The Covalent Structure of a Human γG-Immunoglobulin. VIII. Amino Acid Sequence of Heavy-Chain Cyanogen Bromide Fragments H_5–H_7*

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ABSTRACT: The amino acid sequence of the CNBr fragments H_5–H_7 of the γ chain of the immunoglobulin Eu has been determined. These fragments comprise residues 253–446 of the heavy chain and include all but the first 30 residues of the Fc(t) region. Completion of this sequence has allowed a detailed comparison of all of the constant portions of the molecule.

Two regions of the γ chain named C_H2 (residues 234–341), and C_H3 (residues 342–446) were found to be homologous in sequence to each other, to the previously described C_H1 region of the heavy chain, and to the C_L region of the κ chain. Comparison of the sequences of C_H2 and C_H3 with the reported sequence of corresponding portions of rabbit γ chains showed extensive homologies. These findings support the hypothesis that immunoglobulin chains evolved by means of a series of gene duplications.

Cleavage of the heavy chain of the γG-immunoglobulin Eu with CNBr has been shown to produce seven fragments (Waxdal *et al.*, 1968a,b). CNBr fragments H_1–H_4 contain the Fd(t)[1] region and the first 30 residues of the Fc(t) region (Cunningham *et al.*, 1970). The constant region of the heavy chain begins at residue 115 and its sequence has been completed through residue 252 (Cunningham *et al.*, 1970; Gall *et al.*, 1968). Fragments H_5, H_6, and H_7 make up the remainder of the constant region.

In this paper we describe the determination of the amino acid sequence of CNBr fragments H_5, H_6, and H_7 of protein Eu. The sequence of this portion of the molecule is compared with the complete amino acid sequences of the CNBr fragments H_1–H_4 (Cunningham *et al.*, 1970), with the κ chain from protein Eu (Gottlieb *et al.*, 1970), and with the partial sequence of rabbit Fc fragment (Hill *et al.*, 1967).

Materials and Methods

Preparation of the Fc(t) fragment by limited tryptic digestion of protein Eu has been described (Edelman *et al.*, 1968). The CNBr fragments H_5, H_6, and H_7 were prepared from fully reduced and alkylated Fc(t) (Waxdal *et al.*, 1968b). In one case, fully reduced Fc(t) was alkylated (Cole, 1967) using ethylenimine (K & K Laboratories, Plainview, N. Y.).

Methods of Peptide Isolation and Characterization. General methods of peptide fractionation by ion-exchange chromatography and gel filtration, amino acid analysis, amino-terminal analysis, and amino acid sequence determination have been described (Cunningham *et al.*, 1968; Edelman *et al.*, 1968; Gottlieb *et al.*, 1970). Peptides were also fractionated on columns of SE-Sephadex C-25 (Pharmacia, Uppsala, Sweden) and DEAE-cellulose (DE-52, Whatman, W. and R. Balston, England). Peptides were eluted at 50° from 0.9 × 50 cm columns of SE-Sephadex using a linear gradient from 0.05 M pyridinium-acetate (pH 3.1, 250 ml) to 1.0 M pyridinium-acetate (pH 5.6, 250 ml). DEAE-cellulose columns of the same dimensions were eluted with a linear gradient from 0.05 M Tris-HCl (pH 8.5, 300 ml), to the same buffer (300 ml) which was 1.0 M in NaCl. Paper chromatography was performed using a solvent consisting of *n*-butyl alcohol–acetic acid–H_2O–pyridine (15:3:12:10, v/v) (Bennett, 1967).

Enzymatic Digestions. TRYPSIN. Fully reduced and aminoethylated Fc(t) (700 mg) was suspended in 35 ml of water and digested with 7.0 mg of TPCK-trypsin (Calbiochem, Los Angeles, Calif., B Grade, lot no. 73320; retreated with TPCK just before use) at 37° and pH 8.0 in a pH-Stat. The pH was maintained by addition of 1.0 N NH_4OH. After 1 hr the digestion was terminated by lyophilization. CNBr fragments H_6 and H_7 and chymotryptic peptide H5C3 were digested with 1% (w/w) trypsin in 0.05 M Tris-HCl (pH 8.0) for 4 hr at 37° and then lyophilized.

CHYMOTRYPSIN. Fully reduced and alkylated Fc(t) (180 mg) was suspended in 35 ml of water and digested with 2.1 mg of α-chymotrypsin (Worthington Biochemical Corp., Freehold, N. J., three-times crystallized, CD1-6150-1) at 37° and pH 8.0 in a pH-Stat. The pH was maintained by addition of 1.0 N NH_4OH. After 4-hr digestion the clear solution was lyophilized. Tryptic peptides HT32 and HT37 and Arg-tryptic peptides (see below) AT-1, AT-2, and AT-3 were digested with 1% (w/w) α-chymotrypsin in 0.05 M Tris-HCl

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: Fc(t) and Fab(t), tryptic fragments corresponding to Fc and Fab (World Health Organization, 1964); dansyl, 1-dimethylaminonaphthalene-5-sulfonyl; Asx, aspartic acid or asparagine; Glx, glutamic acid or glutamine; DBDA, 1,3-dinitro-2,4-benzenedisulfonic acid; TPCK, L-1-tosylamino-2-phenylethyl chloromethyl ketone; Gd, guanidine.

TABLE I: Amino Acid Compositions of Tryptic Peptides from CNBr Fragments H_5 to H_7.[a]

	HT17	HT18	HT18a	HT18b	HT19	HT20	HT21	HT22,23	HT22a	HT22b	HT23
Lys		1.1 (1)		1.0 (1)	1.1 (1)	1.0 (1)		2.1 (2)		1.0 (1)	1.1 (1)
His		1.0 (1)		0.9 (1)	0.9 (1)			0.9 (1)		1.0 (1)	
Arg	0.8 (1)					1.0 (1)	1.1 (1)				
CM-Cys[b]		0.7 (1)									
AE-Cys[b]			1.0 (1)								
Asp	0.8 (1)	2.2 (2)		2.0 (2)	2.8 (3)		1.0 (1)	2.2 (2)		2.0 (2)	
Thr	0.9 (1)	1.8 (2)	2.0 (2)			1.0 (1)	0.9 (1)	0.9 (1)		0.9 (1)	
Ser	1.1 (1)	1.2 (1)	0.3	0.9 (1)		0.2	1.1 (1)	1.0 (1)	1.0 (1)		
Glu		3.0 (3)	1.0 (1)	2.1 (2)	1.1 (1)		2.8 (3)	2.0 (2)		1.1 (1)	1.0 (1)
Pro		2.1 (2)	1.0 (1)	1.1 (1)		1.0 (1)					
Gly		0.2	0.3		1.2 (1)	0.2		1.1 (1)		1.1 (1)	
Ala					1.0 (1)			0.2			
Val		5.1 (6)[c]	0.9 (1)	4.2 (5)[c]	2.8 (3)			3.6 (4)	2.7 (3)	1.2 (1)	
Met	0.9 (1)										
Ile	1.0 (1)										
Leu	1.1 (1)	0.2						2.5 (3)	1.1 (1)	2.1 (2)	
Tyr					0.9 (1)		1.9 (2)	0.9 (1)			0.9 (1)
Phe					1.2 (1)						
Trp					(1)			(1)		(1)	
Total residues	7	19	6	13	14	4	9	19	5	11	3
Yield (%)[d]	40	10	15	20	25	20	80	5	20	25	40

	HT24	HT25	HT26	HT27	HT28	HT29	HT30,31	HT30	HT31	HT32	HT33
Lys	1.0 (1)	1.1 (1)	0.9 (1)	1.0 (1)	1.0 (1)		1.0 (1)	0.2	0.9 (1)	1.0 (1)	0.9 (1)
His											
Arg						0.9 (1)	1.0 (1)	0.9 (1)			
CM-Cys	0.8 (1)									0.9 (1)	
AE-Cys											
Asp		1.0 (1)								0.9 (1)	4.0 (4)
Thr		0.2		0.9 (1)			1.7 (2)	0.9 (1)	1.2 (1)	1.1 (1)	
Ser		1.2 (1)	0.2	1.0 (1)			1.0 (1)	1.2 (1)	0.3	1.1 (1)	2.0 (2)
Glu			1.1 (1)			1.0 (1)	4.0 (4)	2.0 (2)	2.0 (2)	1.1 (1)	4.0 (4)
Pro			2.2 (2)			0.9 (1)	2.8 (3)	3.0 (3)		0.2	2.0 (2)
Gly		0.2		0.2		1.0 (1)		0.2	0.3	0.2	2.0 (2)
Ala			1.7(2)		1.0 (1)				0.2		1.0 (1)
Val		1.0 (1)					1.0 (1)	1.0 (1)		1.7 (2)	1.0 (1)
Met									0.8 (1)		
Ile			1.0 (1)	1.0 (1)							0.9 (1)
Leu			1.1 (1)				1.0 (1)	1.0 (1)	0.3	1.9 (2)	
Tyr							0.8 (1)	0.9 (1)			1.7 (2)
Phe											0.9 (1)
Trp											(1)
Total residues	2	4	8	4	2	4	16	11	5	10	22
Yield (%)[d]	25	40	65	50	85	75	15	45	5	20	60

	HT34	HT34a	HT34b	HT35	HT36	HT37	HT37b	HT38	AT3[e]
Lys	0.9 (1)		0.9 (1)	0.9 (1)		1.0 (1)	1.0 (1)		(7)
His						2.8 (3)	2.9 (3)		1.0 (1)
Arg					0.9 (1)				1.0 (1)
CM-Cys						1.0 (1)			(1)
AE-Cys									
Asp	2.0 (2)	2.2 (2)		1.1 (1)		2.1 (2)	1.1 (1)		3.1 (3)
Thr	2.2 (2)	2.0 (2)		1.0 (1)		1.0 (1)	0.9 (1)		2.1 (2)
Ser	3.1 (3)	2.0 (2)	1.1 (1)	0.2	1.1 (1)	1.8 (2)		2.8 (3)	3.5 (3)
Glu	0.8					4.1 (4)	2.1 (2)		4.0 (4)
Pro	2.0 (2)	1.8 (2)						1.1 (1)	2.8 (3)

TABLE I (*Continued*)

	HT34	HT34a	HT34b	HT35	HT36	HT37	HT37b	HT38	AT3[a]
Gly	1.4 (1)	1.1 (1)	0.2			0.9 (1)		1.0 (1)	2.2 (2)
Ala	0.3		0.2			1.0 (1)	1.0 (1)		2.9 (3)
Val	1.2 (1)	1.0 (1)		1.0 (1)		2.1 (2)			4.4 (5)
Met						0.9 (1)			
Ile									1.7 (2)
Leu	2.1 (2)	1.1 (1)	0.9 (1)	1.0 (1)		1.0 (1)	1.2 (1)	2.1 (2)	4.0 (4)
Tyr	1.0 (1)		0.9 (1)			1.0 (1)	0.8 (1)		0.8 (1)
Phe	2.0 (2)	1.8 (2)				0.9 (1)			
Trp						(1)			(1)
Total residues	17	13	4	5	2	23	11	7	43
Yield (%)[d]	25	25	20	50	60	17	80	60	

[a] Values reported are amino acid residues. Amino acids present at a level of 0.1 residue are omitted. The assumed integral numbers of residues are given in parentheses. [b] CM-Cys = carboxymethylated Cys; AE-Cys = aminoethylated Cys. [c] Val values are low because of incomplete hydrolysis of a Val-Val-Val sequence. [d] Yields are based on micromoles of peptides isolated compared with micromoles of protein originally digested with trypsin. [e] Lys residues of this peptide have been modified by the DBDA reagent. DBDA-Lys and CM-Cys are eluted together during amino acid analysis.

(pH 8.0) for 4 hr at 37°. The digests were terminated by lyophilization.

OTHER ENZYMES. Digestions of peptides were performed with DFP-treated carboxypeptidases A and B (Worthington Biochemical Corp., Freehold, N. J.), and subtilisin (Nagarse, Teikoku Chemical Industries, Osaka, Japan), as described by Ambler (1967), Light (1967b), and Smyth (1967). Partial acid hydrolysis of peptides was performed at 110° for 12 hr using 0.01 M HCl (Light, 1967a).

Preparation of Arg-tryptic Peptides. The designation Arg-tryptic peptides is given to those peptides obtained by tryptic digestion of Fc(t) fragments which had been reacted with DBDA to block the ε-amino groups of lysine. This procedure results in selective cleavage at arginine (H. J. Dintzis, personal communication). Fully reduced and carboxymethylated Fc(t) (250 mg) was dissolved in 25 ml of 6 M Gd·HCl–0.1 M Tris-HCl (pH 9.3), and 40 mg of DBDA was added. The mixture was reacted for 16 hr at 50°, and then dialyzed against distilled water. The DBDA derivative of Fc(t) was then digested with trypsin as described above.

To determine the extent of substitution by the DBDA reagent a small portion of the Fc(t) derivative was digested with trypsin for 4 hr. Carboxypeptidase B was then added and the digestion was continued for 4 more hr. This material was submitted to amino acid analysis without prior hydrolysis, and the ratio of free lysine to free arginine was used as a measure of the extent of substitution.

To remove the DBDA blocking group, peptides were dissolved in concentrated NH_4OH which was 0.1 M in ammonium acetate (final pH 10.5), and the solution was incubated at 50° for 1 hr. After removal of the ammonia by evaporation under reduced pressure, the peptides were desalted on Sephadex G-25.

Results

The order of the CNBr fragments which comprise the COOH-terminal 194 residues of the heavy chain is given in Figure 1 (Waxdal *et al.*, 1968a). Because of the large size of this region, it was necessary to employ several approaches in the isolation of the tryptic and chymotryptic peptides. CNBr fragments H_5 and H_6 were not readily obtained in large amounts, presumably because of nonquantitative cleavage of the Met–Thr bond linking these fragments (Schroeder, 1969; Cunningham *et al.*, 1968). For this reason many of the peptides were obtained from digests of Fc(t) rather than from digests of the CNBr fragments.

The sequences of regions corresponding to each of the CNBr fragments will be discussed beginning at the NH_2 terminus of each fragment. Tryptic peptides are numbered consecutively from the NH_2 terminus of the heavy chain (Cunningham *et al.*, 1970). Accordingly, the region spanning CNBr fragments H_5–H_7 contains 23 tryptic peptides commencing with peptide HT17b. Peptides obtained from chymotryptic and peptic digests of the Fc(t) fragment are named on the basis of their location in the appropriate CNBr fragment. Before we present the evidence for the amino acid sequence of these fragments we will consider the isolation of the tryptic and chymotryptic peptides.

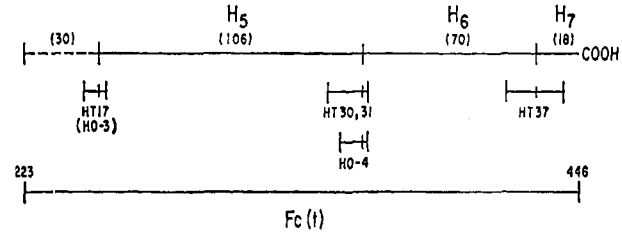

FIGURE 1: CNBr fragments H_5–H_7 of the immunoglobulin Eu. This structure is based upon analysis of the intact CNBr fragments, the Fc(t) fragment, and of tryptic peptides HT17 (HO-3), HT30, 31, and HO-4, and HT37. The number of amino acid residues in each CNBr fragment is indicated in parentheses. Met-253, -358, and -428 of the heavy chain are indicated by vertical lines between CNBr fragments and within peptides. The numbering is that of the complete heavy chain.

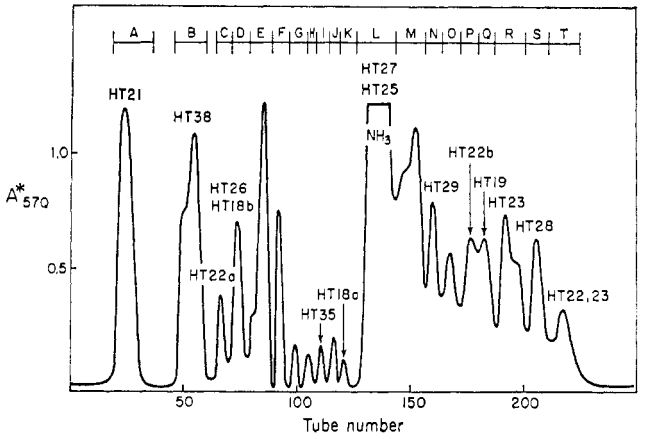

FIGURE 2: Ion-exchange chromatography of a tryptic digest of 25 μmoles of Fc(t) on an Aminex-50B column (1.8 × 50 cm) at 60°. Elution was performed using a linear gradient from 2.0 l. of 0.2 M pyridinium-acetate (pH 3.1) to 2.0 l. of 2.0 M pyridinium-acetate (pH 5.6). The solid line denotes the ninhydrin color yield on an arbitrary scale (A_{570}*) which was determined automatically (Cunningham *et al.*, 1968). Each tube contained 8 ml of effluent.

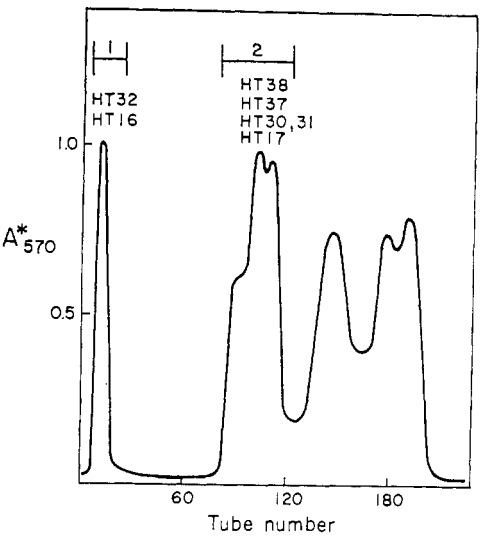

FIGURE 4: Ion-exchange chromatography of tryptic peptides of Fc(t) on a column (0.9 × 15 cm) of AG1X4 at 37°. Peptides were eluted by the method of Schroeder (1967). The solid line represents the ninhydrin color yield (A_{570}*) on an arbitrary scale. Each tube contained 1.8 ml of effluent.

Isolation of Tryptic Peptides. The amino acid compositions and yields of tryptic peptides obtained from Fc(t) and CNBr fragments H_5, H_6, and H_7 are summarized in Table I. Four approaches were used to isolate the tryptic peptides: (1) cation-exchange chromatography of Fc(t) digests, (2) preliminary fractionation of Fc(t) digests on Sephadex G-25 followed by cation-exchange chromatography, (3) initial separation of Fc(t) digests on Sephadex G-50 followed by anion-exchange chromatography, and (4) digestion of the individual CNBr fragments followed by ion-exchange chromatography.

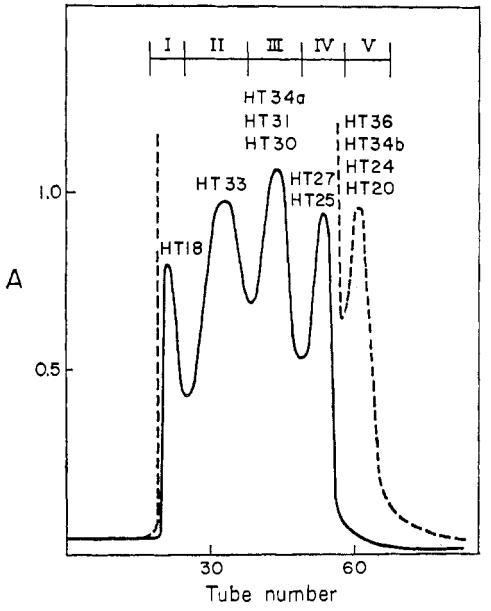

FIGURE 3: Fractionation of a tryptic digest of 8 μmoles of Fc(t) on a column of Sephadex G-25 (1.2 × 200 cm) in 0.01 N NH_4OH. A similar fractionation on Sephadex G-50 yielded essentially the same pattern. The solid and dashed lines represent the absorbance at 280 and 230 mμ, respectively, using a 1-mm path length. The peptides obtained from each fraction by further purification are indicated above their respective peaks. Each tube contained 5.5 ml of effluent.

Figure 2 shows the elution pattern obtained by ion-exchange chromatography of a tryptic digest of fully reduced and aminoethylated Fc(t) on Aminex-50B. Peptides HT19, HT28, and HT35 (Table I) were isolated directly from fractions Q, S, and I, respectively (Figure 2). Peptides HT21, HT38, HT18a, HT29, and HT22,23 were isolated from fractions A, B, K, N, and T, respectively, by chromatography on AG50X4. Chromatography of fraction R on SE-Sephadex yielded peptide HT23. Peptides HT22a and HT22b were obtained directly from fractions C and P. Chromatography of fraction D on AG1X4 yielded HT26 and HT18b. The latter peptide required further purification on AG50X4. Fraction L contained two peptides, HT27 and HT25, which were obtained as a mixture from AG50X4 and separated by paper chromatography.

The initial separations of the tryptic peptides of fully reduced and alkylated Fc(t) by gel filtration on Sephadex G-25 or Sephadex G-50 gave similar patterns (Figure 3). Peptides HT18 and HT33 (Table I) were isolated from fractions I and II (Figure 3), respectively, by chromatography on DEAE-cellulose. Peptides from fraction III (HT30, HT31, and HT-34a), fraction IV (HT25 and HT27), and fraction V (HT20, HT24, HT34b, and HT36) were purified by chromatography on AG50X4.

The fractions obtained by gel filtration of the tryptic peptides of Sephadex G-50 were subjected to a different set of fractionation procedures. Separation of the material corresponding to fractions II and III (Figure 3) on AG1X4 produced four major fractions (Figure 4). Chromatography of fraction 1 (Figure 4) on AG50X4 yielded peptides HT16 (previously denoted FcTn1, Gall *et al.*, 1968) and peptide HT32; similar treatment of fraction 2 yielded peptides HT17, HT37, HT38, and HT30,31.

Three additional peptides were isolated from tryptic digests

TABLE II: Amino Acid Compositions of Chymotryptic Peptides from CNBr Fragments H_6 to H_7.[a]

	H5C1	H5C2	H5C2a	H5C2b	H5C3	H5C4	H5C5	H5C5a	H5C5b	H5C6a[b]	H5C6b	H6C1
Lys	1.1 (1)				2.1 (2)		0.9 (1)		1.0 (1)	(5)	2.0 (2)	1.2 (1)
His	1.0 (1)	0.8 (1)		1.0 (1)			0.9 (1)	1.1 (1)				
Arg	1.0 (1)				0.9 (1)	0.9 (1)				0.8 (1)	0.8 (1)	
CM-Cys	0.8 (1)									(0)		
Asp	2.0 (2)	1.8 (2)	0.9 (1)	1.0 (1)	1.7 (2)		1.8 (2)	0.9 (1)	1.0 (1)	1.1 (1)	0.2	1.5 (1)
Thr	1.7 (2)				2.1 (2)		0.8 (1)	0.8 (1)		0.8 (1)		
Ser	2.0 (2)				1.0 (1)	1.0 (1)	0.2			2.2 (2)	1.1 (1)	0.9 (1)
Glu	3.0 (3)	1.1 (1)		1.2 (1)	3.0 (3)		2.0 (2)	1.0 (1)	1.0 (1)	2.1 (2)	2.7 (3)	1.5 (1)
Pro	2.1 (2)				1.2 (1)					2.8 (3)	1.8 (2)	1.1 (1)
Gly	0.2	1.0 (1)		1.0 (1)			1.1 (1)	0.2	1.0 (1)	1.2 (1)	1.1 (1)	1.3 (1)
Ala					0.9 (1)					3.0 (3)	1.1 (1)	1.0 (1)
Val	5.0 (6)[c]	3.0 (3)		3.0 (3)		2.8 (3)	1.1 (1)	1.0 (1)		0.9 (1)	1.0 (1)	2.1 (2)
Met												
Ile	0.9 (1)				0.2					1.9 (2)	0.9 (1)	0.9 (1)
Leu					0.2	1.1 (1)	1.8 (2)	1.0 (1)	1.0 (1)	1.0 (1)		0.2
Tyr		0.8 (1)	1.1 (1)		2.0 (2)		0.8 (1)		0.8 (1)		0.7 (1)	1.0 (1)
Phe	0.8 (1)											1.0 (1)
Trp		(1)	(1)				(1)	(1)				(1)
Total residues	23	10	3	7	15	6	13	7	6	25	14	13
Yield (%)[d]	23	10	41	15	46	45	20	10	11	25	17	15

	H6C2	H6C3	H6C4	H6C5	H6C6	H6C6a	H6C6b	H6C7	H7C1	H7C2	H7C3
Lys		1.2 (1)		1.0 (1)	1.1 (1)	1.0 (1)				1.0 (1)	
His									2.6 (3)		
Arg					1.1 (1)	1.0 (1)					
CM-Cys								0.7 (1)			
Asp	3.0 (3)	1.9 (2)	0.2		1.9 (2)	1.0 (1)	0.8 (1)		1.1 (1)	0.2	
Thr		1.8 (2)			1.0 (1)	1.0 (1)				0.9 (1)	
Ser	1.0 (1)	2.1 (2)		0.9 (1)	1.0 (1)	1.2 (1)	0.3	2.2 (2)		1.0 (1)	1.9 (2)
Glu	2.9 (3)	0.3			2.0 (2)		1.9 (2)		1.1 (1)	0.9 (1)	
Pro	1.1 (1)	1.9 (2)					0.2				1.1 (1)
Gly	1.1 (1)	1.3 (1)			1.0 (1)	0.2	1.2 (1)				1.1 (1)
Ala		0.3							1.1 (1)		
Val		0.9 (1)	0.2		1.9 (2)	1.0 (1)	1.1 (1)	1.1 (1)		0.3	
Met								0.9 (1)			
Ile											
Leu		1.0 (1)	0.9 (1)	1.1 (1)					1.0 (1)	1.0 (1)	1.0 (1)
Tyr	1.0 (1)		1.0 (1)						0.9 (1)		
Phe		1.0 (1)			0.9 (1)		0.9 (1)				
Trp					(1)	(1)					
Total residues	10	13	2	3	13	7	6	5	8	5	5
Yield (%)[d]	33	25	54	45	8	13	34	20	15	74	23

[a] Values reported are amino acid residues. Amino acids present at a level of 0.1 residue are omitted. The assumed integral numbers of residues are given in parentheses. [b] The Lys residues of this peptide have been modified by the DBDA reagent. DBDA-Lys and CMCys are eluted together during amino acid analysis. [c] Val value is low because of incomplete hydrolysis of a Val-Val-Val sequence. [d] Yields are based on micromoles of peptides isolated compared with micromoles of protein originally digested with chymotrypsin.

of the CNBr fragments. Peptide HT34 was isolated from a digest of fragment H_6 by chromatography on AG50X4. Peptides HT37b and HT38 were obtained from a digest of fragment H_7 by chromatography on AG1X4 (see Table I).

Isolation of Chymotryptic Peptides. The amino acid compositions and yields of chymotryptic peptides are summarized in Table II. All but three of these peptides were obtained from a single digest of Fc(t). The remaining peptides were isolated

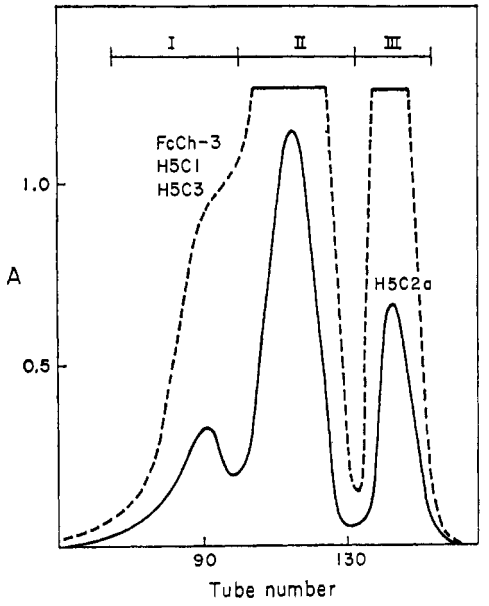

FIGURE 5: Fractionation of a chymotryptic digest of 6 μmoles of Fc(t) on a column (2.1 × 100 cm) of Sephadex G-50 in *n*-propyl alcohol–acetic acid–water (1:2:97, v/v). The absorbance of the effluent at 280 mμ is represented by the solid line and at 230 mμ by the dashed line. Each tube represents 2.7 ml of effluent.

from chymotryptic digests of Arg-tryptic peptides (see Materials and Methods).

The chymotryptic digest of Fc(t) was fractionated by gel filtration on Sephadex G-50 (Figure 5). Fraction I contained peptides FcCH-3 (Gall *et al.*, 1968), H5C1, and H5C3 which were isolated by ion-exchange chromatography on AG1X4. Fraction II (Figure 5) was filtered on Sephadex G-25 to yield H6C4 and a complex mixture of peptides. This mixture was subjected to ion-exchange chromatography on AG1X4 (Figure 6). Ion-exchange chromatography of fraction A (Figure 6) on AG50X4 (Figure 7) yielded peptides FcCH-4 (Gall *et al.*, 1968), H5C4, H5C6b, H6C5, H6C6, H6C6a, and H7C2. Peptides FcCH-2 (Gall *et al.*, 1968), H6C6b, and H7C3 were obtained from fraction E (Figure 6) by chromatography

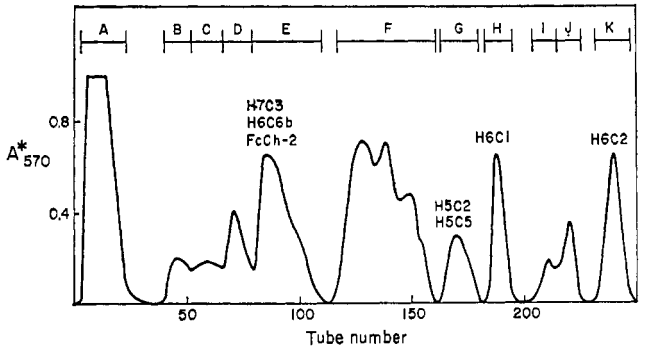

FIGURE 6: Fractionation of the chymotryptic peptides of Fc(t) from fraction II (Figure 5) by ion-exchange chromatography on a column (0.9 × 16 cm) of AG1X4 at 37°. Peptides were eluted by the method of Schroeder (1967). The solid line represents the ninhydrin color yield (A_{570}*) on an arbitrary scale. Each tube contained 1.8 ml of effluent.

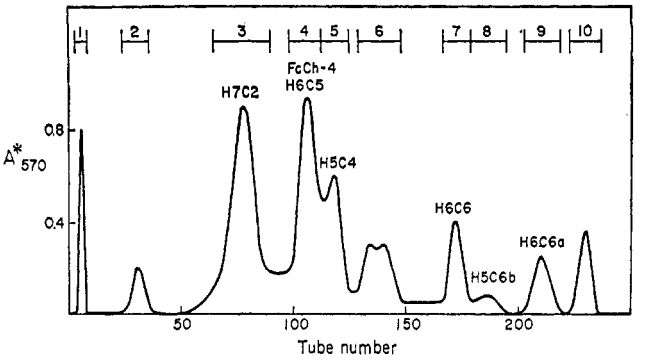

FIGURE 7: Ion-exchange chromatography of peptides from fraction A (Figure 6) on a column (0.9 × 8 cm) of AG50X4 at 37°. The peptides were eluted with a linear gradient from 0.1 M pyridinium-acetate (pH 3.1, 150 ml) to 1.0 M pyridinium-acetate (pH 5.5, 150 ml), followed by another linear gradient from 1.0 M pyridinium-acetate (pH 5.5, 50 ml) to 2.0 M pyridinium-acetate (pH 5.5, 50 ml). The solid line denotes the ninhydrin color yield (A_{570}*) on an arbitrary scale. Each tube contained 1.8 ml of effluent.

on AG50X4. Ion-exchange chromatography of fraction F (Figure 6) on AG50X4 (Figure 8) yielded peptides H5C2b, H5C5a, H5C5b, and H7C1. Peptides H5C2 and H6C5 were isolated from fraction G (Figure 6) by gel filtration on Sephadex G-25 in *n*-propyl alcohol–acetic acid–water (1:2:97, v/v). Peptides H6C1 and H6C2 were obtained directly from fractions H and K (Figure 6), respectively. Peptide H5C2a was obtained directly from fraction III (Figure 5).

Three additional chymotryptic peptides were obtained from digests of the Arg-tryptic peptides AT-1, AT-2, and AT-3. These Arg-tryptic peptides were isolated by ion-exchange chromatography on DEAE-cellulose (Figure 9). The amino acid composition of AT-3 is given in Table I. Chymotryptic digestion of AT-3 followed by gel filtration on Sephadex G-25 in 0.015 N NH_4OH which was 10% in *n*-propyl alcohol, and removal of the DBDA blocking group yielded peptide H5C6a. Peptides H6C3 and H6C7 were isolated from chymotryptic digests of AT-2 and AT-1, re-

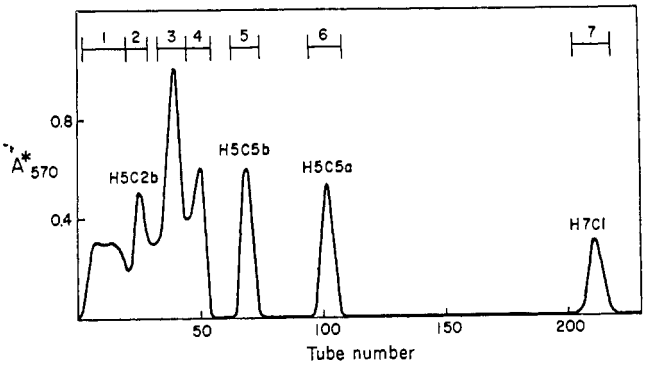

FIGURE 8: Ion-exchange chromatography of peptides from fraction F (Figure 6) on a column (0.9 × 8 cm) of AG50X4 at 37°. The peptides were eluted with a linear gradient from 0.1 M pyridinium-acetate (pH 3.1, 150 ml) to 1.0 M pyridinium-acetate (pH 5.5, 150 ml), followed by another linear gradient from 1.0 M pyridinium-acetate (pH 5.5, 50 ml) to 2.0 M pyridinium-acetate (pH 5.5, 50 ml). The solid line denotes the ninhydrin color yield (A_{570}*) on an arbitrary scale. Each tube contained 1.8 ml of effluent.

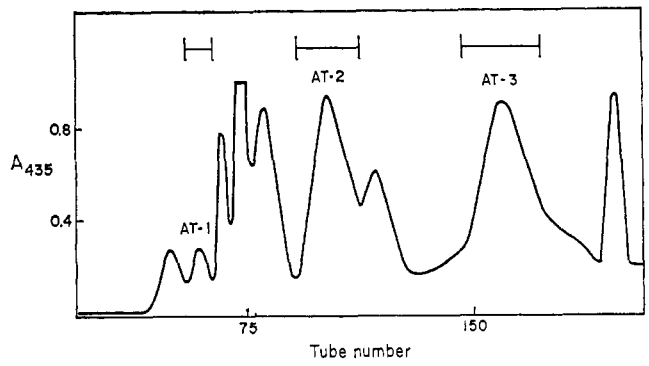

FIGURE 9: Ion-exchange chromatography of Arg-tryptic peptides of Fc(t) on a column of DEAE-cellulose (0.9 × 50 cm) at 37°. The peptides were eluted with a linear gradient from 0.05 M Tris-HCl (pH 8.0, 300 ml), to the same buffer which was made 1.0 M in NaCl (300 ml). The solid line represents the absorbance at 435 mμ. Each tube contained 2.2 ml of effluent.

spectively, by chromatography on AG50X4, followed by removal of the DBDA blocking groups.

Amino Acid Sequence of CNBr Fragment H_5 (Residues 253–358). The data used to determine the sequence of fragment H_5 are presented in Figure 10.

Residues 253–275. Dansyl-Edman analysis of peptide H5C1 indicated that the first four residues are: Ile-Ser-Arg-Thr-. The sequence of peptide HT17 (previously denoted HO-3, Waxdal *et al.*, 1968a) which overlaps CNBr fragments H_4 and H_5 places peptide H5C1 at the NH_2 terminus of H_5. The remaining sequence of H5C1 was deduced from the partial sequences of HT18 and HT18b (Table I).

Residues 276–288. HT19 was the only tryptic peptide from Fc(t) having an NH_2-terminal phenylalanine. Because H5C1 has the sequence Lys-Phe at its COOH terminus, HT19 was assigned the position starting at residue 275. The sequence of HT19 through residue 285 was established by dansyl-Edman analysis of peptides HT19, H5C2, and H5C2a. The sequence of HT19 was completed by analysis of peptide H5C3T1 (Figure 10). This peptide was isolated, along with H5C3T2 and H5C3T3, from a tryptic digest of H5C3 by ion-exchange chromatography on AG50X4. The sum of the amino acid compositions of these peptides accounts for all of the residues of H5C3.

Residues 289–301. Peptide H5C3T1 contains the NH_2-terminal portion of H5C3. Peptides H5C3 and H5C3T3 both have a COOH-terminal tyrosine, thus establishing the order of the three tryptic peptides obtained from H5C3. The sequence from residue 289 to 301 was deduced from the sequences of H5C3T2 (Figure 10) and that of HT21. Peptide HT21 contained carbohydrate and its sequence could not be determined directly. Papain digestion of this peptide gave HT21Pa1, HT21Pa2, and HT21Pa3 (Figure 11), which were isolated by chromatography on AG50X4. Partial acid hydrolysis of HT21Pa1 yielded three peptides which were isolated by chromatography on AG50X4. The compositions and sequence analysis of these peptides establish the sequence of HT21 and suggest that the carbohydrate is attached to Asx residue 297.

Residues 302–320. Peptide H5C4 (Figure 10) has the amino acid sequence Arg-Val-Val-Ser-Val-Leu. Because all other arginine-containing peptides can be unequivocally placed and tryptic peptide HT22,23 has the NH_2-terminal sequence

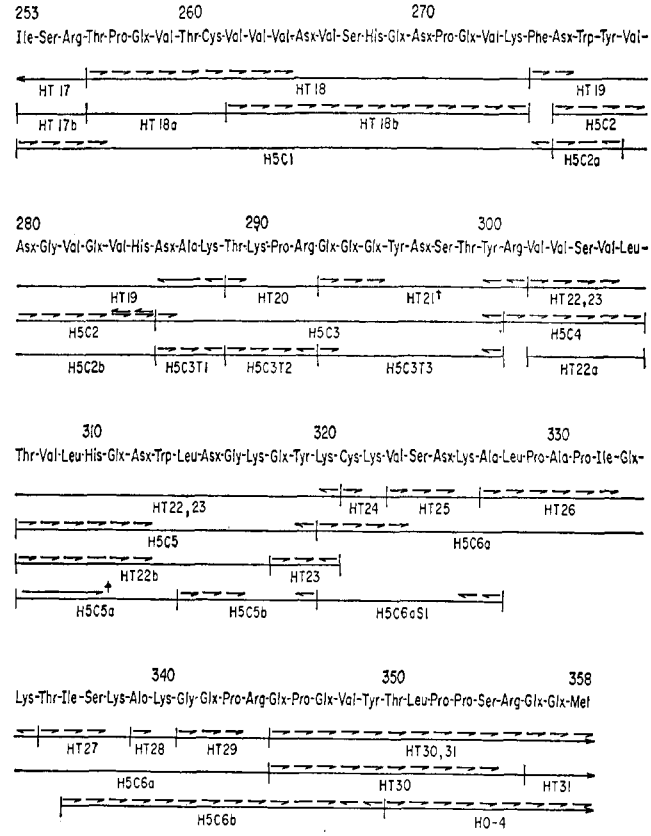

FIGURE 10: Amino acid sequence of CNBr fragment H_5. Peptides used to establish the sequence are represented by solid lines. See Results section for peptide nomenclature. The symbol (—) represents the results of direct sequence analysis by the dansyl-Edman or subtractive-Edman method. Residues which did not yield a detectable dansylamino acid are indicated as (—). The symbol (—) indicates residues removed by digestion with carboxypeptidase A or B. Residue numbers are for the complete heavy chain. (†) See Figure 11. (‡) Residues removed by leucine aminopeptidase digestion.

Val-Val-Ser-Val, H5C4 must overlap peptides HT21 and HT22,23. The sequence of HT22,23 was elucidated by analysis of peptides H5C4, H5C5, H5C5a, H5C5b, HT22b, and HT23.

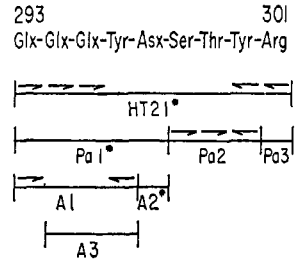

FIGURE 11: Amino acid sequence of peptide HT21, showing the peptide fragments used to establish the sequence. The symbol (*) indicates that the peptide contained carbohydrate as determined by amino acid analysis. See Figure 10 for explanation of symbols (—), (—), and (—). The designation Pa refers to peptides obtained by papain digestion of HT21. The designation A refers to peptides obtained by partial acid hydrolysis of peptide Pa1.

TABLE III: Comparison of Sequence Analysis Compositions with Amino Acid Analysis Compositions.[a]

	H_5		H_6		H_7		Fc(t)	
	Sequence Anal.	Amino Acid Anal.	Sequence Anal.	Amino Acid Anal.	Sequence Anal.	Amino Acid Anal.[b]	Sequence Anal.[c]	Amino Acid Anal.[d]/24,000 g
Lys	10	10.1	5	5.0	1	1.0	18	16.6
His	3	2.7			3	3.0	7	6.9
Arg	5	5.0	1	1.2			6	5.7
Asp	9	8.8	9	8.9	1	1.0	20	19.7
Thr	7	7.0	5	4.9	1	0.9	16	15.0
Ser	7	7.4	8	8.1	3	2.9	20	18.2
Glu	15	15.4	7	7.3	2	2.0	25	25.5
Pro	9	9.0	4	3.9	1	1.0	22	22.0
Gly	3	3.2	4	4.4	1	1.1	10	10.8
Ala	4	4.0	1	1.3	1	1.1	7	8.2
Cys	2	1.8	2	1.8			6	4.7
Val	15	13.8	7	7.1			23	23.3
Met	1	0.9	1	1.0			3	2.9
Ile	3	3.0	1	1.2			4	4.3
Leu	5	5.1	5	5.2	3	3.0	17	17.0
Tyr	5	7.3[e]	3	3.0	1	1.0	9	9.1
Phe	1		4	3.9			7	7.2
Trp	2	(2)	2	(2)			4	3.3
Total residues	106	106.6	70	70.2	18	18.0	224	220.4

[a] Values are reported as amino acid residues. Threonine and serine contents are based on a linear extrapolation of 20- and 72-hr hydrolysates to zero time. Isoleucine and valine values are from 72-hr hydrolysates; Cys is total recovered half-cystine plus *S*-carboxymethylcysteine. [b] Waxdal *et al.* (1968b). [c] Includes residues 223–252 of the Eu heavy chain (Cunningham *et al.*, 1970). [d] Edelman *et al.* (1968). [e] Sum of Tyr, Phe, and glucosamine from carbohydrate.

Residues 321–344. Peptide H5C6a was isolated from a chymotryptic digest of Arg-tryptic peptide AT-3. The composition (Table I) and NH_2-terminal sequence (Val-Val-Ser-Val) of AT-3 indicate that it spans residues 302–344. Although the position of H5C6a cannot be unequivocally assigned on the basis of its single-residue overlap with HT22,23, its position within AT-3 is established by the sequences and compositions of H5C4, H5C5, H5C6a, and AT-3 (Figure 10). The amino acid sequence of peptide H5C6a was deduced by direct sequence analysis of the peptide itself and peptides HT24, HT25, HT26, HT27, HT28, HT29, H5C6b, and H5C6aS1. Peptide H5C6a was also analyzed by the subtractive-Edman procedure. Peptide H5C6aS1 was isolated from a subtilisin digest of H5C6a by ion-exchange chromatography on AG1X4, and its partial sequence was determined by carboxypeptidases A and B digestions after removal of the DBDA blocking group.

Residues 345–360. The complete amino acid sequences of peptides H5C6b (residues 336–349) and HT30,31 (residues 345–360) were determined directly. These data, along with the sequence of peptide HT30, complete the determination of the sequence of CNBr fragment H_5. This sequence is in agreement with the amino acid composition of fragment H_5 (Table III). The position of peptide HO-4 (Waxdal *et al.*, 1968a) is indicated in Figures 11 and 12. The sequence of residues 350–359 is in agreement with that reported for HO-4. However, the composition of this peptide indicated the presence of an additional Ser and Glx residue. The data obtained from HT30, 31, HT31, and H_6 suggest that this discrepancy is due to contamination of HO-4.

Amino Acid Sequence of CNBr Fragment H_6 (Residues 359–428). The amino acid sequence and order of peptides used to determine the sequence are given in Figure 12.

Residues 359–373. The sequence of the first 11 residues of H_6 was determined by dansyl-Edman degradation of the intact CNBr fragment. This orders peptides HT32, HT32C1, and H6C1. Partial sequence determination of these peptides established the sequence through residue 373. Peptide HT32C1 was isolated from a chymotryptic digest of HT32 by gel filtration on Sephadex G-25 in *n*-propyl alcohol–acetic acid–water (1:2:97, v/v). Dansyl-Edman analysis of peptide H6C1 gave an unambiguous sequence (Figure 12). However, the amino acid composition of this peptide (Table II) suggested that it was contaminated with another peptide. Digestion of H6C1 with carboxypeptidase A released 0.2 mole of tyrosine/mole of tryptophan. This finding and the composition of peptide H6C2 (Table II) suggest that H6C1 was contaminated at a level of 20% with a peptide spanning both H6C1 and H6C2.

Residues 374–392. The sequence of H6C1 places HT33. With the exception of Trp-381, the sequence of the first 14 residues of HT33 was determined by the dansyl-Edman method, and the COOH-terminal two residues were determined by digestion with carboxypeptidases A and B. The remainder of the sequence was obtained by dansyl-Edman analysis of peptide H6C2 and of peptides isolated from a

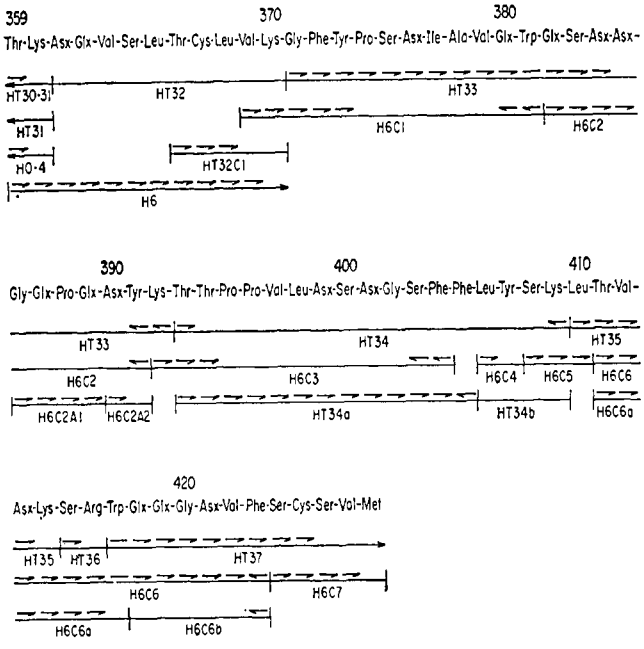

FIGURE 12: Amino acid sequence of CNBr fragment H_6. Peptides used to establish the sequence are indicated by solid lines. See Figure 10 for explanation of symbols (⟶), (—), and (⟝).

partial acid hydrolysate of H6C2 by ion-exchange chromatography on AG50X4. We have previously reported (Rutishauser *et al.*, 1968) that the fifth residue of H6C2 was glutamine or glutamic acid; the correct residue (386) is glycine.

Residues 393–409. The sequence of HT34 was deduced by analysis of HT34a, H6C3, H6C4, and H6C5. Peptide H6C3 is one of two chymotryptic peptides obtained from Fc(t) which has an NH_2-terminal lysine. The position of the other peptide, H5C6a (Figure 10), has already been established. Therefore, H6C3 provides an unambiguous overlap between peptides HT33 and HT34. Although peptides HT34 (Table I) and H6C3 (Table II) were contaminated, the compositions and partial sequences of these peptides were sufficient to place peptides HT34a and HT34b. The positions of peptides H6C4 and H6C5 were then deduced from the sequences and compositions of HT34, HT34a, HT34b, and H6C3.

Residues 410–428. The overlapping sequences of HT34, H6C5, and HT35 establish the position of peptide H6C6. The NH_2-terminal leucine of HT35 is unique among the Fc(t) tryptic peptides, and therefore peptide H6C6 can be placed unequivocally. The sequence of H6C6 was established by analysis of the peptide itself, H6C6a, HT36, and HT37. The remainder of the sequence of CNBr fragment H_6 was established by dansyl-Edman analysis of peptides HT37 and H6C7.

The sequence of H_6 accounts for all of the amino acid residues found by amino acid analysis of the intact CNBr fragment (Table III).

Amino Acid Sequence of CNBr Fragment H_7 (Residues 429–446). Peptide HT37 provides an overlap between CNBr fragments H_6 and H_7 (Figure 13). The sequence of HT37 from residues 429 to 441 was deduced from the sequences of peptides HT37bC1, HT37bC2, and H7C2. Peptides HT37bC1, HT37bC2, and HT37bC3 were isolated from a chymotryptic

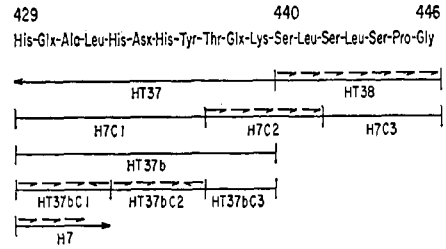

FIGURE 13: Amino acid sequence of CNBr fragment H_7.

digest of HT37b by chromatography on AG1X4 and their order was established by direct sequence analysis of H_7. The determination of the amino acid sequence of H_7 was completed by analysis of the overlapping peptides H7C2 and HT38. The composition of H_7 based on its amino acid sequence agrees with the values obtained by amino acid analysis (Table III).

Discussion

The CNBr fragments H_5–H_7 consist of the last 194 residues of the heavy chain. The amino acid sequence of this region is presented in Figure 14. This sequence together with that of residues 223 to 252 of the Eu heavy chain (Gall *et al.*, 1968; Cunningham *et al.*, 1970) accounts for the entire Fc(t) region of protein Eu (Table III). The determination of the sequences and positions of peptides (Figures 10–13) was in most cases straightforward. Although 5 (HT19, HT21, HT23, HT33, and HT35) of the 22 tryptic peptides were overlapped by only a single residue, each of these could be assigned to a unique position. Four peptides (H6C1, H6C3, HT34, and H7C2) used to establish the sequence were contaminated. Although this might indicate that the sequences in these regions are less reliable, the data obtained from uncontaminated peptides support the sequence shown in Figure 14. Peptide HT18a, HT31, and H6C6b were contaminated at a level of 0.3 residue. These peptides were not essential in establishing the sequence and are included only for completeness. The presence of carbohydrate on Asx residue 297 presented special difficulties but did not prevent the elucidation of the sequence of this region (Figure 11).

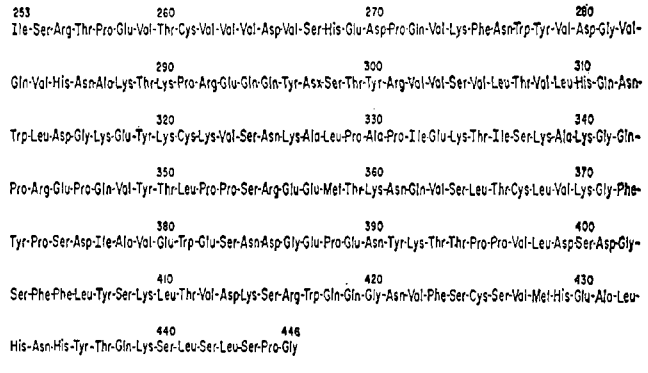

FIGURE 14: The complete amino acid sequence of CNBr fragments H_5–H_7. The assignment of asparaginyl and glutaminyl residues is discussed in another paper of this series (Bennett *et al.*, 1970). Numbering is that of the complete heavy chain.

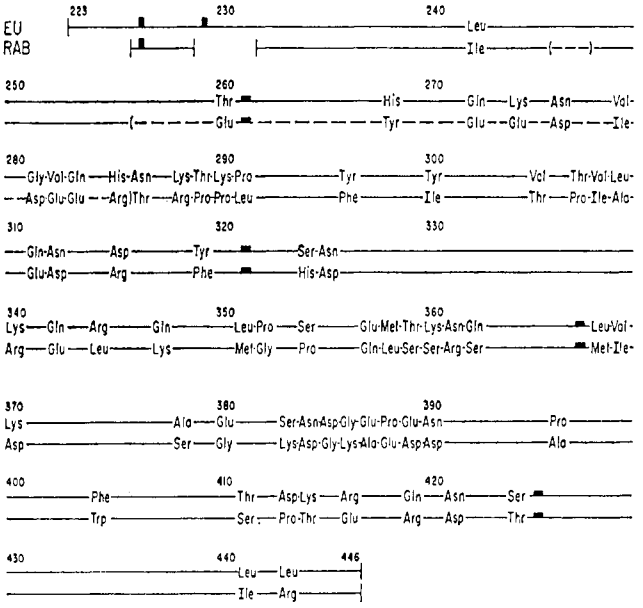

FIGURE 15: Comparison of the amino acid sequences of the Fc(t) region of the human γG-immunoglobulin Eu with the sequence of the Fc region of rabbit γG-immunoglobulin. Solid lines indicate regions of identity, and residues are shown only where sequences differ. Parentheses enclose regions in which complete sequences are unknown; these residues have been aligned for maximum homology and identity is indicated by dashed lines. Half-cystines in interchain and intrachain disulfide bonds are indicated by symbols ▌and ■, respectively. Numbering is that of the Eu heavy chain.

The results of this and previous studies provide the opportunity to look for internal sequence homologies within a single γG-immunoglobulin molecule, as well as to compare the constant regions of Eu with the corresponding regions of immunoglobulins of other species. Such comparisons support the hypothesis (Hill *et al.*, 1966; Singer and Doolittle, 1966) that the immunoglobulin molecule evolved by a series of duplications of a gene sufficiently large to specify a polypeptide of about 100 residues.

The Fc(t) region of protein Eu exhibits internal homology. When the sequence from residue 234 to 341 (the C_H2 region) is aligned with the sequence from residue 342 to the COOH terminus (the C_H3 region), 29 residues or about 30% of the positions compared are identical in the two sequences (Rutishauser *et al.*, 1968). Only three deletions involving a total of five residues are required for this alignment. The heavy chain is 446 residues long, and the constant region begins 115 residues from the NH_2 terminus. The sequence of C_H1 (Cunningham *et al.*, 1970) is homologous to both C_H2 and C_H3 (Edelman *et al.*, 1969). Similar comparisons of the constant region of the Eu light chain (the C_L region; residues 113–214) with C_H1, C_H2, and C_H3 reveals that about 30% of the positions compared are identical (see Edelman, 1970). Furthermore, the sequence of the Fc(t) region of Eu resembles the sequences of the constant regions of human λ and mouse κ chains to about the same extent (Rutishauser *et al.*, 1968).

Several genetic markers have been located in the Fc region of human immunoglobulins (see Natvig and Kunkel, 1968). Homologous peptides from Gm(a+) and Gm(a−) immunoglobulins have been isolated (Thorpe and Deutsch, 1966; Rutishauser *et al.*, 1968; Wang and Fudenberg, 1969). These results and the sequence of Eu indicate that the Gm(a+) marker is associated with the sequence Asp-Glu-Leu from residue 356 to 358 and that in Gm(a−) immunoglobulins the corresponding sequence is Glu-Glu-Met.

Homologous glycopeptides of several γG-immunoglobulins from humans and other species have been isolated and analyzed (Howell *et al.*, 1967). Comparison of these peptides with the corresponding sequence of Eu shows that although residue 296 is tyrosine in Eu, it is phenylalanine in all the other peptides.

A previous analysis has been carried out on the Fc fragment of rabbit immunoglobulin (Hill *et al.*, 1967). A comparison of the Fc(t) region of protein Eu and the partial amino acid sequence of the rabbit Fc fragment is shown in Figure 15. Excluding the region where the sequence of the rabbit protein is incomplete, 69% of the residues compared are identical. Four regions of the sequence are nearly identical in the human and rabbit Fc fragments. From residue 232 to 255, only residue 242 is different and this difference can be accounted for by a single base change in the genetic code. From residue 317 to 339 there are three amino acid substitutions, two of which correspond to single base changes. From residue 391 to 410 and from residue 425 to 446 a total of four differences are found, each involving a single base change. The presence of such regions is not surprising since the Fc fragments from different species have similar biological activities.

Acknowledgments

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

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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

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