The Mode of Binding of Carbohydrate in Ricin D

Shigeru Nanno, Masatsune Ishiguro, Gunki Funatsu and Masaru Funatsu

Laboratory of Biochemistry, Faculty of Agriculture, Kyushu University, Fukuoka 812

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Determination of the amino acid sequences of the three glycopeptides obtained from proteolytic digests of ricin D reveals that the site of carbohydrate linkage is at the Asx residue in all three peptides. Established structures of the glycopeptides are as follows:

**Ala Chain:**
- (glucosamine, mannose)
- Asx-Asx-Gly-Thr
  - (glucosamine, mannose)
- Asx-Asx-Thr-Glu-Pro
- Ile Chain:
  - (glucosamine, mannose)
  - Ile-Asx-Phe

We have isolated three glycopeptides, namely P-1, P-2, and P-3 from the proteolytic digests of ricin D as reported in the previous paper. Since amino acid compositions of P-1 and P-2 have revealed that more than one site of carbohydrate linkage is possible, it is, therefore, attempted to degrade these glycopeptides further in order to understand the number of site and its mode of linkage in each peptide.

**MATERIALS AND METHODS**

**Materials.** Glycopeptides, P-1, P-2, and P-3 were prepared from the proteolytic digests of ricin D as described previously.

**Methods**

- **Amino acid and carbohydrate analyses.** Amino acid and carbohydrate analyses were performed as reported before.
- **Edman degradation.** For subtractive Edman degradation of glycopeptides, a modified method of Konigsberg et al. was used. Glycopeptides (0.1 µmole) dissolved in 2 ml of N-ethylmorpholine buffer (pH 9.0) were incubated with 0.1 ml of phenylisothiocyanate at 37°C for 3 hr. The incubation was carried out in a test tube with a stopper under nitrogen. The reaction mixture was evaporated to dryness under reduced pressure. The residue was washed with each 4 ml of benzene, and then dried in vacuo at 60°C for 5 min. To the residue 0.1 ml of anhydrous trifluoroacetic acid was added and the solution was incubated at room temperature for 1 hr under nitrogen. After the reaction mixture was dried, the residue was dissolved with 4 ml of 0.2 N acetic acid. The solution was then washed four times with each 4 ml of benzene. An aliquot of the aqueous phase was hydrolyzed in 6 N HCl at 100°C for 20 hr for amino acid analysis and the rest was subjected to the next step of the degradation. For the gel filtration (Figs. 1 and 2), this aqueous phase was employed after concentration.

- **Alkali-treatment.** Glycopeptides were treated with 0.5 N NaOH at 0°C for 72 hr for checking of O-glycosyl linkage. After the incubation the peptide solution was neutralized and then hydrolyzed with 6 N HCl at 106°C for 20 hr. The amino acid composition of the hydrolysate was analyzed and the recovery of threonine was calculated.

**RESULTS**

**The structure of glycopeptide P-1**

The subtractive Edman degradation of P-1...

About 0.8 μmole of P-1 was subjected to one cycle of the Edman degradation and the aqueous phase of the degradation-product was applied to a column of 1.7 x 100 cm. Elution was carried out with water and each 3 ml was collected. 0.2 ml of each fraction was analyzed by phenol-sulfuric acid method and 0.1 ml by ninhydrin method after alkaline hydrolysis. •, absorbance at 490 nm (neutral hexose); ▲, absorbance at 570 nm (ninhydrin-colour); ■, absorbance at 263 nm.


About 0.9 μmole of P-2 was subjected to one cycle of the Edman degradation and the aqueous phase of the degradation-product was applied to a column of 1.7 x 100 cm. Elution was carried out with water and each 3 ml was collected. 0.2 ml of each fraction was analyzed by phenol-sulfuric acid method and 0.1 ml by ninhydrin method after alkaline hydrolysis. •, absorbance at 490 nm (neutral hexose); ▲, absorbance at 570 nm (ninhydrin colour); ■, absorbance at 263 nm.

(Table I) showed the amino acid sequence of P-1 to be Asx-Asx-Gly-Thr. It is well known that threonine in the form of O-glycosyl-Thr is destroyed entirely by the alkali-treatment.\(^3\) The recovery of 93% from P-1 after the alkali-treatment suggests that the possible carbohydrate linkage at this site can be ruled out, and therefore it should be at either or both Asx. On the other hand, one glycopeptide fraction P-1-A was obtained by gel filtration of the aqueous phase of the first Edman degradation product of P-1 (Fig. 1). The amino acid and carbohydrate compositions (Table II) showed P-1-A to be the glycopeptide derived from P-1 by the removal of the N-terminal Asx residue. Thus, it was concluded that the carbohydrate in P-1 was linked to the second Asx residue, and hence the structure of P-1 was elucidated to be Asx-Asx(2-glucosamine, 6-mannose)-Gly-Thr.

(Table II) showed the amino acid sequence of P-2 to be Asx-Asx-Gly-Thr. It is well known that threonine in the form of O-glycosyl-Thr is destroyed entirely by the alkali-treatment.\(^3\) The recovery of 93% from P-1 after the alkali-treatment suggests that the possible carbohydrate linkage at this site can be ruled out, and therefore it should be at either or both Asx. On the other hand, one glycopeptide fraction P-2-A was obtained by gel filtration of the aqueous phase of the first Edman degradation product of P-2 (Fig. 2). The amino acid and carbohydrate compositions (Table III) showed P-2-A to be the glycopeptide derived from P-2 by the removal of the N-terminal Asx residue. Thus, it was concluded that the carbohydrate in P-1 was linked to the second Asx residue, and hence the structure of P-1 was elucidated to be Asx-Asx(glucosamine2, mannose6)-Gly-Thr.

The structure of glycopeptide P-2

As shown in Table III, the first and second steps of the subtractive Edman degradation revealed that the N-terminal and the second amino acids were both Asx. Threonine of this glycopeptide was also recovered without loss after the alkali-treatment. Thus, the carbohydrate was supposed to be linked to either Asx or Glx. On the other hand, two com-
TABLE III. TWO CYCLES OF THE SUBTRACTIVE EDMAN DEGRADATION OF P-2

<table>
<thead>
<tr>
<th>Composition\a</th>
<th>Yield</th>
<th>Asp</th>
<th>Thr</th>
<th>Glu</th>
<th>Pro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>75%</td>
<td>1.23</td>
<td>0.89</td>
<td>1.00</td>
<td>0.99</td>
</tr>
<tr>
<td>Step 2</td>
<td>50</td>
<td>0.45</td>
<td>0.88</td>
<td>1.00</td>
<td>1.20</td>
</tr>
</tbody>
</table>

\a Carbohydrate composition is 2 moles of glucosamine and 7 moles of mannose per mole of peptide.

Fig. 3. UV-Spectrum of P-2-A in Water (pH 6.0).

TABLE IV. AMINO ACID AND CARBOHYDRATE COMPOSITION OF P-2-A

\a Hydrolyses were carried out with 6 N HCl at 150°C for 20 h\a and with 4 N HCl at 100°C for 3 hr.\b
\b Calculated from the optical density at 263 nm on the basis of a molar extinction coefficient of 18,000 for PTH-Asn.

<table>
<thead>
<tr>
<th>Asp</th>
<th>Glucosamine</th>
<th>Mannose</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.62\a</td>
<td>1.89 (2)\b</td>
<td>7.00 (7)</td>
</tr>
</tbody>
</table>

\a Carbohydrate composition is 2 moles of glucosamine and 7 moles of mannose per mole of peptide.

TABLE V. SUBTRACTIVE EDMAN DEGRADATION OF P-2-B

<table>
<thead>
<tr>
<th>Composition</th>
<th>Yield</th>
<th>Asp</th>
<th>Thr</th>
<th>Glu</th>
<th>Pro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>92%</td>
<td>0.26</td>
<td>0.92</td>
<td>1.04</td>
<td>1.00</td>
</tr>
<tr>
<td>Step 2</td>
<td>100</td>
<td>0.10</td>
<td>0.12</td>
<td>1.07</td>
<td>1.00</td>
</tr>
<tr>
<td>Step 3\a</td>
<td>89</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.00</td>
</tr>
</tbody>
</table>

\a Analysis was made without acid hydrolysis.

The structure of glycopeptide P-3

The subtractive Edman degradation of P-3 (Table VI) revealed that the amino acid sequence was Ile-Asx-Phe. Asx is the only possible site for carbohydrate linkage. Thus, the structure of P-3 was concluded to be Ile-Asx\(\text{(glucosamine}_2\text{mannose}_4\text{)-Phe.}\)

TABLE VI. SUBTRACTIVE EDMAN DEGRADATION OF P-3

<table>
<thead>
<tr>
<th>Composition\a</th>
<th>Yield</th>
<th>Ile</th>
<th>Asp</th>
<th>Phe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>89%</td>
<td>0.87</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>Step 2\b</td>
<td>48</td>
<td>0</td>
<td>1.00</td>
<td></td>
</tr>
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</table>

\a Carbohydrate composition is 2 moles of glucosamine and 4 moles of mannose per mole of peptide.
\b Analysis was made without acid hydrolysis.

DISCUSSION

As reported previously\b, two glycopeptides, P-1 and P-2, were isolated from the proteolytic digest of Ala-chain, and one glycopeptide, P-3, from Ile-chain. The amino acid sequences of these glycopeptides were eluci-
dated by the subtractive Edman degradation method. As a result, it was found that each of the glycopeptides carried one carbohydrate unit and each carbohydrate unit was linked to a peptide chain through Asx. In each glycopeptide, the attachment of carbohydrate did not disturb the Edman reaction. However, because of the high hydrophilicity of the carbohydrate moiety PTH-Asx-carbohydrate complex was not extracted with benzene and remained in the aqueous phase. This complex was easily isolated by gel filtration and was detected by its characteristic UV-absorption. This was the first time to recognize the attachment of carbohydrate by the isolation and characterization of the PTH-Asx-carbohydrate complex. Since Asx was found to be an amino acid which was directly linked to carbohydrate in each glycopeptide, it is strongly suggested that all of the three carbohydrate units in ricin D are linked to the polypeptide chains by the asparaginyl-glucosamine linkage.\(^4,5\) Both P-1 and P-2 have a general regularity of structure, Asx (carbohydrate)-x-Thr (x is a variable amino acid residue), which is found in general glycoprotein.\(^6\) Whether P-3 can fall into the same category is not known at the present time. This point shall be clarified by the separation of a longer peptide containing this portion and the elucidation of its structure.

REFERENCES