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INCOMPLETE GLYCOSYLATION OF ASN 563 IN MOUSE IMMUNOGLOBULIN M

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Summary: Mouse immunoglobulin IgM was prepared from MOPC 104E ascites fluid and [3H]-mannose labeled tumor cells. The purified protein was used to prepare Fc fragments which were cleaved by cyanogen bromide. Gel filtration allows complete separation of the C-terminal glycosylation site. Amino acid and carbohydrate analyses show that Asn 563 of murine IgM is glycosylated only about 44% of the time.

We are studying murine IgM with a goal of understanding the relationship between protein structure, glycosylation, and carbohydrate processing (1). This system is ideal for these studies since we know the protein sequence and the location of five glycosylation sites on the IgM heavy chain. The protein can be prepared in quantities sufficient for chemical study, and cyanogen bromide cleavage produces four peptide fragments with the five glycosylation sites (1-3). Studies by Kehry et al. and from our laboratory provided indirect evidence that the site at asparagine 563 which is closest to the C-terminus may be incompletely glycosylated (1,3). Both of these studies compared the ratios of tritiated mannose labeling of the peptide containing Asn 563 with the other glycosylation sites. However, the lack of information about the structures of the carbohydrate side chains precludes accurate estimations of the degree of glycosylation.

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Methods: Secreted mouse immunoglobulin (IgM) was collected from F1 mice (DBA x BALB/c) previously injected intraperitoneally with MOPC 104E cells. Approximately 55 ml. of ascites fluid was used. Labeled IgM from the incubation of MOPC 104E cells with tritiated mannose was added as a marker. The pentameric IgM was purified by an initial gel filtration step (Ultragel ACA 22) according to the method of Kehry (2). The protein peak was isolated and precipitated with 50 percent saturated ammonium sulfate. Precipitated protein was collected by centrifugation, dialyzed against distilled water and lyophilyzed. The dried material was resuspended to a 10 mg/ml concentration in 5 M. urea, 0.1 M. Tris-HCl, 0.15 M. NaCl, 0.01 Percent NaN3 (pH 8.0) and incubated at room temperature for 24 h. Trypsin was added under conditions resulting in a single heavy chain cleavage point (4). Digestion was terminated with soybean trypsin inhibitor followed by separation of Fc and Fab fragments by gel filtration over a Biogel A-5M column (2.5 x 100 cm).

Purified Fc was reduced in 10 mM dithiothreitol followed by alklyation in 20 mM iodoacetamide (1). The protein was dialyzed against 0.1 percent trifluoroacetic acid and lyophilized. Cyanogen bromide in 70 percent formic acid was added to obtain cleavage between adjacent glycoslyation sites (5). The cyanogen bromide glycopeptides were fractionated over a gel filtration column of ACA 54 in 3M guanidine HCl, 0.2 N NH4HCO3.

The cyanogen bromide glycopeptide CN8, containing the C-terminal glycoslyation site at Asn 563 was isolated as two fractions. These fractions were divided into several equal aliquots from which amino acid and carbohydrate compositions were determined. Amino acid analysis was performed on samples hydrolyzed in 6N HCl (5) on a Dionex D-300 analyzer equipped with a Na+ microbore column . Carbohydrate analysis was performed on a Hewlett Packard model 5700 A gas chromatograph using glycopeptides hydrolyzed in 2N HCl (1).

Results: Fractionation of isolated heavy chain by cyanogen bromide followed by gel filtration allows separation of four carbohydrate containing peptides (1-3). The smallest carbohydrate containing peptide produced is derived from the C-terminus, and is contaminated by peptides from the non-glycosylated N-terminal part of the heavy chain (2). In order to obtain pure peptide from the C-terminus we used trypsin to produce Fab and Fc fragments. Purified Fc can then be cleaved by cyanogen bromide and fractionated by gel chromatography. Figure 1 is a gel filtration profile of ACA 54 which demonstrates the advantage of the Fc separation technique. The peak labeled CN8 is from the C-terminus of the heavy chain (1-3). The radioactivity incorporated into CN7

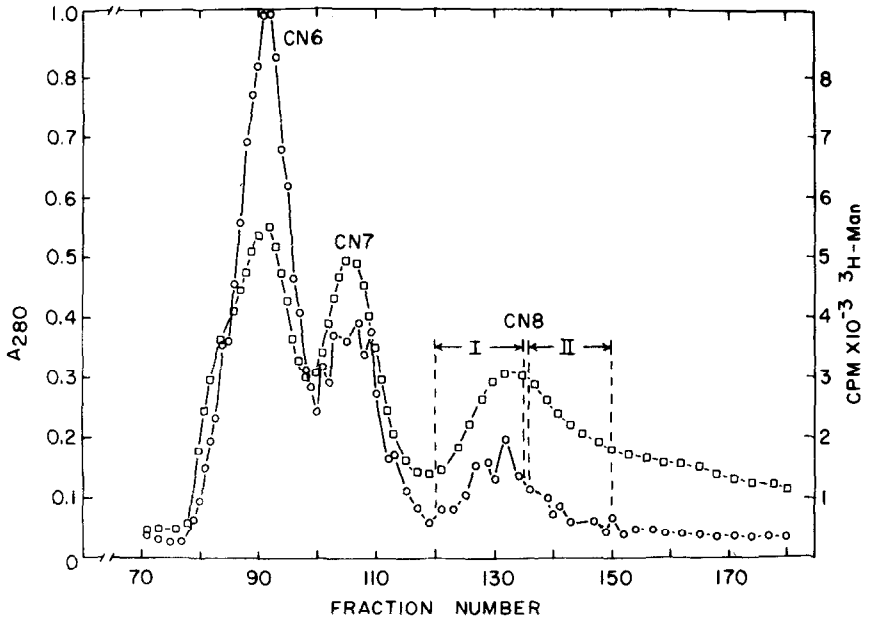

Figure 1 - ACA 54 gel filtration of mouse IgM heavy chain Fc fragment cyanogen bromide cleavage mixture. Purified Fc from MOPC 104E was treated with cyanogen bromide and fractionated in 3M guanidine HCl, 0.2 M NH_4HCO_3 buffer (pH 8.3). Fractions were detected by absorbance at 280 nm (open squares) and by liquid scintillation counting of [3H]-Mannose (open circles).

and CN8 demonstrates how labeling predicts reduced glycosylation for CN8. The incorporation should be identical if the carbohydrate structures are the same. A rather broad peak is observed for CN8 when measured by absorbance. In addition, the majority of the radioactive carbohydrate is associated with the first half of the absorbance peak (fraction I). The second half (fraction II) contains little radioactivity, but includes more than half of the protein by the Lowry assay (Table II).

The isolated CN8 fragment was identified by comparing amino acid compositions of both CN8I and CN8II with that expected from sequence data (Table I). The compositions for CN8I and CN8II are essentially identical, and are very similar to compositions expected from the protein sequence.

Table I

Amino Acid and Carbohydrate Compositions of Isolated Cyanogen Bromide Glycopeptide CN8 Fractions I and II

	[a] CN8		[b] CN8I		[b] CN8II	
Amino Acid	Residues per Mole	Mole Percent	[c] nmol Present	Mole Percent	[c] nmol Present	Mole Percent
Asx	3	5.1	82.6	7.4	153.9	8.0
Thr	9	15.3	122.7	10.9	191.3	10.0
Ser	4	6.8	73.5	6.5	140.6	7.3
Glu	8	13.5	126.1	11.2	208.6	10.8
Pro	5	8.5	81.4	7.3	154.7	8.0
Gly	5	8.5	106.7	9.5	154.3	8.0
Ala	2	3.4	66.5	5.9	125.7	6.5
Val	6	10.2	111.2	9.9	198.9	10.3
Met	0	0.0	4.8	0.4	3.4	0.2
Ile	2	3.4	47.8	4.3	72.6	3.8
Leu	5	8.5	99.8	8.9	179.9	9.3
Tyr	3	5.1	46.6	4.2	76.3	4.0
Phe	2	3.4	41.7	3.7	79.3	4.1
His	2	3.4	33.1	2.9	53.3	2.8
Lys	2	3.4	49.0	4.4	85.0	4.4
Arg	1	1.7	30.0	2.7	57.0	2.9

a Expected values obtained from sequence data (1).
b Determined from amino acid and carbohydrate analyses.
c Based on addition of internal standard (norleucine).

Identical aliquots were hydrolyzed for the determination of carbohydrate and are presented in table I. The amounts of carbohydrate found in CN8I are much higher than CN8II even though fraction II contains more protein. The molar yields of polypeptide calculated from amino acid analyses are remarkably close to that determined by colorimetric assay (6), both fractions varying less than 10 percent.

A comparison of glucosamine ratios to N-linked asparagine was made based on previous determination that 86 percent of CN8 contains a Man6:GlcNac2 "high mannose" structure and remaining material being of "triantennary" type (1). The amount of glycosylation for CN8I based on these criteria is greater than 100 percent (Table II) while that of CN8II is less than 6 percent. The estimated total amount of

Table II

Comparison of Protein and Carbohydrate Contents of Fractions I and II Isolated from Mouse Heavy Chain Cyanogen Bromide Glycopeptide CN8.

Glycopeptide	Total Protein (nmol) [a]	Percent of Total CN8 (I+II)	Total N-linked Asn (nmol) [b]	Total N-linked Glcnac (nmol) [c]	Percent CHO-linked	Percent Total CN8
CN8I	32	36.8	27.6	32.0	116.1	43.78
CN8II	55	63.2	51.3	2.8	5.5	

a Determined by Lowry protein assay (6) and sequence data (2).
b Determined by amino acid composition and sequence data.
c Determined by carbohydrate composition, lectin affinity data (1) and NMR structural determination of major component.

glycosylation of the entire peak consisting of both fractions I and II is less than 44 percent.

Discussion: Cyanogen bromide cleavage of the Fc portion of the heavy chain of IgM allows complete purification of the C-terminus glycopeptide. This peak can be divided into two fractions with identical amino acid compositions but differing in the extent of glycosylation. Fraction I contains 37% of the protein and 91% of the carbohydrate of the C-terminal peptide. Calculations using the protein concentration determined by the Lowry procedure or using the Asn_{11} levels from the amino acid analysis predict 100% and 116% glycosylation respectively. The latter value may be too high because of less than quantitative recoveries from the amino acid analysis. It is very likely that this fraction is nearly completely glycosylated. Fraction II contains the majority of the protein but only 9% of the carbohydrate of CN8. We calculate that only 5-6% of the Asn residues are glycosylated. Over all the C-terminal glycosylation site at Asn 563 is glycosylated 44% of the time.

The incomplete glycosylation of Asn 563 contrasts with the other four sites at Asn 171, 332, 364, and 403 which appear to be fully glycosylated. The reduced glycosylation may be related to the fact that Asn 563 is only 13 amino acids from the COOH terminus. Previous estimates have suggested that a minimum of 30 amino acids must be added beyond an acceptor site for glycosylation to occur (7). We do not know of any consequences for the glycosylation differences. IgD has also been shown to be incompletely glycosylated (8). It would be interesting to know if IgM pentamers contain a random assortment of glycosylated and non-glycosylated C-terminal heavy chains. We would also like to know whether the presence or absence of carbohydrate effects carbohydrate processing at other glycosylation sites. It may be that the glycosylation variability has functional significance or merely provides more information about the complicated events of protein glycosylation.

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