# Dynamical Structure of the Antibody Combining Site As Studied by $^1H$–$^{15}N$ Shift Correlation NMR Spectroscopy†

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ABSTRACT: The Fv fragment, which is a smallest antigen-binding unit of immunoglobulin, has been used for a $^1H$–$^{15}N$ shift correlation NMR study of the dynamical structure of the antibody combining site. Fv has been prepared by clostripain digestion of a mouse anti-dansyl IgG2a monoclonal antibody that lacks the entire $C_H1$ domain. We have previously reported that of the six hypervariable regions, three each from the heavy chain (H1, H2, and H3) and the light chain (L1, L2, and L3), H3 is primarily responsible for the antigen binding in the anti-dansyl Fv fragment. The backbone amide nitrogens of all non-proline amino acid residues in H3 have been *multiply* labeled with $^{15}N$. [$^{15}N$]$T_2$ relaxation times and hydrogen–deuterium exchange rates of the amide groups of the main chain were measured in the absence and presence of ε-dansyl-L-lysine (DNS-Lys). It has been shown that (1) in the absence of DNS-Lys H3 displays a significant degree of internal motion and (2) antigen binding induces a significant change in the dynamical structure of H3.

Immunoglobulin G (IgG),[1] which is a most important member of the group of glycoproteins 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 $C_L$. It is known that the antibody combining site is formed by using six hypervariable regions, three each from the $V_H$ domain (H1, H2, and H3) and the $V_L$ domain (L1, L2, and L3).

The Fv fragment, which is a heterodimer of $V_H$ and $V_L$ domains, is the smallest antigen-binding unit with a molecular weight of 25 000 (Inbar et al., 1972). Preparation of Fv by proteolytic cleavage of the intact IgG protein has been attempted with little success (Inbar et al., 1972; Hochman et al., 1973; Sharon & Givol, 1976). In previous papers, we have shown that Fv can be prepared in high yield by limited proteolysis with clostripain of a short-chain mouse IgG2a anti-dansyl antibody in which the entire $C_H1$ domain is deleted (Igarashi et al., 1990; Takahashi et al., 1991a). A variety of anti-dansyl Fv analogues labeled with $^2H$ in the aromatic rings and with $^{13}C$ and/or $^{15}N$ in the peptide bonds have been prepared and used for multinuclear NMR analyses of Fv in the absence and presence of ε-dansyl-L-lysine (DNS-Lys). On the basis of the multinuclear NMR data we have concluded that H3 is primarily responsible for the antigen binding (Takahashi et al., 1991a,b; Kato et al., 1991b).

Fv fragments have recently been expressed by using *Escherichia coli* (Skerra & Plückthun, 1988; Huston et al., 1988; Ward et al., 1989; Boulot et al., 1990; McManus & Riechmann, 1991; Riechmann et al., 1991) as well as myeloma cells (Riechmann et al., 1988). Wright et al. (1990) have outlined a general strategy for spectral assignments using an anti-lysozyme Fv fragment expressed by myeloma cells. An anti-2-phenyloxazolone Fv expressed by *E. coli* has been uniformly labeled with $^{13}C$ and $^{15}N$ for multinuclear NMR spectroscopy, and the possibility for detailed spectral assignments has been briefly discussed (Riechmann et al., 1991).

The structure of the antibody combining site has been studied on the basis of the X-ray crystallographic data (Mariuzza et al., 1987; Colman, 1988; Davies et al., 1990). However, very little is known about the dynamical structure of the antibody combining site in solution. In the present study, attention will be paid to the dynamical structure of H3 that is primarily responsible for the antigen binding of the anti-dansyl Fv fragment. For this purpose all amide nitrogens of H3 were *multiply* labeled with $^{15}N$. $^1H$–$^{15}N$ shift correlation data on [$^{15}N$]$T_2$ relaxation times and the hydrogen–deuterium exchange rate of the amide protons will be used to discuss the dynamical structure of H3 of the Fv fragment in solution in the absence and presence of DNS-Lys.

## Materials and Methods

*Materials.* L-[$^{15}N$]Tyr was purchased from Isocommerz GmbH, Germany. All other $^{13}C$- and $^{15}N$-labeled amino acids were purchased from ICON Services Inc. The isotope enrichment is 95% or higher for each of the amino acids. Clostripain and ε-dansyl-L-lysine (DNS-Lys) were obtained from Sigma Chemical Co. 4-(Dansylamino)-2,2,6,6-tetramethylpiperidine *N*-oxide was synthesized according to the procedure of Chiniak and Polonski (1973). All other chemicals were of reagent grade and were used without further purification.

*Preparation of Stable-Isotope-Labeled Analogues of the Fv Fragment of the Anti-Dansyl Mouse IgG2a Antibody.* The mouse hybridoma cell line 1B10.7, which produces the short-chain mouse IgG2a monoclonal antibody (Dangl et al., 1982; Igarashi et al., 1990), was adopted to a serum-free

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[1] Abbreviations: CPMG, Carr–Purcell–Meiboom–Gill; DEPT, distortionless enhancement by polarization transfer; H1, H2, and H3, hypervariable region in the $V_H$ domain; L1, L2, and L3, hypervariable region in the $V_L$ domain; DNS-Lys, ε-dansyl-L-lysine; Fv, antigen-binding fragment composed of $V_H$ and $V_L$; HSQC, heteronuclear single-quantum correlation; IgG, immunoglobulin G; NMR, nuclear magnetic resonance; $T_2$, spin–spin relaxation time; TPPI, time-proportional phase incrementation; $V_H$, the variable domain of the heavy chain; $V_L$, the variable domain of the light chain.

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medium (Nissui NYSF 404) and then grown in the medium containing stable-isotope-labeled amino acid(s) (Kato et al., 1989, 1991a). All isotope-labeled analogues of Fv were prepared by clostripain digestion of the short-chain antibody as described previously (Takahashi et al., 1991a). Recombination of the light and heavy chains of the Fv fragment was performed according to the procedure described previously (Kato et al., 1991a; Takahashi et al., 1991a).

*NMR Measurements.* All $^1H$–$^{15}N$ shift correlation spectra presented were recorded with spectral widths of 5600 Hz for $^1H$ and 1260 Hz for $^{15}N$ on a Bruker AM 400 spectrometer equipped with a 5-W BFX-5 X-nuclear decoupler. The pulse sequence for HSQC spectra was as described by Bodenhausen and Ruben (1980). The pulse sequence for the [$^{15}N$]$T_2$ filtering experiment was as described by Kay et al. (1989). 1K data points were used in the $t_2$ dimension, and 96–144 transients were acquired for each of 390 $t_1$ points. $F_1$ quadrature was achieved by TPPI (Marion & Wüthrich, 1983). Prior to 2D Fourier transformation, the acquired data were multiplied by a gauss function in $t_2$ and by a shifted sine-square function in $t_1$ and zero-filled to yield a matrix 512 ($F_2$) × 256 ($F_1$) of the real data points. For hydrogen–deuterium exchange experiments, 32 transients were acquired for each of 128 $t_1$ points. The obtained data matrix was zero-filled once along the $t_1$ direction to a final data matrix of 512 ($F_2$) × 128 ($F_1$). The solvent resonance was suppressed by selective, weak irradiation during the preparation period of 1.2 s and the spin–lock purge pulse (Otting & Wüthrich, 1988). The probe temperature was 30 °C throughout the experiments.

For $^{15}N$ DEPT measurements (Doddrell et al., 1982), the free induction decays were recorded with 8K data points and spectral widths of 2000 and 3000 Hz on Bruker AM 400 and AMX 600 spectrometers, respectively. The interpulse delay for DEPT was set to a shorter time of 4.5 ms, as optimized for sensitivity enhancement by taking short proton $T_2$ relaxation times for the Fv fragment into consideration. An exponential window function with a broadening factor of 4 Hz was used for sensitivity enhancement.

All NMR spectra were measured by using protein solutions concentrated by ultrafiltration to 0.4 mL in 5 mM phosphate buffer, pH 5.0, containing 0.2 M NaCl. The samples were prepared in $H_2O$ that contains 10% $D_2O$. Hydrogen–deuterium exchange of amide protons was followed by observing $^1H$–$^{15}N$ HSQC spectra. A 1 mM protein sample solution was prepared at 4 °C as described above, except that the buffer was prepared with 99.9% $D_2O$.

## Results and Discussion

H3 of the anti-dansyl antibody under investigation corresponds to the underlined part of the peptide sequence -$^{95H}$Ile-Tyr-Tyr-His-Tyr-Pro-Trp-Phe-Ala-Tyr-$^{105H}$Trp-.[2,3] The $^{95H}$Ile-$^{105H}$Trp segment will hereafter be referred to as the H3 segment. In order to obtain structural information about H3, the amide nitrogens of all non-proline residues in the H3 segment, i.e., Ile, Tyr, His, Trp, Phe, and Ala, were *multiply* labeled with $^{15}N$. This has resulted in $^{15}N$ labeling of 53 out of 227 non-proline residues in the Fv fragment. With the multiply labeled Fv analogue, it has become possible to determine the [$^{15}N$]$T_2$ and hydrogen–deuterium exchange rate for the H3 segment under the identical condition. Vide infra.

Table I: [$^{15}N$]$T_2$ Relaxation Times Observed for the H3 Segment in the Absence and Presence of DNS-Lys

| residue | [$^{15}N$]$T_2$ (ms)[a] | |
|---|---|---|
| | –DNS-Lys | +DNS-Lys |
| Ile-95H | 15 (8) | 50 (5) |
| Tyr-96H | 15 (5)[b] | 45 (8)[c] |
| Tyr-97H | 20 (8) | 40 (8) |
| His-98H | 20 (8) | <5 |
| Tyr-99H | 40 (8) | 45 (8)[c] |
| Trp-101H | 30 (10) | 30 (8) |
| Phe-102H | 30 (8) | <5 |
| Tyr-104H | 15 (5)[b] | 45 (10) |
| Trp-105H | 40 (10) | 45 (8) |

[a] Standard deviations are given in parentheses. [b] Resonances are partially overlapped. [c] Resonances are partially overlapped.

Figure 1a shows a $^1H$–$^{15}N$ HSQC spectrum of the multiply labeled Fv fragment observed in the absence of DNS-Lys. Each $^1H$/$^{15}N$ cross peak was assigned to a known amino acid type by using Fv analogues singly labeled with one of the six amino acids used for the multiple labeling. It has shown that all $^1H$/$^{15}N$ cross peaks except for 1 of 11 Ala peaks are observable in the HSQC spectrum. A double-labeling method along with a recombination technique described previously (Kainosho & Tsuji, 1982; Takahashi et al., 1991b; Kato et al., 1989, 1991a,b) was used for spectral assignments. It was also possible to identify the dipeptide bond between two $^{15}N$-labeled amino acid residues by establishing sequential $d_{\alpha N}$ and/or $d_{NN}$ connectivities in $^{15}N$-edited NOESY spectra. So far 37 cross peaks observed in the absence of DNS-Lys have been assigned. The corresponding 37 cross peaks observed in the presence of DNS-Lys have also been assigned. The assignments that have been established are indicated in the figure. The assigned resonances include those for all the H3 segment residues except for Pro-100H and Ala-103H.[3] Details of the spectral assignments will be published elsewhere.

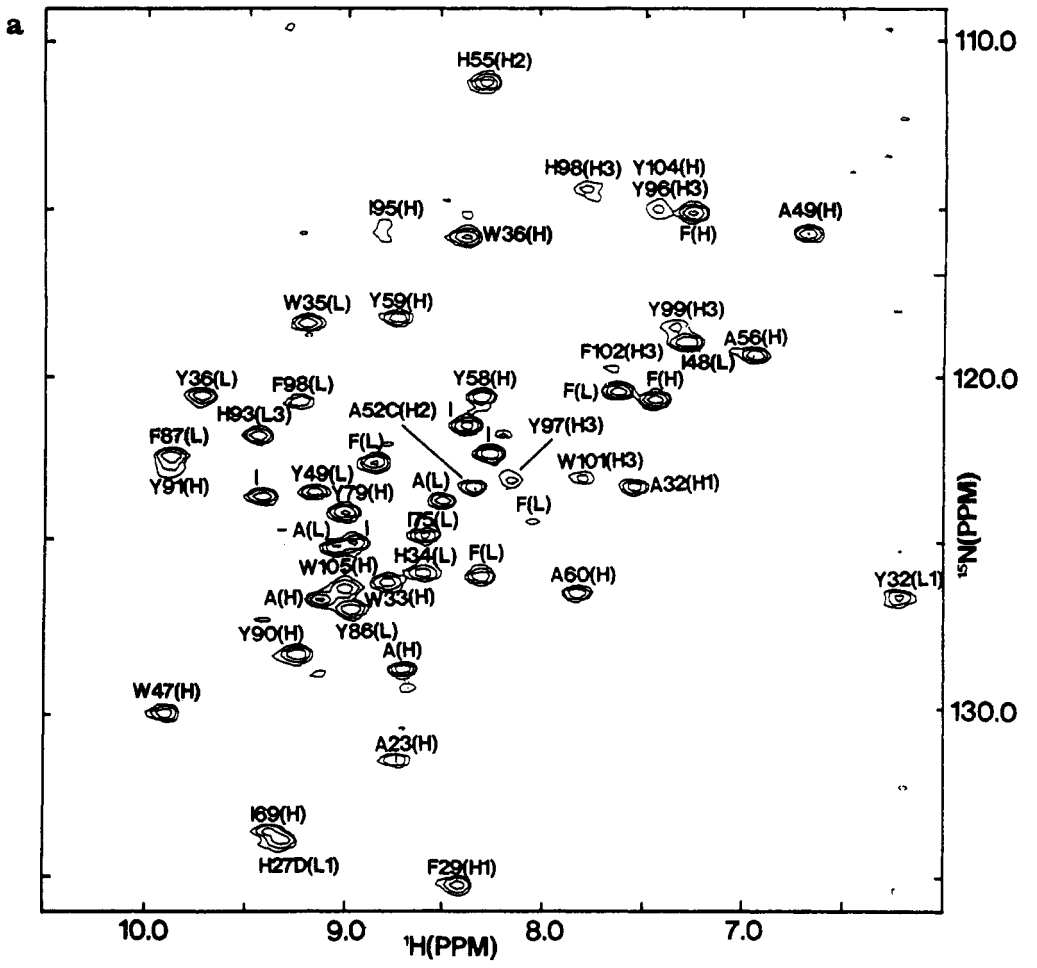
The HSQC spectrum of the multiply labeled Fv fragment was measured in the presence of a spin-labeled hapten, 4-(dansylamino)-2,2,6,6-tetramethylpiperidine *N*-oxide (Dower & Dwek, 1979, and references cited therein; Anglister, 1990, and references cited therein) (data not shown). The result obtained was consistent with our previous conclusion that the H3 segment is primarily responsible for the antigen binding (Takahashi et al., 1991a,b; Kato et al., 1991b).

[$^{15}N$]$T_2$ filtering experiments were performed in the absence and presence of DNS-Lys. In the absence of DNS-Lys, with a 25-ms delay time in the CPMG sequence, the cross peaks originating from Ile-95H, Tyr-96H, Tyr-97H, His-98H, Trp-101H, Phe-102H, and Tyr-104H disappeared (Figure 1b), indicating that these residues possess $T_2$ relaxation times which are significantly shorter than the rest of the residues examined. It should be noted that all of these residues with short $T_2$ relaxation times originate from the H3 segment. The [$^{15}N$]$T_2$ relaxation times were determined by changing the CPMG delay time, and the results obtained for the H3 segment are summarized in Table I. It was shown that in the absence of DNS-Lys the [$^{15}N$]$T_2$ values for the H3 segment residues are in the range 10–40 ms. In the multiply labeled Fv analogue used in the present study, the $^{15}N$ probe has also been introduced over the entire part of the molecule including L1, L2, L3, H1, and H2 and their neighborhood. It was confirmed that all residues other than those originating from the H3 segment possess much longer [$^{15}N$]$T_2$ values, which are in the range 50–70 ms.

$^{15}N$ DEPT spectra were measured at 40 and 60 MHz for an Fv fragment singly labeled with [$^{15}N$]Tyr (Takahashi et

[2] The definition based on the canonical structure (Chothia & Lesk, 1987; Chothia et al., 1989) has been followed.

[3] The convention of Kabat et al. (1987) will be followed for the numbering of the variable regions of the heavy and light chains. Amino acid residues in the heavy and light chains are differentiated by using H and L, respectively.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H98(H3) Y104(H)
Y96(H3)
I95(H)
W36(H)
F(H)
A49(H)
W35(L) Y59(H)
Y99(H3)
A56(H)
F102(H3)
I48(L)
Y36(L) F98(L)
Y58(H)
F(L)
F(H)
H93(L3)
A52C(H2)
F87(L)
F(L)
Y97(H3)
W101(H3)
Y91(H)
Y49(L)
A(L)
A32(H1)
Y79(H)
F(L)
A(L)
I75(L)
W105(H)
H34(L) F(L)
A60(H)
Y32(L1)
A(H)
W33(H)
Y90(H) Y86(L)
A(H)
W47(H)
A23(H)
I69(H)
H27D(L1)
F29(H1)
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9.0
8.0
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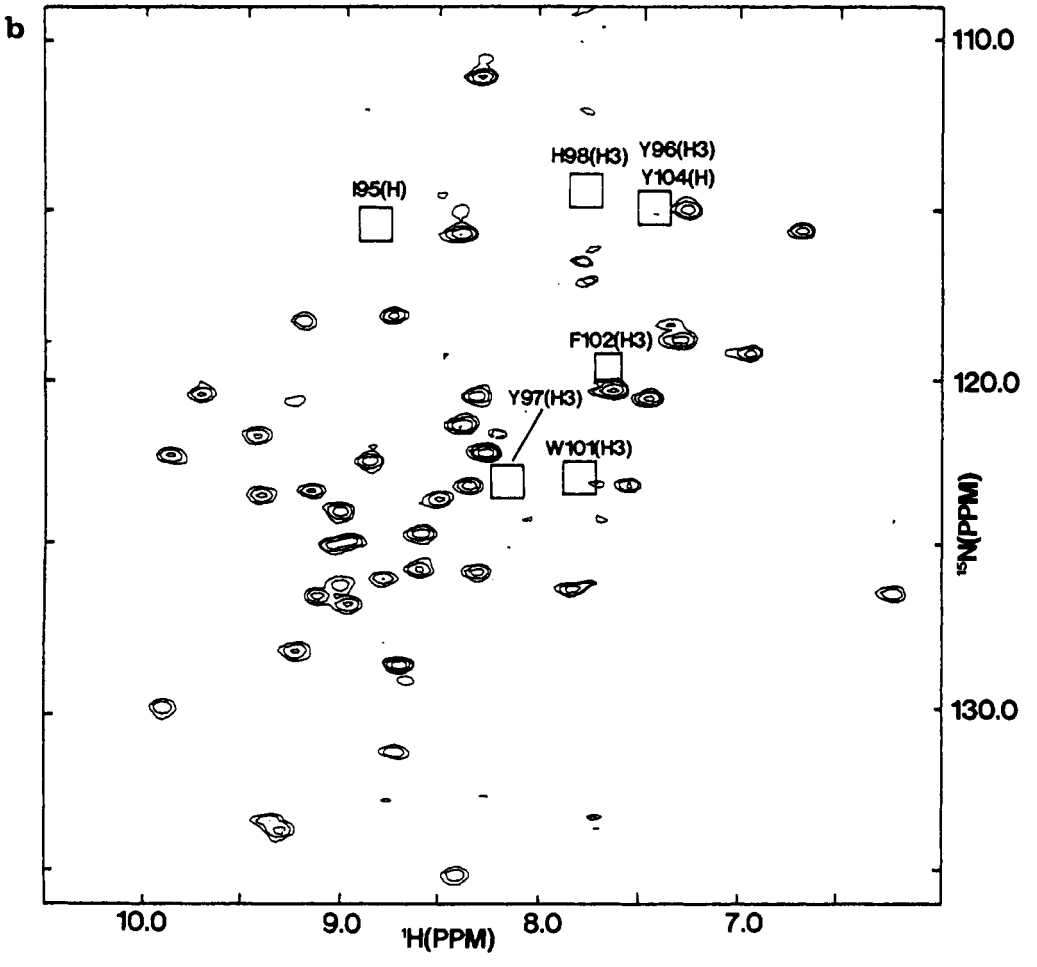
b
H98(H3) Y96(H3)
Y104(H)
I95(H)
F102(H3)
Y97(H3)
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110.0
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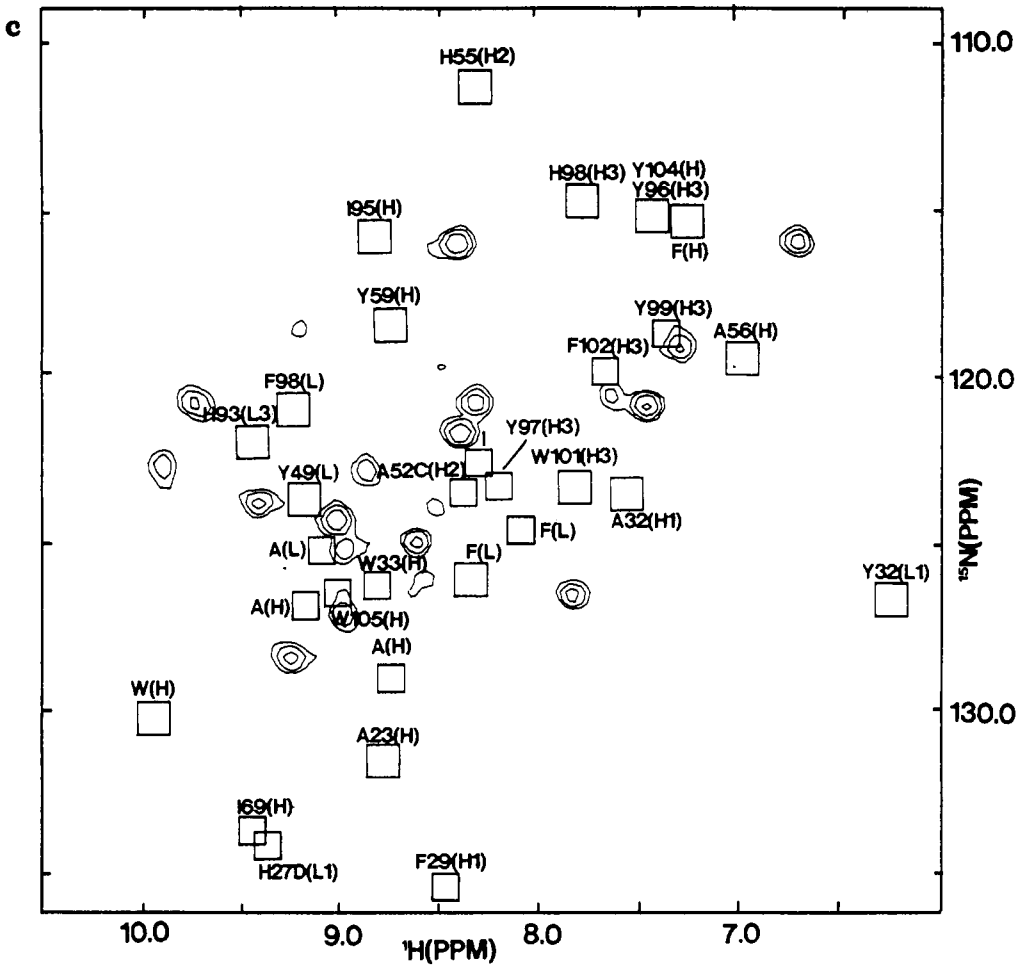


FIGURE 1: $^1H$–$^{15}N$ shift correlation spectra of the anti-dansyl Fv fragment *multiply* labeled with $^{15}N$ at the amide group of Ile, Tyr, His, Trp, Phe, and Ala. Fv was dissolved at a concentration of approximately 1 mM in 5 mM phosphate buffer containing 200 mM NaCl in 90% $H_2O$/10% $D_2O$, pH 4.99. (a) The HSQC spectrum measured in the absence of DNS-Lys. All resonances except for 1 of 11 Ala resonances are observable. Resonances that have been assigned in a site-specific way are identified by using the one-letter abbreviation for the amino acid. Resonances assigned to one of the hypervariable regions are identified as F29(H1) and H93(L3); other assigned resonances are identified with H (heavy chain) and L (light chain) in parentheses, e.g., A60(H) and F98(L), respectively. All other peaks that have not been assigned yet in a site-specific way are labeled, e.g., as F(L) and I; here the origin of each of the peaks, i.e., H or L, whenever available, is given in parentheses. Differentiation between H and L has been made by a recombination experiment. See Materials and Methods. (b) A $[^{15}N]T_2$ filtering experiment performed in the absence of DNS-Lys. The CPMG delay time was set to 25 ms and the interval of the successive refocusing pulses to 2.0 ms. In spectrum b, the peaks that disappeared under the present condition are boxed. (c) A hydrogen–deuterium exchange experiment performed in the absence of DNS-Lys. Fv was incubated in $D_2O$ for 10 h at 30 °C. In the absence of DNS-Lys, 31 peaks disappeared upon incubation in $D_2O$. These peaks are boxed in spectrum c.

al., 1991b). Amide resonances originating from Tyr-96H, Tyr-97H, Tyr-99H, and Tyr-104H of the H3 segment were very much broader in line width than the rest of the resonances. The line broadening observed for the H3 segment was far more pronounced at the higher observing frequency of 60 MHz. This result indicates that the $^{15}N$ resonances for the H3 segment are exchange broadened. We therefore conclude that in the absence of DNS-Lys the H3 segment is undergoing exchange among at least two conformations. It is not possible to quantitatively describe this exchange process, since the number of conformations involved and the chemical shift differences among them are not known.

It has been shown on the basis of X-ray crystallographic data that (1) there are limited numbers of conformations or *canonical structures* for L1, L2, L3, H1, and H2 but (2) H3 is extremely variable in length and amino acid sequence and has no canonical structures (Chothia & Lesk, 1987; Chothia et al., 1989). In view of the NMR results described above, it is possible that upon crystallization H3 is frozen to different conformations for different antibodies.

Hydrogen–deuterium exchange of the amide protons was followed in the absence and presence of DNS-Lys by measuring $^1H$–$^{15}N$ HSQC spectra after Fv had been dissolved in $D_2O$. The HSQC spectrum observed in the absence of DNS-Lys (Figure 1c) indicates that the amide protons of 31 residues were exchanged with deuterium within 10 h. These 31 residues contain all of the H3 segment residues, which except for Ile-95H possess rapid hydrogen–deuterium exchange rates ($k_m > 10^{-1}$ min$^{-1}$). Ile-95H, which exists at an end of the H3 segment, shows slower hydrogen–deuterium exchange with a $k_m$ value of $3 \times 10^{-3}$ min$^{-1}$. The result of the hydrogen–deuterium exchange experiment indicates that in the absence of DNS-Lys solvent is able to access freely exchangeable protons of the H3 segment.

Binding of DNS-Lys dramatically changes the hydrogen–deuterium exchange rate for Ile-95H, Tyr-96H, Trp-101H, Phe-102H, and Tyr-104H to $k_m < 10^{-5}$ min$^{-1}$. No other resonances exhibit such a large change in the hydrogen–deuterium exchange rate. This result is quite consistent with our previous data observed for Tyr-96H and Tyr-104H using an Fv analogue labeled with [$^{15}N$]Tyr (Takahashi et al., 1991b). As Table I shows, $[^{15}N]T_2$ relaxation times for the H3 segment change significantly upon addition of DNS-Lys. No other resonances show $[^{15}N]T_2$ relaxation times which are different

in the absence and presence of DNS-Lys. On the basis of the [$^{15}$N]$T_2$ and hydrogen–deuterium exchange data, we conclude that antigen binding significantly affects the dynamical structure of the H3 segment.

Conformational changes of antibody in association with antigen binding have been discussed on the basis of X-ray crystallographic data (Mariuzza et al., 1987; Colman, 1988; Davies et al., 1990). A significant change in the quaternary structure of Fv upon antigen binding has recently been reported (Bhat et al., 1990). However, it is difficult to extract from the X-ray data information concerning the dynamical aspect of the conformational changes. In the present paper, we have shown on the basis of the NMR data that the dynamical structure of the H3 segment changes significantly upon antigen binding. A combination of X-ray and NMR analyses would therefore be crucial in further understanding the structural basis of antigen recognition.

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## Supplementary Material Available

Three figures showing HSQC spectra of the multiply labeled Fv fragment observed in the presence of DNS-Lys and the spin-labeled hapten and DEPT spectra of the Fv fragment labeled with [$^{15}$N]Tyr (4 pages). Ordering information is given on any current masthead page.

**Registry No.** DNS-Lys, 1101-84-4.

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