Demonstration of an Endo-glycosidase Acting on a Glycoprotein

(Received for publication, April 15, 1971)

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SUMMARY

Enzyme preparations from the culture fluid of Diplococcus pneumoniae released a radioactive oligosaccharide containing mannose and glucosamine from radiolabeled mouse myeloma y-globulin and from its glycopeptide. Glucosaminylitol was recovered from the released oligosaccharide after NaBH4 reduction and acid hydrolysis.

In the last decade, extensive studies have been carried out on the enzymatic hydrolysis of the glycoside linkages in glycoproteins (1). All of the enzymes so far characterized are exoglycosidases releasing monosaccharides from the nonreducing ends of the carbohydrate chains. This communication describes, for the first time, an endo-glycosidase releasing an oligosaccharide from a glycoprotein. To detect this enzyme, carbohydrate radiolabeled myeloma y-globulin and its glycopeptide were used as substrates.

A cell line of mouse plasma cell tumor (M1PC-11 cells, 3 x 10⁶ cells), kindly given by Dr. M. D. Scharff, was cultured for 24 hours in suspension as described by Laskov and Scharff (3) in 100 ml of culture medium containing 20% horse serum and 100 μCi of one of the following radioactive sugars: α-[1-14C]mannose (37.6 mCi per mmole), β-[3H]glucose (4.3 Ci per mmole), α-[6-3H]glucosamine (1.3 Ci per mmole), β-[3H]galactose (231 mCi per mmole). The radiolabeled myeloma y-globulin was precipitated from the culture medium by goat anti-mouse y-globulin serum, after adding syngeneic mouse serum as a source of nonspecific y-globulin as carrier. The immune precipitate was washed four times with 0.15 M NaCl and digested with 20 mg of Pronase as described previously (3). The digested material was mixed with 0.2 volume of phenol to inactivate Pronase and applied to a column of Sephadex G-50 which was equilibrated and eluted with 0.15 M NaCl. The radioactive peak was pooled and used as Pronase-digested y-globulin glycopeptide.2

Carbohydrate-labeled intact myeloma y-globulin was purified from the culture medium by adsorption to a column of DEAE-

1 This research was supported by Grant AI-07280 from the United States Public Health Service and Grant GB-7924 from the National Science Foundation to Dr. S. G. Nathenson.

2 The antigen-antibody complex contained about 55 mg of protein and 100 μCi of [3H]glucosamine-labeled material, 3.2 X 10⁶ cpm; [3H]glucosamine-labeled material, 3.5 X 10⁶ cpm; [3H]galactose-labeled material, 1.8 X 10⁶ cpm; [3H]mannose-labeled material, 1.0 X 10⁶ cpm.

3 The glycopeptide gave a single symmetrical peak corresponding to a molecular weight of 2300 upon Sephadex G-50 column chromatography (3) and moved to negative pole as a single spot by paper electrophoresis at pH 1.9. By paper electrophoresis at pH 6.5, 90% of the radioactive sugars in the glycopeptide were found. The radioactive profile of the glycopeptide from Sephadex G-50 column is not changed after desialylation, the glycopeptide is suggested to have a few sialyl residues.

4 Glycosidase activities were measured as described by Hughes and Jeanloz (4) and specific activities were expressed as micromoles of substrates hydrolyzed per min per mg of protein. Specific activity of β-N-acetylgalactosaminidase in the enzyme preparation was as follows: II, 0.016, III, 0.31. Specific activity of β-galactosidase in the enzyme preparation was as follows: II, 0.0014; III, 0.049.

5 Protein was determined by the method of Lowry et al. (5), with bovine serum albumin as a standard.

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Compounds A and B relative to that of cellobiose was 0.37 and 0.17, respectively. On paper electrophoresis in 1.5 m formic acid (pH 1.9) at 35 volts per cm for 24 hours, Compound A labeled with [3H]fucose or [3H]glucosamine moved 20 cm to the negative pole, while Compound A labeled with [14C]mannose and [3H]glucosamine remained at the origin. Under the same conditions, the intact glycopeptide moved 10 cm to the negative pole. Both Compounds A and B remained at the origin during paper electrophoresis at pH 6.5 (pyridine-acetic acid-water, 10:0:4:89.6). Thus, it is concluded that Compound A is a neutral oligosaccharide, while Compound B carries amino acids. Compound A labeled with [14C]mannose or [3H]glucosamine was digested with 2.3 units of purified α-mannosidase from jack bean meal (0) in 500 μl of 0.05 m acetate buffer, pH 4.5, containing 0.01 m ZnCl₂ for 15 hours at 37°C with toluene. All of the [14C]mannose label in the reaction mixture was identified as free mannose by Sephadex G-25 column chromatography and paper chromatography in Solvent I. The [3H]glucosamine label in the reaction mixture was eluted at the same tubes where free N-acetylglucosaminol was eluted upon Sephadex G-25 column chromatography. The α-mannosidase did not act on the intact glycopeptide or Compound B.

Compound A labeled with [3H]glucosamine was reduced with 2 mg of NaBH₄ in 200 μl of water for 15 hours. After passing the reaction mixture through a column of Dowex 50-X8, H⁺ form, and repeated evaporation with methanol, the reaction product was hydrolyzed with 4 x HCl for 4 hours at 100°C. Paper chromatography of the hydrolysate in Solvent I on either borate-treated or the usual Whatman No. 3MM paper revealed glucosaminitol as the major radioactive spot. On the borate-treated paper, the separation of glucosamine and glucosaminitol was complete, and the recovery of [3H]glucosamine label in the glucosaminitol region was determined to be 80%. This result establishes that the glucosamine residue is present at the reducing end of Compound A. Summarizing the above results, the following structure is proposed for Compound A and B.²

**Compound A:** (Man)₃ → GlcNAc

**Compound B:** Fuc,(Man),GlcNAc → peptide

The action of the enzyme on intact γ-globulin was examined in order to study the nature of enzyme specificity more clearly, and also to know the scope of its application. Intact myeloma γ-globulin (7.5 mg) doubly labeled with [14C]mannose and [3H]fucose was incubated with 0.8 mg of Enzyme Preparation III in 1 ml of 0.1 m Tris-HCl, pH 7.5, containing 0.15 m NaCl at 37°C for 15 hours with toluene. In a separate experiment, [3H]amino acid (reconstituted protein hydrolysate)-labeled myeloma γ-globulin was incubated with enzyme preparation under the same conditions. About 95% of [3H]mannose label in the enzymatic hydrolysate was found in the oligosaccharide region upon Sephadex G-25 column chromatography, and was identified as Compound A by paper chromatography in Solvent I. No release of oligosaccharide was observed when the γ-globulin was incubated without the enzyme. The [3H]fucose label, the [3H]amino acid label, and the rest of the [14C]mannose label contained in the enzymatic hydrolysate were excluded from

Because of the experiments with radiolabeled products, molar ratios of sugars in Compounds A and B are not firmly established.

² The majority of protein is derived from horse serum.
The Sephadex G-25 column, and were eluted at the same position as the intact γ-globulin upon Sephadex G-150 column chromatography. Therefore, it is concluded that the enzyme preparation releases Compound A even from intact γ-globulin without any detectable cleavage of peptide linkages. This result confirms that the release of Compound A from the glycopeptide is not due to cleavage of the carbohydrate amino acid linkage by a previously described amidase named glycopeptidase (7), which requires both free α- and α- carboxyl groups of the carbohydrate-linked asparagine for its action. Furthermore, 0.2 mg of Enzyme Preparation III in 200 μl of 0.05 M sodium phosphate buffer, pH 7.0, released less than 2 nmoles of N-acetylglucosamine from 1 mg of synthetic β-aspartylglycosylamine (the kind gift of Professor I. Yamashina) at 37°C for 24 hours. Under identical conditions, about 60% of the [14C]mannose-labeled glycopeptide (950 cpm) was converted to Compound A within 1 hour. The result again supports the conclusion that the release of Compound A is not due to the cleavage of the carbohydrate-peptide linkage of β-aspartylglycosylamine type by glycopeptidase or hitherto undescribed N-glycosidase, but is instead due to the internal cleavage of the carbohydrate chain by an endo-glycosidase.

The evidence reported in this communication firmly establishes the presence of an endo-glycosidase acting on a glycopeptide. This enzyme is a type of endo-acetylglucosaminidase, probably an endo-β-N-acetylglucosaminidase. The final conclusion as to the precise specificity of this endo-glycosidase can be concluded definitively only after further purification and examination of its activity on different glycopeptides and glycoproteins. Even the possibility that the β-N-acetylglucosaminidase (4) and the endo-glycosidase activities are actually due to a single enzyme cannot be excluded at the present time, for no conclusive experiment showing that this particular β-N-acetylglucosaminidase is a strict exo-glycosidase has ever been published. In any event, this newly discovered endo-glycosidase which has the capacity of acting on an at least one intact glycoprotein should be an excellent tool in many areas of glycoprotein research.

Acknowledgments—The author wishes to express his sincere gratitude to Dr. S. G. Nathenson for encouraging discussions, generous support, and valuable suggestions for the manuscript. He is also much indebted to Dr. M. D. Scharff for the gift of MPC-11 cells, to Dr. S. J. Pancake for careful criticism and helpful suggestions for the manuscript, and to Professor I. Yamashina for the gift of β-aspartylglycosylamine.

REFERENCES
8. From the data described in Footnote 1, about 0.62 mg (3.5 nmoles) of γ-globulin (mixture of mouse and goat γ-globulins) is estimated to be present in 800 cpm of the [14C]mannose-labeled antigen-antibody complex.

The Nature of the Acid Transition of Ribonuclease A

(Received for publication, June 9, 1971)

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SUMMARY

The low temperature acid transition of ribonuclease A has been investigated by using optical rotatory dispersion and circular dichroism. Changes are seen in the mean residual rotation at 228 nm, suggesting some loss of organized secondary structure in the molecule. However, no parallel changes are seen in circular dichroism measurements below 230 nm, indicating that the change in conformation involves only side chain residues.

A blue shift in the tyrosine absorbance band of ribonuclease A at low temperature and low pH was first observed by Hermans and Scheraga (1) and interpreted by them to indicate a discrete, small conformational change between two folded forms of the molecule. However, Tanford has suggested that the observed change actually results from the fact that ribonuclease is at least partially in the thermally unfolded state at all temperatures below pH 2 (2). Such an effect has been observed with α-chymotrypsin (3) and has been ascribed to a decrease of the enthalpy of unfolding of the molecule with temperature, due to large specific heat changes.

![Fig. 1. Optical rotatory dispersion of ribonuclease A.](http://www.jbc.org/Downloadedfrom.jpg)
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