ON THE NATURE OF α-MANNOSIDASE-RESISTANT LINKAGES IN GLYCOPROTEINS

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SUMMARY

The mannose residue in Asn-$(GlcNAc)_2(Man)_1$, a glycosyl-asparagine sequence common to RNase B, ovalbumin, and many other glycoproteins, is resistant to hydrolysis by α-mannosidases from jack bean meal and hen oviduct. This residue is hydrolyzed, however, by a partially purified β-mannosidase fraction from hen oviduct. Similar results were obtained with partially purified α- and β-mannosidases from rat epididymis.

INTRODUCTION

Previously (1), we reported that a glycosyl-Asn isolated from RNase B by proteolytic digestion contained the following composition, Asn-$(GlcNAc)_2(Man)_6$*. Treatment of Asn-$(GlcNAc)_2(Man)_6$ with jack bean meal α-mannosidase released five mannose residues and yielded Asn-$(GlcNAc)_2(Man)_1$ with the resistant mannose linked to the 3 or 4 position of the nonreducing end of di-N-acetyl-chitobiose. A similar Asn-trisaccharide sequence with a jack bean meal α-mannosidase-resistant mannose was isolated from ovalbumin, α-amylase, bromelein (2), and a glycopeptide from rat liver microsomes (3), and may be typical of many other glycoproteins. The most plausible explanation for the resistance of the mannose linkage to hydrolysis involves either

*The abbreviation for Asn-GlcNAc in the glycosyl-asparagine derivatives refers to: 2-acetamido-N-(4-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine.

the anomer function of the bond or the specificity of α-mannosidase for Man → GlcNAc.

Evidence will be presented in this paper suggesting that the reason for the resistance resides in the former proposal. Proof was obtained by the purification of a hen oviduct β-mannosidase capable of rapidly hydrolyzing mannose from Asn-$(GlcNAc)_2(Man)_1$, suggesting a unique function for this enzyme. The enzyme may be more widespread than previously believed. Another oviduct fraction completely devoid of this enzyme, but enriched with respect to α-mannosidase activity, was not capable of affecting the cleavage of mannose.

MATERIALS AND METHODS

The p-nitrophenyl glycosides were obtained from Sigma Chemical Co. Asn-GlcNAc was prepared chemically (4) while Asn-$(GlcNAc)_2$ was provided by Dr. R. Jeanloz (5), Department of Biochemistry, Harvard University Medical School. DONV was a generous gift of Dr. R. Handschumacher, Department of Pharmacology, Yale University Medical School. This compound is not only an inhibitor of asparaginase (6) but is also an inhibitor of glycosyl-asparaginase (7) and was used to conserve the asparagine moiety for the chromatographic detection of glycosyl-Asn derivatives (1).

Glycosyl-Asn derivatives from ovalbumin and RNase B were isolated by the procedure of Huang et al. (8). While ovalbumin yields several fractions by this method, only two, corresponding to Asn-$(GlcNAc)_2(Man)_5$ and Asn-$(GlcNAc)_2(Man)_6$, were found with RNase B. Asn-$(GlcNAc)_2(Man)_1$ was isolated after exhaustive treatment of the glycosyl-Asn derivatives with the α-mannosidase from jack bean meal as described previously (1).

A detailed description for the purification of the α- and β-mannosidases from hen oviduct will be presented in a later publication. The basis for the separation depends on heating at 65° for 60 minutes to inactivate the β-mannosidase and an incubation at pH 11 in the cold to

inactivate the α-mannosidase. The α-mannosidase used was purified approximately 100-fold to a specific activity of 0.25 units/mg protein and the β-mannosidase 200-fold to a specific activity of 0.35 units/mg protein. One unit of mannosidase is defined as the amount of enzyme hydrolyzing one μmoles of p-nitrophenyl mannoside per minute at 37°.

Assays for α- and β-mannosidases, β-N-acetylglucosaminidase, and glycosyl-asparaginase were similar to those described previously (7,9), but modified to conserve on substrate. Mannose release was measured enzymically with a coupled hexokinase-lactate dehydrogenase system (1).

RESULTS AND DISCUSSION

Because of the limited amount of Asn-$(GlcNAc)_2(Man)_1$ that could be obtained from RNase B, a more available source of this compound was sought. A clue was provided by the studies of Huang et al. (8) which suggested the presence of an α-mannosidase-resistant residue in the glycosyl-Asn derivative from ovalbumin. Isolation of Asn-$(GlcNAc)_2(Man)_5$ by their procedure followed by digestion with purified jack bean meal α-mannosidase yielded a compound that eluted in the same region as that reported previously by us for the Asn-$(GlcNAc)_2(Man)_1$ from RNase B (1). Preliminary studies with this compound revealed that an enzyme in hen oviduct extracts was capable of removing the last mannose residue as determined by either the coupled hexokinase-lactate dehydrogenase assay or amino acid analysis of the products (1).

Similarly, exhaustive treatment of Asn-$(GlcNAc)_2(Man)_5$ from RNase B with the jack bean meal α-mannosidase provides a compound that elutes in the same region as Asn-$(GlcNAc)_2(Man)_1$, as shown in Fig. 1C. On further incubation of this glycosyl-Asn derivative with a partially purified β-mannosidase fraction from hen oviduct, complete conversion to a product that chromatographed identically with Asn-$(GlcNAc)_2$ was obtained (Fig. 1D).

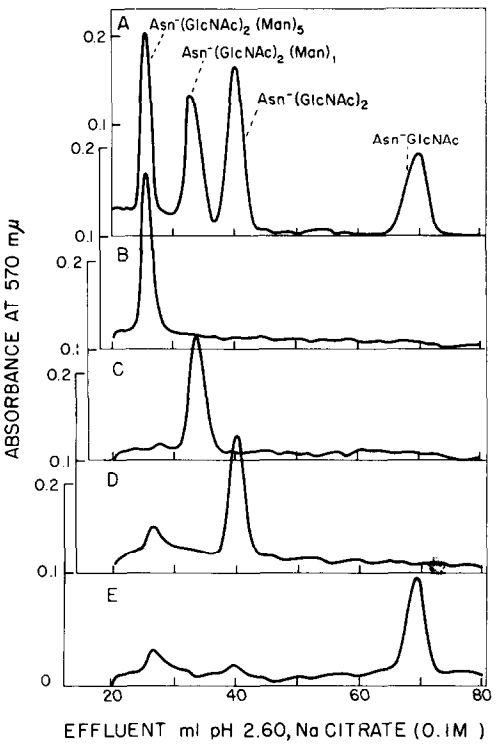

Fig. 1. Chromatographic separation of glycosyl-asparagine derivatives of RNase B after reaction with glycosidases.
Reaction mixtures were placed in individual tubes and contained the following components: Asn-$(GlcNAc)_2(Man)_5$, 0.04 μmole; sodium citrate (pH 4.5), 12.5 μmoles; and enzyme solutions or water to a final volume of 0.25 ml. The tubes were incubated at 37° and the reactions were terminated by dilution to 0.85 ml with 0.2 M sodium citrate, pH 2.2. Aliquots of 0.80 ml were applied to a column of Aminex-H (0.9 X 57 cm, Bio-Rad) in an automatic amino acid analyzer. The column was maintained at 53° and derivatives were eluted with 0.1 M sodium citrate, pH 2.60, containing 3% propanol. The following elution patterns were obtained: (A) Standard mixture of glycosyl-asparagine derivatives, 0.054-0.062 μmole; (B) Asn-$(GlcNAc)_2(Man)_5$, no glycosidase treatment; (C) Asn-$(GlcNAc)_2(Man)_5$ incubated for 12 hours with either jack bean meal α-mannosidase, 11.6 units, or jack bean meal α-mannosidase, 11.6 units, for 12 hours and hen oviduct α-mannosidase, 0.15 units, for 1 hour; (D) Asn-$(GlcNAc)_2(Man)_5$ incubated for 12 hours with jack bean meal α-mannosidase, 11.6 units, and hen oviduct β-mannosidase 0.23 units, for 1 hour; (E) Similar to (D) but following the β-mannosidase treatment, the reaction mixture was incubated with 4.0 units of β-N-acetylglucosaminidase for 4 hours.

The specificity of the hydrolysis was revealed on incubation of Asn-$(GlcNAc)_2(Man)_1$ with a similar number of units of α-mannosidase purified from hen oviduct that are completely free of β-mannosidase activity. It is seen in Fig. 1C that the mannose is not removed from

this glycosyl-Asn derivative. As was shown earlier by us (1), treatment of synthetic and natural Asn-$(GlcNAc)_2$ with β-N-acetylglucosaminidase yields Asn-GlcNAc and was used as a means to identify these compounds. A similar conversion was obtained on incubation of the product formed by the β-mannosidase-enriched fraction with β-N-acetylglucosaminidase (Fig. 1E).

As information on the polysaccharide fractions from glycoproteins becomes more available, the Asn-$(GlcNAc)_2(Man)_1$ residue appears to play an increasingly prominent role. Thus, recent studies by Sinohara et al. (10) and Kornfeld et al. (11) have shown this sequence to be present in silk fibroin and γG myeloma glycoproteins, respectively. In the latter case, studies with a partially purified α-mannosidase fraction from rat epididymis lead the authors to conclude that the Man → GlcNAc linkage was α. Our studies with such fractions indicate that the α-mannosidase can be contaminated with β-mannosidase activity. Thus, on careful separation of the rat epidiymal α- and β-mannosidases by Sephadex G-200 chromatography, little or no cleavage of mannose from Asn-$(GlcNAc)_2(Man)_1$ was obtained with the α-mannosidase fraction. However, the β-mannosidase-containing fraction yielded a pattern identical to that obtained in Fig. 1D with the hen oviduct β-mannosidase fraction.

Results similar to Kornfeld et al. (11) were reported recently by Lee (2) on treatment of Asn-$(GlcNAc)_2(Man)_1$ from ovalbumin with a plant α-mannosidase. While there may be mannosidases with multiple specificities for different anomeric linkages, our results with the partially purified oviduct mannosidases indicate that the α- mannosidase fraction is incapable of hydrolyzing the Asn-$(GlcNAc)_2(Man)_1$ from ovalbumin. In contrast, it is shown in Table 1 that the β-mannosidase fraction completely hydrolyzes the mannose residue. The asparagine-containing products formed during this hydrolysis were identical to those obtained on treatment of Asn-$(GlcNAc)_2(Man)_1$ from RNase B with the β-mannosidase fraction.

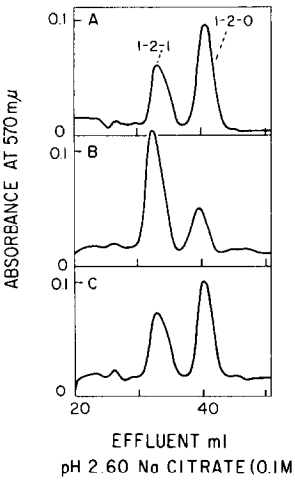

Fig. 2. Effect of p-nitrophenyl α- and β-D-mannopyranosides on the hydrolysis of Asn-$(GlcNAc)_2(Man)_1$ of ovalbumin by a partially purified β-mannosidase fraction from hen oviduct. Reaction mixtures were placed in individual tubes and contained the following components: Asn-$(GlcNAc)_2(Man)_1$, 0.058 μmole; sodium citrate (pH 4.5), 10.0 μmoles; β-mannosidase, 0.045 units; p-nitrophenyl-α-D-mannoside or β-D-mannoside, 1.1 μmoles, and water to a final volume of 0.15 ml. The reactions were incubated at 37° for 30 minutes, and terminated by dilution to 0.85 ml with 0.2 M sodium citrate, pH 2.2, after which they were assayed as in Fig. 1. The following elution patterns were obtained: (A) Asn-$(GlcNAc)_2(Man)_1$ and β-mannosidase; (B) Asn-$(GlcNAc)_2(Man)_1$, β-mannosidase, and p-nitrophenyl-β-D-mannoside; (C) Asn-$(GlcNAc)_2(Man)_1$, β-mannosidase, and p-nitrophenyl-α-D-mannoside.

Since the enzymes used in this study are only partially purified, it is conceivable that the β-mannosidase fraction contains an additional enzyme capable of hydrolyzing Asn-$(GlcNAc)_2\overset{\alpha}{}(Man)_1$ linkages, but not p-nitrophenyl α-D-mannopyranoside. This possibility appears highly unlikely in view of the finding that p-nitrophenyl β-D-mannopyranoside, but not the α-derivative, inhibits the hydrolysis of the mannose from Asn-$(GlcNAc)_2(Man)_1$ (Fig. 2B and 2C).

Although our results suggest rather strongly that the mannose linkage in RNase B and ovalbumin Asn-$(GlcNAc)_2(Man)_1$ is β and may be a common sequence in glycoproteins, final proof must await the isolation and chemical characterization of the Man → GlcNAc disaccharide.

Table I

Hydrolysis of Mannose from Asn-$(GlcNAc)_2(Man)_1$ by α- and β-Mannosidases from Hen Oviduct and Rat Epididymis

Hours	Hen Oviduct		Rat Epididymis	
	α-mannosidase	β-mannosidase	α-mannosidase	β-mannosidase
	Moles Mannose Released/Mole Asn-$(GlcNAc)_2(Man)_1$			
0.4	0	0.85	- *	-
1	0	0.99	0.02	0.19
2	-	-	-	0.30
7	-	-	0.05	0.63
24	-	-	0.17	0.95

*not determined.

The reaction mixtures contained: Asn-$(GlcNAc)_2(Man)_1$, 0.23 μmole; sodium citrate (pH 4.5), 25 μmoles; and enzyme in a volume of 0.500 ml. The tubes were incubated at 37°C and at the indicated times, 0.05 ml aliquots were assayed for mannose. The amount of enzyme added in each case was as follows: hen oviduct, α-mannosidase (β-mannosidase-free), 0.6 units; β-mannosidase (α-mannosidase-free), 0.4 units; rat epididymis: α-mannosidase, 0.08 units containing 0.006 units of β-mannosidase; β-mannosidase, 0.08 units containing 0.008 units of α-mannosidase.

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