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Fb, a New Enzymatic Fragment of Human γG Immunoglobulin†

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ABSTRACT: Extended digestion of human γG immunoglobulin with subtilisin (EC 3.4.4.16) produces several large fragments and a mixture of small peptides. Chemical and immunological characterization of the two principal fragments indicates that one, Fb(s), is composed of the C_L and C_H1 homology regions of the molecule and that the other, Fc′(s), is similar to the Fc′ fragments obtained by digestion of γG immunoglobulins with pepsin or papain. These results are in accord with the hypothesis that the immunoglobulin molecule is composed of compact domains, separated by stretches of relatively extended polypeptide chain, and provide a method of isolating these domains for determination of their individual functions.

Analysis of the complete primary structure of the γG1 immunoglobulin[1] Eu by Edelman *et al.* (1969) led them to propose that antibodies consist of a series of compact domains, and that each domain contains at least one active site serving a specific function in the molecule. Direct evidence to support this hypothesis has been obtained from X-ray crystallographic studies (Sarma *et al.*, 1971; Poljak *et al.*, 1972) and electron micrographs (Green *et al.*, 1971; Dourmashkin *et al.*, 1971) of various immunoglobulins. Several studies of limited enzymatic digestion of immunoglobulins have shown that cleavage of the chains between homology regions can be obtained (Porter, 1959; Turner and Bennich, 1968), consistent with the hypothesis that the chains are composed of compact, tightly folded regions linked by more extended stretches of polypeptide chain. Similarly, Bence-Jones proteins and isolated light chains can be cleaved into constant (C_L) and variable (V_L) halves (Solomon and McLaughin, 1969; Karlsson *et al.*, 1969).

We now report the isolation of a new immunoglobulin fragment from subtilisin digests of both the myeloma protein Eu and normal γG immunoglobulin. The fragment corresponds to the C_L and C_H1 regions of the molecule. The V_L, V_H, hinge, and C_H2 regions are extensively degraded. These results are consistent with the domain hypothesis and provide a method for preparing the C_L and C_H1 domains for the detailed study of their functions within the immunoglobulin molecule.

Materials and Methods

Protein Eu was prepared from plasma by ammonium sulfate precipitation and DEAE-cellulose chromatography (Edelman *et al.*, 1968). Fab(t) and Fc(t) fragments of protein Eu were prepared by tryptic digestion as described by Edelman *et al.* (1968), except that the protein was not reduced or alkylated prior to digestion. Eu light chain was isolated from partially reduced and alkylated Eu by the method of Edelman *et al.* (1968). Normal human immunoglobulin (Cohn fraction II, lot C-842) was obtained from Lederle Laboratories, Pearl River, N.Y.

† From The Rockefeller University, New York, New York 10021. *Received July 26, 1972.* This research was supported in part by Grants AM-04256 and AI-09273 from the National Institutes of Health, U. S. Public Health Service, and by an Independent Study Award (to P. D'E.) from the Woodrow Wilson National Fellowship Foundation. It was presented in part at the 56th Annual Meeting of The Federation of American Societies for Experimental Biology, Atlantic City, N. J., April 9–14, 1972.

[1] The nomenclature used for immunoglobulins and their chains and fragments follows that recommended by the World Health Organization (1964). The nomenclature of immunoglobulin homology regions (V_L, C_L, V_H, C_H1, C_H2, and C_H3) is that of Edelman *et al.* (1969).

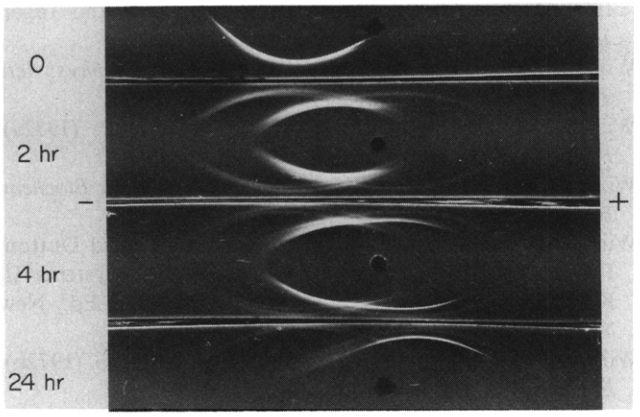

FIGURE 1: Subtilisin digestion of protein Eu. Aliquots taken from the digest after 2, 4, and 24 hr of digestion, as well as an aliquot of the undigested protein (top well), were subjected to immunoelectrophoresis at pH 8.6 (+, anode; −, cathode). The slide was developed with rabbit antiserum against whole protein Eu.

Subtilisin Digestion. Protein to be digested was dissolved (50 mg/ml) in 0.05 M Tris, pH 8.3, and subtilisin (EC 3.4.4.16, protease (subtilopeptidase-A), bacterial type VIII, Sigma Chemical Co., St. Louis, Mo., lot 28B-2340, 1% by weight of the protein) was added. Alternatively, protein was dissolved (50 mg/ml) in 0.15 M sodium chloride, and the pH was adjusted to 8.3. Subtilisin (1% by weight) was added, and the pH was maintained at 8.3 by the automatic addition of 0.5 M sodium hydroxide (Radiometer TTT1b titrator). In both cases, digestion was carried out at room temperature (24–26°) for the times indicated in the text. Where short-term digestion was desired, the enzyme was inhibited by making the digest 0.001 M in phenylmethanesulfonyl fluoride (Schwartz/Mann, Orangeburg, N. Y., lot X1571).

Gel Filtration and Ion-Exchange Chromatography. Gel filtration was carried out at room temperature using either Sephadex G-75 or Bio-Gel P-2. Ion-exchange chromatography was done at room temperature using CM- and DEAE-cellulose (CM52 and DE52, respectively, Whatman, W. and R. Balston, England). The ultraviolet absorbance at 280 and 230 mμ of the effluent fractions from all columns was measured with a Zeiss PMQII spectrophotometer.

Molecular Weights. Molecular weights were determined either in 6.3 M guanidine-HCl (spectrophotometric grade, Heico, Inc., Delaware Water Gap, Pa.), 0.1 M in Tris (pH 8.4) or in 0.1 M Tris (pH 8.4), by the sedimentation equilibrium method (Yphantis, 1964; Nazarian, 1968). Partial specific volumes were calculated from amino acid compositions (McMeekin *et al.*, 1949) and were corrected for the presence of guanidine in the solutions where appropriate (Tanford *et al.*, 1967).

Full Reduction and Alkylation of Proteins. A 5–10-mg/ml solution of protein in 6 M guanidine-HCl–1 M Tris–0.003 M EDTA (pH 8.5) was incubated 1 hr at 50°. A fivefold molar excess of dithiothreitol over protein half-cystine was added and the solution was incubated under N_2 for an additional 2.5 hr at 50°. Iodoacetamide was then added in 20% excess over sulfhydryl groups, and incubation continued at room temperature for 0.5 hr. The resultant peptides were separated from each other and from unreacted reagent by gel filtration on Sephadex G-75 in 6 M guanidine-HCl–0.05 M sodium acetate (pH 5.0). Fractions from this column were desalted by passage over a column of Bio-Gel P-2 in 1 M propionic acid and lyophilized.

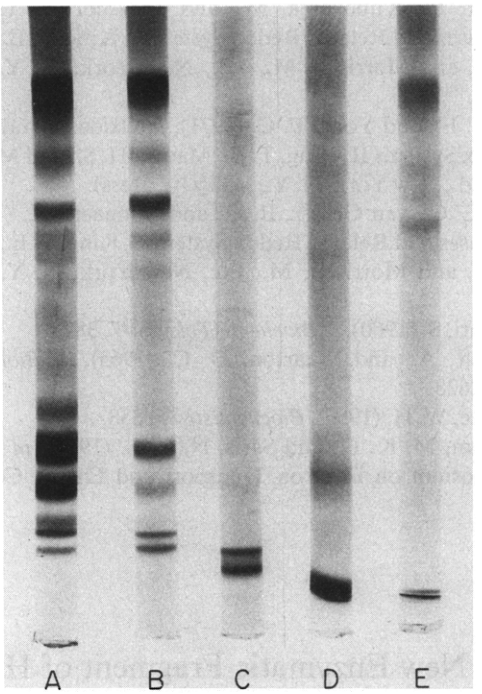

FIGURE 2: Polyacrylamide gel electrophoresis (Davis, 1964) of subtilisin digests of protein Eu. Digests were carried out in: (A) Tris buffer, and in Tris buffer plus (B) 1 M guanidine-HCl, (C) 3 M guanidine HCl, or (D) 6 M guanidine HCl, and (E) in Tris buffer at 55°. Each gel was loaded with 250 μg of digested material. The anode is at the bottom. Gels were stained with Coomassie Brilliant Blue. Under these conditions, intact Eu does not penetrate the running gel. No intact Eu was observed in gels C, D, and E.

Peptide Mapping. Protein was dissolved in 1% ammonium bicarbonate and digested with 1% (w/w) trypsin (trypsin TPCK, Worthington Biochemical Corp., Freehold, N. J., lot 1DA) at 37° for 8–10 hr. The resulting peptides were separated by high-voltage paper electrophoresis at pH 6.5 in pyridinium acetate buffer (Brown and Hartley, 1966) and then at pH 1.9 in acetic acid–formic acid buffer (Schwartz and Edelman, 1963), using Whatman No. 3MM paper. In some cases, a significant amount of the digest was insoluble in the pH 6.5 electrophoresis buffer; this insoluble material was dissolved in 5% formic acid and digested with 1% (w/w) pepsin (Worthington, lot 709) for 4–8 hr at 37°, prior to electrophoresis. Peptides were eluted from the paper with successive washes of 50% pyridine, water, and 1 M acetic acid. The eluates were lyophilized.

Other Techniques. Immunoelectrophoresis was done using the technique of Scheidegger (1955). Immune diffusion was carried out as described by Olins and Edelman (1962). Amino acid analyses and qualitative end-group analyses were done as described by Edelman *et al.* (1968). Polyacrylamide gel electrophoresis was carried out at pH 8.9 using the method of Ornstein and Davis (Davis, 1964), and also in the presence of 0.1% sodium dodecyl sulfate as described by Weber and Osborn (1969). Dialysis was carried out using 23/32 tubing (Arthur H. Thomas Co., Philadephia, Pa.), which retains material with a molecular weight greater than about 8000.

Results

Preliminary experiments indicated that digestion of human γG immunoglobulin with subtilisin yielded fragments which

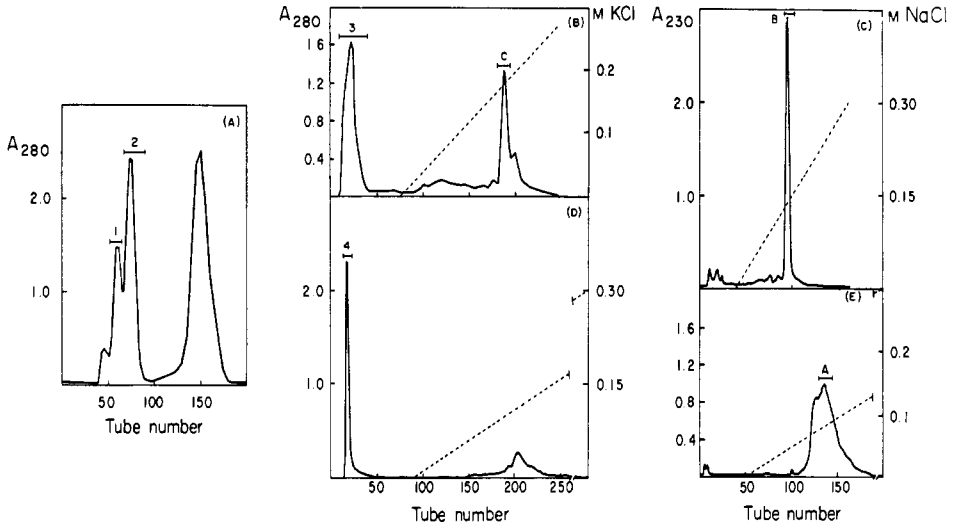

FIGURE 3: Fractionation of subtilisin digest of Eu (500 mg). (A) Gel filtration of whole digest on a column (2.5 × 100 cm) of Sephadex G-75 in 0.01 M Tris, pH 8.0: volume per tube, 3.0 ml; A_{280}, absorbance of effluent at 280 mμ. (B) Ion-exchange chromatography of fraction 2 on DEAE-cellulose: volume per tube, 4.5 ml. (C) Ion-exchange chromatography of fraction 3 on CM-cellulose: volume per tube 4.3 ml; A_{230}, absorbance of effluent at 230 mμ. (D) Ion-exchange chromatography of fraction 1 on DEAE-cellulose: volume per tube, 3.5 ml. (E) Ion-exchange chromatography of fraction 4 on CM-cellulose: volume per tube, 3.0 ml. Peptides were eluted from columns of DEAE-cellulose (1.6 × 30 cm) using a linear gradient from 0.01 M Tris (pH 8.0, 500 ml) to the same buffer (500 ml) which was 0.3 M in KCl. Peptides were eluted from columns of CM-cellulose (1.2 × 15 cm) using a linear gradient from 0.02 M sodium acetate (pH 5.0 400 ml) to the same buffer (400 ml) which was 0.3 M in NaCl.

were not obtained by digestion with papain, pepsin, or trypsin. In order to determine the portions of the molecule represented by these fragments, experiments were carried out on the human γG myeloma protein Eu, whose complete primary structure is known (Edelman *et al.*, 1969).

Digestion of Eu with Subtilisin. The protein is cleaved within 30 min to give fragments resembling Fab and Fc in their mobilities on cellulose acetate and immunoelectrophoresis. After 18–24 hr of digestion, three principal fragments remain (Figure 1). Although components which are immunoelectrophoretically identical with these final digestion products appear within 4 hr of the start of the digest and in increased amounts thereafter, analysis of short-term digests by polyacrylamide gel electrophoresis suggested that many other intermediate products were also present. For this reason, fragments were purified from extended digests, where the number of intermediate products was reduced, at the expense of additional cleavage within the fragments.

In order to determine whether the resistance of these fragments to digestion depended on the conformation of the protein, digests were prepared in the presence of a denaturing agent, guanidine hydrochloride, under conditions where subtilisin has been shown to be active (Stauffer and Sullivan, 1971). Digests were carried out as usual, except that the digestion buffer was 1, 3, or 6 M in guanidine, and that an additional 1% (w/w) of enzyme was added after 12 hr of digestion. After 24 hr, each digest was desalted and freed of small peptides by dialysis against water, and the high molecular weight material remaining was analyzed by polyacrylamide gel electrophoresis (Figure 2). Digests done under native conditions or in the presence of 1 M guanidine gave essentially identical patterns, while no bands were observed in material from digests in 3 or 6 M guanidine. In addition, a digest was also carried out at high temperature (55°). Under these conditions, the protein was almost completely digested to low molecular weight fragments. These results suggest that the resistance to digestion of certain regions of the molecule is due not to some peculiarity of the primary structure, but to the native conformation of the protein.

Purification of Eu Fragments. The principal higher molecular weight fragments were purified from a 24-hr digest as shown in Figure 3. To remove lower molecular weight material, the peptide mixture was first separated by gel filtration on Sephadex G-75 (Figure 3A). The higher molecular weight material, contained in fractions 1 and 2, was purified further by ion-exchange chromatography on DEAE-cellulose (Figure 3B,D). Material which eluted from each DEAE-cellulose column in the initial buffer was purified further by ion-exchange chromatography on CM-cellulose (Figure 3C,E). The major fragments remaining after the extended subtilisin digest were obtained as fractions A, B, and C (Figure 3). Each purified component yielded a single band on immunoelectrophoresis using antiserum against whole protein Eu. The position of each arc corresponds to that of one of the major components seen in the whole digest (Figure 4).

Physical-Chemical and Immunological Properties of the Fragments. Immunodiffusion experiments (Figure 5) indicated strongly that fragments A and B are derived from the Fab region of the molecule, while fragment C is derived from the Fc region. Fragments A and B cross-reacted with Fab(t) (Figure 5A), and fragment B was immunologically deficient to Fab(t) and fragment A. Fragments A and B were immunologically unrelated to purified Eu Fc(t) (not shown). Furthermore, fragments immunologically identical with A and B were obtained from a 24-hr subtilisin digest of Eu Fab(t). As shown in Figure 5C, fragment C shared antigenic determinants with the Fc(t) fragment, but was immunologically deficient with respect to it. Fragment C had no determinants in common with Eu Fab(t) (Figure 5C).

Further immunological and physical-chemical characterization of the major fragment, B, showed that it is approximately half the size of Eu Fab(t) and contains portions of both the light and heavy chains of the molecule. The fragment gave a precipitin line with antiserum against Eu Fab(t) which

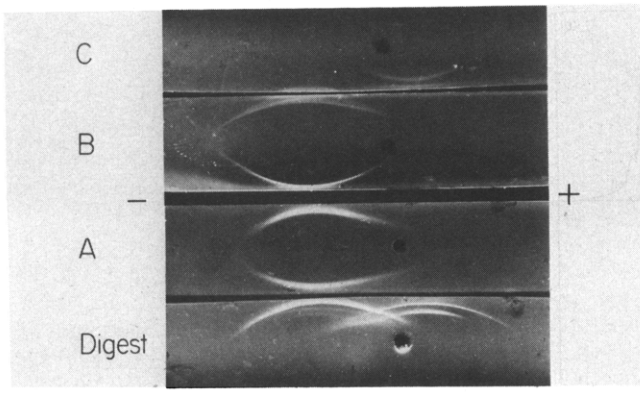

FIGURE 4: Comparison of immunoelectrophoretic patterns of purified fragments (upper three wells) with that of the unfractionated 24-hr digest (bottom well). The slide was developed with rabbit antiserum against protein Eu.

had been exhaustively absorbed with purified Eu light chains (Figure 6). In this reaction, fragment B was still immunologically deficient to Eu Fab(t), suggesting that B contains a portion of the Fd region of the heavy chain.

Fragment B sedimented as a single symmetrical peak with an $s^0_{20,w}$ of 2.1 in 0.01 M Tris–0.15 M NaCl (pH 8.0). The weight-average molecular weight of the fragment determined by sedimentation equilibrium in 0.01 M Tris–0.15 M NaCl (pH 8.0) was 18,000. In 6.3 M guanidine hydrochloride, the weight average molecular weight decreased to 11,000, and, after the addition of 2-mercaptoethanol (final concentration 0.1 M), it decreased further to about 5000. These results suggest that there are internal cleavages in the fragment, as would be expected from the severe digestion conditions. In nondissociating solvents, these internal cleavages seem not to affect the overall structure of the fragment.

In an attempt to obtain this fragment with a minimum of internal cleavages, protein Eu was digested at pH 8.3 for 4 hr, and the digest was fractionated according to the scheme described above. A fragment, B′, resembling fragment B in its properties was isolated in low yield (4%). Fragment B′ was immunologically identical with fragment B, and their amino acid compositions were similar (Table I). The molecular weight of fragment B′, estimated by polyacrylamide gel electrophoresis in sodium dodecyl sulfate, was 24,000. In the presence of 2-mercaptoethanol, the molecular weight decreased to 12,000. These data suggest that fragments B and B′ represent approximately the same portions of the molecule. We believe that the difference in the molecular weights of fragments B and B′ is due to the fact that fragment B has

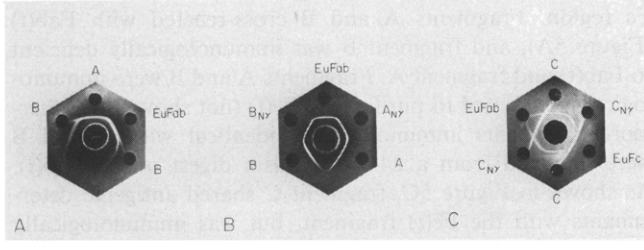

FIGURE 5: Comparison of fragments of Eu and normal human immunoglobulin by immunodiffusion. Subtilisin fragments A, B, and C (Figure 3) prepared from Eu, the corresponding fragments $A_{N\gamma}$, $B_{N\gamma}$, and $C_{N\gamma}$ prepared from normal immunoglobulin, and Fab and Fc fragments, prepared from protein Eu by tryptic digestion, were placed in the outer wells. Rabbit antiserum against protein Eu was placed in the center well in plates A and C; rabbit antiserum against Eu Fab was used to develop plate B.

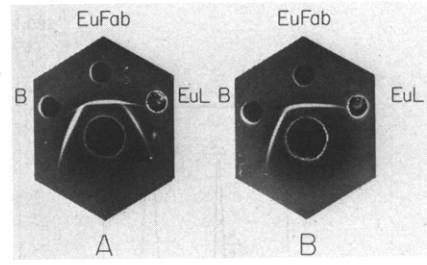

FIGURE 6: Demonstration of heavy chain antigenic determinants in fragment B. Rabbit antiserum against Eu Fab was used to develop plate A, and the same antiserum exhaustively absorbed with Eu light chain (Eu L) was used to develop plate B.

been exposed to subtilisin for a longer time and thus contains internal cleavages which are not present in fragment B′. This interpretation is supported by the fact that the weight average molecular weight of fragment B falls to 5000 after reduction, while the molecular weight of fragment B′ falls only to 12,000.

Because the complete structure of protein Eu is known, each of the fragments derived from the protein could be further characterized to determine more precisely the region of the molecule it represents. These results indicate that the major fragment, B, represents the C_L and C_H1 regions of the molecule. Fragment A seems to be a precursor of B in the digestion process, since the amount of fragment A decreases as the digest proceeds, with a corresponding increase in the amount of fragment B. Fragment B is immunologically deficient to A (Figure 5A). Unlike fragments B and C, A does not appear to be a stable end product of the digestion, and was not further characterized.

TABLE I: Amino Acid Composition of Fragment B.[a]

	Residues/20,800g[b]			
	Calcd from Eu Sequence[b]	B	B′	$B_{N\gamma}$
Lys	15	14.8	15.6	12.8
His	4	4.0	4.2	3.3
Arg	3	2.9	3.1	2.6
Asp	16	17.0	16.9	17.1
Thr	17	16.6	15.5	15.8
Ser	34	27.1	29.8	30.9
Glu	15	16.3	14.8	17.8
Pro	11	12.5	12.8	13.5
Gly	12	10.2	12.9	13.1
Ala	11	12.4	12.4	14.9
Cys/2	6	5.0	4.5	3.2
Val	20	20.0	20.6	15.2
Met	—	—	—	—
Ile	2	1.8	2.0	2.4
Leu	15	14.0	14.7	16.1
Tyr	7	5.4	6.3	7.6
Phe	5	6.5	6.2	8.5
Trp	2			

[a] All values are for 20-hr hydrolysis time. Tryptophan was not determined. [b] Molecular weight and composition are calculated for a fragment consisting of light chain residues 127–214 and heavy chain residues 117–222.

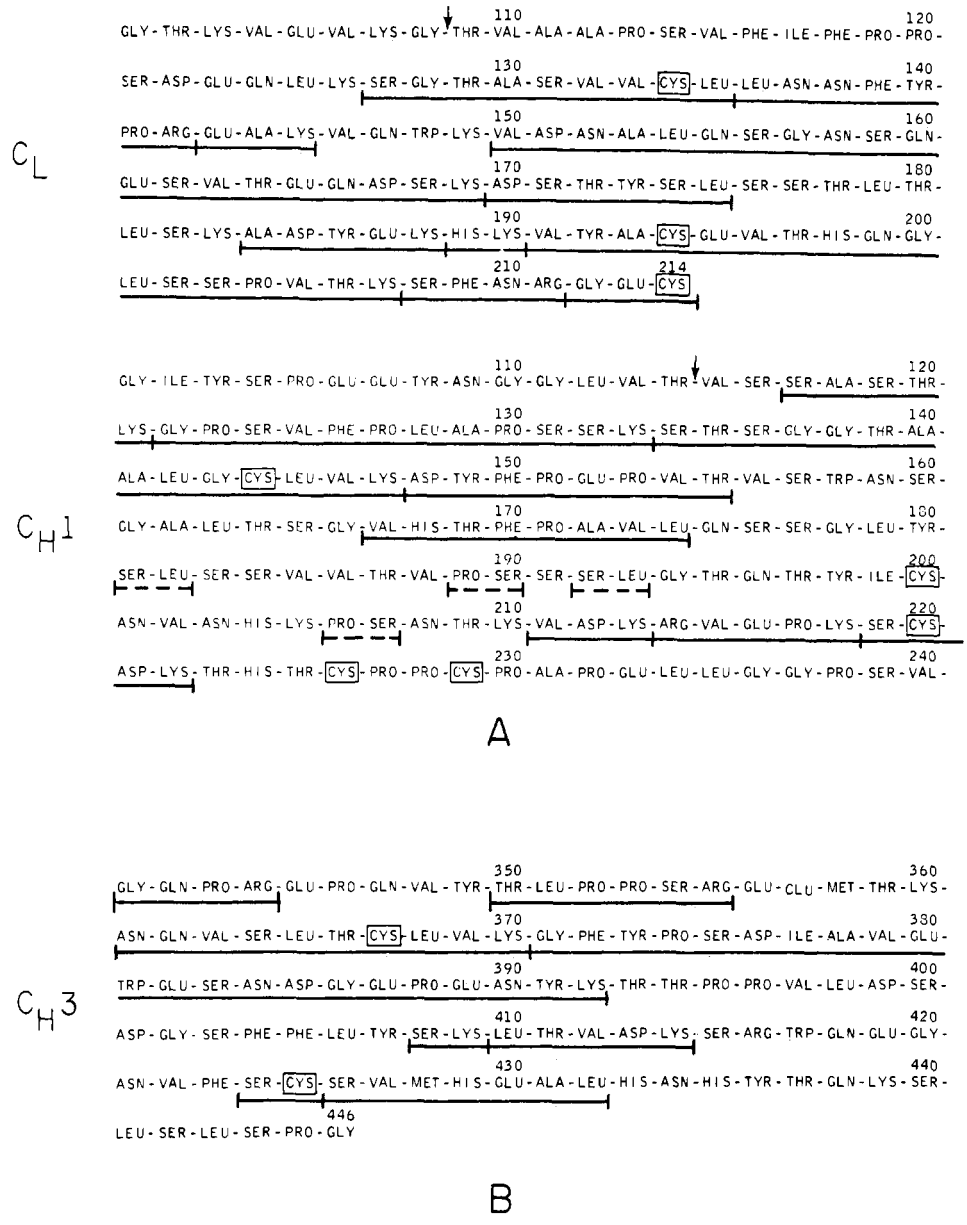

FIGURE 7: Comparison of peptides isolated from peptide maps of fragments B and C with the amino acid sequence of protein Eu (Edelman *et al.*, 1969). Peptides that could be unequivocally located in the sequence are indicated with solid lines; those that could not be placed uniquely are indicated with broken lines. (A) The amino acid sequence of a portion of the C_L and C_H1 homology regions of protein Eu. Numbers indicate the position in the sequence of the whole chain. The arrows indicate the amino termini of the C_L and C_H1 homology regions. Peptides corresponding to the underlined residues were isolated from maps of fragment B. (B) The amino acid sequence of the C_H3 homology region of protein Eu. Peptides corresponding to the underlined residues were isolated from maps of fragment C.

Chemical Characterization of Fragment B. To characterize fragment B more precisely, peptide maps of tryptic and peptic digests of the fully reduced and alkylated fragment were prepared. Peptides were eluted from these maps and their amino acid compositions were determined. Most peptides isolated from the maps could be unequivocally located within the sequence of the molecule on the basis of their compositions and electrophoretic mobilities. All of the peptides could be placed in the C_L and C_H1 regions of the molecule, as shown in Figure 7A. Several dipeptides were obtained whose compositions were consistent with their assignment to the region of the heavy chain between residues 156 and 205. However, the compositions of these peptides are not unique to this region of the molecule, and an unambiguous assignment was not possible. The amino acid composition of fragment B is shown in Table I, together with the amino acid composition calculated for residues 127–214 of the light chain and residues 117–222 of the heavy chain of Eu. The two compositions agree within experimental error. From these data, we conclude that fragment B represents a large portion of the C_L and C_H1 homology regions, from about residue 127 to the carboxyl terminus (residue 214) of the light chain, and from about residue 117 to about residue 222 of the heavy chain. The yield of fragment B, based on the amount of material digested in several different experiments, ranged from 35 to 40%.

Chemical Characterization of Fragment C. The second fragment, C, is derived from the Fc region of the molecule. Peptides were isolated from maps of tryptic and peptic digests of fully reduced and alkylated fragment C. All of these peptides

TABLE II: Amino Acid Composition of Fragment C.[a]

	Residues/10,421g		
	Calcd from Eu Sequence[b]	C	$C_{N\gamma}$
Lys	5	4.8	4.1
His	1	0.6	0.8
Arg	3	2.9	2.5
Asp	9	8.9	10.6
Thr	6	5.6	6.1
Ser	10	9.1	9.7
Glu	13	13.8	13.3
Pro	8	8.2	8.4
Gly	5	5.0	5.9
Ala	2	2.6	3.1
Cys/2	2	1.6	1.0
Val	8	7.7	8.2
Met	2	1.8	1.1
Ile	1	1.1	1.2
Leu	7	6.9	7.6
Tyr	4	4.0	3.7
Phe	4	4.0	4.0
Trp	2		

[a] All values are for 20-hr hydrolysis time. Tryptophan was not determined. [b] Molecular weight and composition are calculated for a fragment consisting of heavy chain residues 341–432.

TABLE III: Proteolytic Fragments of IgG.

Fragment	Domains	Enzyme	Reference
Fab	V_L, C_L, V_H, C_H1	Papain, trypsin	Porter (1959)
Fc	$(C_H2, C_H3)_2$	Papain, trypsin	Porter (1959)
Fd	V_H, C_H1	Papain	Bjork and Tanford (1971)
Fab′	$(V_L, C_L, V_H, C_H1)_2$	Pepsin	Nisonoff *et al.* (1960)
Fc′	C_H3	Papain, pepsin, subtilisin	Utsumi (1969), Turner and Bennich (1968) This paper
Facb	$(V_L, C_L, V_H, C_H1, C_H2)_2$	Plasmin	Connell and Porter (1971)
Fb(s)	C_H1, C_L	Subtilisin	This paper
Fv	V_H, V_L	Pepsin	Inbar *et al.* (1972)
V_L and C_L		Pepsin, trypsin, papain	Solomon and McLaughlin (1969) Karlsson *et al.* (1969) Seon *et al.* (1972)
V_H		Papain	Dammacco *et al.* (1972)
C_H2		(CNBr)	Kehoe and Fougereau (1969)

could be unequivocally placed in the C_H3 region of the molecule, as shown in Figure 7B. The amino-terminal sequence of fragment C as determined by the dansyl–Edman technique is Gly-Glx-Pro, which corresponds to residues 341–343 or 386–388 of the heavy chain. Peptides corresponding to residues 341–344, 350–355, and 361–392 were isolated from this fragment (Figure 7B), suggesting that the N terminus is in fact residue 341. The amino acid composition of fragment C (Table II) indicates that it represents the portion of the Eu heavy chain from residue 341 to 432; this fragment was obtained in about 20% yield. These results indicate that fragment C is similar to the Fc′ fragment which has been obtained by digestion of γG immunoglobulins with pepsin or papain (Utsumi, 1969; Turner and Bennich, 1968).

Nature of Fragments from Normal Immunoglobulin. The large fragments remaining after extended subtilisin digestion of normal human γG immunoglobulin were purified using the fractionation scheme shown in Figure 3. Immunological and chemical tests indicated that these fragments are similar to those obtained from protein Eu. Fragment $B_{N\gamma}$ is immunologically identical were fragment B in its reaction with antisera directed against Eu Fab(t) (Figure 5B), and also with antisera against normal human γG immunoglobulin or against intact Eu. Similarly, fragments A and $A_{N\gamma}$ are immunologically identical. Also, fragments $C_{N\gamma}$ and C give the same reaction with anti-Eu (Figure 5C). As shown in Tables I and II, the amino acid compositions of fragments $B_{N\gamma}$ and $C_{N\gamma}$ are similar to those of B and C, respectively. Fragment $B_{N\gamma}$ was obtained in about 15% overall yield, and fragment $C_{N\gamma}$ was obtained in about 10% yield. These results indicate that the digestion pattern observed is not unique to myeloma protein Eu.

Discussion

The results indicate that extended digestion of human γG immunoglobulins with subtilisin yields two principal fragments. These fragments have been isolated from subtilisin digests of the myeloma protein Eu and characterized on the basis of their immunological properties and primary structure. These data indicate that one fragment, B, represents the C_H1 and C_L regions of the molecule, and that the other, C, represents a well-defined portion of the C_H3 region. A third fragment, A, seems to correspond to a larger portion of the Fab region of the molecule, which is degraded by extended exposure to subtilisin to yield fragment B. Similar fragments are produced by subtilisin digestion of normal human immunoglobulin, suggesting that the digestion pattern observed may be a property of γG immunoglobulins in general. However, the somewhat lower yield of fragments obtained from normal immunoglobulin suggests that the various molecules within this heterogeneous population differ in their susceptibility to digestion with subtilisin. Such differences have previously been observed, *e.g.*, in the proteolytic digestion of individual Bence-Jones proteins to form V_L and C_L fragments (Karlsson *et al.*, 1969; Solomon and McLaughlin, 1969). We propose that fragment B be named Fb(s), to indicate its origin from the constant half of the Fab region of the molecule in the course of subtilisin digestion. We have named fragment C Fc′(s) to indicate its close resemblance to the Fc′ fragments produced by pepsin and papain.

Several different regions of the γG immunoglobulin molecule have been obtained by specific cleavage of immunoglob-

ulins (Table III and Figure 8). As shown by Porter (1959), limited digestion of γG immunoglobulin with papain yields the Fab and Fc fragments (Figure 8B). The major site of cleavage is between the C_H1 and C_H2 regions of the heavy chain. Pepsin digestion yields the Fab′ fragment (Figure 8C) which is a dimer of the Fab region (Nisonoff *et al.*, 1960). The Fc fragment can be degraded further with papain or pepsin to yield the Fc′ fragment (Figure 8F), which corresponds to the C_H3 region of the molecule (Turner and Bennich, 1968; Utsumi, 1969). The fragment Fv (Figure 8G), composed of the V_H and V_L regions, can be produced by peptic digestion of Fab′ (Inbar *et al.*, 1972). Plasmin can be used to prepare the so-called Facb fragment (Figure 8D), which is composed of all of the molecule except the C_H3 region (Connell and Porter, 1971). Isolated immunoglobulin light chains and Bence-Jones proteins (Figure 8H) can be cleaved into variable and constant regions (Solomon and McLaughlin, 1969; Karlsson *et al.*, 1969). Also, rabbit heavy-chain dimers can be cleaved into Fd and Fc regions with papain (Bjork and Tanford, 1971). The V_H domain of a human myeloma protein has been isolated following papain digestion (Dammacco *et al.*, 1972). It has not been possible to isolate a proteolytic fragment corresponding to the C_H2 region of the molecule. However, a CNBr fragment of mouse myeloma protein MOPC 173 corresponding to this region of the molecule has been isolated (Kehoe and Fougereau, 1969). These data emphasize two points: the cleavages produced are specific, and the various domains of the molecule differ in their susceptibility to proteolytic and chemical degradation.

The specificity of the cleavages is striking. In all cases, it occurs between homology regions. Production of Fab, Fab′, Fd, Fc, and Fb(s) all requires cleavage between C_H1 and C_H2. Cleavage of light chains into variable and constant regions, the production of V_H, and the production of Fb(s) all require cleavages between V_L and C_L or between V_H and C_H1, or both. Production of Facb and Fc′ requires cleavage between the C_H2 and C_H3 regions of the molecule.

It is clear that the various homology regions themselves differ greatly in their susceptibility to proteolysis. A large portion of the C_H3 region, for example, is very resistant to digestion by pepsin, papain, and subtilisin. We have shown that the C_L and C_H1 regions together are resistant to subtilisin digestion. The C_H2 region of the molecule, on the other hand, appears to be much more labile. The hinge region is similarly labile, probably because of its relatively extended configuration (Sarma *et al.*, 1971; Poljak *et al.*, 1972). The relative stability of the variable and constant regions of isolated light chains and Bence-Jones proteins seems to vary from protein to protein.

Both specific cleavage and differential susceptibility to proteolysis appear to depend on molecular conformation. Karlsson and collaborators (1969) found that disulfide-linked dimers of Bence-Jones proteins were relatively resistant to digestion, but that prior partial reduction and alkylation of the protein sharply increased the yield of proteolytic fragments. Seon *et al.* (1972) have shown that a Bence-Jones dimer digested with pepsin yielded only V_L at 37° and only C_L at 55°. Our experiments have shown that no large fragments remain after subtilisin digestion of denatured immunoglobulin.

Both the specificity of the cleavages and the differential susceptibility of various regions of the molecule to proteolysis can be explained most easily in terms of the domain hypothesis. All specific cleavages have been observed to occur in the stretches of chain between the homology regions. According to the domain hypothesis, these should be the only regions

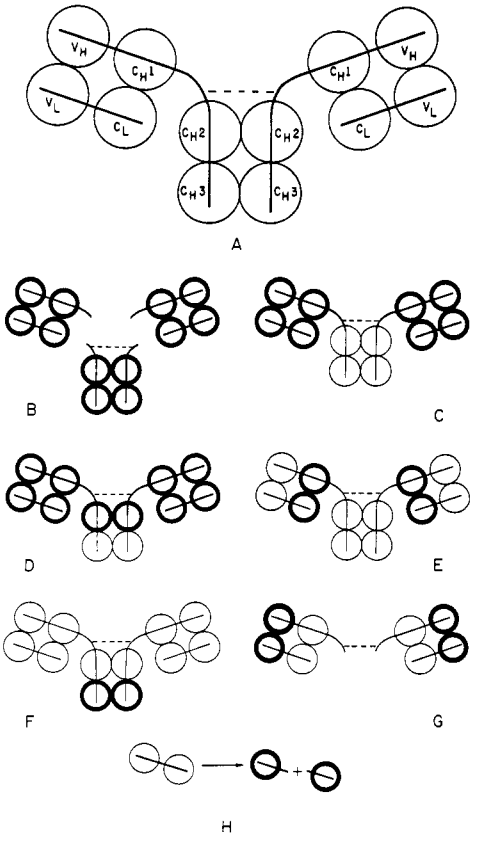

FIGURE 8: Summary of proteolytic fragments of γG immunoglobulin. A diagram representing the whole molecule and indicating the homology regions is given in A. The dotted line indicates the disulfide bonds linking the heavy chains. Fragments which have been obtained by proteolytic digestion (see Table III) are indicated in each case by the heavy lines: (B) Fab (top) and Fc (bottom), (C) Fab′, (D) Facb, (E) Fb, (F) Fc′, (G) Fv, and (H) the production of V_L and C_L from light chains.

of extended polypeptide chain. The hypothesis also suggests that each domain serves a different function. This difference in function should be reflected in the conformations of the various domains. Therefore, the differential susceptibility of the domains to proteolysis is not surprising.

Although the human protein β_2-microglobulin does not appear to be the product of an enzymatic cleavage, its primary structure is homologous to the constant portion of immunoglobulin light chains and to the homology regions of the constant portion of γG1 heavy chains, especially the C_H3 region (Peterson *et al.*, 1972). β_2-Microglobulin contains one intrachain disulfide bond, forming a loop of about 60 residues. These findings suggest that the protein represents a free immunoglobulin domain, possibly serving an effector function similar to that of the C_H3 domain of γG1 immunoglobulins.

A variety of evidence supports the hypothesis that immunoglobulins are arranged in a series of compact domains with each domain connected to the succeeding one by a more extended region of the polypeptide chain. Studies on the proteolytic fragments of immunoglobulins and on molecules such as β_2-microglobulin provide a basis for the detailed study of the various domains. Indeed, with the addition of Fb(s) to the list of immunoglobulin fragments, we can envision the isolation of each of the domains of the molecule for tests of its function, and thus a final test of the domain hypothesis.

Acknowledgment

We thank Professor G. M. Edelman, in whose laboratory this work was done, for his advice and encouragement.

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Chromatographic Separation of *Enhydrina schistosa* (Common Sea Snake) Venom and the Characterization of Two Principal Neurotoxins†

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ABSTRACT: The venom of the common sea snake, *Enhydrina schistosa*, was separated into seven protein zones by gel filtration on Sephadex G-75. The most retarded of the protein zones contained all of the toxic material and accounted for 70% of the soluble venom protein. Further separation of the toxic fraction by gradient chromatography on Bio-Rex 70 in ammonium acetate buffers indicated that the venom preparation studied might contain 16 or more different neurotoxins which together account for 60% of the venom protein, or 45% of the weight of the dried crude starting material. Two principal neurotoxins designated *schistosa* 4 and 5 were isolated in yields corresponding to 14 and 25%, respectively, of the soluble venom protein. Each of the two toxins consists of a single peptide chain of 60 amino acids cross-linked by four disulfide bridges and contains a cysteine residue the free SH group of which can be alkylated with iodoacetate without inactivation. Both toxins have methionine at the amino terminus and the carboxyl terminal sequence Asn-Asn. The two toxins differ in amino acid composition only by substitution of a serine for a proline residue.

Sea snakes, *Hydrophiidae*, are widely distributed in the warmer regions of the Indian and Pacific Oceans. Their venoms are extremely poisonous and primarily neurotoxic in action (Cheymol *et al.*, 1967). Remarkedly large swarms consisting of hundreds of thousands of individuals have been observed. The exclusively aquatic habits of these snakes account for the low frequency of casual unpleasant encounters with humans, but the many thousands caught in nets each year constitute a real occupational hazard to fishermen (Werler and Keegan, 1963; Barme, 1963, 1968; Tu and Tu, 1970).

The venom of the common sea snake, *Enhydrina schistosa*, is among the most toxic snake venoms (Boquet, 1964). The mouse LD_{50} of about 100 μg/kg (Carey and Wright, 1960;

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