A COMPARATIVE STUDY OF BACTERIAL ALANINE AMINOHYDROLASES[1]

Francis J. Behal and James D. Folds
Department of Microbiology
Medical College of Georgia
Augusta, Georgia
30902

Received March 27, 1967

This study is part of a program to investigate the relationship between alanylglycine dipeptidase and arylamidase, each hydrolyzing different, but structurally related substrates, i.e., L-alanylglycine and L-alanine-β-naphthylamine, respectively. These comparative studies were undertaken as an approach to investigating the natural function and role of arylamidases.

The Gram-negative diplococcus Neisseria catarrhalis produces large quantities of each of these two hydrolases. The former has properties generally consistent with alanylglycine dipeptidases reported in microorganisms (Cordonnier, 1961) and mammalian tissues (Ayavou, 1963); the latter has properties consistent with what is now known to be arylamidase (Behal, Klein, and Dawson, 1966; Folds and Behal, 1966). The results obtained in this study indicate that these two enzymes are distinct and unrelated, even though they have a common substrate requirement for N-terminal alanine residues. The biologic role of arylamidase in bacteria remains unclear.

MATERIALS AND METHODS

The methods used for growing N. catarrhalis (ATCC 8176) and preparing cell-free extracts were as previously reported (Folds and Behal, 1966).

[1]Supported in part by U.S.P.H.S. Research Grant CA 06389, and U.S.P.H.S. Training Grant AI 248-01.

Rate of dipeptide hydrolysis was determined by the ninhydrin method (Fleisher, Pankow, and Warmka, 1964). Arylamidase activity was assayed by a modification of the Bratton-Marshall procedure for determining liberated β-naphthylamine (β-NA) (Behal, Klein, and Dawson, 1966). Enzymes were purified by means of salt fractionation, DEAE cellulose chromatography, and calcium phosphate chromatography. The starch gel method (Smithies, 1955) was used for electrophoretic separation of enzymes.

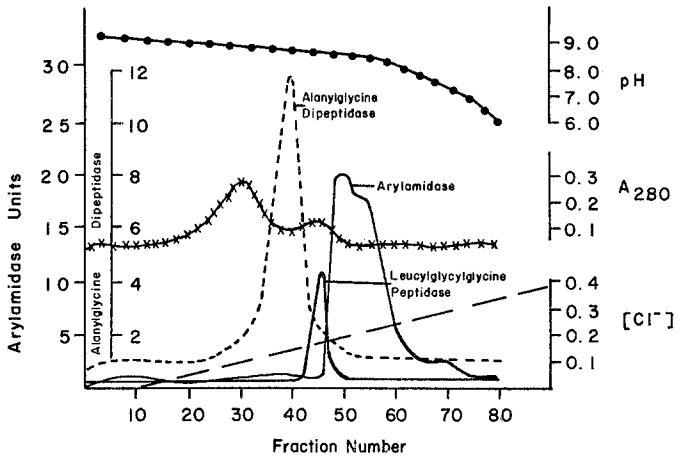
RESULTS AND DISCUSSION

Alanylglycine dipeptidase was separated from arylamidase and a leucylglycylglycine peptidase by column chromatography as shown on Figure I. This procedure gave a 50 fold purification of alanyglycine dipeptidase with a 25% recovery. This purified dipeptidase was characterized with regard to substrate specificity, pH optimum, and metal ion requirement. These data are shown on Table I.

This alanylglycine dipeptidase was similar to leucine aminopeptidase in that divalent cations (Mn^{++} or Mg^{++}) were required for stability of the enzyme. The pH optimum of 7.6 was consistent with that reported for similar hydrolases from other sources as was the spectrum of metal ion activators. Puromycin, at concentrations up to 10.0 μ mole/ml, were not inhibitory; chelating agents (EDTA and citrate) and p-mercuribenzoic acid were powerful inhibitors.

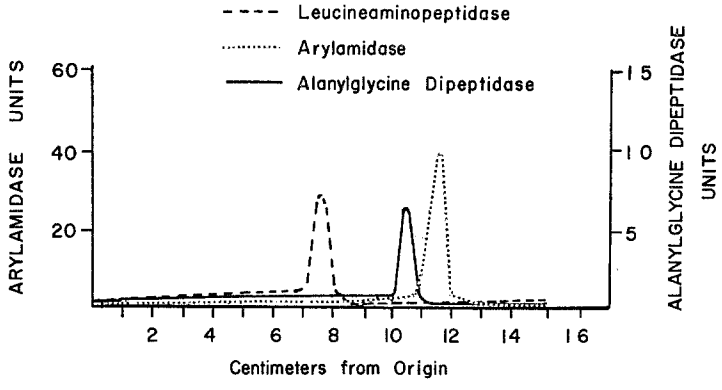
While the nature of the C terminal residue obviously was related to the rate of hydrolysis, it is clear that the N-terminal residue must be of the L configuration. For this reason it was felt that valid comparisons could be made between this hydrolase and arylamidase.

Previously reported (Behal and Folds, 1967) properties for arylamidase from the organism are as follows. No metal ion requirement could be demonstrated; a sharp pH optimum was observed at 7.3 in several buffer species.



COLUMN CHROMATOGRAPHIC ELUTION PROFILE OF ALANYLGLYCINE DIPEPTIDASE, LEUCYLGLYCYLGLYCINE PEPTIDASE AND ARYLAMIDASE ACTIVITY OF N. CATARRHALIS.

Figure 1



ELECTROPHORETIC SEPARATION OF ALANYLGLYCINE DIPEPTIDASE, ARYLAMIDASE AND LEUCINEAMINOPEPTIDASE.

Figure 2

The substrates hydrolyzed, in order of decreasing reactivity, (relative rates as in Table I above) were, alanine-βNA, 100; leucine-βNA, 28; methionine-βNA, 28; α-aspartic acid-βNA, 27; arginine-βNA, 15; glycine, 14; phenylalanine-βNA, 1.0. Those substrates not hydrolyzed were, isoleucine-βNA, valine-βNA, serine-βNA, α and γ-glutamic acid-βNA, β-aspartic acid-βNA, threonine-βNA, histidine-βNA, proline-βNA, cysteine-βNA, and the di-

TABLE I

Properties of Alanyglycine Dipeptidase

Substrate	Rate of Hydrol.*	pH	Rate of Hydrol.*	Cation***	Rate of Hydrol.*
L-alanyglycine	100	6.7	40	Co^{++}	100
L-isoleucylglycine	80				
L-valylglycine	75	7.0	46	Mn^{++}	82
glycylglycine	75				
L-phenylalanylglycine	61				
L-leucylglycine	53	7.3	55	Mg^{++}	60
L-methionylglycine	49	7.6	100	Zn^{++}	0
glycyl-L-leucine	47				
L-serylglycine	46	7.9	68	Ca^{++}	0
L-leucyl-L-leucine	28	8.2	50	Sn^{++}	0
L-alanylglycylglycine	25				
L-alanyl-L-phenylalanine	17	8.5	44	Sr^{++}	0
L-alanyl-L-alanine	10	8.8	40	Be^{++}	0
glycyl-D-leucine	10				
D-alanylglycine	0	9.1	20	Cu^{++}	0
D-leucylglycine	0	9.4	5		0
L-leucylglycylglycine	0				
L-leucyl-L-tyrosine	0				
D-leucyl-L-tyrosine	0				
N-CBZ-L-alanylphenylalanine	0				
L-alanine βNA	0				

* The value, 100 is assigned to that substance or condition in each column giving maximum velocity.

** This value was obtained at pH 7.6 and 7.3 and with and without Co^{++}, Mg^{++} or Mn^{++}.

*** Divalent cations were tested over a range of concentrations from 1.0 to 5.0 μ moles/ml.

and tripeptides shown on Figure I. Puromycin and p-chloromerucribenzoic acid were powerful inhibitors of arylamidase while EDTA was not inhibitory. On the basis of this data and the chromatographic separations described above it was concluded that these two enzymes were distinct.

Further evidence on the non-identity of bacterial alanylglycine dipeptidase, bacterial arylamidase and authenic leucineaminopeptidase (Worthington-swine kidney) was obtained by electrophoresis. Following electrophoresis at 4.0 volts/cm. at 4° C. for 16 hours the starch gels were sliced into longitudinal strips corresponding to the path of

migration of the sample. The strips were then cut into 1 cm. segments which were then mascerated and eluted with phosphate buffer, pH 7.2. The appropriate resulting eluates were then assayed for alanylglycine dipeptidase, and arylamidase and leucineaminopeptidase. These results are shown in Figure 2.

Studies were carried out to localize the alanylglycine dipeptidase and arylamidase activities in N. catarrhalis. Membrane, ribosomal, and soluble fractions were obtained by differential centrifugation (Nomura, 1962). The alanylglycine dipeptidase activity was found to be distributed in the following percentages: 70% membrane; 6% ribosomal, and 24% soluble. Under these conditions, arylamidase was found to be soluble.

A specific rabbit antiserum was prepared to the bacterial arylamidase which inhibited arylamidase in vitro. This antiserum did not inhibit alanylglycine dipeptidase nor did it cross react in Ouchterlony gel diffusion plates with this dipeptidase.

Additional basic differences were noted between the action of alanylglycine dipeptidase and arylamidase in their substrate specificity patterns. While arylamidase had little or no activity on amino acid-βNA with β branching, i.e., valine or isoleucine-βNA (Behal, Asserson, Dawson, and Hardman, 1965), the dipeptidase had considerable activity on isoleucyl-valylglycine. The resistance of β-branched derivitives to hydrolysis has also been noted in the case of aminopeptidases as well (Smith, Spackman, and Polglase, 1952).

It must be concluded that this bacterial alanylglycine dipeptidase is distinct from the bacterial arylamidase, despite the similarity in requirement for N-terminal amino acid residue. Furthermore, no inferences regarding arylamidase function can be made through comparative studies of these two enzymes. Thus, the biologic role of arylamidase remains unclear, but it would appear that it is other than simple cleavage of peptides.

REFERENCES

Ayavou, T. and Cristol, P., 1963 Societe de Biologie, 157:321-354.
Behal, F. J., Asserson, B., Dawson, F. B., and Hardman, J., 1965 Arch. Biochem. Biophys., 111:335-44.
Behal, F. J. and Folds, J. D., 1967 Arch. Biochem. Biophys. In Press.
Behal, F. J., Klein, R., and Dawson, F. B., 1966 Arch. Biochem. Biophys. 115:545-54.
Cordonnier, R., 1961 Compt. Rend., 253:748-50.
Fleisher, G. A., Pankow, M., and Warmka, C., 1964 Clin. Chem. Acta., 9:259-68.
Folds, J. D. and Behal, F. J., 1966 Bact. Proc. p. 105
Nomura, H., Hall, B. D. and Spiegelman, S., 1962 J. Mol. Biol. 2:306.
Smith, E. L., Spackman, D. and Polglase, W. J., 1952 J. Biol. Chem. 199: 801-817.
Smithies, O., 1955 Biochem. J. 61:629-41.