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THE ROLE OF CARBOHYDRATE MOIETIES IN THE ACTIVITY AND PROPERTIES OF AMINOPEPTIDASE FROM PIG KIDNEY*

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SUMMARY

Upon prolonged incubation of particulate aminopeptidase from pig kidney (α-aminoacyl-peptide hydrolase (microsomal), EC 3.4.11.2) with glycosylytic enzymes (sialidase (EC 3.2.1.18), α-mannosidase (EC 3.2.1.24) and β-*N*-acetylglucosaminidase (EC 3.2.1.30)), virtually all of the sialic acid, over 60% of the neutral sugars and 45% of the *N*-acetyl-D-glucosamine originally bound to the aminopeptidase could be split off. The degraded aminopeptidase differs from the native enzyme in its electrophoretic mobility on disc gel and in its solubility properties. However, no differences were found with respect to catalytic activity and substrate specificity. Susceptibility towards inhibition by EDTA or 1,10-phenanthroline was identical to that of the undegraded enzyme.

The results suggest that carbohydrate moieties of aminopeptidase play no significant role in the in vitro activity of aminopeptidase.

INTRODUCTION

Particulate aminopeptidase from pig kidney (α-aminoacyl-peptide hydrolase (microsomal), EC 3.4.11.2) is a glycoprotein which contains glucosamine, galactose, mannose, fucose and sialic acid. The enzyme also contains 2 gatoms of tightly bound zinc per mole (mol. wt 280 000) [2] which is essential for enzymatic activity [3]. Preliminary experiments suggest that at least some of the glycosyl moieties could be removed from the enzyme [2]. Because of the unusually high carbohydrate content and great stability of this aminopeptidase, a study was undertaken on the possible role of the carbohydrate moieties in the activity and certain other properties of the enzyme.

EXPERIMENTAL

Aminopeptidase (50 mg in 5 ml of 0.2 M sodium acetate buffer, pH 4.8) was incubated at 25 °C for 72 h with 0.02 unit of sialidase (EC 3.2.1.18) from *Vibrio*

* A preliminary account of this work was presented at the annual meeting of the Union of Swiss Societies for Experimental Biology at Geneva, May 5 and 6, 1972 [1].

cholerae. Bacterial contamination was prevented by addition of a few drops of toluene. The digest was fractionated on a Sephadex G-25 column, using sodium acetate buffer as eluent. Sialic acid was assayed [4] in the protein-free fractions and in the recovered protein after acid hydrolysis [5]. Essentially all of the sialic acid had been removed from the aminopeptidase (Table I). The activity of the desialated aminopeptidase (31 units/mg, using L-leucine-*p*-nitroanilide as substrate [2]) and the electrophoretic mobility on polyacrylamide gels at pH 8.9 [6] were identical to those of the untreated enzyme.

Desialated enzyme, 22 mg in 4.4 ml of sodium acetate buffer, was incubated at 25 °C with a mixture of 15 units α-D-mannosidase (EC 3.2.1.24) and 1.1 units of β-*N*-acetyl-D-glucosaminidase (EC 3.2.1.30) from jack beans [7] and 3 mg of emulsin (EC 3.2.1.21) (equivalent to 1.8 units of β-D-galactosidase). None of the glycosylases used had any detectable proteolytic activity when tested with hemoglobin [8]. Aminopeptidase activity was determined after 3, 6, 14, 32 and 48 days. In addition, at each time, 0.3-ml aliquots were removed and fractionated (Sephadex G-25) as described above. Cations were removed from the protein-free fractions by passage over a column of Dowex-50 (H^+ form). Reducing sugar was determined by the method of Park and Johnson [9]. The results are depicted in Fig. 1. They show that the removal of approx. 60% of the carbohydrate content produced only an 11% decrease in enzyme activity per volume of reaction mixture.

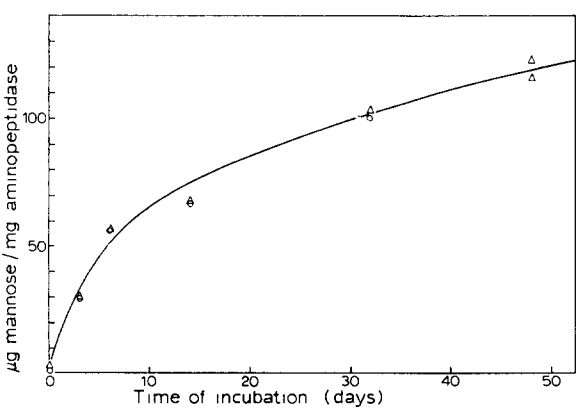

Fig. 1. Release of reducing sugars (expressed as mannose [9]) during incubation of aminopeptidase with glycosylases.

At the end of 48 days, the entire reaction mixture was fractionated and desalted as described above. Thin-layer chromatography demonstrated the presence of D-mannose, D-galactose and *N*-acetyl-D-glucosamine. The results of carbohydrate determinations in the protein-free filtrate and of the modified protein after purification (Sephadex G-200) are shown in Table I. They confirmed that carbohydrate groups, not released by the action of glycosylases, were still associated with the protein. When the partially deglycosylated enzyme was subsequently incubated as described with glycosylases for 45 additional days, there was no further significant loss of galactosyl and mannosyl groups, but the *N*-acetyl-D-glucosamine content of the protein decreased markedly.

TABLE I

CARBOHYDRATE CONTENT OF AMINOPEPTIDASE AND SUGARS RELEASED AFTER INCUBATION WITH GLYCOSYLASES

Sialic acid was determined after acid hydrolysis of the protein [5] according to Warren [4]. Mannose + galactose of the protein and in the protein-free filtrate were estimated by the orcinol–H_2SO_4 technique [10] Glucosamine of the protein was assayed with a Beckman 120 C amino acid analyzer after hydrolysis for 24 h at 110 °C in 3 M methanesulfonic acid in the presence of 0.2% tryptamine [11], and is expressed as *N*-acetyl-D-glucosamine. *N*-Acetyl-D-glucosamine in the protein-free filtrate was determined according to Morgan and Elson (see ref. 12).

	Carbohydrate content of the protein (μg/mg protein)		Sugar released* (μg/mg protein)
	Before incubation	After incubation	
Sialic acid	13.4	0.4	12.4
Mannose + galactose	135	53**	82
		48***	107
N-Acetyl-D-glucosamine	88	61**	32
		49***	47

* Values are corrected for an incubation control containing identical amounts of glycosylases but substituting aminopeptidase with bovine serum albumin.

** Incubation time 48 days.

*** Incubation time 93 days.

The partially deglycosylated enzymes differed from the starting material in regard to several physical properties. Upon disc gel electrophoresis at pH 8.9 [6], deglycosylated enzyme migrated more rapidly towards the anode. Upon electrophoresis in the presence of dodecylsulfate [13], the deglycosylated enzyme exhibited the typical three-band pattern given by the untreated enzyme; however, the mobility of each of the bands was greater. The molecular weight of the deglycosylated enzyme determined from the relative electrophoretic mobility of the subunits in these gels (Fig. 2) was 245 000, and was consistent with a value calculated from the loss of glycosyl moieties. Deglycosylated protein also was less soluble: addition of one-seventh volume of 40% trichloroacetic acid to a 0.12% solution of deglycosylated aminopeptidase precipitated all the enzyme from solution; under identical conditions, no native enzyme was precipitated. Similarly, when a 0.1% solution of enzyme was brought to 70% saturation by addition of solid $(NH_4)_2SO_4$, at least 90% of the original activity could be recovered from the supernatant fluid. An identical treatment precipitated all of the deglycosylated enzyme in the solution; 70% of the initial activity could be recovered from the precipitate.

Regarding the catalytic activities, the untreated and deglycosylated enzymes display a remarkable degree of similarity. Their specific activities were identical when tested with the *p*-nitroanilides of L-leucine, L-alanine, L-phenylalanine and L-glycine. There was no difference in the pattern of inhibition by EDTA and 1,10-phenanthroline. Furthermore, the arginine, histidine and lysine content of both proteins were identical (other amino acids were not determined).

The tryptophan content of the aminopeptidase we had reported originally [2]

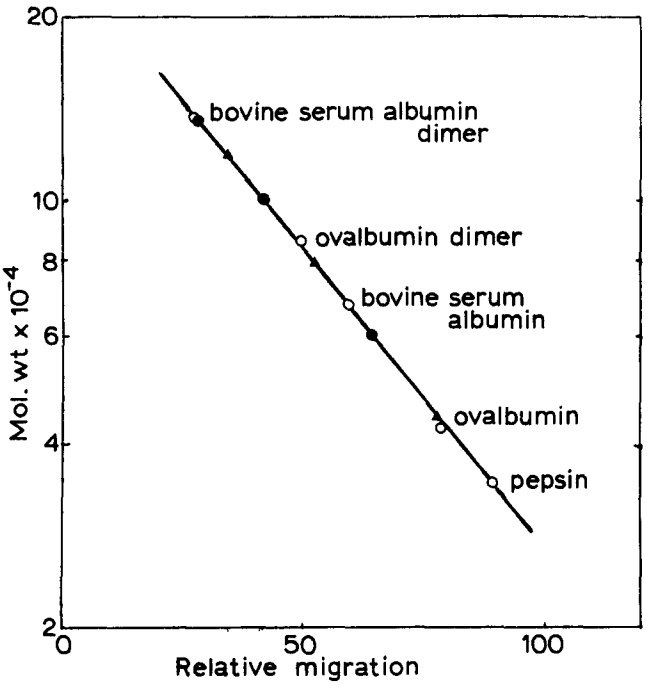

Fig. 2. Plot of relative mobilities obtained for aminopeptidase after incubation with glycosylases as a function of molecular weight following disc gel electrophoresis. Markers (○) were the same as in [2]. Aminopeptidase before (●) and after incubation (▲).

appears incompatible with the molar absorptivity of the protein*. Therefore, the tryptophan content of aminopeptidase was reexamined by two independent methods. Spectrophotometric determination in 6 M guanidine·HCl [14] yielded 60 ± 2 residues per mole. Tryptophan also was determined chromatographically in hydrolysates of the protein done as described above. Tryptophan recovery by this method is a function of the carbohydrate content of the glycoprotein; the true tryptophan content can be determined by extrapolation [11]. Application of this extrapolation to untreated, as well as to partially deglycosylated aminopeptidase, yielded tryptophan contents of 60 and 58 residues per mole, respectively.

DISCUSSION

Enzymic removal of over 50% of the glycosyl residues originally present in aminopeptidase yielded a modified enzyme which had greater gel electrophoretic mobility but lower solubility than the parent enzyme. However, the substrate specificity and specific activity of the enzyme was unchanged. A similar situation has been reported by Plummer and Hirs [15] and Plummer [16], who described several

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naturally occurring ribonucleases which differed only in their carbohydrate contents. The enzymes were identical in regard to amino acid composition, specific activity and substrate specificity.

Since the carbohydrate portion of aminopeptidase does not seem to be essential for enzymic activity in vitro, one might speculate that the glycosyl portion of the molecule fulfils an in vivo requirement, possibly serving to anchor the enzyme to some subcellular structure. Aminopeptidase has been shown to be associated with the kidney plasma membrane [17, 18].

The degradation pattern observed upon treatment of aminopeptidase suggests that sialic acid is indeed terminal and that D-galactose and D-mannose are distal to *N*-acetyl-D-glucosamine. Partially deglycosylated enzyme is remarkably resistant to any further enzymatic deglycosylation. It is not clear whether this is due to the unavailability to the hydrolases of the portions of the polysaccharide side chains proximal to the protein, or to some structural differences between the proximal and distal portions of the chain.

Wetlaufer [19] has pointed out that the observed molar absorptivity of a protein should be greater by no more than 15% than the molar absorptivity calculated from its tryptophan and tyrosine content. On this basis, our previous value of 83 tryptophan residues per mole [2] is too high. New spectrophotometric determination in the intact protein and chromatographic determinations after acid hydrolysis yield values consistent with the tryptophan content calculated from molar absorptivity, and contrast with the values obtained by the method of Bencze and Schmid [20].

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REFERENCES

1 Wacker, H. and Stein, E. A. (1972) Experientia 28, 740
2 Wacker, H., Lehky, P., Fischer, E. H. and Stein, E. A. (1971) Helv. Chim. Acta 54, 473–485
3 Lehky, P., Lisowski, J., Wolf, D. P , Wacker, H. and Stein, E. A. (1973) Biochim. Biophys. Acta 321, 274–281
4 Warren, L. (1959) J. Biol. Chem. 234, 1971–1975
5 Svennerholm, L. (1958) Acta Chem. Scand. 12, 547–554
6 Davis, B. J. (1964) Ann. N.Y. Acad. Sci. 121, 404–427
7 Li, Y.-T. (1967) J. Biol. Chem. 242, 5474–5480
8 Davis, N. C. and Smith, E. L. (1955) in Methods of Biochemical Analysis (Glick, ed.), Vol. 2, pp. 215–257, Interscience, New York
9 Park, J. T. and Johnson, M. J. (1949) J. Biol. Chem. 181, 149–151
10 François, C., Marshall, R. D. and Neuberger, A. (1962) Biochem. J. 83, 335–341
11 Liu T.-Y. and Chang, Y. H. (1971) J. Biol. Chem. 246, 2842–2848
12 Reissig, J. L., Strominger, J. L. and Leloir, L. F. (1955) J. Biol. Chem. 217, 959–966
13 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
14 Edelhoch, H. (1967) Biochemistry 6, 1948–1954
15 Plummer, T. H. and Hirs, C. H. W. (1963) J. Biol. Chem. 238, 1396–1401

16 Plummer, T. H. (1968) J Biol. Chem. 243, 5961–5966
17 Thomas, E. and Kinne, R. (1972) Biochim. Biophys. Acta 255, 114–125
18 George, S. G. and Kenny, A J. (1973) Biochem J. 134, 43–57
19 Wetlaufer, D. B. (1962) Adv. Protein Chem. 17, 303–390
20 Bencze, W. L. and Schmid, K. (1967) Anal. Chem 29, 1193–1196