Sequence Studies on the Constant Region of the Fd Sections of Rabbit Immunoglobulin G of Different Allotype

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The amino acid sequence has been completed for the constant region of the Fd fragments of heavy chains from rabbit IgG (immunoglobulin G) of allotype Aa1 and Aa3. The amino acid sequence given by Fruchter *et al.* [(1970) *Biochem. J.* 116, 249–259] for the constant region of the Fd fragment from Aa1 IgG was extended and in part corrected to give a continuous sequence of 140 residues. No allotype-related sequence variation was found in the constant section of the Fd fragment. This evidence confirms the view that the differences in sequence between the variable regions of Aa1 and Aa3 IgG [Mole *et al.*, (1971) *Biochem. J.* 124, 301–318] are responsible for the allotypic specificities.

It is now well established that both the light and the heavy constituent polypeptide chains of immunoglobulins can be divided into an *N*-terminal portion, the variable (V) region, and a *C*-terminal portion, the constant (C) region (Press & Hogg, 1969; Edelman *et al.*, 1969). Thus differences in primary sequence have been observed in the *N*-terminal 115 residues (V region) of human γ 1 subclass myeloma heavy chains, the *C*-terminal portions (about 330 residues) having an identical primary sequence for a given allelic form (Press & Hogg, 1969; Edelman *et al.*, 1969).

Although the amino acid sequence of the C regions of human γ 1 and μ classes have very low homology, that of associated V regions can be very high (Putnam *et al.*, 1971); indeed, there is good serological (Oudin & Michel, 1969) and chemical (Fudenberg *et al.*, 1971) evidence that heavy chains of different classes can have an identical V region. To explain this and other pertinent data (Gally & Edelman, 1970), it has been logical to assume that an immunoglobulin polypeptide chain is encoded by two separate germ-line genes, a *V* gene (V-region gene) and a *C* gene (C-region gene), which are joined somatically (Dreyer & Bennett, 1965).

In the rabbit immunoglobulin system the serologically defined *a*-locus allotypic markers, a1, a2 and a3, have been shown to be present in all classes of heavy chain, namely γ , μ , and α (Oudin, 1960; Todd, 1963; Feinstein, 1963; Kindt & Todd, 1969). This fact is most easily explained if the *a*-locus specificities are present in the rabbit heavy-chain V region. Direct evidence for this view has come from primary-sequence studies on the three allotypes (Wilkinson, 1969a; Fleischman, 1971; Mole *et al.*, 1971), which have shown correlation of allotype with residues at

several positions within the V region. However, the multiple differences observed do not allow a simple correlation of primary structure with allotype, as is possible with other genetic markers such as the Inv marker (Milstein, 1966; Baglioni *et al.*, 1966). Another approach to the localization of the *a*-locus markers is to study the *C*-terminal regions of the heavy chains from rabbits of different serological specificity. If it were clearly shown that these parts of the polypeptide chain did not differ in sequence, then some of the structural differences observed in the V region must be determining the serological allotypes.

Since it has been shown (Kelus *et al.*, 1961) that the *a*-locus markers are in the Fd portion of the heavy chain, only the *N*-terminal parts of the C region need be considered; therefore we have studied the sequence of the C-1 fragment, derived from the γ chain by CNBr cleavage, which is slightly larger than the Fd fragment (Givol & Porter, 1965). Earlier sequence studies (Cebra *et al.*, 1968b; Fruchter *et al.*, 1970) were carried out on the C-1 fragment derived from 'normal' chain, i.e. the heavy chain from the IgG* of rabbits of unknown *a*-locus allotype. The normal IgG used was, however, typed during the course of these studies by Dr. J. Oudin and shown by him to consist mainly of the a1 specificity (see Wilkinson, 1969a). Further, in structural studies on the V region of normal heavy chain (O'Donnell *et al.*, 1970; Mole, 1971) only peptides thought to be specific for the a1 allotype were isolated.

The approach followed in this study has been to determine the C-region sequence of the C-1 fragment from Aa3 IgG and to compare this with that previously determined for normal (Aa1) IgG. If and when any difference was noted the relevant residues for the C-1 fragment of IgG from rabbits homozygous for the Aa1 specificity were reinvestigated. This has led to some revision of the previously

* Abbreviations: IgG, immunoglobulin G; Cm-Cys, S-carboxymethylcysteine; γ -Glu, pyrrolid-2-one-5-carboxylic acid.

published results, but has shown no distinction between the sequences in the constant region of the Fd section of chains carrying the Aa1 and Aa3 specificities.

Materials and Methods

Materials

IgG was prepared as described by Prahl & Porter (1968) from rabbits of known allotype. Anti-(*a*-locus) antisera were prepared by the method of Kelus & Gell (1967) and shown to be specific by comparison, against a large panel of individual rabbit sera, with anti-(*a*-locus) antisera provided by Professor P. G. H. Gell. After separation from light chain (Fleischman *et al.*, 1963), γ chain was cleaved with CNBr to yield the C-1 fragment (Fruchter *et al.*, 1970), which was then fully reduced and [14 C]alkylated (O'Donnell *et al.*, 1970).

Citraconic anhydride (BDH Chemicals, Poole, Dorset, U.K.) was used to block the ϵ -amino group of lysine residues (Dixon & Perham, 1968). The conditions used for the citraconylation and tryptic digestion of the C-1 fragment and the unblocking of the ensuing tryptic peptides were all as described by Mole *et al.* (1971).

The enzymes trypsin, chymotrypsin and carboxypeptidases A and B were used as previously described (Press *et al.*, 1966). Thermolysin (E. Merck, Darmstadt, Germany) was used under the conditions described by Ambler & Meadway (1968). Pyrrolidonecarboxyl peptidase was the 'A-25 enzyme' described by Doolittle (1970). Before use, the pyrrolidonecarboxyl peptidase activity was assayed by using the substrate L-pyrrolid-2-one-carboxyl-L-alanine ($\square$ Glu-Ala); all conditions were as described by Doolittle (1970). The pyrrolidonecarboxyl peptidase and the substrate $\square$ Glu-Ala were gifts from Dr. R. F. Doolittle.

Enzymic digestion of peptides

The conditions of digestion were varied, depending on the susceptibility of the various peptides to cleavage by the particular enzyme; the conditions used were as follows: tryptic digestion in 1% NH_4HCO_3 buffer, pH 8.1 at 37°C [0.3–0.5% (w/v) peptide] for peptide T2 (200 μg of trypsin/ μmol of peptide for 1 h) and for peptide T3 (500 μg of trypsin/ μmol of peptide for 2 h); chymotryptic digestion in 1% NH_4HCO_3 buffer, pH 8.1, at 37°C [0.2% (w/v) peptide] for peptide T3 (1 mg of chymotrypsin/ μmol of peptide for 4 h), peptide T-T2-LA (500 μg of chymotrypsin/ μmol of peptide for 7 h) and peptide T-T3-LY (700 μg of chymotrypsin/ μmol of peptide for 4 h); thermolytic digestion in 0.1 M-ammonium acetate buffer, pH 8.5, containing 5 mM- CaCl_2 , at 37°C [0.1% (w/v) peptide] for peptide T-T2-LA (1.5 mg of

thermolysin/ μmol of peptide for 3 h), peptide Ts2 (1.6 mg of thermolysin/ μmol of peptide for 4 h) and peptide Ts3 (1.6 mg of enzyme/ μmol of peptide for 2.5 h); peptic digestion of peptide T-T2-LA was carried out as described by Konigsberg & Hill (1962) but for an extended period of 2.5 h.

Analytical methods

High-voltage paper electrophoresis was carried out as described by Crumpton & Wilkinson (1965). Peptides containing no free α -amino group were detected by the hypochlorite-starch-iodide method of Pan & Dutcher (1956), those containing tyrosine by the 1-nitroso-2-naphthol stain (Smith, 1960) and those containing tryptophan by the Ehrlich reagent (Easley, 1965).

Amino acid analysis was carried out as described by Wilkinson (1969b). The dried hydrolysates of peptides containing homoserine were dissolved in 1 ml of pyridine-acetate buffer, pH 6.5, and heated at 100°C for 1 h before analysis (Ambler, 1965). For peptide samples suspected of containing tyrosine 20 μl of 0.1 M-phenol/0.5 ml of 6 M-HCl was added before hydrolysis to minimize destruction of this amino acid (Sanger & Thompson, 1963).

The dansyl-Edman procedure was used as described by Gray (1967) with the modifications suggested by Hartley (1970). Dns-amino acids were resolved by t.l.c. on polyamide sheets (5 cm $\times$ 5 cm) (Woods & Wang, 1967), 0.3–1.0 nmol of the sample being applied to each side. The peptide sequence obtained was confirmed by subjecting a sample of the residual peptide to amino acid analysis after every two or three steps of degradation.

The position of radioactive *S*-carboxymethylcysteine-containing peptides after separation by high-voltage electrophoresis on paper was determined by overnight radioautography with Kodak BB-54 X-ray film. The origin, in the primary sequence of C-1 fragment, of radioactive *S*-carboxymethylcysteine-containing peptides in column effluents was determined by tryptic-chymotryptic digestion of a portion of any radioactive fraction followed by paper electrophoresis at pH 3.5. After overnight radioautography the origin of the radioactive peptide could be determined by comparison with the known properties of the tryptic-chymotryptic intrachain *S*-carboxymethylcysteine-containing peptides of C-1 fragment (O'Donnell *et al.*, 1970).

Results

The previous study by Fruchter *et al.* (1970) showed that tryptic digestion of trifluoroacetylated fragment C-1 released three peptides, T2, T3 and T7, which together made up the C-region sequence. Satisfactory arginine-overlap peptides and good homology

with the human IgG1 protein Daw (Press & Hogg, 1969) confirmed the ordering of the peptides as T3-T7-T2. Comparison of the elution profiles on Sephadex chromatography of tryptic digests of citraconylated Aa1 and Aa3 fragment C-1 showed no differences in the gross properties of the arginine- or methionine-(homoserine-)containing peptides derived from the C region nor the presence of extra arginine-containing peptides derived from this region in Aa3. Thus, if any differences in primary sequence exist between Aa1 and Aa3 C regions, they are to be found in the peptides T3, T7 and T2.

In terms of the numbering system of Mole *et al.* (1971) and Fruchter *et al.* (1970) (see Fig. 4) peptide T3 spans residues 95–170, peptide T7 residues 171–176 and peptide T2 residues 177–253. The final purification of these peptides was as described by Cebra *et al.* (1968a).

Aa3 peptide T7 (residues 171–176)

The small hexapeptide T7 had an amino acid composition and sequence identical with that of peptide T7 from normal fragment C-1 and that of Aa2 fragment C-1 (Fleischman, 1973).

Aa3 peptide T3 (residues 95–170)

Peptide T3 has been shown to contain the major hypervariable region of the V-region at its *N*-terminus (Fruchter *et al.*, 1970), although most of the peptide's sequence lies in the C-region. Tryptic digestion of unblocked peptide T3 releases the peptides Ts3

(residues 124–149) and T-T3-LY (residues 150–170) in excellent yield. However, the great heterogeneity associated with the *N*-terminal sequence (95–110) of peptide T-T3-LB (residues 95–123) had previously created great difficulty in isolating in high yield peptides from the region 110–123. This region has therefore been reinvestigated fully for both allotypes Aa1 and Aa3. Tryptic digestion of Aa3 peptide T3 gave an elution diagram such as the one shown in Fig. 1. Peptide Ts3 was confined to the large radioactivity peak B (Fig. 1), final purification being

Fig. 1. Elution profile of a tryptic digest of Aa3 peptide T3

Gel filtration was conducted on a column (1.8cm × 220cm) of Sephadex G-50 in 0.05M-NH₃. The flow rate was 30ml/h with a fraction size of 5ml. —, *E*₂₃₀; ····, radioactivity (c.p.m./ml of eluate).

Table 1. Isolation and amino acid compositions of thermolytic peptides of Aa3 peptide Ts3

Compositions are given as mol of residues/mol of peptide; values less than 0.1 are omitted. Percentage yield is expressed versus the highest-yield peptide obtained. Mobilities at pH3.5 are relative to lysine (+1), aspartic acid (−1) and ϵ -Dnp-lysine (0); at pH1.9 mobility is relative to ϵ -Dnp-lysine (1.0).

Amino acid	Ts3	Thermolytic peptides					
		Ts3-Th-6A	Ts3-Th-6B	Ts3-Th-6C	Ts3-Th-5A	Ts3-Th-5B	Ts3-Th-3A
Lys	0.9	—	—	—	0.8	—	—
CmCys	2.5	—	—	—	—	0.7	1.9
Asp	1.0	—	—	—	—	—	1.0
Thr	2.7	1.0	—	—	—	—	1.7
Ser	2.6	—	0.9	—	—	—	1.6
Pro	3.3	—	1.0	1.1	—	—	2.0
Gly	2.0	—	—	—	—	1.0	1.0
Ala	1.9	—	1.0	—	—	—	0.9
Val	2.8	1.0	—	0.9	0.9	—	—
Leu	3.0	—	—	—	1.0	1.0	1.0
Phe	0.8	—	—	1.0	—	—	—
Total residues	26	2	3	3	3	3	12
Percentage yield		36	25	63	28	59	100
Mobility at pH3.5		+0.11	+0.091	+0.06	+0.91	−0.49	−0.55
Mobility at pH1.9					+2.6		
Radioautography						+++	+

achieved by paper electrophoresis at pH6.5 (Cebra *et al.*, 1968b). Chymotryptic digestion of Aa3 peptide Ts3 gave two peptides with the same electrophoretic properties, compositions and partial sequences as the peptides C-Ts3-A and C-Ts3-B previously isolated from normal peptide Ts3 (Cebra *et al.*, 1968b). A complete sequence for Aa3 peptide Ts3 (Fig. 2), showing it to be identical with that of Aa1 peptide Ts3, was achieved by thermolytic digestion; the isolation procedure for the thermolytic peptides, together with their compositions, is given in Table 1. The radioactivity associated with peptide Ts3-Th-3A was of interest, as alkylation of whichever cysteine residue of the Cys-Cys sequence was involved in the heavy-light interchain bridge was thought to be complete on partial reduction and alkylation with non-radioactive iodoacetic acid (i.e. before separation of light chains and heavy chains). It was hoped that the decrease in radioactivity of the peptide on sequential Edman degradation would enable us to determine which cysteine residue formed the heavy-light interchain bridge; however, the respective specific radioactivities of the two cysteine residues were found to be equal.

The C-terminal arginine-containing peptide of peptide T3, peptide T-T3-LY (residues 150-170), was isolated from peak C (Fig. 1) by paper electrophoresis at pH6.5. Smaller amounts of the peptides T-T3-LY-V and T-6b (see Fig. 2), produced by cleavage at the asparagine residue of peptide T-T3-LY on tryptic digestion (Fruchter *et al.*, 1970), were isolated from fractions D and E respectively. Sequence determination of the chymotryptic peptides of Aa3 peptide T-T3-LY gave a sequence (Fig. 2) identical with that previously determined for normal peptide T-T3-LY; the composition and properties of the chymotryptic peptides are given in Table 2. The positioning of asparagine in peptide T-T3-LY was based on the presence of a stoichiometric amount of aspartic acid in the analysis of peptide T-T3-LY-V, this amino acid being only fractional in that of peptide C-T3-LY-A, which nonetheless gave a good yield of tryptophan on carboxypeptidase A digestion. Assignment of acid or amide status to Glx- or Asx-containing peptides was based on their mobility at pH6.5 (Offord, 1966). There were no differences in sequence between the Aa3 and normal Ts3 and T-T3-LY peptides.

The search for tryptic peptides from the region T-T3-LB (residues 95-123) was performed by assuming them to be lysine-containing peptides. A calculation of the ^{14}C specific radioactivity of purified peptide Ts3 showed that the lysine content of peak B (Fig. 1) was completely accounted for by this peptide. Lysine present in the analyses of peaks A, C, D and E (Fig. 1) could not, however, be accounted for by the small amount of radioactivity present, nor by the presence of peptide T-T3-LY, which contains no lysine. Paper electrophoresis of samples from peaks

Table 2. Isolation and amino acid compositions of chymotryptic peptides of Aa3 peptides T-T3-LY and T-T3-LB
Compositions are given as mol of residues/mol of peptide; values less than 0.1 are omitted. Mobilities are expressed as in Table 1 for pH1.9 and pH3.5; pH6.5 mobilities are relative to lysine (+1) aspartic acid (-1) and ϵ -Dnp-lysine (0).

Amino acid	T-T3-LY			T-T3-LB						
	C-T3-LY-A	C-T3-LY-B	C-T3-LY-C	C-T3-LB	C-T3-LB-1	C-T3-LB-2	C-T3-LB-2A	C-T3-LB-2B	C-T3-LB-2C	
Lys	—	—	—	0.9	—	1.0	0.8	0.9	1.1	
Arg	—	0.8	0.9	—	—	—	—	—	—	
Asp	0.3	1.0	1.0	—	—	—	—	—	—	
Thr	1.9	1.9	1.0	1.8	1.1	0.9	—	—	—	
Ser	—	0.9	—	1.9	—	1.9	1.7	1.7	1.0	
Glu	1.0	—	—	1.2	—	1.0	1.0	1.0	1.0	
Pro	2.3	—	—	1.9	0.9	1.2	1.1	0.9	0.8	
Gly	1.0	1.8	1.3	2.8	1.9	1.0	1.1	1.1	1.1	
Val	1.9	1.0	1.0	1.7	—	1.9	0.9	—	—	
Leu	1.0	1.0	—	1.0	1.0	—	—	—	—	
Tyr	1.1	—	—	—	—	—	—	—	—	
Trp	+	—	—	—	—	—	—	—	—	
Total residues	11	9	5	14	5	9	7	6	5	
Mobility at pH1.9	—	—	1.90	—	1.30	—	—	—	—	
Mobility at pH3.5	—	+0.28	—	+0.34	—	+0.47	+0.42	+0.46	+0.48	
Mobility at pH6.5	-0.19	0	0	—	—	+0.37	—	—	—	

A and C showed no distinct arginine-negative peptide spot, but peaks D and E contained a novel basic peptide (C-T3-LB-2 in Fig. 2). However, if samples from peaks A and C were subjected to chymotryptic digestion before electrophoresis, a basic peptide of properties similar to that from peaks D and E was revealed. It therefore appeared that peaks A and C contained a larger form of peptide C-T3-LB-2 that did not give a distinct spot on paper electrophoresis, presumably owing to heterogeneity at its *N*-terminus.

As the products of chymotryptic digestion of peptide T-T3-LY were well characterized (Table 2), peaks A, C, D and E were pooled and subjected to chymotryptic digestion, and the major peptides were isolated: the properties of those peptides isolated that were not derived from peptide T-T3-LY are given in Table 2 and their sequences are given in Fig. 2 (peptide C-T3-LB).

The peptides comprising peptide C-T3-LB were obtained in good yield; on the basis of the yield from paper of peptide C-T3-LY-A (100%), the respective yields of peptides C-T3-LB-2, C-T3-LB-1 and C-T3-LB were 120, 85 and 15%. Peptides C-T3-LB-2A, C-T3-LB-2B and C-T3-LB-2C were obtained from a larger preparation in yields of 10% or less, and were only purified from each other (and peptide C-T3-LB-2) after prolonged paper electrophoresis at pH 6.5 (3000 V for 2.5–3 h). Although other peptide material was indeed apparent in the isolation of the peptides, no other peptides in significant yield could be purified, presumably because of heterogeneity.

The sequence obtained for the region C-T3-LB (Fig. 2), although very homologous to the human γ 1 sequence in this region (Press & Hogg, 1969), is different from that previously determined (Fruchter *et al.*, 1970; Cebra *et al.*, 1968b). There was therefore the possibility that the sequence for Aa1 and Aa3 peptides T3 differed in this region. We repeated the studies with Aa1 peptide T3, and it was found that basic peptides of composition and electrophoretic mobility identical with those of the Aa3 peptides C-T3-LB, C-T3-LB-2 and C-T3-LB-2A (see Table 2) were present in good yield. The sequence of Aa1 peptide C-T3-LB-2 was fully determined and that of Aa1 peptide C-T3-LB for eight residues, therefore overlapping into the Aa1 peptide C-T3-LB-2 sequence; their sequences were identical with those derived for the Aa3 peptides (Fig. 2).

Thus, from residues 110–170, peptides T3 from both allotypes Aa1 and Aa3 were found to be identical in sequence.

Sequence of Aa3 peptide T2 (residues 177–253)

Cebra *et al.* (1968b) showed that tryptic digestion of unblocked normal peptide T2 (residues 177–253)

released four major peptides T-T2-SN (residues 214–216), T-T2-SA (residues 250–253), Ts2 (residues 217–250) and T-T2-LA (residues 177–213). It seemed apparent from a trial digestion of Aa3 peptide T2 that considerable enzymic breakdown of the large peptides T-T2-LA and Ts2 occurred under the conditions used by Cebra *et al.* (1968b); indeed, they were unable to isolate peptide T-T2-LA intact. It was found that a digestion time of 1 h (instead of 12 h) with a slightly smaller amount of trypsin was sufficient to achieve complete digestion of peptide T2 while preserving most of the Ts2 and T-T2-LA peptide molecules intact.

After digestion of Aa3 peptide T2 it was fractionated on a Sephadex G-50 column in 0.05 M-NH₃. The amino acid compositions of the isolated Aa3 peptides T-T2-SN, T-T2-SA and Ts2 were consistent with the sequence for the Aa1 peptides. However, the composition of peptide Aa3-T-T2-LA (Table 4) suggested it to be several residues larger than the Aa1-T-T2-LA peptide described by Fruchter *et al.* (1970).

The major peptides isolated from the small-molecular-weight fraction were the peptides Aa3-T-T2-SA and T-T2-SN, which had the respective sequences Asp-Thr-Leu-HSer and Val-Asp-Lys; they were therefore identical in sequence with the equivalent peptides from Aa1 peptide T2. Also present in the small-peptide fraction were two tyrosine-containing peptides, which, although present in low yield, were shown to be derived from peptide T-T2-LA (see below).

The sequence of Aa3 peptide Ts2 (residues 217–250) was determined from peptides isolated from chymotryptic or thermolytic digests. Only the peptides used in aligning the sequence of peptide Ts2 are described in Table 3, although other peptides consistent with this sequence (Fig. 3) were isolated. Peptide Aa3-Ts2-Th-4B formed an overlap peptide between peptides T-T2-SN and Ts2 and, although isolated in low yield (<15%), indicated that tryptic digestion of peptide T2 was not quite complete under the conditions described above.

The only inconsistency found between the sequence of peptide Ts2 described here and that reported for the normal Ts2 peptide (Cebra *et al.*, 1968b) was in the peptide Aa3-Ts2-C-4, which had the sequence Ile-Phe-Pro-Pro-Lys-Pro-Lys (instead of Ile-Phe-Lys-Pro-Pro-Pro-Lys). Again, this discrepancy was checked in the Aa1 peptide, and the sequence of peptide Aa1-Ts2-C-4 was identical with that we describe here for the Aa3 peptide. As well as showing this area to be exactly homologous to the equivalent region in the human γ 1 sequence (see Fig. 4), it also explains why the expected chymotryptic split in this peptide, between the Phe-Lys-bond, did not occur; the Phe-Pro bond in the revised sequence is resistant to chymotryptic cleavage.

Aa3 peptide T-T2-LA (residues 177–213) is the *N*-

Table 3. Isolation and amino acid compositions of chymotryptic and thermolytic peptides of Aa3 Ts2 peptides

Compositions are given as mol of residues/mol of peptide; values less than 0.1 are omitted. Mobilities are expressed as in Table 2.

Amino acid	Chymotryptic peptides		Thermolytic peptides			
	Ts2-C-3	Ts2-C-4	Ts2-T	Ts2-Th-3	Ts2-Th-4A	Ts2-Th-4B
Lys	—	1.7	1.0	1.0	—	1.0
CmCys	—	—	1.5	0.4	—	—
Asp	—	—	—	—	—	1.1
Thr	—	—	1.7	0.9	—	0.9
Ser	1.1	—	1.0	1.0	1.2	—
Glu	—	—	1.0	1.2	—	—
Pro	1.0	3.2	4.2	4.5	1.1	—
Gly	1.9	—	—	—	2.0	—
Val	0.9	—	—	—	—	1.0
Ile	—	0.9	—	—	—	—
Leu	—	—	0.8	0.9	1.0	—
Phe	0.9	1.0	—	—	—	—
Total residues	6	7	12	10	5	4
Mobility at pH 1.9					1.42	1.49
Mobility at pH 3.5	+0.22		+0.08	+0.31	+0.23	+0.36
Mobility at pH 6.5		+0.68				

terminal tryptic peptide of peptide T2, and was isolated in two forms by Fruchter *et al.* (1970), owing to the susceptibility of the *N*-terminal glutamine residue to deamidation to form the pyrrolid-2-one-5-carboxylic acid-containing peptide. The differences in the isolation procedures for the tryptic peptides of fragment C-1 introduced by Mole *et al.* (1971), especially the fractionation of the large tryptic peptides on a column of Sephadex G-50 in 50% formic acid, have made the conversion into the pyrrolid-2-one-5-carboxylic acid derivative virtually complete (95%). It was therefore convenient to isolate peptide T-T2-LA from DEAE-Sephadex A-25, the peptide being eluted from the column with 0.005M-NH₄HCO₃, pH 8.5, completely free of any Ts2 peptide.

The amino acid composition of Aa3 peptide T-T2-LA (Table 4) indicated that it was made up of 34–36 residues, 4–6 more than accounted for by Fruchter *et al.* (1970) in the sequence of normal peptide T-T2-LA. By difference, a leucine residue, two serine residues and two valine residues (determined after a 72h acid hydrolysis) were the additional residues. In the course of sequence analysis Aa3 peptide T-T2-LA did prove to be longer, by at least five residues, than the sequence described for normal peptide T-T2-LA; moreover, the sequence obtained was at variance in many positions. The sequence of Aa3 peptide T-T2-LA is given in Fig. 3, together with the constituent peptides used in its derivation; the isolation and compositional data for these peptides are given in Table 4.

As stated above, two tyrosine-containing peptides were isolated from the small-peptide fraction of the tryptic digest of peptide T2, together with the peptides T-T2-SA and T-T2-SN; the yield of each peptide was 8% of that of T-T2-SA. Peptide T-T2-LA-T1 (see Fig. 3) was unreactive to dansylation and was concluded to be the blocked (pyrrolid-2-one-5-carboxylic acid-containing) *N*-terminal peptide of peptide T2; carboxypeptidase A digestion showed the *C*-terminal sequence to be Leu-Tyr. The remainder of the sequence of peptide T-T2-LA-T1 was determined by using the pyrrolidonecarboxyl peptidase enzyme described by Doolittle (1970). After incubation with pyrrolidonecarboxyl peptidase, the peptide was re-isolated by paper electrophoresis, although a subsequent experiment showed this to be unnecessary, and gave the sequence Ser-Ser-Gly-Leu-Tyr.

Peptide T-T2-LA-T2, which also had a blocked *N*-terminus, had additional serine and leucine residues over the composition of peptide T-T2-LA-T1; leucine was released by carboxypeptidase A. This second leucine residue had been missed by Fruchter *et al.* (1970), who had not been able to isolate a peptide overlapping the tyrosine residue.

As thermolysin cleaves on the *N*-terminal side of leucine residues, digestion of peptide T-T2-LA with this enzyme was used in an attempt to overlap from this leucine residue into the sequence deduced to be *C*-terminal to the tyrosine residue in the earlier studies. However, the only peptides isolated that could be assigned to the *N*-terminal region of peptide T-T2-LA were the peptides [Glu-Ser-Ser-Gly

Fig. 3. Amino acid sequence of Aa3 peptide T2

Sequence determination from the *N*-terminal end was by the dansyl-Edman or subtractive Edman techniques, shown by $\longrightarrow$, the removal of *N*-terminal pyrrolid-2-one-5-carboxylic acid **Glu** by pyrrolidonecarboxyl peptidease, shown by $\dashrightarrow$, and hydrolysis from the C-terminal end by carboxypeptidase A, shown by $\longleftarrow$, or carboxypeptidase B, shown by $\dashleftarrow$.

(T-T2-LA-Th-4C) and Leu-Tyr-Ser (T-T2-LA-Th-5A); no other leucine-containing peptide was isolated.

The thermolysin digest did, however, contain the peptides that comprise the C-terminal half of the T-T2-LA peptide. The peptides T-T2-LA-Th-1, T-T2-LA-Th-3A, T-T2-LA-Th-5B and T-T2-LA-Th-5C were characterized as shown in Fig. 3, and gave the C-terminal sequence of peptide T-T2-LA as Val-Ala-His-Pro-Ala-Thr-Asn-Thr-Lys. This sequence was as described by Fruchter *et al.* (1970), except for the insertion of the proline residue after histidine; this discrepancy presumably derives from an analytical error. The *N*-terminal extension of this sequence was provided by the peptides T-T2-LA-Th-2, T-T2-LA-Th-4A and T-T2-LA-Th-4B (see Fig. 3). The *N*-terminal valine residue of peptide T-T2-LA-Th-4A constituted another insertion into the sequence assumed for this region, on compositional data, in the earlier studies; further, the sequence Val-Thr-Ser-Ser-Ser-Gln-Pro differs from the equivalent sequence determined for normal peptide T-T2-LA, which had the Val-Thr sequence inverted and a valine residue in the position of the second serine residue.

Subtraction of the sequence derived from the thermolytic peptides from the composition of peptide T-T2-LA indicated 8-10 missing residues, which included the second leucine and two or three valine residues. As these residues are specific to thermolysin cleavage, it seemed probable that the presence of three of four susceptible bonds in eight or so residues had given rise to a large number of partially digested peptides, which were therefore present in the digest in yields too low for purification to be achieved. It has been shown by Konigsberg & Hill (1962) that an enzyme of wide specificity, such as pepsin, will hydrolyse a particular peptide linkage, in a given peptide, at a higher rate than other potentially sensitive bonds. Thus they demonstrated the specific cleavage of the Leu-Ala bond in a peptide, by using 0.002% (w/v) pepsin, whereas all other linkages, susceptible under more stringent conditions, remained intact. Peptide T-T2-LA was therefore treated with pepsin under similar conditions, and the digest was fractionated on a Sephadex G-50 column in 0.05M-NH₃.

This procedure gave only two fractions containing peptide material, the first containing peptide T-T2-LA-PA and the second a mixture of the peptides T-T2-LA-PB-1 and T-T2-LA-PB-2 (Fig. 3); the composition and properties of these peptides are given in Table 4. There was no evidence of any release of di- or tri-peptides or free amino acids, and it was therefore deduced that the whole of the sequence of Aa3 peptide T-T2-LA was contained in the three peptides isolated.

Peptide T-T2-LA-PB-2 was blocked and its sequence was not determined; however, the compo-

	110	120	130
RK 17	Trp-Gly-Pro-Gly-Val-Leu-Val-Thr-Val-Ser-Ser-Gly-Val (	Lys-Ala-Pro-Ser-Val-Phe-Pro-Leu-Ala-Pro-Cys (	Gly-Asp
Rabbit	Gly-Pro-Gly-Thr-Leu-Val-Thr-Val-Ser-Ser-Gly-Gln-Pro-Lys-Ala-Pro-Ser-Val-Phe-Pro-Leu-Ala-Pro-Cys-Cys-Gly-Asp-		
Daw	Trp-Gly-Gln-Gly-Ile-Leu-Val-Thr-Val-Ser-Ser-Ala-Ser-Thr-Lys-Gly-Pro-Ser-Val-Phe-Pro-Leu-Ala-Pro-Ser-Ser-Lys-Ser-		
	140	150	160
Rabbit	Thr-Pro-Ser-Ser-Thr-Val-Thr-Leu-Gly-Cys-Leu-Val-Lys-Gly-Tyr-Leu-Pro-Glu-Pro-Val-Thr-Val-Thr-Trp-Asn-Ser-		
Daw	Thr-Ser-Gly-Gly-Thr-Ala-Ala-Leu-Gly-Cys-Leu-Val-Lys-Asp-Tyr-Phe-Pro-Glu-Pro-Val-Thr-Val-Ser-Trp-Asn-Ser-		
	170	180	
Rabbit	Gly-Thr-Leu-Thr-Asp-Gly-Val-Arg-Thr-Phe-Pro-Ser-Val-Arg-Gln-Ser-Ser-Gly-Leu-Tyr-Ser-Leu-Ser-Ser-		
Daw	Gly-Ala-Leu-Thr-Ser-Gly-Val-His-Thr-Phe-Pro-Ala-Val-Leu-Gln-Ser-Ser-Gly-Leu-Tyr-Ser-Leu-Ser-Ser-		
	190	200	210
Rabbit	Val-Val-Ser-Val-Pro-Val-Thr-Ser-Ser-Ser-Gln-Pro-Val-Thr-Cys-Asn-Val-Ala-His-Pro-Ala-Thr-Asn-Thr-Lys-		
Daw	Val-Val-Thr-Val-Pro-Ser-Ser-Leu-Gly-Thr-Gln-Thr-Tyr-Ile-Cys-Asn-Val-Asn-Val-His-Lys-Pro-Ser-Asn-Thr-Lys-		
	220	230	
Rabbit	Val-Asp-Lys-Thr-Val-Ala-Pro-Ser-Thr-Cys-Ser-Lys-Pro- ——— Thr-Cys-Pro-Pro- ——— Pro-Glu-Leu-Leu-		
Daw	Val-Asp-Lys-Lys-Val-Gln-Pro-Lys-Ser-Cys-Asp-Lys-Thr-His-Thr-Cys-Pro-Pro-Cys-Pro-Ala-Pro-Glu-Leu-Leu-		
	240	250	253
Rabbit	Gly-Gly-Pro-Ser-Val-Phe-Ile-Phe-Pro-Pro-Lys-Pro-Lys-Asp-Thr-Leu-Met		
Daw	Gly-Gly-Pro-Ser-Val-Phe-Leu-Phe-Pro-Pro-Lys-Pro-Lys-Asp-Thr-Leu-Met		

Fig. 4. Comparison of rabbit γ chain sequence with that of human $\gamma 1$ chain

The human sequence is that of the $\gamma 1$ chain of the pathological human IgG1 protein Daw (Press & Hogg, 1969). Identical residues are underlined, assuming deletions to give maximum homology. Numbering is as used in the Daw protein sequence. The sections represented by the major peptides discussed in the paper are: peptide T3, residues 110-170; peptide T7, residues 171-176; peptide T2, residues 177-253. The RK 17 antibody sequence is part of that described by Strosberg *et al.* (1972).

Table 4. Isolation and amino acid compositions of tryptic, peptic and thermolytic peptides of Aa3 peptide T-T2-LA

Compositions are given as mol of residues/mol of peptide; values less than 0.1 are omitted. Mobilities are expressed as in Table 2.

Amino acid	Tryptic peptides				Peptic peptides				Thermolytic peptides				Thermolytic peptides			
	LA-T1	LA-T2	LA-P-A	LA-P-B-1	LA-P-B-2	LA-Th-1	LA-Th-2	LA-Th-3A	LA-Th-4B	LA-Th-4A	LA-Th-4C	LA-Th-5A	LA-Th-5B	LA-Th-5C		
Lys	1.1	—	0.9	—	—	1.0	—	1.0	—	—	—	—	—	1.0		
His	1.0	—	0.9	—	—	0.8	—	—	—	—	—	—	—	—		
CmCys	0.7	—	0.8	—	—	—	—	—	—	—	—	—	—	—		
Asp	2.4	—	2.0	—	—	1.2	—	—	—	—	—	—	—	—		
Thr	4.3	—	3.9	—	—	1.8	0.9	—	—	—	—	—	—	0.8		
Ser	8.4	2.7	4.9	2.8	—	0.2	2.6	—	—	—	—	—	—	—		
Glu	2.3	1.1	1.3	—	—	1.1	1.1	—	—	—	—	—	—	—		
Pro	2.8	1.0	2.4	—	—	1.1	1.2	—	—	—	—	—	—	—		
Gly	1.2	1.3	—	—	—	—	—	—	—	—	—	—	—	—		
Ala	2.4	—	1.8	—	—	2.0	—	—	—	—	—	—	—	—		
Val	5.8	—	5.2	0.8	—	0.9	1.0	—	—	—	—	—	—	—		
Leu	2.0	2.0	—	1.0	—	—	—	—	—	—	—	—	—	—		
Tyr	0.9	0.9	—	1.0	—	—	—	—	—	—	—	—	—	—		
Total residues	34-36	8	24-25	6	5	9	7	4	4	4	4	3	2	2		
Mobility at pH1.9						+2.1	+0.25	+2.3				+1.26				
Mobility at pH3.5																
Mobility at pH6.5	-0.39	-0.33	+0.21	-0.76	-0.76	0	0	-0.42	+0.22	-0.91	+0.46	0	+0.68			

sition of this peptide was consistent with the sequence of the first five residues of peptide T-T2-LA. Peptide T-T2-LA-PB-1 had the sequence Tyr-Ser-Leu-Ser-Ser-Val and therefore overlapped the second leucine residue of peptide T-T2-LA. The major *N*-terminal sequence of peptide T-T2-LA-PA was Val-Ser-Val-Pro, and therefore the sequence Ser-Leu-Ser-Ser-Val-Val needs to be inserted in the sequence Tyr-Ser-Val-Pro proposed, but not overlapped at the tyrosine residue, by Fruchter *et al.* (1970). The presence of the Val-Val sequence was supported by the detection of Dns-Val-Val on analysis of the *N*-terminus of peptide T-T2-LA-PA and of a small amount of valine on dansylation after the first Edman step, indicating that a small proportion of the T-T2-LA-PA peptides began Val-Val. The fifth residue of peptide T-T2-LA-PA could not be confidently assigned, although the sixth residue was threonine. The overlap of the sequence of peptide T-T2-LA-PA into that derived for peptide T-T2-LA-Th-2 was therefore ambiguous, and the possibility remains that the sequence of peptide T-T2-LA given in Fig. 3 may be longer by one or two residues inserted between the Pro-Val sequence given for residues 191 and 192. Although the derived sequence is in agreement with the composition of peptide T-T2-LA, the presence of an extra serine and/or threonine residue in the sequence is possible.

An attempt was made to resolve this problem when the Aa1-T-T2-LA peptide sequence was reinvestigated by subjecting the peptide to chymotryptic digestion. Peptides identical with peptides Aa3-T-T2-LA-T1 and Aa3-T-T2-L-Th-5C were isolated, as well as the peptides Ser-Ser-Ser-Gln-Pro-Val(Thr, CmCys, Asn) and Val-Ala-His-Pro-Ala-Thr(Asn), which confirmed that the sequences of the C-terminal half of peptide T-T2-LA were identical for both the Aa1 and Aa3 allotypes. However, as was the case with the thermolysin digestion of the Aa3 peptide, no small peptides could be isolated from the region between the tyrosine residue and the C-terminal peptides described above.

The limited peptic-digestion procedure used for Aa3 peptide T-T2-LA was repeated with the small amount of the Aa1 peptide remaining, and peptides equivalent to Aa3 peptides T-T2-LA-PA and T-T2-LA-PB-1 were isolated. The compositions and properties of these peptides from both allotypes were identical, although characterization of the Aa1 peptides was restricted to *N*-terminal analysis.

From these data it is concluded that, although the sequence derived for Aa3 peptide T-T2-LA differed in many positions from that described for this peptide from normal (Aa1) C-1 fragment, these differences were due to errors in sequence analysis, and that the peptides derived from Aa3 or Aa1 fragment C-1 are identical.

Discussion

The sequence described in this study for residues 110–253 of the rabbit γ chain, although showing identity for the Aa1 and Aa3 allotypes studied, shows substantial variance in the regions 110–123 and 183–200 compared with the findings obtained in previous studies (Cebra *et al.*, 1968b; Fruchter *et al.*, 1970). That these differences were due to allotype variation is unlikely, as it appears that the material used in these previous studies was largely of the Aa1 allotype (Wilkinson, 1969a). Plausible reasons for these discrepancies are, however, indicated from the present study. Thus, in deriving a sequence for the region 116–123, Fruchter *et al.* (1970) isolated, by paper electrophoresis of a chymotryptic digest of peptide T3, a large peptide presumed to span the region 116–145. In the present study chymotryptic digestion of the region T-T3-LB (residues 95–123) yielded five different peptides with C-terminal lysine, four of these peptides only being separated after a long paper-electrophoresis run (3000V for 2.5h). Such a separation would be impossible if the peptides in question were each provided with an identical extension of 22 residues (i.e. the region 124–145), and therefore the large peptide isolated by Fruchter *et al.* (1970) must have been a mixture of peptides and hence could easily provide conflicting sequence data. A similar explanation can be invoked for the differences in the sequence derived for the region 183–200, which, in the previous study, was also based on data from rather large chymotryptic peptides.

A comparison of the corrected rabbit γ chain sequence and that of the γ 1 chain of the human IgG1 myeloma protein Daw (Press & Hogg, 1969) for residues 109–253 is shown in Fig. 4. Except for the four deletions in the rabbit 'hinge' region around residue 230, expected, perhaps, because such deletions have also to be assumed to homologize the human γ chain subclass sequences in this region (Pink *et al.*, 1970), the rabbit and human γ 1 sequences appear identical in length. However, there is still some uncertainty as to the rabbit sequence around residue 191, which was not properly overlapped, and an additional one or two residues may yet have to be inserted at this point. The alignment given in Fig. 4 shows that 67% of the residues of the human and rabbit sequences for this region are identical.

The homology shown between the rabbit sequence and that of protein Daw between residues 110 and 115 is perhaps fortuitous because, although these residues are not part of the hypervariable region, differences can occur between the sequences of protein Daw and of other myeloma proteins up to residue 116 (Press & Hogg, 1969; Edelman *et al.*, 1969). This would presumably explain the difference at residue 113 (see Fig. 4) between the sequence found in the present study and that of the rabbit antibody RK 17 (a corrected version of that given by

Strosberg *et al.*, 1972; A. D. Strosberg, personal communication), an anti-pneumococcal antibody of restricted heterogeneity, although no heterogeneity of sequence could be confidently ascribed to this region in the Aa1 and Aa3 IgG pools described in the present paper. The difference between our results and the sequence of antibody RK 17 at residue 121 is more difficult to explain, as human sequence data indicate that the C region starts after residue 116. Although protein RK 17 is of the a2 allotype, this difference does not appear to be related to a difference at the *a* locus, as our findings for allotypes a1 and a3 show no C-region sequence differences. Further, the Aa2 protein R2690 (Fleischman, 1973) has a sequence identical with the one that we have found for residues 124–176. As suggested by Fleischman (1973), it is possible that the V region extends beyond residue 116 or that the sequence of antibody RK 17 represents a minor subclass of rabbit γ chain. These differences could also be due to an as yet undefined C-region allotype not related to the *a*-locus specificity.

The fact that the region 110–123 was isolated in equivalent yield to other chymotryptic peptides of peptide T3 indicates that the tryptophan residue at position 109 present in the RK 17 and Daw proteins is present also in Aa1 and Aa3 pooled IgG. However, no distinct tryptophan-containing peptide could be isolated from this region of peptide T3, and it is concluded that the major hypervariable region of the rabbit heavy chain is immediately N-terminal to this residue, as is the case in human γ chains (see Putnam *et al.*, 1971). As the rabbit V-region sequence has been completed up to residue 94 (Mole *et al.*, 1971), it would appear that virtually all the residues unaccounted for in our studies are those comprising the major hypervariable region of the V region (Putnam *et al.*, 1971; Strosberg *et al.*, 1972).

It is therefore concluded that the C regions of rabbit γ -chain Fd fragment are identical for both allotypes Aa1 and Aa3. Although the maintenance of the *a*-locus specificities within different classes of heavy chain throughout evolutionary divergence would seem to be an unlikely event, the uniqueness of the *a*-locus system of the rabbit demanded the investigation of such a possibility. It is further concluded that the differences in amino acid sequence described in the V regions of rabbit γ chain of differing *a*-locus allotype (Wilkinson, 1969a; Mole *et al.*, 1971; Fleischman, 1971) are responsible, wholly or in part, for the different serological specificities.

The following paper (Mole, 1975) further defines one of the V-region sequence differences proposed to be specific for either the a1 or the a3 allotype (Mole *et al.*, 1971) and compares the chemical inheritance of this peptide with the *a*-locus serotype.

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