Preparation of the Fv Fragment from a Short-Chain Mouse IgG2a Anti-Dansyl Monoclonal Antibody and Use of Selectively Deuterated Fv Analogues for Two-Dimensional ^{1}H NMR Analyses of the Antigen–Antibody Interactions†

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ABSTRACT: The Fv fragment, a univalent antigen-binding unit with a molecular weight of 25 000, has successfully been prepared in high yield by limited proteolysis with clostripain of a short-chain mouse IgG2a anti-dansyl monoclonal antibody in which the entire C_H1 domain is deleted [Igarashi, T., Sato, M., Takio, K., Tanaka, T., Nakanishi, M., & Arata, Y. (1990) *Biochemistry 29*, 5727–5733]. The Fv fragment obtained is stable at room temperature and retains its full antigen-binding capability. It has been shown that selective deuterium labeling of the Fv fragment, which is half the size of the Fab fragment, provides ^{1}H NMR spectral data at a sufficient resolution for a detailed structural analysis of the antigen-combining site. NOESY spectra of an Fv analogue, in which all aromatic protons except for His C2′-H and Tyr C3′,5′-H had been deuterated, were measured in the presence of varying amounts of dansyl-L-lysine. On the basis of the NOESY data obtained, it was possible to assign all the ring proton resonances for the dansyl group that is bound to the Fv fragment. It was also possible to obtain information about His and Tyr residues of the Fv fragment in the absence and presence of the antigen. On the basis of the NMR data obtained, we have shown that at least two Tyr residues along with one of the amide groups are directly involved in antigen binding. The mode of interaction of the dansyl ring with these residues in the Fv fragment has briefly been discussed.

Immunoglobulin G (IgG),[1] which is an important member of the group of proteins that function as antibodies, consists of two identical heavy chains and two identical light chains. The heavy chains are composed 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. Papain digestion of the whole antibody molecule gives the Fab and Fc fragments. Antigen recognition is carried by the Fab fragment, which consists of the light chain (V_L and C_L) and the N-terminal half (V_H and C_H1) of the heavy chain. A variety of effector functions are expressed by the Fc region that is composed of two C_H2 and two C_H3 domains.

We are studying the structure of IgG (M_r 150 000) by NMR spectroscopy in an effort to understand the processes of molecular recognition involved in a variety of antibody functions. It has been shown that ^{1}H NMR is useful in obtaining information about the flexible part in IgGs and their proteolytic fragments (Arata et al., 1980; Endo & Arata, 1985; Ito & Arata, 1985). However, due to the increased molecular size, it is virtually impossible to follow the standard procedure used for the structural analysis of smaller proteins (Wüthrich, 1986). Thus efforts are being made to explore appropriate systems and methods in order to extract relevant information concerning antibody structure and functions as extensively and as selectively as possible.

Selective deuteration was first used by Jardetzky and coworkers in order to obtain information by ^{1}H NMR about the structure of proteins in solution at an atomic level (Markley et al., 1968). Multinuclear NMR techniques, which use proteins singly or doubly labeled with ^{2}H, ^{13}C, and ^{15}N, are now in extensive use for the determination of detailed solution structures of proteins of $M_r \lesssim 20000$ (Markley, 1989). In order to cope with difficulties originating from the large size of IgGs and their fragments, we are developing strategies that heavily rely on the use of stable-isotope labeling. We have already shown that (1) direct observation is possible of each of the carbonyl carbon resonances of IgGs and their fragments selectively labeled with ^{13}C at the carbonyl carbon, (2) all of these carbonyl carbon resonances can be unambiguously assigned, and (3) the assigned carbonyl carbon resonances can be used as sensitive probes for domain–domain interactions (Kato et al., 1989a, 1991). Another approach is to use multinuclear NMR techniques for a detailed analysis of the structure of functional sites of IgGs. For this purpose, a variety of functional fragments are being used, and we report in this paper the first of a series of our NMR studies along this line.

The structure of the antibody-combining site has been studied by NMR using selectively deuterated Fab fragments [Anglister, 1990, and references cited therein]. It has recently been reported that a combination of selective deuterium labeling and two-dimensional transferred nuclear Overhauser effect difference spectroscopy is effective in extracting information about interactions between a peptide of cholera toxin and the Fab fragment of one of antibodies raised against it (Anglister & Zilber, 1990). Even with the use of selectively deuterated analogues, the size of the Fab fragment (M_r 50 000) has hampered detailed analysis of the mechanism of antigen

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[1] Abbreviations: CDR, complementarity-determining region; C_H1, a constant domain of the heavy chain; C_L, the constant domain of the light chain; DNS-Lys, ε-dansyl-L-lysine; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; Fab, antigen-binding fragment; Fv, heterodimer of V_H and V_L; IgG, immunoglobulin G; IgG2a(s), a short-chain mouse IgG2a anti-dansyl monoclonal antibody that lacks the entire C_H1 domain; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, two-dimensional NOE correlated spectroscopy; V_H, the variable domain of the heavy chain; V_L, the variable domain of the light chain.

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recognition by NMR. For example, it is of great interest to compare the structure of the antigen-combining site in the absence and presence of the antigen. However, information that is needed to discuss this problem was not available in the above approach because all the measurements had been made with the antigen-combining site saturated with the antigen. This was necessary in order to circumvent the problem of spectral complexity due to the large size of the Fab fragment.

The Fv fragment, which is a heterodimer of V_H and V_L domains, is a smallest antigen-binding unit (M_r 25 000) and is of great interest for NMR analyses of the molecular mechanism of antigen recognition. Inbar et al. (1972) first succeeded in preparing the Fv fragment by pepsin digestion of a mouse myeloma IgA(λ_2) protein MOPC 315 [see also Hochman et al. (1973)]. However, various attempts have so far been made without success to obtain Fv fragments by proteolysis of whole IgG molecules with other types of the light chain (Sharon & Givol, 1976).

We have recently reported a structural study of a mouse IgG2a anti-dansyl monoclonal antibody in which the entire C_H1 domain is deleted (Igarashi et al., 1990). This short-chain IgG2a antibody is designated IgG2a(s). In the present paper, we will show that limited digestion with clostripain of the IgG2a(s) antibody gives the corresponding Fv fragment in high yield. We have already established a procedure for selectively labeling the antibody with ^2H, ^{13}C, and ^{15}N (Kato et al., 1989a,b, 1991). Thus a variety of Fv analogues have now become available to us in large quantities for structural analyses by NMR.

Successful attempts have recently been reported to express and secrete Fv fragments from *Escherichia coli* (Skerra & Plückthun, 1988; Ward et al., 1989) as well as from myeloma cells (Riechmann et al., 1988). X-ray structural analyses of Fv fragments derived by gene technology have been started (Glockshuber et al., 1990a; Boulot et al., 1990). Preliminary NMR studies of an Fv fragment expressed in myeloma cells have also been reported by Wright et al. (1990), who outlined a general strategy for spectral assignments by the combined use of stable-isotope labeling and a variety of two-dimensional techniques.

In the present work, we will show that by selective deuterium labeling of the Fv fragment, which is half the size of the Fab fragment, detailed spectral information becomes available about the antigen-combining site in the absence and presence of the antigen. It was also possible to determine the chemical shifts for the ring protons of the dansyl ring bound to the Fv fragment. On the basis of the NMR data obtained, we will briefly discuss the mode of interaction of the antigen with the Fv fragment.

Materials and Methods

Materials. Tyr-*2′,3′,5′,6′-d_4* was prepared from phenol-d_5 through an enzymatic reaction using β-tyrosinase (Nagasawa et al., 1981). Tyr-*2′,6′-d_2* was prepared by treatment at 110 °C of Tyr-*2′,3′,5′,6′-d_4* in 6 M HCl for 2 h. His-*α,2′,4′-d_3* and Trp-*2′,4′,5′,6′,7′-d_5* were prepared according to the procedures described in the literature (Matthews et al., 1977). Phe-*2′,3′,4′,5′,6′-d_5* was purchased from CIL. Clostripain was obtained from Sigma Chemical Co.

Preparation of Selectively Deuterated IgG2a(s). The IgG2a(s) protein was purified as described previously (Igarashi et al., 1990). The mouse hybridoma cell line, 1B10.7, that produces IgG2a(s) (Dangl et al., 1982) was adapted to a serum-free medium (Nissui NYSF 404) and then grown in the medium containing Tyr-*2′,6′-d_2* along with His, Phe, and Trp perdeuterated in the aromatic rings (Kato et al., 1989a,b, 1991).[2] After cell growth, the cell supernatant was concentrated with a Minitan ultrafiltration system (Millipore) and then applied to an Affi-Gel protein A column (Bio-Rad). The resultant filtrate was concentrated by ultrafiltration. A typical yield was 45 mg of purified IgG2a(s) per liter of cell culture. A different type of an IgG2a(s) analogue, in which Tyr-*2′,6′-d_2* is replaced by Tyr-*2′,3′,5′,6′-d_4* was also prepared in a similar way.

Preparation of the Fv Fragment. The IgG2a(s) protein was dissolved in 100 mM Tris-HCl and 150 mM NaCl, pH 7.5, at a concentration of 10 mg/mL. Proteolytic digestion was carried out basically according to the procedure used by Tsunenaga et al. (1987) for cleavage of the "switch region" of κ-type Bence-Jones proteins. Clostripain was dissolved in a digestion buffer that contained 5 mM DTT. The enzyme to substrate ratio was 1:100 (w/w). Digestion was performed at 37 °C for 6 h. The reaction was stopped by adding iodoacetamide at a final concentration of 30 mM and incubating the reaction mixture for 20 min at 4 °C in the dark. The reaction mixture was then extensively dialyzed against a Mono Q buffer (20 mM Tris-HCl, pH 8.0) that was subsequently used for the purification of the digestion products. The dialyzate was charged onto a Mono Q HPLC column, and the pass-through fraction was collected. The digestion product thus collected was charged onto a Mono S HPLC column equilibrated with 50 mM sodium acetate at pH 5.0. The concentration of NaCl was increased by a gradient elution to 500 mM in 20 min. The major fraction was collected, and the purity of the Fv fragment obtained was checked by SDS–polyacrylamide gel electrophoresis (see Figure 1). The yield of the Fv fragment from 45 mg of IgG2a(s), which is typically obtained from a 1-L culture of hybridoma cells, was 8 mg. It has been confirmed that the Fv preparation obtained in the present work is sufficiently stable at 30 °C during a long-term signal accumulation for two-dimensional NMR measurements. Selectively deuterated Fv analogues were prepared in the same way.

Recombination of the Light and Heavy Chains of the Fv Fragment. The two kinds of deuterated analogues of the IgG2a(s) antibody prepared as described above by using Tyr-*2′,6′-d_2* and Tyr-*2′,3′,5′,6′-d_4* are designated [Tyr-d_2]IgG and [Tyr-d_4]IgG, respectively. The heavy (light) chain isolated from [Tyr-d_2]IgG was recombined with the light (heavy) chain obtained from [Tyr-d_4]IgG. Recombination was performed basically according to the procedure described by Björk and Tanford (1971a–c). [Tyr-d_2]IgG was reduced by 10 mM DTT at room temperature for 1 h in 1.5 M Tris-HCl, pH 8.5, containing 2 mM EDTA and then alkylated by adding 22 mM iodoacetic acid. The reaction mixture was dialyzed against 10 mM phosphate buffer, pH 7.2, containing 150 mM NaCl. The reduced and alkylated [Tyr-d_2]IgG was denatured by dialyzing it against 6 M guanidinium chloride, pH 7.0. Separation of the heavy and light chains was performed on a Pharmacia Superose 12 HPLC column equilibrated with 6 M guanidinium chloride, pH 7.0. The heavy and light chains were isolated from [Tyr-d_4]IgG in the same way. For recombination, the 6 M guanidinium chloride solution of the heavy (light) chain obtained from [Tyr-d_2]IgG was combined with that of the light (heavy) chain obtained from [Tyr-d_4]IgG, and the combined solution was dialyzed against 5 mM acetate

[2] It was observed that the C2′ position of the imidazole ring of the His residues of the labeled proteins is always protonated even if His-*2′,4′-d_2* is used. Presumably proton–deuterium exchange had occurred at the C2′ position during the incubation of the hybridoma cells in the synthetic medium.

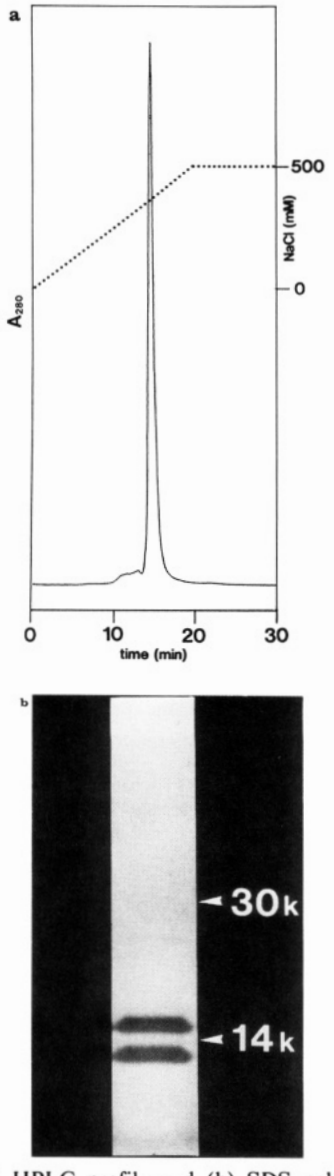


FIGURE 1: (a) HPLC profile and (b) SDS–polyacrylamide gel electrophoretogram of the Fv fragment used in the present study. HPLC was performed on a Mono S column equilibrated with 50 mM sodium acetate, pH 5.0. The concentration of NaCl was increased by a linear gradient to 500 mM in 20 min. The major fraction was collected. The purity of the Fv fragment thus obtained was checked by SDS–polyacrylamide gel electrophoresis (15%).

buffer, pH 5.5. During dialysis, 100 mM phosphate buffer containing 1.5 M NaCl was gradually added to the dialyzate over a period of 7–8 h, so that the final concentration was 10 mM phosphate and 150 mM NaCl, pH 7.0. The recombined preparation of the IgG2a(s) antibody was finally purified on a Pharmacia Superose 12 HPLC column equilibrated with 10 mM phosphate buffer, pH 7.2, containing 150 mM NaCl. Limited digestion of the recombined IgG2a(s) with clostripain was performed as in the case of the native antibody.

Binding Constant of the Fv Fragment. The binding constant was determined by observing the fluorescence of the dansyl residue bound to the Fv fragment. Excitation was at 335 nm and emission was observed at 500 nm. Fluorescence measurements were made at 30 °C on a Shimadzu RF-5000 spectrofluorophotometer. The dissociation constant K_d thus obtained was 1.2×10^{-8} M. The IgG2a(s) antibody is a member of a family of switch variant antibodies developed by Dangl et al. (1982). These antibodies have identical V_H, V_L, and C_L domains but different heavy-chain constant regions. Fab fragments with the V_H and V_L domains that are identical with those of the Fv fragment were prepared from an IgG2a antibody, which is another member of the switch variant family and retains the C_H1 domain intact (Kato et al., 1991). The K_d determined for the Fab fragment was 1.4×10^{-8} M.

NMR Measurements. For ^{1}H NMR measurements, the Fv solution was concentrated to a final volume of 0.4 mL with 5 mM phosphate buffer, pH 7.4, containing 0.2 M NaCl in D_2O. The final concentration of the protein was typically 0.8 mM. One-dimensional spectra were measured by using a Bruker AM-400 spectrometer operating at 400 MHz. All two-dimensional spectra were recorded at 500 MHz with a JEOL JNM-GSX 500 spectrometer. NOESY spectra were observed in the pure-phase mode (States et al., 1982), where 256 increments of 2K data points were recorded with 256 transients. The data obtained were zero-filled once along the t_1 direction to a final data matrix of 512 × 1K words. Mixing times of 80 and 150 ms were used for the measurements. The Gauss function was used for the apodization. The solvent resonance of HDO was suppressed by selective irradiation during the relaxation delay, which was taken to be 1.0 s. The probe temperature was 30 °C throughout the experiment.

RESULTS

Preparation of the Fv fragment and Its Deuterated Analogues. As Figure 1 shows, the Fv fragment prepared by clostripain digestion of the IgG2a(s) antibody is sufficiently pure as assessed by HPLC and SDS–polyacrylamide gel electrophoresis. An IgG2a(s) analogue, [Tyr-d_2]IgG, was digested by clostripain to prepare Fv fragments that are selectively deuterated with His-4′-d_1, Phe-2′,3′,4′,5′,6′-d_5, Trp-2′,4′,5′,6′,7′-d_5, and Tyr-2′,6′-d_2.[2] This type of Fv analogue will hereafter be designated [^{2}H]Fv.

One-Dimensional ^{1}H NMR Spectra of [^{2}H]Fv in the Presence of Varying Concentrations of DNS-Lys. Figure 2a shows the spectrum of [^{2}H]Fv observed in the absence of the antigen. We have also prepared a different type of Fv analogue, in which the aromatic rings of Phe, Trp, and Tyr are all perdeuterated, and compared its spectrum with that of [^{2}H]Fv (data not shown). It was thus confirmed that resonances a–e and resonances A–M originate from the His and Tyr residues of [^{2}H]Fv, respectively. In the absence of the antigen, peaks B and C and peaks F and G are observed as single peaks. The anti-dansyl Fv fragment used in the present work contains 5 His and 13 Tyr residues.[3] Thus all of the His and Tyr resonances are observed in the spectrum shown in Figure 2a, although some of the Tyr resonances are significantly broadened.

On addition of DNS-Lys, the [^{2}H]Fv spectrum changed to a great extent. As Figure 2 shows, resonances a–e and A–M decrease in intensity without changing their chemical shifts, and a number of new resonances appear instead with a concomitant gain in intensity. In the following discussion, we will focus our attention to resonances b′, D′, I′, and J′. Attention will also be paid to resonances 2′, 3′, 4′, 6′, 7′, and 8′.

At a [^{2}H]Fv to DNS-Lys molar ratio of 100:120, additional sharp resonances are clearly observed (Figure 2d). It has been

[3] Sequence data of the V_H region of the antibody used in the present study have been given previously (Dangl, 1986). Sequence data of the V_L region were kindly provided by Professor L. A. Herzenberg, Stanford University, and Dr. V. T. Oi, Becton Dickinson Immunocytometry Systems, prior to publication. The convention of Kabat et al. (1987) has been followed for the numbering of the V_H and V_L regions of the switch variant antibodies. The numbering system used for the constant regions is based on protein Ag for the light chain (Putnam, 1969) and on protein Eu for the heavy chain (Edelman et al., 1969).

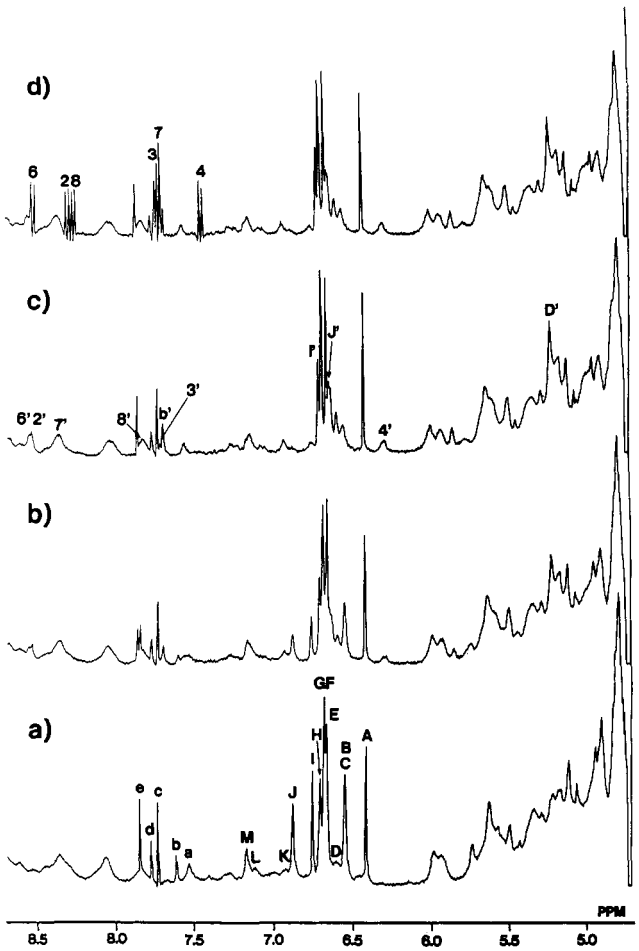
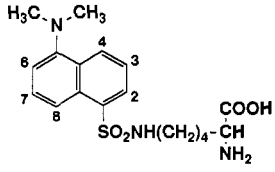


FIGURE 2: The 400-MHz ^1H NMR spectra of [^2H]Fv in the presence of varying concentrations of DNS-Lys. The molar ratios of [^2H]Fv to DNS-Lys were (a) 100:0 (no antigen), (b) 100:50, (c) 100:100, and (d) 100:150. [^2H]Fv was dissolved at a concentration of approximately 0.8 mM in 5 mM phosphate buffer containing 200 mM NaCl in D_2O, pH 7.4. The probe temperature was 30 °C. The accumulation parameters were as follows: spectral width, 5000 Hz; data points, 8K; pulse repetition time, 1.0 s; number of accumulations, 1024.

Chart I: Numbering of the Ring Protons of DNS-Lys



confirmed that these resonances originate from the ring protons of DNS-Lys in the free state and are numbered in the spectrum according to Chart I.

The above results indicate that DNS-Lys is *slowly* exchanging between the free and bound states. On the basis of NOESY data, we will show that (1) resonances 2′, 3′, 4′, 6′, 7′, and 8′ originate from protons 2, 3, 4, 6, 7, and 8 of DNS-Lys bound to the Fv fragment, respectively, and (2) resonances b′, D′, I′, and J′ are due to His and Tyr residues of the Fv–DNS-Lys complex. In the following, resonances originating from the Fv–DNS-Lys complex will be referred to by adding a prime to the letters and numbers that are used for the free [^2H]Fv and DNS-Lys, respectively. The letters b, D, I, and J will also be used to refer to the corresponding His and Tyr residues themselves.

NOESY Spectra of [^2H]Fv Observed in the Presence of Varying Concentrations of DNS-Lys. Figure 3a shows a NOESY spectrum of [^2H]Fv measured in the absence of the antigen. The NOESY spectrum changed to a great extent with an increasing concentration of DNS-Lys. Panels b, c, and d of Figure 3 show the NOESY spectra of [^2H]Fv observed in the presence of the antigen with [^2H]Fv to DNS-Lys molar ratios of 100:50, 100:100, and 100:120, respectively. A number of cross-peaks are observed in Figure 3a–d at different positions. As discussed below, these cross-peaks are due to NOE correlation and chemical exchange.

Identification of His and Tyr Residues Originating from the Fv–DNS-Lys Complex. Figure 3b shows a NOESY spectrum observed at a [^2H]Fv to DNS-Lys molar ratio of 100:50. As panels a and c of Figure 3 show, six cross-peaks labeled in Figure 3b with Ex1, Ex2, Ex3, Ex4, Ex5, and Ex6 disappear on increasing or decreasing the concentration of DNS-Lys. Comparisons of the spectra shown in Figure 2 and Figure 3b indicate that Ex1, Ex2, Ex3, and Ex4 connect b and b′, J and J′, I and I′, and D and D′, respectively. Under the experimental conditions used for the measurement of the NOESY spectrum shown in Figure 3b, the antigen-combining site of [^2H]Fv is on average half-filled with the antigen. We therefore conclude that (1) Ex1–Ex6 are exchange cross-peaks originating from [^2H]Fv and (2) resonances b′, D′, I′, and J′ are due to His b, Tyr D, Tyr I, and Tyr J of the Fv–DNS-Lys complex, respectively. Ex5 and Ex6 are exchange cross-peaks of unidentified origin. All other His and Tyr residues of the Fv fragment do not apparently give exchange cross-peaks. It is possible that chemical shift differences in the absence and presence of the antigen are small for these residues.

Tyr D shows a large upfield shift of 1.38 ppm, giving resonance D′ in the presence of the antigen. As Figure 4c shows, resonance D′ is not observable when the aromatic rings of Phe, Trp, and Tyr are all perdeuterated. It has thus been confirmed that resonance D′ is actually due to the C3′,5′-H protons of Tyr D.

Identification of the DNS-Lys Resonances in the Bound State. At a [^2H]Fv to DNS-Lys molar ratio of 100:120, additional cross-peaks Ex(2), Ex(4), Ex(7), and Ex(8) are observed (Figure 3d). It should be noted that these cross-peaks are observable only when an excess amount of the antigen exists in solution. A comparison of Figure 2 and Figure 3d clearly indicates that Ex(2), Ex(4), Ex(7), and Ex(8) are exchange cross-peaks between 2 and 2′, 4 and 4′, 7 and 7′, and 8 and 8′, respectively. On the basis of the spectral assignment given for the ring protons of DNS-Lys in the free state (Figure 2d), we assign resonances 2′, 4′, 7′, and 8′ to protons 2, 4, 7, and 8 of DNS-Lys in the bound state, respectively. In the NOESY spectrum shown in Figure 3d, protons 3 and 6 of DNS-Lys do not apparently give any detectable exchange cross-peaks. This is presumably because chemical shifts for protons 3 and 6 are virtually identical for the free and bound states (see Figure 2).

Six additional cross-peaks are observed both in panel c and panel d of Figure 3. The spectral data given in Figures 2 and 3c,d clearly indicate that all of these cross-peaks originate from the ring protons of DNS-Lys in the bound state. These cross-peaks are labeled accordingly as 2′–3′, 2′–4′, 3′–4′, 6′–7′, 6′–8′, and 7′–8′ (Figure 3c). It has thus been confirmed that resonances 3′ and 6′ (Figure 2c) are due to protons 3 and 6 of DNS-Lys in the bound state, respectively. Existence of cross-peaks other than 2′–4′ and 6′–8′ may be attributed to an intramolecular NOE. It is possible that cross-peaks 2′–4′ and 6′–8′ arise as a result of spin diffusion.

NOE Cross-Peaks between [^2H]Fv and DNS-Lys in the Fv–DNS-Lys Complex. It is observed that the Fv–DNS-Lys

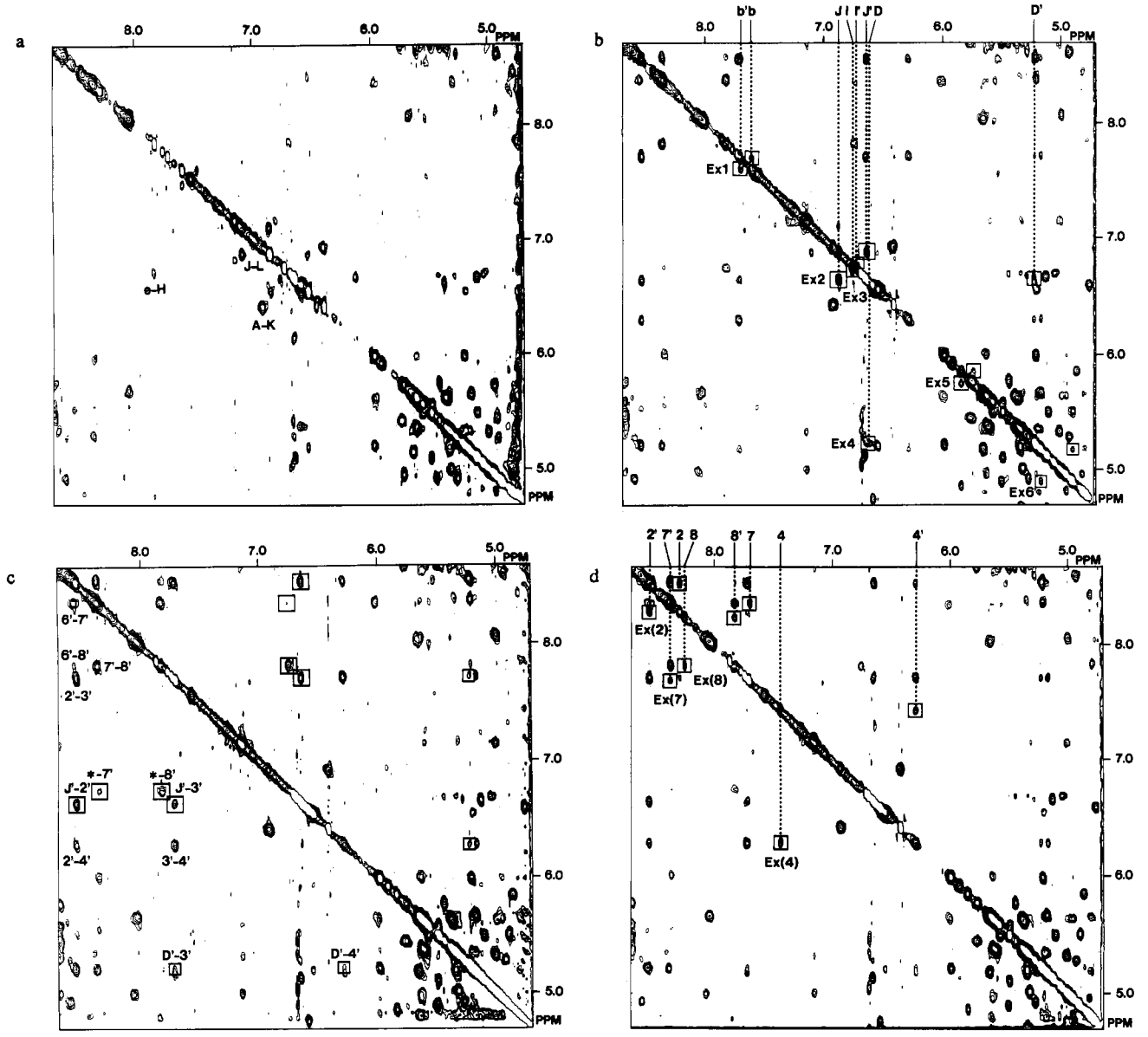


FIGURE 3: The 500-MHz NOESY spectra of [^{2}H]Fv measured in the absence and presence of the antigen. A mixing time of 150 ms was used for the measurements. Details of the two-dimensional measurements are described under Materials and Methods. Other experimental conditions are as in Figure 2. The [^{2}H]Fv to DNS-Lys molar ratios were (a) 100:0 (no antigen), (b) 100:50, (c) 100:100, and (d) 100:120. (a) Intramolecular NOE peaks observed between Tyr A and Tyr K, Tyr J and Tyr L, and His e and Tyr H are labeled as A–K, J–L, and e–H, respectively. (b) Six exchange cross-peaks observed are identified by squares labeled with Ex1–Ex6. (c) NOE cross-peaks observed between Tyr and DNS-Lys in the Fv–DNS-Lys complex are shown by squares labeled with D′–3′, D′–4′, *–7′, *–8′, J′–2′, and J′–3′. Intramolecular NOE cross-peaks for DNS-Lys in the bound state are simply labeled as 2′–3′, which represents the number of protons on the DNS ring. (d) Ex(2)–Ex(8) are exchange cross-peaks observed for ring protons 2, 4, 7, and 8 of DNS-Lys in the free and bound states.

complex gives intramolecular NOE cross-peaks, which are identified in Figure 3c as D′–3′, D′–4′, J′–2′, J′–3′, *–7′, and *–8′. Use of a shorter mixing time of 80 ms gave virtually the identical two-dimensional NOE data (data not shown). It has been shown that resonances 2′, 3′, 4′, 7′, and 8′ originate from protons 2, 3, 4, 7, and 8 of DNS-Lys in the bound state, respectively. D′ and J′ have already been assigned to Tyr D and Tyr J in the Fv–DNS-Lys complex. A signal marked with an asterisk is most clearly observed in Figure 4. Figure 4c shows that this signal exists even when the aromatic rings of Phe, Trp, and Tyr are perdeuterated. It is therefore quite likely that this signal is due to one of the amide protons of the Fv fragment that persists in the D_2O solution. It has also been confirmed by a one-dimensional NOE experiment that irradiation of proton 8 of DNS-Lys in the bound state actually gives rise to the NOE enhancement for this amide proton (see Figure 4d).

Assignments of Tyr Resonances to either the Light Chain or the Heavy Chain. For this purpose, we have basically followed the procedure described by Anglister et al. (1985). Two kinds of IgG2a(s) analogues, in which the Tyr ring of either the heavy chain or the light chain is perdeuterated, were prepared by recombination experiments using [Tyr-d_2]IgG and [Tyr-d_4]IgG. The recombined IgG2a(s) analogues thus obtained were subjected to clostripain digestion. ^{1}H NMR spectra of an Fv analogue, in which the aromatic rings of His, Trp, and Tyr of the light chain are perdeuterated, were measured in the absence and presence of the antigen (see Figure 4). This result indicates that resonances D (D′) and J (J′) originate from the heavy chain.

Discussion

Preparation and Properties of Fv Fragments. Since Inbar et al. (1972) first reported the preparation of the Fv fragment

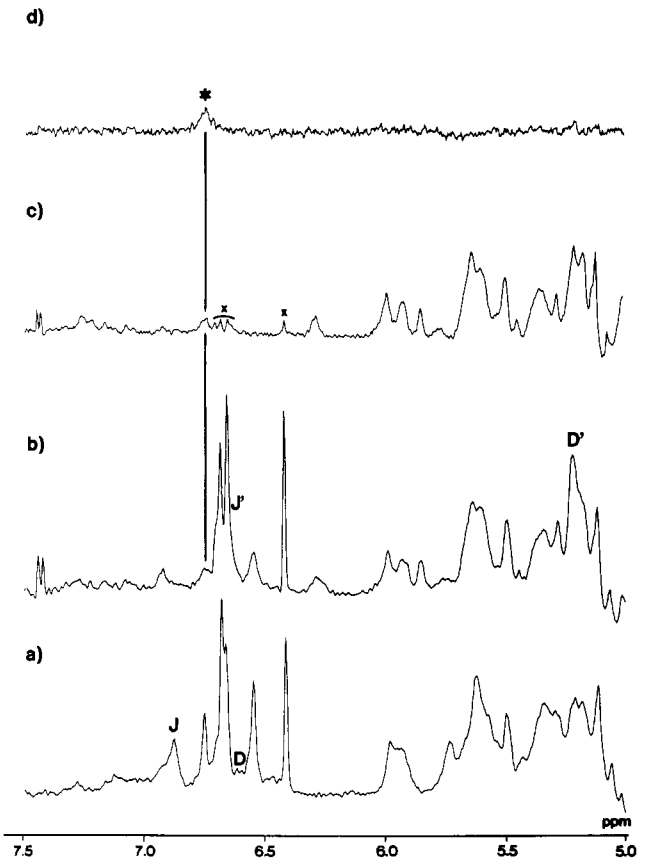


FIGURE 4: The 400-MHz ^{1}H NMR spectra of a recombined Fv analogue in which Phe and Trp are perdeuterated and Tyr residues of the light and heavy chains are labeled with Tyr-*2′,3′,5′,6′-d*$_4$ and Tyr-*2′,6′-d*$_2$, respectively. Measurements were made (a) in the absence of DNS-Lys and (b) in the presence of an excess amount of DNS-Lys. (c) The 400-MHz ^{1}H NMR spectrum of an Fv analogue in which the aromatic rings of His, Trp, and Tyr have been perdeuterated. This spectrum was measured in the presence of an excess amount of DNS-Lys. Small resonances marked with an x originate from residual protons at positions 3′ and 5′ of Tyr-*2′,3′,5′,6′-d*$_4$, which was used for the culture of hybridoma cells. It should be noted that this analogue does not give resonance D′ observed in (b). (d) NOE difference spectrum observed by irradiating proton 8 of the DNS ring. Spectral conditions are as in Figure 2.

of a mouse myeloma IgA(λ_2) protein MOPC 315, many attempts have so far been made without success to prepare Fv fragments by proteolysis of other IgG antibodies. In the present work, we have shown that the IgG2(s) antibody, in which the entire C_H1 domain is deleted (Igarashi et al., 1990), can be cleaved by clostripain in a selective way, resulting in high yields of the corresponding Fv fragment. It has been confirmed that the Fv fragment thus obtained retains its full antigen-binding activity.

In the IgG2a(s) antibody, the V_H domain is directly connected to the hinge region (Igarashi et al., 1990), which is in general highly susceptible to proteolytic digestion. As shown by Tsunenaga et al. (1987), the human κ-type light chain can selectively be cleaved by clostripain at the C-terminal side of the Arg residue in the switch region. The IgG2a(s) antibody possesses Arg at position 218 in the hinge region and at position 108 in the switch region of the light chain (κ-type).[3] These are probably the reason that the IgG2a(s) antibody has been selectively cleaved, giving the Fv fragment in high yield.

The results of the present experiment are reminiscent of interesting work reported by Sharon and Givol (1976), who have shown that the Fv fragment can be obtained by limited digestion of a recombinant protein composed of V_L and the heavy chain. They also suggested a possibility of using another type of recombinant protein, which is composed of V_H and the light chain. Although this method is extremely attractive, we would in practice have to overcome difficulties in fragmentation for the production of sufficient quantities of Fv fragments. The difficulties have most likely arisen from the use of papain for digestion of the recombinant protein. In view of the results of the present experiments, it is quite likely that digestion with clostripain rather than with papain of a recombinant protein, which is composed of V_H and the intact light chain, can provide us with a general method of preparing Fv fragments. We would obviously need a clostripain cleavage site in the switch region of the light chain. It should be noted that most mouse light chains are of κ-type, which possess Arg at position 108 in the switch region.

Fv fragments have become available by gene technology (Riechmann et al., 1988; Skerra & Plückthun, 1988; Ward et al., 1989). Glockshuber et al. (1990b) have reported that the recombinant Fv fragment of mouse myeloma IgA McPC603, which was expressed and secreted by *E. coli*, is extremely unstable at low protein concentration with a half-life of denaturation of 1.3 h at 37 °C. By contrast, the Fv fragment used in the present work is fully stable at 30 °C at a concentration of 1 mM, and virtually no deterioration in quality of the protein was experienced during a long-term data accumulation for two-dimensional NMR measurements. It appears that the stability of Fv fragments is sensitively affected by the difference in the structure of the antigen-combining site.

Interaction between Fv and DNS-Lys. As Figure 3c shows, Tyr D and Tyr J of the Fv–DNS-Lys complex give NOE cross-peaks with protons 3 and 4 and protons 2 and 3 of the DNS ring, respectively. The pH titration experiments have shown that the chemical shift for resonance D′ is pH dependent with a pK_a of 6.3, which is identical with that observed for His b (data not shown). The other four His residues gave pK_a values that are lower than 6. This suggests that His b exists in close spatial proximity to Tyr D. In addition, protons 7 and 8 of the DNS ring of the Fv–DNS-Lys complex give rise to an NOE with an amide proton of the Fv fragment (see Figures 3c and 4). This amide proton is resistant to proton–deuterium exchange upon prolonged incubation of the Fv fragment in D_2O, suggesting that the corresponding amide group is shielded from the solvent and is involved in the network of hydrogen bonding.

Figure 3b shows that the chemical shifts of the resonances originating from His b, Tyr D, and Tyr J are significantly affected by the addition of the antigen. It should particularly be noted that the Tyr D resonance is shifted upfield by 1.38 ppm. As Figure 3d shows, proton 4 of the DNS ring exhibits a large upfield shift of 1.15 ppm upon binding with the Fv fragment. As the NOE data show, proton 4 of the DNS ring is in close spatial proximity to the Tyr D ring. It is therefore quite likely that the large chemical shifts observed upon complex formation have been induced by the mutual ring current of the aromatic rings of Tyr D and DNS. In view of the fact that both the Tyr D and DNS protons show high-field shift, it is quite likely that in the Fv–DNS-Lys complex the aromatic rings of Tyr D and DNS are stacking with each other. On the basis of these results, we suggest that Tyr D, Tyr J, His b, and the amide group form a hydrophobic environment interacting with the antigen.

It is known that the antigen-combining site of the antibody molecule is constructed by three CDR loops from the heavy chain and three CDR loops from the light chain. In the

anti-dansyl Fv fragment used in the present study, seven Tyr residues exist in the CDR loops, i.e., Tyr 58 and Tyr 59 in CDR 2 of the heavy chain, Tyr 96, Tyr 97, Tyr 99, and Tyr 104 in CDR 3 of the heavy chain, and Tyr 32 in CDR 1 of the light chain.[3] ^{13}C NMR spectra were measured with ^{13}C Fv analogues in which the carbonyl carbon of the Tyr or Trp residue is selectively labeled with [1-^{13}C]Tyr or [1-^{13}C]Trp. It was observed that the chemical shifts for the carbonyl carbon resonances of Tyr 97, Tyr 99, Tyr 104, Trp 101, and Trp 105 are selectively affected to a large extent by the addition of the antigen (manuscript in preparation). This result indicates that, in the case of the anti-dansyl Fv fragment used in the present work, CDR 3 of the heavy chain is primarily responsible for the antigen binding. On the basis of the recombination experiment, we have shown that resonances D and J originate from the heavy chain. We therefore suggest that resonances D and J originate from the CDR 3 loop of the heavy chain. In order to assess a contribution to the observed chemical shift from other amino acid residues in the antigen-combining site, similar NOESY experiments using different types of deuterated Fv analogues are under way.

Structure of Fv in the Absence and Presence of DNS-Lys. Figure 3a shows that three NOE cross-peaks, His e–Tyr H, Tyr A–Tyr K, and Tyr J–Tyr L, are observed in the absence of the antigen. It should be noted that upon antigen binding the His e–Tyr H and Tyr J–Tyr L cross-peaks disappear and only the Tyr A–Tyr K cross-peak remains to be observed (see Figure 3c). It should also be noted that the line width for Tyr D, which is broad in the absence of the antigen, becomes much sharper in the presence of the antigen (see Figure 2). It appears that the structure of the Fv fragment is significantly affected by the antigen binding.

Anglister and Zilber (1990) have used two-dimensional transferred NOE difference spectroscopy for a ^1H NMR study of antigen–antibody interactions in an antipeptide monoclonal antibody. In their NMR analyses, both the antigen and the Fab fragment of the antipeptide antibody had been selectively deuterated, and resonances originating from the side chains of amino acid residues in the antigen–antibody complex had been extracted by taking the difference between the spectra of the antigen–antibody complex measured in the absence and presence of an excess amount of the antigen. On the basis of the difference spectral data, they discussed how the antigen is interacting with the antigen-combining site. In their experiment, however, the Fab fragment was always saturated with the antigen in order to make interpretation of the two-dimensional spectral data for the protein with a molecular weight of 50000 less ambiguous. Therefore, it was not possible to compare the conformation of the antigen-combining site in the absence and presence of the antigen.

Concluding Remarks. In the present study, we have shown that use of the selectively deuterated Fv fragment, which is half the size of the Fab fragment, makes it possible to collect ^1H NMR spectral data at a resolution sufficient for the structural analysis of the antigen-combining site in the absence and presence of the antigen. For a better understanding of antigen recognition by the Fv fragment, we will have to use as many *assigned* spectroscopic probes as possible. Multinuclear NMR analyses using a variety of ^{13}C- and ^{15}N-labeled Fv analogues are in progress in our laboratory.

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Registry No. DNS-Lys, 28217-24-5; Tyr, 60-18-4.

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Oligomeric Domain Structure of Human Complement Factor H by X-ray and Neutron Solution Scattering†

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ABSTRACT: Factor H is a regulatory component of the complement system. It has a monomer M_r of 150000. Primary structure analysis shows that the polypeptide is divided into 20 homologous regions, each 60 amino acid residues long. These are independently folding domains and are termed "short consensus repeats" (SCRs) or "complement control protein" (CCP) repeats. High-flux synchrotron X-ray and neutron scattering studies were performed in order to define its solution structure in conditions close to physiological. The M_r of factor H was determined as 250000–320000 to show that factor H is dimeric. This structure is maintained at concentrations between 1 and 11 mg/mL in the pH range 5–9. Zn^{2+} ions are an inhibitor of C3b cleavage by factor I, a reaction in which factor H acts as a cofactor. Additions of Zn^{2+} to factor H caused it to form oligomers containing 4–10 monomers. The radius of gyration R_G of native factor H by X-rays or by neutrons in 0% or 100% ^2H_2O buffers is not measurable but is greater than 12.5 nm. Two cross-sectional radii of gyration $R_{XS\text{-}1}$ and $R_{XS\text{-}2}$ were determined as 3.0–3.1 and 1.8 nm, respectively. Analyses of the cross-sectional intensities show that factor H is composed of two distinct subunits. The $R_{XS\text{-}1}$ corresponds to the cross-sectional properties of both subunits and exhibits an unusual radiation dependence on the X-ray flux. Since $R_{XS\text{-}2}$ is close to the corresponding R_{XS} of C4b binding protein (91% of which is formed from SCR/CCP domains), it is inferred that the SCR/CCP domains of factor H and C4b binding protein have similar solution structures. The use of hydrodynamic spheres to reproduce literature sedimentation coefficients of 5.5–5.6 S showed that these were compatible with a V-shaped arrangement of two rods (36 spheres each, length 87 ± 5 nm) joined at an angle of 5°. The use of a similar arrangement of 244 spheres arranged in two rods (length 77 nm) to fit the experimental X-ray and neutron scattering curves showed that the two rods are joined at an angle of 5°. This model corresponds to an actual R_G of 21–23 nm. The separation between each SCR/CCP in factor H is close to 4 nm. In the solution structure of factor H, the SCR/CCP domains are in a highly extended conformation.

In the alternative pathway of the complement cascade of immune defense, factor H (0.2–0.6 mg/mL in plasma) is a regulatory component that serves as the cofactor for the factor I mediated cleavage of component C3b into iC3b. Factor H also accelerates the decay of the C3 convertases of the alternative pathway and inhibits their formation. Both roles are mediated by the noncovalent binding of factor H to free or surface-bound C3b or to C3b in its binary complex with factor Bb and ternary complex with Bb and properdin. The interaction between factor H and C3b therefore plays a central role in complement activation and control. Analogous roles in the complement cascade are performed on C3b or its homologue C4b by the membrane-bound complement proteins CR1 (complement receptor type 1), decay accelerating factor, and membrane cofactor protein, and by the soluble protein C4BP (C4b binding protein) [see Reid et al. (1986), Sim et al. (1987), and Reid and Day (1989) for recent reviews].

The most frequently occurring structural domain found in complement components is termed the short consensus repeat and occasionally as the short complement repeat (SCR); it is also known as the complement control protein (CCP) repeat (Sim & Perkins, 1989). Sequences for mouse and human factor H show that both are entirely formed from 20 SCR/CCPs (Kristensen & Tack, 1986; Ripoche et al., 1988). The C3b binding site of factor H and the cofactor activity for factor I both reside within the N-terminal (M_r 38000) tryptic fragment of factor H (Alsenz et al., 1985), which arises from a single proteolytic cleavage within the sixth SCR/CCP (Ripoche et al., 1988). A truncated form of factor H containing only the first seven SCR/CCPs also has cofactor activity for factor I (Ripoche et al., 1988; Misasi et al., 1989). The N-terminal five SCR/CCPs in factor H thus constitute a functionally active region.

Knowledge of the physical structures of both C3b and factor H is required to understand their roles. For C3, evidence from sedimentation data, electron microscopy, and neutron and

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