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The Structure of a Glycopeptide Purified from Porcine Thyroglobulin

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1. The structure of a purified glycopeptide isolated from porcine thyroglobulin was studied by sequential hydrolysis with specific glycosidases, by periodate oxidation and by treatment with galactose oxidase. 2. Sequential hydrolysis with several combinations of neuraminidase, α-L-fucosidase, β-D-galactosidase, β-*N*-acetyl-D-glucosaminidase and α-D-mannosidase presented the evidence for the following structure. 3. The monosaccharide sequence of the peripheral moiety of the heteropolysaccharide chain was sialic acid→galactose→*N*-acetylglucosamine. Some of the galactose residues were non-reducing end-groups with the sequence galactose→*N*-acetylglucosamine. 4. After removal of the peripheral moiety composed of sialic acid, fucose, galactose and *N*-acetylglucosamine, α-mannosidase released 1.4mol of mannose/mol of glycopeptide, indicating that two of the three mannose residues were located between peripheral *N*-acetylglucosamine and internal *N*-acetylglucosamine or mannose. 5. Periodate oxidation and sodium borohydride reduction confirmed the results obtained by enzymic degradation and gave information concerning the position of substitution. 6. Based on the results obtained by enzymic hydrolysis and periodate oxidation together with the treatment with galactose oxidase, a structure is proposed for the glycopeptide.

In the preceding paper (Fukuda & Egami, 1971), a procedure for the isolation and fractionation of glycopeptides from porcine thyroglobulin was reported. Several of them were obtained in a state that showed the presence of only one component when subjected to paper electrophoresis at pH 3.6.

The present paper deals with the structural analysis of one of these glycopeptides, with special reference to the structure of the carbohydrate moiety, which has been examined by sequential degradation with specific glycosidases, by periodate oxidation and by treatment with galactose oxidase.

EXPERIMENTAL

Materials. Thyroglobulin glycopeptide V (fraction V) was prepared as described in the preceding paper (Fukuda & Egami, 1971). The chemical composition of this glycopeptide, which appeared to have only one component by high-voltage electrophoresis, is shown in Table 1.

Clostridium perfringens PB6K was given by Dr Kameyama of the National Institute of Health, Tokyo, Japan.

Glycosidases. α-D-Mannosidase (EC 3.2.1.24), α-L-fucosidase and β-D-galactosidase (EC 3.2.1.23) were purified from the liver of *Turbo cornutus* as described previously (Muramatsu & Egami, 1967; Iijima, Muramatsu & Egami, 1969). *N*-Acetyl-β-D-glucosaminidase (EC 3.2.1.30) was purified from the liver of *Charonia lampas* (M. Fukuda & F. Egami, unpublished work). α-L-Fucosidase purified from the liver of *Ch. lampas* was prepared by Mr Y. Iijima of this laboratory. Neuraminidase (EC 3.2.1.18) was purified from *C. perfringens* PB 6K (Cassidy, Jourdian & Roseman, 1965) by using porcine submaxillary mucin as substrate. Assays of other glycosidases were carried out with *p*-nitrophenyl glycosides as described previously (Fukuda, Muramatsu & Egami, 1969). The activity of these purified glycosidases is given in Table 2.

Chemical analysis. The quantitative determination of the individual neutral sugars and amino acids of glycopeptides was carried out as described in the preceding paper (Fukuda & Egami, 1971). Hexosamine was determined by the borate-catalysed Morgan–Elson reaction (Strominger, Park & Thompson, 1959) or by using the amino acid analyser after hydrolysis in 4M-HCl at 100°C for 4–6h.

Digestion with purified glycosidases. For the digestion of glycopeptides, the purified glycosidases (Table 2) were concentrated to 0.01vol. by pressure dialysis. A portion of the glycopeptide solution (containing 0.5–1mg) was freeze-dried in a test-tube. After being dried, 10–30μl of the concentrated enzyme solution and 10μl of buffer solution (see below) were pipetted into the tube. After the addition of a few drops of toluene, the reaction mixture was incubated at 37°C for 24h unless otherwise specified.

For the stepwise release of monosaccharides from the glycopeptide, the enzyme in the incubation mixture was inactivated by immersing the tube in a boiling-water bath for 1min, and the sample was freeze-dried, if necessary, before the next enzyme was added.

The buffer solutions used were as follows: 0.5M-sodium

acetate buffer, pH4.5, containing 0.15% of bovine serum albumin for neuraminidase; 0.2M-sodium acetate buffer, pH4.0, containing 0.5M-NaCl for α-L-fucosidase, β-D-galactosidase and α-D-mannosidase; 0.2M-citrate–phosphate buffer, pH4.0, containing 0.5M-NaCl for β-*N*-acetyl-D-glucosaminidase.

Tubes containing enzyme without substrate were treated in the same way as a control.

The liberated sialic acid and *N*-acetylglucosamine were directly determined by the thiobarbituric acid method (Aminoff, 1961) and Morgan–Elson reaction (Reissig, Strominger & Leloir, 1955), respectively. The released neutral sugars and *N*-acetylglucosamine were quantitatively determined by g.l.c. as described in the preceding paper (Fukuda & Egami, 1971). When *N*-acetylglucosamine was determined the column temperature was increased at a rate of 3°C/min from 162 to 210°C after 25min from injection.

Periodate oxidation of glycopeptide. Periodate oxidation of the glycopeptide was carried out in 0.05M-sodium metaperiodate in the presence of 0.02M-sodium acetate buffer, pH4.0, at 4°C in the dark, and the consumption of periodate was determined spectrophotometrically by using the extinction at 260nm (Montgomery, Wu & Lee, 1965). The sodium metaperiodate/glycopeptide molar ratio was approx. 100:1. After the oxidation an equal volume of 0.1M-borate buffer, pH8.0, containing 0.53M-$NaBH_4$ was added. The reduction was continued at 4°C for 20h. The periodate-oxidized–borohydride-reduced glycopeptide solution was passed through a column of Amberlite IR-120 (H^+ form) and the effluent and washes were evaporated to dryness in a vacuum rotator with addition of methanol. The products that remained were analysed for carbohydrate constituents.

Polyols were determined as follows. The product was hydrolysed in 1M-HCl at 100°C for 3h. The hydrolysate was diluted, then a fixed amount of trimethylolpropane [$C_2H_5C(CH_2OH)_3$] was added (Yamaguchi, Ikenaka & Matsushima, 1970). It was passed through a column of Amberlite IRA-400 (HCO_3^- form) and evaporated to dryness at 40°C.

The dried materials were trimethylsilylated and g.l.c. of trimethylsilyl derivatives was carried out with a glass column (3mm×200cm, 4% SE-30 on Gas-Chrom CLH). The column temperature was 80°C at the start, and increased at a rate of 2°C/min.

Standard samples of glycerol, erythritol and propane-1,2-diol (see below) were used for the determination of the relative detector response. Samples of threitol and propane-1,2-diol were prepared by Smith degradation (Smith & Unrau, 1959) from β-1,4-galactan of pectin (Wako Pure Chemicals, Japan) and fucose, respectively.

Treatment of galactose oxidase. Samples were incubated with galactose oxidase (Worthington) and catalase (prepared by Mr K. Yoshida of this Department from bovine blood cells) in 0.1M-phosphate buffer, pH7.0, at 37°C for 24h under toluene. The concentration of the substrate was about 1μmol as galactose/ml. The galactose oxidase was present at a concentration of 0.8mg/ml, and catalase at 0.04mg/ml (Spiro, 1967). Controls containing either enzyme or the substrate were also prepared. At the end of the incubation, the digest was hydrolysed in 0.5M-H_2SO_4 at 100°C for 8h, and neutral sugars were determined by g.l.c. as described in the preceding paper (Fukuda & Egami, 1971).

Table 1. *Chemical composition of glycopeptide V*

Component	Composition (mol/mol of glycopeptide)
Sialic acid*	2.40
Fucose	1.00
Galactose	2.96
Mannose	2.93
Glucosamine	4.92
Aspartic acid†	1.35
Threonine†	0.65
Serine†	0.46

* Sialic acid is expressed as *N*-acetylneuraminic acid.

† In addition to these amino acids, small amounts of glutamic acid (0.27mol/mol of glycopeptide), proline (0.22) and glycine (0.32) were also present.

RESULTS

Enzymic hydrolysis of glycopeptide. It is expected that specific exoglycosidases can release monosaccharides only from the non-reducing ends of

Table 2. *Activity of purified glycosidases used in enzymic hydrolysis*

All activities are expressed as μmol of *p*-nitrophenol liberated/min per ml of enzyme solutions under the conditions described by Fukuda *et al.* (1969).

Enzyme preparation	Activity: α-L-Fucosidase	β-D-Galactosidase	*N*-Acetyl-β-D-glucosaminidase	α-D-Mannosidase
α-L-Fucosidase (*T. cornutus*)	0.56	0.07	0.03	0.01
α-L-Fucosidase (*Ch. lampas*)	1.04	0.001	0.001	0.06
β-D-Galactosidase (*T. cornutus*)*	0.03	2.16	0.108	0.03
N-Acetyl-β-D-glucosaminidase (*Ch. lampas*)	0.002	0.04	0.45	0.001
α-D-Mannosidase (*T. cornutus*)†	<0.001	0.08	0.008	1.60

* β-D-Galactosidase preparation contained no activity against *p*-nitrophenyl α-D-galactoside.

† α-D-Mannosidase preparation contained no β-D-mannosidase activity.

Table 3. *Enzymic liberation of monosaccharide from glycopeptide V*

For the stepwise liberation of monosaccharide from glycopeptide V with the use of two or more enzymes, the enzyme in the incubation mixture was inactivated by immersing the tube in a boiling-water bath for 1 min, and the next enzyme was then added. The other details are described in the Materials and Methods section. Blank spaces mean values less than 0.1 μmol.

Expt.	Monosaccharides liberated (mol/mol of glycopeptide)				
	Sialic acid	Fuc	Gal	GlcNAc	Man
1. Neuraminidase only	2.4				
2. α-L-Fucosidase only*	0.1	0.5	0.5		
3. Neuraminidase + β-D-galactosidase	2.4		2.6		
4. α-L-Fucosidase + β-D-galactosidase	0.2	0.14	0.78		
5. β-D-galactosidase + *N*-acetyl-β-D-glucosaminidase†	0.1	0.13	0.76	0.76	
6. Neuraminidase + β-D-galactosidase + *N*-acetyl-β-D-glucosaminidase	2.4		2.5	2.2	
7. Neuraminidase + α-L-fucosidase + β-D-galactosidase + *N*-acetyl-β-D-glucosaminidase + α-D-mannosidase	2.4	0.32	2.8	2.5	1.4

* In this experiment only, α-L-fucosidase preparation from *Ch. lampas* was used and incubation time was 48 h.

† *N*-Acetyl-β-D-glucosaminidase was added at 24 h incubation of β-D-galactosidase without heat treatment. The total incubation time was 72 h.

heterosaccharide moieties. Therefore, the sequence of monosaccharides can be determined by sequential enzymic digestion.

The results obtained by specific glycosidases are summarized in Table 3, and these together with periodate oxidation provide evidence for the following structure.

(1) The peripheral moiety of the glycopeptide contains three side chains; one terminates with galactose and the other two terminate with sialic acid.

(2) The monosaccharide sequences of side chains are either galactose→*N*-acetyl glucosamine or sialic acid→galactose→*N*-acetylglucosamine. Non-reducing terminal *N*-acetylglucosamine residues are absent, because this sugar was found to be completely resistant to periodate oxidation (see below).

(3) As the release of *N*-acetylglucosamine was almost equal to that of galactose in the experiment with β-D-galactosidase and *N*-acetyl-β-D-glucosaminidase, *N*-acetylglucosamine of this side chain has no substituent except galactose.

(4) Since galactose was almost quantitatively released without appreciable release of fucose in Expts. 3 and 6 of Table 3, most of galactose is not substituted with fucose. But the release of *N*-acetylglucosamine was smaller than that of galactose and it was increased by pretreatment with fucosidase (cf. Expts. 6 and 7, Table 3). This indicates that a part of *N*-acetylglucosamine is probably substituted with fucose.

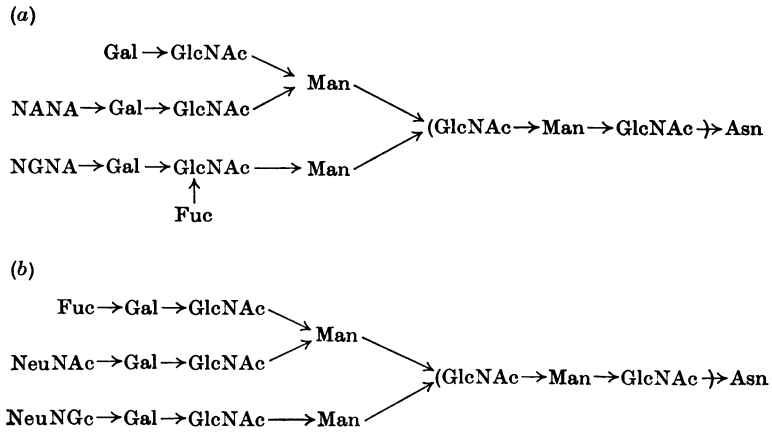
Therefore, taking into consideration the deductions made in (3) and (4) above, it is concluded that fucose is attached to *N*-acetylglucosamine of a part of the side chains which terminate with sialic acid (Scheme 1*a*).

(5) In addition to the linkage of fucose→*N*-acetylglucosamine, some of the fucose (about 0.2 mol/mol of glycopeptide) may be joined to galactose that is not substituted with sialic acid (Scheme 1*b*). This is suggested by the fact that release of galactose was increased by fucosidase pretreatment and some fucose was released by a small amount of α-L-fucosidase (experiments with β-D-galactosidase and *N*-acetyl-β-D-glucosaminidase).

From these results, included with α-D-mannosidase digestion, the possible structures of the glycopeptide consonant with enzymic digestion are shown in Scheme 1. Approx. 80% of the glycopeptide molecules have the structure of Scheme 1(*a*) and the other 20% of the molecules have the structure of Scheme 1(*b*).

Unexpectedly, the release of fucose in the experiment with α-L-fucosidase and β-D-galactosidase was small and almost equal to that in the experiment with β-D-galactosidase and *N*-acetyl-β-D-glucosaminidase. Digestion was continued for 72 h without heat treatment in the latter experiment, whereas the incubation time with α-L-fucosidase was 24 h in the former experiment.

This could be explained in the following way. α-L-Fucosidase has a narrow specificity with regard to the attachment of fucose and can hydrolyse fucose attached to *N*-acetylglucosamine which is further substituted with galactose only with difficulty (fucose in Scheme 1*a*), but it may hydrolyse rather easily fucose attached to galactose without any other substitution (fucose in Scheme 1*b*). Thus,



Scheme 1. Possible structures of glycopeptide V consonant with enzymic degradation. NeuNAc, *N*-Acetylneuraminic acid, NeuNGc, *N*-glycollylneuraminic acid.

a small amount of α-L-fucosidase, which contaminated the β-D-galactosidase preparation, hydrolysed fucose (Scheme 1*b*) on prolonged incubation, and also α-L-fucosidase in Expt. 3 of Table 3 hydrolysed only the same fucose under this condition.

This assumption was supported by the fact that the release of fucose was increased by removal of galactose substituent on *N*-acetylglucosamine (cf. Expts. 4 and 7 of Table 3). A similar result was obtained with β-galactosidase of *Trichomonas foetus* (Harrap & Watkins, 1970).

The α-L-fucosidase preparation from *Ch. lampas* contained only a little β-galactosidase activity (0.1%) compared with α-L-fucosidase activity by using *p*-nitrophenyl glycoside as substrate. However, it released equal amounts of fucose and galactose from thyroglobulin glycopeptide.

This may be due to the fact that β-galactosidase has a higher activity for some natural substrates than for synthetic substrate, and so dual activity of β-galactosidase against galactoside of thyroglobulin is higher than its activity against *p*-nitrophenyl β-D-galactoside. A similar result was reported with β-xylosidase of almond emulsin (Scocca & Lee, 1969).

The existence of fucose at the non-reducing end was indicated by partial acid hydrolysis with 10M-trifluoroacetic acid at room temperature for 20 h (Yasuda, Takahashi & Murachi, 1970). Fucose was the only neutral monosaccharide found by g.l.c.

Periodate oxidation. The glycopeptide was oxidized with 0.05M-sodium metaperiodate at 4°C for 22 h and the consumption of periodate reached a plateau at this time. The composition of sugars in the native and oxidized glycopeptide is shown in Table 4. The oxidation of the glycopeptide resulted in almost complete loss of galactose. However, one of the three mannose residues and all of the *N*-acetylglucosamine residues were resistant to periodate oxidation.

Table 4. *Carbohydrate composition of glycopeptide V after periodate oxidation and sodium borohydride reduction*

Results are expressed as mol/mol of glycopeptide.

Component	Native	After periodate oxidation
Sialic acid	2.40	0
Fucose	1.00	0
Galactose	2.96	0.10
Mannose	2.93	1.35
Glucosamine	4.92	4.92

Determination of the reaction products such as propane-1,2-diol, glycerol and erythritol was carried out by g.l.c. After reduction and acid hydrolysis, 1 mol of glycopeptide produced 0.9 mol of propane-1,2-diol, 4.4 mol of glycerol and 0.5 mol of erythritol.

This result is consistent with that obtained on the destruction of sugars by periodate oxidation.

Thus, it is concluded that the oxidation of 3 mol of galactose yielded 3 mol of glycerol, 2 mol of mannose yielded 1 mol of glycerol and 1 mol of erythritol, and 1 mol of fucose yielded 1 mol of propane-1,2-diol.

Galactose oxidase treatment. Incubation with galactose oxidase was performed to determine whether C-6 of galactose in the glycopeptide is unsubstituted. This enzyme is known to oxidize galactose at C-6 (Avigad, Amaral, Asensio & Horecker, 1962).

After this treatment about one of the three galactose residues was oxidized although recovery of mannose was quantitative. This indicated that one of the three galactose residues was unsubstituted at C-6.

DISCUSSION

From the results presented in this paper, the structure shown in Scheme 2 is proposed for the major part of glycopeptide V molecules.

The sequence of monosaccharides in Scheme 2 was based mainly on the results of the sequential degradation by purified glycosidases. It clearly indicates that the monosaccharide sequence of this glycopeptide was either galactose→*N*-acetylglucosamine or sialic acid→galactose→*N*-acetylglucosamine. Fucose was linked to the latter side chain at the position of *N*-acetylglucosamine.

The inner core of glycopeptide V consists of three mannose and two *N*-acetylglucosamine residues. After removal of the peripheral moiety composed of sialic acid, fucose, galactose and *N*-acetylglucosamine, 1.4 mol of mannose was released by α-D-mannosidase (Expt. 7, Table 3). This leads to the conclusion that two of the three mannose residues are located between peripheral *N*-acetylglucosamine and internal *N*-acetylglucosamine or mannose residues. The fact that the amount of mannose released was 1.4 and not 2 mol was probably due to incomplete elimination of fucose.

The results obtained by periodate oxidation and sodium borohydride reduction were quite consistent with those in enzymic degradation and yielded more detailed information about the position of substitution. All the galactose residues were oxidized and yielded glycerol, indicating that possible attachment to galactose was at C-2 or C-6. Galactose oxidase treatment indicates that two of three galactose residues were probably substituted at C-6.

Since *N*-acetyl-lactosamine [β-D-Gal-(1→4)-D-GlcNAc] has been isolated from thyroglobulin in a reasonable yield (Spiro & Spiro, 1968), it is likely that a β-(1→4)-linkage is the main linkage between galactose and *N*-acetylglucosamine. However, the presence of a (1→3)-linkage cannot be completely excluded.

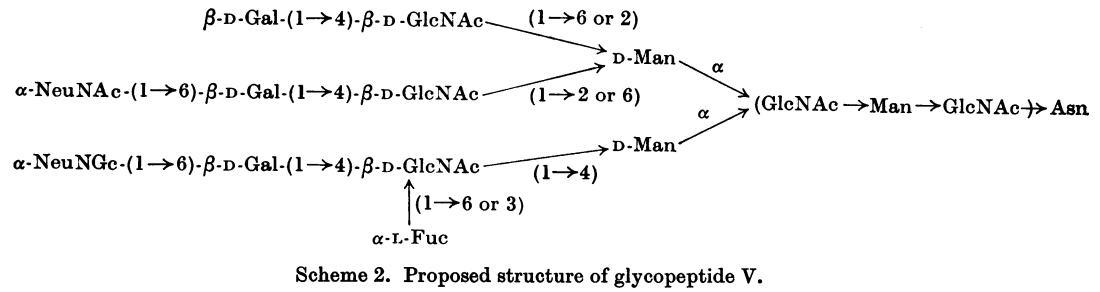
The location of the periodate-resistant mannose residues could not be made with any certainty. In Scheme 2, the innermost mannose was assumed to be resistant to periodate oxidation. One of the mannose residues susceptible to periodate oxidation was substituted at C-4 and the other was at C-2 and C-6. The former yielded erythritol and the latter glycerol.

Finally, some *N*-acetylglucosamine residues are linked to a β-amido group of asparagine, based on the results in the preceding paper (Fukuda & Egami, 1971).

The information so far obtained is not enough to exclude other possibilities from that shown in Scheme 2. Indeed, there are a few alternatives, particularly concerning the location of the mannose moiety. Although mannose resistant to periodate oxidation can be located by controlled Smith degradation (Smith & Unrau, 1959), the separation of unoxidized glycopeptide from oligosaccharide released was unsuccessful by paper electrophoresis.

Preliminary results of studies on the structural relationship between glycopeptide V and *N*-acetylglucosamine–mannose glycopeptide (Fukuda & Egami, 1971) suggest that the latter is not a precursor of a more complex carbohydrate unit and that the two types of carbohydrate units have a different basic structure. This is supported by the results obtained by Arima, Spiro & Spiro (1970). The structure of glycopeptide V appears to be similar to the glycopeptides from orosomucoid (Wagh, Bornstein & Winzler, 1969), with some similarities to the glycopeptides from fetuin (Spiro, 1964) and myeloma globulin (Dawson & Clamp, 1968).

The utility of sequential degradation with specific glycosidases for determination of sequence and nature of linkage is quite evident from the present study. Moreover, the enzymic analysis can be carried out with smaller amounts of materials, i.e. less than the 20 mg used in the present study, when the determination of monosaccharide produced can be performed on a micro scale.



Scheme 2. Proposed structure of glycopeptide V.

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