Completion of the Analysis of the Primary Structure of the Variable Domain of a Homogeneous Rabbit Antibody to Type III Pneumococcal Polysaccharide

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The amino acid sequence between residues 70 and 116 of the V (variable) region of the H (heavy) chain derived from rabbit antibody BS-5, specific for type III pneumococcal polysaccharide, was determined. The sequence of this section of the H chain which includes the hypervariable residues 94 to about 112 was unique, although minor variant sequences present in the H chain preparation would not have been detected by the techniques used in this work. Taken together with the known sequences of the *N*-terminal 69 residues of H chain BS-5 (Jaton & Braun, 1972) and of the V region of the light chain (Jaton, 1974*b*), the data establish the complete sequence of the V domain of a rabbit immunoglobulin G. The V region of H chain BS-5 is compared with the basic sequences of the three human V region subgroups known to date, with one mouse H chain, and with guinea-pig pooled H chains. Even though chains from guinea pig and mouse clearly belong to the subgroup III of variability (V_{HIII}), rabbit H chain BS-5 (allotypic variant a_1) appears more closely related to the subgroup V_{HII} than to the subgroups V_{HIII} or V_{HI} . The homology between V_L and V_H regions of antibody BS-5 (28%) is not greater than that observed between the V_H region of antibody BS-5 and the V_L regions of different rabbit antibodies.

The molecular basis of antibody specificity has been shown to reside in the primary structure of both heavy (H)* and light (L) chains of the antibody molecule (Haber, 1964; Whitney & Tanford, 1965; Freedman & Sela, 1966; Jaton *et al.*, 1968). H and L chains consist of a variable *N*-terminal part (V region) of 105–120 residues and a constant *C*-terminal portion (C region) which comprises the remainder of the chain (for review, see Milstein & Pink, 1970).

Comparative studies of a large number of amino acid sequences of myeloma proteins have revealed the presence of distinctive hypervariable zones within the V_L and V_H regions (Kabat & Wu, 1971). It is generally accepted that these hypervariable zones are likely to define the shape of the antibody-combining site (for review, see Givol, 1973).

Direct evidence for the localization of the site in the V regions comes from the demonstration that fragment Fv (V_H+V_L regions) of the mouse myeloma protein MOPC 315 retains full antigen-binding activity (Inbar *et al.*, 1972).

* Abbreviations: IgG, immunoglobulin G; H and L chains, heavy and light chains of IgG; V and C regions, variable and constant regions of IgG respectively; CmCys and $\square\text{Glu}$ (in amino acid sequences and Tables), *S*-carboxymethylcysteine and pyrrolidonecarboxylic acid respectively; V_{HI} , V_{HII} and V_{HIII} , subgroups I, II and III respectively of V regions of H chains.

Homogeneous rabbit antibodies to bacterial polysaccharide antigens (Krause, 1970; Haber, 1971; Braun & Jaton, 1974) are appropriate probes to tackle the structural analysis of the site (Fleischman, 1971; Jaton & Braun, 1972; Jaton & Haimovich, 1974; Jaton, 1974*a,b*; Chen *et al.*, 1974). The knowledge of the amino acid sequence of the V domain (V_H+V_L regions) will facilitate the investigation of the three-dimensional structure of the combining site and is a prerequisite for detailed studies on the interaction of the antigenic determinant with amino acid residues in the site.

This report describes the determination of the amino acid sequence of residues 70–116 of the H chain derived from the homogeneous rabbit antibody BS-5, specific for type III pneumococcal polysaccharide. These data taken together with the known sequences of the *N*-terminal 69 residues of H chain BS-5 (Jaton & Braun, 1972) and of the V region of the corresponding L chain (Jaton, 1974*b*) establish the complete sequence of the V domain, which includes the antigen-combining site of a naturally elicited antibody molecule.

Experimental

Materials

Enzymes, chemicals and reagents used for the characterization of the peptides and for the sequence

determination were described previously (Jaton, 1974a) except *NN*-dimethylbenzylamine and propan-1-ol, sequanal grade, which were obtained from Pierce Chemical Co., Rockford, Ill., U.S.A.

Methods

Antibody production and purification. Rabbit BS-5 was immunized with type III pneumococcal vaccine and the antibody was isolated as described by Jaton & Braun (1972).

The preparation of the *N*-terminal half of H chain BS-5 (fragment C1), the isolation of the *N*-terminal peptide from fragment C1, and the citraconylation of CNBr fragments were done as described by Jaton & Braun (1972) and Jaton (1974a).

Cyclization of glutamine into pyrrolidonecarboxylic acid. This was done by the method of Melville (1935) by dissolving the peptide that had glutamine as *N*-terminal residue in 7M-guanidine hydrochloride, pH 2.5, and incubating it at 37°C for 40h. Excess of salts was removed by dialysis against water and the peptide freeze-dried.

Analytical methods. Preparative high-voltage paper electrophoresis, detection of peptides, enzymic digestion, amino acid analyses and the determination of sequences of small peptides by the 'dansyl-Edman' procedure (Gray, 1967) were done as described by Jaton (1974a).

Amino acid sequence analysis of large peptides was performed in the Beckman sequencer model 890B equipped with an undercut cup and N₂ flush. The Quadrol buffer was replaced by the semi-volatile *NN*-dimethylbenzylamine buffer (Hermanson *et al.*, 1972). This buffer was prepared by mixing 6ml of dimethylbenzylamine with 27ml of water and 25ml of propan-1-ol. The pH was adjusted to 9.5 by addition of a few drops of acetic acid. The volatile buffer programme was based on that supplied by Beckman, Spinco Division ('Fast Peptide-DMAA no. 090872') and contains the following alterations: the delivery time of phenyl isothiocyanate was extended for 1s in order to ensure that a large excess of coupling reagent covered completely the peptide film. The heptane (solvent for phenyl isothiocyanate) was dried down completely before delivering the volatile buffer which was calibrated to rise up to the undercut (halfway-up). *NN*-Dimethylbenzylamine was delivered once as no substantial evaporation of the buffer was apparent during the coupling reaction time (20min) at the operating temperature of 55°C. The line carrying the buffer (reagent bottle R₄) was purged with benzene contained in reagent bottle R₅. After thorough washing of the reaction mixture with benzene in bottle S₁ (250s), extensive drying steps with rough and fine vacuum pumps, coupled with N₂ flushing, were performed. Only one cleavage reaction with heptafluorobutyric acid was employed;

after completion of the reaction, the drying steps were carried out while the cup was spinning at low speed in order to keep the protein or peptide film at the bottom of the cup. This precaution avoids peptide material being carried above the undercut level during extraction of the thiazolinone derivative by the butylchloride.

The determination of the sequence of lysine-containing peptides by using 4-sulphophenyl isothiocyanate to minimize losses of peptides (Braunitzer *et al.*, 1971) and the identification of phenylthiohydantoin derivatives were carried out as previously described (Jaton, 1974a). In some instances, phenylthiohydantoin derivatives were also assayed by t.l.c. particularly for the identification of lysine, glutamic acid/glutamine and aspartic acid/asparagine residues (Edman, 1970).

Results

Cleavage of H chain BS-5 with CNBr

The partial amino acid sequence of H chain BS-5 has been reported for residues 1–69 (Jaton & Braun, 1972). The objective of the present work is to establish the rest of the sequence of the V region up to the beginning of the C region (about residue 116). It was previously shown that CNBr fragment C1 of H chain BS-5 (*N*-terminal half of the H chain) contained one internal homoserine residue at position 33, in addition to the *C*-terminal homoserine residue at position 253 (Fruchter *et al.*, 1970). Fragment C1 was then split by CNBr into two fragments, which were held together by a disulphide bond. The smaller *N*-terminal fragment (residues 1–33) was separated from the larger *C*-terminal peptide (residues 34–253) after full reduction, alkylation with iodo[2-¹⁴C]-acetate and chromatography on a column (2cm × 200cm) of Sephadex G-75 developed in 5M-guanidine hydrochloride. Three fractions A, B and C were recovered successively. The large CNBr fragment (residues 34–253) was identified in fraction A, the *N*-terminal 33-residue peptide in B and excess of salts in C respectively. The *N*-terminal 33-residue peptide was obtained in higher yield (70%) than when the separation was carried out in 0.02M-NH₃ as previously reported (Jaton & Braun, 1972).

Characterization of large arginine-containing peptides from fragment C1 of H chain BS-5

On the basis of the amino acid composition and partial *N*-terminal sequence determination, fragment C1 contains 6 arginine residues at positions 9, 37, 65, 93, 170 and 176 (Jaton & Braun, 1972; Fruchter *et al.*, 1970).

Fragment A (residues 34–253; 2.2 μmol) was citraconylated and digested with trypsin; small peptides were separated from the larger arginine-containing peptides of the digest by gel filtration on Sephadex

Tryptic peptides		Residue position in the sequence
T-A ₄	Thr-Phe-Pro-Ser-Val-Arg	170-176
T-A ₅	Gly-Trp-Val-Arg	33-37
T-A _{2a}	Ala-Pro-Ser-Val-Phe-Pro-Leu-Ala(Pro ₂ , CmCys ₃ , Gly ₂ , Asp ₁ , Thr ₃ , Ser ₂ , Leu ₂ , Val ₁) Val-Lys	124-149
T-A _{2b}	Gly-Tyr-Leu-Pro-Glu-Pro-Val(Thr ₄ , Val ₁ , Asp ₂ , Ser ₁ , Gly ₂ , Leu ₁ , Trp ₁) Val-Arg	150-170
T-A _{3T₁}	Thr-Ser-Leu-Pro-Thr-Glu-Asp-Thr-Ala-Thr-Tyr-Phe-CmCys-Ala-Arg	79-93
T-A _{3T₂}	Phe-Thr-Ile-Ser-Lys	66-70
T-A _{3T₃}	Gly-Arg	64-65
T-A _{3T_{N2}}	Thr-Ser-Thr-Thr-Val-Asp-Leu-Lys	71-78
T-A _{3T_{N3}}	Glu(Ala ₁ , Pro ₁ , Gly ₁)-Lys	38-42
Chymotryptic peptide		
T-A _{3Ch₁}	Phe-CmCys-Ala-Arg	90-93

Fig. 1. Sequence determination of some peptides from fragment C1 of H chain BS-5

The amino acid sequence was established by the 'dansyl-Edman' procedure (—) and by digestion with carboxypeptidases A and B (—). —, Dansyl residue identified without hydrolysis. The amino acid compositions and mobilities of the peptides are given in Table 1. Amide residues were assigned on the basis of electrophoretic mobility of peptides at pH 6.5 (Offord, 1966).

Fig. 2. Isolation of the large arginine-peptides from a tryptic digest of citraconylated, reduced and $[2-^{14}\text{C}]$ alkylated fragment A of H chain BS-5

The column (2 cm $\times$ 200 cm) of Sephadex G-50 was developed in 5 M-guanidine hydrochloride, pH 5.5. The material under the hatched area in each peak was used for further characterization. —, E_{280} ; - - - -, radioactivity (c.p.m./20 μl of eluate). See the text for details.

G-25 (fine grade) in 0.2 M- NH_3 . They were purified by high-voltage paper electrophoresis at pH 6.5. Two peptides were obtained and their sequence was determined by the 'dansyl-Edman' procedure (Fig. 1). One of the two, designated T-A₄, had the sequence Thr-Phe-Pro-Ser-Val-Arg and the other, T-A₅, the sequence Gly-Trp-Val-Arg. The basic peptides T-A₅ and T-A₄ occupy positions 34–37 (Jaton & Braun, 1972) and 170–176 (Fruchter *et al.*, 1970), respectively in fragment C1.

The larger peptides in the breakthrough fraction of the Sephadex G-25 run were further fractionated by gel filtration on Sephadex G-50 in the presence of 5 M-guanidine hydrochloride. Three fractions CT-A₁, CT-A₂ and CT-A₃ were obtained (Fig. 2); as fractions CT-A₁ and CT-A₂ were cross-contaminated, the material under the hatched area in Fig. 2 was pooled and further purified in the citraconylated form, on DEAE-Sephadex A25 by using a gradient of ionic strength in NH_4HCO_3 buffer, as suggested by Cebra *et al.* (1968). The amino acid composition of the purified peptides is given in Table 1. Peptide CT-A₁ contains about 68 amino acids including one homoserine residue, but no arginine and no tryptophan residues. Homoserine was determined by digestion with carboxypeptidase A to be the C-terminal residue, as expected, whereas no clear-cut N-terminal residue could be identified by the dansyl procedure, except for traces of glutamic acid/glutamine. Its analysis agrees well with that reported for the C-terminal peptide T₂ of fragment C1 (Cebra *et al.*, 1968); it is therefore the C-terminal peptide of fragment C1.

Fraction CT-A₂ contains a peptide of about 75 amino acids including tryptophan residues and one histidine residue (Table 1). Arginine was released from the C-terminal end by carboxypeptidase B; the N-terminal residue was found to be mainly glutamic acid/glutamine by the dansyl technique.

On the basis of the recovery of 650 nmol of fraction CT-A₁ and 510 nmol of fraction CT-A₂, an average yield of 30% and 23% respectively was calculated.

One cycle of Edman degradation indicated that fraction CT-A₃ contains a mixture of at least 2 peptides as demonstrated by the identification of 2 major N-terminal residues phenylalanine and glutamine and a minor N-terminal leucine residue. Arginine and a trace of homoserine were released by digestion with carboxypeptidases A+B.

Amino acid sequence of peptide CT-A₂

Citraconyl peptide CT-A₂ (200 nmol) was subjected to automated Edman degradation by using the semi-volatile dimethylbenzylamine buffer instead of Quadrol in order to minimize losses of peptide during the washing cycles; 23 successful degradations were carried out (Fig. 3). Glutamine was found to be the N-terminal residue. The absolute yield of phenylthiohydantoin-glutamine derivative at the first step was 27% (calculated from the recovery of glutamic acid after back hydrolysis). This recovery is relatively low since yields of phenylthiohydantoin derivatives at the N-termini of various proteins and peptides vary between 25 and 70% (Braun & Jaton, 1973; Niall, 1973). In peptide CT-A₂, one cannot exclude the possibility of partial cyclization of the glutamine residue into pyrrolidonecarboxylic acid, which would make Edman degradation impossible. Fig. 4 shows the yields of phenylthiohydantoin derivatives at selected steps in the degradation of peptide CT-A₂ as a function of step number. It can be seen that there is a rapid drop in yield, probably as a result of extensive extraction of peptide in the benzene and/or butylchloride. The repetitive yield calculated on the basis of the recovery of leucine-19 relative to leucine-8 and of valine-13 relative to valine-6 averaged 86–89% in 2 successive runs. A single

Fig. 3. Amino acid sequence of the rabbit H chain BS-5 in the region 64-116

Residues 66-81 and 94-116 were identified by the sequencer. Overlapping peptides extending between positions 64 and 93 are shown. T and Ch are tryptic and chymotryptic peptides respectively. The sequence between residues 34 and 69 has been determined previously (Jaton & Braun, 1972).

residue was found at each degradation step, thus indicating the high degree of homogeneity of the H chain BS-5. The remainder of peptide CT-A₂ (approx. 100nmol) was used for further analysis. After decitraconylation at low pH (Jaton, 1974b), the peptide, which became partially insoluble at pH 8, was digested with trypsin and the soluble digest was electrophoresed at pH6.5; two peptides, T-A_{2a} and T-A_{2b}, were readily isolated: their amino acid compositions and mobilities are shown in Table 1 and their partial N-terminal sequences in Fig. 1. By homology with the known sequence of the constant domain C_{H1} of the rabbit H chain (Fruchter *et al.*, 1970), it is apparent that the radioactive peptide T-A_{2a} extends between positions 124 and 149, whereas the arginine-containing peptide T-A_{2b} is the C-terminal end of peptide CT-A₂. The insoluble material from the tryptic digest was further digested with chymotrypsin and the digest was run at pH6.5 and 3.5. No evidence for the presence of variant peptides in the N-terminal segment of peptide CT-A₂ was found. Peptide CT-A₂ extends therefore between positions 94 and 170, i.e. it contains the third hyper-variable section of the rabbit H chain in its N-terminal

Fig. 4. Quantitative yields of phenylthiohydantoin-amino acids obtained at selected steps of the automated degradation of fragment CT-A₂

Yields were computed by comparing peak heights of sample with relevant standard derivatives by g.l.c. (○). The absolute yield of phenylthiohydantoin-glutamine at the first step was 27% on the basis of 200nmol of fragment CT-A₂ (see the text for details).

part and the beginning of the constant region in its C-terminal portion.

Sequence of fraction CT-A₃

As fraction CT-A₃ contains 2 major N-terminal residues, phenylalanine and glutamine, advantage was taken of the presence of the N-terminal glutamine residue which was cyclized into pyrrolidone-carboxylic acid (see under 'Methods'), thus making it unreactive in the Edman reaction. The acid-treated fraction CT-A₃ (3mg), whose citraconyl groups were also removed by the cyclization procedure, was subjected to automated Edman degradation by using 4-sulphophenylisothiocyanate. A major residue could be detected (Fig. 3) together with 10 to 15% of a second residue at most of the first 16 degradation steps performed. This indicates that fraction CT-A₃ contains also a small amount of peptide identified as peptide 10-33, resulting from the contamination of fraction A by the N-terminal CNBr fragment 1-33.

The whole fraction CT-A₃ (10mg) was decitraconylated and further digested with trypsin. Six peptides were detected from paper electrophoresis at

pH 6.5 and 3.5, and 5 of them were isolated. The uncharacterized peptide was the only one of the digest which stained with Ehrlich reagent. It was assumed that this is the peptide 43-63 of known sequence (Jaton & Braun, 1972); the data are compiled in Table 1 and the sequences shown in Fig. 1. The partial N-terminal sequence of the acid-treated fraction CT-A₃ determined by the sequencer overlaps successively peptides T-A₃T₂, T-A₃T_{N2} and T-A₃T₁ and establishes their order (Fig. 3). The N-terminal sequence of fraction CT-A₃ also overlaps the sequence of the first 69 residues determined previously (Jaton & Braun, 1972), thus indicating that fraction CT-A₃ contains one arginine peptide of 28 residues which extends between positions 66 and 93 (Fig. 3). The other major peptide of the fraction, characterized by the N-terminal glutamine residue occupies positions 38-65; its sequence has been previously reported (Jaton & Braun, 1972).

All the constituent arginine-containing peptides of the CNBr fraction A were accounted for; the partial N-terminal sequence of the first 69 residues of H chain BS-5 overlaps successively

Table 1. Amino acid composition of some tryptic and chymotryptic peptides isolated from the region 64-116 of fragment C1 derived from H chain BS-5

Values are residues/molecule of peptide. Values in parentheses are integral values confirmed by sequence analysis. The chymotryptic peptide T-A₃Ch₁ was derived from peptide T-A₃T₁. N.D., not determined; Hse, homoserine.

	Tryptic peptides											Chymo- tryptic peptide
	T-A ₄	T-A ₅	T-A _{2a}	T-A _{2b}	T-A ₃ T ₁	T-A ₃ T ₂	T-A ₃ T ₃	T-A ₃ T _{N2}	T-A ₃ T _{N3}	CT-A ₁	CT-A ₂	T-A ₃ Ch ₁
Lys			1.1			1.1 (1)		1.1 (1)	1.2	5.1	2.3	
His										0.8	0.7	
Arg	1.0 (1)	1.0 (1)		1.0	0.9 (1)		1.0 (1)			0	1.0	1.1 (1)
CmCys			2.5		0.9 (1)					2.4	2.3	0.9 (1)
Asp			1.0	2.3	1.0 (1)			1.0 (1)		4.1	4.6	
Thr	1.0 (1)		3.0	4.1	3.8 (4)	1.1 (1)		2.7 (3)		8.4	10.3	
Ser	1.2 (1)		3.3	1.4	1.2 (1)	1.2 (1)		1.2 (1)	1.0	9.3	5.5	
Glu			0.0	1.3	1.1 (1)					2.9	2.4	
Pro	0.9 (1)		4.0	1.8	0.8 (1)				1.0	11.5	7.8	
Gly		1.1 (1)	2.2	3.5			1.2 (1)		1.3	4.5	10.5	
Ala			2.0	0.2	1.8 (2)				0.9	3.3	2.7	1.0 (1)
Val	0.9 (1)	1.0 (1)	2.4	2.6				0.9 (1)		7.2	10.2	
Ile			0.0			1.0 (1)				1.2	0.2	
Leu			3.1	2.0	1.1 (1)			1.0 (1)		4.3	8.4	
Tyr				1.0	0.9 (1)					1.2	1.2	
Phe	1.1 (1)		1.0		1.0 (1)	1.0 (1)				1.8	2.0	1.0 (1)
Hse										0.8	0	
Trp*		+ (1)	—	+	—					—	+	
Total	6.1		25.6	20.2	14.4	5.4	2.2	7.9	5.4	68.7	72.1	4.0
residues												
Mobility	+0.41	+0.49	−0.28	−0.13	−0.34	+0.48	+0.78	0	0	N.D.	N.D.	0
at pH6.5												

* Qualitative determination with Ehrlich reagent.

antibodies (Jaton, 1974b; Appella *et al.*, 1973; Chen *et al.*, 1974).

Fig. 5 shows that V_H regions contain highly conservative sections (A, D, H and J), 'subgroup specific' sections (B and F), three hypervariable sections (C, E and I) and one variable section (G). The distribution of the conservative zones between the hypervariable sections C-E and G-I, and at the N- and C-terminal portions of the V region, may be a necessary requirement for the conservation of the three-dimensional structure of the entire V domain by optimal H-L chain interactions (Bourgois & Fougereau, 1970).

The presence of only one basic residue, histidine, in the middle of the third hypervariable section of the H chain in antibody BS-5 is of interest (Fig. 6): the antigenic determinant for which antibody BS-5 is specific is cellobiuronic acid (glucuronic acid-glucose), which contains one carboxyl group/disaccharide unit (Campbell & Pappenheimer, 1966). Ionic linkages between antigen and basic amino acid residues in the active sites of antibodies to type III and VIII have been postulated (Speyer *et al.*, 1973). Type III antisera may contain antibodies of high affinity ($K_a = 10^7$ litre/mol) as measured with the hexasaccharide hapten which contains three glucuronic acid residues, thus suggesting that the site of the antibodies is capable of binding more than one carboxyl group. There is no evidence at the moment that the unique histidine residue in the site of the antibody BS-5 would be able to interact with one of the carboxyl groups of the hexasaccharide determinant by ionic linkage. Other types of linkages might contribute substantially to the total binding energy, e.g. co-operative hydrogen-bonding to the hydroxyl groups of the antigenic determinant. The presence of the essentially hydrophilic stretches contributed by the first and second hypervariable zones of the H chain and by the third hypervariable section of the L chain (Fig. 6) in the binding site of the antibody BS-5 should provide sufficient energy for the specific interaction with the antigen. Other reports also suggest that antibodies to negatively charged hapten contain amino acid residues of the opposite charge in their combining site (Pressman *et al.*, 1970; Grossberg *et al.*, 1973). Koo & Cebra (1974) have identified an affinity-labelled lysyl residue in the second hypervariable section of the H chain of antibodies to the azobenzenearsonate group. The authors suggest that this residue may be a contact residue in the binding site of the antibody.

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