Light, A. (1967b), *Methods Enzymol. 11*, 426.
Natvig, J. B., and Kunkel, H. G. (1968), *Ser. Haematol. 1*, 66.
Prahl, J. W. (1967), *Biochem. J. 105*, 1019.
Rutishauser, U., Cunningham, B. A., Bennett, C., Konigsberg, W. H., and Edelman, G. M. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 1414.
Schroeder, W. A. (1967), *Methods Enzymol. 11*, 361.
Schroeder, W. A. (1969), *Arch. Biochem. Biophys. 130*, 551.
Singer, S. J., and Doolittle, R. F. (1966), *Science 153*, 13.
Smyth, D. G. (1967), *Methods Enzymol. 11*, 421.
Thorpe, N. O., and Deutsch, H. F. (1966), *Immunochemistry 3*, 329.
Wang, A., and Fudenberg, H. H. (1969), *J. Mol. Biol. 44*, 493.
Waxdal, M. J., Konigsberg, W. H., and Edelman, G. M. (1968b), *Biochemistry 7*, 1967.
Waxdal, M. J., Konigsberg, W. H., Henley, W. L., and Edelman, G. M. (1968a), *Biochemistry 7*, 1959.
World Health Organization (1964), *Bull. W. H. O. 30*, 447.

The Covalent Structure of a Human γG-Immunoglobulin. IX. Assignment of Asparaginyl and Glutaminyl Residues*

Carl Bennett,† William H. Konigsberg, and Gerald M. Edelman‡

ABSTRACT: The locations of the asparaginyl and glutaminyl residues in the amino acid sequence of the human γG1-immunoglobulin Eu have been determined by subjecting the purified peptides to high-voltage paper electrophoresis or to complete enzymatic hydrolysis followed by amino acid analysis.

Studies on the determination of the amino acid sequence of the γG1-immunoglobulin Eu have been reported in the preceding papers of this series (Cunningham *et al.*, 1968; Gottlieb *et al.*, 1970; Cunningham *et al.*, 1970; Rutishauser *et al.*, 1970). The methods chosen for analysis of the amino acid sequences and compositions of peptides required the use of acid hydrolysis, a procedure which destroys amides. As a result, the position of asparaginyl and glutaminyl residues could not be determined directly. The purpose of this paper is to present the evidence used to establish the positions of these residues. Peptides which had been isolated previously were submitted to paper electrophoresis for determination of their mobilities or were degraded under conditions which maintained the integrity of asparaginyl and glutaminyl residues. Certain peptides contained a large number of Asx[1] and Glu residues and therefore had to be subjected to special treatment.

Materials and Methods

The isolation and purification of the peptides used have been described in previous papers of this series (Cunningham *et al.*, 1968; Gottlieb *et al.*, 1970; Cunningham *et al.*, 1970; Rutishauser *et al.*, 1970).

Leucine aminopeptidase, α-chymotrypsin, subtilisin, DFP-treated carboxypeptidase A, and carboxypeptidase B were obtained from Worthington Biochemical Corp., Freehold, N. J. Pronase was obtained from Calbiochem, Los Angeles, Calif. Aminopeptidase M was purchased from Henley and Company, New York, N. Y. Chymotrypsin C was a gift from J. E. Folk and streptococcal proteinase was a gift from S. Moore and W. H. Stein. All chemicals used were reagent grade except for pyridine which was refluxed with ninhydrin and distilled before use.

Enzymatic Digestions:

1. LEUCINE AMINOPEPTIDASE. A solution of the enzyme (0.5 mg/ml) in 5 mM sodium barbital buffer–0.25 mM in $MgCl_2$, pH 8.5, was dialyzed against distilled water for 1 hr. A 100-μl aliquot of the enzyme solution was added to approximately 30 nmoles of the peptide in 100 μl of 50 mM sodium barbital buffer–2.5 mM $MgCl_2$, pH 8.5. The mixture was incubated at 37° and digestion was terminated after 3 hr by the addition of 700 μl of 0.3 M lithium citrate buffer, pH 2.2.

2. PRONASE AND AMINOPEPTIDASE M. A solution of pronase (0.5 mg/ml, 100 μl) was added to 30 nmoles of the peptide in 100 μl of 50 mM sodium barbital buffer, pH 7.7. The mixture was incubated for 16 hr at 37°. A solution of aminopeptidase M (0.5 mg/ml, 10 μl) in the same barbital buffer was then added and the mixture was incubated for 4 hr at 37°. The digestion was terminated by addition of 0.3 M lithium citrate buffer, pH 2.2.

3. CARBOXYPEPTIDASES A AND B. A stock solution of a mixture of carboxypeptidase A and carboxypeptidase B was prepared by dissolving 50 μl of each enzyme suspension in 500 μl of 1 M lithium chloride and adding 500 μl of 1 M $NaHCO_3$. The solution was dialyzed against 0.01 M $NaHCO_3$ for 2 hr. This

* From the Department of Biochemistry, Yale University, New Haven, Connecticut, and The Rockefeller University, New York, New York. *Received December 8, 1969.* This work was supported in part by grants from the National Science Foundation (GB 6655 and GB 8371) and the U. S. Public Health Service (GM 12607, AM 04256, and AI 09273).

† Postdoctoral Fellow of the U. S. Public Health Service.

‡ Reprint requests may be addressed to G. M. Edelman, The Rockefeller University, New York, N. Y. 10021.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: Asx, aspartic acid or asparagine; Glx, glutamic acid or glutamine.

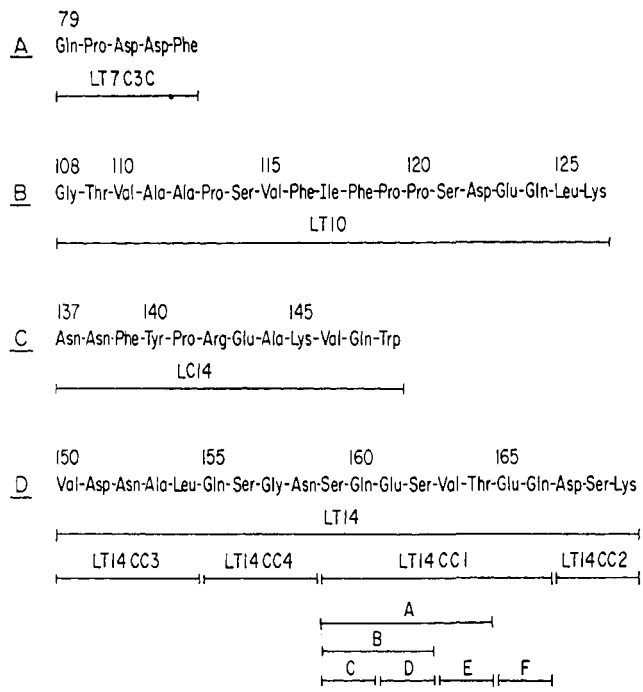

FIGURE 1: Amino acid sequence of peptides from the Eu light chain. Numbers above the sequence indicate the positions in the amino acid sequence from the NH_2 terminus of the chain (Cunningham *et al.*, 1968; Gottlieb *et al.*, 1970). Peptides LT14CC1 through LT14CC4 (Figure 1D) were obtained from a chymotrypsin C digest of LT14. Peptides A through F (Figure 1D) were obtained from a digest of LT14CC1 with streptococcal proteinase.

stock solution was stable for 3 weeks at −4°. The stock solution (50 μl) was added to about 30 nmoles of the peptide in 200 μl of 0.05 M sodium barbital buffer, pH 8.5. For digestion of large peptides, sodium dodecyl sulfate was added to a final concentration of 0.08%. The digestion was carried out at 35° for various lengths of time, and was terminated by the addition of 600 μl of 0.3 M lithium citrate buffer, pH 2.2. The solutions were centrifuged and the amino acid compositions of the supernatants were determined directly in the amino acid analyzer.

4. CHYMOTRYPSIN. Chymotrypsin (1%, w/w) was added to a solution of the peptide in 1 ml of 0.1 M NH_4HCO_3. Digestion was carried out at 37° for 3 hr and was terminated by lyophilization.

5. CHYMOTRYPSIN C. A solution of chymotrypsin C (1 mg/ml in 0.1 M NH_4HCO_3, pH 8.0) corresponding to 1% (w/w) of the peptide and 1 mg of 1,10-phenanthroline hydrochloride (Toda and Folk, 1969) were added to the peptide in 1 ml of 0.1 M NH_4HCO_3, pH 8.0. After 4 hr at 25° the digestion was terminated by lyophilization.

6. DIGESTION WITH STREPTOCOCCAL PROTEINASE AND SUBTILISIN. Digestion was performed according to procedures previously described (Gerwin *et al.*, 1966; Weber and Konigsberg, 1967).

Paper electrophoresis was performed in tanks under varsol (Savant, Hicksville, N. Y.) at pH 6.5 (pyridine–acetic acid–water, 25:1:375, v/v), and at pH 4.7 (pyridine–acetic acid–water, 1:1:38, v/v) and pH 1.9 (formic acid–acetic acid–water, 22:87:1871, v/v) (Schwartz and Edelman, 1963). A known mixture of amino acids (10 nmoles) was used as a standard. Peptides were located by spraying with 0.025% ninhydrin in 95% ethanol. The mobility of each peptide relative to that of aspartic acid was measured, and the net charge on the peptide was calculated from this mobility and from the known molecular weight (Offord, 1966). Electrophoresis of peptides containing histidine was carried out at both pH 6.5 and pH 4.7 because the imidazole ring is only partially charged at 6.5 but becomes fully charged at pH 4.7. For preparative purposes, acidic and neutral peptides were eluted from the paper with 50% pyridine and basic peptides were eluted with 50% acetic acid.

Ion-Exchange Chromatography. Separation of the peptides produced by digestion with chymotrypsin C or subtilisin was carried out on columns of AG50X4 (Cunningham *et al.*, 1968). Other buffer gradients have also been described (gradients I and II, Weber and Konigsberg, 1967).

Amino Acid Analysis. Amino acid analyses were carried out as previously described (Edelman *et al.*, 1968) except that asparagine and glutamine were determined using the lithium citrate system (Benson *et al.*, 1967).

The dansyl–Edman (Cunningham *et al.*, 1968) and the Edman (Jeppson and Sjöquist, 1967) techniques have previously been described.

Results and Discussion

Two principal methods were used in the present studies: high-voltage paper electrophoresis and amino acid analysis after prolonged enzymatic digestion. An estimate of the net charge of a peptide can be obtained from its electrophoretic mobility and molecular weight (Offord, 1966). At pH 6.5, aspartyl and glutamyl residues are negatively charged while asparaginyl and glutaminyl residues are uncharged; thus the net charge may be used as a measure of the number of acidic side chains in the peptide. Alternatively, the peptide may be subjected to prolonged enzymatic hydrolysis with pronase and aminopeptidase M. This procedure preserves asparaginyl and glutaminyl residues, which may then be measured by amino acid analysis (Benson *et al.*, 1967). One or both of these methods were usually sufficient for assignment of the positions of asparaginyl and glutaminyl residues. For some peptides, however, enzymatic digestion with leucine aminopeptidase or carboxypeptidase or degradation by the Edman technique were necessary.

The results of all of the studies are summarized in Tables I, II, and III. In most cases, the assignments are self-evident and require no further discussion. Peptides which presented particular difficulties will be discussed separately for light and heavy chains. Isolation and placement of the light-chain peptides have been described by Cunningham *et al.* (1968) and Gottlieb *et al.* (1970). Similar descriptions of the heavy-chain peptides have been given by Cunningham *et al.* (1970) and Rutishauser *et al.* (1970).

Light Chain. Data used for the assignment of asparaginyl and glutaminyl residues in the light chain are summarized in Table I. Four regions of the light-chain sequence required special attention; the sequences of these regions are presented in Figure 1.

An acidic peptide (LT7C3C) with the composition Glx, 1.1; Pro, 1.1; Asx, 1.9; Phe, 0.8 (Figure 1A, residues 79–83) was isolated by high-voltage paper electrophoresis at pH 6.5 after extensive chymotryptic digestion of peptide LT7C3. The

TABLE I: Assignment of Amides in the Light Chain.

Residue Number	Result	Peptides Used	Residues Spanned	Net Charge at pH 6.5[a]		Amino Acid Analysis[b]			
				Found	Calculated	Asp	Asn	Glu	Gln
1	Asp[c]	LT1	1–18			1.5	0.1	0.2	1.6
3	Gln	LT1	1–18						
6	Gln	LT1	1–18						
17	Asp	LT1	1–18						
27	Gln	LT3C1	25–32	0	0		0.7	0.1	1.0
30	Asn	LT3C1	25–32						
37	Gln	LC6	36–48	+3 (0.59)	+3 (0.58)				1.5
38	Gln	LC6	36–48						
55	Glu	LT6	51–61	0	0			0.9	
70	Glu	LT7C1	62–71					0.8	
79[l]	Gln	LT7C3C	79–83	−2 (0.72)	−2 (0.69)				
81[l]	Asp	LT7C3C[d]	80–83	−2 (0.82)	−2 (0.80)				
82[l]	Asp	LT7C3C[d]	80–83						
89	Gln	LC11[k]	87–91	0	0	0.1		0.1	1.4
90	Gln	LC11[k]	87–91						
92	Asn[e]	LT7C5	92–96	0	0	0.8	0.8		
94	Asp	LT7C5	92–96						
100	Gln	LT8	97–103	+1 (0.36)	+1 (0.32)				0.9
105	Glu	LT9	104–107	0	0				
122[l]	Asp	LT10	108–126	−1 (0.15)	−1 (0.17)	0.8		0.9	0.8
123[l]	Glu	LT10	108–126						
124[l]	Gln[f]	LT10	108–126						
137[l]	Asn[e]	LC14	137–148			0.1	1.5	0.8	0.7
138[l]	Asn	LC14	137–148						
143[l]	Glu	LC14	137–148						
147[l]	Gln	LC14	137–148						
151[l]	Asp[c]	LT14CC3[g]	150–154	−1 (0.38)	−1 (0.41)	0.8	0.8		
152[l]	Asn	LT14CC3[g]	150–154						
155[l]	Gln	LT14CC4[g]	155–158	0	0		0.7		0.8
158[l]	Asn	LT14CC4[g]	155–158						
160[l]	Gln	LT14CC1C[h]	159–160	0	0				
161[l]	Glu	LT14CC1D[h]	161–162	−1 (0.70)	−1 (0.71)				
165[l]	Glu	LT14CC1F[h]	165–166	−1 (0.58)	−1 (0.63)				
166[l]	Gln[i]	LT14CC1[g]	159–166						
167[l]	Asp	LT14CC2[g]	167–169	0	0	0.9			
170	Asp	LT15	170–183			0.9			
185	Asp	LT16	184–188			0.9		0.8	
187	Glu	LT16	184–188						
195	Glu	LC19SP1[j]	193–197					0.9	
199	Gln	LC19SP2[j]	198–201						0.7
210	Asn	LT19	208–211	+1 (0.44)	+1 (0.41)		0.9		
213	Glu	LT20[k]	212–214	−1 (0.51)	−1 (0.52)				

[a] The numbers in parentheses are the measured and calculated mobilities of each peptide. [b] Numbers in each column indicate the moles of amino acid obtained per mole of peptide after enzymatic digestion. [c] Identified as PTH-Asp. [d] Peptide was obtained after one step of the Edman degradation of LT7C3C. [e] Identified as PTH-Asn. [f] Determined by treatment of LT10 with carboxypeptidases A and B. [g] Peptides were obtained from a chymotrypsin C digest of LT14 (residues 150–169). [h] Peptides were isolated from a streptococcal proteinase digest of LT14CC1 (residues 159–166). [i] This assignment was based on the release of glutamine when LT14CC1 was digested with carboxypeptidase A. [j] Obtained from a streptococcal proteinase digest of LC19. [k] The cysteinyl residue is blocked with a carboxamidomethyl group. [l] The assignment of this residue is discussed in the text.

net charge of peptide LT7C3C was −2 (Table I). One step of the Edman degradation yielded PTH–Gln and the resulting tetrapeptide (residues 80–83, Table I) still had a net charge of −2, indicating that both residues 81 and 82 were aspartic acid.

Digestion of peptide LT10 (residues 108 to 126, Figure 1B)

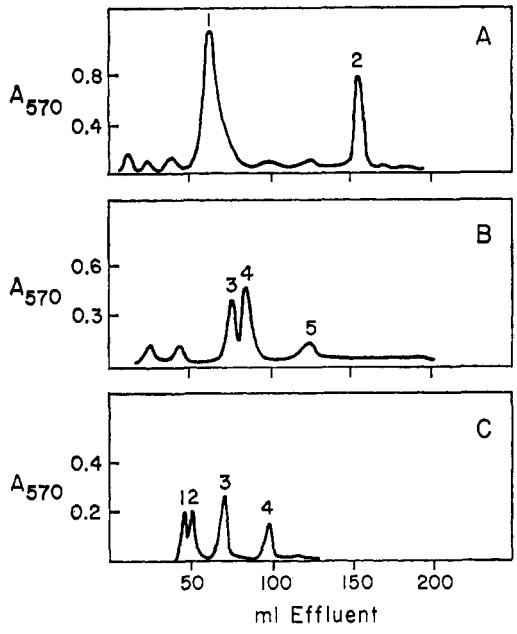

FIGURE 2: Chromatography on AG50X4. (A) Ion-exchange chromatography of peptides obtained after digestion of peptide LT14 with chymotrypsin C. Gradient I was used (see Materials and Methods). (B) Chromatography of material from fraction I (Figure 2A) using gradient II. (C) Chromatography of peptides obtained after digestion of fraction 4 (Figure of 2B) with streptococcal proteinase. Gradient II was used.

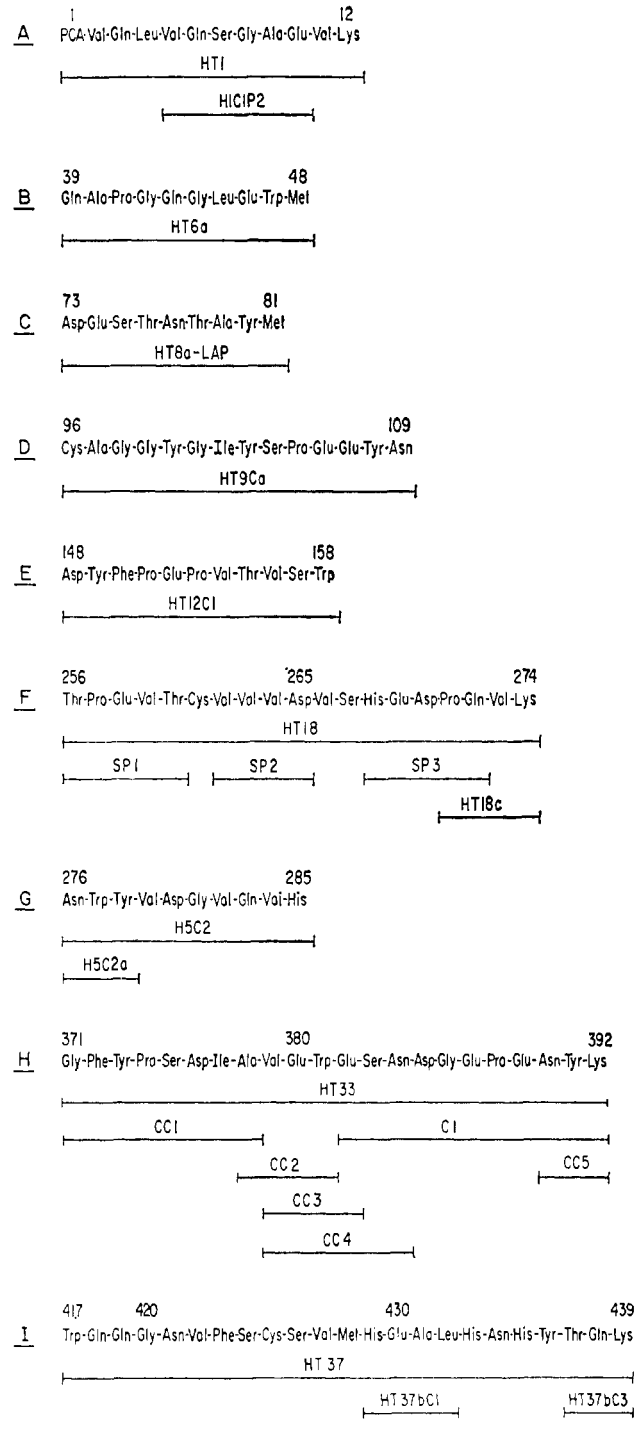

FIGURE 3: Amino acid sequence of peptides from the Eu heavy chain. Numbers above the sequence indicate the positions in the amino acid sequence from the NH_2 terminus of the chain (Cunningham *et al.*, 1970; Rutishauser *et al.*, 1970). Peptides SP1 through SP4a (Figure 3F) were obtained from a digest of HT18 with streptococcal proteinase. Peptides HT33CC1 through HT33CC5 were obtained from a digest of HT33 with chymotrypsin C. Peptide HT33C1 was obtained from a digest of HT33 with chymotrypsin.

with pronase and aminopeptidase M released one residue each of aspartic acid, glutamic acid, and glutamine. The electrophoretic mobility of LT10 (Table I) is consistent with this composition. Treatment of the peptide with carboxypeptidases A and B yielded lysine, leucine, and glutamine. From these data, we conclude that residue 124 (Figure 1B) is glutamine and that residues 122 and 123 are aspartic acid and glutamic acid, respectively.

Treatment of the peptide LC14 (residues 137 to 148, Figure 1C) with pronase and aminopeptidase M indicated that it contained two residues of asparagine and one residue each of glutamic acid and glutamine (Table I). Residues 137 and 138 therefore are asparagine. Residue 137 was also identified as PTH-asparagine after one step of the Edman degradation. Digestion of LC14 with carboxypeptidase A released 0.6 residue of tryptophan and 0.8 residue of glutamine, indicating that position 147 is Gln. Residue 143 must therefore be glutamic acid.

The assignment of an amide or a free acid to each of the nine positions in peptide LT14 (residues 150 to 169, Figure 1D) was made on the basis of analyses of smaller peptides derived from this tryptic peptide. A mixture of peptides from a chymotrypsin C digest of peptide LT14 was partially resolved by chromatography on AG50X4 (Figure 2A). Fraction 2 contained a single peptide, LT14CC2 (Asx, 1.0; Ser, 1.1; Lys, 0.9; Figure 1D). The sequence of this peptide was determined as Asx-Ser-Lys by the subtractive Edman procedure. Peptide LT14CC2 was neutral at pH 6.5 and released one residue of aspartic acid after treatment with aminopeptidase M (Table I), indicating that residue 167 is aspartic acid. The material from fraction I (Figure 2A) was further purified by ion-exchange chromatography on AG50X4 (Figure 2B). Fraction 3 from this chromatogram yielded peptide LT14CC3 (Asx, 1.9; Ala, 0.9; Val, 1.0; Leu, 1.1; Figure 1D). High-voltage electrophoresis of this peptide at pH 6.5 indicated that it contained one acidic residue, and digestion with amino-

TABLE II: Assignment of Amides in CNBr Fragments H_1 through H_4.

Residue Number	Result	Peptides Used	Residues Spanned	Net Charge at pH 6.5[a]		Amino Acid Analysis[b]			
				Found	Calculated	Asp	Asn	Glu	Gln
3[g]	Gln	HT1	1–12					0.7	2.2
6[g]	Gln	HT1	1–12						
10[g]	Glu	H1C1P2	5–10	−1 (0.37)	−1 (0.38)			0.9	0.8
39[g]	Gln	HT6a	39–48					0.9	1.9
43[g]	Gln	HT6a	39–48						
46[g]	Glu	HT6a	39–48						
59	Asn	HT6c	55–63	+1 (0.26)	+1 (0.26)		[c]		0.6
62	Gln	HT6c	55–63						
65	Gln	HT7	64–67	+1 (0.45)	+1 (0.42)				0.9
73[g]	Asp	HT8a-LAP	73–81	−2 (0.52)	−2 (0.50)	0.8	0.9	0.7	
74[g]	Glu	HT8a-LAP	73–81						
77[g]	Asn	HT8a-LAP	73–81						
82	Glu[d]	HT8b	82–87						
89	Glu	H4P1	87–93			0.8		0.9	
90	Asp	H4P1	87–93						
106	Glu[d]	HT9C3	104–121						
107	Glu[d]	HT9C3	104–121						
109[g]	Asn	HT9Ca	96–109	−2 (0.37)	−2 (0.36)				
148[g]	Asp	HT12C1	148–158	−2 (0.34)	−2 (0.41)	0.9		[c]	
152[g]	Glu	HT12C1	148–158						
159	Asn	HT12C2	159–168				0.9		
175	Gln	HT12C4	175–180	0	0				0.9
196	Gln	HT12C5	181–198	0	0				0.8
201	Asn	HT12C6	199–210	+3 (0.63)[e]	+3 (0.61)		2.7		
203	Asn	HT12C6	199–210						
208	Asn	HT12C6	199–210						
212	Asp	HT13a	211–213	0	0				
216	Glu	HT14	215–218	0	0				
221	Asp	HT15	219–222	0	0				
233	Glu	H-H-2[f]	223–241	0[e]	0				
249	Asp	HT17	240–255	0	0	0.7			

[a] The numbers in parentheses are the measured and calculated mobilities of each peptide. [b] Numbers in each column indicate the moles of amino acid obtained per mole of peptide after enzymatic digestion. [c] This residue was not released by enzymatic digestion, presumably because it is adjacent to a prolyl residue. [d] Identified as PTH-Glu. [e] Electrophoresis was carried out at pH 4.7 because histidine was present. [f] Gall *et al.* (1968). [g] The assignment of this residue is described in the text.

peptidase M followed by amino acid analysis showed that aspartic acid and asparagine were present in equal quantity. Two steps of the Edman degradation yielded PTH-Val followed by PTH-Asp. These results indicate that residue 151 is aspartic acid and that residue 152 is asparagine. Fraction 5 (Figure 2B) yielded peptide LT14CC4 (Asx, 0.9; Ser, 1.2; Glx, 0.9; Gly, 1.1; Figure 1D). Digestion of this neutral peptide (Table I) with aminopeptidase M released 0.8 residue of glutamine and 0.7 residue of asparagine. Therefore residue 155 is glutamine and residue 158 is asparagine.

Peptide LT14CC1 (residues 159–166, Figure 1D) was obtained from fraction 4 (Figure 2B). In order to obtain smaller peptides comprising this region, peptide LT14CC1 was digested with streptococcal proteinase. Separation of this digest by ion-exchange chromatography on AG50X4 (Figure 2C) yielded peptides A through F (Figure 1D). Peptides A, B, and C (Figure 1D) were obtained by high-voltage paper electrophoresis of fraction 3 (Figure 2C) at pH 1.9. Peptides D and F were obtained from fractions 1 and 2, respectively. Fraction 4 contained peptide E. The amino acid sequences of peptides A through E were determined by the dansyl–Edman procedure. The net charges of peptides C and D (Table I) indicate that residue 160 is glutamine and residue 161 is glutamic acid. Peptide F yielded only glutamic acid after acid hydrolysis, but its net charge (Table I) indicated that it contained one residue each of glutamic acid and glutamine. Positive Edman degradation liberated PTH-glutamic acid, indicating that residue 165 is glutamic acid and residue 166 is glutamine. This result was confirmed by digestion of LT14CC1 with carboxypeptidase A. These data complete the assignment of amides in peptide LT14 (Figure 1D).

Heavy Chain. The data used for the assignment of the positions of glutamic acid, glutamine, aspartic acid, and asparagine in the heavy chain are presented in Tables II and III.

TABLE III: Assignment of Amides in CNBr Fragments H_5 through H_7.

Residue Number	Result	Peptides Used	Residues Spanned	Net Charge at pH 6.5[a]		Amino Acid Analysis[b]			
				Found	Calculated	Asp	Asn	Glu	Gln
258[o]	Glu	HT18SP1[c]	256–260	−1 (0.42)	−1 (0.40)				
265[o]	Asp	HT18SP2[c]	262–265			0.7			
269[o]	Glu	HT18SP3[c]	268–272	−1[d] (0.34)	−1 (0.37)	*e*		0.9	*e*
270[o]	Asp	HT18SP3[c]	268–272						
272[o]	Gln	HT18c[f]	271–274	+1 (0.33)	+1 (0.42)				
276[o]	Asn	H5C2a	276–278				0.9		
280[o]	Asp	H5C2	276–285	0[d]	0	0.9	0.9		
283[o]	Gln	H5C2	276–285						
286	Asn	H5C3T1	286–288				0.8		
293	Glu[g]	HT21	293–301						
294	Gln	HT21A1	293–296					0.9	1.9
295	Gln	HT21A1	293–296						
297	Asx[h]	HT21	293–301						
311	Gln	H5C5a	307–313	+1[d] (0.17)	+1 (0.29)		0.6		0.4
312	Asn	H5C5a	307–313						
315	Asp	H5C5b	314–319	−1 (0.37)	−1 (0.33)	0.8		0.8	
318	Glu	HT23	318–320	0	0			1.0	
325	Asn	HT25	323–326				0.7		
333	Glu	HT26	327–334	0	0			0.8	
342	Gln	HT29	341–344	+1 (0.47)	+1 (0.45)				
345[o]	Glu	HT30C1[i]	345–349	−1 (0.38)	−1 (0.36)				
347[o]	Gln	HT30C1[j]	346–349	0	0				
356	Glu	HO4a[k]	350–358					1.7	
357	Glu	HO4a[k]	350–358						
361	Asn	HT32SP1[l]	361–365	0	0		0.9		0.8
362	Gln	HT32SP1[l]	361–365						
376[o]	Asp	HT33CC1[m]	371–378			0.8			
380[o]	Glu	HT33CC2[m]	378–381					0.8	
382[o]	Glu	HT33CC3[m]	379–382					1.7	
384[o]	Asn	HT33CC4[m]	379–384				0.6	1.7	
385[o]	Asp	HT33C1[n]	382–392	−3 (0.63)	−3 (0.64)				
387[o]	Glu[g]	HT33C1[n]	382–392						
389[o]	Glu[g]	HT33C1[n]	382–392						
390[o]	Asn	HT33CC5[m]	390–392	+1 (0.47)	+1 (0.48)		0.8		
399	Asp	HT34a	393–404	−2 (0.43)	−2 (0.44)				
401	Asp	HT34a	393–404						
413	Asp	HT35	410–414	0	0	0.8			
418[o]	Gln	HT37	417–439				2.0	1.0	3.0
419[o]	Gln	HT37	417–439						
421[o]	Asn	HT37	417–439						
430[o]	Glu	HT37bC1	429–432	0[d]	0			0.8	
434[o]	Asn	HT37	417–439				2.0	1.0	3.0
438[o]	Gln	HT37bC3	437–439						0.9

[a] The numbers in parentheses are the measured and calculated mobilities of each peptide. [b] Numbers in each column indicate the moles of amino acid obtained per mole of peptide after enzymatic digestion. [c] These peptides were derived from a streptococcal proteinase digest of HT18 (residues 256–274). [d] Electrophoresis was carried out at pH 4.7 because histidine was present. [e] This residue was not released by enzymatic digestion, presumably because it is adjacent to a prolyl residue. [f] Obtained from a tryptic digest of Fc(t). See text. [g] Identified as PTH-Glu. [h] The carbohydrate is attached to this residue. [i] This peptide was obtained from a chymotryptic digest of HT30 (residues 345–355). [j] This peptide was obtained after one step of the Edman degradation of peptide HT30C1 (residues 345–349). [k] Isolated by diagonal electrophoresis (Tang and Hartley, 1967) of a tryptic and chymotryptic digest of Fc(t). [l] This peptide was obtained from a streptococcal proteinase digest of HT32 (residues 361–370). [m] These peptides were isolated from a chymotrypsin C digest of HT33 (residues 371–392). [n] This peptide was obtained from a chymotryptic digest of HT33 (residues 371–392). [o] The assignment of this residue is discussed in the text.

Most of the assignments were straightforward, but eight peptides presented special difficulties which require discussion.

The NH_2-terminal tryptic peptide the of heavy chain, HT1 (residues 1–12, Figure 3A), contained one glutamic acid and two glutamines (Table II). It had previously been established that residue 3 is glutamine (Cunningham *et al.*, 1970). Peptide HlClP2 (residues 5–10; Figure 3A, Table II) contained one residue each of glutamine and glutamic acid. Treatment of this peptide with carboxypeptidase A at pH 5.5 released one residue of glutamic acid (residue 10), and digestion with leucine aminopeptidase released one residue each of valine, glutamine (residue 6), and serine.

Peptide HT6a (residues 39–48, Figure 3B) contained one glutamic acid and two glutamines. PTH-Gln was identified after one step of the Edman degradation, indicating that residue 39 is glutamine. Digestion of HT6a with carboxypeptidase A at pH 8 released one residue each of homoserine and tryptophan. After lowering the pH to 5.5 and the addition of more enzyme, 0.7 residue of glutamic acid (position 46) was released after 6 hr. Therefore, residue 46 is glutamic acid and residue 43 must be glutamine.

Peptide HT8a-LAP (residues 73–81, Figure 3C) contained one residue each of aspartic acid, glutamic acid, and asparagine. Residues 73 and 74 were assigned as aspartic acid and glutamic acid because PTH-Asp and PTH-Glu were identified after the first and second steps, respectively, of Edman degradation. Therefore, residue 77 was assigned as asparagine.

Electrophoresis of a chymotryptic digest of HT9 gave peptide HT9Ca (residues 96–109, Figure 3D), which had a net charge of −2. Residues 106 and 107 were determined as PTH-Glu by Edman degradation of peptide HT9C3 (residues 104–121). Therefore residue 109 must be asparagine.

Digestion of peptide HTl2Cl (residues 148–158, Figure 3E) with aminopeptidase M released the aspartic acid at position 148. Because the net charge of this peptide was −2, residue 152 must be glutamic acid.

The remaining data in Table II complete the assignment of asparaginyl and glutaminyl residues in the region corresponding to CNBr fragments H_1 through H_4. Similar data for the region corresponding to CNBr fragments H_5 through H_7 are given in Table III.

The assignment of asparaginyl and glutaminyl residues in the region from residue 256 to 274 (peptide HT18, Figure 3F, Table III) depended on the isolation of smaller fragments corresponding to this region. Peptides from a streptococcal proteinase digest of HT18 (Figure 3F, Table III) were separated by ion-exchange chromatography on AG50X4. Peptide SP1 (residues 256–260) had a net charge of −1, and therefore residue 258 must be glutamic acid. Pronase and aminopeptidase M digestion of peptide SP2 (Figure 3F, residues 262–265) released 0.7 residue of aspartic acid (residue 265, Table III). Peptide SP-3 (Figure 3F, residues 268–272) had a net charge of −1 at pH 4.7 indicating that two residues must be present as free acids. Treatment of this peptide with aminopeptidase M released 0.9 residue of glutamic acid in accord with the sequence His-Glu-Asx-Pro-Glx. The electrophoretic mobility of peptide HTl8c (Figure 3F) indicated that residue 272 is glutamine; residue 270 therefore must be aspartic acid. Peptide HT18c was isolated in high yield from a tryptic digest of Fc(t). As tryptic cleavage of an Asp–Pro bond seems unlikely, this peptide probably resulted from nonenzymatic cleavage (E. L. Smith, 1969, personal communication) during the isolation procedures.

Digestion of peptide H5C2 (residues 276–285, Figure 3G) with pronase and aminopeptidase M released 0.9 residue of asparagine and 0.9 residue of aspartic acid. For unknown reasons, neither glutamic acid nor glutamine was released. A similar digestion of peptide H5C2a (residues 276–278, Figure 3G, Table III) showed that residue 276 is asparagine, and therefore residue 280 must be aspartic acid. Peptide H5C2, which contained one histidine, was neutral at pH 4.7, indicating that residue 283 is glutamine.

In order to determine the positions of the asparagine and glutamine in the region from residues 371 to 392 (Figure 3H), peptide HT33 was digested with chymotrypsin C. The resulting peptides (CC1 to CC5, Figure 3H) were fractionated by chromatography on AG50X4. The assignments of aspartic acid to position 376, glutamic acid to positions 380 and 382, and asparagine to positions 384 and 390 were straightforward from the amino acid compositions (Table III) of pronase and aminopeptidase M digests of peptides CC1, CC2, CC3, CC4, and CC5 (Figure 3H). Because peptide HT33C1 (Figure 3H) had a net charge of −3 at pH 6.5 (Table III), the remaining positions (residues 385, 387, and 389) must be the free acids. To confirm these assignments, PTH-glutamic acid was identified from the Edman degradation of HT33C1 in the positions corresponding to residues 387 and 389.

Digestion of peptide HT37 (Figure 3I) with pronase and aminopeptidase M yielded two residues of asparagine, one residue of glutamic acid, and three residues of glutamine (Table III). Residues 421 and 434 therefore are asparagine. The amino acid composition and net charge of peptide HT37bC1 (Figure 3I, Table III) indicate that residue 430 is glutamic acid; thus residues 418, 419, and 438 must be glutamine. The amino acid composition of peptide HT37bC3 (Table III) confirms these assignments. Together with the data in Table III, this completes the assignments of the asparaginyl and glutaminyl residues in the light and heavy chains of protein Eu. Some sources of possible error are considered below.

Many of these studies were carried out on peptides which were obtained from CNBr fragments and thus had been exposed to 70% formic acid for 4 hr at room temperature. In addition, many purification steps were required to obtain some of the peptides. Therefore, it is possible that some amide groups were lost prior to analysis. No evidence indicates that such deamidation occurred to any significant extent.

The sequence Asx-Gly appears in three places in the Eu heavy chain (residues 109–110, 385–386, and 401–402). In the determination of the amino acid sequences of peptides containing residues 109, 385, and 401, the Edman degradation was severely limited or actually halted at these positions. This suggests that the rearrangement of the α-peptide bond to the β peptide or the corresponding imide (see Weber and Konigsberg, 1967; also Jackson and Hirs, 1970) occurs readily. Although this rearrangement probably took place during the Edman degradation, we cannot rigorously exclude the possibility that rearrangement occurred during the isolation of the peptides. Such a rearrangement may alter the electrophoretic mobility of a peptide and hence we assign residue 109 as asparagine and residues 385 and 401 as aspartic acid with some reservation.

Of the 116 positions in the sequence of Eu at which the assignment of an amide or a free acid was required, 23 were

made solely on the basis of electrophoretic mobility. Mobilities were measured for a total of 49 peptides. The observed mobility of each peptide was within ten per cent of the calculated mobility in all but three cases. For these three, additional evidence was obtained to confirm the assignments. In no case did the electrophoretic mobility of a peptide suggest an assignment different from that indicated by other methods.

References

Benson, J. V., Jr., Gordon, M. J., and Patterson, J. A. (1967), *Anal. Biochem. 18*, 228.

Cunningham, B. A., Gottlieb, P. D., Konigsberg, W. H., and Edelman, G. M. (1968), *Biochemistry 7*, 1983.

Cunningham, B. A., Rutishauser, U., Gall, W. E., Gottlieb, P. D., Waxdal, M. J., and Edelman, G. M. (1970), *Biochemistry 9*, 3161.

Edelman, G. M., Gall, W. E., Waxdal, M. J., and Konigsberg, W. H. (1968), *Biochemistry 7*, 1950.

Gall, W. E., Cunningham, B. A., Waxdal, M. J., Konigsberg, W. H., and Edelman, G. M. (1968), *Biochemistry 7*, 1973.

Gerwin, B. I., Stein, W. H., and Moore, S. (1966), *J. Biol. Chem. 241*, 3331.

Gottlieb, P. D., Cunningham, B. A., Rutishauser, U., and Edelman, G. M. (1970), *Biochemistry 9*, 3155.

Jackson, R. L., and Hirs, C. H. W. (1970), *J. Biol. Chem. 245*, 637.

Jeppsson, J. O., and Sjöquist, J. (1967), *Anal. Biochem. 18*, 264.

Offord, R. E. (1966), *Nature (London) 211*, 591.

Rutishauser, U., Cunningham, B. A., Bennett, C., Konigsberg, W. H., and Edelman, G. M. (1970), *Biochemistry 9*, 3171.

Schwartz, J. H., and Edelman, G. M. (1963), *J. Exp. Med. 118*, 41.

Tang, J., and Hartley, B. S. (1967), *Biochem. J. 102*, 593.

Toda, H., and Folk, J. D. (1969), *Biochim. Biophys. Acta 175*, 427.

Weber, K., and Konigsberg, W. H. (1967), *J. Biol. Chem. 242*, 3563.

The Covalent Structure of a Human γG-Immunoglobulin. X. Intrachain Disulfide Bonds*

W. Einar Gall and Gerald M. Edelman

ABSTRACT: The arrangement of the 12 intrachain disulfide bonds in the human γG-immunoglobulin Eu has been determined. The first intrachain bond in each light (κ) chain is formed by half-cystinyl residues at positions 23 (designated I, numbering from the NH_2 terminus) and 88 (II), and the second is formed by half-cystines at positions 134 (III) and 194 (IV). The four intrachain bonds in each heavy (γ1) chain are formed by half-cystines at positions 22 (I) and 96 (II), 144 (III) and 200 (IV), 261 (VIII) and 321 (IX), and 367 (X) and 425 (XI). Each disulfide loop in the C_L and C_H regions of the molecule contains from 57 to 61 residues; the V_L loop contains 66 residues and the V_H loop contains 75 residues. These data indicate that the intrachain bonds form loops which have a linear and periodic arrangement and provide additional evidence for the hypothesis that immunoglobulins evolved by gene duplication.

A previous paper in this series (Gall *et al.*, 1968) described the location and arrangement of the interchain disulfide bonds in the human γG1-immunoglobulin Eu. A linear arrangement of all of the intrachain disulfide bonds of the molecule was first proposed by Waxdal *et al.* (1967). We now present proof of this arrangement.

Peptides containing intrachain disulfide bonds have been isolated from Bence–Jones proteins and light chains of other immunoglobulins by Milstein and coworkers (for a review, see Milstein, 1966b), and the location of these peptides in the light chains has been suggested by comparison with amino acid sequences obtained in several laboratories. Peptides containing some of the intrachain disulfide bonds have been isolated from the four subclasses of γ chains (Frangione and Milstein, 1967; Pink and Milstein, 1967; Frangione *et al.*, 1968, 1969), and these authors also suggested a linear arrangement for these bonds. The results of the present studies, together with those on the interchain bonds (Gall *et al.*, 1968) and the determination of the complete amino acid sequence of protein Eu (Cunningham *et al.*, 1968; Gottlieb *et al.*, 1970; Cunningham *et al.*, 1970; Rutishauser *et al.*, 1970), establish the exact location of all of the disulfide bonds in the molecule.

Materials and Methods

Purification of the immunoglobulin Eu and preparation of the CNBr fragments have been described (Edelman *et al.*,

* From The Rockefeller University, New York, New York 10021. *Received December 8, 1969.* This work was supported in part by grants from the National Science Foundation (GB 8371) and the U. S. Public Health Service (AM 04256 and AI 09273).