Specificity of \textit{Aeromonas} Aminopeptidase toward Amino Acid Amides and Dipeptides*

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SUMMARY

The substrate requirements of \textit{Aeromonas} aminopeptidase toward NH$_2$-terminal residues were determined by use of amides representing all classes of amino acids, and the effects of the adjacent residue on hydrolysis were assessed by use of leucyl dipeptides in which the identity of the COOH-terminal residue was varied. The enzyme was specific for substrates possessing bulky, hydrophobic residues in the NH$_2$ terminus; of 20 amides tested, measurable rates of hydrolysis were found only for leucinamide, norleucinamide, norvalinamide, isoleucinamide, valinamide, methioninamide, and phenylalaninamide. Of these, leucinamide showed the highest ratio of $k_{cat}$: $K_m$(app). Among the dipeptides, Leu-Met had the highest $k_{cat}$: $K_m$(app) ratio and the leucyl peptides with arginine, phenylalanine, tryptophan, and tyrosine in the COOH terminus exhibited successively lower ratios. The selectivity of the aminopeptidase for large hydrophobic residues in the penultimate position was emphasized by the fact that low $k_{cat}$: $K_m$(app) ratios were exhibited toward Leu-Ala, Leu-Gly, and Leu-OMe.

Evidence from previous work (1, 2) suggested that the substrate specificity of \textit{Aeromonas} aminopeptidase was different from that of aminopeptidases previously described (3–8). We thus were prompted further to investigate the catalytic specificity of this enzyme which is particularly interesting because it has the lowest molecular weight (29,500) of any aminopeptidase yet characterized, does not consist of subunits, and is a zinc metalloenzyme (2). The present investigation was therefore undertaken to determine the influence exerted by the NH$_2$-terminal and penultimate residues of substrates on hydrolysis. The former was investigated by use of amino acid amides in order to assess the influence of the NH$_2$-terminal residue, uncomplicated by other substituents, whereas the effects of penultimate residues were investigated with leucyl dipeptides in which the identity of the COOH-terminal residue was varied to include all classes of amino acids.

EXPERIMENTAL PROCEDURE

Materials—Substrates, obtained from Mann and Cyclo, were thoroughly dried over P$_2$O$_5$ before use and were tested for purity by thin layer chromatography or high voltage electrophoresis. Samples of all compounds which proved susceptible to cleavage by the aminopeptidase were subjected to acid hydrolysis followed by thin layer chromatography or high voltage electrophoresis to verify the identity of component residues. \textit{Aeromonas} aminopeptidase with a specific activity of approximately 350 units per mg was prepared as previously described (2), devoid of endopeptidase activity and homogeneous by polyacrylamide gel electrophoresis at pH 8.6.

Enzyme Assays—L-Leucinamide$^1$ was the substrate used for standard enzyme assays, and 1 unit of aminopeptidase was defined as that quantity of enzyme which catalyzed the hydrolysis of 1 pmole of leucinamide per min at pH 8.0 and 25$^\circ$. The hydrolysis of amide, dipeptide, and ester substrates was measured from the decrease in absorbance at 230 nm in a Gilford model 299 recording spectrophotometer, the cuvette compartment of which was maintained at 25+1$^\circ$; enzyme and substrate solutions were equilibrated at this temperature prior to being used in the assays. Assays in 1-cm cuvettes were performed by adding 50 $\mu$l of enzyme solution to 2.95 ml of substrate, both in 50 mM Tris-HCl buffer, pH 8.0; the solutions were immediately mixed and the decrease in absorbance was recorded for about 5 min. The contribution of the bond in each substrate to the molar extinction at 230 nm was determined by subtracting the sum of molar extinctions of the hydrolitic products from that of the substrate and the resulting values were used to calculate the extent of hydrolysis. Reaction velocities were calculated from the initial slopes of tracings of absorbance against time (apparent zero order rate) and expressed as micromoles of substrate hydrolyzed per min per ml of enzyme solution. Substrates that showed no discernible change in absorbance after 5 min of incubation with the enzyme were qualitatively evaluated by incubation with the aminopeptidase for 6 hours at 25$^\circ$, then subjected to high voltage electrophoresis to detect any hydrolysis.

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$^1$ The abbreviations used are: Leu-NA, L-leucyl-$\beta$-naphthylamide; Leu-OMe, leucine methyl ester; Phe-OMe, phenylalanine methyl ester. Dipeptides are designated with the usual three letter abbreviations for the constituent residues. Unless otherwise indicated, all amino acid residues were of the $L$ configuration.

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Kinetic values were calculated from the results of duplicate assays run on five dilutions from each of three individual 20 mm stock solutions, with enzyme concentrations being varied as necessary. Thus, the values for $k_{\text{cat}} \left( V_{\text{max}}/E \right)$ (E = enzyme concentration) and $K_{\text{m(app)}}$ (apparent $K_m$) represent at least 30 individual assays. Calculations of $K_{\text{m(app)}}$ and $V_{\text{max}}$ (maximum velocity of an enzyme-substrate reaction) were made from Lineweaver-Burk double reciprocal plots analyzed by the method of least squares. Values for $k_{\text{cat}}$ were obtained by dividing $V_{\text{max}}$ by the concentration of enzyme in micromoles per ml of reaction mixture.

Other Procedures—Concentrations of aminopeptidase solutions were determined spectrophotometrically at 278 nm by use of the value $E_{1cm}^\text{nm} = 14.4$ (9). Thin layer chromatography was performed on glass plates coated with Adsorbosil 1 and activated by heating at 100° for 1 hour; the chromatograms were developed with 77% ethanol. One-dimensional high voltage electrophoresis was done as described previously (9) with a formic acid-acetic acid-water (1:4:45) buffer at pH 1.9 and Varsol as the coolant.

RESULTS AND DISCUSSION

Influence of NH2-terminal Residue—In preliminary experiments, amides representative of all classes of amino acids were tested at 20 mm concentration, and only seven, all of which contained neutral, bulky residues in the NH2 terminus, were cleaved at measurable rates. The identities of the susceptible substrates, along with all others tested, are shown in Table I. A minimum chain length is obviously required for susceptibility to the aminopeptidase as the amides of $\alpha$-aminobutyric acid, alanine, and glycine showed no activity as substrates. Iso glutamine and isoasparagine were not hydrolyzed, probably because the negative charge on these amides prevented cleavage, but in contrast, activity could be discerned qualitatively when the positively charged amides of arginine and lysine were tested. Determination of the kinetic constants $k_{\text{cat}}$ and $K_{\text{m(app)}}$ for the susceptible substrates (Table I) revealed that leucinamide not only had the highest $k_{\text{cat}}$ value, but its $k_{\text{cat}}/K_{\text{m(app)}}$ ratio was 4-fold greater than any other amide.

Bender and Kézdy (10) have proposed that the value $k_{\text{cat}}/K_{\text{m(app)}}$ is probably always a meaningful kinetic constant in correlating structure and specificity, and from certain simple assumptions one can show that for substrates with the same $k_{\text{cat}}$, differences in their $K_{\text{m(app)}}$ values are indicative of different enzyme-substrate affinities. Table I reveals similar $k_{\text{cat}}$ and $K_{\text{m(app)}}$ values for the unbranched substrates, norleucinamide and norvalinamide, and for the branched pair, isoleucinamide and valinamide. In both examples, the 6-carbon compound was bound more effectively than the 5-carbon substrate. The marked influence exerted by the position of a $\alpha$-amino acid amides that the enzyme failed to hydrolyze when tested at 20 mm concentration were alaninamide, serinamide, threoninamide, prolaminamide, histidinamide, isoglutamine, isoasparagine, $\alpha$-NH2-butyramide, and glycaminamide. Activity toward lysinamide, argininamide, tyrosinamide, and tryptophanamide was detectable by thin layer chromatography or high voltage electrophoresis after a 6-hour incubation at 25°C.

TABLE I

Kineti values for Aeromonas aminopeptidase toward some L-amino acid amides

| Substrate | $K_{\text{m(app)}} \pm \text{S.E.}$ | $k_{\text{cat}} \pm \text{S.E.}$ | $k_{\text{cat}}/K_{\text{m(app)}}$ |
|-----------|-------------------------------|-------------------------------|-------------------------------|
| Leu-Met   | 5.1 ± 1.0 220.0 ± 18.3         | 3.7 ± 8.8 153.3               |
| Norleucinamide | 6.3 ± 0.5 60.0 ± 8.3         | 9.5               |
| Norvalinamide | 10.8 ± 2.7 66.7 ± 9.7         | 6.2               |
| Isoleucinamide | 1.6 ± 0.5 0.2 ± 1.2         | 0.2               |
| Valinamide | 2.4 ± 0.9 6.5 ± 0.8           | 2.7               |
| Methioninamide | 14.6 ± 3.4 32.0 ± 3.7       | 2.2               |
| Phenylalaninamide | 1.8 ± 0.1 6.2 ± 0.3      | 3.4               |

a. $\alpha$-Amino acid amides that the enzyme failed to hydrolyze when tested at 20 mm concentration were alaninamide, serinamide, threoninamide, prolaminamide, histidinamide, isoglutamine, isoasparagine, $\alpha$-NH2-butyramide, and glycaminamide. Activity toward lysinamide, argininamide, tyrosinamide, and tryptophanamide was detectable by thin layer chromatography or high voltage electrophoresis after a 6-hour incubation at 25°C.

Effects of penultimate residue on activity of Aeromonas aminopeptidase toward leucyl dipeptides and analogous substrates

Kinetic values shown were obtained from at least six replicate analyses on each of five substrate concentrations so chosen that no substrate inhibition was evident.

| Substrate | $K_{\text{m(app)}} \pm \text{S.E.}$ | $k_{\text{cat}} \pm \text{S.E.}$ | $k_{\text{cat}}/K_{\text{m(app)}}$ |
|-----------|-------------------------------|-------------------------------|-------------------------------|
| Leu-Met   | 0.35 ± 0.06 53.7 ± 6.8         | 153.3               |
| Norleucinamide | 0.39 ± 0.07 39.3 ± 4.9       | 100.8               |
| Norvalinamide | 0.86 ± 0.09 72.0 ± 9.7      | 83.7               |
| Isoleucinamide | 0.96 ± 0.2 58.0 ± 9.0      | 60.4               |
| Valinamide | 1.50 ± 0.33 85.4 ± 4.8         | 56.0               |
| Methioninamide | 0.18 ± 0.06 8.3 ± 0.5      | 46.3               |
| Phenylalaninamide | 0.38 ± 0.1 16.5 ± 0.33     | 43.4               |
| Leu-Ala   | 0.64 ± 0.07 15.2 ± 0.63         | 23.8               |
| Leu-Arg   | 1.0 ± 0.08 15.4 ± 1.0          | 15.4               |
| Leu-Phen  | 7.3 ± 2.2 31.6 ± 6.2           | 4.3                |
| Leu-NAba  | 0.43 ± 0.05 29.6 ± 2.6         | 68.0               |
| Leu-OMe   | 12.2 ± 2.8 20.4 ± 2.0          | 1.7                |
| Phe-OMe   | 3.9 ± 0.8 41.6 ± 6.1           | 10.7               |

a. Dipeptides not hydrolyzed by the aminopeptidase were Leu Glu, Leu-Pro, p-Leu-l-Leu, and L-Leu-p-Leu.

b. Assayed in 1-mm cuvettes to compensate for high absorbance. The volumes of enzyme and substrate were one-tenth those used in the standard assays.

c. Assayed by measuring the decrease in absorbance at 248 nm.

Specific, as indicated by the failure of p-leucinamide to inhibit the hydrolysis of the l enantiomorph.

Influence of Penultimate Substituents—Values for $K_{\text{m(app)}}$ and $k_{\text{cat}}$ of leucyl dipeptides are shown in Table II which reveals that rates of hydrolysis ($k_{\text{cat}}$) were highest for those containing aromatic residues, methionine, or arginine in the penultimate position. The presence of glutamic acid or proline residues in
the adjacent position rendered the dipeptide nonsusceptible to hydrolysis, as had the presence of these residues in the NH₂ terminus. Enantiomeric specificity also is apparent in the penultimate position as evidenced by the failure of L-Leu-M-Leu to serve as a substrate or to inhibit the hydrolysis of either L-leucinamide or D-Leu-L-Leu. The latter observation suggests the L-Leu-D-Leu was not bound by the catalytic site on the aminopeptidase. Dipeptides generally had lower \( K_m \) values than the amino acid amides, probably because the adjacent amino acid residue enhanced substrate binding. Differences in enzyme-substrate affinity among the dipeptides were evident, as Leu-Met and Leu-Trp had similar \( k_{cat} \) values but differed substantially with respect to \( K_m \); a similar situation existed for Leu-Ile, Leu-Val, and Leu-Ala. Introduction of leucine in the penultimate position resulted in the lowest \( k_{cat} \) of any dipeptide tested, and the influence of the position of a methyl group on the penultimate residue is evident from a comparison of the kinetic values for Leu-Leu and Leu-Ile.

Our previous work on the isolation and characterization of Aeromonas aminopeptidase was done with Leu-NA as the substrate (1, 2) and it is interesting to compare the kinetic values of this compound with those of the dipeptides and amino acid amides. Although \( k_{cat} \) for Leu-NA was not unusually high, \( K_m \) was lower than that of any amino acid amide (Table I) and in fact, was lower than more than half of the dipeptides, probably because the bulky \( \beta \)-naphthylamine moiety enhanced binding to the enzyme. The methyl esters of leucine and phenylalanine were hydrolyzed at rates comparable to those shown by a number of dipeptides, but due to its extraordinarily high \( K_m \), Leu-OMe had the lowest \( k_{cat}:K_m \) ratio of any substrate tested and it is apparent that the structural differences between Leu-OMe and leucinamide exert profound effects on both binding and the catalytic process. In contrast, Phe-OMe had a higher \( k_{cat}:K_m \) ratio than phenylalaninamide because of the low \( K_m \) of the latter.

In addition to the aminopeptidase, Aeromonas proteolytica produces an extracellular neutral proteinase (11-13) with a specificity toward substrates to which bulky hydrophobic resi
dues contribute the nitrogen atom of the peptide bond (12). Hydrolysis of a peptide chain by the endopeptidase thus liberates a fragment having an NH₂-terminal residue that is particularly susceptible to the aminopeptidase. The pattern is analogous to that found in bovine pancreatic juice in which chymotryptic cleavage liberates products most susceptible to carboxypeptidase A and hydrolysis by trypsin yields preferred substrates for carboxypeptidase B. Such complementary specificities between endopeptidases and exopeptidases of the same cellular origin may prove to be of frequent occurrence in nature.

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REFERENCES

1. Prescott, J. M., and Wilkes, S. H. (1966) Arch. Biochem. Biophys., 117, 328.
2. Prescott, J. M., Wilkes, S. H., Wagner, F. W., and Wilson, K. J. (1971) J. Biol. Chem., 246, 1756.
3. Smith, E. L., and Spackman, D. H. (1955) J. Biol. Chem., 212, 255.
4. Glenner, G. G., McMillan, P. J., and Folk, J. E. (1962) Nature, 194, 867.
5. Horne, V. K., Martin, K. K., and Glenner, G. G. (1966) Arch. Biochem. Biophys., 114, 567.
6. Wachsmuth, E. D., Fritz, I., and Pfleiderer, G. (1966) Biochemistry, 6, 176.
7. Minamurra, N., Yamamoto, T., and Fukumoto, J. (1966) Agr. Biol. Chem., 30, 180.
8. Yost, V. M. (1970) J. Biol. Chem., 245, 4660.
9. Tanksley, T. D., Jr., Neumann, H., Lyman, C. M., Pace, C. N., and Prescott, J. M. (1970) J. Biol. Chem., 245, 6456.
10. Bender, M. L., and Kézdy, F. J. (1965) Annu. Rev. Biochem., 34, 49.
11. Merkel, J. R., Traganza, E. D., Mukherjee, B. B., Griffin, T. B., and Prescott, J. M. (1964) J. Bacteriol., 87, 1227.
12. Wilkes, S. H., Mukherjee, R. R., Wagner, F. W., and Prescott, J. M. (1969) Proc. Soc. Exp. Biol. Med., 131, 382.
13. Griffin, T. B., and Prescott, J. M. (1970) J. Biol. Chem., 245, 1548.
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