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Glycopeptides from Human κ-Chains

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Glycopeptides have been isolated from tryptic digests of κ-type light chains separated from human myeloma proteins obtained from the serum of two patients, Car and Rai. The glycopeptides are derived from the variable region of the chain in both cases, but from different sections. On the basis of homology it is deduced that glycopeptide from Car, κI type, is derived from position 25–31 whereas that from Rai, κII type, is from position 62–77, their sequences being respectively Ala-Ser-Gln-Asn-Ile-Ser and Phe-Ser-Gly-Ser-Gly-Ser-Gly(Thr,Asp)Phe-Thr-Leu-Asx-Ile-Ser-Arg. The significance of the results is discussed in connexion with the nature of the attachment site of carbohydrate to protein.

Immunoglobulins G are formed by two heavy chains and two light chains covalently linked by interchain disulphide bonds (Porter, 1962). Their carbohydrate content is close to 3% in several species. The major part of this is attached to a site in the *C*-terminal half of the heavy chain in rabbit (Nolan & Smith, 1962; Fleishman, Porter & Press, 1963) and man (Chaplin, Cohen & Press, 1965). The function of the carbohydrate is unknown, but since it was found attached to the Fc fragment it was considered to play no part in antigen–antibody interaction. It might be associated with some of the other biological properties of the molecule such as antigenic specificity, skin sensitization or passage through membranes. More recently attachment of the carbohydrate to other parts of the molecule has been reported in heavy chains and light chains (Abel, Spiegelberg & Grey, 1968). In some cases such carbohydrate has been shown to be covalently attached and glycopeptides have been isolated from one mouse κ-chain (Melchers, Lennox & Facon, 1966), from two human λ-type Bence-Jones proteins (Edmunson *et al.* 1968) and from the *N*-terminal section of a heavy-chain-disease protein (Frangione & Milstein, 1969). In all the instances cited the glycopeptides were isolated either from urinary Bence-Jones proteins or from abnormal heavy chains. We report here the attachment of carbohydrate in two human κ light chains that were isolated from intact immunoglobulin G myeloma proteins present in the serum of patients Car and Rai.

MATERIALS AND METHODS

Proteins. The myeloma proteins were kindly supplied by Dr C. Ropartz (Rai) and Dr B. Frangione (Car).

Carboxymethylated chains were prepared by the procedure of Fleishman, Pain & Porter (1962) by using iodoacetate as the blocking agent and fractionating on a Sephadex G-100 column in 5% formic acid. The carbohydrate content of the isolated light chains (Table 3) was kindly determined by Dr J. R. Clamp of the University of Bristol by the method of Clamp, Dawson & Hough (1967). In this method the glycopeptide, together with internal standards, was heated in 1.5M-HCl in methanol at 90°C for 24h and after re-*N*-acetylation was subjected to g.l.c.

Total reduction and alkylation with iodo[^{14}C]*acetate.* This was carried out on the separated light chains as described by C. Milstein (1968). The solution was dialysed against large volumes of 1% (w/v) NH_4HCO_3 and then digested with trypsin. The tryptic digest was fractionated as described in the Results section and a large number of peptides was purified. The purification procedure of the peptides described in this paper is detailed in Table 1.

Tryptic digestion. This was done in 1% (w/v) NH_4HCO_3, pH 8.0 (enzyme/substrate ratio 1:100), at 37°C for 4h and the approximate final concentration of protein was 9mg/ml.

Chymotryptic digestion, This was carried out in 1% (w/v) NH_4HCO_3, pH 8.0 (enzyme/substrate ratio 1:150), at 37°C for 2h, the approximate final concentration of protein being 11mg/ml.

Pronase digestion. A 100nmol portion of glycopeptide was dissolved in 0.2ml of 0.05M-NH_4HCO_3, pH 7.9. Then 100μg of pronase was added to start the digestion and 100μg of fresh enzyme was added every day. Digestion was continued for 3 days at 37°C. After 41h of digestion, when the last addition of pronase was made, 20μg of leucine aminopeptidase and 20μg of carboxypeptidase A were added. Trypsin (batch TR3F 6401) and chymotrypsin (not numbered) were crystallized salt-free preparations from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Carboxypeptidase A (batch CoADFP 9KA) was a suspension from Worthington Biochemical Corp. The enzyme was washed according to the procedure described by Ambler (1967). Pronase

Table 1. *Some properties of glycopeptides and peptides related to them from proteins Car and Rai*

In purification procedure the numbers refer to the pH at which high-voltage paper electrophoresis was done and BAWP refers to descending paper chromatography in butan-1-ol–acetic acid–water–pyridine (15:3:12:10, by vol.). Mobilities were calculated as described by C. P. Milstein (1968). Glucosamine values were not corrected for acid-hydrolysis losses. N.D., Not determined.

	Protein Car				Protein Rai				
Peptide	TCN 2	TCA1a	TCA2a	TCA1aPa	TA2a	TA12a	TA2aCA2	TA2aCA3	TA2aCN
Purification procedure ...	6.5 and 3.5	6.5, BAWP and 2.1	6.5, BAWP and 2.1	6.5	6.5 and BAWP	6.5 and BAWP	6.5	6.5	6.5 and 3.5
Mobility 6.5 ...	0	0.15	0.27	0.20	0.22	0.10	0.19	0.35	0
Mobility 3.5 ...	0.16	—	—	—	—	—	—	—	0.12
N-Terminus ...	Ala	Ala	Ala	N.D.	N.D.	N.D.	Thr	Phe	Thr
	Amino acid composition (mol of amino acid/mol of peptide)								
Arg	—	—	—	—	1.1	0.9	1.0	—	0.9
Asp	0.9	0.9	0.9	1.0	1.9	1.7	1.1	1.1	1.1
Thr	—	—	—	—	2.0	1.9	1.0	1.0	0.9
Ser	2.2	2.3	2.2	—	4.1	3.8	1.1	2.7	1.2
Glu	1.1	1.0	1.0	1.1	—	—	—	—	—
Gly	0.5	0.1	0.2	—	2.9	2.9	—	3.0	—
Ala	0.8	0.9	0.9	—	—	—	—	—	—
Val	—	—	—	—	—	—	—	—	0.2
Ile	0.8	1.0	0.9	—	1.1	1.1	1.0	—	0.9
Leu	—	—	0.1	—	1.1	1.0	1.0	—	1.0
Phe	—	—	—	—	1.8	1.7	—	1.9	—
Glucosamine	—	1.6	1.1	1.9	+	+	1.0	—	0.4

was from Calbiochem, Los Angeles, Calif., U.S.A. Leucine aminopeptidase (batch no. L-9876) was from Sigma Chemical Co., St Louis, Mo., U.S.A.

High-voltage paper electrophoresis and paper chromatography. These were carried out as described by Milstein & Feinstein (1968).

Identification and location of peptides on paper. The radioactive peptides were located by radioautography. The non-radioactive peptides were located by dipping a strip of paper in ninhydrin–cadmium reagent (Heilmann, Barrollier & Watzke, 1957). The strip of paper containing the peptide was cut out and sewn to a new sheet of paper, which was subjected either to descending paper chromatography or to paper electrophoresis as detailed in Table 1. Peptides were eluted from the paper strip with 1% acetic acid.

Total acid hydrolysis. Peptide samples (15–30nmol) were hydrolysed in 0.2ml of constant-boiling HCl in evacuated sealed tubes at 105°C for 19h.

Amino acid composition. Quantitative amino acid analyses were carried out in an automatic Locarte amino acid analyser (Spackman, Stein & Moore, 1958). Glucosamine values included in Table 1 were obtained by integrating the area of the peaks obtained in the automatic amino acid analyser and correlating them to standards of known concentration.

Edman degradations and N-terminal analyses. The procedure described by Gray (1967) was used but the DNS-amino acids were identified as described by Milstein & Feinstein (1968).

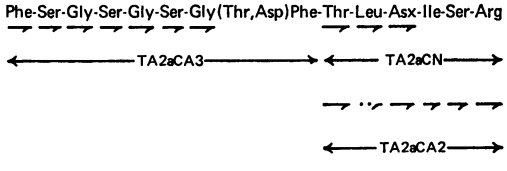

Fig. 1. Sequence of peptide Rai TA2a. Sequence determination from the *N*-terminal end was by the dansyl–Edman technique (→); .. ⌐ not determined.

RESULTS

The radioactive labelled light chain of protein Car after tryptic digestion and filtration through Sephadex G-50 gave four radioactive fractions and one non-radioactive one. The radioactive fraction eluted first from the column was a mixture of large peptides. It was digested with chymotrypsin and dried in a desiccator two or three times until free of salt. The digest was subjected to high-voltage paper electrophoresis at pH 6.5 and the derived peptides were purified and analysed. Some of these peptides contained glucosamine, which was revealed in the amino acid analysis. The procedure used to purify these peptides is detailed in Table 1. The presence of other sugars in the peptides was not investigated. Among the acidic peptides two were found that had the same amino acid composition except for the content of glucosamine, which was lower in the more acid one, peptide TCA2a (Table 1). Among the neutral peptides there was one that had the same amino acid composition but did not contain glucosamine, peptide TCN2. The sequence of peptide TCN2 determined by the dansyl–Edman technique was Ala-Ser-Gln-Asn-Ile-Ser. The mobility of the peptide at pH 6.5 (Table 1) indicates that the aspartic acid and glutamic acid which are formed on acid hydrolysis were originally present as asparagine and glutamine residues respectively. Peptide TCA1a was digested with pronase as described in the Materials and Methods section and the product thus obtained subjected to paper electrophoresis at pH 6.5. The composition of peptide TCA1aPa (see Table 1), isolated from this run, proves that the carbohydrate is bound either to the glutamine or to the asparagine residue. When the dansyl-Edman technique was applied to peptide TCA1a the following sequence was obtained: Ala-Ser-Glx-?-Ile-Ser. Residue 4 was not detected by

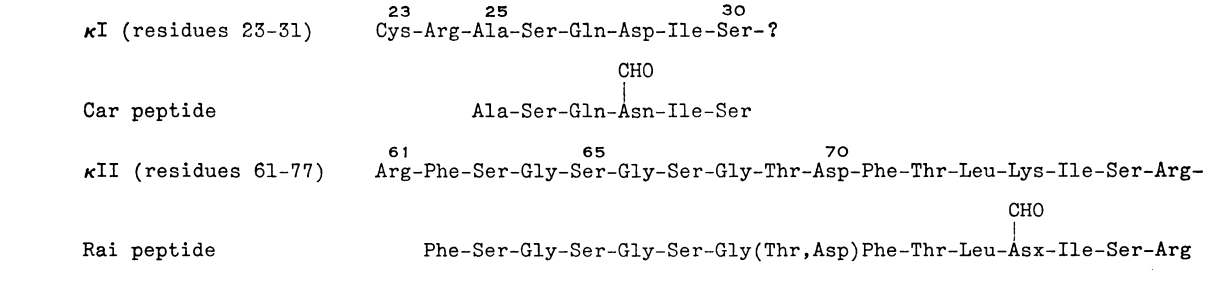

Fig. 2. Comparison of the isolated peptides of κ chains from proteins Car and Rai with the most homologous stretches of their corresponding basic sequences. Car is a κI protein (Milstein *et al.* 1969*a*) and Rai a κII (Milstein *et al.* 1969*b*). The basic sequences are taken from Milstein (1969). Question mark (?) indicates positions that cannot be represented by a single residue. CHO indicates the suggested position of carbohydrate (see the text).

the dansyl method. An attempt to release specifically the carbohydrate from the residual peptide, after three Edman degradations, was made by following the procedure of Tsugita & Akabori (1959) based on partial hydrolysis with 2M-hydrochloric acid for 90min at 100°C. The product was dansylated and total hydrolysis released DNS-isoleucine and DNS-serine but not DNS-aspartic acid. Attempts to identify the point of attachment of the carbohydrate by subtractive Edman degradation of peptide TCA1a were unsuccessful.

Light chain of protein Rai was labelled with iodo[^{14}C]acetate, digested with trypsin and subjected to high-voltage paper electrophoresis at pH6.5. Among the acid peptides were two that contained glucosamine and had the same amino acid composition, peptides TA2a and TA12a (Table 1). Both peptides were pooled and digested with chymotrypsin for 7h as described in the Materials and Methods section, and the dried salt-free digest was subjected to high-voltage paper electrophoresis at pH6.5. Two acidic and one neutral peptides were obtained, peptides TA2aCA2, TA2aCA3 and TA2aCN respectively (Table 1). The sequence of peptide TA2aCA3 was determined as shown in Fig. 1. Phenylalanine is placed as *C*-terminal only on the basis of chymotrypsin specificity. Peptides TA2aCN and TA2aCA2 had the same amino acid composition except for the glucosamine, which was present in a smaller amount in the neutral peptide (Table 1). The difference in the glucosamine content of the two peptides could be interpreted as being one residue since the glucosamine values in Table 1 are not corrected for hydrolysis losses and it is well known that hexosamines are less stable to acid hydrolysis than are the great majority of amino acids. Constant-boiling hydrochloric acid has been shown to destroy 47% of glucosamine in 20h at 110°C (Nolan & Smith, 1962); we have used constant-boiling hydrochloric acid for 19h at 105°C. The sequences of peptides TA2aCN and TA2aCA2 are shown in Fig. 1.

DISCUSSION

Protein Car is a κI (Milstein, Milstein & Feinstein, 1969*a*) whereas protein Rai is a κII (Milstein, Milstein & Jarvis, 1969*b*) light chain. On the grounds of homology the glycopeptide of protein Car seems to be derived from residues 25–31 whereas the glycopeptide of protein Rai seems derived from residues 72–77 (Fig. 2). It seems most likely that the carbohydrate in protein Car is attached to the asparagine residue at position 28, which is exactly the same position at which carbohydrate is attached in the mouse κ light chains (Melchers, 1969), whereas in protein Rai it is attached to peptide TA2aCA2, which includes the aspartic acid or asparagine residue at 74 (Fig. 2), a residue that represents a variant from the one normally found at the same position. However, peptide TA2aCA2 also contains threonine and serine and the possibility that the carbohydrate is attached to one of these residues cannot be excluded. No difficulty was found in identifying the aspartic acid or asparagine residue of peptide TA2aCA2 of protein Rai but we were unable to identify residue 4 in peptide TCA1a of protein Car. In both cases the aspartic acid or asparagine residue is followed by the same amino acids, isoleucylserine, and the only difference is the preceding amino acid, which is glutamine in protein Car and leucine in protein Rai. Cyclization of glutamine to give a blocked *N*-terminal is not excluded. A comparison of the amino acid sequence of these two glycopeptides with glycopeptides from other immunoglobulins is shown in Table 2.

We have made no attempt to establish the nature of the chemical bond between the carbohydrate group and the peptide chain. There are many cases known where *N*-acetylglucosamine is joined by its C-1 to the amide nitrogen of asparagine (Neuberger, Gottschalk & Marshall, 1966), and the present case may be similar. The occurrence of the sequence Asn-X-$^{\text{Ser}}_{\text{Thr}}$ in the polypeptide chain has been suggested to be a necessary but not sufficient condition for glycosylation to occur (Eylar, 1965; Neuberger & Marshall, 1968; Hunt & Dayhoff, 1970). Both the present examples may support this hypothesis. In each, the carbohydrate moiety appears on the variable part of the light chain when the variant sequence Asx-X-Ser is formed. The carbohydrate composition is similar in both proteins (J. R. Clamp, unpublished work; Table 3) even though the moieties occur in different segments.

Table 2. *Glycopeptides isolated from different Bence-Jones, myeloma and heavy-chain-disease proteins*

Protein	Sequence	Reference
Mouse κ chain (MOPC 46)	Ala-Ser-Glu-Asx-Ile-Ser-Asn	Melchers (1969)
Human λ Hul	Gln-Gln-Glu-Asp-Glu(Ala,Thr)Tyr	Edmunson *et al.* (1968)
Human λ Nev	Ser-Glu-Asp-Glu-Ala-Asp-Tyr	Edmunson *et al.* (1968)
Human κ Car	Ala-Ser-Gln-Asn-Ile-Ser	This paper
Human κ Rai	Phe-Thr-Leu-Asx-Ile-Ser-Arg	This paper
Human Fc	Glx-Glx-Phe-Asp-Ser-Thr-Tyr	Howell, Hood & Sanders (1967)
Zuc	Pro-Gly-Gly-Ser-Ser-Glu-Pro-Lys	Frangione & Milstein (1969)

Table 3. *Carbohydrate composition of some immunoglobulin G light chains*

The compositions were kindly determined by Dr J. R. Clamp with the use of g.l.c. (Clamp *et al.* 1967).

	Carbohydrate composition (molar proportions)					
	Fucose	Mannose	Galactose	Glucosamine	Galactosamine	Sialic acid
Human Car κ chain	0.6	2.3	1.3	2.6	0	0.8
Human Rai κ chain	0.4	2.4	1.5	2.2	0	0.7

What role the carbohydrate plays in the immunoglobulins is unknown, but its presence in light chains is more common than previously thought. The fact that it is observed in the variable part raises the question of whether it plays any part in the antibody-combining site. If the carbohydrate is present in the vicinity of the combining site its effect on combining specificity seems unavoidable. In fact residue 28 is very near (through the disulphide bridge) to the stretch of higher variability of the chain believed to be involved in the active site (Milstein & Pink, 1970). The fact that the involved peptides are found in different forms (presumably differing in sialic acid content) and even lacking carbohydrate could add further variability to the protein surface. This type of heterogeneity had been noticed previously (Frangione & Milstein, 1969) and it is not known if the differences in carbohydrate content result from incomplete synthesis of the carbohydrate side chain, or incomplete carbohydrate removal after secretion, or a combination of both.

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