Primary Structure of the C_H3 Homology Region from Guinea Pig IgG2 Antibodies†

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ABSTRACT: The amino acid sequence has been determined for an 85-residue cyanogen bromide fragment from the fourth quarter of the heavy chain of strain 13 guinea pig IgG2. This fragment, C-4, contains an intrachain disulfide bond and occurs adjacent to the carboxyl-terminal octadecapeptide of the heavy chain. The sequence of this fragment is about 51 and 62% homologous with corresponding sequences from rabbit IgG and human IgG1 heavy chains, respectively. Further, the guinea pig protein has two additional residues in this region compared with the human IgG1 or rabbit IgG immunoglobulins and these extra residues occur in a region noticeably nonhomologous among these species. With these data, the primary structural analysis of the constant homology regions of γ_2 chain (~N-120 to ~N-446) from guinea pig IgG2 is completed.

The elucidation of the sequence of the cyanogen bromide fragment, C-4, reported here, completes the determination of primary structure for the carboxyl-terminal 327 residues (N-120 to N-446) of the γ_2 chain including all of the constant homology regions. The only other immunoglobulin heavy chains to have had the amino acid sequence of their constant regions completely or almost completely determined are the *normal* rabbit γ chain (Hill *et al.*, 1967; Cebra *et al.*, 1968a,b; Appella *et al.*, 1971), a human γ_1 chain from a myeloma protein (Edelman *et al.*, 1969), a human γ_4 chain from a myeloma protein (Pink *et al.*, 1970), and a human μ chain from a protein obtained from a patient with macroglobulinemia (Putnam *et al.*, 1973; Watanabe *et al.*, 1973). Knowing the sequence of the constant part of guinea pig γ_2 chain may be particularly useful in view of the association of distinct secondary biologic activities with different isotypes of guinea pig IgG (Ovary *et al.*, 1963; Bloch *et al.*, 1963; Oliveira *et al.*, 1970). A comparative primary structural analysis of the γ_1 chain from the other major guinea pig IgG isotype is currently underway.

It may be significant for attempts to relate structure to function that the amino acid sequences known for the immunoglobulin constant regions of different species and of different isotypes are the least similar in the homology region containing the fragment C-4 described here.

Materials and Methods

Serum Donors. All sera used for the preparation of IgG2 were obtained from our own colony of strain 13 inbred guinea pigs.

Preparation of C-4. Fragment C-4 was isolated from a CNBr digest of γ_2 chain prepared from partially reduced and carboxymethylated strain 13 IgG2 as outlined previously (Birshtein *et al.*, 1971a).

Automated Sequential Degradation. Several preparations of C-4 (1.0–1.5 μmol) were submitted to automatic sequential degradation in the Beckman Model 890-B sequencer. Identification of the amino acid residues at each position was by procedures previously described (Birshtein and Cebra, 1971; Koo and Cebra, 1974).

Isolation of Tryptic Peptides. Fragment C-4 (0.8–2.0 μmol) was digested with trypsin (L-1-tosylamido-2-phenylethyl chloromethyl ketone treated, Worthington Biochemical Corp.) at 37° for 6 hr (enzyme–substrate, 1:50 w/w). A precipitate present after digestion was removed by centrifugation. The soluble tryptic peptides were fractionated on a column of Sephadex G-50 (2.6 × 255 cm) equilibrated in 0.05 M NH_4OH (Figure 1). Pools of effluent from the G-50 column were freeze-dried or brought to a small volume by rotary evaporation. Peptides in pools 1 and 2 were further purified on small columns (1.2 × 22 cm) of DEAE-Sephadex A-25 initially equilibrated in 0.05 M NH_4HCO_3 (see Figure 2). Peptides were eluted with a linear gradient of a solution of increasing ionic strength generated with a two-chamber gradient maker. One chamber contained 250 ml of 0.05 M NH_4HCO_3, the other 250 ml of 0.35 M NaCl–0.05 M in NH_4HCO_3. Pools from the A-25 column were rotary evaporated to a small volume and petides in them were desalted by passage of the concentrated solutions through a column of Sephadex G-10 (2.6 × 58.5 cm) equilibrated in 0.05 M NH_4OH. Peptides found in pools 3 and 4 could be separated directly by high-voltage paper electrophoresis at either pH 3.6 or 6.5.

Isolation of Thermolytic Peptides. Fragment C-4 (3 μmol) was dissolved in 0.05 M NH_4HCO_3 (pH 8.0) and digested with 300 μg of thermolysin (Calbiochem) for 7 hr at 40°. The digest was applied to a column of Sephadex G-25, fine (2.6 × 24 cm) equilibrated in 0.05 M NH_4OH (Figure 3). Pools were freeze-dried or brought to a small volume by rotary evaporation. Peptides in pools 1 and 2 were further fractionated by ion-exchange chromatography on columns of Dowex 1-X2 as previously described (Birshtein *et al.*, 1971b). Components of pools 3, 4, and 5 (Figure 3) and of pools from the Dowex 1 columns were further resolved by high-voltage paper electrophoresis at either pH 3.6 or 6.5.

Other Enzymatic Digests. Pepsin, carboxypeptidase A, and carboxypeptidase B were obtained from Worthington and used as previously described (Turner and Cebra, 1971). Aminopeptidase M was supplied by Rohm and Haas and used as described (Tracey and Cebra, 1974).

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TABLE I: C-4 and Peptides Derived from It by Tryptic Digestion.[a]

	C-4[c]	T11-12	T11	T10	T8	T7	T6	Ch-T6-1	Ch-T6-2	Ch-T6-3	T5	T4
Lys	6.0	1.0	1.0	1.0	1.0	1.0	0.98			1.0	1.0	
His	0.96											
Arg	2.0	0.99										
CM-Cys	2.0											1.0
Asp	9.7	2.1	0.99				3.0	2.6			1.0	1.0
Thr	6.1	0.99					1.0	0.87			0.89	1.8
Ser	9.8	1.9	1.0	1.0	0.94		1.6	1.1		1.0		1.8
Glu	6.2	1.1	1.0		1.1	1.0	1.1	1.0				1.0
Pro	7.0	2.9			1.0		2.0	2.0				
Gly	2.3						1.1	1.5				1.1
Ala	4.2						1.1	1.1				1.0
Val	9.0	1.1			1.9		0.30				1.0	2.0
Ile	3.7						0.98	0.75				
Leu	5.0	1.9	.99				1.0		1.0		1.0	
Tyr	4.6	0.93				.91	2.0	0.77	0.89			0.98
Phe	2.9						1.0	1.2				
Hsr	+											1.0
Trp	+											+
Mobility[b]												
pH 3.6			+0.16		+0.53	+0.56					+0.61	
pH 6.5		−0.13	−0.32	+0.72	+0.13	+0.05	−0.28	−0.54	0	+0.79	+0.08	−0.40

[a] Compositions are reported as moles of amino acid per mole of peptide. [b] Mobility given relative to lysine = +1.0, aspartic = −1.0, neutral amino acid = 0. [c] Values for valine, isoleucine, and leucine are from a 72-hr hydrolysis. Values for serine and threonine are from extrapolation to zero hydrolysis time. All other values are from an 18-hr hydrolysis.

Isolation of Chymotryptic Peptides. Fragment C-4 (1.0 μmol) was dissolved in 0.05 M NH_4HCO_3 (pH 8.0) and digested with 200 μg of chymotrypsin (Worthington) for 8 hr at 37°. The entire digest was fractionated by preparative high-voltage electrophoresis at pH 6.5.

Other Methods. High-voltage paper electrophoresis of peptides at pH 3.6 and 6.5 and amino acid sequence analysis of component peptides were carried out as previously described (Turner and Cebra, 1971; Birshtein *et al.*, 1971a). Wherever possible, enzymatic digestion of peptides with aminopeptidase M, followed by amino acid analysis, was used to determine whether Asx or Glx in peptides was present as the free acid or amide. Where enzymatic digestion was not possible, assignment was deduced from the mobility of the peptide at pH 6.5. Nomenclature of peptides is according to Turner and Cebra (1971).

Results

Tryptic Peptides. Tryptic peptides of C-4 were initially fractionated on a column of Sephadex G-50 as shown in Figure 1. The major peptide in pool 1 was further purified on a column of DEAE-Sephadex A-25 as described in Materials and Methods. Only a single peptide, T6, was eluted from the A-25 column at a salt concentration of approximately 0.2 M. The amino acid compositions of all isolated tryptic peptides are given in Table I. The amino-terminal sequence of T6 was determined as shown in Figure 6. A chymotryptic digestion of T6 followed by preparative high-voltage electrophoresis at pH 6.5 gave the three peptides whose compositions are shown in Table I. Peptide Ch-T6-3, with a lysyl residue, was of necessity from the carboxyl-terminal part of T6 and must have the sequence Ser-Lys. A single Edman degradation step on peptide Ch-T6-2 showed the sequence to be Leu-Tyr. A time course analysis of digests of Ch-T6-1, from the amino-terminal end of T6, using carboxypeptidase A gave a carboxyl-terminal sequence of -Tyr-Phe.

Pool 2 from the G-50 column (Figure 1) was treated identically to pool 1. The elution profile from the subsequent A-25 column is shown in Figure 2. Peptides T11-12 and T4 were isolated from pools 1 and 2, respectively. The amino acid sequence of peptide T11-12 has been previously published (Benjamin *et al.*, 1972). The presence of homoserine in peptide T4 indicated that it was the carboxyl-terminal peptide of C-4. The results of sequential degradation and carboxypeptidase A digestion of T4 are shown in Figure 6.

Other tryptic peptides were purified by preparative high-

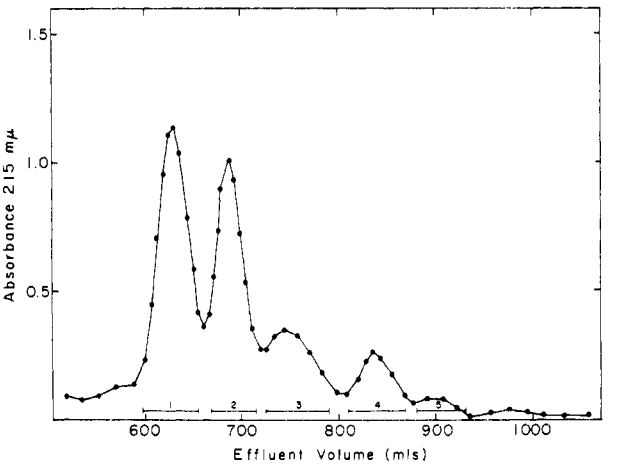

FIGURE 1: Fractionation of components in a tryptic digest of C-4 (2 μmol) on a column of Sephadex G-50, fine (2.6 × 255 cm) equilibrated in 0.05 M NH_4OH. Fraction size was 5.3 ml.

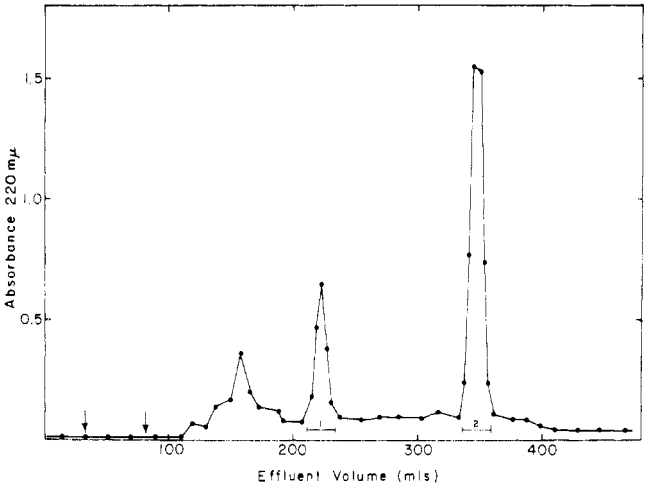

FIGURE 2: Separation of tryptic peptides contained in pool 2 (Figure 1) on a column of DEAE-Sephadex A-25 (1.2 × 22 cm). A salt gradient was used to elute peptides as described in the text. The first arrow indicates application of the sample to the column and the second arrow indicates the start of the gradient. Fraction size was 3.8 ml.

voltage electrophoresis. Peptides T11, T8, and T5 were isolated from pool 3 (Figure 1) and peptides T10 and T7 from pool 4. The sequences of these peptides were determined as shown in Figure 6.

Tryptic peptide T9, which was expected to encompass the region between tryptic peptides T10 and T8, was found to be present in a precipitate formed during tryptic digestion. Amino acid analysis of the precipitate showed the presence of carboxymethylcysteine, histidine, and arginine residues characteristic of T9. The precipitate, however, was resistant to further enzymatic digestion and was not studied further.

Automated Sequential Degradation of C-4. A Beckman sequencer was used to obtain information on the proper alignment of the tryptic peptides at the amino terminus of C-4. Residues identified with the aid of the sequencer are indicated in Figure 6. Tryptic peptides T12, T11, T10, T8, and T7 were thus positioned in order and their sequences, determined by manual methods, were confirmed. In addition, most of the residues occurring in the region between peptides T10 and T8 were also identified.

Thermolytic Peptides. Peptides from a thermolytic digest of C-4 were initially fractionated on a column of Sephadex G-25 fine as shown in Figure 3. Peptides in pool 1 were further fractionated by ion-exchange chromatography on a column of Dowex 1-X2 (Figure 4). Final purification of peptides following the Dowex column was by high-voltage electrophoresis. Compositions of the resulting peptides are given in Table II. The pool from the Dowex column that they were isolated from is also given. Determination of the sequences of these peptides is shown in Figure 6. One thermolytic peptide, Th35, was further digested with pepsin and the two resulting peptides were separated by high-voltage electrophoresis at pH 6.5. Th35 provided a peptide overlapping and thus aligned tryptic peptides T7 and T6. The peptic fragments of Th35 in conjunction with Th36 permitted completion of sequence determination for tryptic peptide T6.

Pool 2 from the G-25 column (Figure 3) was treated identically to pool 1. The elution profile of the resulting Dowex column is shown in Figure 5 and compositions of isolated peptides are given in Table III along with their pool of origin from the Dowex column. Both peptides Th39 and Th40 overlapped tryptic peptides T5 and T4. Th42 was useful for determining the final sequence of tryptic peptide T4.

Peptides in pools 4 and 5 from the G-25 column (Figure 3) were isolated in purified form after high-voltage electrophoresis. Compositions of these peptides as well as their pools of origin are given in Table IV. Sequence data on these peptides are found in Figure 6.

Thermolytic peptides, as can be most easily seen in Figure 6, allowed identification of residues in the region between tryptic peptides T10 and T8 which were not identified from sequencer data. Most peptides overlapping tryptic peptides were isolated from the thermolytic digest and thermolytic peptides provided data establishing the final sequence of all isolated tryptic peptides.

Chymotryptic Peptides. The thermolytic digest did not provide a peptide overlapping tryptic fragments T6 and T5. To obtain such a peptide, a chymotryptic digest of C-4 was directly subjected to preparative high-voltage electrophore-

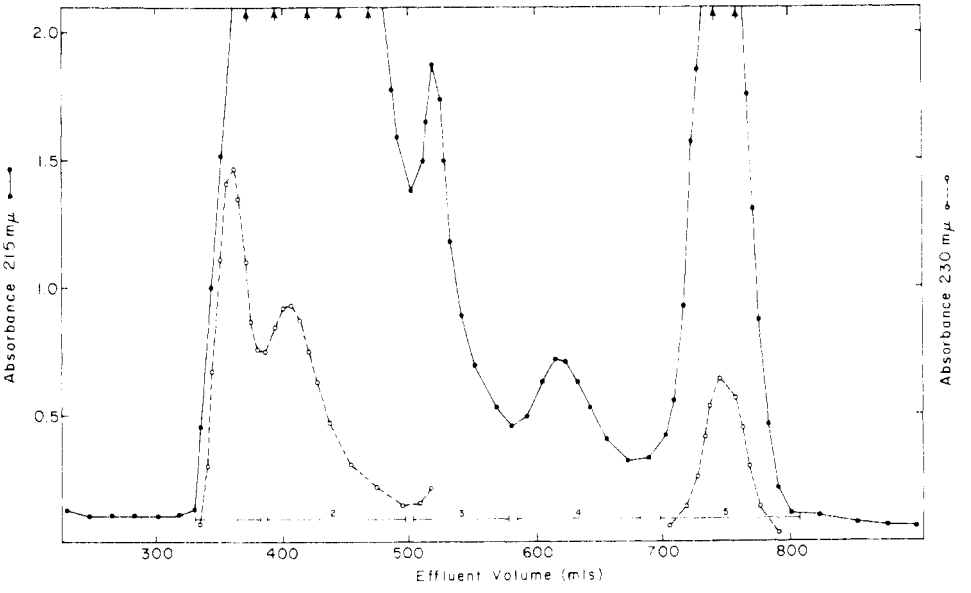

FIGURE 3: Fractionation of components in a thermolytic digest of C-4 (3 μmol) on a column of Sephadex G-25, fine (2.6 × 240 cm) equilibrated in 0.05 M NH_4OH. Fraction size was 5.0 ml.

TABLE II: Thermolytic Peptides from C-4.[a]

	Th33	Th34	Th35	Pe-Th35-1	Pe-Th35-2	Th36
Lys		1.1	0.95	0.99		
Arg	0.89					
Asp	1.0		3.0	1.0	2.0	1.1
Thr			0.89	0.89		
Ser	0.96	0.96	1.0		1.0	1.0
Glu		2.1	1.2	1.0		
Pro	0.99		2.4	2.0		
Gly			1.0		1.1	0.98
Ala			0.98		1.0	0.94
Val	0.98	0.98				
Ile			0.90	0.96		
Tyr			1.7	0.93	0.93	1.1
Mobility						
pH 3.6	+0.68	+0.53				
pH 6.5	+0.46	−0.03		+0.13	−0.80	−0.43
Pool of origin						
(Figure 4)	1	4	7			6

[a] Compositions and mobilities are expressed as indicated in Table I.

TABLE III: Thermolytic Peptides from C-4.[a]

	Th27	Th29	Th30	Th31[b]	Th32	Th39	Th40	Th42
Lys						0.97	1.0	
Arg	0.93							
CM-Cys		1.2	1.1					0.97
Asp	1.0			1.1	0.96	1.1	1.0	
Thr	0.93	0.92	0.80			0.93		1.1
Ser	1.1	0.95				1.1	0.77	1.3
Glu	0.99							
Pro	2.0				1.0			
Ala					0.95			
Val	0.84	2.0	1.2			0.96	0.99	1.0
Ile				1.9				
Leu	1.0			1.0		0.76		
Tyr	0.83							0.97
Phe					1.5			
Mobility								
pH 3.6	+0.26	−0.15	−0.24	+0.31	+0.03	+0.47	+0.53	−0.02
pH 6.5					−0.26		+0.05	
Pool of origin								
(Figure 5)	4	5	5	2	5	2	2	7

[a] Compositions and mobilities are expressed as indicated in Table I. [b] Amino acid composition of Th31 was determined after hydrolysis for 72 hr.

sis at pH 6.5. The compositions of the isolated peptides, Ch41 and Ch42, are given in Table V and their placement in the final sequence of C-4 is shown in Figure 6. Both peptides Ch41 and Ch42 provided the missing overlapping peptide.

Discussion

The entire amino acid sequence of the constant region of the heavy chain of guinea pig IgG2 has now been established. If the "switch" point in the guinea pig heavy chain occurs at a position homologous to that found for human heavy chains, the length of the constant region for the γ_2 chain is 332 residues, spanning five cyanogen bromide fragments and part of a sixth. A single sequence has been found for the entire length of the constant region, any amino acid interchanges due to allotypic differences presumably having been eliminated by the use of an inbred strain of guinea pigs. The constant region sequences of the heavy chains of guinea pig, rabbit, and human IgG1 are very similar. In only two sections of the constant region is there a noticeable nonhomologous primary structure—one occurs directly before the "hinge" region (Turner and Cebra, 1971) and the other in fragment C-4 as discussed below.

While a single sequence has been presented for C-4 in Figure 6, the possibility exists of a Val/Ser interchange in the tryptic peptide T6. Several different preparations of T6

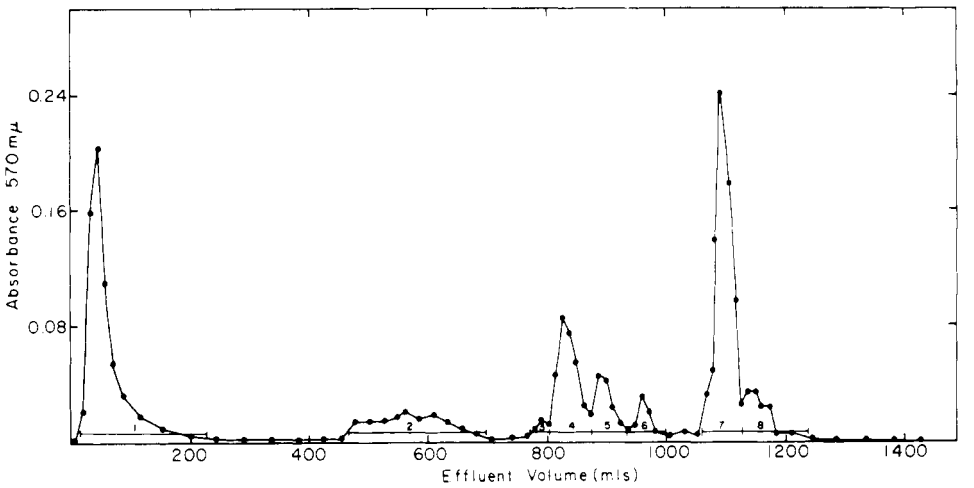

FIGURE 4: Separation of the thermolytic peptides contained in pool 1 (Figure 3) on a column of Dowex 1-X2. Details of the procedure are found in Birshtein *et al.* (1971b). A portion of the effluent was automatically analyzed by the ninhydrin reaction after alkaline hydrolysis. The absorbance of the reaction mixture was monitored at 570 mμ. Fractions were collected for 7.5 min each.

TABLE IV: Thermolytic Peptides from C-4.[a]

	Th28	Th37	Th38	Th41
Lys	2.1		1.1	
Asp				0.98
Thr				0.96
Ser	2.0		0.90	
Glu				1.0
Gly				1.5
Ala				1.3
Leu	1.0	0.95		
Tyr			0.91	
Phe		1.1		
Trp				+
Mobility				
pH 3.6	+0.96	+0.42	+0.81	0
Pool of origin				
(Figure 3)	4	5	5	5

[a] Compositions and mobilities are expressed as indicated in Table I.

TABLE V: Chymotryptic Peptides from C-4.[a]

	Ch40	Ch41	Ch42	Ch43	Ch44
Lys	1.0	1.0	2.0		
Arg	0.98				
CM-Cys					1.0
Asp	1.0		1.1	1.1	
Thr			1.0*	0.91	0.94
Ser	2.1	0.85	1.7		0.99
Glu	1.9			1.1	
Pro	1.1				
Gly				1.1	
Ala	0.89		0.98		
Val	1.9		1.1	0.95	1.1
Leu		1.1	1.1		
Tyr	1.1			0.87	
Hsr					1.0
Trp				+	
Mobility					
pH 3.6	+0.47	+0.83		−0.40	−0.20
pH 6.5	+0.07	+0.52	+0.27	−0.36	−0.50

[a] Compositions and mobilities are expressed as indicated in Table I.

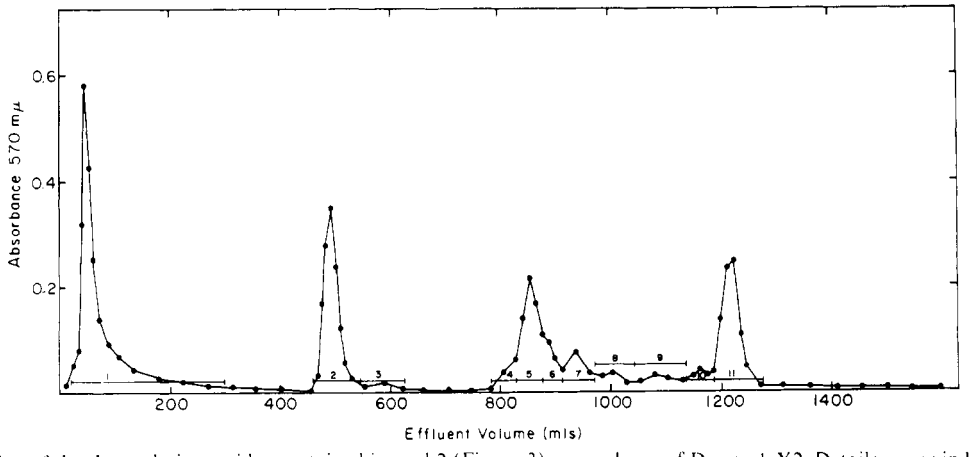

FIGURE 5: Separation of the thermolytic peptides contained in pool 2 (Figure 3) on a column of Dowex 1-X2. Details are as in legend for Figure 4.

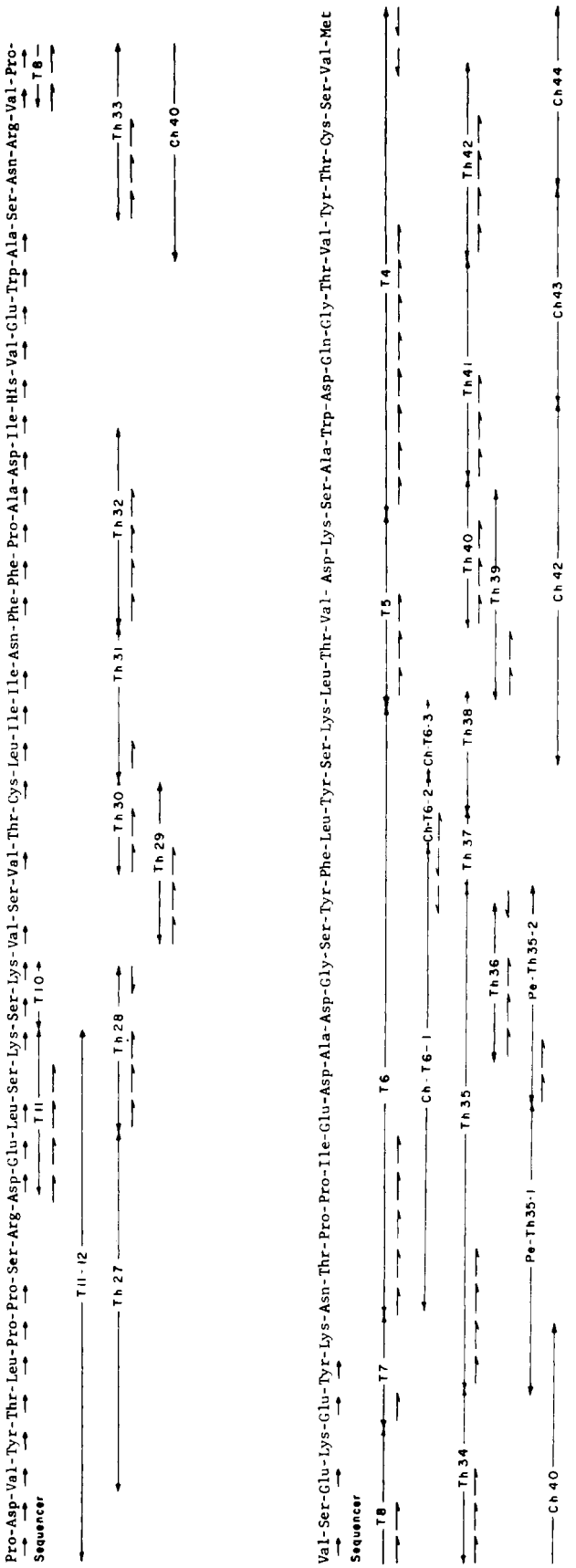

FIGURE 6: A schematic representation of the procedures and resulting data used to determine the sequence of C-4. Exact compositions of the peptides are given in Tables I-V. Symbols used are: (—), removal of a residue by the Edman degradation procedure; (—) identification of a residue by treatment with carboxypeptidase A; (—) identification of a residue by treatment with carboxypeptidase B; (→) removal of a residue by the automatic sequencer.

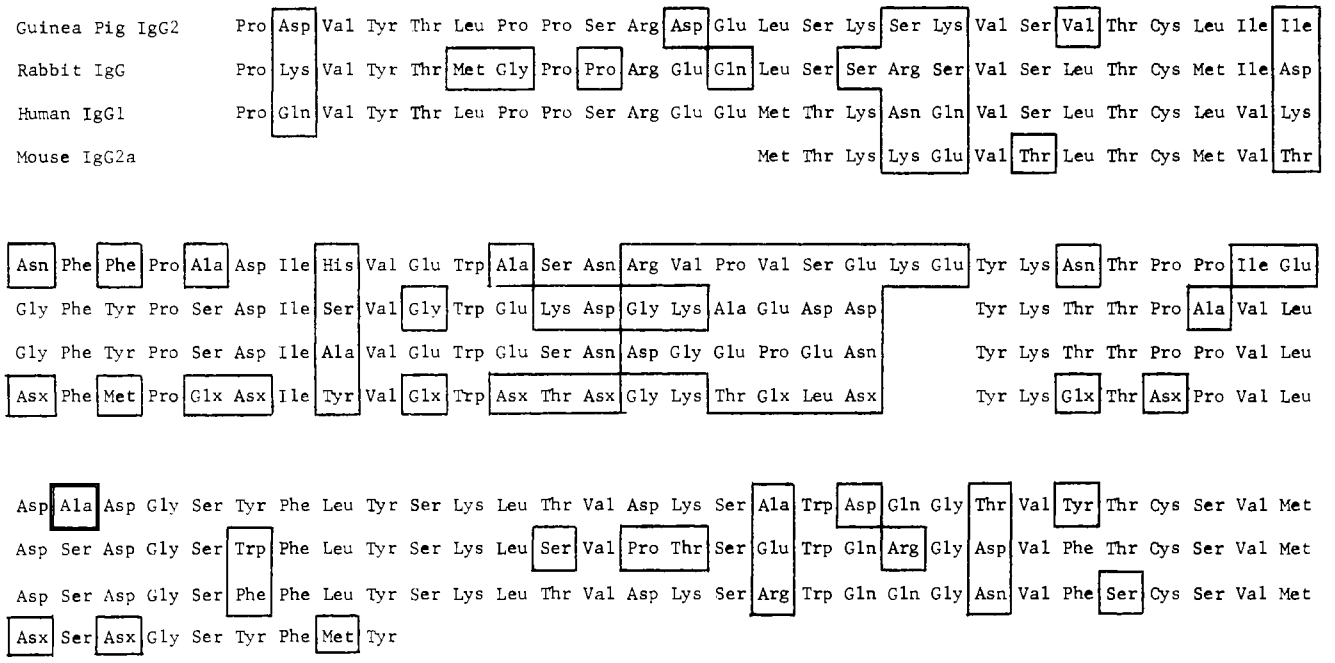

FIGURE 7: A comparison of the amino acid sequences from the fourth quarter of γ chain from IgG of several species. The sequences shown are taken from Edelman *et al.* (1969) for human IgG1, Hill *et al.* (1967) for rabbit, and Bourgois *et al.* (1970) for mouse IgG2a. A closed rectangle is placed around each residue at a given position which is present in only one species. For purposes of comparison, Asx and Glx are considered nonidentical with the corresponding acid or amide.

have consistently given the amino acid analysis shown in Table I. Peptide T6 has been isolated by preparative high-voltage electrophoresis and by ion-exchange chromatography on DEAE-Sephadex A-25, both methods yielding identical peptides. A chymotryptic digest of T6 was not helpful in locating the possible interchange due to the poor analysis gotten of peptide Ch-T6-1. Thermolytic peptides from this region failed to show any extra valyl residue and the question of whether the valine may indicate an isotypic variant or was due to a contaminating peptide was not pursued further.

As with other cyanogen bromide fragments of the heavy chain, the sequence of C-4 was very homologous with both rabbit γ- and human γ_1-chain sequences. Figure 7 shows, however, a short segment of polypeptide almost completely dissimilar among the three species of heavy chains. In addition, the guinea pig heavy chain is found to have two more residues in this region than in this same region of either rabbit or human γ chain. In the guinea pig γ_1 chain, this region has also been found to be nonhomologous with other sequences and to contain four fewer residues than the guinea pig γ_2 chain (T. Trischmann, unpublished data). A partial sequence of the mouse myeloma MOPC 173 is also shown in Figure 7. The sequence of the heavy chain of this γ_{2a} myeloma is homologous with that of the rabbit γ chain at four out of six positions in this region. As more sequences become available for this region, it will be easier to decide if a marked lack of homology does occur in this short stretch.

While no secondary biological properties have been ascribed to the fourth quarter or C_H3 homology region of the guinea pig heavy chain, Yasmeen *et al.* (1973) have found that the ability to adhere to the plasma membrane of macrophages appears to correlate with the C_H3 domain of human IgGs. Minta and Painter (1972) have evidence that the pFc′ fragment obtained from a peptic digest of human IgG1, which comprises the C_H3 homology region, may be able to compete with intact antibody for globulin binding sites on mast cells. The ability to isolate cyanogen bromide fragments C-3 and C-4 from the guinea pig γ_2 chain without breaking the interchain disulfide bond present in each fragment (Birshtein and Cebra, 1971) may prove useful as C-3 and C-4 comprise respectively most of the C_H2 and C_H3 homology regions. It may therefore be possible to allow each homology region to dimerize and from the C_H2/C_H2 and C_H3/C_H3 domains (Poljak *et al.*, 1972) which may be necessary for secondary biological activity. As distinct secondary biological activities have been associated with the IgG1 and IgG2 subclasses of the guinea pig (Ovary *et al.*, 1963; Bloch *et al.*, 1963; Oliveira *et al.*, 1970), these fragments may lend themselves to studies aimed at identifying the homology region or domain associated with each particular activity. Together with the sequence of the γ_1 chain constant region, the primary structure of C-3 and C-4 from γ_2 chain should be of value to those who seek to correlate primary structure with secondary biological activities of antibody.

Acknowledgments

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Calcium Uptake and Membrane Potential in Mitochondria†

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ABSTRACT: The effect of uncouplers and the ionophores A23187, X-537A, nigericin, and valinomycin on calcium concentration gradients in rat liver mitochondria has been investigated. The calcium gradients under various conditions were compared with rubidium concentration gradients. Calcium concentration gradients were estimated from the extent of uptake as measured by changes in murexide absorption or by ^{45}Ca distribution and the matrix water content, under conditions in which Ca^{2+} binding and internal precipitation inside mitochondria are minimized. All the tested ionophores in very high concentration cause release of calcium. However, in low concentrations A23187 is without effect, valinomycin causes Ca^{2+} release (which depends on external potassium), and nigericin increases the rate and extent of Ca^{2+} uptake. This stimulation is observed only at low nigericin concentrations, low external calcium concentrations, and in the absence of anions such as acetate or phosphate. In the presence of valinomycin, $^{86}Rb^+$ distribution shows good correlation with Ca^{2+} distribution over a wide range of values which were obtained by varying uncoupler concentration or potassium concentration. The Ca^{2+} concentration ratio is always higher than the Rb^+ ratio, approximately obeying the relation $\log([Ca^{2+}]_{in}/[Ca^{2+}]_{out}) = 2 \log([Rb^+]_{in}/[Rb^+]_{out})$. Assuming that rubidium distribution in the presence of valinomycin is governed by membrane potential obeying the Nernst equation, these results are interpreted as evidence that calcium uptake in mitochondria is an electrogenic process driven by membrane potential with a net charge transfer of 2.

The reaction of calcium ions with mitochondria has been the subject of investigation from various standpoints for over a decade. Mainly from the studies of the groups of Lehninger (Lehninger *et al.,* 1967) and Chance (Chance, 1965), it is now well established that Ca^{2+} accumulation by mitochondria is an energy-linked process in which the energy can be derived from electron transport or ATP hydrolysis. More recently, attention has been focused on the existence of a specific Ca^{2+} carrier localized in the inner membrane of mitochondria. The Ca^{2+} binding properties of the proposed carrier (Reynafarje and Lehninger, 1969), its inhibition by lanthanides (Mela, 1968), and its kinetic behavior (Vinogradov and Scarpa, 1973) have been studied in great detail.

However, the nature of the driving force for the Ca^{2+} accumulation against concentration gradients in mitochondria has not been established. Several models have been suggested to describe the coupling between the energy producing metabolic reactions and the energy utilizing transport process. The earlier models were based on direct coupling between the metabolic reactions and the transport process, thus forming a calcium pump (Chance, 1965). However, various other models were considered in which the coupling is not direct but mediated through the transport of other ions, either directly or electrically (for recent discussion, see Lehninger, 1973).

A gradient of major importance, which is formed in energized mitochondria, is that of the proton electrochemical potential which is composed of a membrane potential ($\Delta\psi$) and a concentration gradient (ΔpH). An important role for this gradient for energy conversion in mitochondria has

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