Isotope-Edited Nuclear Magnetic Resonance Study of Fv Fragment of Anti-Dansyl Mouse Monoclonal Antibody: Recognition of the Dansyl Hapten†

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ABSTRACT: An isotope-edited proton nuclear magnetic resonance study is reported of Fv, which is the smallest antigen recognition unit composed of V_H and V_L domains. Fv has been obtained by clostripain digestion of a short-chain anti-dansyl mouse IgG2a monoclonal antibody [Igarashi, T., Sato, M., Katsube, Y., Takio, K., Tanaka, T., Nakanishi, M., & Arata, Y. (1990) *Biochemistry 29*, 5727–5733]. A variety of stable-isotope-labeled anti-dansyl Fv analogues have been prepared. The aromatic proton resonances for all Tyr residues of the Fv fragment have been assigned in the absence and presence of ε-dansyl-L-lysine by means of isotope-edited homonuclear and heteronuclear two-dimensional NMR experiments. On the basis of the established assignments, it has been concluded that the dansyl ring is bound through Tyr-96H and Tyr-104H to both ends of H3, the third hypervariable region of the heavy chain. We also suggest that the antigen binding results in the formation of a hydrophobic core comprising the dansyl ring and the aromatic rings of Tyr-96H and Tyr-104H.

The antibody combining site is composed of six hypervariable regions, three each in the V_H domain (H1, H2, and H3) and the V_L domain (L1, L2, and L3).[1] The Fv fragment, which is a heterodimer of V_H and V_L domains, is the smallest antigen binding unit, having a molecular weight of 25 000. We have reported that (1) limited clostripain digestion of a short-chain mouse IgG2a anti-dansyl monoclonal antibody, which lacks the entire C_H1 domain, gives the Fv fragment in a high yield and (2) the Fv fragment thus prepared retains full antigen binding capability (Igarashi et al., 1990).

We recently reported a series of stable-isotope-aided NMR studies on switch variant anti-dansyl antibodies. By use of the intact IgGs and their proteolytic fragments selectively labeled with ^{13}C at the carbonyl carbon of the peptide bond, it has been shown that carbonyl carbon resonances can be observed separately and the site-specific assignment of each of these resonances is possible (Kato et al., 1989, 1991a,b). We are also using the anti-dansyl Fv fragment and developing a multinuclear NMR approach in order to elucidate the static and dynamic structures of the antibody combining site (Kato et al., 1991a; Takahashi et al., 1991a,b, 1992).

In the previous work, we studied the interaction of ε-dansyl-L-lysine (DNS-Lys) with the antibody combining site of the Fv fragment, in which all aromatic protons except for His C2′-H and Tyr C3′,5′-H had been deuterated (Takahashi et al., 1991a). Two-dimensional (2D) NOESY experiments have indicated that two Tyr residues of the Fv fragment are in close spatial proximity to the dansyl ring. In order to proceed further, we have to assign all the Tyr resonances. However, in the case of proteins of $M_r \gtrsim 20\,000$, assignment of the side-chain proton resonances becomes generally difficult due to broadening and severe overlapping of spectral lines.

In the present paper, we report the assignment of the aromatic proton resonances originating from all of the Tyr residues of the anti-dansyl Fv fragment. Isotope-edited homonuclear and heteronuclear 2D NMR experiments were performed using a variety of Fv analogues. On the basis of the NMR data obtained, we discuss the mode of interaction of DNS-Lys with the antibody combining site of the Fv fragment. A possible role of the Tyr residues in antigen binding is briefly discussed.

MATERIALS AND METHODS

Materials. Tyr-*3′,5′-d*$_2$, 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). L-[1-^{13}C]Tyr and L-[1,2-$^{13}C_2$]Tyr were prepared by enzymatic coupling of phenol with [1-^{13}C]pyruvate and [1,2-$^{13}C_2$]pyruvate, respectively, in the presence of CH_3COONH_4 (Nagasawa et al., 1981). Tyr-*2′,3′,5′,6′-d*$_4$ was prepared in the same way using phenol-*d*$_5$ (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 3 h. Phe-*2′,3′,4′,5′,6′-d*$_5$, L-[^{15}N]Tyr, and L-[3′,5′-$^{13}C_2$]Tyr were purchased from CIL, Isocommerz GmbH, and ICON Services Inc., respectively. The isotope enrichment is 95% or higher for each of the amino acids. Clostripain, ε-dansyl-L-lysine (DNS-Lys), and 4-amino-2,2,6,6-tetramethylpiperidine N-oxide (AmTEMPO) were obtained from Sigma Chemical Co. 4-(Dansylamino)-2,2,6,6-tetramethylpiperidine N-oxide (DNS-AmTEMPO) was synthesized according to the procedure of Chiniak and Polonski (1973). The purity of DNS-AmTEMPO was checked by thin-layer chromatography. All other chemicals were of reagent grade and used without further purification.

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[1] Abbreviations: AmTEMPO, 4-amino-2,2,6,6-tetramethylpiperidine N-oxide; C_H1, C_H2, and C_H3, constant domains of the heavy chain; C_L, constant domain of the light chain; DNS-Lys, ε-dansyl-L-lysine; HPLC, high-performance liquid chromatography; FR1(H), FR2(H), FR3(H), and FR4(H), framework regions in the V_H domain; FR1(L), FR2(L), FR3(L), and FR4(L), framework regions in the V_L domain; Fv, heterodimer of V_H and V_L; HOHAHA, homonuclear Hartmann–Hahn spectroscopy; HSQC, heteronuclear single-quantum correlation; H1, H2, and H3, hypervariable regions in the V_H domain; IgG, immunoglobulin G; L1, L2, and L3, hypervariable regions in the V_L domain; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, two-dimensional NOE correlated spectroscopy; V_H, variable domain of the heavy chain; V_L, variable domain of the light chain.

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Preparation of Stable-Isotope-Labeled Analogues of the Anti-Dansyl Fv Fragment. The mouse hybridoma cell line 1B10.7, which produces the short-chain mouse IgG2a monoclonal antibody (Dangl et al., 1982), was adapted to a serum-free medium (Nissui NYSF 404) and then grown in a medium containing stable-isotope-labeled amino acid(s) (Kato et al., 1989, 1991a). All Fv analogues were prepared by clostripain digestion of the short-chain antibody as described previously (Takahashi et al., 1991a). The purity of the protein preparations used for NMR measurements was checked by SDS–polyacrylamide gel electrophoresis. Recombination of the light and heavy chains of the Fv fragment was performed according to the procedures described previously (Kato et al., 1991a; Takahashi et al., 1991a).

Interaction of DNS-Lys and DNS-AmTEMPO with the Fv Fragment. The dissociation constants for DNS-Lys and DNS-AmTEMPO were determined by monitoring the fluorescence of the dansyl residue bound to the Fv fragment, with excitation at 335 nm and emission at 500 nm. Fluorescence measurements were made at 30 °C with a Shimadzu RF-5000 spectrofluorophotometer. The dissociation constants of the Fv fragment obtained for DNS-Lys and DNS-AmTEMPO were 1.4×10^{-8} M and 9.5×10^{-8} M, respectively.

NMR Measurements. 2D-HOHAHA (Bax & Davis, 1985b) and ROESY (Bax & Davis, 1985a) spectra were measured with a mixing time of 25 ms, and ^1H–^{13}C HSQC spectra were recorded using the pulse sequence of Bodenhausen and Ruben (1980). A total of 512 and 128 blocks were acquired with a JEOL JNM-GSX 500 spectrometer with data points of 2K for 2D-HOHAHA and ^1H–^{13}C HSQC experiments, respectively. $^{15}N(\omega_2)$-Half-filtered HOHAHA and $^{13}C(\omega_1)$-half-filtered NOESY spectra were recorded with the pulse sequence of Otting and Wüthrich (1990) with Bruker AM-400 and JEOL JNM-GSX 500 spectrometers, respectively. Data points of 2K were used in the t_2 dimension and 256–512 transients were acquired for each of 400 t_1 points. The isotope-filter delay was 4.5 ms for ^{15}N-filtered and 2.9 ms for ^{13}C-filtered experiments. The mixing times for the HOHAHA and NOESY measurements were 20 and 100 ms, respectively.

All two-dimensional spectra were obtained in the pure absorption mode with time-proportional phase incrementation (TPPI) (Marion & Wüthrich, 1983) for the $^{15}N(\omega_2)$-half-filtered HOHAHA measurements and by the method of States et al. (1982) for the 2D-HOHAHA, ^1H-^{13}C HSQC, and ^{13}C-(ω_1)-half-filtered NOESY measurements. Prior to 2D Fourier transformation, the data acquired were multiplied by a Gauss function in t_2 and by a shifted sine square function in t_1 and then zero-filled once along the t_1 direction.

^{13}C NMR spectra were recorded at 125 MHz with a JEOL JNM-GSX 500 spectrometer. The free induction decay was recorded with 32K data points and a spectral width of 24 000 Hz. For resolution enhancement, the free induction decay was multiplied by a Gauss function prior to Fourier transformation. For the ^{13}C selective decoupling experiment, a second irradiation channel was used.

For all NMR measurements except for the $^{15}N(\omega_2)$-half-filtered HOHAHA measurement, the protein solutions were concentrated by ultrafiltration to a final volume of either 0.4 or 2.0 mL in 5 mM phosphate buffer, pH 7.4, containing 0.2 M NaCl. The samples were prepared in D_2O. $^{15}N(\omega_2)$-Half-filtered HOHAHA spectra were measured using a protein solution in 5 mM phosphate buffer, pH 5.0, in H_2O that contained 10% D_2O. The final concentrations of the proteins were typically 0.8–1.0 mM. The solvent resonance was suppressed by selective irradiation during the relaxation delay, which was taken to be 1.2 s. The probe temperature was 30 °C throughout the experiment.

Table I: Abbreviations for the Fv Analogues Used in the Present Study.

Fv analogue	labeled with
[^{2}H]Fv	Tyr-2′,6′-d_2, Phe-2′,3′,4′,5′,6′-d_5, Trp-2′,4′,5′,6′,7′-d_5, and His-α,2′,4′-d_3[a]
[2′,6′-H]Fv	Tyr-3′,5′-d_2, Phe-2′,3′,4′,5′,6′-d_5, Trp-2′,4′,5′,6′,7′-d_5, and His-α,2′,4′-d_3[a]
[H]Fv	Phe-2′,3′,4′,5′,6′-d_5, Trp-2′,4′,5′,6′,7′-d_5, and His-α,2′,4′-d_3[a]
[N]Fv	[^{15}N]Tyr
[3′,5′-C]Fv	[3′,5′-$^{13}C_2$]Tyr
[2-C]Fv	[2-^{13}C]Tyr, Phe-2′,3′,4′,5′,6′-d_5, Trp-2′,4′,5′,6′,7′-d_5, and His-α,2′,4′-d_3[a]
[1,2-C]Fv	[1,2-$^{13}C_2$]Tyr

[a] The C2′ position of the imidazole ring of each of the His residues of the labeled proteins was always protonated even if His-α,2′,4′-d_3 had been used (Kato et al., 1989).

Table II: Distribution of Tyr Residues in the Anti-Dansyl Fv Fragment[a]

Tyr	location	Tyr	location
Tyr-58H	H2	Tyr-99H	H3
Tyr-59H	H2	Tyr-104H	H3
Tyr-79H	FR3(H)	Tyr-32L	L1
Tyr-90H	FR3(H)	Tyr-36L	FR2(L)
Tyr-91H	FR3(H)	Tyr-49L	FR2(L)
Tyr-96H	H3	Tyr-86L	FR3(L)
Tyr-97H	H3		

[a] Residues in the heavy and light chains are denoted by H and L, respectively.

RESULTS

Strategy for the Assignment of the Aromatic Proton Resonances Originating from the Tyr Residues of the Fv Fragment. The stable-isotope-labeled analogues of the Fv fragment used in the present experiment are listed in Table I. Spectral assignments were made as follows:

(1) Use of [3′,5′-C]Fv, [^{2}H]Fv, [2′,6′-H]Fv, and [H]Fv. The proton resonances originating from the aromatic ring of each of the Tyr residues of the Fv fragment were identified by ^1H–^{13}C HSQC, 2D-HOHAHA, and ROESY experiments.

(2) Use of [N]Fv, [1,2-C]Fv, and [2-C]Fv. The Cα protons originating from the Tyr residues were assigned in a site-specific way by $^{15}N(\omega_2)$-half-filtered HOHAHA, selective ^{13}C decoupling, and ^1H–^{13}C HSQC experiments. The aromatic proton resonances were connected to the Cα proton resonances by measuring the $^{13}C(\omega_1)$-half-filtered NOESY spectrum of [2-C]Fv.

Identification of the Aromatic Proton Resonances of the Tyr Residues. The Fv fragment used in the present study contained 13 Tyr residues, nine in V_H and four in V_L (see Table II). By following the assignment procedure described above, the 3′,5′ and 2′,6′ proton resonances of the Tyr residues were identified in the absence and presence of DNS-Lys.

Figure 1a shows the aromatic region of the ^1H spectrum of [^{2}H]Fv observed in the absence of DNS-Lys. In order to distinguish resonances originating from the 3′,5′ protons of the Tyr residues from the residual amide proton resonances, the ^1H–^{13}C HSQC spectrum of [3′,5′-C]Fv was measured. Figure 1b shows the aromatic region of the ^1H–^{13}C HSQC spectrum of [3′,5′-C]Fv observed in the absence of DNS-Lys. In this spectrum, 14 cross peaks were observed, including K and K′. In the ROESY spectrum of [^{2}H]Fv observed in the absence of DNS-Lys with a mixing time of 25 ms, one exchange cross peak was detected between K and K′ at 6.43 and 6.93

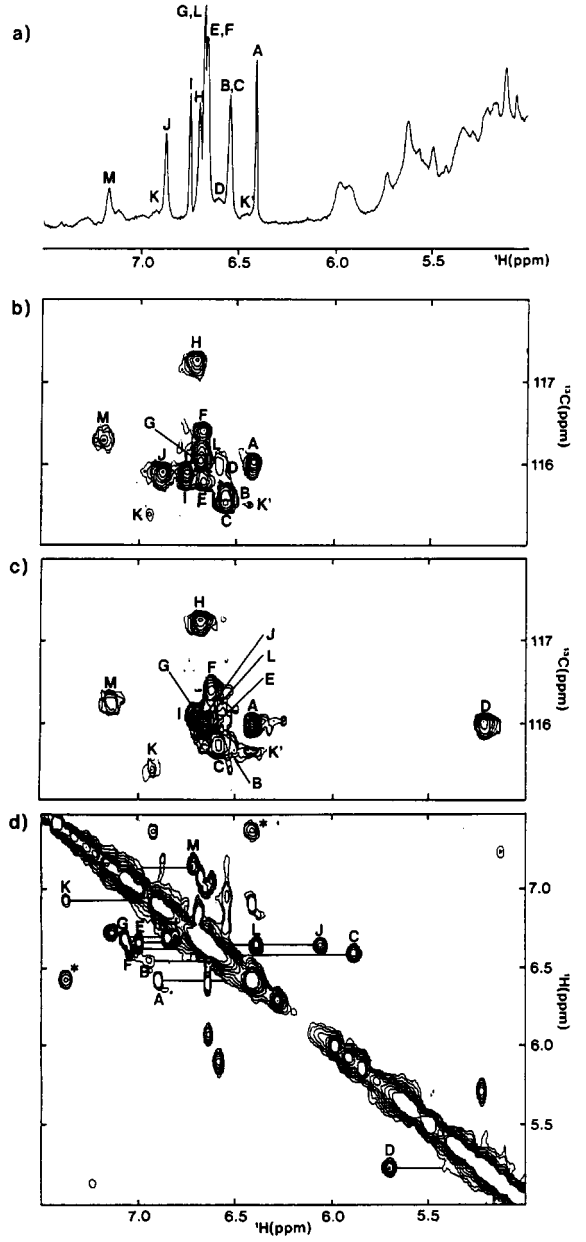

FIGURE 1: Aromatic regions of the NMR spectra of anti-dansyl Fv analogues. The protein samples were dissolved at a concentration of approximately 1.0 mM in 5 mM phosphate buffer containing 200 mM NaCl in D_2O at pH 7.4. The probe temperature was 30 °C. (a) ^1H NMR spectrum of [^2H]Fv. ^1H–^{13}C HSQC spectra of [3′,5′-C]Fv were observed in the absence (b) and presence (c) of DNS-Lys. (d) 2D-HOHAHA spectrum of [H]Fv observed in the presence of DNS-Lys. The mixing time was 25 ms. The spin system of the Tyr residues is connected by solid lines. The peaks denoted by the asterisks are the exchange peaks between the 2′,6′ protons of Tyr-K (see text).

ppm (data not shown). This indicates that peaks K and K′ originate from the same Tyr residue (Tyr-K). It is likely that the aromatic ring of Tyr-K is in slow equilibrium between two states. In view of the results described above, we conclude that in the ^1H spectrum of [^2H]Fv (Figure 1a) the resonances originating from the 3′,5′ protons of all 13 Tyr residues can be detected, although some of the resonances are significantly broadened. Other broad signals in Figure 1a are presumably due to the amide protons that persist in D_2O.

Furthermore, the results of the V_H and V_L recombination experiment indicated that peaks C, F, H, and M are due to V_L and all other peaks to V_H (data not shown).

We have also been able to identify the 3′,5′ proton resonances of the Tyr residues in the ^1H–^{13}C HSQC spectrum obtained in the presence of DNS-Lys (Figure 1c). The correspondence of each pair of cross peaks observed in the absence and presence of DNS-Lys has previously been established by means of exchange NOESY experiments (Takahashi et al., 1991a).

Figure 1d shows the 2D-HOHAHA spectrum of [H]Fv observed in the presence of DNS-Lys. The chemical shifts of the 2′,6′ proton resonances originating from the Tyr residues of the Fv fragment were determined by observing the HOHAHA transfer from the 3′,5′ protons to the 2′,6′ protons. In order to confirm the chemical shifts of the 2′,6′ proton resonances of Tyr-K, we measured the ROESY spectrum of [2′,6′-H]Fv in the presence of DNS-Lys with a mixing time of 25 ms. In the ROESY spectrum, one exchange cross peak was detected at 6.42 and 7.38 ppm (data not shown). We therefore conclude that the chemical shifts of the 2′,6′ proton resonances of Tyr-K in the presence of DNS-Lys are 6.42 and 7.38 ppm. The 2′,6′ proton resonances observed in the absence of DNS-Lys were identified in the same way.

Site-Specific Assignment of the Cα Proton Resonances of the Tyr Residues. The spectral assignment of the Cα proton resonances of the Tyr residues was made as follows. Here we present the results obtained in the presence of DNS-Lys. In previous studies, we assigned the amide proton resonances observed in the ^1H–^{15}N HSQC spectra of the Tyr residues of the Fv fragment (Takahashi et al., 1991b, 1992). It was possible to connect the Cα proton resonances to the corresponding amide proton resonances on the basis of the ^{15}N-(ω_2)-half-filtered HOHAHA spectrum. In order to detect the amide proton resonances of the Tyr residues, the ^{15}N-(ω_2)-half-filtered HOHAHA spectrum of [N]Fv in the presence of DNS-Lys was measured at pH 5.0 in H_2O that contained 10% D_2O (Figure 2). All NH_i–$C\alpha H_i$ cross peaks of the Tyr residues except for Tyr-97H and Tyr-99H are observable in this spectrum.[2] On the basis of the results of the $^{15}N(\omega_2)$-half-filtered HOHAHA experiment, the assignment of the Cα protons at pH 7.4 in the presence of DNS-Lys was performed using the ^1H–^{13}C HSQC spectrum of [2-C]Fv in D_2O.

In the case of the Tyr-97H and Tyr-99H resonances, no NH_i–$C\alpha H_i$ cross peaks were observed in the $^{15}N(\omega_2)$-half-filtered HOHAHA spectrum. Therefore, for the assignment of these resonances, we started from the carbonyl carbon resonances that had already been assigned (Kato et al., 1991b). The assignment of the Cα proton resonances of Tyr-97H and Tyr-99H was established by means of selective ^{13}C decoupling and ^1H–^{13}C HSQC experiments performed on [1,2-C]Fv (data not shown).

In this way, all of the Cα protons resonances originating from the Tyr residues were assigned in a site-specific way. Figure 3 shows the ^1H–^{13}C HSQC spectrum of [2-C]Fv observed in the presence of DNS-Lys at pH 7.4 and 30 °C with the established assignments. The same procedure was followed for the spectral assignments in the absence of DNS-Lys.

Site-Specific Assignment of the Aromatic Proton Resonances of the Tyr Residues. Figure 4 shows the region between the aromatic proton and the Cα proton resonances of the $^{13}C(\omega_1)$-half-filtered NOESY spectrum of [2-C]Fv observed in the presence of DNS-Lys. In this spectrum, NOE cross

[2] The sequence data for the V_H region of the antibody used in the present work were provided by J. L. Dangl (Ph.D. Thesis, Stanford University, 1986). The sequence data for 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 antibody. Amino acid residues in the heavy and light chains are indicated by suffixes H and L, respectively; e.g., Tyr-96H and Tyr-49L.

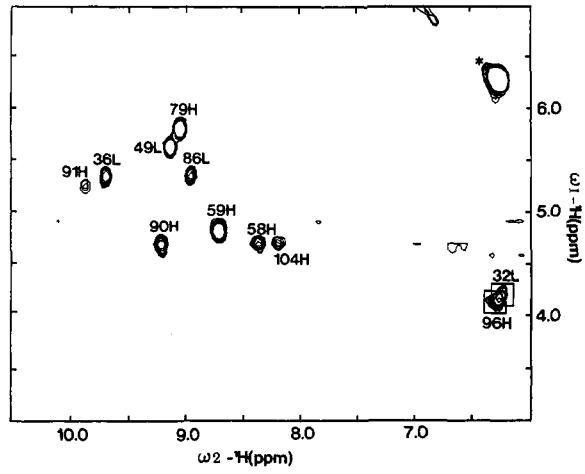

FIGURE 2: Amide and Cα proton region of the $^{15}N(\omega_2)$-half-filtered HOHAHA spectrum of [N]Fv observed in the presence of DNS-Lys. The measurement was made in H_2O containing 10% D_2O at pH 5.0 and 30 °C. The mixing time was 20 ms. The peak denoted by the asterisk is the diagonal peak for Tyr-32L and Tyr-96H.

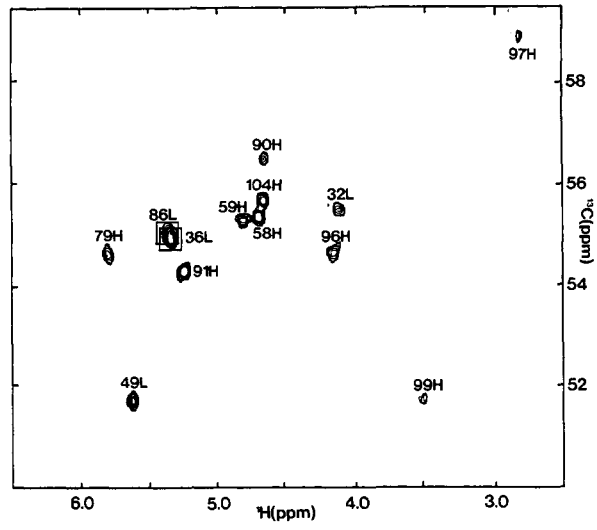

FIGURE 3: ^1H–^{13}C HSQC spectrum of [2-C]Fv observed in the presence of DNS-Lys in D_2O at pH 7.4 and 30 °C.

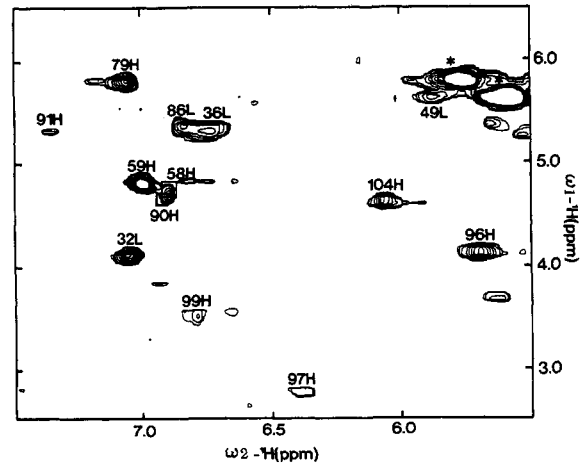

FIGURE 4: Portion of the $^{13}C(\omega_1)$-filtered NOESY spectrum of [2-C]-Fv observed in the presence of DNS-Lys in D_2O at pH 7.4 and 30 °C. The mixing time was 100 ms. The peaks denoted by the asterisks are the diagonal peaks for Tyr-49L and Tyr-79H.

peaks between the Cα protons and the aromatic protons originating from the Tyr residues that were labeled with ^{13}C at the Cα position were extracted. It should be noted that in [2-C]Fv all of the aromatic protons except for those of the Tyr residues and the C2′ proton of His residues had been deuterated.[3] Therefore, it was possible to specify intraresidue NOE cross peaks between the Cα protons and the aromatic protons of the Tyr residues. The NOE cross peaks between the 2′,6′ protons and the Cα protons of the Tyr residues can be observed in the $^{13}C(\omega_1)$-half-filtered NOESY spectrum (Figure 4).

At this stage, all the aromatic protons of the Tyr residue of the Fv fragment in the presence of DNS-Lys have been assigned. The spectral assignments in the absence of DNS-Lys were accomplished in the same way. The established assignments have been confirmed by observing the NOE cross peaks between the 2′,6′ protons and the Cβ protons in the HMQC–NOESY spectrum for the Fv fragment labeled with [3-^{13}C]Tyr (data not shown). The ^1H and ^{13}C chemical shifts of the Tyr residues are summarized in Table III. In Table IIIA, spectral assignments for the 3′,5′ proton resonances (Figure 1a) are summarized in the two columns on the left.

Tyr-K was assigned to Tyr-91H, which is buried in the core region of V_H. The spectral data for the Cα and amide protons of Tyr-91H indicate that the backbone of Tyr-91H does not exist in two states. We therefore suggest that the exchange process observed for the aromatic ring of Tyr-91H is due to a slow flip-flop motion of the aromatic ring about the Cβ–Cγ bond.

Binding of DNS-AmTEMPO to [H]Fv. DNS-AmTEMPO was used as a paramagnetic probe (Dower & Dwek, 1979). The dissociation constant of the Fv fragment for DNS-AmTEMPO is 9.5×10^{-8} M, which is approximately 7 times larger than that for DNS-Lys.

Figure 5a shows the aromatic region of a 2D-HOHAHA spectrum of [H]Fv observed at a [H]Fv to DNS-AmTEMPO molar ratio of 100:120. The cross peaks originating from Tyr-96H, Tyr-97H, Tyr-99H, Tyr-104H, Tyr-32L, and Tyr-49L disappeared upon addition of DNS-AmTEMPO.

For determination of the binding sites for DNS-AmTEMPO in the Fv fragment, L-ascorbic acid was added to the Fv-DNS-AmTEMPO solution as a reductant in order to quench the effect of the electron spin of DNS-AmTEMPO. In the 2D-HOHAHA spectrum of the [H]Fv-DNS-AmTEMPO complex observed in the presence of L-ascorbic acid, the cross peaks originating from Tyr-96H, Tyr-97H, Tyr-99H, Tyr-104H, Tyr-32L, and Tyr-49L reappeared (Figure 5b). The chemical shifts of protons at the 3′,5′ and 2′,6′ positions of Tyr-96H, Tyr-97H, Tyr-99H, Tyr-104H, Tyr-32L, and Tyr-49L are almost identical to those observed in the presence of DNS-Lys (see Figure 1d). We therefore conclude that both DNS-AmTEMPO and DNS-Lys bind to the antibody combining site of the Fv fragment in a similar way.

DISCUSSION

Interaction of DNS-Lys with the Fv Fragment. We previously showed that the 3′,5′ protons of two Tyr residues (Tyr-J and Tyr-D) originating from V_H give NOE cross peaks with the 2 and 3 and with the 3 and 4 protons of the dansyl ring, respectively (Takahashi et al., 1991a). On the basis of the results of the present study, Tyr-J and Tyr-D have been unambiguously assigned to Tyr-104H and Tyr-96H, respectively. We therefore conclude that the dansyl ring is bound to both ends of H3 of the Fv fragment (see Table IV). The present DNS-AmTEMPO binding experiment results indicate that Tyr-96H, Tyr-97H, Tyr-99H, and Tyr-104H, which are located in the H3 loop, along with Tyr-32L and Tyr-

[3] The C2′ position of the imidazole ring of each His residue of the labeled proteins was always protonated even if His-α,2′,4′-d_3 had been used (Kato et al., 1989).

Table III: ^1H and ^{13}C Chemical Shifts[a] of the Tyr Residues Observed in the Absence and Presence of DNS-Lys

(A) ^1H Chemical Shifts

peak	residue	αH			2′,6′ H			3′,5′ H		
		–[b]	+	diff	–	+	diff	–	+	diff
A	58H	4.71	4.69	–0.02	6.92	6.90	–0.02	6.41	6.42	0.01
B	90H	5.21	5.21	0.00	6.96	6.96	0.00	6.55	6.55	0.00
C	49L	5.59	5.58	–0.01	5.96	5.90	–0.06	6.55	6.59	0.04
D	96H	4.35	4.16	–0.19	6.87	5.72	–1.15	6.60	5.22	–1.38
E	59H	4.80	4.80	0.00	7.02	7.01	–0.01	6.66	6.66	0.00
F	32L	4.07	4.12	0.05	7.05	7.05	0.00	6.68	6.63	–0.03
G	79H	5.78	5.78	0.00	7.07	7.08	0.01	6.68	6.68	0.00
H	86L	5.37	5.35	–0.02	6.88	6.86	–0.02	6.70	6.69	–0.01
I	99H	3.66	3.46	–0.20	6.88	6.82	–0.06	6.76	6.70	–0.06
J	104H	5.14	4.65	–0.49	7.09	6.07	–1.02	6.88	6.65	–0.23
K	91H[c]	4.64	4.64	0.00	7.40	7.38	–0.02	6.93	6.91	–0.02
K′	91H[c]				6.43	6.42	–0.01	6.43	6.42	–0.01
L	97H	3.14	2.82	–0.32	6.61	6.40	–0.21	6.68	6.65	–0.03
M	36L	5.36	5.32	–0.04	6.76	6.72	–0.04	7.17	7.14	–0.03

(B) ^{13}C Chemical Shifts

residue	αC			3′,5′ C		
	–	+	diff	–	+	diff
58H	55.3	55.3	0.0	116.0	116.0	0.0
90H	56.5	56.5	0.0	115.6	115.6	0.0
49L	51.7	51.7	0.0	115.5	115.7	0.2
96H	53.8	54.6	0.8	116.0	116.0	0.0
59H	55.2	55.2	0.0	115.8	115.9	0.1
32L	55.4	55.4	0.0	116.4	116.4	0.0
79H	54.6	54.6	0.0	116.0	116.0	0.0
86L	55.1	55.0	–0.1	117.2	117.2	0.0
99H	51.0	51.7	0.7	115.9	116.1	0.2
104H	54.7	55.7	1.0	115.9	116.1	0.2
91H[d]	54.4	54.2	–0.2	115.4	115.4	0.0
91H[d]				115.5	115.6	0.1
97H	58.3	58.9	0.6	116.1	116.0	–0.1
36L	55.0	54.9	–0.1	116.3	116.2	–0.1

[a] Chemical shifts are expressed in parts per million relative to DSS. [b] Chemical shift differences in the absence (–) and presence (+) of DNS-Lys at pH 7.4 and 30 °C. [c] The aromatic ring proton resonances were observed individually. [d] The carbon resonances of the 3′, 5′ position of the aromatic ring were observed individually.

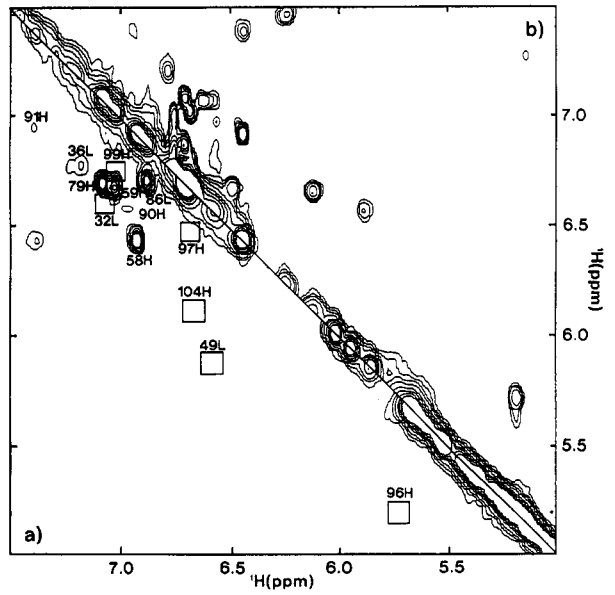

FIGURE 5: Aromatic region of the 2D-HOHAHA spectrum of the [H]Fv-DNS-AmTEMPO complex observed in the absence (a) and presence (b) of L-ascorbic acid. The measurements were made in D_2O at pH 7.4 and 30 °C. The mixing time was 25 ms. In (a), the cross peaks that disappeared upon formation of the complex are boxed.

49L exist within 15 Å from the paramagnetic center of the spin label hapten [Dower & Dwek (1979) and Anglister (1990) and references cited therein].

The crystal structure of the Fv fragment in the absence of DNS-Lys has recently been determined at 2.5-Å resolution

Table IV: Comparison of the Amino Acid Sequences of the H3 Regions of 10 Anti-Dansyl IgM Antibodies and That of the Anti-Dansyl Fv Fragment[a]

clone (IgM)	sequence
	H3
DBF1-386.5	A R - - A S T A P - - Y Y A M D Y W G
DBF1-235.4	A R - - Y D Y G S - - - Y A M D Y W G
DB2-101.1	A R - F Y Y Y G S S - - Y A M D Y W G
DB1-314.3	T - Y Y Y D Y E G - - - - - F A Y W G
DF4-12.6	T - - P Y Y Y G S S G - - - F A Y W G
DBF1-608.1	T - I Y Y D Y D G - - - - - F A Y W G
DF8-611.1	A R I L Y Y G N S - - - - - F D Y W G
DF4-29.4	A R C P Y D S - - - - - W Y F D V W G
DB1-310.9	T - S P T M T L A - - - - W F A Y W G
DB1-453.2	A R E G - - - - - - - - - - - A Y W G
	96 H3 104
Fv	T G I Y Y H Y P - - - - - W F A Y W G

[a] The 10 IgM sequences are from Chua et al. (1987). Fv is aligned for maximum coincidence with the IgM sequences.

(Satow et al., private communication). This indicates that in the absence of DNS-Lys (1) the aromatic ring of Tyr-96H

is in close spatial proximity to that of Tyr-104H and (2) Tyr-97H, Tyr-99H, Tyr-32L, and Tyr-49L are located within 10 Å from Tyr-96H and Tyr-104H. Thus, the NMR data obtained in the present work are quite consistent with the crystal data.

It has been observed that upon addition of DNS-Lys a large chemical shift change is induced in the aromatic proton and Cα proton resonances for Tyr-96H and Tyr-104H (see Table III). We suggest that this large chemical shift change is primarily due to the ring current effect induced by DNS-Lys binding. It should be noted that the addition of DNS-Lys results in large upfield shifts of 1.15 and 1.38 ppm for the resonances of the 2′,6′ protons and 3′,5′ protons of Tyr-96H, respectively. No other Tyr resonances exhibit such large changes in chemical shift. Thus, it is quite likely that Tyr-96H is stacked on the dansyl ring of DNS-Lys.

Formation of the Hydrophobic Core in the H3 Loop upon DNS-Lys Binding. Upon the addition of DNS-Lys to the Fv fragment, the hydrogen-deuterium exchange rates for the amide protons of Tyr-96H and Tyr-104H become dramatically low (Takahashi et al., 1991b). On the basis of the results obtained in the present study, we suggest that at both ends of H3 of the Fv fragment a hydrophobic core is formed comprising the dansyl ring and the two aromatic rings of Tyr-96H and Tyr-104H.

Comparison of the antibody sequence data has demonstrated that a large number of Tyr residues exist in the hypervariable region (Kabat et al., 1987; Mian et al., 1991). It has been suggested that amphipathic amino acids, such as Tyr and Trp, can change the hydrophilic environment of the antibody combining site to a hydrophobic one upon antigen binding (Mian et al., 1991). In the case of the anti-dansyl Fv fragment used in the present study, it is possible that Tyr-96H and Tyr-104H changed the hydrophobicity of the antibody combining site in the absence and presence of DNS-Lys.

We have recently demonstrated by means of a transferred NOE experiment that the entire molecule of DNS-Lys tightly binds to the anti-dansyl antibody and that DNS-Lys takes on a folded conformation in the antibody combining site (Kim et al., 1991). We have also found that the antigen combining site of the anti-dansyl antibody can be used as a chiral auxiliary for a stereoselective organic reaction. On the reduction of $NaBH_4$ of 5-dansylaminolevulinic acid, the yield of the *S*-enantiomer increased in the presence of the antibody. It is possible that the hydrophobic core induced by the antigen binding plays an important role in increasing the yield of the *S*-enantiomer in a stereoselective reaction.

Mode of Recognition of the Dansyl Moiety. The hybridoma cell lines that produce IgM antibodies with specificity for the dansyl moiety have been studied by sequencing single-stranded cDNAs (Chua et al., 1987). It has been suggested that the anti-dansyl affinity of these IgM antibodies is associated primarily with H3. However, due to variations in both the length and amino acid content of H3 for all of these anti-dansyl IgM antibodies, it was not possible to specify the amino acid residues that participate in the recognition of the dansyl moiety. It has been demonstrated that, in the case of the Fv fragment used in the present study, Tyr-96H and Tyr-104H, which are located at both ends of H3, are responsible for the recognition of the dansyl ring. Table IV summarizes the amino acid sequences of H3 for 10 hybridomas which produce anti-dansyl IgM antibodies (Chua et al., 1987). This indicates that in the IgM antibodies, except for DBF1-386.5, DB1-310.9, and DB1-453.2, Tyr residues are encoded at both ends of H3 as in the case of the Fv fragment. We therefore suggest that the two Tyr residues at both ends of H3 play an important role in the recognition of the dansyl ring.

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