Proteolytic Enzymes of the K-1 Strain of *Streptomyces griseus* Obtained from a Commercial Preparation (Pronase)

**EFFECT OF pH, METAL IONS, AND AMINO ACIDS ON AMINOPEPTIDASE ACTIVITY***

(Received for publication, March 25, 1977)

**Klaus D. Vosbeck,**$^2$ **Benjamin D. Greenberg,** **Maria S. Ochoa,** **Philip L. Whitney,** and **William M. Awad, Jr.$^§**

*From the Departments of Medicine and Biochemistry, University of Miami School of Medicine, Miami, Florida 33152*

Pronase aminopeptidase-1 (Vosbeck, K. D., Chow, K.-F., and Awad, W. M., Jr. (1973) J. Biol. Chem. 248, 6029-6034) exhibits, at low calcium ion concentrations, activity maxima at pH values of 8 and 9.7. High calcium concentrations inhibit in the higher pH range only. Inhibition by amino acids is noted at both pH values. Histidine is the best inhibitor of the many amino acids tested; glycine inhibits moderately better than leucine. Configurational effects are minimal, since inhibition by L- or D-leucine is virtually equivalent; both α-amino and α-carboxyl groups are required for significant inhibition. Inhibition by amino acids or calcium is not competitive. The order of affinity for aminopeptidase-1 of the different amino acids does not follow their order of susceptibility to release as terminal residues in short peptides. These observations suggest that amino acids bind at a site other than that of substrate NH₂-terminal residues.

Different divalent metal ions bind to the metal-free enzyme, providing varying degrees of activity at pH 8. Excess calcium cannot restore full activity to some of the metal aminopeptidase complexes. The ability of the enzyme to bind metals with such different ionic radii suggests that the enzyme exhibits some conformational flexibility.

The metal content of the native enzyme could not be determined since the usual purification scheme required the application of EDTA. Acetylation of a partially purified preparation in calcium-containing, but zinc-free buffers permitted the purification of an aminopeptidase derivative without exposure to chelating agents. This preparation contained 0.78 g-atom of zinc and 0.30 g-atom of calcium/mol of enzyme. This suggests that the native protein is a zinc enzyme and that some exchange of calcium for zinc occurs during purification procedures.

The purification and preliminary characterization of two aminopeptidases in pronase have been described; it has been suggested that these enzymes may serve as useful agents in studies on the primary structure of polypeptide chains (2, 3). The present studies were carried out to determine the best conditions for utilization of one of the aminopeptidases, noting in particular the effects of various buffers and metals. It was observed earlier that calcium provided the highest activity after EDTA treatment. In fact calcium restores more activity than originally present in the native enzyme, even though the protein had been processed in calcium-containing buffers. This led to the consideration that certain metals present in pronase might inhibit or provide lesser activity to the aminopeptidases. If such were the case, treatment with EDTA followed by calcium ion addition could have permitted superactivation of the enzymes. The following investigations support this interpretation; however, as noted below, complicated effects of calcium ions and protons upon the activity were noted. To discern the metal content of the native enzyme, it became necessary to develop a purification scheme without prior EDTA treatment.

**EXPERIMENTAL PROCEDURES**

The miniprint supplement contains all experimental details.’

**RESULTS AND DISCUSSION**

An earlier report described the pH dependency of activity for the calcium complexes of pronase aminopeptidases (2). The finding of a double peak (at pH values of 8.0 and 9.7) for maximal activity was observed consistently at low calcium concentrations. Later studies gave marked variations in activity in the higher pH range and this soon was correlated with differences in the calcium concentrations. As Fig. 1 reveals, a wide range in calcium concentrations (at values in excess of the AP-1* concentration) affects the activity only slightly at

*This investigation was supported by United States Public Health Service Grants NIH-GM-22468 and NIH-GM-22011. This is Paper XII of a series. Paper XI is Ref. 1. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Present address, Biozentrum der Universität, Basel, Switzerland.

§ To whom all inquiries should be directed.

**REFERENCE**

1. Experimental data as well as Table I, Figs. 1a, 2a, and 3a, some of the "Results and Discussion," and all the references are presented as a miniprint supplement immediately following this paper. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 77M429, cite author(s), and include a check or money order for $1.55 per set of photocopies.

2. The abbreviations used are: AP-1, aminopeptidase-1 from pronase (2); LeuNHNp, L-leucine-p-nitroanilide.
pH 8, whereas at pH values of 9.5 or greater the enzyme's activity is reduced sharply with increasing metal content. Studies carried out at pH values of 8.0 and 9.5 revealed in each case that no activity was found in the absence of calcium.

The observation of two pH optima for aminopeptidase activity was noted earlier under certain conditions in the case of the swine kidney enzyme (4). In contrast, studies with Aeromonas and human liver alanine aminopeptidases have revealed only single peaks of activity as a function of the hydrogen ion concentration (5, 6). The fall in activity between pH values of 10 and 11 in the present case is not due to loss of stability, since pronase aminopeptidase has been demonstrated previously to be stable to pH 11 (2). The differential effect of increasing metal concentrations on aminopeptidase activity at pH 10 as compared to pH 8 appears to be a unique property for the pronase enzyme. Since metal ion is absolutely required for activity, the conclusion follows that the inhibition by calcium at higher pH values reflects binding of the metal at a second site. Thus a high affinity site for calcium provides activity whereas a low affinity site contributes to enzyme inhibition. The apparent $K_D$ of about 9.2 (Fig. 1) associated with the inhibitory effect of the 2nd calcium ion may reflect either metal binding to enzyme or to enzyme-substrate complex, or may be due to the compound effect of both calcium binding and a hydroxyl group interaction with the calcium.

Preliminary studies were carried out with different amine buffers to determine the best conditions for application of AP-1 in the hydrolysis of polypeptide substrates. Marked differences were noted on the activity with various buffers at pH values of both 8.0 and 9.5; Fig. 2 shows the inhibition patterns as a function of the concentration of the different buffers. Histidine is the best inhibitor of the many compounds tested. Glycine is a slightly better inhibitor than leucine; the inhibitions by $d$- or l-leucine are virtually equivalent. The inhibition by $\beta$-alanine or $\gamma$-aminobutyrate is very poor; the dipeptide, glycylglycine, shows only 50% inhibition at a concentration of 0.1 M. Glycylglycine is a very poor substrate for AP-1 (3); the amounts of glycine generated would be too low to affect the enzyme's activity during the short periods of assays of activity. Finally Tris-HCl appears to be a poor inhibitor.

The inhibition of aminopeptidase activity by amino acids was demonstrated earlier in the cases of human liver alanine and swine kidney microsomal aminopeptidases (7, 8). With the former enzyme inhibition was competitive at low amino acid concentrations, but at higher concentrations mixed competitive and uncompetitive inhibition was noted. Hydrophobic amino acids were the best inhibitors. Glycine and l-histidine inhibited 80 and 3 times, respectively, less than L-leucine. $d$-Leucine inhibited 30 times less than L-leucine. $\beta$-Alanine and $\gamma$-aminobutyrate were significantly better inhibitors than glycine. The substrate used in these studies was Leu-NHNp.

In the study with the liver enzyme, the substrates were L-alanyl-$\beta$-naphthylamide, L-leucyl-L-leucine, and L-alanyl-L-alanyl-$l$-alanine; inhibition with amino acids was noncompetitive, mixed, and competitive for the three substrates, respectively. The following were the significant findings for individual amino acids: hydrophobic amino acids were the best inhibitors; L-histidine inhibited very poorly while 10 mM glycine demonstrated no significant inhibition, $\beta$-alanine was a good inhibitor; and 10 mM $d$-leucine provided very little inhibition. The conclusions were that the enzyme had several sites for binding amino acids in NH$_2$-terminal sequences and that the enzyme site which bound free amino acids preferentially was probably the site which bound the antepenultimate residue. To confirm that this site preferentially bound large hydrophobic residues, it was demonstrated that Gly-Gly-L-Phe was 5 times better an inhibitor against L-alanyl-$\beta$-naphthylamide than was Gly-Gly-L-Ala.

In the present study, the striking finding is the marked inhibition by l-histidine in contrast to the observations noted above. Furthermore, glycine is a slightly better inhibitor than leucine and in the case of leucine, little difference between optical isomers is noted. Clearly both an $a$-amino group and an $a$-carboxyl group are required for good inhibition, since $\beta$-alanine and $\gamma$-aminobutyrate have little effect on enzyme activity at low concentrations. The pattern of inhibition as a function of concentration by these latter two compounds is not representative of binding to a single site. The order of reactivity as inhibitors for the many amino compounds in this investigation may be related in part to their differential ability to chelate metals.
As described in the supplement the present investigations indicate very strongly that the native metal in pronase aminopeptidase is probably zinc. Exchange of some of the zinc by calcium occurred during either the preliminary steps of commercial preparation (9) or the purification steps described in this report. The finding of zinc with the protein is not unexpected, since this metal has been found in all other aminopeptidases where metal studies have been carried out (5, 10-13). The determination of whether this observation is a manifestation of divergent evolutionary development of this class of enzymes must await sequence studies. The other aminopeptidases are not activated by calcium; where metal activation has been demonstrated usually magnesium, manganese, and cobalt have been implicated. Swine kidney aminopeptidase at pH 8.6 reveals increasing activity as manganese and magnesium ion is increased from $10^{-5}$ M to $10^{-3}$ M. Pronase aminopeptidase is an extracellular enzyme in contrast to the other cases and the possibility exists that activation with calcium reflects an adaptation to the availability of extracellular calcium in contrast to the low intracellular concentrations of this metal (14).

Acknowledgments—We wish to express our appreciation for the encouragement and support that Mr. and Mrs. George Joannou have extended to the efforts of this laboratory and for the guidance of Dr. A. H. Brady in the circular dichroism measurements. We are grateful for the help in the atomic absorption analyses provided by Dr. Bruce Cameron, Dr. Fazal Ahmad, and Mrs. Paulette Smariga of the Papanicolau Cancer Research Institute, Miami, Fla.

REFERENCES

For list of references cited in this article, see p. 260.
Pronase Aminopeptidase

**Fig. 25**

**Fig. 25** demonstrates the enzymatic activity of pronase A on various substrates. The activity is measured as the release of free amino acids from the peptide bond. The values are plotted against time and are expressed as milligrams of free amino acids per hour. The y-axis represents the concentration of free amino acids, and the x-axis represents time in hours.

**Table 1**

| Substrate | Reaction Rate | Reaction Efficiency | Productivity after 60 min |
|-----------|--------------|---------------------|--------------------------|
| xylose    | 100          | 10                  | 10                       |
| xylobuf   | 90           | 9                   | 9                        |
| xylobuf   | 80           | 8                   | 8                        |
| xylobuf   | 70           | 7                   | 7                        |
| xylobuf   | 60           | 6                   | 6                        |
| xylobuf   | 50           | 5                   | 5                        |
| xylobuf   | 40           | 4                   | 4                        |
| xylobuf   | 30           | 3                   | 3                        |
| xylobuf   | 20           | 2                   | 2                        |

The data were analyzed using an enzyme activity calculator, and the results were plotted as a graph. The graph shows a clear correlation between the reaction rate and the final product concentration. The productivity after 60 minutes is also calculated, showing a consistent increase in product concentration over time.

**Fig. 26**

**Fig. 26** shows the effect of various pronase A concentrations on the activity of the enzyme. The y-axis represents the concentration of free amino acids, and the x-axis represents the concentration of pronase A. The data points are plotted as a line graph, showing a linear relationship between the two variables.

**Fig. 27**

**Fig. 27** illustrates the effect of pH on the activity of pronase A. The y-axis represents the concentration of free amino acids, and the x-axis represents the pH. The data points are plotted as a line graph, showing a peak activity at a specific pH value.

**Fig. 28**

**Fig. 28** depicts the effect of temperature on the activity of pronase A. The y-axis represents the concentration of free amino acids, and the x-axis represents the temperature. The data points are plotted as a line graph, showing a peak activity at a specific temperature.
Proteolytic enzymes of the K-1 strain of Streptomyces griseus obtained from a commercial preparation (Pronase). Effect of pH, metal ions, and amino acids on aminopeptidase activity.

K D Vosbeck, B D Greenberg, M S Ochoa, P L Whitney and W M Awad, Jr

J. Biol. Chem. 1978, 253:257-260.