Hydrolysis of Di- and Oligopeptides by Human Liver Arylamidase[1] (35404)

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The use of L-leucine-β-naphthylamide (leucine-βNA) instead of L-leucylglycine as a specific substrate for leucine aminopeptidase (LAP) was generally accepted until other enzymes that hydrolyzed leucine-βNA and other amino acid-βNA very rapidly were isolated. These other hydrolytic enzymes, subsequently designated arylamidases, differed from LAP in that they hydrolyzed amino acid-βNA very much more rapidly than leucylglycine. They also differed from LAP in their pH optima and divalent cation requirements. Arylamidases appear to be ubiquitous in nature but their *in vivo* role is uncertain.

We recently reported the purification of an arylamidase from human liver, some of its characteristics, and its catalytic action on a variety of amino acid-βNA (1). Subsequently, we have extended our knowledge of the specificity of this enzyme by investigating its action on a number of di- and oligopeptides. These are probably more closely related to the natural substrates of the enzyme and, therefore, might provide some insight into the biological function of arylamidases. These investigations are reported below. Although the specificity of human liver arylamidase is rather broad, a definite pattern of specificity has emerged from our studies which makes the enzyme readily distinguishable from other aminopeptidases (2–11) and makes possible certain speculations regarding the nature of binding of substrates at the active site.

Materials and Methods. Substrates were obtained from Mann Research Laboratories, New York, N.Y., the International Chemical and Nuclear Co., Burbank, Calif. and from Fox Chemical Co., Los Angeles, Calif.

Arylamidase was purified 2120-fold from human liver as previously described (1). Ultracentrifugal and electrophoretic data indicated the enzyme preparation contained a single protein.

The rate of liberation of β-naphthylamine (βNA) from amino acid-βNA was determined as previously described (1). The rate of hydrolysis of dipeptides was determined as described by Fleisher *et al.* (12). All assays were carried out at pH 6.8. The substrate concentration was 1.0 mM in all cases.

One-dimensional chromatography was carried out at 27° on Whatman No. 1 filter paper using the ascending technique described by Williams and Kirby (13). The chromatograms were developed with butanol: acetic acid:water 4:1:5 (upper phase) or with phenol saturated with water, then allowed to dry at room temperature. Chromatograms developed with phenol were washed with ether to remove remaining phenol. Liberated amino acids and peptides were then visualized by spraying the chromatograms with 0.25% Ninhydrin in acetone and heating them at 100° for 10 min.

Results. The influence of substrate structure on susceptibility to arylamidase catalyzed hydrolysis was studied. Table I shows the effect of different N-terminal amino acid residues on the rate of hydrolysis of dipeptides and amino acid-βNA. The highest rates of hydrolysis were observed with substrates having a nonpolar or basic amino acid as the N-terminal residue. Furthermore, substrates with straight side chains on the N-terminal residue were hydrolyzed more rapidly than substrates with branched side chains, and substrates with γ-branched side chains were hydrolyzed more rapidly than those with

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TABLE I. Rates of Hydrolysis of Substrates with Varying N-Terminal Residues.[a]

N-Terminal	C-Terminal			
	β-Naphthylamine	*p*-Nitroaniline	Glycine	Alanine
L-Alanine	9.95 (100)	0.99 (100)	1.87 (100)	3.88 (100)
L-Phenylalanine	6.25 (62.8)		1.64 (87.7)	
L-Norvaline			1.09 (58.3)	
L-Norleucine			0.68 (36.5)	
L-Methionine	5.72 (57.5)			
L-Leucine	3.62 (36.4)	0.40 (40)	0.47 (25.2)	2.19 (56.4)
L-Arginine	2.79 (28.0)			
L-Tryptophan	1.92 (19.2)			
Glycine	1.36 (13.7)		0.00 (0.0)	
L-Lysine	1.34 (13.4)			
L-Serine	0.59 (6.0)		0.11 (6.0)	
L-Threonine	0.57 (5.7)			
L-Glutamic acid	0.37 (3.7)			
L-Valine	0.28 (2.8)		0.00 (0.0)	
L-Isoleucine	0.28 (2.8)		0.13 (6.7)	

[a] Rates of hydrolysis in all cases are expressed in nanomoles per milliliter per minute. In all cases the incubation mixtures were identical except for the variation of substrate. The numbers in parentheses are normalized values with the substrate hydrolyzed most rapidly in a given column being assigned the value, 100.

β-branched side chains. Substrates having N-terminal L-alanine residues were hydrolyzed most rapidly (for a given C-terminal residue) with L-alanyl-βNA being the most rapidly hydrolyzed of all the substrates tested. L-Leucylglycine, the preferred substrate of leucine aminopeptidase (EC 3.4.1.1.) was hydrolyzed only 4.5% as rapidly as L-alanyl-βNA.

Table II shows the effect of different C-terminal residues on the rate of hydrolysis of dipeptides. It was again observed that nonpolar or basic residues were associated with the higher rates of hydrolysis. Of this group of substrates, L-alanyl-L-tryptophan was the one most rapidly hydrolyzed. Also since L-alanyl-L-valine was hydrolyzed quite rapidly, a β-branch in the side chain of the C-terminal residue appears to be of considerably less importance than in the case of an N-terminal residue. L-Alanyl-L-proline was totally resistant to hydrolysis by arylamidase.

The susceptibility of L-alanyl-L-alanineamide to arylamidase catalyzed hydrolysis was determined by paper chromatography. Alanine and alanineamide were the initial products as shown in Fig. 1. These were formed at a rate comparable to that of the hydrolysis of L-alanyl-L-alanine by arylamidase. The subsequent hydrolysis of the re-

TABLE II. Rates of Hydrolysis of Substrates with Varying C-Terminal Residues.[a]

C-Terminal	N-Terminal	
	Alanine	Leucine
β-Naphthylamine	9.95 (100)	
L-Tryptophan	8.55 (85.9)	
L-Leucine	2.32 (23.3)	3.07 (100)
L-Valine	4.04 (40.6)	
L-Alanine	3.88 (39.0)	2.19 (71.3)
L-Phenylalanine	3.66 (36.8)	
L-Histidine	3.76 (37.8)	
L-Serine	1.92 (19.3)	
L-Glutamic acid	1.62 (16.3)	
Glycine	1.52 (15.3)	0.47 (15.3)
L-Aspartic acid	0.97 (9.75)	
p-Nitroaniline	0.99 (9.95)	
L-Proline	0.00	

[a] Rates of hydrolysis in all cases are expressed in nanomoles per milliliter per minute. In all cases the incubation mixtures were identical except for the variation of substrate. The numbers in parentheses are normalized values with the substrate hydrolyzed most rapidly in a given column being assigned the value, 100.

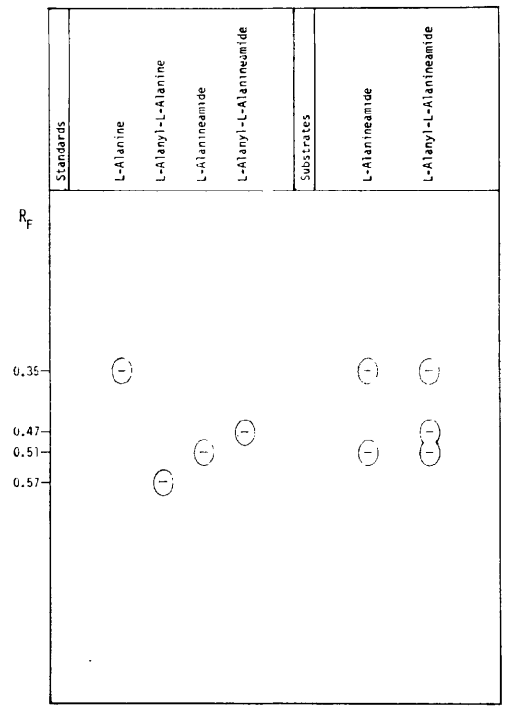

FIG. 1. Composite chromatogram showing products resulting from arylamidase catalyzed hydrolysis of amides. Chromatograms were developed with *n*-butanol:acetic acid:water (4:1:5; v:v:v) upper phase.

sultant alanineamide to alanine and ammonia occurred at a very much slower rate.

To characterize further the specificity and mode of action of human liver arylamidase, the hydrolysis of the optical isomers of alanylalanine and alanylalanylalanine was investigated. When the four optical isomers of alanylalanine were incubated with arylamidase, and aliquots of the reaction mixture were chromatographed, only L-alanyl-L-alanine was found to be hydrolyzed. After short incubation L-alanyl-L-alanyl-L-alanine yielded alanine and alanylalanine; after further incubation only alanine was found. When L-alanyl-L-alanyl-D-alanine was used as a substrate, alanine and alanylalanine were the only products, even after prolonged incubation. L-Alanyl-D-alanyl-L-alanine was completely resistant to hydrolysis by arylamidase. When tetra-L-alanine was the substrate the initial products were alanine and tri-alanine with alanylalanine appearing somewhat later. After extended incubation only alanine was found in the incubation mixture. These results are shown in the composite chromatogram in Fig. 2.

Discussion. A study of arylamidase action on a group of peptides composed of naturally occurring amino acids only was undertaken to provide further insight into the *in vivo* action of this enzyme since amino acid-βNA are not encountered as normal metabolites. As expected, only those peptides with L-amino acid residues in the N-terminal position were hydrolyzed.

The nature of the side chain of the N-terminal residue was an important factor in the rate at which various peptides were hydrolyzed. In the first group of experiments, this residue was varied and the C-terminal residue was fixed. Substrates with N-terminal L-alanine residues were hydrolyzed most rapidly. Substrates having N-terminal amino acid residues with γ-branched side chains (*e.g.*, L-leucylglycine) were hydrolyzed more rap-

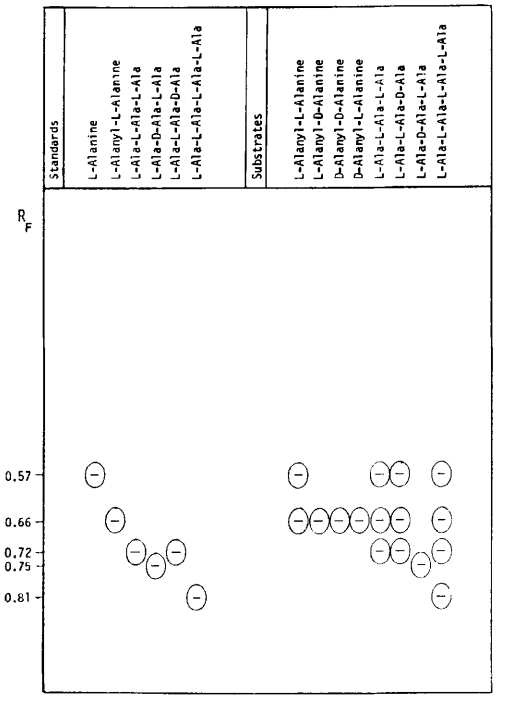

FIG. 2. Composite chromatogram showing products resulting from arylamidase catalyzed hydrolysis of stereoisomers of alanine di- and oligopeptides. Chromatograms were developed with phenol saturated with water.

idly than substrates with β-branched side chains (*e.g.*, L-valylglycine and L-isoleucylglycine). Substrates with straight side chains on the N-terminal residue (*e.g.*, L-norleucylglycine and L-norvalylglycine) were hydrolyzed more rapidly than those with either β branched or γ-branched side chains. Glycylglycine, a substrate with no side chains at all, was extremely resistant to arylamidase catalyzed hydrolysis. These results, in addition to the kinetic data reported previously for amino acid-βNA (1) suggest very strongly that a hydrophobic region exists at, or very near, the active site of arylamidase, which interacts with the side chain of the N-terminal residue in attachment of substrate to enzyme.

Our earlier studies on the hydrolysis of optical isomers of alanylalanyl-β-naphthylamide by arylamidase (1) indicated that the enzyme was an exopeptidase. The action of arylamidase on the stereoisomers of alanylalanine, alanylalanylalanine and on L-alanyl-L-alanyl-L-alanyl-L-alanine supports this conclusion. Also, from these studies, it appears that an amino acid of the D-configuration in either the N-terminal or penultimate residue renders a peptide resistant to arylamidase catalyzed hydrolysis since neither D-alanyl-L-alanine, nor L-alanyl-D-alanyl-L-alanine was hydrolyzed by arylamidase. A D-isomer at a position other than N-terminal or penultimate appears to be without effect since the N-terminal residue of L-alanyl-L-alanyl-D-alanine was hydrolyzed by arylamidase.

To be susceptible to arylamidase catalyzed hydrolysis, a substrate must have an unsubstituted α-amino group of the L-configuration (1). Since L-alanineamide is susceptible and L-alanyl-L-proline is not susceptible to arylamidase, substrates for the enzyme can be described in general terms as α-amino acid amides with at least one unsubstituted hydrogen on the amide nitrogen. Monosubstituted amides such as peptides and amino acid-βNA are hydrolyzed more rapidly than unsubstituted amides. This may indicate a point of attachment for the substituent group at the active site especially since the nature of the side chain of the C-terminal residue of dipeptides so markedly influences the rate of hydrolysis. It is therefore, possible to postulate three points of interaction between dipeptide and enzyme, namely, the α-amino group, the N-terminal side chain and the C-terminal side chain.

Summary. Arylamidase catalyzed hydrolysis of a variety of dipeptides and oligopeptides was investigated. The N-terminal residue must be an α-amino acid of the L-configuration. The penultimate residue must also be of the L-configuration. Substrates with straight or γ-branched nonpolar side chains on the N-terminal residue were most susceptible to arylamidase catalyzed hydrolysis. Substrates with β-branched or polar side chains on the N-terminal residue were quite resistant to arylamidase catalyzed hydrolysis. Substrates having nonpolar side chains on the penultimate residue were hydrolyzed quite rapidly regardless of the branch point. Amino acid amides are hydrolyzed by arylamidase.

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