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# Proton Nuclear Magnetic Resonance Studies of Human Immunoglobulins: Conformation of the Hinge Region of the IgG1 Immunoglobulin†

Yoji Arata,* Mayumi Honzawa, and Akira Shimizu

ABSTRACT: The conformation of the hinge region of the human IgG1 immunoglobulin has been investigated by making use of His-224 in the hinge region as a *built-in* proton nuclear magnetic resonance (NMR) probe. Human myeloma IgG1($\kappa$) proteins Ogo and Yot and human polyclonal IgG were used along with their Fab and F(ab')$_2$ fragments for the assignment of the His-224 signals. The titration behavior of His-224 of the intact IgG and the fragments was compared. It was shown that the titration curves for the intact IgG and the F(ab')$_2$ fragments are identical and quite similar to those for the histidine residue in small peptides. By contrast, the Fab fragments give titration curves which are quite different from those for the intact IgG and the F(ab')$_2$ fragments. Conclusions derived may be summarized as follows: (1) in the intact IgG1, the hinge peptide is fully exposed to the solvent and exhibits internal motion which is much more rapid than the Fab segmental motion with respect to Fc; (2) at the loss of the Fc portion of the IgG, the conformation of the hinge peptide in the F(ab')$_2$ fragments remains unchanged; (3) the heavy–heavy interchain interactions involving the two disulfide bridges do not play the primary role in determining the conformation of the hinge region in the intact IgG as well as in the F(ab')$_2$ fragments; (4) the existence of a small stretch of peptide fragment Thr-225–Leu-234 is essential in maintaining the conformation of the hinge region of the intact IgG and the F(ab')$_2$ fragments; (5) in the Fab fragments, as a result of cleavage of a major portion of the hinge peptide, the C-terminal part of the heavy chain including His-224 is partially folded back toward the globular portion of the polypeptide chains; and (6) the hinge peptide in the Fab fragments still retains a degree of flexibility which is similar to that in the intact IgG and the F(ab')$_2$ fragments.

Proteins of the human immunoglobulin G (IgG)[1] class can be differentiated into four subclasses (IgG1 through IgG4), each with a distinctive heavy chain, $\gamma$1, $\gamma$2, $\gamma$3, and $\gamma$4; light chains can be either of $\lambda$ or $\kappa$ type, regardless of its heavy chain subclass.[2,3] The $\gamma$ chains consist of four homology units, $V_H$, $C_H1$, $C_H2$, and $C_H3$, whereas the light chains are divided into

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[1] Abbreviations used: DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid sodium salt; IgG, immunoglobulin G; NMR, nuclear magnetic resonance.

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

[3] The numbering system used in the present paper is based on protein Ag for the $\kappa$ chain (Putnam, 1969) and on protein Eu for the $\gamma$1 chain (Edelman et al., 1969).

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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 cleavage can give rise to Fab, $F(ab')_2$, and Fc fragments [see, e.g., Kabat (1976)]. On the basis of hydrodynamic and spectroscopic observations, it has been established that segmental flexibility exists between Fab and Fc portions of antibody molecules; the main structural site of this property is the hinge region (Noelken et al., 1965; Yguerabide et al., 1970; Cathou, 1978).

In previous papers we have reported $^1H$ nuclear magnetic resonance (NMR) studies of the solution conformation of the constant domain of the light chain (Arata et al., 1978a; Arata & Shimizu, 1979; Shimizu et al., 1980). We have also shown that the Oz, Kern, and Mcg isotypic markers can be identified on the basis of the $^1H$ NMR chemical shifts (Shimizu et al., 1980). In the present paper, we report the assignment of the $^1H$ NMR signals of His-224, which exists in the hinge region of the IgG1 immunoglobulin. Human myeloma proteins IgG1($\kappa$) Ogo and Yot and normal human IgG as well as their Fab and $F(ab')_2$ fragments were used for the assignment. Titration behavior of the hinge region histidine in the intact IgG and the fragments was compared. These results will be discussed in terms of the conformation and flexibility of the hinge region of the IgG molecules.

## Materials and Methods

*Preparation and Purification of IgG Proteins and Their Fragments.* IgG proteins Ogo and Yot were isolated from the sera of patients with multiple myeloma. Polyclonal human IgG was obtained from Kabi, Stockholm (lot no. 72536). IgG was also isolated from a patient with polycythemia. The IgG was purified on a column of DEAE-cellulose (Whatman DE-52) equilibrated with 0.01 M Tris-HCl buffer, pH 8.0. The serum was equilibrated and eluted in the same buffer with increasing molarity of KCl to 0.1 M. The IgG fraction was collected and purified further by gel filtration on Sephadex G-100 in 0.1 M Tris-HCl, 0.15 M NaCl, and 0.05% $NaN_3$ (pH 8.0). Fab fragments were prepared from IgG Ogo, IgG Yot, and normal human IgG. Digestion with papain (Sigma, lot no. P-3125, twice crystallized) was performed according to the method of Porter (1958) except that digestion was carried out for 60 min. The fragments were separated by DEAE-cellulose column chromatography and purified by gel filtration as described above for IgG. $F(ab')_2$ fragments Ogo and Yot were prepared by digesting the corresponding IgG with pepsin according to the method of Turner et al. (1970). The IgG dissolved in 0.1 M sodium acetate, pH 4.5, buffer at a protein concentration of 2% was digested with pepsin (Sigma, lot no. P-7012, twice crystallized) for 24 h at 37 °C. The enzyme/IgG ratio was 1:100. The fragments were purified on a DEAE-cellulose column followed by gel filtration as described above for IgG.

*Digestion of the Fab Fragments with Carboxypeptidase A.* Fab Ogo (16 mg) was dissolved in 3 mL of 0.2 M *N*-ethylmorpholine acetate (pH 8.5), and 0.8 mg of carboxypeptidase A DFP (Sigma, type II, lot no. C-6510) was added; the mixture was incubated for 3 h at 37 °C (Steiner & Porter, 1967). An aliquot was separated from the reaction mixture, acidified by adding concentrated HCl, and lyophilized, and the amount of amino acids released was quantitated. It was confirmed that 0.91 nmol of histidine and 0.87 nmol of threonine were released from 1 nmol of Fab Ogo; the amount of other amino acids was less than one-tenth that of the histidine and threonine. The main portion of the digest was dialyzed against distilled water at 4 °C and lyophilized. This fraction will be referred to as Fab Ogo (CPase) in the text.

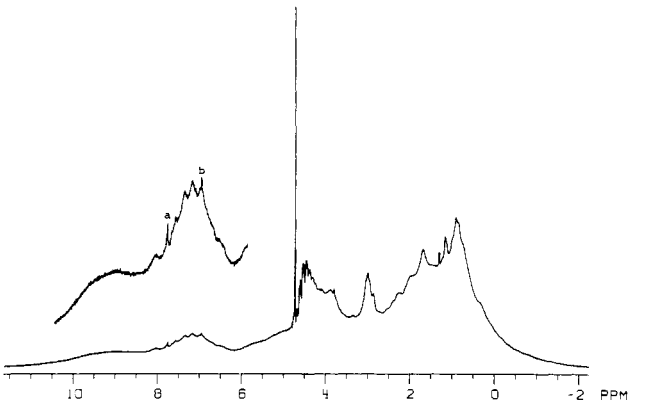


FIGURE 1: The 360-MHz $^1H$ NMR spectrum of IgG1($\kappa$) Ogo (10 mg in 0.3 mL of 0.2 M NaCl/$D_2O$, pH 7.86). Chemical shifts are in parts per million from external DSS (5% in $D_2O$). The probe temperature was 29 °C.

Fab Yot was treated in the same way; 0.95 nmol of histidine and 0.93 nmol of threonine were recovered from 1 nmol of Fab Yot.

*Purity and Subclass Identification of the Proteins.* Purity of IgG and the fragments was checked by immunoelectrophoresis and agar gel electrophoresis. Whole human antisera (Hoechst–Behring) was used for the purity check by immunoelectrophoresis. IgG Ogo, IgG Yot, Fab Ogo, $F(ab')_2$, and normal human IgG employed in the present experiment showed a single spot and a single arc on agar gel electrophoresis and immunoelectrophoresis, respectively. Type and class of IgG were identified by Ouchterlony methods with specific antisera (Hoechst–Behring). The subclass of IgG was determined with subclass specific antisera purchased from Nordic Immunological Laboratories (lot no. 16-476, 3-1279). Purified IgG OgO and IgG Yot reacted with anti-IgG1 sera but not with other subclass specific antisera. It was also confirmed that both of these proteins have the $\kappa$ type light chains.

*Reduction and Alkylation of $F(ab')_2$ Ogo.* $F(ab')_2$ Ogo was reduced with 10 mM dithiothreitol in 0.2 M Tris-HCl and 0.1 M NaCl (pH 8.2) for 1 h at 25 °C and alkylated with 22 mM monoiodoacetamide for 30 min at 4 °C. The reduced and alkylated $F(ab')_2$, which is monomeric, will be referred to as Fab′ in the text.

*NMR Measurements.* Ten milligrams of each protein was dissolved in 0.3 mL of 0.2 M NaCl/$D_2O$. The pH was adjusted with 0.3–0.5 M DCl or NaOD. All pH values reported in this paper are uncorrected meter readings of $D_2O$ solutions made with an electrode standardized by using $H_2O$ buffers. $^1H$ NMR spectra were obtained at 100 MHz with a JEOL PS-100 spectrometer in the correlation mode (Arata & Ozawa, 1976; Arata et al., 1978b) and at 360 MHz with a Bruker HXS-360 spectrometer in the pulse–FT mode. In the correlation mode at 100 MHz, typically 2000 transients (512 Hz/0.5 s) were accumulated to improve the signal-to-noise ratio. All chemical shifts are given in parts per million from external DSS (5% in $D_2O$). The probe temperature was 29 °C throughout the experiment.

## Results and Discussion

*Assignment of Signals of His-224 of the Hinge Region.* Figure 1 shows an example of the $^1H$ NMR spectrum of human myeloma protein IgG1($\kappa$) Ogo. Only a small number of narrow signals can be observed. Signals a and b are virtually the only ones which are narrow in line width and can be observed throughout the entire pH range 4–9 examined.

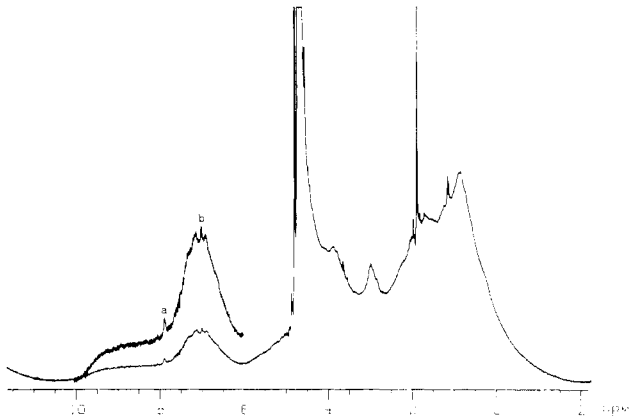

FIGURE 2: The 360-MHz $^1H$ NMR spectrum of polyclonal human IgG (Kabi), pH 7.22. Other conditions are the same as in Figure 1. Sharp signals observed around 1.8 ppm are probably due to contaminated small molecules.

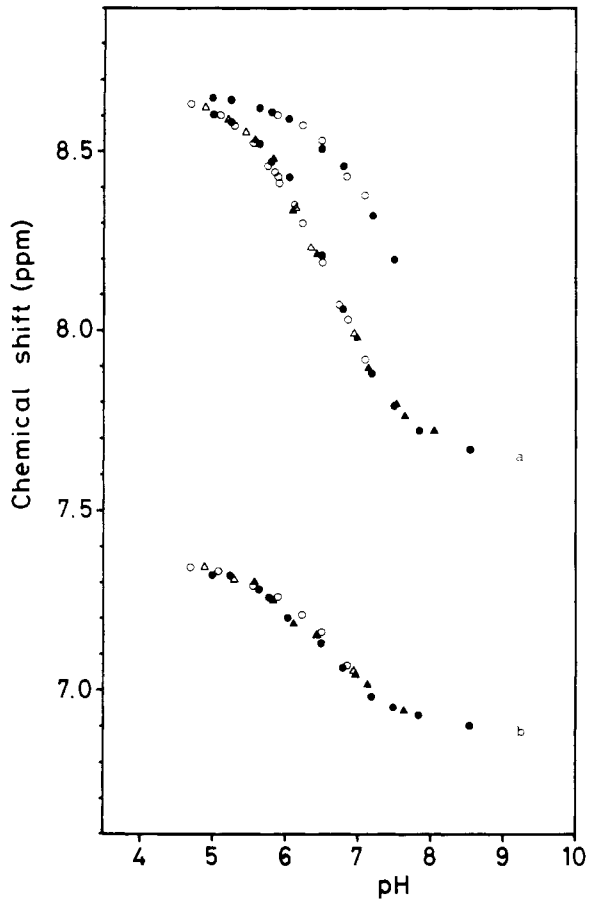


FIGURE 3: The pH dependence of the chemical shifts for signals a and b: IgG1($\kappa$) Ogo (●); IgG1($\kappa$) Yot (○); polyclonal IgG (Kabi) (▲); polyclonal IgG from a patient with polycythemia (△). Titration data for His-189($\kappa$) of IgG1($\kappa$) Ogo and IgG1($\kappa$) Yot are also included in the figure (see text).

IgG1($\kappa$) Yot and two kinds of human polyclonal IgG, one from a commercial source and the other from a patient with polycythemia, also give signals a and b. The spectrum observed at pH 7.22 of polyclonal IgG is reproduced in Figure 2. The pH dependence of the chemical shifts of signals a and b is given in Figure 3; the four kinds of IgG proteins examined give the identical titration curves for signals a and b. These results strongly suggest that signals a and b are due to the C2- and C4-H protons of the histidine residue which belongs to the constant part of the IgG molecule.

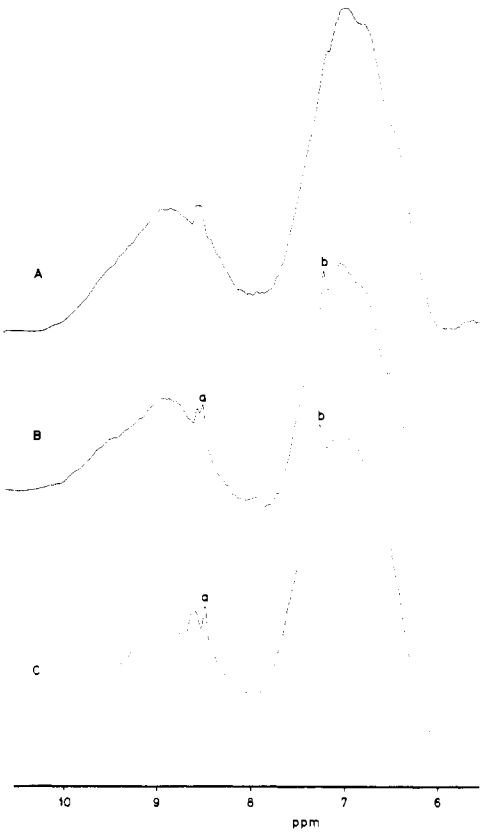


FIGURE 4: The 100-MHz $^1H$ NMR spectra of fragments obtained from IgG1($\kappa$) Ogo. (A) Fab(CPase), pH 5.65; (B) Fab, pH 6.14; (C) $F(ab')_2$, pH 5.72. Ten milligrams of each protein was dissolved in 0.3 mL of 0.2 M $NaCl/D_2O$. Chemical shifts are in parts per million from external DSS (5% in $D_2O$). The probe temperature was 29 °C. Notations of the fragments are described under Materials and Methods. See also Figure 6.

The Fab and $F(ab')_2$ fragments of IgG1($\kappa$) Ogo, IgG1($\kappa$) Yot, and polyclonal IgG also give a pair of well-resolved signals in the aromatic region; representative spectra are given in Figure 4. The pH dependence of the chemical shifts for these fragments is plotted in Figure 5 where titration curves for signals a and b observed in Figure 3 for the intact IgG proteins are also given (dotted lines a and b). It should be noted in Figure 5 that $F(ab')_2$ Ogo and $F(ab')_2$ Yot give titration curves which coincide with those for signals a and b observed in the intact IgG proteins. This result indicates that signals a and b do *not* originate from the Fc portion. The $F(ab')_2$ fragments of IgG1 contain a histidine residue at position 224 in the hinge region. The pertinent portion of the amino acid sequence of the hinge region of human IgG1 is illustrated in Figure 6. For the assignment of signals a and b, we used Fab (CPase) which, as Figure 6 shows, has lost the peptide segment Thr-223–Leu-234 in the $F(ab')_2$ fragment (see Materials and Methods). Figure 4A shows that signals a and b disappeared on the proteolytic cleavage.[4] A broad signal observed in Figure 4 at low field in common in the Fab(CPase) as well as in the Fab and $F(ab')_2$ fragments is a superposition of more than one peak which contains the signal from His-189 of the $\kappa$ chain. As we describe later, the His-189($\kappa$) signal remains unchanged in the intact IgG as well as in the Fab, Fab(CPase), and $F(ab')_2$ fragments. In view of the results described above, it may now be concluded that signals a and b are due to His-224 of the hinge region of IgG1.

[4] In Figure 4A, the presence of small signals a and b may be noticed, which presumably are due to an unreacted fraction of the Fab fragment. See Materials and Methods.

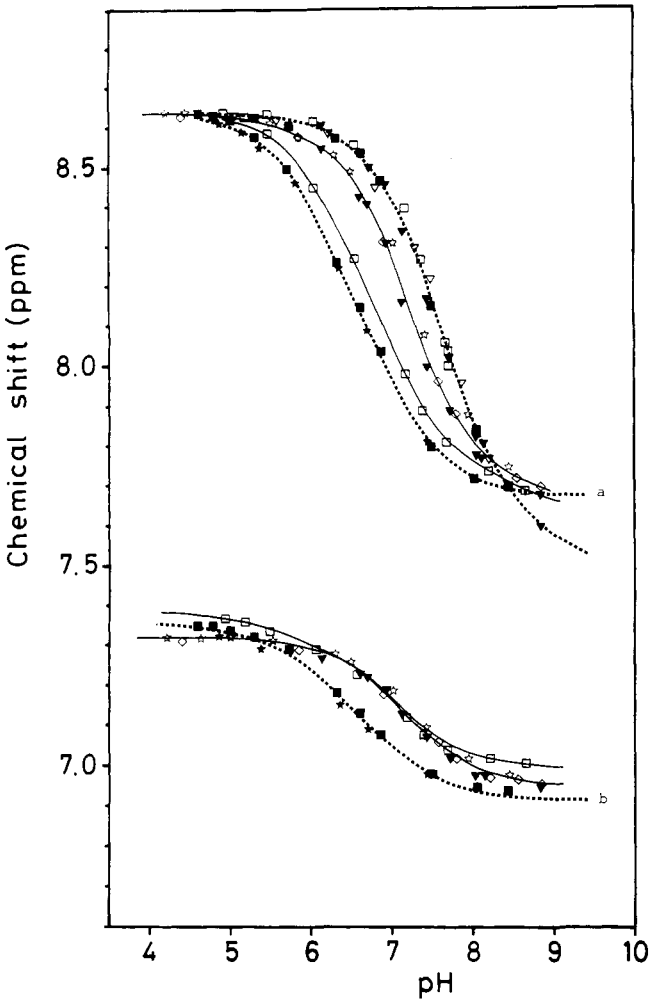


FIGURE 5: The pH dependence of the chemical shifts of the C2- and C4-H protons of His-224($\gamma$1) and of the C2-H proton of His-189($\kappa$) of the IgG fragments. $F(ab')_2$ Ogo (■); $F(ab')_2$ Yot (★); Fab Ogo (▼); Fab Yot (☆); Fab from polyclonal IgG (◇); Fab' Ogo (□); Fab Ogo (CPase) (▽). Notations of the fragments are described under Materials and Methods. See also Figure 6. Curves a and b are drawn by using the data presented in Figure 2 for the C2- and C4-H protons of His-224 of the hinge region of IgG1, respectively. Curve $\kappa$ is based on our previous results for the C2-H proton of His-189 of $\kappa$-type Bence-Jones proteins (Arata & Shimizu, 1979).


FIGURE 6: Human IgG1 hinge region.

*Conformation of the Hinge Region.* It has been established by using a variety of physicochemical techniques that (1) segmental flexibility exists in the intact IgG as well as in the $F(ab')_2$ but not in the Fab fragments, (2) the Fab portions are flexible with respect to each other and to Fc in the $F(ab')_2$ fragment and the intact IgG, respectively, and (3) the primary site of flexibility is the hinge region (Noelken et al., 1965; Valentine & Green, 1967; Yguerabide et al., 1970; Cathou, 1978). In view of these findings, we use the His-224 NMR signals as a built-in probe to discuss the conformation of the hinge peptide of the human IgG1 and the Fab and $F(ab')_2$ fragments.

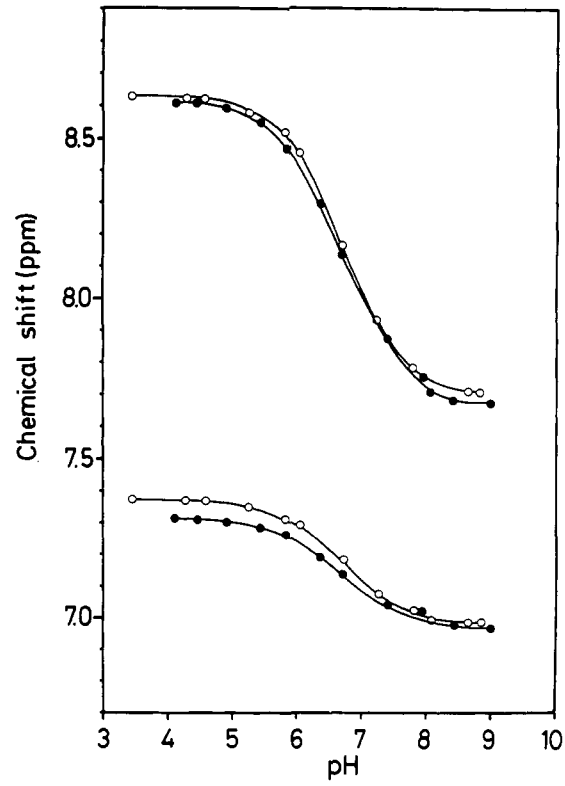


FIGURE 7: The pH dependence of the chemical shifts of the C2- and C4-H protons of histidine peptides. Peptide I (○), Ser-His-Arg; peptide II (●), Ser-Tyr-Ser-Cys-Gln-Val-His-Glu-Gly-Ser-Thr-Val-Glu-Lys. See Arata et al. (1978a). Each peptide was dissolved in 0.2 M $NaCl/D_2O$. Chemical shifts are in parts per million from external DSS (5% in $D_2O$). The probe temperature was 29 °C.

As Figure 5 shows, His-224 in the $F(ab')_2$ fragments gives the titration curves which coincide with those for the intact IgG1($\kappa$). This suggests that the conformation of the hinge region in the intact IgG1 changes very little in the $F(ab')_2$ fragments at the loss of the Fc portion of the molecule. It is of interest that the titration curves for the intact IgG1 and the $F(ab')_2$ fragments are quite similar to those of small histidine peptides [peptides I and II in our previous work (Arata & Shimizu, 1979)] which were obtained by tryptic cleavage of a human $\lambda$ chain. The titration curves for peptides I and II (see Figure 7), which are quite similar to those for *N*-acetyl-L-histidine methylamide (Tanokura et al., 1978), may be taken as representing the histidine residue which is exposed to solvent and has a sufficient degree of flexibility in solution. In the cases of His-224 in the intact IgG and the $F(ab')_2$ fragments, the $pK_a$ value is 6.5 and the limiting values of the chemical shifts are 8.65 and 7.65 ppm (C2-H) and 7.35 and 6.90 ppm (C4-H). The $pK_a$ values of histidine residues in both peptides I and II are 6.5, and the limiting values of the chemical shifts for the C2- and C4-H protons of these peptides are also quite similar to those observed for His-224 in IgG1($\kappa$) Ogo and IgG1($\kappa$) Yot and their $F(ab')_2$ fragments. These results along with the observation that the His-224 signals are among a very small number of well-resolved ones observed in the intact IgG molecules (see Figures 1 and 2) strongly suggest that in the intact IgG1 as well as in the $F(ab')_2$ fragments, the hinge peptide, without tight folding, is exposed to solvent and is highly flexible.

As Figure 5 shows, reduction and alkylation of the heavy–heavy interchain disulfide bridges at positions 226 and 229 of $F(ab')_2$ Ogo results in only a small increase in the $pK_a$ value of His-224. This indicates that cleavage of the heavy–heavy interchain disulfide bridges in the $F(ab')_2$ fragment does not induce any major change in conformation of the hinge region; i.e., even in the Fab′ fragment, which is monomeric, the conformation of the hinge region is basically the same as that in the intact IgG molecules. This suggests that the heavy–heavy interchain interaction involving the two disulfide bridges does not play a primary role in maintaining the conformation of the hinge peptide. However, it should also be noted that in Figure 5 there is a significant difference in the limiting shifts for the C4-H titration curves for $F(ab')_2$ and Fab′. We have observed in the case of human λ light chains and their constant fragments that the C4-H proton is more sensitive than the corresponding C2-H proton to changes of the environments in which the histidine residue is located in the protein molecules [see Figures 2 and 4 in Shimizu et al. (1980)]. It appears that the environment surrounding His-224 in the hinge region of Fab′ is not quite the same as in the case of the intact IgG1 and its $F(ab')_2$ fragments. By contrast, the Fab fragment, where His-224 is at the C terminus of the γ1 heavy chain, gives the titration curves which are shifted from the corresponding ones in the intact IgG and its $F(ab')_2$ fragments to the high pH side by 0.7 pH unit (see Figure 5). This means that the existence of a small stretch of peptide segment, Thr-225–Leu-234, is essential in maintaining the extended conformation which is characteristic of the hinge region in the intact IgG and the $F(ab')_2$ fragments. We suggest that in the Fab fragments, as a result of cleavage of most of the hinge peptide, the C-terminal portion including His-224 is partially folded back toward the globular portion of the polypeptide chains. However, as we discuss below, a degree of flexibility which is similar to that in the intact IgG as well as in the $F(ab')_2$ fragments is still retained in the Fab fragments.

In the human γ chains, the hinge region contains an unusual abundance of proline [see, e.g., Kabat (1976)]. It was suggested that the prolyl residues fold in a conformation resembling a poly(L-proline) helix, keeping apart the Fab and Fc portions and facilitating rotation of Fab with respect to Fc in the IgG molecule (Yguerabide et al., 1970). It was reported that, in the case of rabbit IgG, the rotational correlation time for the Fab segmental motion is 33 ns, whereas the correlation time for the tumbling of the whole antibody molecule is 168 ns (Yguerabide et al., 1970). In the IgG, the $^1H$ NMR line widths for His-224 of the hinge region are one order of magnitude smaller than those for almost all other amino acid residues in the protein. No significant change was observed in the line widths of the His-224 signals when the whole IgG was cleaved into the $F(ab')_2$, Fab′, and Fab fragments. This means that in the hinge peptide a significant degree of flexibility exists in common with the intact IgG as well as with the Fab and $F(ab')_2$ fragments. Even in the case of the Fab fragment, which gives some additional signals presumably due to a smaller size of the molecule, the His-224 signals are distinctively narrower than the rest of the observed signals, including that of His-189(κ) which is exposed to the solvent (Arata & Shimizu, 1979); from the NMR criterion the hinge peptide in the Fab fragment is far more flexible than any other part of the molecule. It may now be concluded that in the intact IgG and in the $F(ab')_2$ fragments the hinge peptide exhibits internal motion which is much more rapid than the Fab seqmental motion with respect to Fc and to each other, respectively. We suggest that the Fab segmental motion is based on the *intrinsic* flexibility of the hinge peptide which is retained even in the Fab fragment where, in addition to Fc, most amino acid residues of the hinge peptide including all proline residues are cleaved.

*Other Histidine Residues in the $C_L$ and $C_H1$ Domains.* At pH ≲6, IgG1(κ) Ogo and IgG1(κ) Yot give, in addition to the His-224 signals, signals which also titrate in the aromatic region. Due to a severe overlapping of the signals, which are generally broad, it is difficult to follow the titration curves for most of these signals. One of these signals is fairly easy to follow, however, and the pH dependence of its chemical shift is given in Figure 3. The same signal is observed in the Fab and $F(ab')_2$ fragments; as Figure 5 shows, the pH dependence of the chemical shifts is identical for the intact IgG as well as for the Fab and $F(ab')_2$ fragments. Also drawn in Figure 5 is the titration curve κ which has been obtained for the C2-H proton of His-189 of κ-type Bence-Jones dimers (Arata & Shimizu, 1979). Comparison of these titration data clearly established the assignment of the His-189(κ) signal. The result obtained here is consistent with X-ray structural studies which have shown that there is a close resemblance in the conformation of the constant domain of the light chain in the Bence-Jones dimer and that in the Fab fragment (Poljak et al., 1973; Edmundson et al., 1975). Above pH 6, the light chain signals become difficult to observe with an increase in pH. This is due to a gradual broadening. By contrast, in the case of the light chain dimer, no such broadening was observed (Arata & Shimizu, 1979). Even at low pH, the light-chain His-189 signal is much broader in the Fab fragment than in the light-chain dimer.

There are five histidine residues in the constant portion of the $F(ab')_2$ fragment of IgG1(κ): His-170 and His-208 in the $C_H1$ domain, His-224 in the hinge region, and His-189 and His-198 in the constant domain of the κ chain. As described above, the signals due to His-224 and His-189(κ) have been identified. According to the X-ray structural data on the Fab fragment of IgG1(λ) New (Poljak et al., 1973), His-198(λ) and His-208 (γ1) occupy equivalent environments in the $C_L$ and $C_H1$ domains, respectively; both histidine residues are oriented toward the interior of the *immunoglobulin fold*.[5] Presumably His-198 is located in an equivalent environment in the λ and κ chains. It has been shown by $^1H$ NMR that His-198 in the λ as well as in the κ chain dimers gives a broad C2-H proton signal and has an unusually low $pK_a$ value of ≲4.0 (Arata & Shimizu, 1979). In view of these results, it is quite likely that signals due to His-198(κ) and His-208(γ1) cannot be observed, especially in the intact IgG. His-170(γ1) belongs to an antiparallel segment of the immunoglobulin fold of the $C_H1$ domain and, although it is not oriented toward the interior of the immunoglobulin fold, it is located inside the whole IgG molecule (Poljak et al., 1973).[5] Therefore, it is likely that the His-170(γ1) signals are also subject to a significant line broadening. As described above, in the Fab and $F(ab')_2$ fragments, titrating signals other than those of His-224 and His-189(κ) can be observed in the aromatic region below pH 6. Apparently these signals are due to the above-mentioned histidine residues in the $C_L$ and $C_H1$ domains. In the aromatic region of the intact IgG1, some additional signals become observable at low pH, apparently due to a decrease in line width. By comparing these signals with those observed in the Fc fragments, we were able to show that some of these signals

[5] Atomic coordinates were obtained from the Protein Data Bank (Bernstein et al., 1977). Plotter drawings of the protein structure were made by using program STDRAW developed by Dr. Y. Mitsui and coworkers at University of Tokyo.

originate from histidine residues in the Fc portion of the IgG molecule. It appears that the intact IgG1 and the fragments acquire an increased degree of flexibility at low pH.

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# Roles of Phospholipid and Detergent in Soluble Protein Activation of Squalene Epoxidase†

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ABSTRACT: "Soluble protein factor" (SPF) from hog liver stimulates hepatic microsome-associated squalene epoxidase in the presence of phosphatidylglycerol or phosphatidylserine. When SPF and phosphatidylglycerol are preincubated for 30 min at 37 °C before addition to the epoxidase system, this stimulation is abolished. On Sephadex chromatography of the protein–phospholipid mixture, both components appear in the void volume, whereas SPF alone is retarded on the column. These results suggest formation of a SPF–phosphatidylglycerol complex. Treatment of the complex with Tween 80 restores the stimulatory effects of SPF on squalene epoxidase. The stimulation of microsomal squalene epoxidase by SPF was abolished by pretreatment of the membrane with low concentrations of deoxycholate or by solubilizing the enzyme with Triton X-100, implying that an intact membrane system is required for SPF sensitivity. SPF has been purified 1200-fold from hog liver.

Several soluble proteins that stimulate microsomal enzymes involved in the late stages of hepatic cholesterol biosynthesis have been described (Tchen & Bloch, 1957; Ritter & Dempsey, 1970; Srikantaiah et al., 1976; Spence & Gaylor, 1977). Rat hepatic tissue has been the source of soluble proteins in all previous investigations. In contrast, information on such soluble proteins in hepatic tissue other than that of the rat has been missing. In particular, a rat liver cytoplasmic protein, termed "supernatant protein factor" (SPF),[1] is shown to stimulate microsomal squalene epoxidase (Tai & Bloch, 1972; Saat & Bloch, 1976). Although homogeneous SPF can be obtained from this tissue (Ferguson & Bloch, 1977), the yield is low, not exceeding ~2 mg/kg of rat liver. Since the scarcity of the rat material could be a major hindrance to the study of the mechanism of SPF stimulation, it seemed of importance to search for a more convenient source. Hog liver was found to contain moderate SPF activity, whereas no SPF was demonstrable in extracts of fresh or frozen beef liver. This paper accounts for the partial purification and characterization of SPF from hog liver and demonstrates the requirement of anionic phospholipids for the SPF response. Toward the un-

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[1] Abbreviations used: SPF, supernatant protein factor; $SPF_{Ac}$, crude SPF preparation, 45–75% acetone precipitate (as described under Methods) from hog liver; AMO-1618, (trimethylammonio)-5-carvacryl 1-piperidinecarboxylate chloride; $S_{78}$, 78000*g* supernatant from hog liver; DTT, dithiothreitol; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; NADPH, reduced nicotinamide adenine dinucleotide phosphate; FAD, flavin adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid.

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