A fluorimetric assay for aminopeptidase W

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A novel two-step enzyme-linked assay for aminopeptidase W is described and validated by comparison with other assays. L- α -Glutamyl-L-tryptophan (Glu-Trp) is a favoured substrate for this enzyme. With the use of glutamate dehydrogenase (EC 1.4.1.2) in a second step, the assay measured the release of free glutamate from L- α -glutamyl-L-tryptophan by the increase in NADH fluorescence. In the presence of 5 mM-1,10-phenanthroline and 50 μ M-cilastatin the contribution of other membrane peptidases, in particular aminopeptidases N and A and microsomal dipeptidase in kidney, was very small. Residual cytosolic activities hydrolysing Glu-Trp were sensitive to inhibition by 2.5 mM-N-ethylmaleimide. The activity of aminopeptidase W was unaffected by these inhibitors. There was good correlation between the fluorimetric assay and those in which the free tryptophan released by kidney membrane fractions was determined by h.p.l.c. or the aminopeptidase W was measured immunoradiometrically with a monoclonal antibody.

INTRODUCTION

Aminopeptidase W is a newly discovered member of the group of membrane peptidases (for review see Kenny *et al.*, 1987) located in the brush borders of the kidney and intestine. It was first isolated as a glycoprotein recognized by a monoclonal antibody, GK5C1, which had been generated after immunizing a mouse with pig kidney microvilli (Gee & Kenny, 1985a). When the detergent-solubilized pig kidney membranes were subjected to immuno-affinity chromatography, a dimeric protein of 130 kDa subunits with a topology typical of other microvillar membrane hydrolases (i.e. a stalked globular protein) was isolated. An extended search for an enzymic activity for this putative hydrolase was rewarded when the isolated protein was shown to hydrolyse some peptides by an aminopeptidase attack, but unlike other membrane aminopeptidases it did not hydrolyse aminoacyl substrates such as Ala-NH-Mec (Gee & Kenny, 1985b). Although dipeptides are attacked more rapidly than extended peptides, it is not a strict dipeptidase. The side-chain specificity permits a wide range of amino acid residues in the P₁ position but favours bulky side chains in the P₁' position (Gee & Kenny, 1987). Thus Xaa-Trp dipeptides are well hydrolysed, with Glu-Trp being the best substrate so far tested (K_m 568 μ M; k_{cat} 6773 min⁻¹; k_{cat}/K_m = 11.9 min⁻¹· μ M⁻¹). In a survey of 32 dipeptides, Trp was the most favourable P₁' residue followed, in order, by Phe, Tyr, Leu and Gly; hence this preference for Trp justifies the proposed name of aminopeptidase W.

Aminopeptidase W has been assayed antigenically by an immunoradiometric assay and enzymically by h.p.l.c. analysis, monitoring the appearance of free tryptophan at 214 nm. but neither assay is very convenient for routine use. We report here an enzyme-linked fluorimetric assay originally devised to measure gliadin digestion (Bruce & Woodley, 1981), which depends on the determination by glutamate dehydrogenase of the free glutamate released when Glu-Trp is hydrolysed.

EXPERIMENTAL

Materials

Glutamate dehydrogenase (EC 1.4.1.2) was a 3-times crystallized preparation from bovine liver in 50% glycerol (approx. 45 units/ml; BDH Chemicals, Poole, Dorset, U.K.). Glu-Trp was obtained from Bachem, Bubendorf, Switzerland. NAD⁺ and ADP were also from BDH Chemicals. Cilastatin [MK 0791, monosodium Z-S-(6-carboxy-6-[[2,2-dimethyl-(s)-cyclopropyl]carbonylamino]hex-5-enyl)-L-cysteine] was kindly given by Dr. H. Kropp, Merck, Sharp and Dohme Research Laboratories, Rahway, NJ, U.S.A. Renal dipeptidase (EC 3.4.13.11) was kindly given by Dr. N. Hooper.

Tissue preparations and peptidase

Kidney microvillar membranes were prepared from pig kidney cortex as previously described (Booth & Kenny, 1974). Intestinal microvillar membranes were prepared from rat jejunum (Kessler *et al.*, 1978). A supernatant fraction from papain-treated microvillar membranes was also used (Louvard *et al.*, 1973). Peptidases were purified from pig kidney as previously described: aminopeptidase W (by immuno-affinity chromatography; Gee & Kenny, 1985b), aminopeptidase N (Matsas *et al.*, 1985) and microsomal dipeptidase (Hooper *et al.*, 1987).

Fluorimetric assay of Glu-Trp hydrolysis

Eppendorf tubes (1.5 ml) contained 10 μ l of enzyme preparation with 90 μ l of 0.1 M-Tris/HCl buffer, pH 8.0, containing 5 mM-1,10-phenanthroline, 50 μ M-cilastatin and 0.5 mM-Glu-Trp. The mixture was incubated at 37 °C for 15 min and the reaction stopped by heating at 100 °C for 4 min. The tubes were cooled to 0 °C, and 10 μ l of glutamate dehydrogenase in 50% glycerol (4.5 units) and 1 ml of 0.5 M-glycine/0.5 M-hydrazine buffer, pH 8.6, containing 0.5 mM-NAD⁺ and 0.1 mM-ADP

Abbreviations used: i.r.m.a., immunoradiometric assay; -NH-Mec, 7-amino-4-methylcoumarylamide.

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were added. The tubes were then incubated at 37 °C for 15 min and fluorescence was determined with a Perkin-Elmer fluorimeter (model 1000 or LS3), with excitation at 365 nm and emission at 460 nm. A blank, in which the initial incubation mixture was heated immediately on addition of the enzyme or tissue sample, provided a correction for any free glutamate.

I.r.m.a.

This was performed as previously described (Gee & Kenny, 1985b).

Other peptidase assays

Aminopeptidase N (EC 3.4.11.2) and aminopeptidase A (EC 3.4.11.7) were assayed fluorimetrically with Leu-NH-Mec and α -Glu-NH-Mec as substrate respectively, as previously described (Fulcher & Kenny, 1983). Microsomal dipeptidase (EC 3.4.13.11) was assayed by h.p.l.c. with Gly-D-Phe as substrate (Hooper *et al.*, 1987).

Determination of protein

Protein was determined colorimetrically with Coomassie Blue (Peterson, 1983) or by the Lowry method with bovine serum albumin as standard.

H.p.l.c. analysis for aminopeptidase W

This was performed either as described previously (Gee & Kenny, 1985b) with a gradient of acetonitrile to resolve tryptophan from Glu-Trp or by an isocratic method eluting the column with 30 % (v/v) acetonitrile/0.1 M-NaClO₄/0.085 % H₃PO₄ and a 254 nm detector.

RESULTS AND DISCUSSION

Assay of purified aminopeptidase W

Over the range 0–10 ng of enzyme (Fig. 1a) the formation of glutamate was linear up to 25 % hydrolysis (12.5 nmol of glutamate released). The specific activity derived from this experiment (20.4 μ mol of Glu-Trp hydrolysed/min per mg of protein) is comparable with that previously reported (22.2 μ mol/min per mg) for another batch of the purified enzyme assayed by h.p.l.c. (Gee & Kenny, 1987).

Recovery of activity of purified aminopeptidase W added to papain-solubilized microvillar proteins

A sample of papain-solubilized extract of intestinal microvillar membranes released 3.75 ± 0.35 nmol of glutamate; a sample of the purified enzyme released 13.8 ± 0.3 nmol and the two together released 18.0 ± 0.97 nmol, i.e. 97 % recovery of the two activities. Thus the enzyme can be assayed quantitatively in crude extracts.

Effects of the inhibitors on peptidase and glutamate dehydrogenase activities

Glu-Trp was selected as the standard substrate for aminopeptidase W because it has the highest specific activity and $k_{\text{cat.}}/K_m$ value of any dipeptides tested (Gee & Kenny, 1987). But it may also be hydrolysed by other peptidases, in particular by three enzymes found in many membranes, aminopeptidases N and A and microsomal dipeptidase. It was necessary to minimize the contribution of these and other metallopeptidases by selective inhibition. Pure samples of aminopeptidase W, aminopeptidase N and microsomal dipeptidase were available

to check the effects of inhibitors in the assay mixture (Table 1). Since no purified sample of aminopeptidase A was available, its activity was determined in kidney microvillar membranes with α -Glu-NH-Mec as substrate in the presence and in the absence of 1 mM-Ca²⁺. Neither EDTA nor 1,10-phenanthroline caused any diminution of aminopeptidase W activity even at a concentration of 10 mM (Gee & Kenny, 1987). However, EDTA was a much less effective inhibitor for aminopeptidase N, and hence 1,10-phenanthroline was the preferred chelator. Microsomal dipeptidase was poorly inhibited by 5 mM-1,10-phenanthroline, and it was necessary to include 50 μ M-cilastatin (Campbell *et al.*, 1984) to exclude any significant contribution from this enzyme. The determination of glutamate over the range 10–50 nmol by glutamate dehydrogenase was unaffected by these inhibitors (Fig. 1b).

Kidney cytosol contained substantial amounts of other enzyme activities that hydrolysed Glu-Trp. In one sample the activity (no inhibitors) was 130 nmol/min per mg of protein, decreased to 16.5 nmol/min per mg by

Fig. 1. Fluorimetric assay of purified aminopeptidase W

See the Experimental section for details. (a) Assay of aminopeptidase W linearity was lost when 25 % of Glu-Trp had been hydrolysed. (b) Determination of glutamate by glutamate dehydrogenase: ●, no additions; ■, in the presence of 5 mM-1,10-phenanthroline and 50 μ M-cilastatin. The deviation from linearity above 50 nmol of glutamate could be minimized by extending the incubation time with glutamate dehydrogenase from 15 min to 25 min (○).

Table 1. Effect of inhibitors on renal microvillar peptidases

Aminopeptidases N and W and microsomal dipeptidase were samples of purified enzymes, but aminopeptidase A was determined on microvillar membranes. The assay for microsomal dipeptidase was by h.p.l.c., the other activities were assayed by fluorimetric methods. (See the Experimental section for details.) Activities are relative to those in the absence of inhibitor (%).

Enzyme	Inhibitor ... Concn. ...	Activity (%)							
		1,10-Phenanthroline				Cilastatin			
		0.1 mM	1 mM	5 mM	10 mM	1 μ M	10 μ M	50 μ M	100 μ M
Aminopeptidase W		92	92	88	84	95	102	98	93
Aminopeptidase A		83	38	14	0	—	—	—	—
Aminopeptidase N		39	0	0	0	96	76	62	54
Microsomal dipeptidase		92	78	56	54	62	16	4	3

Fig. 2. Effect of Ca^{2+} on the hydrolysis of Glu-Trp and α -Glu-NH-Mec by a rat intestinal microvillar preparation

The supernatant fraction after papain treatment of the microvillar fraction was employed (see the Experimental section). The results of fluorimetric assays for aminopeptidase A with α -Glu-NH-Mec (○) and for aminopeptidase W with Glu-Trp (●) are expressed as the increase in activity compared with the control (= 1). No inhibitors were present.

5 mM-1,10-phenanthroline and to 6.5 nmol/min per mg (95 % inhibition) by the further addition of 2.5 mM-*N*-ethylmaleimide. Aminopeptidase W was unaffected by this concentration of *N*-ethylmaleimide.

Contribution of peptidases other than aminopeptidase W to the hydrolysis of Glu-Trp in the absence of inhibitors

Glu-Trp is a poor substrate for aminopeptidase N, being hydrolysed at 1.3 % of the rate with aminopeptidase W (Gee & Kenny, 1987). Even if this assessment is adjusted for the greater abundance of aminopeptidase N in renal or intestinal microvillar membranes (4–6-fold more), the contribution amounts to less than 10 %. Aminopeptidase A might be expected to be more efficient since peptides with *N*-terminal Glu are its favoured substrates. The hydrolysis of Glu-NH-Mec by purified aminopeptidase A was stimulated 4-fold by Ca^{2+} (Danielson *et al.*, 1980), and the hydrolysis of this substrate by a rat intestinal microvillar preparation (Fig. 2) illustrates this effect of Ca^{2+} . However, the hydrolysis of Glu-Trp by the same preparation showed only a 22 % stimulation by Ca^{2+} . Since aminopeptidase W is not affected by Ca^{2+} , this small stimulation is probably an indication of the contribution of aminopeptidase A, which in this case may be calculated to be less than 6 %. On the other hand, Glu-Trp is a good substrate for microsomal dipeptidase, an enzyme with a wide distribution including kidney and intestine (Hirota *et al.*, 1986) and exhibiting a very broad specificity in hydrolysing dipeptides. The hydrolysis of

Table 2. Correlation of aminopeptidase W activity in pig kidney fractions assayed by three methods

See the Experimental section for details of assays. The h.p.l.c. assay included 5 mM-EDTA in place of phenanthroline, which obscured the product peaks. The fractions were prepared as described previously (Booth & Kenny, 1974), P4 being the microvillar fraction and P2 the fraction before the second MgCl_2 treatment. The units are expressed as nmol of Glu-Trp hydrolysed/min, except for the i.r.m.a. values, which are arbitrary. The values in parentheses are expressed relative to fraction P4.

Fraction	Specific activity		
	I.r.m.a. (arbitrary units mg of protein)	H.p.l.c. assay (units/mg of protein)	Fluorimetric assay (units/mg of protein)
Homogenate	7 (8)	16 (17)	20 (22)
P2	37 (43)	46 (49)	42 (47)
P4	87 (100)	92 (100)	90 (100)

Glu-Trp by the purified dipeptidase was 5 $\mu\text{mol}/\text{min}$ per mg of enzyme, about 20% of the rate with the usual assay substrate, Gly-D-Phe. If uninhibited, it may therefore be the main additional peptidase in microvillar membranes contributing to the hydrolysis of Glu-Trp.

Determination of aminopeptidase W in crude tissue extracts by three independent assays

A homogenate and two pelleted fractions (both enriched to different degrees in microvilli) were assayed for aminopeptidase W by i.r.m.a., the fluorimetric assay and the h.p.l.c. assay (Table 2). No *N*-ethylmaleimide was included in the incubations, so the values for the enzymic assays in the homogenate are inflated by a contribution from cytosolic peptidases. There is good agreement between all three assays for the membrane fractions P2 and P4.

Conclusion

We conclude that aminopeptidase W can be assayed in preparations derived from kidney and intestine by a simple two-step fluorimetric method. Other microvillar membrane peptidases contributed little to the hydrolysis of the substrate, Glu-Trp, and these activities could be effectively eliminated by 1,10-phenanthroline and cila-statin. Residual cytosolic activity hydrolysing Glu-Trp was sensitive to *N*-ethylmaleimide.

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