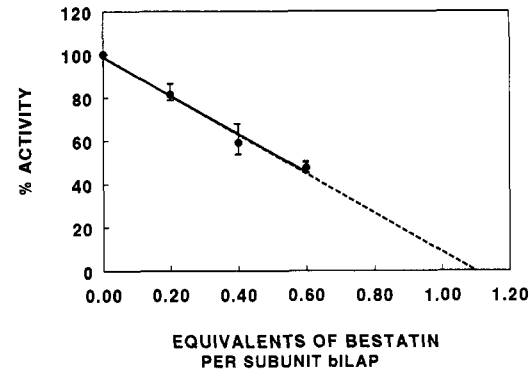


FIGURE 1: Determination of the number of bestatin bound per subunit of blLAP. Plot of percent activity vs equivalents of bestatin per subunit of blLAP. Maximal velocities for the hydrolysis of LeuGlyGly were obtained for assays containing 1.3×10^{-8} M blLAP hexamers and 0, 2×10^{-8}, 4×10^{-8}, or 6×10^{-8} M bestatin. The data plotted indicate the average percent activity for three assays each run in duplicate. Bars indicate the range of activities.

RESULTS

Specificity of the Assay. Physiological substrates for aminopeptidases appear to be di- and tripeptides or polypeptides (Botbol & Scornik, 1991; Squire et al., 1991), and kinetic data using aminoacyl-*p*-nitroanilides are frequently inconsistent with data obtained using peptides (Taylor et al., 1981; Hanson & Frohne, 1976; Delange & Smith, 1971). Accordingly, an assay was developed using LeuGlyGly as substrate. In order to do so, it was essential to demonstrate that only the LeuGly bond is cleaved to a significant extent during the course of the assay.

Bovine lens LAP-catalyzed hydrolysis of LeuGlyGly was followed by thin-layer chromatography. The R_f values for Leu, LeuGly, Gly, GlyGly, and LeuGlyGly were 0.73, 0.70, 0.43, 0.31, and 0.64, respectively. The R_f values for the products of the hydrolysis of LeuGlyGly by blLAP after 15 h were 0.73, 0.31, and 0.65, representing Leu, GlyGly, and unhydrolyzed LeuGlyGly, respectively. No free glycine was detected. This indicates that within the time of these experiments, cleavage of the glycylglycine bond of LeuGlyGly is insignificant. The Michaelis constant, K_m, for LeuGlyGly is 1.6 mM (±0.14 SEM) and is in agreement with prior reports using blLAP in which one zinc ion was replaced by a magnesium ion (Taylor et al., 1981). When concentrations of substrate exceeded 10 mM, some inhibition was noted.

Number of Binding Sites for Bestatin. Description of the mechanism of action is dependent upon knowledge of the number of substrate, and presumably inhibitor, binding sites for blLAP hexamer. Reasonable extrapolations from the two descriptions of other aminopeptidases suggest this could be from one to six (Wilkes & Prescott, 1985). First, the number of bestatin molecules bound per subunit was determined by titrating the enzyme with bestatin (Figure 1) (Wilkes & Prescott, 1985). The intersection of the extrapolated line at 1.1 indicates that 1 equiv of bestatin is bound per subunit of blLAP.

Second, this value was corroborated by utilizing molecular sieve chromatography to isolate blLAP saturated with bestatin. The blLAP–bestatin sample showed no activity. The amount

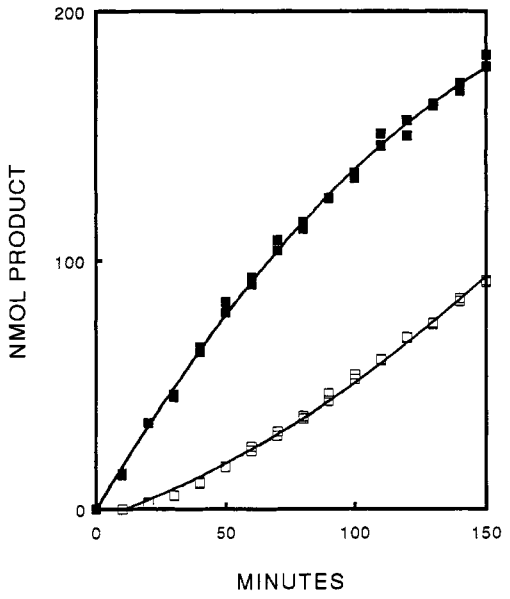


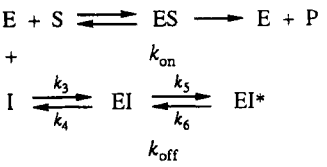
FIGURE 2: Association and dissociation of bestatin with blLAP. Upper curve: blLAP (1.7×10^{-9} M subunits) (final concentration) was added to a solution of 9.5 mM LeuGlyGly and 10^{-8} M bestatin (final concentration), and samples were tested for activity at the times indicated as described under Materials and Methods. Lower curve: blLAP was incubated with bestatin for 20 min. This solution was then added to a solution of substrate. Activity determinations and the final concentration of bestatin were as indicated for the upper curve.

of bestatin initially present in completely inhibited blLAP was determined after diluting the sample 100-fold to restore activity. This activity level was then compared to the activity of standard blLAP solutions comprised of similar concentrations of LAP but with known amounts of bestatin. An average of 32.7% activity was restored to fully inhibited blLAP after dilution. This indicated a concentration of approximately 1 (±0.37 SEM) equiv of bestatin per subunit of blLAP. Taken together, these data indicate that there is 1 equiv of bestatin bound per subunit.

Bestatin Is a Slow Binding Inhibitor of blLAP. Next it was necessary to determine the type of inhibition of BlLAP by bestatin. Three approaches were used to do this. (1) Enzyme was added to a solution containing LeuGlyGly and bestatin (Figure 2, upper line). (2) A solution containing enzyme and inhibitor was added to a solution containing substrate and inhibitor such that there was no change in the concentration of inhibitor (data not shown). (3) A solution containing enzyme and inhibitor was added to substrate (Figure 2, lower line). In all cases, the final concentration of inhibitor was the same.

When blLAP was added to solutions of LeuGlyGly containing bestatin, the initial rapid rate of evolution of product decreased by 71% after approximately 110 min. The $t_{1/2}$ for establishing steady-state levels of the EI* complex was approximately 30 min under these conditions. As expected, when the bestatin:blLAP ratio increased $t_{1/2}$ was reduced. That the reduced activity is not due to substrate depletion is established by the following: (1) by a calculation that during the assay less than 4% of the available substrate was consumed; (2) by observation that the final velocity observed in the assay shown by the upper curve is equivalent to the velocity observed when the inhibitor concentration does not change (data not shown), and the same velocity is observed in assays in which LAP was preincubated with an excess of bestatin and then added to the substrate (lower curve) ($p > 0.7$ by Student's t-test). In all of these assays, the final bestatin concentration in the assay tube was equal. In the latter instance, the reaction

Scheme I



progress curve turns upward as the high inhibitor concentration initially present in the preincubation mixture is diminished. The $t_{1/2}$ for the deformation of the tightly bound complex, EI*, is approximately 22 min. The regain in activity shown by the lower curve indicates that the inhibiton of blLAP by bestatin is reversible. This conclusion was supported by the restoration of blLAP activity by diluting the enzyme/inhibitor mixture and by restoration of activity upon dialyzing the blLAP inhibited by bestatin or azidobestatin (Taylor et al., 1992). Similar half-lives have been found in multiple repetitions of this experiment and in similar assays using *p*-azidobestatin (Taylor et al., 1992). The similarity of the rate constants obtained when [I]:[E] ratios were 10 or 100 provides further justification for use of kinetic analysis for a slow binding inhibitor.

Bestatin Is a Competitive Inhibitor of blLAP. Cha (1976) derived methods to analyze pre-steady-state kinetic data for slow binding inhibitors and to identify the type of inhibition. A simple scheme which describes slow binding inhibition is shown in Scheme I where the dissociation constant for the initial (EI) collision complex is $K_i = k_3/k_4$ and the dissociation constant, K_i^*, for the final complex, EI*, is $k_{off}/k_{on} = k_4k_6/k_3k_5$ (Cha, 1976; Morrison & Walsh, 1988; Williams & Morrison, 1979). k_{on} and k_{off} are the rate constants for formation and deformation of EI*. K_i^* can either be calculated from the ratio of k_{off}/k_{on} as obtained from pre-steady-state experiments or be determined directly using data from both phases of the reaction (see below). Progress curves were plotted for assays of blLAP with various concentrations of LeuGlyGly and bestatin (Figure 3). The data were fitted with quadratic equations (correlation coefficient >0.98), and velocities were calculated at various times. The apparent first-order constant, λ, and the apparent second-order rate constant for formation of the enzyme–inhibitor complex, β, were determined according to Cha (1976). Fitting the data for β in a reciprocal form, $1/\beta = 1/k_{on} + S/k_{on}K_m$, indicates a straight-line plot (Figure 3E, $r = 0.96$). The linearity of the $1/\beta$ vs S/K_m plot indicates that bestatin is a competitive inhibitor. Since S/K_m values were as high as 2.5, a significant level of noncompetitive inhibition would have been observed as curvature in the line (Cha, 1976).

For competitive inhibitors, it is possible to derive rate and binding constants for the major steps of the reaction pathway shown in Scheme I using pre-steady-state and steady-state portions of the progress curves (eq 1a and 2a; Morrison & Walsh, 1988). The data shown in Figure 2 were used for

$$v_s = V_{max}S/[K_m(1 + I/K_i^*) + S] \quad (1a)$$

$$v_0 = V_{max}S/[K_m(1 + I/K_i) + S] \quad (2a)$$

these calculations since those experiments allow for observation of a prolonged steady state. K_i and K_i^* respectively are 1.1×10^{-7} M and 1.3×10^{-9} M. These data indicate that bestatin and LAP are bound approximately 84-fold more tightly in the final EI* complex than in the initial collision complex.

The apparent rate constant, $k = 3.4 \times 10^{-4}$ s^{-1}, is determined from the intersection of the tangent and the asymptote of the progress curve (Morrison & Walsh, 1988). The rate constant

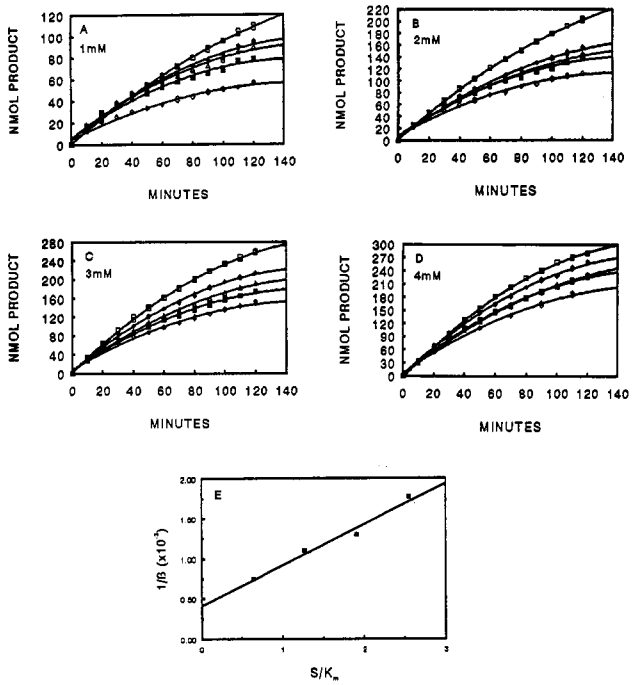


FIGURE 3: Bestatin is a competitive inhibitor of the hydrolysis of LeuGlyGly. Assays of blLAP were done with final concentrations of (panel A) 1.0, (panel B) 2.0, (panel C) 3.0, and (panel D) 4.0 mM LeuGlyGly and inhibitor final concentrations of 2×10^{-8}, 4×10^{-8}, 5×10^{-8}, 6×10^{-8}, and 8×10^{-8} M bestatin. (Panel E) $1/\beta$ vs S/K_m. As indicated under Results, β was determined from the slopes of plots shown in panels A–D.

for the formation of the final complex is obtained from $k_5 = k_6(K_i/K_i^* - 1) = 1.5 \times 10^{-2}\ s^{-1}$. This is in reasonable agreement with the value of k_5 calculated from k_{on} [2.38 (±0.49) × 10^3 s^{-1} M^{-1}] obtained from three duplicate sets of pre-steady-state data and the diffusion-controlled rate (10^7 M^{-1} s^{-1}). The rate constant for deformation of the final complex from the initial collision complex is obtained from the expression $k_6 = kv_s/v_0 = 2 \times 10^{-4}\ s^{-1}$.

Finally, the rate constant for the dissociation of the collision complex, k_4 (1 s^{-1}), is obtained from $K_i = k_4/10^7\ M^{-1}\ s^{-1}$. This is corroborated by a value of k_{off} of 8.29 (±1.4) × 10^{-5} s^{-1}. These data indicate that bestatin is a slow binding inhibitor of blLAP and that the slow achievement of steady state involves slow deformation of the EI* (see below).

DISCUSSION

Bovine lens LAP appears to be prototypical for many of the bestatin-inhibitable aminopeptidases, and structural information regarding this aminopeptidase is better developed than for other aminopeptidases (Burley et al., 1992; Wallner et al., submitted for publication; Taylor et al., 1982b; Taylor, 1993a,b). Yet full exploitation of the structural information to elucidate a mechanism of action for blLAP has been limited because the number of binding sites per subunit was never clearly identified. Bestatin seemed a logical candidate with which to determine the number of binding sites on LAP since (1) its structure is analogous to rapidly cleaved physiological peptide substrates and it is a competitive inhibitor of peptide hydrolysis, (2) it is the most tightly bound LAP inhibitor known, and (3) it had previously been used to quantify active sites in other aminopeptidases (Wilkes & Prescott, 1985).

Many prior investigations (Table I) of aminopeptidases utilized aminoacyl-*p*-nitroanilides as substrates because they display large chromophoric changes upon hydrolysis and are readily available. However, recent work indicated that (1) the relative rates of hydrolysis of specific aminoacyl-*p*-nitroanilides do not always parallel relative rates for the removal of that amino acid from peptides (Taylor et al., 1981), (2) small peptides are physiological substrates for aminopeptidases in vivo (Botbol & Scornik, 1991; Squire et al., 1991; Taylor et al., 1992), and (3) the binding site might extend beyond the S_1, S'_1 sites (Schechter & Berger, 1967). Consequently, an assay which employs the tripeptide LeuGlyGly as substrate was adapted (Taylor et al., 1981; Nicholson & Kim, 1975). This assay is more sensitive, displays rapid velocities typical of peptide hydrolysis, and can accommodate large numbers of tubes with automated sample handling, and the substrate fills an extended binding site. Kinetic analysis is facilitated since, during the time of the assays used here, blLAP cleaved LeuGlyGly only at the LeuGly bond (Taylor et al., 1981; Wolff & Resnik, 1963; Smith & Hill, 1960).

Similar Dissociation Constants for the Dissociation of Bestatin from BlLAP and Other Aminopeptidases. The requirement of approximately 110 min (formation) or 80 min (dissociation) to achieve steady state in the assays shown in Figure 2 and the linearity of the plot of $[S]/K_m$ vs $1/\beta$ indicate that bestatin is a slow binding inhibitor of blLAP and that bestatin competes with substrate for the same binding site on blLAP. This knowledge allowed use of pre-steady- and steady-state data to further describe the binding process.

The slow rearrangement of EI* of the rapidly formed initial complex, EI, and the even slower deformation of EI* to EI fulfill definitions of slow binding inhibitors (Morrison & Walsh, 1988) and are consistent with (1) data regarding the association between azidobestatin and blLAP (Taylor et al., 1992) and (2) the slow competitive binding of bestatin to virtually all other aminopeptidases tested (Table I). A similar accommodation of enzyme may also explain the delay in hydrolysis of the rapidly cleaved aminoacyl substrate leucylamide (data not shown).

Because of structural similarity, it might be expected that some of the enzymes listed in Table I might have similar K_i^* values for bestatin (Taylor, 1993a). hkLAP and blLAP share 92% homology of primary sequence. Both are hexamers of identical monomers (Taylor et al., 1984a,b). *Aeromonas* aminopeptidase (possibly hkLAP and others) and blLAP appear to share similar metal ion constellations at the active site (i.e., two metal ions in close proximity to each other and to the inhibitor; Wilkes & Prescott, 1985). The K_i^* value for binding of bestatin to blLAP falls within the range of from 5.8×10^{-10} to 4.1×10^{-6} M (Rich et al., 1984; Suda et al., 1976a,b) for bestatin and all other bestatin-inhibited aminopeptidases studied to date (Table I).

Further comparison of binding of bestatin to LAPs was initially made difficult since the K_i^* noted by Rich for hkLAP is approximately 34-fold higher than that noted by Wilkes and Prescott for the same enzyme (Rich et al., 1984; Wilkes & Prescott, 1985). The reasons for these discrepancies become clearer when the equations which are used to calculate K_i^* are recast (eq 1b and 2b) to emphasize relationships between

$$K_i^* = K_m I/[V_{max}S/v_s - K_m - S] \quad (1b)$$

$$K_i = K_m I/[V_{max}S/v_0 - K_m - S] \quad (2b)$$

K_i and V/v_0, or between K_i^* and V/v_s. It is observed that a change of only 5-fold in the ratio of V/v results in differences in K_i or K_i^* of over 40-fold. In various laboratories, differences in experimental technique (i.e., different metal ion content of the enzyme, time of assay, substrate, etc.) could result in small differences in V/v. Thus, it is plausible that aminopeptidases share modes of bestatin binding, which may be even more

Table I: Dissociation Constants for Bestatin and Aminopeptidases

enzyme	type of inhibn	K_i* (M)	K_i (M)	substrates	$\sim K_m$	M^{2+}	ref
mouse ascites tumor dipeptidase	compet, slow	2.7×10^{-9}		dipeptides	mM	Mg^{2+}	Patterson (1989)
aminopeptidase M	compet, slow	4.1×10^{-6}	7×10^{-6}	LpNA	mM	Zn^{2+},Mg^{2+}	Rich et al. (1984), Ocain & Rich (1988)
	not slow	1.4×10^{-6}		LpNA			Wilkes & Prescott (1985)
hog kidney cytosolic leucine aminopeptidase	compet, slow	2×10^{-8}		LpNA	mM	Zn^{2+},Mg^{2+}	Rich et al. (1984)
	compet, slow	2×10^{-8}		LpNA	mM	Zn^{2+},Mg^{2+}	Ocain & Rich (1988)
	compet, slow	5.8×10^{-10}		LpNA	mM	Zn^{2+},Mn^{2+}	Wilkes & Prescott (1985)
	compet, slow	2×10^{-8}	$\sim 10^{-8}$	LeuGlyGly	mM	Zn^{2+},Zn^{2+}	Hakamian & Taylor (not shown)
		2×10^{-8}		LβNA		Zn^{2+},Zn^{2+}	Nishizawa et al. (1977)
Aeromonas	compet, slow	1.8×10^{-8}		LpNA	mM		Wilkes & Prescott (1985)
bovine lens leucine aminopeptidase	compet, slow	1.3×10^{-9}	1.1×10^{-7}	LeuGlyGly			this work
bovine lens leucine aminopeptidase azidobestatin	compet, slow	4×10^{-9}	10^{-8}	LeuGlyGly	mM		Taylor et al. (1992)
aminopeptidase B	compet, slow	6×10^{-8}		LβNA			Suda et al. (1976b)
arginine aminopeptidase	compet	1.4×10^{-8}		ArgβNA	mM		Harbeson & Rich (1988)

similar than suggested by the data in Table I, despite their diverse specificity.

Number of Bestatin Bound per Hexamer. The data shown in Figure 1 indicate that one bestatin is bound per subunit of blLAP. Full occupancy of subunits by bestatin was also indicated in the direct bestatin binding assays. This is consistent with the identical structure of the monomers (Burley et al., 1992). A ratio of 1:1 bestatin/monomer was also obtained in *Aeromonas* aminopeptidase (Wilkes & Prescott, 1985) and yeast aminopeptidase I (Rohm, 1984), and preliminary experiments indicate that there is also one bestatin per protomer or six bestatin bound per hkLAP hexamer (Taylor and Peltier, unpublished results). It would appear to be in contrast with reports that only one bestatin is sufficient to inactivate cytosolic hkLAP provided by the same supplier (Wilkes & Prescott, 1985).

Given the homologies between blLAP and hkLAP noted above, the difference in the equivalents of bestatin bound per subunit of blLAP and hkLAP appears enigmatic. A plausible explanation for the apparent difference between hkLAP and blLAP binding of bestatin may be the difficulty in measuring protein concentrations in the highly variable commercial hkLAP preparations. In this laboratory, hkLAP concentrations measured by OD_{280} and amino acid analysis differed by as much as 9.6-fold.

Mode of Bestatin Binding and Mechanism of Peptide Hydrolysis. The consistency of data that each subunit can bind a bestatin allowed use of NMR (Taylor et al., 1982b), photoaffinity labeling (Taylor et al., 1992), and X-ray diffraction (Burley et al., 1992) information to propose a mode of binding of bestatin and to infer a mechanism of hydrolysis of peptide substrates (Figure 4). Photoaffinity labeling indicated that the active site is in the carboxyl-terminal third of the subunit. Diffraction data corroborated this and indicated which residues are used to ligand the zinc ions and which residues comprise the substrate binding pocket. NMR studies showed that the carbonyl oxygen of substrate analogs is within one hydration radius of Mn^{2+} in blLAP in which the more readily exchangeable Zn^{2+} has been replaced by Mn^{2+}. Thus, the scissile carbonyl oxygen of substrates or the C-2 OH of bestatin (required for tighter binding than peptides) are shown coordinated to the zinc ion located in the readily exchangeable cation binding site in the native enzyme. Presumably, this Zn^{2+} is liganded by Lys-250, Asp-273, and Glu-334 (and possibly Arg-336) (Burley et al., 1990, 1992) and provides electrophilic interaction with the substrate carbonyl to initiate the reaction and/or stabilize the tetrahedral intermediate (Figure 4B) (Bryce & Rabin, 1964; Nishizawa et al., 1977). Additional stabilization could be achieved via the ϵNH_3 of Lys-262. The α-amino group (probably unprotonated under physiological conditions) of bestatin and substrates also appears to be coordinated to this ion. The backbone of the inhibitor is stabilized in the enzyme by hydrogen bonds involving Asp-273 and Leu-360.

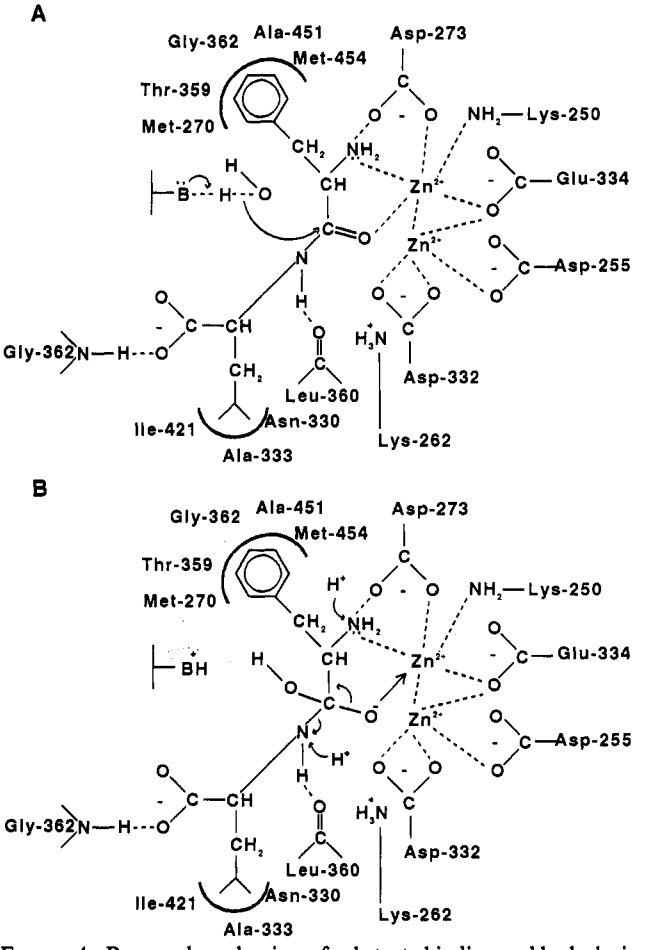


FIGURE 4: Proposed mechanism of substrate binding and hydrolysis by blLAP. By analogy to binding observed for bestatin, the Phe and Leu side chains are found within pockets formed by Met-270, Thr-359, Gly-362, Ala-451, and Met-454 and by Ala-330, Ala-333, and Ile-421, respectively. The more readily exchanged metal ion (thought to be involved in catalysis) is found uppermost and linganded as shown. (A) Hydrolysis is initiated by electrophilic attraction between the carbonyl oxygen of the scissile peptide bond and this zinc ion. Hydroxyl, from water or a zinc ligand, provides nucleophilic attack at the C of the scissile carbonyl. (B) The intermediate is stabilized by liganding to the zinc as well as with the hydrogen bonds indicated (see Discussion). Collapse of the tetrahedral intermediate results in hydrolysis (see arrows). The increased pK of the α-NH_2 results in protonation and release of the amino acid.

Hydrolysis also involves nucleophilic attack at the carbon of the scissile carbonyl. There are no enzyme nucleophiles in

the area of the scissile peptide bond. This is consistent with our inability to inactivate the enzyme using affinity labels which require a nucleophile [reviewed in Taylor et al. (1992)]. Accordingly, general base catalysis is suggested for the mechanism of hydrolysis of peptides (Nishizawa et al., 1977). The most plausible nucleophile would appear to be H_2O or OH^- stabilized by zinc. Upon hydrolysis, an increase in pK of the α-NH_2 to greater than 9 would result in protonation and release of products from the active site.

These data also allow rationalization of several unexpected observations. Thioamide derivatives did not show enhanced binding to hkLAP (Ocain & Rich, 1988). The LAP used for that work was in the Zn^{2+},Mg^{2+} form. Assuming that hkLAP and blLAP have the same metal ion distribution, it would appear that Mg^{2+} and not Zn^{2+} was in the readily exchanged site. Since Mg^{2+} does not have the same binding affinity for sulfur-containing compounds as Zn^{2+}, this might explain the unexpected decrease in apparent affinity found for thioamides in the hkLAP. Several compounds which were anticipated to be inhibitors were activators. These included Leu- and Ala-*p*-aminobenzenesulfonates, which are also substrates, as well as orthanilic and sulfanilic acids (Taylor et al., 1981) and an amastatin analog (Rich et al., 1984). The reasons for this should become clear when higher resolution diffraction data including these derivatives are available.

We recently proposed that human LAP, hlLAP, blLAP, hkLAP, bkLAP, the *xerB* gene product aminopeptidases I and A, and prolyl aminopeptidase form a mechanistically similar family of peptidases (Wallner et al., submitted for publication) in which all but one of the residues needed to bind metal ions or bestatin are conserved. The similarity of the binding constants and slow mode of inhibition of many of the peptidases indicated in Table I corroborates this prediction and allows further hypothesis that these enzymes employ a similar mechanism of action. Site-directed mutagenisis is being employed to test the hypotheses regarding the mechanism.

Many uses of bestatin and biological functions for aminopeptidases have been noted [reviewed in Taylor et al. (1992) and Taylor (1993b)]. Most of these depend on binding of bestatin to aminopeptidases. The information presented here should help to further elucidate mechanisms of action of blLAP and other similar aminopeptidases, expand the uses of bestatin derivatives, and aid in the design of other modulators of this class of enzymes.

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