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Carbon-13 NMR Study of Switch Variant Anti-Dansyl Antibodies: Antigen Binding and Domain-Domain Interactions†

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ABSTRACT: A ^{13}C NMR study is reported of switch variant anti-dansyl antibodies, which possess the identical V_H, V_L, and C_L domains in conjunction with highly homologous but not identical heavy-chain constant regions. Each of these antibodies has been selectively labeled with ^{13}C at the carbonyl carbon of Trp, Tyr, His, or Cys residue by growing hybridoma cells in serum-free medium. Spectral assignments have been made by following the procedure described previously for the switch variant antibodies labeled with [1-^{13}C]Met [Kato, K., Matsunaga, C., Igarashi, T., Kim, H., Odaka, A., Shimada, I., & Arata, Y. (1991) *Biochemistry 30*, 270-278]. On the basis of the spectral data collected for the antibodies and their proteolytic fragments, we discuss how ^{13}C NMR spectroscopy can be used for the structural analyses of antigen binding and also of domain-domain interactions in the antibody molecule.

Immunoglobulin G (IgG),[1] which is a multifunctional glycoprotein with a molecular weight of 150K, 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. Each of these homology units forms a characteristic domain structure known as an *immunoglobulin fold* that is rich in antiparallel β-sheets.

In the expression of a variety of antibody functions, domain-domain interactions play a crucial role. The antigen-binding site is constructed by V_H and V_L domains, whereas a variety of effector functions are carried out by the Fc region that is composed of two C_H2 and two C_H3 domains. Therefore, it is essential to understand how each of these domains interacts and behaves in the process of the expression of a variety of antibody functions.

We have recently reported a ^{13}C NMR study of a mouse monoclonal antibody specifically labeled with [1-^{13}C]Met (Kato et al., 1989). It has been shown that, even with the intact IgG with a molecular weight of 150K, the line widths of methionyl carbonyl carbon resonances are sufficiently narrow, and therefore, a double-labeling method developed by Kainosho and Tsuji (1982) can be applicable to site-specific resonance assignments. In the double-labeling method the carbonyl carbon of one type of amino acid (X) is labeled with ^{13}C and the α-nitrogen of another type of amino acid (Z) is

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[1] Abbreviations: C_L, constant region of the light chain; C_H1, C_H2, C_H3, constant regions of the heavy chain; CDR, complementarity-determining region; CDR1(H), CDR2(H), CDR3(H), CDR in the V_H domain; CDR1(L), CDR2(L), CDR3(L), CDR in the V_L domain; DNS-Lys, ϵ-dansyl-L-lysine; Fab, antigen-binding fragment; Fab*, a three-domain fragment composed of V_H, V_L, and C_L; Fc, fragment composed of the C-terminal halves of the heavy chains; FR, framework region; FR1(H), FR2(H), FR3(H), FR in the V_H domain; FR1(L), FR2(L), FR3(L), FR in the V_L domain; Fv, antigen-binding fragment composed of V_H and V_L; IgG, immunoglobulin G; IgG2a(s), a short-chain mouse IgG2a monoclonal antibody that lacks the entire C_H1 domain; [W]IgG, IgG labeled with [1-^{13}C]Trp (similar notations are used for other ^{13}C-labeled analogues); [H, Y]IgG, IgG that is doubly labeled with [1-^{13}C]His and [^{15}N]Tyr (similar notations are used for other doubly labeled analogues); NMR, nuclear magnetic resonance; V_H, variable region of the heavy chain; V_L, variable region of the light chain.

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labeled with ^{15}N. Resonances originating from the X-Z dipeptide can be identified by ^{13}C–^{15}N coupling ($^1J_{CN} \sim 15$ Hz).

The antibody used in the above work is a member of switch variant IgG family developed by Dangl et al. (1982). A fascinating feature of these antibodies is that they possess the identical V_H, V_L, and C_L in conjunction with different heavy chain constant regions, providing us with an interesting possibility of discussing the relationship between domain–domain interactions and antibody functions. We have therefore extended the application of ^{13}C NMR spectroscopy to a panel of switch variant antibodies, which are of the IgG1, IgG2a, and IgG2b subclasses. Hereafter these antibodies will simply be referred to as IgG1, IgG2a, and IgG2b. The panel of switch variant antibodies used also contains an interesting short-chain IgG2a antibody, in which the entire C_H1 domain is deleted (Dangl et al., 1982; Dangl & Herzenberg, 1982; Oi et al., 1984; Igarashi et al., 1990). The short-chain IgG2a antibody will be designated as IgG2a(s). We have shown that all of the methionyl carbonyl carbon resonances of the switch variant IgG1, IgG2a, IgG2a(s), and IgG2b antibodies can be assigned with use of a variety of techniques involving limited proteolysis, recombination of labeled (unlabeled) heavy chains and unlabeled (labeled) light chains, and the double-labeling technique (Kato et al., 1991).

In the present paper, we will use a variety of analogues of the switch variant antibodies and their proteolytic fragments for the molecular structural study of antigen binding and also of domain–domain interactions. In order to obtain information about the antigen-binding site, which is composed of six CDR loops, three each from the heavy and light chains, we will use analogues labeled at the carbonyl carbon with Trp, Tyr, or His. It is known that one Trp and two Cys residues are conserved in the *pin region* of all immunoglobulin domains (Lesk & Chothia, 1982). We therefore also use an analogue labeled with [1-^{13}C]Cys.

Fab and Fc fragments have been prepared by papain digestion from each of the labeled antibodies. Limited digestion of the IgG2a(s) antibody gives in high yield the Fv fragment, which is a heterodimer of the V_H and V_L domains (Takahashi et al., 1991a). A three-domain Fab* fragment comprising the V_H, V_L, and C_L domains was also obtained from the IgG2a(s) antibody (Kato et al., 1991). Spectral assignments will be made by following the general strategy that has been established in our previous work (Kato et al., 1989, 1991). The ^{13}C data obtained for a variety of labeled proteins will be used along with those reported previously for the switch variant antibodies labeled with [1-^{13}C]Met (Kato et al., 1991) for the structural analyses of antigen binding and also of domain–domain interactions in the antibody molecule.

Materials and Methods

Materials. L-[1-^{13}C]Tyr and L-[1-^{13}C, ^{15}N]Tyr were prepared by an enzymatic coupling of phenol with [1-^{13}C]pyruvate in the presence of CH_3COONH_4 and [^{15}N]NH_4Cl, respectively (Nagasawa et al., 1981). L-[1-^{13}C]Cystine was kindly provided by Dr. N. Sugita. L-[^{15}N]Leu, L-[α-^{15}N]Lys·2HCl, DL-[^{15}N]Met, L-[^{15}N]Tyr, and L-[^{15}N]Val were purchased from Isocommerz GmbH, Germany. L-[^{15}N]Pro was purchased from CIL. All other ^{13}C- and ^{15}N-labeled amino acids were purchased from ICON Service Inc., USA. The isotope enrichment is 95% or higher for each of these amino acids. Gly-L-Gln was kindly supplied by Dr. Y. Minamoto. Clostripain, papain, and ϵ-dansyl-L-lysine (DNS-Lys) were from Sigma. All other chemicals were of reagent grade and were used without further purification.

Cell Lines and Stable-Isotope Labeling. Switch variant cell lines 27-44 (IgG1), 27-13.6 (IgG2a), 27-1B10.7 (IgG2a(s)), and 27-35.8 (IgG2b) (Dangl et al., 1982; Dangl & Herzenberg, 1982) were kindly provided by Professor L. A. Herzenberg and Dr. V. T. Oi. Hybridoma cells adapted to a serum-free medium (Nissui NYSF 404) were grown in tissue culture flasks at 37 °C in a humidified atmosphere of 5% CO_2/95% air.

Protocols for the preparation of antibodies selectively labeled with a stable isotope have been described previously (Kato et al., 1991). In incorporating the ^{15}N-labeled amino acids except [α-^{15}N]Lys and [^{15}N]Thr, isotope dilution had to be suppressed. For the incorporation of [^{15}N]Ala, 16 mg of β-chloro-L-alanine and 60–70 mg of L-[^{15}N]Ala were added to 1 L of the medium that contained DL-*threo*-β-hydroxyaspartic acid at a concentration of 0.5 mM. For the labeling with [^{15}N]Gly and [^{15}N]Ser, 16 mg/L β-chloro-L-alanine and 100 mg/L L-Ala were added and unlabeled Gly and L-Ser in the medium were replaced by 50 mg/L [^{15}N]Gly and 29.5 mg/L L-[^{15}N]Ser, respectively. Double labeling with [^{15}N]Pro has successfully been achieved by using Gly-L-Gln (500 mg/L) in place of Glu and Gln. For the double labeling of all other antibodies, 16 mg/L β-chloro-L-alanine and 100 mg/L L-Ala were added to the medium. Incorporation of [^{15}N]Asn has been attempted without success. After cell growth, the cell supernatant was concentrated with a Millipore Minitan ultrafiltration system and then applied to an Affi-Gel protein A column (Bio-Rad). A typical yield was 10–40 mg of purified antibody/L of the cell culture.

Limited Proteolysis of IgG Proteins. Fab and Fc fragments of the switch variant IgG1, IgG2a, and IgG2b antibodies were prepared by papain digestion as described previously (Kato et al., 1991). Clostripain digestion was used for the preparation of the Fab* and Fv fragments (Kato et al., 1991; Takahashi et al., 1991a).

NMR Measurements. For NMR measurements, the protein solutions were concentrated to a final volume of 2 mL in 5 mM phosphate buffer, pH 7.4, containing 0.2 M NaCl and 3 mM NaN_3 in D_2O unless otherwise stated. A 10-mm NMR sample tube was used with a final protein concentration of 0.1–0.4 mM. NMR measurements were made on a Bruker AM 400 spectrometer. ^{13}C NMR spectra were recorded at 100 MHz with use of a Waltz 16 composite pulse decoupling sequence. The free induction decay was recorded with 32K data points and a spectral width of 24000 Hz. For resolution enhancement, the free induction decay was multiplied by the Gaussian window function prior to Fourier transformation. Chemical shifts are given in parts per million from internal dioxane. The probe temperature was 30 °C.

Molecular Modeling. The molecular model for the anti-dansyl Fv fragment was calculated by starting from the X-ray crystallographic data obtained for the Fab of mouse McPC603 myeloma IgA antibody (Satow et al., 1986). The program NEC BIOCES[E] has been used for the calculation.

Results

Each of the switch variant antibodies has been labeled with ^{13}C at the carbonyl carbon of the Trp, Tyr, His, or Cys residue. Fab and Fc fragments were prepared from the labeled IgG1, IgG2a, and IgG2b antibodies. A variety of Fv and Fab* analogues were obtained by clostripain digestion of labeled IgG2a(s) proteins. The labeled antibodies and their fragments will be designated using the one-letter abbreviation for the amino acid, e.g., [W]IgG1, [Y]Fab, [H]Fab*, and [C]Fv. In each of the spectra, resonances are coded in alphabetical order starting from the lowest field resonance. Overlapped peaks are coded with different letters.

Table I: Resonance Assignments for Trp, His, Tyr, and Cys Residues in the Fv Fragment

residue	domain	residue no.	X[a]	location	assignment[b]
Trp	V_H	33	Met	CDR1(H)	e
		36[c]	Val	FR2(H)	
		47[c]	Val	FR2(H)	
		101	Phe	CDR3(H)	f
		105	Gly	FR4(H)	a
	V_L	35	Tyr	FR2(L)	c
His	V_H	55	Ala	CDR2(H)	b
		98	Tyr	CDR3(H)	c
	V_L	27D	Ser	CDR1(L)	a
		34	Trp	CDR1(L)	e
		93	Val	CDR3(L)	d
Tyr	V_H	58[d]	Tyr	CDR2(H)	
		59	Ala	CDR2(H)	d
		79[e]	Leu	FR3(H)	
		90[d]	Tyr	FR3(H)	
		91	Cys	FR3(H)	g
		96[d]	Tyr	CDR3(H)	
		97	His	CDR3(H)	b
		99	Pro	CDR3(H)	l
		104	Trp	CDR3(H)	i
	V_L	32[e]	Leu	CDR1(L)	
		36[e]	Leu	FR2(L)	
		49	Lys	FR2(L)	f
		86	Phe	FR3(L)	a
Cys	V_H	22	Ala	FR1(H)	b
		92	Thr	FR3(H)	c
	V_L	23	Arg	FR1(L)	d
		88	Ser	FR3(L)	a

[a] Residue that directly follows in the amino acid sequence; e.g., Trp-33H is followed by Met-34H. [b] Resonance lines observed for Trp, His, Tyr, and Cys residues are identified as shown in Figure 1 and parts A, B, and C of Figure 2, respectively. Site-specific assignments have been established except those given in the following footnotes. [c] Trp-36H, Trp-47H: b or d (Figure 1). [d] Tyr-58H, Tyr-90H, Tyr-96H: c, h, or k (Figure 2B). [e] Tyr-79H, Tyr-32L, Tyr-36L: e, j, or m (Figure 2B).

Spectral Assignments. A general strategy for the resonance assignments reported previously for Met analogues of the switch variant antibodies and their fragments (Kato et al., 1991) will be followed for all of the ^{13}C-labeled proteins used in the present work. The procedure for the assignments will be described in some detail for [W]Fv.

(i) [W]Fv. The Fv fragment contains six Trp residues (see Table I). As Figure 1 shows, [W]Fv gives, in the absence of ε-dansyl-L-lysine (DNS-Lys), the six resonances a, b, c, d, e, and f, where peaks b and e are overlapping with peaks c and f, respectively. The ^{13}C NMR spectra of [W, G]Fv, [W, V]Fv, and [W, Y]Fv measured in the absence of DNS-Lys are also included in Figure 1. It was observed that resonances indicated by the arrows are split into a doublet with $^1J_{CN}$. Comparisons of these results with the sequence data summarized in Table I have led to the assignments of a, b, c, and d to Trp-105H, Trp-36H (or Trp-47H), Trp-35L, and Trp-47H (or Trp-36H), respectively.[2,3] Trp-36H and Trp-47H cannot be differentiated by the double-labeling method because both of them are followed by Val.

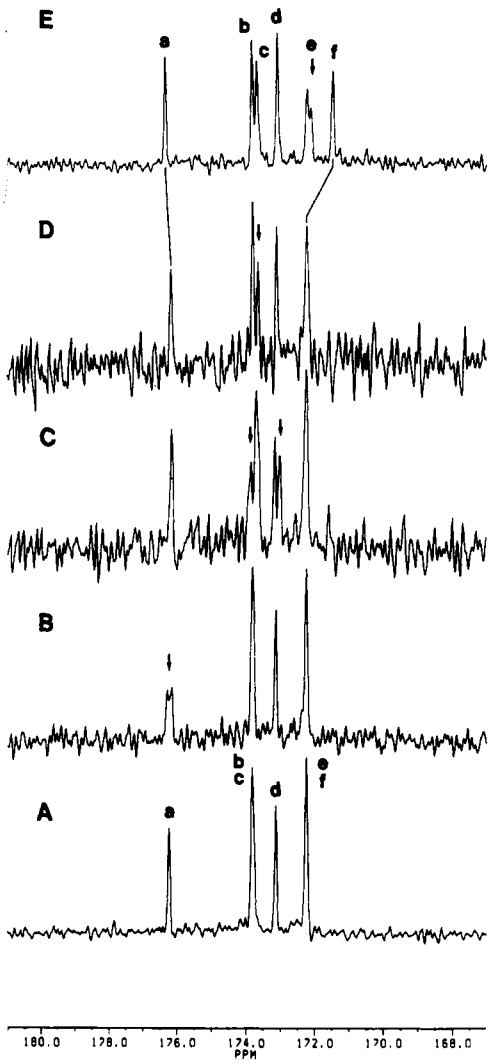

FIGURE 1: 100-MHz ^{13}C NMR spectra of (A) [W]Fv, (B) [W, G]Fv, (C) [W, V]Fv, (D) [W, Y]Fv, and (E) [W, M]Fv. Spectra A–D were measured in the absence of DNS-Lys whereas spectrum E was measured in the presence of a 1.1-fold molar excess of DNS-Lys. The arrows indicate the resonances split into a doublet due to $^1J_{CN}$. Spectral assignments are summarized in Table I. All sample solutions were made in 5 mM phosphate buffer containing 0.2 M NaCl in D_2O. Concentrations and the pH of the sample solutions were 0.1–0.2 mM and 7.4, respectively. The probe temperature was 30 °C; 13 000–77 000 transients were accumulated for each spectrum, where 32K data points and a spectral width of 24 000 Hz were used with a delay time of 0.3 s.

No splitting was observed in the spectra of [W, M]Fv and [W, F]Fv (data not shown). Apparently two overlapping resonances possess almost identical chemical shifts for peaks e and f, and the splitting due to $^1J_{CN}$ is exceeded by the line width of the two overlapped resonances. However, the results of the double-labeling experiments described above indicate that the overlapping peak coded with e and f is a superposition of Trp-33H and Trp-101H resonances. The Trp-33H resonance can clearly be identified in the spectrum of [W, M]Fv measured in the presence of DNS-Lys (see Figure 1E). The ^{13}C data given in Figure 1 indicate that Trp-101H and Trp-105H give chemical shifts that are significantly different in the absence and presence of DNS-Lys. In the case of the remaining four Trp residues, chemical shift changes induced by the addition of DNS-Lys were less than 0.1 ppm. It should be noted that Trp-101H and Trp-105H are located in the

[2] Sequence data of the V_H region of the switch variant antibodies used in the present study has been given by J. L. 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.

[3] The numbering system used in the present paper for the constant region is based on human myeloma protein Eu for the heavy chain (Edelman et al., 1969) and mouse myeloma protein MOPC21 for the light chain (Kabat et al., 1987). 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. Amino acid residues in the heavy and light chains are identified by H and L, respectively, e.g., Trp-101H and Trp-35L.

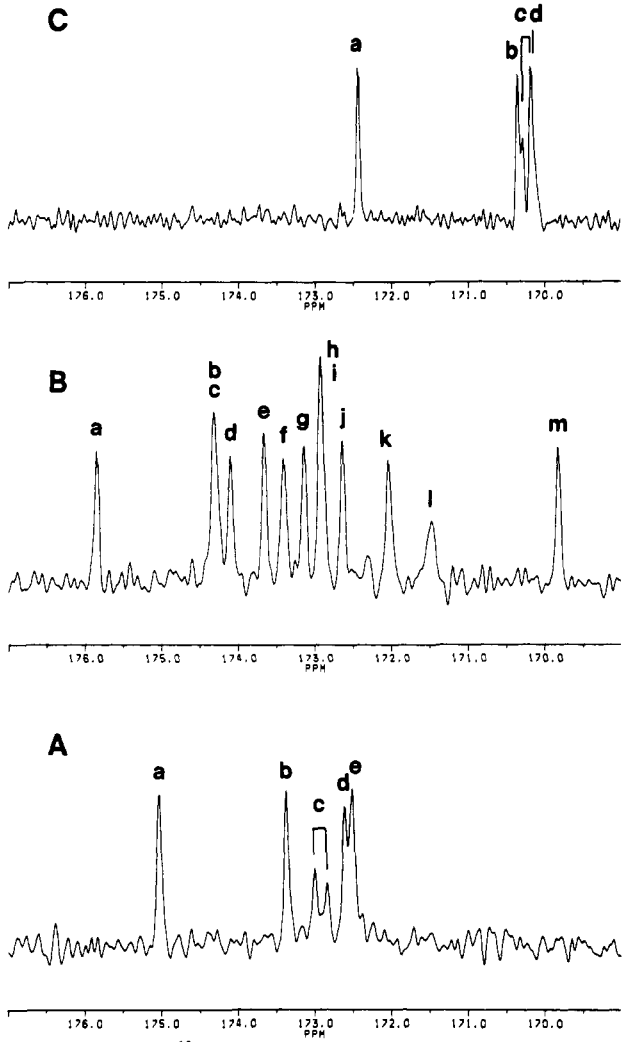

FIGURE 2: 100-MHz ^{13}C NMR spectra of (A) [H, Y]Fv, (B) [Y]Fv, and (C) [C, T]Fv. Spectral assignments are given in Table I. Sample solutions were made in 5 mM phosphate buffer containing 0.2 M NaCl in 90% H_2O/10% D_2O. Concentrations and the pH were 0.2–0.4 mM and 7.4, respectively. Other experimental conditions are as in Figure 1.

CDR3(H) loop and at the junction of CDR3(H) and FR4(H), respectively.

The result of assignments of the Trp resonances is summarized in Table I.

(ii) [H]Fv. The Fv fragment contains five His residues, all of which belong to the CDR loops (see Table I). The ^{13}C spectrum of [H, Y]Fv observed in the absence of DNS-Lys is given in Figure 2A, where resonance c is split into a doublet. Table I indicates that among the five His residues His-98H is the only one that is directly linked by a peptide bond to Tyr. Therefore, resonance c can unambiguously be assigned to His-98H. Assignments of all other His resonances have been made in a similar way, and the result is summarized in Table I. Resonance c was shifted upfield by 0.22 ppm upon addition of DNS-Lys. It is to be noted that His-98H is located in CDR3(H).

(iii) [Y]Fv. The ^{13}C spectrum of [Y]Fv observed in the absence of DNS-Lys is given in Figure 2B. In the absence of the antigen, peaks b and h are overlapped with peaks c and i, respectively. Among 13 Tyr residues that exist in the Fv fragment, three of them are followed in the amino acid sequences by another Tyr. There also exist three Tyr-Leu dipeptides in the amino acid sequences. Therefore, site-specific assignments were not possible for these six Tyr residues.

Table II: Resonance Assignments for Trp Residues in the IgG1, IgG2a, and IgG2b Antibodies and Fab* fragment

		assignment[a]			
domain	residue no.	Fab*	IgG1	IgG2a	IgG2b
V_H	33	g	h	i	i
	36[b]				
	47[b]				
	101	h	i	j	j
	105	a	a	b	b
V_L	35	e	e	f	f
C_L	148	c	c	d	c
	163	b	b	c	a
C_H1	158		f	a	d
	194		–[d]	g	g
C_H2	277		n	n	n
	313		j	k	k
C_H3	381[c]			m	l
	383[c]			–[d]	–[d]
	417[c]			l	m

[a] Resonances are identified with the alphabetic codes shown in Figures 3 and 4. Site-specific assignments have been established except those given in the following footnotes. [b] Trp-36H, Trp-47H: d or f (Fab*), d or g (IgG1), e or h (IgG2a), and e or h (IgG2b). [c] Trp-381H, Trp-383H, Trp-417H (IgG1): k, l, or m. [d] Trp is replaced here by other amino acids.

Results of spectral assignment are summarized in Table I.

The chemical shifts for Tyr-97H, Tyr-99H, and Tyr-104H, all of which belong to the CDR3(H) loop, were affected to a large extent upon antigen binding; a downfield shift of 0.53 ppm, an upfield shift of 0.62 ppm, and an upfield shift of 0.90 ppm were observed for Tyr-97H, Tyr-99H, and Tyr-104H, respectively. It is also observed that DNS-Lys induces a small but significant shift of <0.2 ppm for resonances c, e, f (Tyr-49L), and g (Tyr-91H).

(iv) [C]Fv. The Fv fragment possesses four Cys residues, two each from the V_H and V_L domains. ^{13}C NMR spectra were measured in the absence and presence of DNS-Lys, and all of the Cys resonances have been assigned by the double-labeling method. Table I summarizes the result of the assignment.

The spectrum of [C, T]Fv measured in the absence of DNS-Lys is given in Figure 2C, where the Cys-92H resonance is split into a doublet. It was observed that the Cys-22H resonance is shifted downfield by 0.12 ppm in the presence of the antigen. A small but significant upfield shift was also observed in the case of the Cys-92H resonance. The remaining two resonances originating from the light chain, i.e., Cys-23L and Cys-88L, did not show any significant shift upon antigen binding.

(v) Assignments of ^{13}C Resonances of IgGs to Fab and Fc. Figure 3 compares the ^{13}C NMR spectra of [W]IgG1, [W]-IgG2a, and [W]IgG2b with those of their digestion products Fab and Fc. In each pair of Fab and Fc, the observed resonances have been coded in alphabetical order starting from the lowest field resonance for Fab, which is followed by that for Fc. The ^{13}C data indicate that the carbonyl carbon resonances observed for the intact antibodies are simply a superposition of those for the corresponding Fab and Fc fragments. This is consistent with our previous result obtained by using the panel of the switch variant antibodies labeled with [1-^{13}C]Met (Kato et al., 1991). On the basis of these spectral data we assign the ^{13}C resonances observed for the intact antibodies to the corresponding Fab and Fc fragments.

(vi) Fab and Fab.* Figure 4 compares Trp resonances observed for [W]Fv, [W]Fab*, and a panel of [W]Fab proteins derived from the IgG1, IgG2a, and IgG2b antibodies. Each of the resonances observed for [W]Fv is conserved in

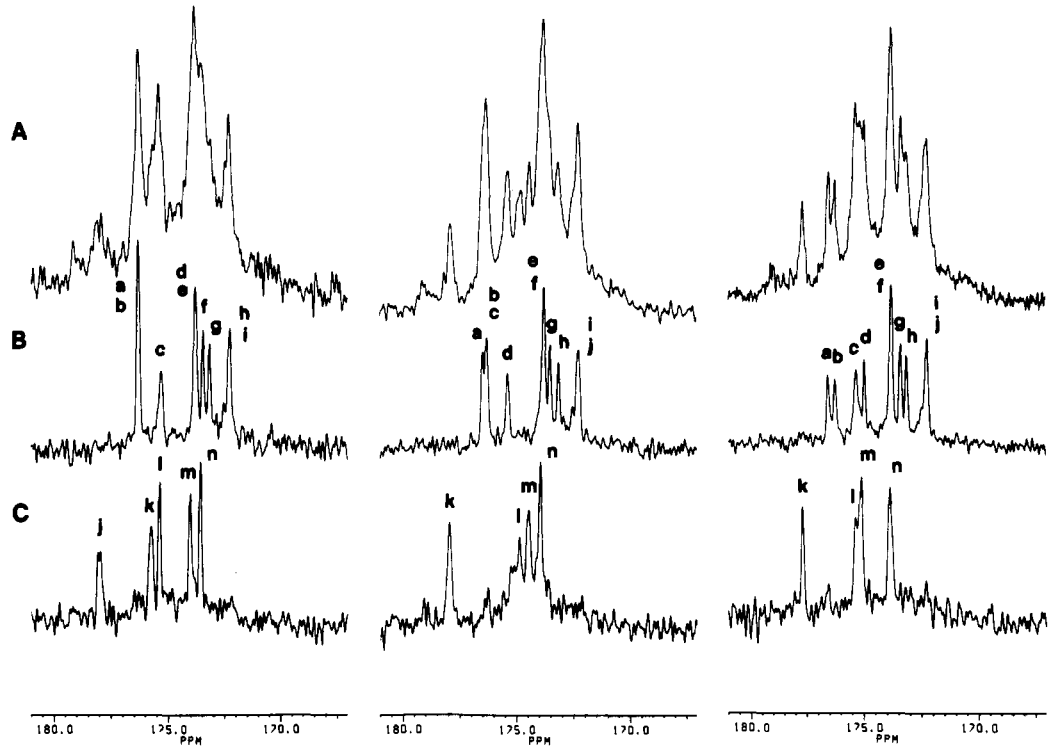

FIGURE 3: Comparisons of 100-MHz ^{13}C NMR spectra of switch variant anti-dansyl antibodies IgG1, IgG2a, and IgG2b labeled with [1-^{13}C]Trp and the Fab and Fc fragments derived from each of the IgGs: (A) [W]IgG, (B) [W]Fab, and (C) [W]Fc. Spectral assignments are summarized in Table II. Sample solutions were made in 5 mM phosphate buffer containing 0.2 M NaCl in D_2O. Protein concentrations and the pH of the sample solutions were in the ranges 0.1–0.4 mM and 7.1–7.4, respectively; 26 000–120 000 transients were accumulated for the measurements. For each measurement, 32K data points were used with a spectral width of 24 000 Hz and a delay of 0.3 s. In each set of Fab and Fc fragments, resonance lines were coded alphabetically starting from the signal at the lowest field. The probe temperature was 30 °C.

the spectra of Fab* and the Fab fragments, giving the identical chemical shifts. On the basis of these results, all the resonances originating from the constant region (either C_H1 or C_L) of the Fab and Fab* fragments have been identified. Site-specific assignments of most of the resonances originating from the constant domains (C_H1, C_H2, C_H3, and C_L) have been established by the double-labeling method, and the results are summarized in Table II.

Discussion

Use of the Carbonyl Carbon Chemical Shifts for Mapping Antigen-Binding Sites. The ^{13}C data obtained so far by using the carbonyl carbon resonances of Trp, Tyr, His, and Cys as spectroscopic probes are summarized in Figure 5 with the use of a molecular model of Fv. Our previous data obtained with Met analogues (Kato et al., 1991) are also included in Figure 5. Chemical shift changes of larger than 0.3 ppm were observed for the Tyr-97H, Tyr-99H, Trp-101H, and Tyr-104H resonances. It was observed that the addition of DNS-Lys also induces a significant shift of larger than 0.1 ppm in the case of His-98H and Trp-105H resonances. It is to be noted that the spectral changes observed in the presence of DNS-Lys are confined to the CDR3(H) loop, which possesses the amino acid sequence

-Ile^{95}-Tyr-Tyr-His-Tyr-Pro-Trp-Phe-Ala-Tyr-Trp^{105}-

We have recently reported a ^1H NMR study of the interaction of the Fv fragment with DNS-Lys (Takahashi et al., 1991a). Two-dimensional NOESY experiments were performed with the use of selectively deuterated Fv analogues, and it has been concluded that the aromatic rings of two Tyr residues are interacting with the dansyl ring of DNS-Lys. We have further demonstrated on the basis of a chemical modification experiment that one of these two Tyr residues is Tyr-97H or Tyr-99H in the CDR3(H) loop (Takahashi et al., 1991b). We therefore conclude that the observed change in chemical shift of the carbonyl carbon resonances is detecting the antigen binding and that the CDR3(H) loop is primarily responsible for the antigen binding in the switch variant antibodies used in the present study. This conclusion is further supported by measurements of ^1H–^{15}N shift correlation spectra of Fv labeled with [^{15}N]Tyr (Takahashi et al., 1991b).

The antigen binding also affected the chemical shifts of other residues, e.g., Tyr-49L and Cys-22H. We have previously reported that a small downfield shift of 0.14 ppm is induced by the antigen binding in the case of Met-34H, which is located in the CDR1(H) (Kato et al., 1991).

It is known that two of the Cys residues are conserved in all immunoglobulin domains including V_H and V_L, forming the core of the *pin region* (Lesk & Chothia, 1982). The present ^{13}C data show that the binding of DNS-Lys affects the chemical shifts of the Cys-22H resonance and, to a lesser extent, of the Cys-92H resonance. It should be noted that no significant change in chemical shift was observed for Cys-23L and Cys-88L, which exist in the *pin region* of V_L. Cys-92H is close to the CDR3(H) loop in the amino acid sequence. It is of interest that upon antigen binding the resonance of Cys-22H, which is far apart from the CDR3(H) loop but is bonded to Cys-92H through the disulfide bridge, shows a chemical shift change that is larger than that observed for Cys-92H.

In addition to the two Cys residues mentioned above, one Trp residue is known to be conserved in the *pin region* of all immunoglobulin domains (Lesk & Chothia, 1982). In the case of [W]Fv, Trp-36H and Trp-35L, which exist in the *pin region*, show little or no change in chemical shift upon antigen binding.

Carbonyl Carbon Resonances as Spectroscopic Probes for the Analyses of Domain–Domain Interactions. The Fab* and

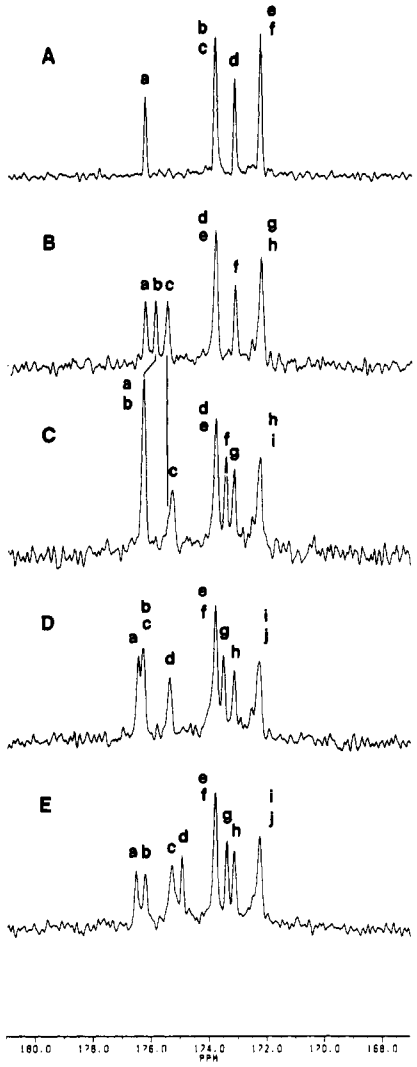

FIGURE 4: Comparisons of the ^{13}C NMR spectra of (A) [W]Fv, (B) [W]Fab*, (C) [W]Fab (IgG1), (D) [W]Fab (IgG2a), and (E) [W]Fab (IgG2b). Each resonance line is coded as in Figures 1 and 3. Sample solutions were made in 5 mM phosphate buffer containing 0.2 M NaCl in D_2O. Other experimental conditions are as in Figure 3.

Fab fragments used in the present study had been prepared from the panel of switch variant antibodies, in which the V_H, V_L, and C_L domains are identical. Therefore, as far as the amino acid sequences are concerned, the only difference in the Fab* and Fab fragments is the C_H1 domain. In the Fab* fragment, the entire C_H1 domain is missing, whereas the three Fab fragments possess different C_H1 domains that are highly homologous but not quite identical in the amino acid sequences. Therefore, the Fab* and Fab fragments would constitute an ideal system in discussing domain–domain interactions in the antibody. In the following, the ^{13}C NMR data obtained for the Trp-148L and Trp-163L resonances will be used along with those for Met-18H and Met-175L, which had been reported previously (Kato et al., 1991).

(*i*) *Trp-148L.* Comparisons of the ^{13}C spectra given in Figure 4 indicate that deletion of the C_H1 domain results in a downfield shift of 0.2 ppm for Trp-148L. It is known that Trp-148L is a member of the homologous Trp residues, which are conserved and exist in the *pin region* of immunoglobulin domains (Lesk & Chothia, 1982). It is of interest that the chemical shift of Trp-148L, which is deeply buried inside the immunoglobulin fold of the C_L domain, sensitively reflects the absence and presence of the C_H1 domain. However, very little

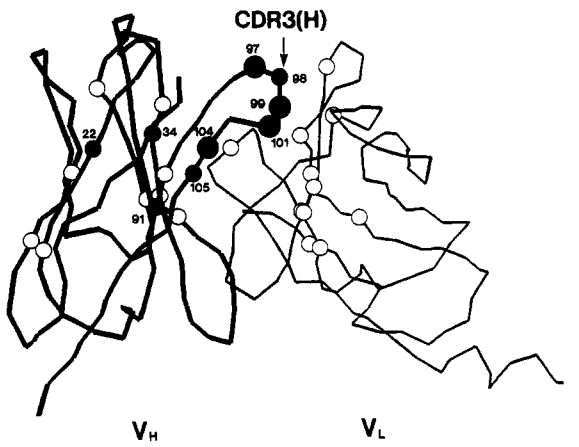

FIGURE 5: Summary of the ^{13}C NMR measurements obtained with use of the carbonyl carbon resonances of Trp, Tyr, His, Cys, and Met as spectroscopic probes for the antigen binding. The effects of the antigen on the chemical shifts for one of these spectroscopic probes are indicated on the molecular model of the Fv fragment. Chemical shift changes observed are larger than 0.3 ppm (large closed circles), 0.1–0.3 ppm (small closed circles), less than 0.1 ppm (open circles). The molecular model was based on the X-ray crystallographic data obtained for the Fab of mouse McPC603 myeloma antibody (Satow et al., 1986). Among six Tyr resonances whose assignments have not been completed, all three Tyr resonances originating from the Tyr-Leu dipeptide were shifted less than 0.1 ppm in the presence of DNS-Lys and therefore are included as such in the figure.

differences in chemical shift were observed for the three Fab preparations of different subclasses.

(*ii*) *Trp-163L.* The Trp-163L resonance observed for Fab* is shifted upfield by 0.4–0.6 ppm from those for the Fab fragments that retain the C_H1 domain (see Figure 4). It should be noted that, in marked contrast to what was observed for Trp-148L, the three kinds of the Fab fragments obtained from the IgG1, IgG2a, and IgG2b antibodies show small but significant differences in the Trp-163L chemical shift.

(*iii*) *Met-175L.* In previous work, we have shown that the Met-175L resonance for the Fab* fragment shows a large downfield shift of 1.6 ppm from those for the Fab fragments (Kato et al., 1991). Significant shifts were also observed among the Fab fragments prepared from the IgG1, IgG2a, and IgG2b antibodies. It has been confirmed that the Met-175L chemical shifts do not change at all on recombination of the heavy and light chains, which involves reduction and alkylation of the interchain disulfide bridges and denaturation and renaturation of the proteins (Kato et al., 1991). This means that the differences in the Met-175L chemical shift observed for the intact antibodies are *not* due to a different degree of distortion in domain–domain interactions induced in each of the antibodies by different combinations of interdomain disulfide bridges.

(*iv*) *Met-18H.* We have previously shown that the Met-18H chemical shifts are solely dependent upon the absence or presence of the C_H1 domain (Kato et al., 1991). The Met-18H chemical shifts were identical for the Fab fragments of the IgG1, IgG2a, and IgG2b antibodies but were significantly different from those for the Fv fragment, the Fab* fragment, and the intact IgG2a(s).

(*v*) *Domain–Domain Interactions.* The X-ray crystallographic structure of the Fab fragment of McPC603, a mouse myeloma IgA(κ) protein, has been reported by Satow et al. (1986). The C_L domain of McPC603 is identical in the amino acid sequence with that for the switch variant antibodies used in the present work. The crystal structural study shows that (1) Trp-148L exists in the *pin region* of the C_L domain, (2) Met-175L and Trp-163L are located on the junction of the

C_H1 and C_L domains, and (3) Met-18H exists on the surface of the V_H but does not make any contact with the amino acid residues that belong to the C_H1 domain.

The Met-175L and Trp-163L chemical shifts observed for the Fab* fragment are different to a large extent from those for the Fab fragments. It is quite likely that this is due to deletion of the C_H1 domain. It should also be noted that small but significant differences were observed for the Met-175L and Trp-163L of the three kinds of the Fab fragments. The amino acid sequences of the V_H, V_L, and C_L domains are identical for all the switch variant antibodies examined in the present work. In addition, the amino acid sequences for the C_H1 domain of the IgG1, IgG2a, and IgG2b antibodies have a homology of more than 80% to each other. In the case of the switch variant antibodies used in the present work, there exist a few amino acid substitutions on the junction of the C_L and C_H1 domains. However, the amino acid residues in the C_H1 domain that are directly involved in contact with Met-175L and/or Trp-163L are all conserved for the switch variant antibodies and McPC603. It may therefore be concluded that substitution of the amino acid residue(s), which is(are) not located on the junction of the C_L and C_H1 domains, is responsible for the observed shifts for the Met-175L and Trp-163L resonances. We suggest that the carbonyl carbon chemical shift sensitively reflects a subtle difference in the mode of the domain–domain interactions in the different switch variant antibodies.

It should also be noted that small but significant shifts are observed for the Met-18H and Trp-148L resonances for the Fab* fragment as compared with those for the Fab fragments. We suggest that deletion of the C_H1 domain causes a conformational change in the C_L domain and part of the V_H domain.

The above results observed for the Met-18H, Met-175L, Trp-148L, and Trp-163L chemical shifts indicate that the carbonyl carbon chemical shifts can be a sensitive probe for the discussion of *lateral* as well as *longitudinal* domain–domain interactions, which play a crucial role in the construction of functional regions of the antibody molecule (Huber, 1980).

Concluding Remarks. On the basis of the ^{13}C along with ^1H NMR data, we have concluded that the CDR3(H) loop is primarily responsible for the antigen binding. The results of the present investigation indicate that the ^{13}C chemical shifts of the carbonyl carbon can be used for the identification of the *paratope* of the antibody.

It is of interest that binding of a small hapten like DNS-Lys induced a small but significant change in chemical shift for a variety of residues that are apart from the antigen-binding site. For example, the effect of the antigen binding was observed to extend to Cys-22H, which exists in the *pin region* of the immunoglobulin fold. Another example is Tyr-49L, which belongs to FR2(L). Although more experiments are obviously needed to discuss the nature of the ^{13}C chemical shift data in a more quantitative way, it may be concluded that this line of approach would provide us with invaluable information about the change in antibody structure in the absence and presence of the antigen.

In the present work it has been concluded that ^{13}C chemical shifts for the carbonyl carbon resonances are sensitive to the subtle difference in the way in which domains construct the functional sites of the antibody. On the basis of the crystal data reported for four different immunoglobulin molecules, Miller (1990) has discussed similarities and differences in the way in which immunoglobulin constant domains interact with each other. A fascinating feature of the switch variant family used in the present study is that these antibodies share the identical V_H, V_L, and C_L domains, thus providing us with an ideal system for the discussion of domain–domain interactions. Further ^{13}C study is now in progress in our laboratory for the better understanding of the static and dynamic structure of immunoglobulin molecules.

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