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Human Liver Alanine Aminopeptidase. Inhibition by Amino Acids†

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ABSTRACT: Human liver alanine aminopeptidase is inhibited by L-amino acids having hydrophobic side chains such as Phe, Tyr, Trp, Met, and Leu. Blocking of the amino group or the carboxyl group greatly reduces the inhibitory capacity of the amino acid. Kinetic studies demonstrate that inhibition of hydrolysis of the substrate L-Ala-β-naphthylamide is of the noncompetitive type. Inhibition of the substrate L-Leu-L-Leu is of the mixed type. Inhibition of the substrate L-Ala-L-Ala-L-Ala is of the competitive type. These changes in the mechanism of inhibition are thought to be the result of the binding of the amino acid to the third residue binding site on the enzyme. This is the part of the active center to which the third residue from the amino end of a peptide substrate is normally bound. The inhibitor constants of several alanine oligopeptides are shown to decrease with increasing length through L-Ala-L-Ala-L-Ala-L-Ala, demonstrating that alanine aminopeptidase is a multisited enzyme with three and possibly four residue sites per active center. The inhibitor constant for Gly-Gly-L-Ala is five times the value of that for Gly-Gly-L-Phe suggesting that indeed the third residue site preferentially binds large hydrophobic residues.

The detailed study of the catalytic properties of human tissue and serum aminopeptidases has largely been ignored even though much interest has been demonstrated in regard to their potential clinical importance. Consequently, studies of the mechanism of catalysis by alanine aminopeptidase have been initiated in the authors' laboratories. This enzyme is stimulated by cobaltous ion (Behal et al., 1966; Smith et al., 1965) and is different from leucine aminopeptidase (Behal et al., 1966) which also is present in human liver. Alanine aminopeptidase has recently been shown to be a zinc metalloenzyme containing one atom of zinc for each subunit of molecular weight of 120,000 (Garner and Behal, 1974). Chelators were shown to inhibit via binding to the zinc; however, the zinc was not removed except by drastic treatment. Chelators were also shown to be competitive inhibitors of hydrolysis. During these studies, it was observed that certain amino acids were also inhibitors of the enzyme. It was the purpose of this investigation to determine the structural features of amino acids governing inhibition and to attempt to elucidate the mechanism of inhibition.

Experimental Procedure

Materials. Human liver alanine aminopeptidase was purified to greater than 92% homogeneity by an improved procedure based on the procedures of Little (1970) and Starnes and Behal (1974). Human liver aminopeptidase was re-

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Table I: Summary of Purification of Human Liver Alanine Aminopeptidase.

Step	Protein (mg)	Activity (units)	Specific Activity (units/mg)
I. Autolysis	132,000[a]	2620	0.020
II. Bio-Glas 200	592[a]	3340	5.65
III. Sephadex G-200	91[a]	1870	20.5
IV. DEAE-cellulose	17.2[b]	1200	70

[a] Based on A_{280}/A_{260}. [b] Based on E_{280} (1%) = 17.5.

leased from homogenized liver (1500*g*) in 3 l. of 0.05 *M* phosphate buffer (pH 6.86) at 37° during a 24-hr autolysis. After filtration and centrifugation to remove solid material, the extract was made 1.0 *M* in ammonium sulfate and applied to a Bio-Glas 200 (Bio-Rad) column (15 cm wide × 6 cm long, a sintered glass funnel) equilibrated in 1.0 *M* ammonium sulfate. Most of the material absorbing at 260 and 280 nm passed through unretarded. Most of the colored material was retained. Activity was eluted with water. The water fraction (2.2 l.) was again made 1.0 *M* in ammonium sulfate and applied to a second Bio-Glas 200 column (5 cm wide × 15 cm long) also equilibrated in 1.0 *M* ammonium sulfate. The adsorbed activity was eluted with 0.02 *M* ammonium sulfate. This fraction, concentrated from 450 to 30 ml by pressure dialysis, was chromatographed by gel filtration on Sephadex G-200 (5.0 × 90 cm; buffer: 0.05 *M* borate (pH 8.0)–0.5 *M* NaCl). The active fractions were collected, concentrated, dialyzed, and finally chromatographed on a DEAE-cellulose column (0.9 × 60 cm) with a 1000-ml linear NaCl gradient (0–0.2 *M*) in 0.05 *M* imidazole buffer (pH 7.0). A summary of a typical preparation is described in Table I. This procedure was found to be applicable to the purification of this enzyme from human kidney in comparable yields, except that the autolysis was allowed to continue for 72 hr.

Water was passed through two high purity deionization columns before use.

Peptides were the products of Sigma or ICN, purity was ascertained by thin-layer chromatography (TLC) or chromatography on the medium or long column of a Beckman Model 121 automatic amino acid analyzer, were found to be of acceptable quality, and were used without further treatment. D-Leu-L-Tyr had the same properties as L-Leu-L-Tyr on TLC and was not hydrolyzed by aminopeptidase, in contrast to L-Leu-L-Tyr. Amino acids were the products of Calbiochem. Salts and buffers were purchased as the highest grade available and used without further purification.

Methods. Enzymic hydrolysis of L-alanyl-β-naphthylamide (L-Ala-βNA)[1] was determined by measuring the amount of β-naphthylamine released during a 10-min incubation by diazotization or, in kinetic studies, by measuring the continuous release of β-naphthylamine at 340 nm. These assays have been described (Garner and Behal, 1974). All assays were performed in potassium phosphate buffer, 0.05 *M* (pH 6.86), containing 0.1–0.5 μg of enzyme in 1 ml at 37°. Enzymic hydrolysis of peptide substrates was determined in the above reaction mixture either by measuring the continuous decrease in absorption at 220–230 nm or by measuring the amount of amino acids released on a Beckman Model 121 automatic amino acid analyzer. Enzymic reactions destined for the amino acid analyzer were made 0.2 *M* in acetic acid at the end of an appropriate incubation time with 0.1 μg of enzyme. All spectroscopic measurements were made on a Beckman Acta III recording uv/vis spectrophotometer. K_i determinations were made by plotting v vs. v/S (Webb, 1963).

Table II: Inhibition of Aminopeptidase by Amino Acids and Amino Acid Derivatives.[a]

Amino Acid	Concn (m*M*)	Relative Activity (v/v_c)
1. None		1.00
2. Group I[b]	10	0.9–1.05
3. Group II	5	0.7–0.9
4. Group III	5	0.4–0.5
5. Group IV	5	0.05–0.2
6. D-Leu (III)[c]	10	0.94
7. D-Phe (IV)[c]	10	0.75
8. L-Leu-NH_2 (III)	20	0.90
9. L-Met-NH_2 (IV)	10	0.50
10. *N*-Chloroacetyl-L-Leu (III)	10	0.75
11. *N*-Chloroacetyl-L-Tyr (IV)	10	0.44

[a] Activity was measured by diazotization of the released β-naphthylamine from 1.0 m*M* L-Ala-βNA in the presence of 0.25 μg of enzyme. [b] Group I: L-Glu, L-Asp, Gly, L-Asn, L-Ser, L-Pro, L-Thr; group II: L-Lys, L-Gln, L-Val, L-Arg, L-Ala, L-His, L-Ile; group III: L-Leu, β-Ala; group IV: L-Met, L-Phe, L-Tyr, L-Trp. [c] Taken from group III or group IV, respectively.

Results

Inhibition of Alanine Aminopeptidase by Amino Acids and Amino Acid Derivatives. Alanine aminopeptidase is inhibited by a variety of amino acids and amino acid derivatives. The results are shown in Table II. Of the simple amino acids, the most potent inhibitors are those amino acids having a large hydrophobic side chain especially L-Trp, L-Tyr, L-Met, and L-Phe (group IV). Inhibition by β-Ala (group III) was unexpected since it cannot be considered to be a hydrophobic amino acid and is structurally similar to the less inhibitory amino acids. Inhibition by β-Ala appears, thus far, to be by the same mechanism as by hydrophobic amino acids. Other amino acids were not inhibitory (group I) or only slightly inhibitory (group II). The decreased inhibition by D-amino acids, *N*-chloroacetyl L-amino acids, and by L-amino acid amides demonstrates the specificity of the enzyme binding site for hydrophobic L-amino acids having free carboxyl and amino groups.

Several aliphatic amines and carboxylic acids were tested as possible analogs of hydrophobic amino acids and were found to be potent inhibitors of the enzyme. Their mechanism of inhibition, which is different from that of amino acids, is under investigation.[2]

Kinetics of Inhibition of Alanine Aminopeptidase. In an effort to determine the mechanism by which amino acids inhibit alanine aminopeptidase, the kinetics of the inhibition of hydrolysis of several substrates were investigated. L-Phe, L-Tyr, L-Met, L-Trp, β-Ala, and L-Leu inhibit the hydroly-

[1] Abbreviations used are: L-Ala-βNA, L-alanyl-β-naphthylamide; K_i, inhibitor constant, i.e., the dissociation constant for the inhibitor–enzyme complex; $L\text{-Ala}_2$, L-Ala-L-Ala; $L\text{-Ala}_2\text{-NH}_2$, L-Ala-L-Ala-$NH_2$; $L\text{-Ala}_3$, L-Ala-L-Ala-L-Ala; L-Ala-pNA, L-Ala-*p*-nitroanilide.

[2] C. W. Garner and F. J. Behal, manuscript in preparation.

Table III: Inhibition Constants for Amino Acid Inhibitors of Alanine Aminopeptidase.[a]

Inhibitor	Substrate	Inhibition Type	K_i (mM)
1. L-Phe	L-Ala-βNA	Noncompet.[b]	0.74 (0.18)[c]
2. L-Tyr	L-Ala-βNA	Noncompet.	0.47 (0.07)
3. L-Met	L-Ala-βNA	Noncompet.	0.80 (0.21)
4. L-Met-NH_2	L-Ala-βNA	Compet.	1.8 (0.72)
5. L-Leu	L-Ala-βNA	Noncompet.	3.37 (0.05)
6. L-Leu	$L\text{-Leu}_2$	Mixed	2.65 (0.17)
7. L-Leu	$L\text{-Ala}_3$	Compet.	1.60 (0.27)
8. L-Tyr	$L\text{-Ala}_3$	Compet.	2.1 (1.30)

[a] Assays were performed at pH 6.86 and at 37°. The reactions were followed by the increase in absorption at 340 nm (lines 1–5), by the decrease in absorption at 220 nm (lines 6, 7), or by the appearance of products on the amino acid analyzer (line 8). [b] Noncompet. = noncompetitive; Compet. = competitive. [c] K_i values were calculated from plots of v vs. v/S. Values in parentheses represent the average deviation from the mean for 2–3 determinations.

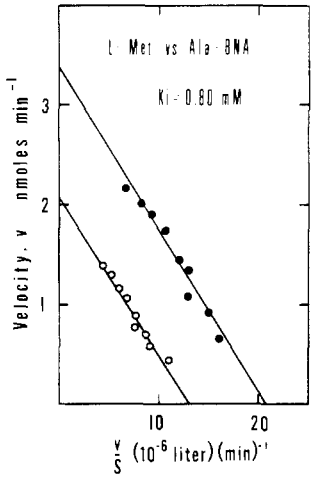

FIGURE 1: The hydrolysis of L-Ala-βNA in the presence (○) or absence (●) of 0.5 mM L-Met. Activity was measured by the increase in absorption at 340 nm with time in the presence of 0.3 μg of enzyme. Other components of the reaction mixture are given in Methods.

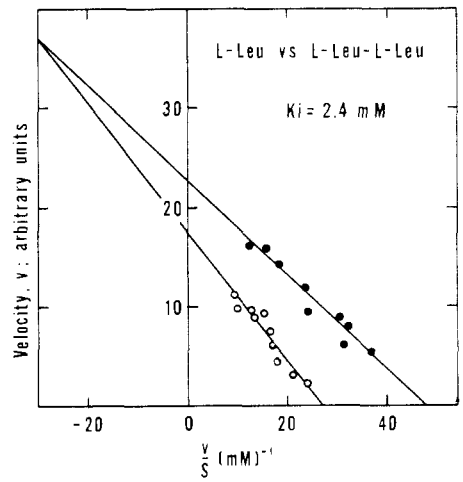

FIGURE 2. The hydrolysis of $L\text{-Leu}_2$ in the presence (○) or absence (●) of 2 mM L-Leu. Activity was measured by the decrease in absorbance at 220 nm with time with 0.5 μg of enzyme. Other experimental details are given in Methods.

sis of the simple substrates L-Ala-βNA or L-Phe-βNA in the noncompetitive manner. Shown in Figure 1 are the data for L-Met inhibition of L-Ala-βNA hydrolysis. Its K_i is 0.8 mM. The K_i for other amino acid inhibitors are shown in Table III.

The hydrolysis of the substrates $L\text{-Ala}_2$, $L\text{-Ala}_2\text{-NH}_2$, and $L\text{-Leu}_2$ also was inhibited by hydrophobic amino acids. With these substrates, the inhibition was found to be of the mixed type, i.e., with characteristics of both noncompetitive and competitive types. Shown in Figure 2 is the inhibition of $L\text{-Leu}_2$ by L-Leu.

In contrast, inhibition of the hydrolysis of the substrate $L\text{-Ala}_3$ by L-Leu or L-Tyr is of the competitive type. The data for L-Leu are shown in Figure 3. The data for L-Tyr inhibition of Ala_3 (Table III) were collected on an amino acid analyzer. All the expected products were observed but no transpeptidation products were found.

The inhibitor constants for several amino acids and derivatives are shown in Table III. Aromatic amino acids, L-methionine, β-alanine, and L-leucine, had K_i values near 1 mM. All other amino acids tested had K_i values in excess of 10 mM. Taurine, a sulfonic acid analog of β-alanine, was not inhibitory. Methioninamide was less inhibitory than methionine, but more importantly, its inhibition was of the competitive type. Other peptides such as L-Leu-L-Tyr, D-Leu-L-Tyr, $L\text{-Ala}_3$, and $L\text{-Ala}_4$ were competitive inhibitors of L-Ala-βNA hydrolysis (data not shown).

The ability of a peptide to inhibit the hydrolysis of L-Ala-βNA is a function of its length. The values of K_i for oligopeptides containing only L-Ala, shown in Table IV, can be seen to decrease until the length of the peptide reached $L\text{-Ala}_4$. Not only does inhibitory power increase with increasing peptide length, but also it increases with the size and degree of hydrophobicity of the R group of the third residue. As seen in Table IV, Gly-Gly-L-Phe is five times more inhibitory than Gly-Gly-L-Ala.

Discussion

Numerous amino acids have been found to inhibit human liver alanine aminopeptidase. The requirements for binding to the inhibitor site include: (1) a free amino group; (2) a free carboxyl group; (3) the L configuration at the α carbon; and (4) a hydrophobic R group such as with Leu, Met, Phe, Tyr, and Trp.

Alanine aminopeptidase was shown by Little and Behal (1971) to prefer dipeptide substrates having hydrophobic residues especially in C-terminal position. Thus, it is not unreasonable, in retrospect, that the products of the hydrolytic reaction be inhibitory. Such end product inhibition would be expected to occur by binding into either the site for the N-terminal residue or the site for the C-terminal residue of dipeptides. In an effort to determine if this is the case, the mechanism of inhibition of alanine aminopeptidase by hydrophobic amino acids was investigated.

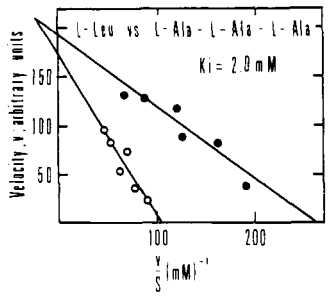

FIGURE 3: The hydrolysis of L-Ala$_3$ in the presence (○) or absence (●) of 3 mM L-Leu. Activity was measured by the decrease in absorbance at 230 nm with time with 0.5 μg of enzyme. Other experimental details are given in Methods.

When L-Ala-βNA was used as substrate, hydrophobic amino acids were found to be noncompetitive inhibitors. Therefore, it is unlikely that hydrophobic amino acids inhibit via binding in the same site occupied by the substrate L-Ala-βNA. When the substrate was L-Leu$_2$ or L-Ala$_2$, the inhibition by amino acids was found to be of the mixed type. With a tripeptide substrate such as L-Ala$_3$, the inhibition was of the competition type. This change in mechanism presently can best be interpreted as the result of binding of the inhibitor amino acid to the third residue site of the active center, shown schematically in Figure 4. Binding at this locus is consistent with the above observations of the mechanisms of inhibition. This model predicts noncompetitive inhibition for amino acid amide substrates, because the substrate and inhibitor should be able to occupy their respective sites without direct steric interference. Competitive inhibition for tripeptide substrates would also be predicted since the binding of the inhibitor amino acid would prevent binding of a tripeptide substrate by direct steric interaction. Mixed inhibition observed with dipeptides is consistent with the steric interaction between the ammonium group of the inhibitory amino acid and the carboxylate group of the dipeptide. The degree of interaction must be small enough to allow both the inhibitor and the substrate to bind, but large enough to prevent their binding in an undisturbed manner. These explanations for the observed kinetics can be visualized by superimposing line D onto lines A, C, and B, respectively, of Figure 4.

Other, more complex, explanations of the observed patterns of inhibition are possible; however, binding of inhibitory amino acids to the first or second subsites seem definitely eliminated. The third subsite has been selected because only with tripeptides (and presumably longer peptides) does the inhibition become competitive.

Since a third site has been postulated, the question can be raised concerning the independent evidence for its existence. Such evidence can be seen in Table III. The values of K_i for a series of L-alanine-containing oligopeptides can be seen to decrease with increasing peptide length through L-Ala$_4$. The decrease in K_i, i.e., the increase in binding affinity, likely is the result of the increase in the number of binding points. The existence of a fourth site based on these data must be stated cautiously since it is possible that the decrease in K_i in going from L-Ala$_3$ to L-Ala$_4$ is due simply to the masking of the terminal carboxyl group of L-Ala$_3$. A fifth site is unlikely since L-Ala$_5$ was bound with nearly the same affinity as L-Ala$_4$.

Values of K_i for two tripeptides differing only in the size and hydrophobicity of the R group of the third residue indicate that indeed, the third binding site preferentially binds

Table IV: Inhibition Constants for Peptide Inhibitors of Alanine Aminopeptidase.[a]

Inhibitor	Substrate	K_i (mM)
1. Leu$_2$	L-Ala-βNA	0.23 (0.08)[b]
2. Leu$_3$	L-Ala-βNA	0.054 (0.012)
3. Ala$_2$	L-Ala-βNA	1.9 (0.3)
4. Ala$_3$	L-Ala-βNA	0.32 (0.01)
5. Ala$_4$	L-Ala-βNA	0.089 (0.018)
6. Ala$_5$	L-Ala-βNA	0.068 (0.020)
7. Gly-Gly-L-Ala	L-Ala-pNA	7.0 (0.7)
8. Gly-Gly-L-Phe	L-Ala-pNA	1.4 (0.2)

[a] Reactions were followed by measuring the increase in absorbance at 340 or 410 nm with L-Ala-βNA or L-Ala-pNA, respectively, in the presence of 0.2 μg of enzyme. Other experimental details are described in Methods. [b] Values in parentheses represent the average deviation from the mean for between 2 and 5 determinations.

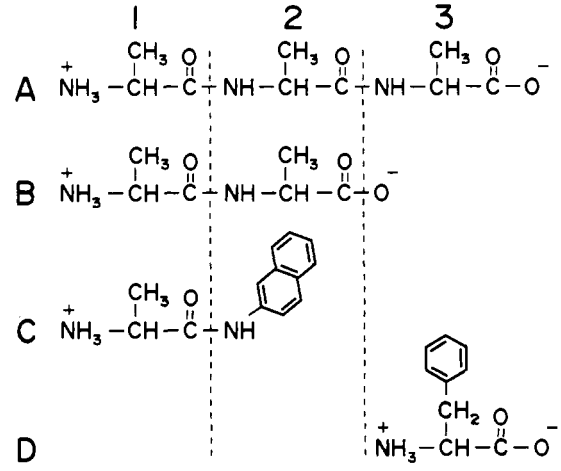

FIGURE 4: Schematic model for the binding of substrates and L-Phe to alanine aminopeptidase subsites. Details are described in the text.

large hydrophobic residues. This is in keeping with the suggestion that hydrophobic amino acids bind at the third residue site. A more thorough determination of the topography of this site is under way using a series of tripeptides varying only in the R group of the third residue from the amino end.

A large increase in the rate of hydrolysis of peptide bonds when dipeptides were lengthened to tripeptides was observed with a similar aminopeptidase isolated from rat kidney (Felgenhauer and Glenner, 1966). This suggests that this enzyme too has a third residue binding site.

Multiple sites within an active center have been demonstrated for several other enzymes, including lysozyme (Dickerson and Geis, 1969), carboxypeptidase (Abramowitz et al., 1967), and chymotrypsin (Segal et al., 1971).

The inhibition of another aminopeptidase (from pig kidney) by amino acids has also been observed (Wachsmuth et al., 1966). Hydrophobic L-amino acids were found to have the lowest K_i values. Amino acid amides were less inhibitory than the corresponding amino acid except with amides such as glycinamide. Further characterization of the inhibition was not reported.

Alanine aminopeptidase also hydrolyzes amino acid amides at rates comparable to peptides, although K_m values are higher (C. W. Garner, unpublished results). This aminopeptidase also hydrolyzed all peptides tested (except D-Leu-L-Tyr). Apparently all substrates studied bind the ac-

tive site in the same fashion, i.e., the ammonium group of each amino acid amide or peptide binds into the same residue site. This is based on the observation that plots of pK_i vs. pH are identical for amino acid amides, dipeptides, and tripeptides.[2]

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