Proton Nuclear Magnetic Resonance Study of Human Immunoglobulins G1 and Their Proteolytic Fragments: Structure of the Hinge Region and Effects of a Hinge-Region Deletion on Internal Flexibility

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ABSTRACT: A high-resolution nuclear magnetic resonance (NMR) study is reported of human IgG immunoglobulins. Two kinds of myeloma proteins, IgG1 Ike-N and IgG1 Dob, were used in this study. IgG1 Ike-N has an intact hinge, whereas the 15-residue segment Glu-216–Pro-230, which includes the disulfide-linked central region of the hinge Cys-226–Pro-227–Pro-228–Cys-229, is missing in IgG1 Dob [Steiner, L. A., & Lopes, A. D. (1979) *Biochemistry 18*, 4054–4067]. It was observed that, upon saturation of the backbone CH proton resonances, *spin diffusion* is prevalent, resulting in a marked decrease in intensity of NMR signals that arise from rigid portions of the IgG molecules. NMR spectra thus obtained were compared for IgG1 Ike-N and IgG1 Dob. On the basis of the NMR measurements, it was possible to identify a number of resonances that originate from the hinge region of IgG1 Ike-N. This indicates that spin diffusion extends much more slowly to the hinge region. The rate of saturation transfer through spin diffusion was compared for the two kinds of IgG proteins. It was shown that IgG1 Dob, which still retains a part of the hinge segment from Ala-231 on, loses a significant degree of internal flexibility as compared to IgG1 Ike-N. On the basis of the NMR measurements for the intact IgG1 Ike-N and IgG1 Dob as well as those of the Fab, $F(ab')_2$, Fab', and Fc fragments of IgG1 Ike-N, it is concluded that (1) segments Lys-222–Thr-225 and Pro-230–Leu-234, which precede and follow the disulfide-linked Cys-Pro-Pro-Cys part, respectively, are highly flexible, (2) the Pro-230–Leu-234 segment has an extended conformation and is less flexible than the Lys-222–Thr-225 segment, (3) having the Lys-222–Thr-225 segment is essential for the IgG1 molecules to express a significant degree of flexibility, and (4) the two segments become comparable in flexibility when inter heavy chain disulfide bridges in the Cys-Pro-Pro-Cys part are reduced and alkylated. Without irradiation for the presaturation of the backbone CH proton resonances, it was possible to observe His resonances for the Fc region of the intact IgG1 molecules. On the basis of the pH titration data for His-435, which exists in the interdomain C_H2–C_H3 contact, we have shown that the way in which the C_H2 and C_H3 domains contact with each other in the IgG1 molecules changes very little (1) on cleavage of the Fab part, (2) on reduction and alkylation of the interchain disulfide bridges, and (3) on deletion of most of the hinge segment.

Immunoglobulins that mediate antibody function consist of two distinct regions, i.e., Fab and Fc.[1] The Fab region carries a recognition site for antigenic determinants, whereas the Fc region reacts with receptors of a variety of effector systems. Proteins of the human immunoglobulin G (IgG) class can be differentiated into four subclasses (IgG1 through IgG4), each with a distinctive heavy chain, $\gamma1$, $\gamma2$, $\gamma3$, or $\gamma4$; light chains can be of either λ or κ type, regardless of its heavy-chain subclass.[2] The γ chains consist of four homology units, V_H, C_H1, C_H2, and C_H3, whereas the light chains are divided into two homology units, V_L and C_L. The hinge region, which is a peptide segment between the C_H1 and C_H2 domains, is highly susceptible to attack by proteolyses, and the cleavage can give rise to Fab, $F(ab')_2$, and Fc fragments [see, e.g., Kabat (1976)].

Hydrodynamic and spectroscopic techniques have been used to demonstrate that segmental flexibility exists between Fab and Fc portions of the IgG molecule. It has been suggested that the main structural site of this property is the hinge region (Noelken et al., 1965; Yguerabide et al., 1970; Cathou, 1978; Pecht, 1982). In a previous paper we have reported the assignment of the proton nuclear magnetic resonance (NMR) signals of the His-224,[3] which exists in the hinge region of the IgG1 immunoglobulins (Arata et al., 1980) (see Figure 1). Titration behavior of the hinge-region histidine in the intact IgG1 and its proteolytic fragments was compared, and these results were discussed in terms of the conformation of the hinge region.

Akasaka (1983) has reported that information about the gross dynamic structure of large proteins with slow tumbling motion can be obtained by observing how *spin diffusion* extends to various parts of the molecules. The relaxation of the backbone α protons and side-chain protons that are held rigidly in the molecular framework of the large proteins is most likely dominated by the *flip-flop* transition, which is propagated through the entire array of the protons. This process is called spin diffusion (Bothner-By, 1979; Jardetzky & Roberts, 1981). When spin diffusion is prevalent, the nuclear Overhauser effect becomes completely nonspecific; continuous irradiation of any one of the protons in the rigid parts would result in saturation of the entire NMR spectrum. By contrast, spin diffusion extends much more slowly to the flexible parts with rapid

[1] Abbreviations: C1, the first component of complement; CPase, carboxypeptidase; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid sodium salt; Fab, antigen-binding fragment; Fc, fragment composed of the C-terminal halves of the heavy chains; Fc RA, reduced and alkylated Fc; HPLC, high-performance liquid chromatography; IgG, immunoglobulin G; IgG RA, reduced and alkylated IgG; NMR, nuclear magnetic resonance; PTH, phenylthiohydantoin; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

[2] The nomenclature for immunoglobulin G and its fragments is as recommended in *Bull. W. H. O.* (1964).

[3] The numbering system used is based on protein Eu for $\gamma1$ chain (Edelman et al., 1969).

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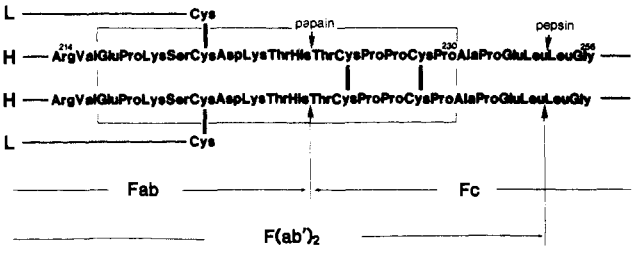

FIGURE 1: Human IgG1 hinge region. In the ordinary IgG1, Cys-220 forms a disulfide bridge to the light chain, and Cys-226 and Cys-229 form disulfide bridges with the other heavy chain. In IgG1 Dob, the residues indicated in the box are missing from the heavy chain.

side-chain and segmental motions, because with an increase in flexibility the flip-flop transition becomes less effective as the relaxation mechanism. Intuitively, the flexible parts are thermally separated from the rigid parts that have been *heated* uniformly with the radio-frequency irradiation. The selectivity of saturation transfer, which has actually been observed in myosin (Akasaka et al., 1978), is of particular interest in the case of immunoglobulins where different biological functions are carried by different regions of the molecules.

Campbell et al. (1973) have reported several types of difference spectroscopy to improve the resolution of a complex NMR spectrum by subtracting the broadened spectrum from the original one. It has also been demonstrated by Campbell et al. (1975) that observation of the Fourier-transformed spin-echo spectrum makes it much easier to separate signals with narrow line widths from the severely overlapped broad background. These methods should also be useful in extracting information concerning the flexible parts of the immunoglobulin molecules.

In this present study, differential effects of saturation transfer were analyzed and compared by using two kinds of human myeloma IgG1 proteins, Ike-N and Dob. IgG1 Ike-N has an intact hinge, whereas the 15-residue segment Glu-216–Pro-230, which includes the disulfide-linked central region of the hinge Cys-Pro-Pro-Cys, is missing in IgG1 Dob (Steiner & Lopes, 1979) (see Figure 1). Fab, $F(ab')_2$, Fab′, and Fc fragments of IgG1 Ike-N were used along with intact IgG1 Ike-N and IgG1 Dob to study the structure of the hinge region. Protein Ike-N and protein Dob were compared to discuss the internal flexibility of the IgG1 molecules. It has been reported that His-435 in the Fc fragment reflects sensitively the way in which the C_H2 and C_H3 domains contact with each other (Shimizu et al., 1983). We will show that, without irradiation for the presaturation of the backbone CH proton resonances, the intact IgG1 molecules also give His resonances for the Fc region including that of His-435. On the basis of pH titration data for His-435 of the intact IgG1, we will discuss how the existence of the Fab region, the inter heavy chain disulfide bridges, or the hinge segment influences the longitudinal interaction between the C_H2 and C_H3 domains.

Materials and Methods

Materials. Myeloma protein IgG1 (κ) Ike-N was isolated from the plasma of a patient with multiple myeloma by precipitation with 44% saturated ammonium sulfate. The precipitated protein was dissolved in 0.01 M Tris-HCl buffer at pH 8.0 and dialyzed against the same buffer. The dialyzate was then subjected to ion exchange chromatography on a DE-52 (Whatman) column equilibrated with the same buffer. By increasing the molarity of KCl to 0.1 M, the IgG was eluted. The IgG fractions were collected and dialyzed against distilled water and then lyophilized. An ammonium sulfate precipitate of IgG1 (κ) Dob, which was kindly provided by Dr. Lisa A. Steiner of the Department of Biology, Massachusetts Institute of Technology, was dissolved in a small amount of 0.1 M Tris-HCl at pH 8.0 and then passed through a DE-52 column equilibrated in the same buffer.

Fab and Fc fragments were prepared by digesting IgG1 Ike-N with papain. Digestion was carried out for 5 h at 30 °C in the absence of cysteine.[4] The enzyme to IgG ratio was 1:100. The fragments were separated by gel filtration on a Sephacryl S-300 column and then purified by ion exchange chromatography on DE-52 resin. Fab and Fc fractions were collected, dialyzed against distilled water, and then lyophilized. $F(ab')_2$ fragment was prepared by digesting IgG with pepsin according to the method of Turner et al. (1970). IgG1 Ike-N dissolved in 0.1 M sodium acetate buffer, pH 4.5, at a protein concentration of 2% was digested with pepsin for 35 h at 37 °C. The enzyme to IgG ratio was 1:100. The $F(ab')_2$ fragment thus obtained was purified by gel filtration on a Sephacryl S-300 column.

Fc or $F(ab')_2$ fragment that was dissolved in 0.15 M Tris-HCl buffer containing 0.15 M NaCl, pH 8.0, at a concentration of 1% was reduced with 20 mM dithiothreitol for 2 h at 25 °C under nitrogen and then alkylated with 80 mM iodoacetate for 1 h at 4 °C in the dark. Reduced and alkylated Fc or $F(ab')_2$ fragment was dialyzed against distilled water in the dark and lyophilized. In the case of IgG1 Ike-N, the concentration of dithiothreitol was 75 mM, and that of iodoacetate was 125 mM. Under this condition, all interchain disulfide bridges are reduced and alkylated. Purity of the proteins used in this experiment was checked by HPLC, immunoelectrophoresis, and SDS–polyacrylamide gel electrophoresis.

NMR Measurements. A total of 2–10 mg of each protein was dissolved in 0.3 mL of 0.2 M $NaCl/D_2O$. The pH was adjusted with 0.3 M DCl or NaOD. All pH values reported in this paper are uncorrected meter readings of D_2O solutions made with an electrode standardized with H_2O buffers. ^1H NMR measurements were performed at 30 °C on Bruker WH-270 and WM-400 spectrometers operating at 270 and 400 MHz, respectively. For the experiments of saturation transfer, different levels of radio-frequency pulses were applied for a certain period of time, typically 0.1–0.5 s, before acquisition of the free-induction decay. Unless otherwise stated, the frequency for presaturation was set to that of the HDO resonance. This resulted in saturation of the backbone CH proton resonances. Irradiation at the center of the aliphatic region gave essentially the same result; in this case, however, information about this part of the spectrum was inevitably lost. All chemical shifts are given in parts per million (ppm) from external DSS (5% in D_2O). The probe temperature was 30 °C throughout the experiments.

Ultracentrifugal Measurements. Sedimentation velocity experiments were performed at 30 °C with a Beckman Model E analytical ultracentrifuge. Single-sector cells with a 12-mm optical path and quartz window were used. The D_2O solutions of IgG1 Ike-N and IgG1 Dob used for the NMR measurements were placed in a standard cell and a wedge cell, respectively. The speed of centrifugation was 56 000 rpm, and

[4] In the proteolytic cleavage of IgG1 proteins with papain, an addition of cysteine resulted in a mixture of Fc fragments with heterogeneous N termini; a substantial amount of Fc fragments that lack the disulfide-linked Cys-Pro-Pro-Cys part is produced. An Fc fragment used in the present work was prepared in the absence of cysteine. It was confirmed that the Fc fragment is homogeneous on SDS–polyacrylamide gel electrophoresis (nonreducing condition) and that the N-terminal sequence is Thr-Cys-Pro-Pro as determined by HPLC analysis of PTH-amino acids.

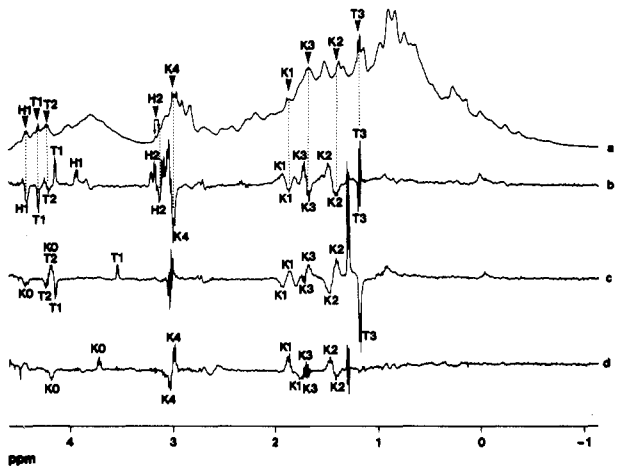

FIGURE 2: The 400-MHz ^1H NMR spectra of Fab Ike-N (8 mg in 0.3 mL of 0.2M $NaCl/D_2O$, pH 8.0). The HDO peak was presaturated in the gated-decoupling mode for 0.5 s at a radio-frequency level of $\gamma H_2/(2\pi)$ = 70 Hz. A sampling pulse of 60° was applied after the presaturating pulse with an interval of 5 ms to allow for the dead time of the receiver. After the spectrum of Fab Ike-N was measured, CPase A was added to the sample solution at an enzyme to substrate ratio of 1:4000. After the addition of the enzyme, spectra of Fab(CPase A, 0 h), Fab(CPase A, 0.5 h), and Fab(CPase A, 9 h) were recorded at 0, 0.5, and 9 h, respectively. CPase B was then added at an enzyme to substrate ratio of 1:4000, and the solution was incubated at 30 °C. Spectra of Fab(CPase B, 0 h) and Fab(CPase B, 1 h) were recorded at 0 and 1 h, respectively. (a) Intact Fab Ike-N; (b) Fab(CPase A, 0.5 h) – Fab(CPase A, 0 h); (c) Fab(CPase A, 9 h) – Fab(CPase A, 0.5 h); (d) Fab(CPase B, 1 h) – Fab(CPase B, 0 h). The free-induction decay was recorded with 8K data points and a spectral width of ±2500 Hz. Typically, 600 transients were acquired, and a line broadening of 0.5 Hz was applied prior to Fourier transformation. The difference spectrum was obtained by subtraction of transformed spectra. Assignments were as follows: K, Lys-222; T, Thr-223; H, His-224. See also Table I.

the progress of the sedimenting boundary was followed by using the schlieren optical system with exposure at 4-min intervals.

RESULTS

NMR Spectral Data for Hinge-Region Resonances. NMR spectra of Fab, $F(ab')_2$, Fab', and Fc fragments of IgG1 Ike-N were observed with the HDO peak and, therefore, with the backbone CH proton peaks, presaturated for 0.5 s with a radio-frequency pulse at a level of $\gamma H_2/(2\pi)$ = 360 Hz.

Figure 2a shows an example of NMR spectra of Fab Ike-N. After the measurements of the spectra, the sample solution was incubated at 30 °C in the presence of CPase A with an enzyme to substrate ratio of 1:4000. After the addition of the enzyme, spectra of Fab(CPase A, 0 h), Fab(CPase A, 0.5 h), and Fab(CPase A, 9 h), i.e., spectra of Fab Ike-N incubated with CPase A for 0, 0.5, and 9 h, respectively, were recorded. Spectra b and c of Figure 2 are the difference spectra Fab-(CPase A, 0.5 h) – Fab(CPase A, 0 h) and Fab(CPase A, 9 h) – Fab(CPase A, 0.5 h), respectively. In Figure 2b, two sets of the α-CH and β-CH_2 proton resonances are observed for His; one is from His-224 at the C terminal (negative peaks), and the other corresponds to the free amino acid cleaved off by digestion (positive peaks). The difference spectrum shown in Figure 2b also indicates that, upon cleavage of His-224, chemical shifts for Lys-222 as well as those for Thr-223, which is the new C terminal, change significantly. In Figure 2c, the α-CH, β-CH, and γ-CH_3 proton resonances of Thr-223 (negative peaks) are observed. Positive peaks for Thr originate from the free amino acid cleaved off by digestion. No His resonances are observed in Figure 2c, indicating that Fab-(CPase A, 0.5 h) has lost His-224 completely. In the presence

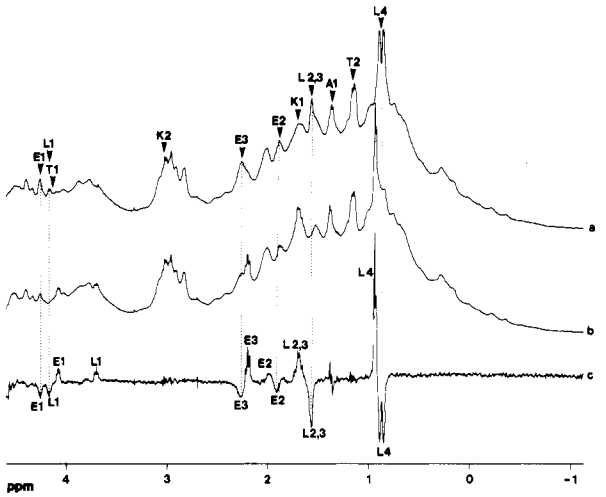

FIGURE 3: The 400-MHz ^1H NMR spectra of $F(ab')_2$ Ike-N (8 mg in 0.3 mL of 0.2 M $NaCl/D_2O$, pH 8.0). The HDO peak was presaturated for 0.5 s at a level of $\gamma H_2/(2\pi)$ = 70 Hz. After the measurements of the spectrum of $F(ab')_2$, the sample solution was incubated at 30 °C with CPase A (enzyme to substrate ratio = 1:4000). Spectra of $F(ab')_2$(CPase A, 0 h) and $F(ab')_2$(CPase A, 1 h) were recorded at 0 and 1 h, respectively. (a) Spectrum of $F(ab')_2$(CPase A, 0 h); (b) spectrum of $F(ab')_2$(CPase A, 1 h); (c) difference spectrum $F(ab')_2$(CPase A, 1 h) – $F(ab')_2$(CPase A, 0 h). Spectral conditions are as described in Figure 2. Assignments were as follows: K, Lys-222; T, Thr-223 and Thr-225; A, Ala-231; E, Glu-233; L, Leu-234. See also Table I.

of CPase A, no further change in spectra was observed. CPase B was added at this stage to the sample solution at an enzyme to substrate ratio of 1:4000. The solution was incubated at 30 °C and spectra of Fab(CPase B, 0 h) and Fab(CPase B, 9 h) were recorded. Figure 2d gives the difference spectrum Fab(CPase B, 9 h) – Fab(CPase B, 0 h). The ϵ-CH_2, δ-CH_2, γ-CH_2, β-CH_2, and α-CH proton resonances of Lys-222 can be identified by spin decoupling. On the basis of these results, all of the CH resonances of His-224, Thr-223, and Lys-222 from the C-terminal segment of Fab Ike-N can be assigned, and the results of the assignments are summarized in Figure 2a.

Figure 3a gives a spectrum of $F(ab')_2$ Ike-N. Assignments of C-terminal resonances were again made by proteolysis with CPase A. Figure 3b shows the spectrum of $F(ab')_2$(CPase A, 1 h). Figure 3c gives the difference spectrum $F(ab')_2$(CPase A, 1 h) – $F(ab')_2$(CPase A, 0 h), where the δ-CH_3, γ-CH, β-CH_2, and α-CH proton resonances of Leu-234 at the C terminal can be identified (negative peaks). Resonances are also observed for Glu-233 in the difference spectrum. Chemical shift data for the Glu-233 resonances, which appeared on cleavage of Leu-234, are different from those of a free glutamate. This means that the Glu resonances (positive peaks) are those of Glu-233 at the C terminal. Glu-233 can be cleaved off by treating $F(ab')_2$(CPase A, 1 h) with CPase Y (data not shown). These observations lead to the assignments of all of the CH proton resonances of Leu-234 and Glu-233 of the $F(ab')_2$ fragment. In addition, spin-decoupling experiments demonstrated that there exist one lysine, one histidine, two threonines, and one alanine that give resolved resonances in the aliphatic region of the spectra. The Fab' fragment, which was obtained by reduction and alkylation of interchain disulfide bridges of the $F(ab')_2$ fragment, gives much narrower resonances for all of these residues (data not shown). Comparisons of the data for the $F(ab')_2$ and Fab' fragments with those for the Fab fragment made it possible to assign the Lys-222, Thr-223, and His-224 resonances. It was also possible to identify Thr-225 and Ala-231 resonances that are

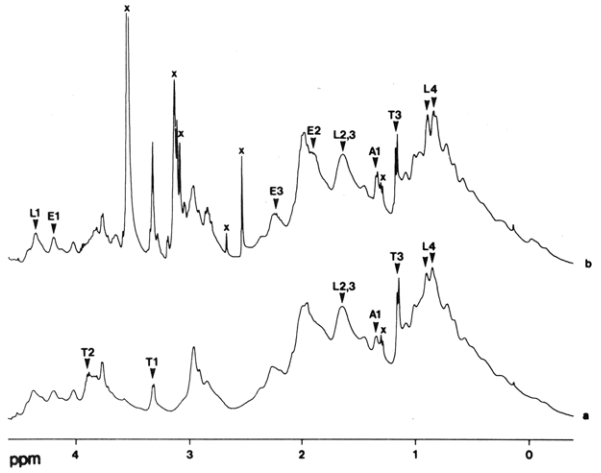

FIGURE 4: The 400-MHz ^1H NMR spectra of Fc Ike-N (8 mg in 0.3 mL of 0.2 M $NaCl/D_2O$). The HDO peak was presaturated for 0.5 s at a level of $\gamma H_2/(2\pi) = 70$ Hz. (a) Intact Fc Ike-N (pH 8.52); (b) reduced and alkylated Fc Ike-N (pH 8.49). For each measurement, 2000 transients were accumulated. Other spectral conditions are as described in Figure 2. Assignments were as follows: T, Thr-225; A, Ala-231; E, Glu-233; L, Leu-234. See also Table I. The peaks marked × are due to low molecular weight contaminants; most peaks observed in (b) between 2.5 and 3.5 ppm are due to EDTA.

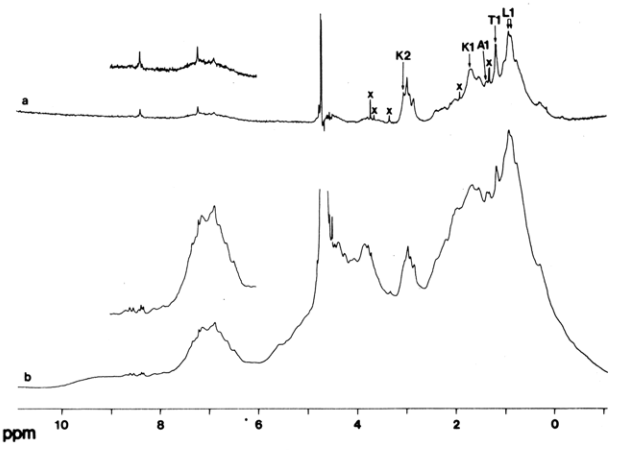

FIGURE 5: The 400-MHz ^1H NMR spectra of IgG1 Ike-N (5 mg in 0.3 mL of 0.2 M $NaCl/D_2O$, pH 6.09) (a) with and (b) without presaturation of the HDO peak. For presaturation, a radio-frequency pulse was applied for 0.5 s at a level of $\gamma H_2/(2\pi) = 360$ Hz. For each measurement, 3000 transients were accumulated. Other spectral conditions are as described in Figure 2. Assignments were as follows: K, Lys-222; T, Thr-223 and Thr-225; A, Ala-231; L, Leu-234. See also Table I. The peaks marked × are due to low molecular weight contaminants.

missing in the spectra of the Fab fragment.

In the case of the Fc fragment, which retains about half of the hinge region, a relatively small number of well-resolved peaks can be observed as shown in Figure 4. Resonances originating from a threonine residue can clearly be identified by spin decoupling. The chemical shifts of the α-, β-, and γ-proton peaks of this threonine residue titrate with pH, indicating that this residue is Thr-225, which exists at the N terminal. In addition, a doublet of the CH_3 group is observed at 1.35 ppm. It was confirmed by spin-decoupling experiments that this CH_3 group couples to a proton at 4.58 ppm. These chemical shift data coincide with those for Ala-231 observed in the case of the $F(ab')_2$ fragment. In addition, resonances that are due to a Leu residue were identified by spin decoupling. Reduction and alkylation of the Fc fragment give a better resolution for the Ala-231 doublet; furthermore, it was possible to identify resonances originating from the β and γ protons of a Glu residue.

In Figure 5, we give spectra of the intact IgG1 Ike-N. The spectra were observed in the presence and absence of a presaturating pulse applied at the HDO peak. Upon saturation of the HDO peak, which presumably causes saturation of the backbone CH proton resonances, spin diffusion extends to the rigid parts of the entire IgG molecule, resulting in a dramatic decrease in the intensity of the broad-band signals. A most striking effect of saturation transfer may be noticed in the two sharp peaks observed in the aromatic region. In our previous paper (Arata et al., 1980), we have assigned these signals to the C2-H and C4-H protons of His-224 of the hinge region. In Figure 6, the chemical shifts of the C2-H and C4-H proton signals observed for Ike-N are plotted as a function of pH. The C2-H proton of His-224 gives a pH titration curve that is virtually identical with those observed for histidine residues in small peptides that were obtained by tryptic digestion from human λ light chains (Arata et al., 1980). $F(ab')_2$ Ike-N gives pH titration curves for the C2-H and C4-H protons of His-224 that coincide with those for the intact IgG1 Ike-N [see also Arata et al. (1980)].

Figure 5 shows that, upon saturation of the backbone CH proton signals, a substantial number of signals remain to be observed in the aliphatic region. In Figure 7 is given an NMR

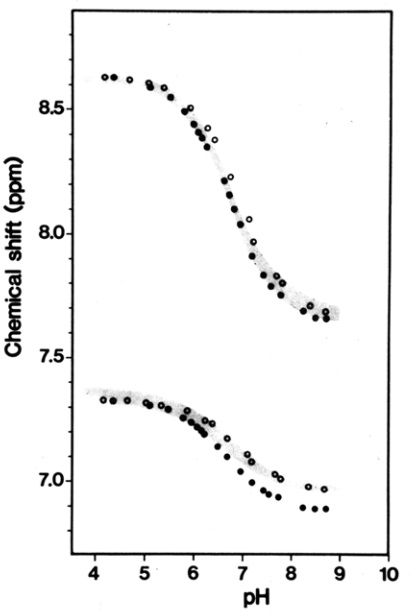

FIGURE 6: pH dependence of the chemical shifts of the C2-H and C4-H protons of His-224 observed for IgG1 Ike-N: (closed symbols) intact IgG1 Ike-N; (open symbols) reduced and alkylated IgG1 Ike-N. $\mu = 0.2$, 30 °C. The shaded area is drawn by using data observed for histidine peptides obtained from Bence–Jones protein Ak (Arata et al., 1980).

spectrum of IgG1 Dob that is known to lack most of the hinge (Steiner & Lopes, 1979) (see Figure 1). Comparisons of the spectra observed for IgG1 Ike-N and IgG1 Dob indicate that signals that survived under the condition of strong presaturation in the case of IgG1 Ike-N are missing in the spectrum of IgG1 Dob. This finding demonstrates that these signals originate from the hinge region. It was possible to identify by spin-decoupling experiments resonances of the CH_3 protons of threonine and the δ-CH_2 and ϵ-CH_2 protons of lysine. These residues are presumably those of the Lys-222–Thr-225 segment of the hinge region (see Figure 1). It was also possible to identify resonances of the CH_3 protons of alanine and leucine. Chemical shift data for these resonances coincide with those of the Ala-231 and Leu-234 resonances observed in the case of the $F(ab')_2$ fragment.

Table I: Chemical Shifts for Hinge-Region Amino Acid Residues of Human IgG1 Immunoglobulin and Its Proteolytic Fragments[a]

	IgG	IgG RA	$F(ab')_2$	Fab'	Fab	Fc	Fc RA
Lys-222							
β-CH_2					1.75		
γ-CH_2					1.40		
δ-CH_2	1.71	1.71	1.71	1.71	1.71		
ϵ-CH_2	3.03	3.01	3.04	3.01	3.01		
Thr-223							
α-CH				4.34	4.31		
β-CH		4.16	4.18	4.16	4.23		
γ-CH_3	1.17	1.17	1.18	1.15	1.18		
His-224							
α-CH					4.43		
β-CH	*b*	*b*	*b*	*b*	*b*		
C2-H	*b*	*b*	*b*	*b*	*b*		
C4-H	*b*	*b*	*b*	*b*	*b*		
Thr-225							
α-CH				4.34		*b*	*b*
β-CH		4.16	4.18	4.16		*b*	*b*
γ-CH_3	1.17	1.17	1.18	1.15		*b*	*b*
Ala-231							
α-CH	4.58	4.58	4.58	4.58		4.58	4.58
β-CH_3	1.35	1.34	1.35	1.35		1.35	1.35
Glu-233							
α-CH			4.26				4.20
β-CH_2		1.90	1.90	1.90			1.89
γ-CH_2		2.25	2.26	2.27			2.22
Leu-234							
α-CH			4.18	4.16			4.36
β-CH_2			1.57	1.56		1.64	1.64
γ-CH			1.57	1.56		1.64	1.64
δ-CH_3	0.86	0.84	0.85	0.85		0.85	0.83
	0.90	0.90	0.89	0.89		0.90	0.88

[a] Chemical shifts are in parts per million from external DSS (5% in D_2O). μ = 0.2, 30 °C. [b] pH dependent.

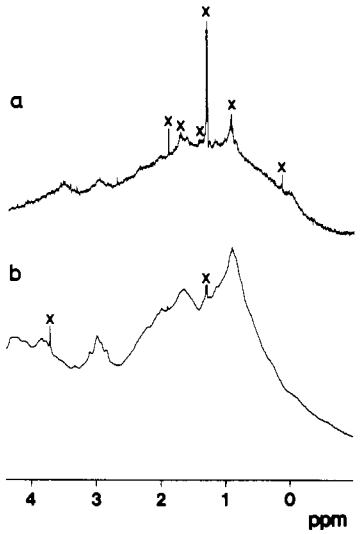

FIGURE 7: The 400-MHz ^1H NMR spectra of IgG1 Dob (1 mg in 0.3 mL of 0.2M $NaCl/D_2O$, pH 6.06) observed (a) with and (b) without presaturation of the HDO resonance. For presaturation, a radio-frequency pulse was applied for 0.5 s at a level of $\gamma H_2/(2\pi)$ = 360 Hz. For each measurement, 10000 transients were accumulated. Other spectral conditions are as described in Figure 2. The peaks marked × are due to low molecular weight contaminants. Vertical expansion is different for spectra a and b.

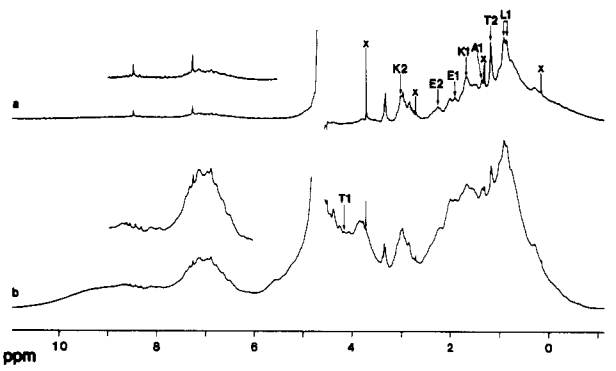

FIGURE 8: The 400-MHz ^1H NMR spectra of reduced and alkylated IgG1 Ike-N (5 mg in 0.3 mL of 0.2 M $NaCl/D_2O$, pH 6.11) (a) with and (b) without presaturation of the HDO peak. For presaturation, a radio-frequency pulse was applied for 0.5 s at a level of $\gamma H_2/(2\pi)$ = 360 Hz. For each measurement, 3000 transients were accumulated. Other spectral conditions are as described in Figure 2. Assignments were as follows: K, Lys-222; T, Thr-223 and Thr-225; A, Ala-231; E, Glu-233; L, Leu-234. See also Table I. The peaks marked × are due to low molecular weight contaminants.

As Figure 6 shows, a significant difference is observed for the His-224 C4-H proton titration curves that were observed for the IgG1 and the small peptides. It should be noted that, on reduction and alkylation of the interchain disulfide bridges of IgG1 Ike-N, the titration curve for the C4-H proton also becomes very close to those observed for the simple peptides. It was also observed that the reduced and alkylated IgG1 Ike-N gives narrower line widths for the hinge-region resonances described above. In this case, the β- and γ-proton resonances of a Glu residue were identified. The β-CH signal corresponding to the CH_3 signal of Thr at 1.17 ppm can also be identified (see Figure 8). Chemical shift data observed for the hinge-region resonances for the intact IgG1 and its proteolytic fragments are summarized in Table I.

Comparisons of Internal Flexibility of IgG1 Ike-N and IgG1 Dob. The effect of saturation transfer was compared for IgG1 Ike-N and IgG1 Dob in Figure 9, where the signal intensities observed at 1.1 ppm are plotted as a function of the duration of the presaturating pulse whose intensity was held fixed. Use of signal intensities observed at 0.9 and 1.9 ppm gave essentially the same results. No significant concentration dependence was observed in the range of 10–25 mg/mL. As Figure 9 shows, the rate of saturation transfer is significantly different for the two IgG1 proteins; the rate is 30% greater for IgG1 Dob than for IgG1 Ike-N. The sedimentation ve-

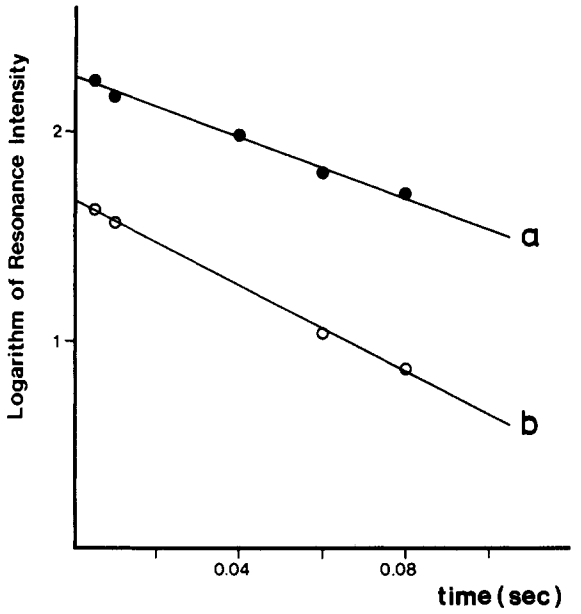

FIGURE 9: Rate of saturation transfer observed for (a) IgG1 Ike-N and (b) IgG1 Dob. In both (a) and (b), protein concentrations were 1 mg in 0.3 mL of 0.2 M $NaCl/D_2O$. The HDO peak was irradiated at a level of $\gamma H_2/(2\pi)$ = 70 Hz, and signal intensities at 1.1 ppm were measured as a function of duration of the presaturating pulse. A sampling pulse of 60° was applied after the presaturating pulse with an interval of 5 ms. For each measurement, 10000 transients were accumulated. Other spectral conditions are as described in Figure 2. Resonance intensities $I(t) - I(\infty)$ (in arbitrary logarithmic units) are plotted, where $I(t)$ is a signal intensity observed with a presaturation pulse applied for a given duration t and $I(\infty)$ is the equilibrium signal intensity observed under the radio-frequency irradiation with a sufficient length of time.

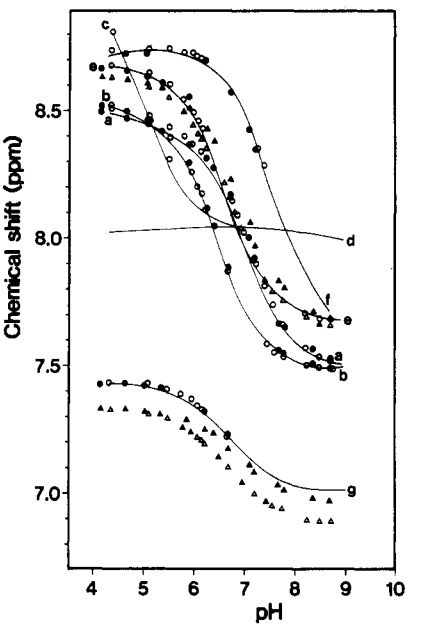

FIGURE 10: pH dependence of the chemical shifts of the C2-H and C4-H protons of histidine residues in the Fc region of IgG1 Ike-N. μ = 0.2, 30 °C. Open and closed symbols represent titration data for the intact and reduced and alkylated IgG1, respectively. Solid lines are based on the titration data observed for the Fc fragment (Shimizu et al., 1983). Data for His-224 in the hinge region are also included (open and closed triangles). Assignments were as follows: d, His-429; f, His-435; e and g, His-433 (C2-H and C4-H protons, respectively).

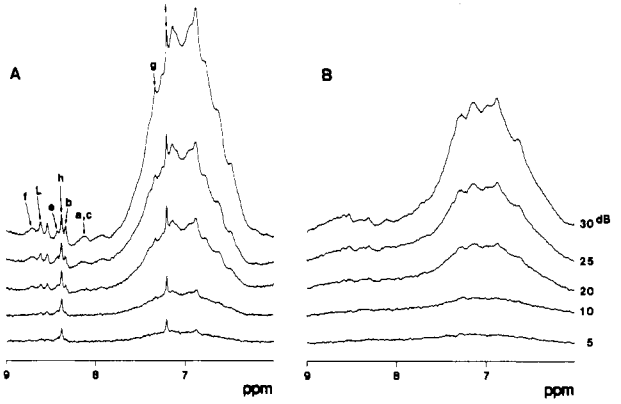

FIGURE 11: Effect of level of the radio-frequency pulse on the spectra of (A) IgG1 Ike-N (5 mg in 0.3 mL of 0.2 M $NaCl/D_2O$, pH 6.09) and (B) IgG1 Dob (1 mg in 0.3 mL of 0.2 M $NaCl/D_2O$, pH 6.09). The HDO peak was presaturated for 0.5 s in the gated-decoupling mode. The level of the radio-frequency pulse [$\gamma H_2/(2\pi)$] at 5 dB is approximately 360 Hz and is reduced by a factor of 2 for each 6 dB of attenuation. The number of transients accumulated for each of the spectra in (A) was 3000 and for that in (B) was 10000. Other spectra conditions are as described in Figure 2. The peaks marked h and i arise from the C2-H and C4-H protons of His-224 in the hinge region. The peak marked L is due to His, which is at position 189 in the κ chain. The peaks a–g originate from the histidine residues in the Fc region; assignments for peaks e–g are given in Figure 10.

locity experiments using the D_2O solutions used for the NMR analyses showed that both proteins Ike-N and Dob sediment as a single component with 6.6 and 6.9 S (corrected to a solvent with density and viscosity of H_2O at 20 °C), respectively.

NMR Spectra of Histidine Residues in Fc Region. In the absence of the presaturating pulse, intact IgG1 Ike-N gives below pH 6 some additional peaks in the aromatic region of the spectra (see Figure 5). With an increase in pH, most of these peaks become broader in line width and eventually become unobservable. We have previously reported the pH titration data for all six histidine residues that exist in the Fc fragment of human IgG1; assignments for resonances of His-429, His-433, and His-435, in the C_H3 domain have been made (Shimizu et al., 1983). Figure 10 gives the pH titration data for intact IgG1 Ike-N, which are compared with the data reported previously for the human Fc fragment. It should be noted that the data observed for the intact IgG1 are in quite good agreement with those of the Fc fragment. Assignments for the three C_H3 domain His resonances are also given in Figure 10. Similar results were obtained for IgG1 Dob. However, the line widths of these histidine peaks are much broader than those observed for IgG1 Ike-N (see Figure 11). On reduction and alkylation of interchain disulfide bridges, a slight decrease in line width, but no significant change in the pH titration curves, was observed for the Fc His signals for both proteins Ike-N and Dob.

Figure 11 shows how the Fc His resonances for IgG1 Ike-N and IgG1 Dob change in intensity on increasing the power of presaturating pulse. Differential effects of saturation transfer are clearly observed; the His-433 resonance is among those that disappeared at late stage. With an increase in power of the presaturating pulse, all the Fc resonances eventually disappeared, leaving in the aromatic region only the C2-H and C4-H proton resonances for His-224 in the hinge region.

Discussion

It has been shown that the presence of the hinge region affects to a great extent the result of X-ray crystallographic analyses of immunoglobulins. X-ray crystallographic studies of human myeloma protein IgG1 Kol have shown that the central region of the hinge consists of two parallel disulfide-linked poly(L-proline) helixes formed by the segment Cys-Pro-Pro-Cys and no significant residual density was visible beyond the Cys-Pro-Pro-Cys part (Marquart et al., 1980).

Although segment Cys-220 to Thr-225 is in an open, helical, solvent-accessible conformation with little inherent stability, the electron density is well-defined to Cys-229. It was shown that the Cys-220–Thr-225 segment is stabilized through crystal packing interactions. It was also reported that in the Fc fragment no interpretable electron density could be found for residues Thr-223 to Gly-237 (Deisenhofer, 1981). This experiment has been performed on an Fc fragment obtained from pooled human sera. A similar observation about the disorder of the N-terminal sequence in Fc has been reported by Mariuzza et al. (1983), who used a heavy-chain disease protein that has a homogeneous N-terminal sequence starting at Asp-221.

As Figures 5 and 6 show, the C2-H and C4-H protons of His-224 give extremely sharp resonances, and the pH titration data for this residue are virtually identical with those of simple histidine peptides. We have so far examined by ^{1}H NMR a number of histidine residues that exist in different parts of IgG1 immunoglobulins and discussed on the basis of the pH titration data the microenvironments of these residues (Arata et al., 1978, 1980; Arata & Shimizu, 1979; Shimizu et al., 1980, 1983). These NMR data show that, aside from His-224, His-433 that exists in the C_H3 domain in the Fc fragment is most exposed to solvent. X-ray crystallographic study of the Fc fragment has shown that His-433 is completely exposed to solvent (Deisenhofer, 1981). Figure 11 shows that in the absence of the strong presaturating pulse one can observe the C2-H and C4-H proton resonances of His-433 for the intact IgG1 Ike-N. As Figure 10 shows, the pH titration curves observed for these protons are quite similar to those of His-224. Figure 11 also demonstrates that, among His residues of the Fc region, His-433 exists in a flexible environment. In spite of this apparent close similarity for the His-224 and His-433 resonances, the His-433 resonances eventually disappear, as Figure 11 shows, upon extensive saturation of the backbone CH proton resonances; the His-224 resonances change very little in intensity under the same condition. This indicates that, when spin diffusion extends to the entire part of the molecule, simple exposure to solvent is not quite sufficient for amino acid residues to retain the intensity of their NMR resonances. In view of these results, we conclude that in solution a hinge segment, on which His-224 is located, is undergoing an extensive degree of internal motion. Consistent with this are the present observations that in the case of IgG1 Ike-N virtually all resonances, which survive the extensive saturation of the backbone CH proton signals, originate from the hinge region.

The titration curves for His-224 of the intact IgG coincide with those of $F(ab')_2$. In addition, the chemical shift data observed for Lys-222, Thr-223, Thr-225, Ala-231, and Leu-234 of intact IgG1 Ike-N are in good agreement with those of the $F(ab')_2$ fragment. These results strongly suggest that the hinge segment of the intact IgG takes a conformation that is essentially the same as that of the $F(ab')_2$ fragment. By contrast, as we have previously reported (Arata et al., 1980), the His-224 titration curves for Fab are quite different from those for $F(ab')_2$. In Figure 3, we have shown the difference spectrum $F(ab')_2$(CPase A, 1 h) − $F(ab')_2$(CPase A, 0 h), which gives only resonances for Leu-234 and Glu-233. By contrast, the difference between spectra of Fab fragments with and without C-terminal residues is complicated, giving a number of signals that do not correspond to any of the C-terminal residues (see Figure 2b–d). These results demonstrate that (1) in the $F(ab')_2$ fragment the C-terminal Leu-234 and Glu-233 have little contact, if any, with the rest of the molecule and (2) the C-terminal segment Lys-222-Thr-223-His-224 in the Fab fragment is interacting with other amino acid residues. We suggest that in the Fab fragment the hinge segment, which is much shorter than that of $F(ab')_2$ and the intact IgG, is partially folded back toward the main body of the Fab molecule. We also suggest that in $F(ab')_2$ and IgG the hinge segment, to which His-224 is bonded, is free from such interaction. It appears that the existence of the Cys-Pro-Pro-Cys part that is followed by the Pro-230–Leu-234 segment is necessary in maintaining the Lys-222–Thr-225 segment in a conformation that exists in the intact IgG as well as the $F(ab')_2$ fragment. These results appear to be closely related to the highly flexible nature of the Lys-222–Thr-225 segment.

Ala-231 gives identical chemical shifts for the α and β protons of the intact IgG, $F(ab')_2$, Fab', and Fc. This is of particular interest in view of the fact that the chemical shift data for the alanine residue reflect the direct information about the polypeptide backbone. It should also be noted that Leu-234 gives chemical shifts that are quite similar for all types of the molecules mentioned above. On the basis of these observations, we conclude that the structure of the segment Pro-230–Leu-234 in the intact IgG is essentially the same as that of $F(ab')_2$ and Fc.

In marked contrast to what was observed for the Fab and $F(ab')_2$ fragments, the Fc fragment exhibits only a limited number of well-resolved peaks even when spin diffusion is prevalent. The segmental flexibility involving the Pro-230–Leu-234 appears to be restricuted as compared to that for the segment preceding the Cys-Pro-Pro-Cys part. We suggest that the poly(L-proline) helix extends in the C-terminal direction, resulting in the formation of an extended conformation of the Pro-230–Ala-231–Pro-232 segment.

A deletion of residues Glu-216–Pro-230 of the hinge region is known in three myeloma proteins: Dob (Steiner & Lopes, 1979), Lec (Rivat et al., 1976), and Mcg (Fett et al., 1973). X-ray crystallographic studies have shown that, in marked contrast to what was reported for IgG1 Kol, proteins Dob and Mcg show an ordered Fc region (Silverton et al., 1977; Edmundson et al., 1970). It was suggested that the mobility of the Fc portion of the IgG molecule, relative to the Fab arms, is reduced as a result of the hinge-region deletion.

IgG1 Ike-N gives resonances that are due to the histidine residues in the Fc region. It should also be noted that another His signal, which originates from the Fab region, is observable; on the basis of the pH titration data for Bence–Jones proteins (Arata & Shimizu, 1979), this signal (marked L in Figure 11A) can be assigned to His that is at position 189 of the light chain. The line widths of these histidine signals observed for the intact IgG1 Ike-N are much smaller than what is expected from the increase in size of the molecule. In the absence of the presaturating pulse, signals due to the histidine residues in the Fab and Fc regions are also observable in the case of intact IgG1 Dob. See Figure 11B. However, the line widths of these peaks are much broader than those observed for IgG1 Ike-N, which retains the hinge segment. The sedimentation velocity experiments using the D_2O solutions used for the NMR analyses showed that no significant degree of aggregation occurs in protein Dob, as well as in protein Ike-N, under the present experimental conditions for the NMR measurements. This is consistent with what has been reported by Terry et al. (1968). As Figure 9 shows, the rate of saturation transfer observed for IgG1 Dob is much greater than that for IgG1 Ike-N. These results indicate that in solution (1) the hinge region contributes significantly to the internal flexibility of the normal (i.e., with an intact hinge) immunoglobulins and (2) the hinge-region deletion in protein Dob actually results

in a significant decrease in the mobility of the Fc portion of the IgG molecule, relative to the Fab arms. It should be noted that protein Dob still retains a part of the hinge region from Ala-231 on. We suggest that IgG1 molecules have to have the flexible Lys-222–Thr-225 segment *intact*, which is essential for the hinge to express a sufficient degree of flexibility.

It has been shown that IgG1 Dob lacks the ability of binding to the first component of complement C1 and also is unable to bind to Fc receptors. Klein et al. (1981) have suggested that a hinge-region deletion results in a closer contact of the Fab and Fc regions, thus making the Fc region unaccessible to C1 or Fc receptors. The present NMR results are consistent with their model.

The C2-H and C4-H proton chemical shifts of His-224 become identical with those observed for oligopeptides only when the inter heavy chain disulfide bridges are reduced and alkylated (see Figure 6). This suggests that upon reduction and alkylation the hinge segment acquires another degree of freedom of motion, due especially to the increase in the flexibility in the segment that follows the $(Cys\text{-}Pro\text{-}Pro\text{-}Cys)_2$ part. As suggested above, this part is extended and has a lower degree of segmental flexibility in the native form of IgG as compared with that for the segment that precedes the $(Cys\text{-}Pro\text{-}Pro\text{-}Cys)_2$ part. On reduction and alkylation of the inter heavy chain disulfide bridges, the hinge region amino acid residues including Ala-231 and Leu-234 give much sharper signals (see Figure 8). It appears that the two parts of the hinge, which precedes and follows the $(Cys\text{-}Pro\text{-}Pro\text{-}Cys)_2$ part, become comparable in flexibility when the inter heavy chain disulfide bridges are reduced and alkylated.

We have previously shown that His-435 in the Fc fragment can be a sensitive probe in studying the way in which the C_H2 and C_H3 domains contact with each other (Shimizu et al., 1983). Figure 10 indicates that the pH titration data observed for His-435 of the Fc fragment agree quite well with those for IgG1 Ike-N. Similar results were obtained for IgG1 Dob (data not shown). Reduction and alkylation do not cause any significant change in the His-435 titration data. On the basis of these observations we suggest that the way in which the C_H2 and C_H3 domains contact with each other changes very little (1) on cleavage of the Fab portion of the IgG molecule, (2) on reduction and alkylation of inter heavy chain disulfide bridges, and (3) on deletion of most of the hinge region. The present result is consistent with the report that binding of IgG1 Dob to protein A from *Staphylococcus aureus* is entirely normal (Klein et al., 1981). It has been established that the site of protein A binding to Fc is located at the junction between the C_H2 and C_H3 domains [Deisenhofer, 1981; see also Shimizu et al. (1983)].

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