The Carbohydrate Moiety of Bovine Pancreatic Deoxyribonuclease*

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SUMMARY

Preceding experiments by Price and his associates establish that bovine pancreatic deoxyribonuclease is a glycoprotein which contains glucosamine and mannose. In order to determine the mode of attachment of the carbohydrate to the enzyme molecule, chromatographically purified deoxyribonuclease has been digested with Pronase. Gel filtration of the digest yielded a fraction containing all of the carbohydrate in association with a mixture of the dipeptide and tetrapeptide sequences Ser-Asp and Ser-Asp-Ala-Thr. Removal of serine by the Edman degradation demonstrated that all of the carbohydrate is in association with aspartic acid. Analyses of the glycopeptides showed 1 residue of ammonia, 2 residues of glucosamine, and 5 of mannose to be present per residue of aspartic acid. Since only 2 residues of glucosamine (assumed to occur as the N-acetyl derivative) are present in the parent molecule, it is concluded that the saccharide moiety is attached at a single position on the enzyme and through an aspartamido-hexose linkage.

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When chromatographically purified bovine pancreatic deoxyribonuclease was analyzed for amino acids in the experiments of Price et al. (1), the chromatograms showed the presence of glucosamine. Quantitative results indicated 2 residues of amino sugar per molecule of molecular weight 31,000, accompanied by at least an equal number of residues of mannose. This result showed deoxyribonuclease to be a glycoprotein and prompted a comparison of these findings with those which have been obtained on the two ribonucleases from the same tissue, one of which possesses a carbohydrate side chain.

In the initial research on the ion exchange chromatography of pancreatic ribonuclease, Hirs, Moore, and Stein (2) found two enzymatically active fractions, A and B. The major component, ribonuclease A, was the subject of structural studies which led to the elucidation of the full sequence (3-6). The nature of ribonuclease B remained unknown until Plummer and Hirs (7) isolated the enzyme from pancreatic juice (cf. Greene, Hirs, and Palade (8)) and showed it to be the same as ribonuclease A with the addition of a saccharide side chain attached to asparagine at position 34 in the sequence.

The experiments to be described in this communication were designed to study the composition and disposition of the carbohydrate moiety of deoxyribonuclease.

EXPERIMENTAL PROCEDURE

Preparation of Deoxyribonuclease—Bovine pancreatic DNase, amorphous DH grade, was purchased from Worthington and purified on sulfoethyl-Sephadex (1). DNase activity was measured by a modification (1) of the hyperchromicity assay of Kunitz (9).

Hydrolysis of DNase with Pronase—Pronase, grade B, was purchased from Calbiochem. Purified, salt-free DNase (42 mg) was dissolved in 0.01 M calcium chloride (8.0 ml) and the pH was adjusted to pH 8.1 with 0.1 M sodium hydroxide. Digestion, initiated with 1% by weight of Pronase, was performed at the same pH and at 37° in a pH-stat. An additional, equal amount of Pronase was added after 5 hours and the digestion was continued for a total of 18 hours. Toluene was added as a preservative. The lyophilized digest, dissolved in 1% acetic acid, was fractionated on a column (2 X 40 cm) of Sephadex G-25 equilibrated in the same solvent. A flow rate of 12 ml per hour was maintained by a Milton Roy miniPump. Peptides were located by subjecting 0.1-ml portions of the effluent fractions to ninhydrin analysis after alkaline hydrolysis (10).

Analyses for Carbohydrate—The phenol-sulfuric acid procedure (11) served for detection of carbohydrate in the effluent from column fractionations; 0.1- to 0.2-ml portions of the effluent fractions to ninhydrin analysis after alkaline hydrolysis (10).

The neutral hexose content of the isolated glycopeptides was determined by hydrolysis in 2 N sulfuric acid at 100° in evacuated tubes at concentrations of less than 0.1% to minimize amino acid-carbohydrate interactions (12). Hydrolysates were diluted to 0.25 N in sulfuric acid and passed in sequence through columns (0.9 X 6 cm) of Dowex 50-X2 (200 to 400 mesh) in the H+ form and Dowex 1-X2 (200 to 400 mesh) in the formate form (13). Neutral sugar in the effluent was measured by the ferricyanide...
method of Park and Johnson (14). Control hydrolyses in 2 N 

were applied. After the breakthrough peak had emerged, elution was effected with a concentration gradient by the procedure of Price et al. (1). Flow rate about 40 ml per hour; 5-ml effluent fractions collected; temperature, 22°. Protein content was monitored by measuring the absorbance at 280 μm using 1-cm cells (O). Portions (0.2 ml) of the effluent fractions were removed for carbohydrate determination with the phenol-sulfuric acid procedure (absorbance at 490 μm, O).

FIG. 1. Measurement of carbohydrate content of effluent fractions when amorphous bovine pancreatic deoxyribonuclease is chromatographed on sulfoethyl-Sephadex. The column (3 × 40 cm) was equilibrated with 0.2 M acetic acid-sodium acetate buffer, pH 4.7. Approximately 500 mg of DNase were applied. After the breakthrough peak had emerged, elution was effected with a concentration gradient by the procedure of Price et al. (1). Flow rate about 40 ml per hour; 5-ml effluent fractions collected; temperature, 22°. Protein content was monitored by measuring the absorbance at 280 μm using 1-cm cells (O). Portions (0.2 ml) of the effluent fractions were removed for carbohydrate determination with the phenol-sulfuric acid procedure (absorbance at 490 μm, O).

The hexosamine content of hydrolysates of DNase was determined on a 55-cm column of the amino acid analyzer (15, 16) using an early buffer shift to elute valine at the breakthrough of the pH 4.25 buffer and to separate the hexosamines from the aromatic amino acids. The hexosamine content of the glycopeptide fraction was determined on the short column of the amino acid analyzer following hydrolysis in 4 N HCl at 100° in evacuated tubes. To ensure that no fragment resulting from incomplete hydrolysis of the peptide was eluted in the same position as hexosamine, the amino acid analyses of this hydrolysate and one hydrolysis of the peptide was eluted in the same position as hexosamine, the amino acid analyses of this hydrolysate and one hydrolysate of the peptide. The molar ratios of the amino acids in this hydrolysate and one from a 22-hour hydrolysis at 110° in 6 N HCl were compared. After correction for destruction of serine and threonine, no difference could be found and it was concluded that hydrolysis of the small peptide was complete after 6 hours in 4 N HCl.

It was verified that neither glucosamine nor hexosamine was destroyed under the hydrolytic conditions used for their liberation from the glycopeptide.

Sialic acid, released by a neuraminidase from Clostridium perfringens purchased from Worthington, was determined by the thiobarbituric acid method of Warren (17). Samples of human glycoprotein, Fraction VI, purchased from Calbiochem, were used as controls with all estimations.

For the identification of monoasaccharides, samples were hydrolyzed in 4 N HCl at 100° for 5 hours in evacuated tubes and the hydrolysates were repeatedly evaporated to dryness with water. The residue was taken up in 75 μl of water and applied to Whatman No. 1 chromatography paper. Descending irrigation was with ethyl acetate-pyridine-water, 10:4:3 (v/v) (18), and the dried chromatograms were visualized with a silver nitrate-sodium hydroxide dip (19).

Amino Acid Analyses—Amino acids were determined by the automatic procedure of Spackman, Stein, and Moore (15, 16) with the addition of a range card, as described by Hamilton (20), for increased sensitivity. Samples were prepared for analysis by hydrolysis in 2 ml of glass-distilled 6 N HCl at 110° for 24 hours in evacuated tubes (21).

Sequential Degradation of Peptides—The subtractive Edman method was employed along the lines of the modifications described by Konigsberg and Hill (22) and by Dopheide and Jones (23).

Determination of Amide Nitrogen—Samples were hydrolyzed in 4 N HCl for 4 hours in an evacuated tube at 100°. The ammonia content of the hydrolysate was determined on the short column of the amino acid analyzer by the method of Smyth, Stein, and Moore (24). Controls were run to correct for ammonia already present in the unhydrolyzed peptide solution and the reagents.

RESULTS

The purification of amorphous bovine deoxyribonuclease on sulfoethyl-Sephadex C-25 (1) produced a typical separation of two enzymically active peaks designated by Price et al. (1) as DNase A and DNase B (Fig. 1). The carbohydrate concentration in the fractions, monitored by the phenol-sulfuric acid procedure showed a close correspondence with the protein content in both of the areas in which enzymic activity was found. Fractions containing DNase A were pooled, dialyzed against distilled water, and lyophilized. This material was used for the characterization of the carbohydrate moiety.

Characterization of Carbohydrate Moiety of DNase A—DNase A was digested with Pronase at pH 8.1 and 37° until approximately half of the peptide bonds in the protein had been cleaved. Chromatography on Sephadex G-25 in 1% acetic acid (Fig. 2) yielded a forerunning fraction which contained all of the phenolsulfuric acid-positive, and very little ninhydrin-positive, material. This fraction contained 93% of the glucosamine present in the DNase A; glucosamine was absent in the remaining pooled fractions. Hydrolysis of this glycopeptide in 4 N HCl at 100° for 5 hours followed by paper chromatography revealed the major carbohydrates to be glucosamine and mannose. Glucose and galactose were also detected, but were only present in trace amounts (i.e. each less than 5% of the mannose content). Since glucosamine was the only neutral hexose present in significant quantity, values for neutral hexose were taken to represent mannose. The molar ratios of components in this glycopeptide fraction were Asp, 1.00; Thr, 0.69; Ser, 0.92; Ala, 0.73; glucosamine, 1.73; and mannose, 4.9. No sialic acid could be detected either in association with this glycopeptide or with DNase A. Since the analysis indicated that the peptide moiety was not homogeneous, the glycopeptide mixture was further incubated with Pronase at pH 8.0 and 37° for 72 hours followed by isolation of the glycopeptide on Sephadex G-25. The molar ratios of the amino acids became Asp, 1.00; Thr, 0.18; Ser, 0.87; Ala, 0.20. No hexosamine or hexose was lost during this procedure; it was tentatively concluded that the carbohydrate was present in association with a dipeptide of aspartic acid and serine and a tetrapeptide containing, in addition, threonine and alanine. Evidence that the
The amino acid removed is indicated by boldface. The above analysis is consistent with the presence of the tetrapeptide sequence, Ser-Asp-Ala-Thr, and a dipeptide sequence, Ser-Asp.

**Characterization of Carbohydrate Moiety of DNase B**—Reference to Fig. 1 shows that the smaller of the two enzymatically active peaks, DNase B, also contains phenol-sulfuric acid-positive material. The ratio of absorbances at 280 and 490 nm are similar across the two peaks. Isolation of the carbohydrate by digestion of DNase B with Pronase, followed by chromatography on Sephadex G-25, produced a similar elution profile to that shown in Fig. 2 for DNase A. Again the only sugars present were mannose and glucosamine and they occurred in the same molar ratio and were associated with the same amounts of aspartic acid, threonine, serine, and alanine as found with DNase A. The sole difference appeared to be in the sialic acid content; intact DNase B analyzed for 0.2 residue per molecule whereas the amount associated with the isolated glycopeptide had dropped to 0.06 mole per mole of aspartic acid. These results indicate that sialic acid is an impurity and is probably not associated with the heptasaccharide moiety, but may be part of a contaminating glycoprotein eluted in the same position as DNase B, which is subject to less retardation on sulfoethyl-Sephardex than DNase A.

**DISCUSSION**

The evidence presented shows that the carbohydrate moiety of bovine pancreatic deoxyribonuclease is attached at a single point on the polypeptide chain through an amide bond involving the \(\beta\)-COOH of aspartic acid. In all probability this is the \(N\)-\(\beta\)-aspartylglycosyamine bond described by Marks, Marshall, and Neuberger (25) for hen egg albumin, and since then frequently found to occur in glycoproteins (26, 27).

The number of glucosamine residues, presumed to be N-acetylated, was found to be 1.73. The difference between this result and 2.00 may arise from the fact that under the conditions of acid hydrolysis used to release hexosamine from the oligosaccharide a small proportion of glycosidic bonds may become resistant to cleavage (28), since decylation of \(N\)-acytylgulcosamine residues prior to hydrolysis of the glycosaminide bond will produce a relatively stable glycosidic bond. It also follows that, unless the \(N\)-acytylgulcosamine residues are adjacent, a small proportion of mannose will remain undetected. The carbohydrate is then approximately a heptasaccharide comprising 2 \(N\)-acytylgulcosamine and 5 mannose residues, although the possibility of microheterogeneity has not been eliminated. Price et al. (1), who used a sample purified from crystalline deoxyribonuclease, have found only 2 mannose and 2 glucosamine residues to be present. The method of preparation of DNase, therefore, may give rise to glycoproteins the saccharide moiety of which may vary in chain length as Plummer and Hirs (7) have observed for RNase B. It will be desirable to study the DNases in pancreatic juice in order to learn whether the isolation procedures currently in use produce alterations in carbohydrate content.

The similarity in the carbohydrate moieties of DNase and RNase extends to the respective tripeptide sequences at the points of attachment, which are Asn-Ala-Thr and Asn-Leu-Thr, respectively. Eylar (29) has commented on the frequency with which threonine occurs near asparagines at the points of attachment of the saccharide chain in glycoproteins. There is only one Asn-X-Thr sequence in the 124-residue chain of bovine pancreatic RNase; Jackson, Reinhold, and Hirs (30) have recently found that the carbohydrate in porcine RNase is also linked to an Asn-X-Thr sequence. Further conclusions on the uniqueness of the sequence at the point of attachment of carbo-
hydrate in DNase await the elucidation of the full amino acid sequence of the enzyme.

REFERENCES

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