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ENZYMATIC AND IMMUNOLOGICAL PROPERTIES OF THE PROTEASE FORM OF AMINOPEPTIDASES N AND A FROM PIG AND RABBIT INTESTINAL BRUSH BORDER

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Summary

Immunological homology was shown between the active site regions of pig and rabbit aminopeptidases N and between those of the corresponding aminopeptidases A. However, no homology was detectable between the aminopeptidases N and A (EC 3.4.11.-) in a given species. The dimeric structure of pig aminopeptidases did not significantly modify their catalytic properties in aqueous solution compared to those of the monomeric rabbit enzymes. Only a slight difference in binding conditions was noted in the case of aminopeptidases N.

Aminopeptidase A activity towards acidic substrates was enhanced by physiological concentrations of Ca^{2+} while that towards neutral substrates was considerably reduced. Therefore, acidic amino acid residues in proteins and peptides may be assumed to be mostly split off in vivo by aminopeptidase A, neutral residues by aminopeptidases N and basic residues by both enzymes.

The respective specificity of aminopeptidases A and N for acidic and neutral amino acid residues was found to be mainly due to a more productive binding mode of the substrate rather than to a better affinity.

A remarkable cooperation appears to exist between pancreatic endopeptidases and carboxypeptidases for protein degradation in the intestine. The specificity of the first enzyme is mostly governed by the residues on the carboxyl side of the scissile peptide bonds and these residues correspond fairly well to the specificity of the second enzyme. In contrast, the specificity of the

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intestinal [1—3] and pancreatic peptidases are complementary, thus allowing complete protein breakdown into constitutive amino acids.

The major brush border peptidases present in intestinal and renal absorbing cells are two aminopeptidases denoted N and A assumed to be mostly active on neutral and acidic N-terminal amino acid residues in proteins and peptides [1,4—7]. The N and A enzymes represent, respectively, 8% and 3.5% of the pig intestinal brush border proteins [4,6]. Both amount to 3.5% of the proteins in the rabbit [5,7]. Aminopeptidases N from gut [4], kidney [8—9] and liver [10] are homologous enzymes [9,11]. In human kidney and liver [9], porcine kidney and intestine [12,13] and in rat intestine [14], the N enzymes have been shown to be symmetrical dimers containing 1 gatom Zn^{2+}/subunit. By contrast, the enzyme from rabbit intestine [5] and probably also that from rabbit kidney [15] are monomeric with 1 gatom Zn^{2+}/mol. The effect of these different structures on activity has not yet been elucidated. While several reports have already been published on the enzymatic properties of aminopeptidases N, the role of Zn^{2+} in catalysis and the topography of the active site [16,21] relatively few data are presently available on aminopeptidases A, which have been more recently purified from pig and rabbit intestine [6,7] and from pig kidney [22]. In pig, the A enzyme is a dimeric protein in which Zn^{2+} is replaced by Ca^{2+} [22] while it is also monomeric in rabbit [7].

The aim of this work was to evaluate the role played by the dimeric structure of the pig aminopeptidases compared to the monomeric structure of the rabbit enzymes. The respective specificities of aminopeptidases N and A from these two species were also compared in the presence or absence of added Ca^{2+}, using a variety of synthetic *p*-nitroanilides and peptides as substrates. Moreover, the immunological homology between the N and A enzymes was investigated.

Material and Methods

Materials

Amino acids, peptides and the *p*-nitroanilides of L- or D-alanine and of L-leucine were purchased from Bachem. L-Glutamic acid α-*p*-nitroanilide was from Merck.

Methods

Purification of enzymes and antibodies. The so-called detergent forms of aminopeptidases N and A were first extracted by Emulphogen treatment of pig and rabbit intestinal mucosa [5,6] and then converted into the corresponding protease forms by trypsin digestion. These latter forms were purified to homogeneity as described [4—7]. The preparations served for guinea pig immunization. Total immunoglobulins were prepared from the resulting immunsera [23].

Enzyme kinetics. Hydrolysis of amino acid *p*-nitroanilides was measured spectrophotometrically at 410 nm [4]. Peptide hydrolysis was followed by determination of the liberated amino acids in a Beckman automatic analyzer Model 120 C. Standard conditions were used in most cases. For the peptide Leu-Gly-Gly, full separation of leucine and Gly-Gly required column equilibration with a 0.2 M citrate buffer, pH 3.54, and elution by 0.8 M citrate, pH 3.80. Inhibition of Ala-Gly-Gly hydrolysis by alanine or Ala-Gly was followed

by chromatography of the Gly-Gly peptide in 0.2 M citrate pH 3.54.

The kinetic parameters (K_m and V) of *p*-nitroanilide or peptide hydrolysis were derived from Lineweaver-Burk reciprocal plots using at least eight substrate concentrations near the expected K_m. The catalytic constants k_{cat} were calculated using the following molecular weight values: 245 000 for porcine aminopeptidases N and A [4,6], 125 000 for rabbit aminopeptidase N [5] and 185 000 for rabbit aminopeptidase A [7].

The inhibitory effect of amino acids and peptides was tested by measuring $K_{m,i}$ and V_i at three inhibitor concentrations. When the inhibition was competitive ($V_i = V$), the inhibition constant K_i was calculated using the equation:

$$K_i = \frac{K_m[I]}{K_{m,i} - K_m}$$

The K_i values, which never differed by more than 10%, were averaged.

Results and Discussion

Immunological homology between intestinal aminopeptidases

During the immunological assays between the four enzymes (pig and rabbit aminopeptidases N and A) and their respective antibodies, three cases should be considered. At first, each enzyme was completely precipitated by its own specific antibodies and inhibition was about 50% (Fig. 1). Moreover, no precipitation was observed when an enzyme of a certain type (A or N) in a given species was incubated with an antibody raised against the same enzyme from another species (for example, rabbit aminopeptidase N and anti-pig aminopeptidase N). But, substantial inhibition, almost as strong as above, (Fig. 1) showed that cross reactions occurred in this case. Cross reactions between the aminopeptidases N and between the aminopeptidases A from pig and rabbit were also demon-

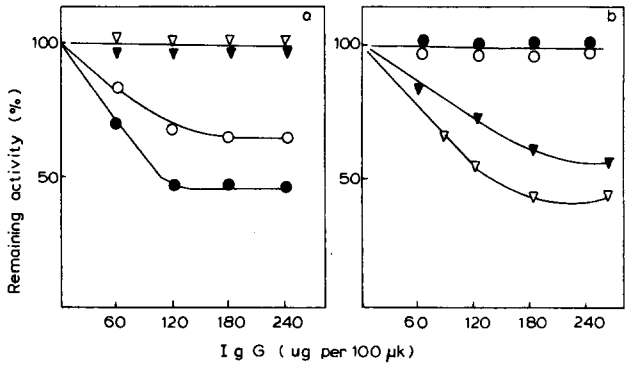

Fig. 1. Inhibition of pig and rabbit aminopeptidases N and A by their antibodies. The enzymes (12 µg) in 100 µl of a 10 mM phosphate buffer, pH 7.4/0.15 M NaCl were incubated for 4 h at 37° C, and then overnight at 4° C with various concentrations of immunoglobulins (IgG) from guinea pig against pig aminopeptidase N (a) or rabbit aminopeptidase A (b). The substrates were alanine *p*-nitroanilide for the N enzymes and glutamic acid α-*p*-nitroanilide for the A enzymes. ●——● and ▲——▲, pig aminopeptidases N and A, respectively; ○——○ and △——△, rabbit aminopeptidases N and A, respectively.

strated by immunofluorescence labeling (Feracci, H., Bernadac, A., Benajiba, A., Provost, A. and Maroux, S., unpublished data).

Finally, no cross reaction was observable between aminopeptidases N and A irrespective of the species from which they were extracted. As shown by Fig. 1, anti-pig aminopeptidase N antibodies did not inhibit pig and rabbit aminopeptidase A and reciprocally. No precipitation was observed.

These data are consistent with the existence of a significant immunological homology between the active site regions of the same enzyme originating from different species, but not between the aminopeptidases N and A in a given species. Chemical homology and consequently immunological homology cannot be expected in this latter case since the N enzymes are Zn^{2+}-dependent while the A enzymes are Ca^{2+}-dependent [4,5,22].

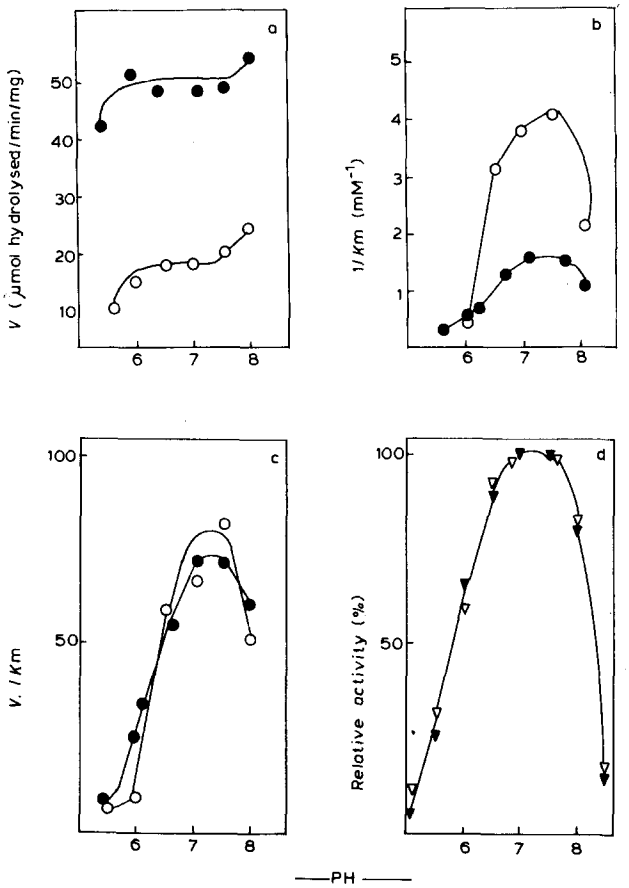

Fig. 2. Kinetic parameters of the hydrolysis of alanine or glutamic acid *p*-nitroanilide, respectively, by aminopeptidase N (Fig. 2a—c) and aminopeptidase A (Fig. 2d). ●———●, pig aminopeptidase N; ○———○, rabbit aminopeptidase N; ▲———▲, pig aminopeptidase A; △———△, rabbit aminopeptidase A. The reactions were performed at 37°C in 50 mM phosphate buffer for the N enzymes or in 0.1 M Tris-HCl buffer/25 mM $CaCl_2$ for the A enzymes.

pH dependence of p-nitroanilide hydrolysis by dimeric or monomeric aminopeptidases

The pH dependence of the kinetic parameters of alanine *p*-nitroanilide hydrolysis by pig and rabbit aminopeptidases N is illustrated by Fig. 2a—c. No significant difference between the two enzymes could be detected in the whole investigated pH range. Activity was maximal at pH 7.0—7.5 in both cases.

The hydrolysis of glutamic acid α-*p*-nitroanilide by pig and rabbit aminopeptidases A was also compared at various pH values in the presence of Ca^{2+}. As shown by Fig. 2d, the results were also similar for the two enzymes.

Ca^{2+} effect on the activity of aminopeptidases N and A towards synthetic and peptide substrates

An activating effect of Ca^{2+} on aminopeptidase A has been reported several years ago by the first authors who characterized this type of enzyme in the brush border membrane [24]. As for intestinal aminopeptidase N [5] and kidney aminopeptidase A [22], the A enzyme activity from pig and rabbit intestine was observed here to be completely abolished by a 24 h dialysis against 5 mM EDTA/50 mM phosphate, pH 6.0. Moreover, the assays depicted in Fig. 3 showed that addition of 5 mM $CaCl_2$ enhanced the A enzyme activity towards acidic substrates 2-fold, but induced an almost complete inhibition with neutral substrates. Due to this dual effect, the action of aminopeptidases A is probably restricted in vivo to acidic N-terminal residues.

The mechanism of the very large specificity difference existing between the N and A enzymes was also explained by the data reported in Table I. The much higher catalytic efficiency of the A enzymes for acidic substrates was shown to

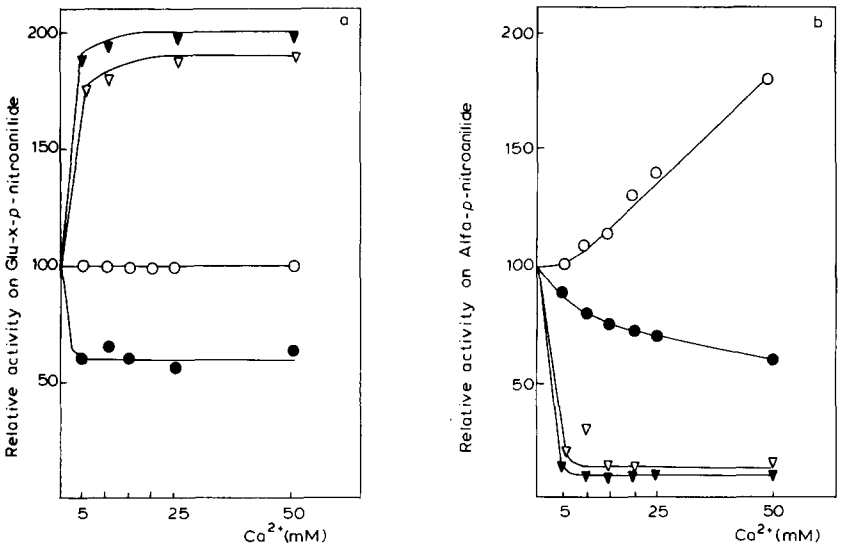

Fig. 3. Ca^{2+} effect on the hydrolysis of acidic and neutral substrates by intestinal aminopeptidases N and A. In all assays, the substrate (glutamic acid α-*p*-nitroanilide on the left; alanine *p*-nitroanilide on the right) was dissolved (1.5 mM) in 0.1 M Tris-HCl buffer, pH 7.0, containing various concentrations of $CaCl_2$. ●———● and ○———○, aminopeptidase N from pig and rabbit, respectively; ▲———▲ and △———△, aminopeptidase A from pig and rabbit, respectively.

TABLE I

KINETIC PARAMETERS OF PEPTIDES AND *p*-NITROANILIDES HYDROLYSIS BY AMINOPEPTIDASES N AND A

The reactions were performed at 37°C in a 50 mM phosphate buffer pH 7.0, except for the assays marked by an asterisk which were carried out at the same temperature and pH, but in a 0.1 M Tris-HCl buffer/26 mM $CaCl_2$. n.d., not determined.

	Aminopeptidase N						Aminopeptidase A					
	Rabbit			Pig			Rabbit			Pig		
	K_m (mM)	k_{cat} (s^{-1})	$\frac{k_{cat}}{K_m}$	K_m (mM)	k_{cat} (s^{-1})	$\frac{k_{cat}}{K_m}$	K_m (mM)	k_{cat} (s^{-1})	$\frac{k_{cat}}{K_m}$	K_m (mM)	k_{cat} (s^{-1})	$\frac{k_{cat}}{K_m}$
*Glutamic acid α-*p*-nitroanilide							0.85	135	158	0.98	208	212
*Glu-Ala-Ala	1.3	27	20.7	4.8	86	18	0.48	243	506	0.47	228	485
Glu-Ala-Ala	7.7	17	2.2	12.5	115	9	6.0	205	34	5.0	250	50
Glycine *p*-nitroanilide	1.4	15	10.7	2.6	44	17	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Gly-Gly-Gly	7.0	175	25	21	510	24	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Leucine *p*-nitroanilide	0.65	63	100	0.72	152	211	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Leu-Gly-Gly	0.15	30	200	0.13	75	577	0.5	3	6	1.2	2	1.7
Alanine *p*-nitroanilide	0.3	40	133	0.87	222	255	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ala-Gly-Gly	1.8	112	62	1.6	440	275	2.2	13	6	4.0	9.0	2.2
Ala-Gly-Gly-Gly	2.0	110	55	1.4	440	314	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ala-Ala-Ala	0.5	138	276	0.83	394	475	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Lys-Ala-Ala	0.18	58	322	0.35	128	365	0.6	16	27	0.06	25	415

mainly result from a better k_{cat} and this effect was still amplified by the already mentioned affinity increase mediated by Ca^{2+}. The most significant overlap between both enzymes is their common ability to split lysine and probably other basic substrates.

Comparison between pig dimeric and rabbit monomeric enzymes

Table I also shows that the catalytic efficiency and affinity were not modified in the pig dimeric enzymes by more than 2—4-fold compared to the monomeric rabbit enzymes and this difference, if any, was not considered significant, especially if the porcine enzymes are symmetrical dimers with two independent active sites/mol. However, it must be stressed here that little is known at the present time about the real associations existing between molecules or subunits in the brush border membrane. The above evidence does not exclude the possibility that enzymes, which are monomeric in aqueous solutions are actually in an aggregated state in situ to acquire full efficiency for catalysis and transport.

Inhibition of aminopeptidase N by amino acids and peptides.

The possibility that the binding mode of *p*-nitroanilides and model peptides to aminopeptidase N may be different was suggested by the following observations. (a) The hydrolysis of the first substrates was slightly less affected than that of the second by the nature of the N-terminal amino acid (Table I). (b) The hydrolysis of *p*-nitroanilides was inhibited by an excess of substrate, especially in the case of the porcine enzyme, while that of peptide substrates was not inhibited. (c) As shown by Table II, the inhibition induced by free amino acids and peptides was competitive when the substrate was itself a peptide. With *p*-nitroanilides, competitive inhibition was only observed in the case of the pig enzyme and peptide inhibitors. In all other cases (pig enzyme + amino acids; rabbit enzyme + amino acids or peptides), the inhibition was of a non-competitive mixed type (simultaneous variations of K_m and V [25]). Therefore, *p*-nitroanilides and peptides may be assumed either to bind at two independent enzyme sites, or to require two different sets of subsites within a unique binding site.

It was noteworthy that, irrespective of the inhibition type and the origin of the enzyme, leucine and Leu-Gly were more potent inhibitors than alanine and Ala-Gly. In the case of non-competitive inhibition, this difference was mainly due to an effect on the K_m values rather than on V values.

The assays also permitted us to compare the effect of a given peptide taken successively as substrate (Tables I and III) or inhibitor of the hydrolysis of alanine *p*-nitroanilide (Tables II and III). In this manner, the K_m value was found to be very close to the K_i value, thus proving a posteriori that the K_m values of aminopeptidase-catalyzed reactions may, for all practical purposes, be regarded as affinity constants.

Stereospecificity of aminopeptidase N

It has been proposed that the binding site of aminopeptidase N for peptides is composed of at least three subsites interacting with the first three residues of the chain [21]. The stereospecificity of these subsites was tested using as substrates a series of peptides containing a D-alanine residue at different positions.

TABLE II

INHIBITION OF RABBIT AND PIG AMINOPEPTIDASES N BY AMINO ACIDS AND PEPTIDES

The symbol NCM indicates non-competitive mixed type inhibition: $K_{m,i}$ and V_i are calculated for an inhibitor concentration of 5 mM. NI means that the compound tested was not inhibitory up to a concentration of 25 mM.

Inhibitor	Substrate	Rabbit				Pig			
		Inhibition type	K_i (mM)	$K_{m,i}$ (mM)	V_i	Inhibition type	K_i (mM)	$K_{m,i}$ (mM)	V_i
Glycine	Alanine *p*-nitroanilide	NI		0.30	19	NI		0.9	53
Alanine	Alanine *p*-nitroanilide	NCM		0.25	4.8	NCM		0.9	27
Leucine	Alanine *p*-nitroanilide	NCM		1.3	3.6	NCM		2.1	23
Ala-Gly	Alanine *p*-nitroanilide	NCM		0.2	7.5	Competitive	4.5		
Leu-Gly	Alanine *p*-nitroanilide	NCM		0.8	3.6	Competitive	0.65		
Ala-Gly-Gly	Alanine *p*-nitroanilide	NCM		0.4	5.5	Competitive	1.3		
Alanine	Ala-Gly-Gly	Competitive	3.12			Competitive	5.9		
Leucine	Ala-Gly-Gly	Competitive	0.37			Competitive	0.7		
Ala-Gly	Ala-Gly-Gly	Competitive	1.53			Competitive	4.0		

TABLE III

STEREOSPECIFICITY OF AMINOPEPTIDASES N FROM PIG AND RABBIT INTESTINE

Inhibition was tested shaking as substrate alanine *p*-nitroanilide at the usual concentration. See Table II for the abbreviations.

Substrate and inhibitor	Rabbit			Pig		
	k_{cat} (s^{-1})	K_m (mM)	K_i (mM)	k_{cat} (s^{-1})	K_m (mM)	K_i (mM)
L-Ala-L-Ala-L-Ala	138	0.5	n.d.	394	0.83	0.60
L-Ala-L-Ala-D-Ala	82	1.1	n.d.	230	1.35	n.d.
L-Ala-D-Ala-L-Ala	0	—	NI	0	—	NI
D-Ala-Gly-Gly	0	—	NI	0	—	NI
L-Ala-L-Ala	70	1.8	—	190	2.2	3.1
L-Ala-D-Ala	0	—	NI	0	—	NI
D-Ala-L-Ala	0	—	NI	0	—	NI
L-Ala	—	—	NCM	—	—	NCM
D-Ala	—	—	NI	—	—	NI
L-Alanine *p*-nitroanilide	50	0.3	n.d.	222	0.87	n.d.
D-Alanine *p*-nitroanilide	0	—	NI	0	—	NCM

Evidence presented in Table III showed that hydrolysis was completely stopped by the D-stereo-isomer at the N-terminal or second position. Therefore, the first two enzyme subsites may be assumed to be fully functional and strictly stereospecific.

When located at the third position, the isomer was only slightly inhibitory, thus suggesting a much more minor role for the still hypothetical third subsite. The importance of the first two subsites for correct substrate binding was confirmed by the finding that L-alanine *p*-nitroanilide hydrolysis was not inhibited by D-alanine, D-alanine *p*-nitroanilide and the peptides D-Ala-L-Ala, D-Ala-Gly-Gly, L-Ala-D-Ala and L-Ala-D-Ala-L-Ala-.

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