Structural Studies of Human γD Myeloma Protein*

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ABSTRACT: The structure of a γD myeloma protein of λ light-chain type was studied. The myeloma protein was composed of two δ and two λ chains linked by two interheavy–light- and only a single interheavy–heavy-chain disulfide bond. The molecular weight of the δ chain was 60,000 and that of the γD calculated from the data of the δ and λ chains was 166,000 and 172,000 when measured by sedimentation equilibrium. The γD contained 11.3% carbohydrate, all localized at the δ chain. When the carbohydrate was taken into consideration, the molecular weight of the peptide portion of the δ chain was found to be the same as that of the γ chain—50,000.

The carbohydrate of the δ chain was distributed among at least three glycopeptides; two containing glucosamine as the characteristic amino sugar were localized in the Fc fragment and one containing galactosamine as the sole amino sugar was localized in the inter–Fd–Fc region of the δ chain. Papain, in the absence of reducing agent, fragmented the γD on the N-terminal side of the glycopeptide containing galactosamine. Cleavage by trypsin and plasmin and the "spontaneously" occurring fragmentation were localized on the C-terminal side of this glycopeptide. No free N-terminal amino acid was detected on the δ chain. Methods which have been shown to identify the C-terminal residues of the γ, α, and μ chains failed to identify the C terminus of the δ chain, suggesting that the C-terminal residues of the δ chain might differ from those of the other immunoglobulin heavy chains.

Immunoglobulin D (γD) has recently been described as a new class of human immunoglobulin (Rowe and Fahey, 1965a; Rowe and Fahey, 1965b). Compared with other immunoglobulins, γD is a relatively minor component, its average concentration in the serum being only about 30 μg/ml. Patients suffering from multiple myeloma only rarely produce γD myeloma proteins, the frequency being about 2% (Fahey *et al.*, 1968; Hobbs and Corbett, 1969; Fishkin *et al.*, 1970). In contrast to γG and γA myeloma proteins and λM macroglobulins, γD myeloma proteins are predominantly of λ light-chain type. The function of γD is unknown and only little information is available on its structure. It has been shown that γD is composed of heavy and light polypeptide chains, has a sedimentation rate of about 7 S and can be fragmented by papain into Fab and Fc fragments (Rowe and Fahey, 1965a). In the present study, additional biochemical characteristics of γD were obtained through the analyses of a γD myeloma protein.

Materials and Methods

γD Myeloma Protein. Plasma of a patient (May) suffering from multiple myeloma was collected by plasmaphoresis into acid–citrate dextrose solution. Immediately following separation of the plasma from the cells, 1 g of ε-aminocaproic acid dissolved in 5 ml of water was added to the plasma bag containing approximately 400 ml of plasma. The plasma was stored at −20°. Urine specimens from the patient were dialyzed against water and lyophilized. The γD myeloma protein was isolated from the plasma by DEAE-cellulose chromatography followed by Sephadex G-200 gel filtration. The bulk of the γG was eluted from the column using 0.015 M phosphate-buffer, pH 8.0, and the γD myeloma protein together with other protein by stepwise increase of molarity to 0.035 M. This fraction was concentrated and applied to a Sephadex G-200 column equilibrated with pH 7.0 phosphate-buffered 0.15 M NaCl (saline). As previously reported, the γD was eluted from the Sephadex G-200 column between γM and γG (Rowe and Fahey, 1965a). ε-Aminocaproic acid to a final concentration of 5 mM was added to all buffers in order to prevent digestion of the γD by serum proteases. The γD preparations were analyzed for purity by immunoelectrophoresis at a concentration of 10–20 mg/ml using antiwhole human serum obtained from Hyland Laboratories, Los Angeles, Calif. Only traces of an α-2 protein were demonstrated in the γD preparations by this method.

The concentration of γD in the serum or plasma of the patient was determined by radial diffusion (Mancini *et al.*, 1965) using rabbit antisera specific for the γD Fc fragment. Isolated γD myeloma protein preparations of known protein nitrogen content served as standards. Protein nitrogen determinations were carried out by a modified method of the micro-Kjeldahl technique using a Technicon Auto Analyzer (Ferrari, 1960). The nitrogen content of the myeloma protein was 15%, calculated on the basis of its amino acid composition and sugar content. The extinction coefficient of the myeloma protein dissolved in saline was $E_{280}^{1\%,\ 1cm}$ 14.5. The extinction coefficient did not change significantly when ε-aminocaproic acid was added to the saline and therefore the concentration of all γD preparations containing ε-aminocaproic acid was determined using the extinction coefficient of 14.5.

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A γA_1 myeloma protein and a γM macroglobulin were isolated by Pevikon block electrophoresis (Müller-Eberhard, 1960) followed by Sephadex G-200 gel filtration from the serum of patients having either multiple myeloma of γA_1 type or macroglobulinemia. Normal human γG was obtained as Cohn fraction II from the American National Red Cross and further purified by DEAE-cellulose chromatography using 0.01 M phosphate buffer, pH 8.0.

Isolation of Heavy and Light Polypeptide Chains. The proteins dissolved in 0.5 M Tris buffer, pH 8.2, were partially reduced at a concentration of 0.02 M dithiothreitol for 1 hr at room temperature and alkylated by addition of 0.05 M iodoacetamide for 1 hr at 4°. The heavy and light chains were separated by gel filtration on Sephadex G-100 columns equilibrated with 1 N acetic acid in the cold. Fd fragments were isolated from reduced and alkylated tryptic Fab fragments by Sephadex G-100 gel filtration in 1 N acetic acid. The first protein peak eluted contained the Fd fragment and the second peak the light chains.

Molecular weight determinations of fully reduced and alkylated heavy and light chains of γD, γG, and γA were carried out according to the method described by Andrews (1965) using a Sephadex G-200 column equilibrated with 8 M urea in 0.05 M propionic acid.

The number of moles of free disulfide groups were measured by determining the moles of carboxymethylated cysteine[1] (CMCys)/mole of heavy and light chains isolated from mildly reduced γD which had been alkylated with 0.5 μCi of ^{14}C-labeled iodoacetamide/mg of protein. Following dialysis and separation of the polypeptide chains, the peak tubes containing either heavy or light chains were analyzed both for protein nitrogen contents and for ^{14}C radioactivity. The number of moles of CMCys/mole of δ chains were calculated by assuming the molar specific activity of the light chains to be equal to 1 CMCys/mole of λ chains. In addition, the number of moles of CMCys/mole of δ chain were determined by amino acid analysis of the reduced and alkylated δ chain.

Enzymatic Digestion. Digestion of γD with twice-crystallized papain (Worthington Biochemicals, Freehold, N. J.) in the presence of reducing agent was carried out according to the method described by Porter (1959), except that the incubation time was reduced to 1 hr. Papain digestion in the absence of reducing agent was performed similarly, except that both cysteine and EDTA were omitted from the digestion mixture. Trypsin digestion was performed at pH 8.0 using 1% w/w trypsin treated with L-(tosylamido-2 phenyl)ethyl chloromethyl ketone (Worthington Biochemicals) at room temperature and the digestion was terminated by addition of 2% w/w soy bean trypsin inhibitor. Pepsin digestion was carried out as described by Nisonoff *et al.* (1960). Highly purified plasminogen (obtained through the courtesy of Dr. Fletcher Taylor) was converted into plasmin immediately before use by incubation with 3000 units of streptokinase (Varidase, Lederle, Inc.) per 1 mg of plasminogen for 10 min at 37°. Plasmin (1% w/w) was added to γD dissolved in saline and incubated for 1 hr at 37°. As a control γD was similarly incubated with streptokinase alone which did not result in fragmentation of the γD. Following incubation of γD with the different enzymes, the mixtures were cooled to 0° and immediately applied to a Sephadex G-100 column equilibrated with saline in the cold. The protein peaks eluted from the G-100 column were concentrated and analyzed by immunoelectrophoresis using antisera specific for λ light chains and λD Fc fragments. The Fab and Fc fragments were purified by DEAE-cellulose chromatography. The Fab fragments were eluted from the DEAE column with 0.015 M phosphate buffer, pH 8.0, and the Fc fragments were eluted from the column using 0.3 M phosphate buffer, pH 8.0.

Digestion with neuraminidase was carried out by addition of 50 units of neuraminidase (Behringwerke, Germany)/mg of protein dissolved in 0.05 M acetate buffer, pH 5.5, containing 0.01 M calcium chloride and incubation for 24 hr at 37°.

Cyanogen Bromide Cleavage. Cyanogen bromide cleavage of the δ chain was carried out in 70% v/v formic acid for 24 hr at room temperature as previously described (Prahl, 1967).

Starch Gel Electrophoresis. Vertical starch gel electrophoresis was performed either by employing a 0.05 M glycine buffer, pH 8.8 (Fahey, 1963), or an acid urea–formate buffer, pH 3.5 (Poulik, 1960).

Ultracentrifugation. Analytical ultracentrifugation was carried out on isolated γD preparations of different protein concentrations using a Beckman Model E analytical ultracentrifuge equipped with schlieren and interference optics. The sedimentation rate of the protein was determined in a single experiment by the standard procedure (Schackman, 1959) to give an extrapolated value for $s^0_{20,w} = 7$ S, using γD protein concentrations of 5.2 and 10.3 mg per ml. The molecular weight of freshly isolated γD was determined by sedimentation equilibrium according to the method described by Yphantis (1964). The partial specific volume, $\bar{v}$, of the γD was calculated to be 0.717 on the basis of its amino acid composition and sugar content (Cohn *et al.*, 1943).

Carbohydrate Analysis. All carbohydrate analyses of γD and its subunits were performed on the peaks of protein eluted from the Sephadex columns. The phosphate-buffered saline or 1 N acetic acid eluted from the column before and after elution of protein was used as a blank. The methods used to determine the various sugars have been described previously (Abel *et al.*, 1968). Hexoses were determined by the orcinol reaction (Svennerholm, 1956), fucose by the cysteine reaction (Dische and Schettles, 1948), glucosamine and galactosamine using a 12-cm column of Aminex A5 on the short column of a Beckman amino acid analyzer, and sialic acid by the thiobarbituric acid assay (Warren, 1959). The standards used consisted of 0.058–0.233 μmole/ml of an equimolar mixture of galactose and mannose; 0.05 and 0.1 μmole of D-glucosamine, 0.012–0.048 μmole/ml of L-fucose, and 0.0485–0.194 μmole/ml of crystalline *N*-acetylneuraminic acid. All values given in the tables represent average values of duplicate determinations of at least three different protein preparations.

Peptide Mapping and Amino Acid Analyses. Peptide mapping, elution of peptides, and analyses of peptides for amino acid composition were carried out as previously described (Grey and Kunkel, 1967). Amino acid analyses were performed using a Beckman Model 120C amino acid analyzer. Tryptophan was determined according to the method

[1] Abbreviation used is: CMCys, carboxymethylated cysteine.

described by Opienska-Blauth *et al.* (1963). N-Terminal amino acids were determined by the method described by Stark and Smyth (1963) and by using the Sanger reagents (Sanger, 1949). Carboxy-terminal amino acids of the δ chain were determined by digestion with carboxypeptidase A or B (Abel and Grey, 1967) and by hydrazinolysis as previously described (Press *et al.*, 1966). Both α and γ chains were used as controls.

Iodination. Trace labeling of the proteins with ^{131}I was carried out according to a modified chloramine T method (McConahey and Dixon, 1966) using 20 μg of chloramine T/mg of protein. The γD myeloma protein was labeled in the presence of 5 mM of ε-aminocaproic acid which did not interfere with the uptake of iodine. No fragmentation of the γD myeloma protein occurred during the labeling as demonstrated by autoradioimmunoelectrophoresis of the labeled γD.

Results

γD Myeloma Protein May. The isolated γD myeloma protein May appeared on examination by starch gel electrophoresis at pH 8.8 as a single relatively broad band of β electrophoretic mobility (Figure 1). When analyzed by immunoelectrophoresis it reacted specifically with an anti-γD antiserum obtained from the National Institutes of Health Reference Center and with a rabbit antiserum to human λ light chains. The studies reported in this paper were all performed on γD isolated from a single plasmaphoresis specimen in which the γD concentration was 7.5 mg/ml. In first attempts to isolate the γD myeloma protein, spontaneous degradation of the γD into Fab- and Fc-like fragments occurred. This degradation was completely prevented by the addition of 5 mM ε-aminocaproic acid to all buffers. Once isolated, the myeloma protein was relatively stable and did not fragment when stored either at 0 or at −20° for at least 1 week. However, to be sure to avoid any spontaneous fragmentation, the γD preparations were stored in the presence of 5 mM ε-aminocaproic acid. The isolated myeloma protein migrated as a single homogeneous peak in the analytical ultracentrifuge with an extrapolated value for $s^0_{20,w}$ of 7.0 S. Following mild reduction and alkylation, the γD myeloma protein could be separated into heavy and light chains by gel filtration on a Sephadex G-100 column equilibrated with 1 N acetic acid. The protein was eluted in the heavy-chain peak (78%) and in the light-chain peak (22%). The analysis of the γD and the reduced and alkylated γD by urea acid–starch gel electrophoresis is shown in Figure 1. The native γD showed a single band and the reduced and alkylated γD showed two bands, a cathodal band corresponding to the light chains, and a more slowly moving band corresponding to the heavy chains. The molecular weights of the fully reduced and alkylated γD heavy (δ) and light (λ) chains were determined by gel filtration using a Sephadex G-200 column equilibrated with 8 M urea–propionic acid (Table I). By this method, molecular weights of 60,000 and 23,000 were obtained for the δ and λ chains, respectively, assuming molecular weights of 52,000 for the normal human γ chains and 23,000 for the normal human light chains which were used as markers. Assuming a four chain model, the molecular weight of γD calculated from these data would be 166,000. Using the same Sephadex column, molecular weights of 55,000 and 69,000 were obtained

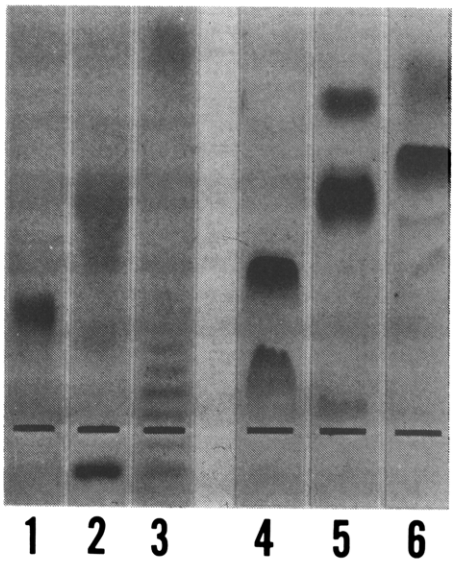

FIGURE 1: Starch gel electrophoresis employing a glycine buffer, pH 8.8, anode on top (1–3); employing 8 M urea formate buffer, pH 3.5, anode at the bottom (4–6): (1) and (4) γD May; (2) γD digested for 1 hr with papain in the absence of reducing agent; (3) γD digested for 2 min with trypsin; (5) reduced and alkylated γD; (6) reduced and alkylated normal γG. The point of application appears as a solid bar.

for human α and μ chains, respectively. The molecular weight of the γD myeloma protein was also determined by sedimentation equilibrium ultracentrifugation. Six determinations on two different preparations of γD were made. No evidence of heterogeneity was discernible by examination of the In fringe displacement *vs.* rad^2 plot, nor was any concentration dependency observed. A $\bar{v}$ of 0.717 was calculated from the amino acid and sugar compositions. The molecular weights obtained from these data ranged from 170,000 to 174,600 with a mean of 172,000.

The amino acid composition expressed as moles of amino acids/mole of γD and δ chain compared with that of γ chains of 5 γG myeloma proteins is shown in Table II. As compared with γ chains of the five myeloma proteins, the δ chain May had a relatively high content or arginine, glutamic acid, and alanine. The fully reduced and alkylated δ chain contained 10 moles of CMCys. When the number of moles of CMCys was determined following mild reduction of γD sufficient to cleave only the interchain disulfide bonds, the δ chains contained 2.1 moles of CMCys. The relatively low content of CMCys/mole of mildly reduced δ chain was further shown by determining the uptake of ^{14}C-labeled iodoacetamide. In three different experiments, the heavy chain was labeled with 2.1–2.5 moles of [^{14}C]iodoacetamide/mole of polypeptide chain and the light chain with 0.9–1.1 moles.

The carbohydrate content of the γD myeloma protein is shown in Table III. The myeloma protein contained 11.3% carbohydrate, all of which was localized to the heavy chain. Besides neutral sugars, the γD contained both galactosamine and glucosamine and a relatively large quantity of sialic acid. No significant amounts of fucose were detected.

Fragmentation of γD. Fragments obtained following digestion of γD with papain, trypsin, plasmin, pepsin, and following "spontaneous" degradation of γD were studied.

TABLE I: Molecular Weight Determinations of Intact γD May and Its Polypeptide Chains by Equilibrium Sedimentation and Gel Filtration.

Protein	No. of Determinations	Sedimentation Equilibrium[a] Protein Concn (mg/ml)	$\bar{v}$	rpm	Wt-Av Mol Wt
γD May (prep 1)	3	0.25–1.0	0.717 (calcd)	14,290	172,300 (170,100–174,600)
γD May (prep 2)	3	0.2–0.8	0.717 (calcd)	14,290	171, 600 (170,700–172,400)

Material	$\sqrt{K_d}$	Gel Filtration[b] Mol Wt (Lit. Value)	Calcd Mol Wt
δ chain May	0.2726		60,000
σ chain May	0.4802		23,000
γ chain (human)	0.3233	52,000	
L chain (human)	0.4842	23,000	
μ chain (human)	0.2321		69,000
α chain (human)	0.3054		55,000

[a] Samples in phosphate-buffered saline (pH 7.2) were run at a controlled temperature of 20° in a Spinco Model E ultracentrifuge equipped with Rayleigh interference optics. Molecular weights were calculated from the graphically averaged plot of ln C *vs.* r^2 (Yphantis, 1964) and are presented as the average of the indicated number of determinations. The range of values is indicated in parentheses. Solvent density was taken as 1.0 for all calculations. [b] Molecular weights were calculated from a plot of $(K_d)^{1/2}$ *vs.* $(MW)^{1/2}$ (Andrews, 1965). $K_d = (V_e - V_0)/V_i$.

TABLE II: Amino Acid Composition of γD and δ Chain May, γ Chains of 5 γG Myeloma Proteins, and the Peptides of the δ Chain Containing the Inter-Heavy (H–H) and Interheavy–Light (H–L) Disulfide Bonds and of 3 Glycopeptides of the δ Chain (G–I, G–II, G–III).

	γD[a]	δ[a]	γ[b]	H–H	H–L	G–I	G–II	G–III
Lys	72	21	25–29					0.8
His	30	11	5–11	0.9		1.4		
Arg	58	23	12–17		1.0	1.0	1.0	1.0
Asp	92	32	34–39		1.3	0.9	1.0	1.1
Thr	118	41	33–40	1.5			1.1	3.4
Ser	146	46	40–47	1.0	1.2	3.3	1.0	2.3
Glu	170	61	36–41	2.1	1.6	2.8	0.6	2.7
Pro	122	42	29–42	3.0	1.2		0.6	1.8
Gly	106	32	26–32	1.2	1.7	1.1		1.6
Ala	112	38	16–20		0.7		1.3	4.8
Val	102	33	38–41	0.9	0.8			0.7
Met	16	8	4–8					
Ile	32	8	6–9		0.7			
Leu	110	40	30–36	1.0	1.0		1.6	0.7
Tyr	50	13	13–20	0.5				
Phe	40	11	12–15		0.6			
Trp	16	7	7–10					
Glu N	24	12	10[c]			+[d]	+[d]	
Gal N	14	7	0[c]					+[d]
CMCys (total)		10	11[c]	+[d]	+[d]			
CMCys (labile)		2.3	4–5					

[a] Average of 3 determinations mole/mole of γD (155,000) and δ chain (50,000). [b] Range of 5 γG myeloma proteins (Prahl, 1967). [c] Values obtained for normal γ chains treated under the same conditions as δ chain. [d] Yields were variable and low so that no quantitation was possible.

TABLE III: Carbohydrate Content of the γD Myeloma Protein May and Its Proteolytic Fragments.[a]

	Hexoses	Glucosamine	Galactosamine	Sialic Acid	Fucose
γD	47	23	13	15	0.5
L chain	0	0	0	0	0
Fab papain ± reduction	0	0	0	0	Nt[c]
Fc papain − reduction	51	+[b]	+	+	Nt
Fc papain + reduction	32	+	−	+	Nt
Fab trypsin	15	−	+	+	Nt
Fc trypsin	28	+	−	+	Nt

[a] Average of triplicate determination. Moles of sugar/mole of protein assuming molecular weights of 166,000 for γD and 55,000 for Fab and Fc fragments. [b] Yields were variable so that no quantitation was possible: (+) positive reaction; (−) negative reaction. [c] Nt, not tested.

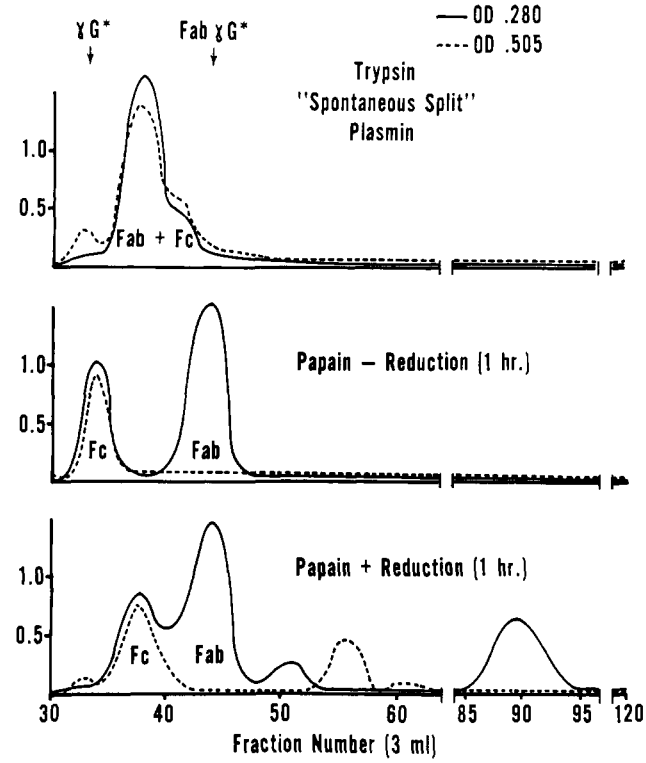

FIGURE 2: Elution of γD digested with various enzymes from a Sephadex G-100 column equilibrated with phosphate-buffered 0.015 M NaCl, pH 7.0. The hexose content of the eluates determined by the orcinol reaction was measured at 0.505 μ.

The fragments were isolated by Sephadex G-100 gel filtration and DEAE-cellulose chromatography and analyzed by starch gel and immunoelectrophoresis. Fab fragments were identified antigenically by their reaction with an anti-λ light-chain antiserum and Fc fragments by their reaction with anti-γD class-specific antiserum. Papain fragmented γD into Fab and Fc fragments both in the presence and the absence of reducing agent. The Fab fragments thus obtained were eluted from the Sephadex G-100 column at a position similar to that of Fab fragments obtained from normal γG (Figure 2). The Fc fragments obtained following digestion with papain in the absence of reducing agent were eluted from the Sephadex G-100 column together with ^{131}I-labeled γG used as marker. In contrast, the Fc fragments obtained following digestion with papain in the presence of reducing agent were eluted in a position between γG and Fab fragments. In addition to the formation of Fab and Fc fragments, papain digestion in the presence of reducing agent resulted in the release of small peptides (including a glycopeptide) which were eluted from the Sephadex column after the Fab fragments (Figure 2). The starch gel electrophoresis of γD digested with papain in the absence of reducing agent is shown in Figure 1. The papain Fab fragment showed a single band with a cathodal mobility and the Fc fragment had a more anodal mobility than intact γD. The carbohydrate content of the papain fragments of γD is summarized in Table III. The Fab fragments did not contain carbohydrate. The Fc fragments obtained following digestion with papain in the absence of reducing agent contained all the carbohydrate present in the γD. In contrast, the Fc fragments obtained following digestion with papain in the presence of reducing agent contained only about two-thirds of the neutral sugars of γD, sialic acid, and glucosamine as the sole amino sugar. Because of the limited quantity of γD myeloma protein available, only hexose determinations could be performed repeatedly and therefore no quantitative data are given in Table II for glucosamine, galactosamine, and sialic acid.

Digestion of γD with trypsin for 2 min resulted in the formation of Fab and Fc fragments differing from the papain fragments of γD. The tryptic Fab and Fc fragments were eluted from the Sephadex G-100 column together in a position between γG and γG Fab fragments used as markers (Figure 2). The Fab fragments showed about eight distinct bands by starch gel electrophoresis all of which had an intermediate electrophoretic mobility unlike the Fc fragments which had a very anodal mobility (Figure 1). Treatment of the tryptic Fab fragments with neuraminidase reduced the number of bands to one, the most cathodal band, indicating the multiple banding was the result of heterogeneity of different molecules with respect to the sialic acid contents. In addition to sialic acid, the tryptic Fab fragments contained about one-third of the neutral sugars of the γD myeloma protein and galactosamine as sole amino sugar (Table III). The tryptic Fc fragments contained about two-thirds of the neutral sugars present in γD and in addition sialic acid and glucosamine as the sole amino sugar.

Fragments obtained following digestion of γD with plasmin and following "spontaneous" fragmentation of γD were indistinguishable from the tryptic Fab and Fc fragments both by starch gel electrophoresis and elution of the Sephadex G-100 column. Digestion of γD with pepsin for 18 hr resulted in the formation of mainly dialyzable peptides and was therefore not further studied.

Peptide Maps and Peptide Analysis of γD. In order to localize the position of the interchain disulfide bonds with

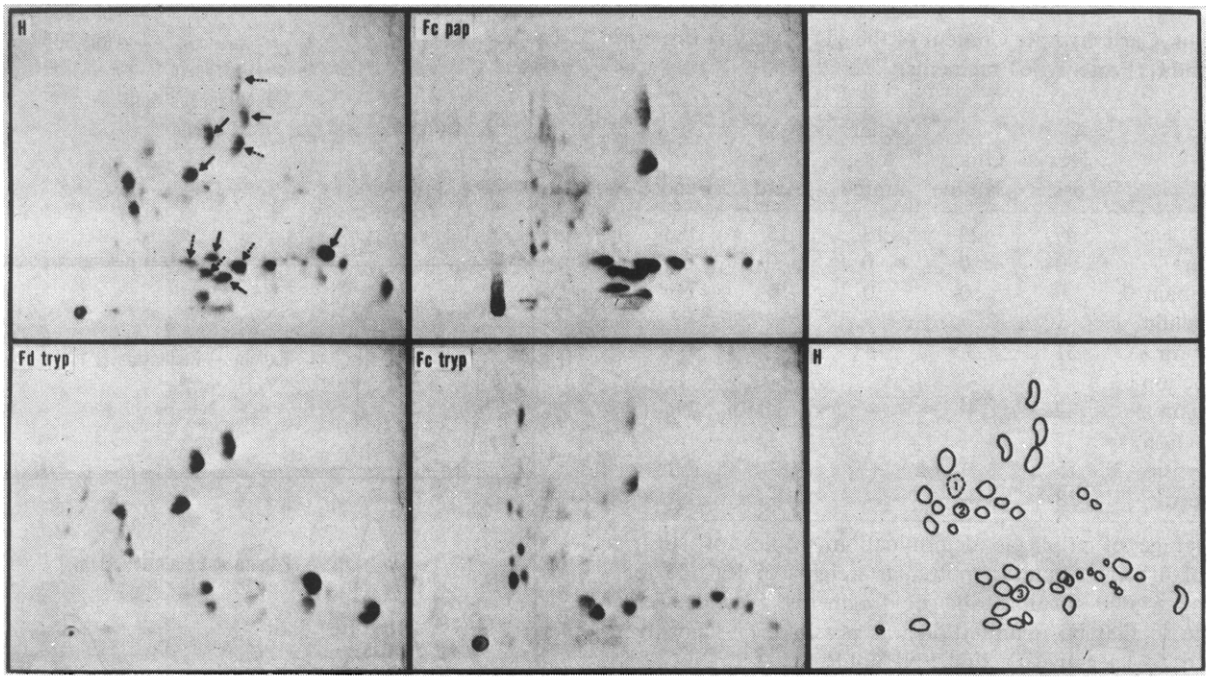

FIGURE 3: Tryptic peptide maps of the γD heavy chain (H) and γD Fc and Fd fragments. The Fc fragments were obtained either following digestion with papain in the absence of reducing agents (Fc pap) or following brief digestion with trypsin (Fc tryp). The Fd fragments (Fd tryp) were isolated from reduced and alkylated tryptic Fab fragments by Sephadex G-100 acid gel filtration. Solid arrows on the H-chain map indicate some of the more easily recognized peptides of the Fd fragment and broken arrows indicate some of the peptides of the Fc fragment. Autoradiography of maps of partially reduced and [^{14}C]iodoacetamide-alkylated material revealed only 2 radioactively labeled peptides (1 and 2 on the composition map). Peptide 1 was present on the map of the Fd fragments and therefore must contain the cysteine involved in the interheavy–light disulfide bond and peptide 2 was on the map of the Fc fragments and must therefore contain the cysteine involved in the interheavy–heavy-chain disulfide bond (see text). Peptides 3 and 4 were present both on the maps of the papain Fc fragments and tryptic Fd fragments, suggesting that they came from the inter-Fd–Fc region of the δ chain.

respect to the fragments of γD, autoradiographic analyses were performed on peptide maps of γD polypeptide chains and of the papain and trypsin Fab and Fc fragments which had been mildly reduced and alkylated with [^{14}C]iodoacetamide. Fd fragments isolated from the tryptic Fab fragments were analyzed in a similar manner. The radioactively labeled peptides were eluted from the maps and analyzed for their amino acid composition. The glycopeptides, which characteristically did not migrate on chromatography and were thereby localized in the line of application on the paper, were also eluted from the maps and analyzed for amino acid and amino sugar composition. The δ chain contained two radioactively labeled peptides, one of which was ninhydrin negative (Figure 3), and the light chains contained one labeled peptide. The Fc fragment contained one labeled peptide corresponding to one of the labeled peptides of the δ chain. The papain and tryptic Fab fragments contained two labeled peptides, one corresponding to the peptide also present in the Fd fragment and one corresponding to the labeled peptide of the light chains. Therefore, the labeled peptide present in the Fd fragment represented the peptide containing the cysteine of the heavy–light-interchain disulfide bond. Both papain and trypsin Fc fragments contained one labeled peptide in a position corresponding to one of the labeled peptides of the δ chain. This peptide apparently contained the cysteine of the interheavy-chain disulfide bond. The amino acid composition of the two peptides containing either the interheavy or heavy–light-chain disulfide bonds is shown in Table II. Neither one of these peptides contained an amino sugar. The peptide containing the interheavy-chain disulfide bond contained 3 proline residues out of 14 amino acid residues. Three glycopeptides were isolated from the map of the δ chain. As seen in Table II, two contained glucosamine and one galactosamine as sole amino sugar. None of the glycopeptides contained a high number of proline residues. The peptide map of the papain Fc fragment (obtained following digestion in the absence of reducing agent) showed more peptide spots than the map of the tryptic Fc fragment (Figure 3). Two of these additional peptides present on the map of the papain Fc fragment were found on the map of the tryptic Fd fragment, suggesting that they came from the inter-Fd–Fc region of the δ chain. These two peptides contained 7 and 8 amino acids, respectively, but no proline residues.

N- and C-Terminal Amino Acids. Attempts to determine the N- and C-terminal amino acids of the δ chain were unsuccessful. The cyanate method of Stark and Smyth did not demonstrate a free N-terminal residue nor could any dinitrophenyl derivatives be identified using the Sanger reagents. No amino acids were released from the δ chain following digestion with carboxypeptidases A and B, even if the digestion was carried out in the presence of 2 M urea. Hydrazinolysis did not establish the identity of the C-terminal residue, since it yielded 2 moles of serine and 1 mole of glycine/mole of δ chain. The yield of 2 moles of serine and 1 mole of glycine might be artifactual, however, it was not found when α chains (which yielded 1 mole of tyrosine) were examined under the same conditions. Cyanogen bromide cleavage

of the δ chain did not give rise to the formation of the characteristic octadecapeptide C-terminal fragment found in human γ chains nor the octapeptide found in human α or μ chains.

Discussion

A schematic model of the structure of γD, based on the data obtained from the γD myeloma protein May, is shown in Figure 4. The γD is composed of four polypeptide chains, two λ light and two δ heavy chains as indicated from the mass ratio of the heavy and light polypeptide chains and the respective molecular weights. The polypeptide chains are probably linked by three disulfide bonds, two interheavy–light- and one interheavy- chain disulfide bond. The δ chain contains a relatively large amount of carbohydrate, which is distributed among a minimum of three glycopeptides. One glycopeptide containing galactosamine as characteristic amino sugar has been localized in the inter-Fd–Fc region and two glycopeptides containing glucosamine as sole amino sugar are located in the Fc fragment. Papain in the absence of reducing agent fragments γD on the N-terminal side of the glycopeptide containing galactosamine and trypsin, plasmin, and the "spontaneous" fragmentation of γD occurs on the C-terminal side of this glycopeptide.

The most striking difference between γD and other classes of immunoglobulins appears to be the enzymatic cleavage into Fab and Fc fragments. In contrast to γG, which is digested by papain and trypsin at a very similar site (Rutishauser *et al.*, 1968), the γD was digested by these enzymes at readily distinguishable sites as shown by the absence and presence of carbohydrate on the respective Fab fragments. The comparative peptide mapping of the Fd fragments obtained following tryptic digestion and Fc fragments produced by papain digestion suggested that the sites of cleavage are at least 15 amino acid residues apart. Furthermore, the γD myeloma protein was very susceptible to proteolysis. Incubation with various enzymes for only brief periods of time cleaved all γD into Fab and Fc fragments and prolonged incubation of γD with these enzymes resulted, in some instances, in complete degradation of these fragments. The γD even fragmented "spontaneously" unless it was stored in the presence of ε-aminocaproic acid. The spontaneously occurring fragmentation was most likely the result of plasmin digestion, since ε-aminocaproic acid is known to inhibit relatively specifically plasmin formation (Alkjaersig *et al.*, 1959) and since the fragments obtained from γD digested with purified plasmin were indistinguishable from the spontaneously formed fragments. The reason for the different sites of cleavage of papain and trypsin and for the great susceptibility of γD to proteolysis is unknown. It has been shown for other immunoglobulin classes (Hill *et al.*, 1966; Abel and Grey, 1969) that the inter-Fd–Fc region is rich in proline residues preventing a highly ordered structure in this area of the molecule and thereby rendering this region accessible to the action of a variety of enzymes. The peptides isolated from the inter-Fd–Fc region of the γD were not particularly rich in proline residues, except for the peptide containing the interheavy-chain disulfide bond. However, this peptide contained fewer proline residues than the corresponding peptide in γG (Hill *et al.*, 1966; Edelman *et al.*, 1969) and it therefore appears unlikely that

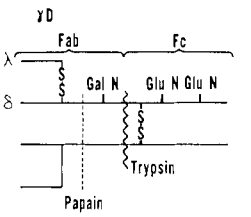

FIGURE 4: Schematic model of γD based on the data obtained from the γD myeloma protein May: Gal N, glycopeptide containing galactosamine as characteristic amino sugar; Glu N, glycopeptide containing glucosamine as the sole amino sugar.

the presence of proline residues in the inter-Fd–Fc region is responsible for the greater susceptibility of γD to proteolysis. The amino acid sequence of the inter-Fd–Fc region of the δ chain, presently under investigation, might lead to a better understanding of the reasons for the susceptibility of this immunoglobulin class to proteolysis.

Although the structure of γD taken as a whole is quite distinct from that of other immunoglobulins, certain individual structural features show striking similarities to one or another class of immunoglobulin. The high carbohydrate content of γD is similar to that found in human γM (Müller-Eberhard *et al.*, 1956) and γE (Bennich and Johansson, 1967). The glycopeptide containing galactosamine localized in the inter-Fd–Fc region appears in a position homologous to a galactosamine containing glycopeptide found thus far only in human γA_1 (Ko *et al.*, 1967; Abel and Grey, 1969) and rabbit γG (Smyth and Utsumi, 1967). The presence of only a single interheavy-chain disulfide bond is unique among human immunoglobulins but has been shown in rabbit γG (Palmer and Nisonoff, 1964). As in all other mammalian immunoglobulins, no free N-terminal amino acid could be identified in the γD heavy chain. The methods which have led to the positive identification of the C-terminal residues of the γ, α, μ, and ε chains have failed to identify the C-terminal residues in the case of the δ chain, suggesting that the C-terminal residues of the δ chain may be different from those of the heavy chains of the other immunoglobulin classes. Obviously, more studies on the structure, and particularly the amino acid sequence, of the δ chain will be necessary in order to determine the structure of the C terminus as well as to establish the degree of homology between γD and the other immunoglobulin classes.

The molecular weight for γD obtained by sedimentation equilibrium was 172,000. This figure is in good agreement with the molecular weight of 166,000 obtained by gel filtration of the completely reduced and alkylated heavy and light chains. If the 15% of carbohydrate of the δ chain is taken into consideration the molecular size of the peptide portion of the δ chain is similar to that of the γ chain—50,000. Previously reported values for the molecular weight of γD have been somewhat higher than those reported in this study: 180,000 (Rowe *et al.*, 1969) and 200,000 (Heiner *et al.*, 1969). The reason for this discrepancy is not clear. Since γD has a great tendency to aggregate, the higher values could be a result of a small quantity of aggregates. Also, the partial specific volume used in the previous studies was not stated. The calculated $\bar{v}$ for the May protein was 0.717, which is lower than that seen in other proteins not so rich

in carbohydrate. If a higher $\bar{v}$ was incorrectly assumed in the other studies a concomitantly higher molecular weight would have been calculated.

The unique structure of γD probably reflects a specific biological activity of γD antibodies. However, the biological function of γD is thus far unknown and antibody activity has only recently been described in the γD immunoglobulin class (Gleich *et al.*, 1969; Heiner *et al.*, 1969). Further studies on the biological activity and structure of γD will be necessary before a correlation between structure and function of γD can be established.

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