Complete Assignment of the Methionyl Carbonyl Carbon Resonances in Switch Variant Anti-Dansyl Antibodies Labeled with [1-^{13}C]Methionine†

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ABSTRACT: A ^{13}C NMR study is reported of switch variant anti-dansyl antibodies developed by Dangl et al. [(1982) *Cytometry 2*, 395-401], who had used the fluorescence-activated cell sorter to select and clone these variants. These switch variant antibodies possess the identical V_H, V_L, and C_L domains in conjunction with different heavy chain constant regions. In the present study, switch variant antibodies of IgG1, IgG2a, and IgG2b subclasses were used along with a short-chain IgG2a antibody, in which the entire C_H1 domain is deleted. The switch variant antibodies were specifically labeled with [1-^{13}C]methionine by growing hybridoma cells in serum-free medium. Assignments of all the methionyl carbonyl carbon resonances have been completed by using the intact antibodies along with their fragments and recombined proteins in which either heavy or light chain is labeled. A double labeling method [Kainosho, M., & Tsuji, T. (1982) *Biochemistry 21*, 6273-6279] has played a crucial role in the process of the spectral assignments. The strategy used for the assignments has been described in detail. In incorporating ^{15}N-labeled amino acids into the antibodies for the double labeling, isotope dilution caused a serious problem except in the cases of [α-^{15}N]lysine and [^{15}N]threonine, both of which cannot become the substrate of transaminases. It was found that β-chloro-L-alanine is most effective in suppressing the isotope scrambling. So far, spectral assignments by the double labeling method have been possible with ^{15}N-labeled Ala, His, Ile, Lys, Met, Ser, Thr, Tyr, and Val. On the basis of the results of the present ^{13}C study, possible use of the assigned carbonyl carbon resonances for the elucidation of the structure-function relationship in the antibody system has been briefly discussed.

Immunoglobulin G (IgG),[1] which has a molecular weight of 150K, consists of two identical heavy chains and two identical light chains. The heavy chains consist 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. In the expression 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 by the Fc region that is composed of two C_H2 and two C_H3 domains.

Dangl et al. (1982) have generated a family of switch variants, which produce mouse anti-dansyl monoclonal antibodies with the identical V_H, V_L, and C_L in conjunction with different heavy chain constant regions. This family of switch variant antibodies, which had been selected and cloned by using the fluorescence-activated cell sorter, contains an interesting *short-chain* IgG2a variant, the molecular weight of which is 10K smaller than that of the normal IgG2a heavy chain (Dangl et al., 1982; Dangl & Herzenberg, 1982; Oi et al., 1984). In a previous paper (Igarashi et al., 1990) we have shown that the entire C_H1 domain is deleted in this short-chain IgG2a antibody, which will hereafter be designated as IgG2a(s).

We have recently reported a ^{13}C NMR study of an IgG2b antibody, which is a member of the switch variant family described above (Kato et al., 1989). The carbonyl carbon of the Met residues of the IgG2b antibody was specifically labeled with [1-^{13}C]Met. It has been shown that even with the intact antibody with a molecular weight of 150K (1) the carbonyl carbon resonances can be observed separately, (2) each of the carbonyl carbon resonances can be assigned to either the Fab or Fc region, and (3) a double labeling method developed by Kainosho and co-workers (Kainosho & Tsuji, 1982; Kainosho et al., 1985) is applicable to site-specific resonance assignments.

In the present paper we used switch variant IgG1, IgG2a, IgG2a(s), and IgG2b anti-dansyl antibodies. Hereafter, these antibodies will simply be referred to as IgG1, IgG2a, IgG2a(s), and IgG2b. Each of these antibodies was specifically labeled with [1-^{13}C]Met. Fab and Fc fragments have been prepared by papain digestion of the IgG1, IgG2a, and IgG2b antibodies. A limited proteolysis of the IgG2a(s) antibody, in which the entire C_H1 domain is missing, gives in an excellent yield the Fv fragment, which is a heterodimer of the V_H and V_L domains (Takahashi et al., 1991). A three-domain Fab* fragment comprising the V_H, V_L, and C_L domains was also obtained from the IgG2a(s) antibody.

The strategy adopted in the present study for the site-specific assignments of the methionyl carbonyl carbon resonances will

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[1] Abbreviations: C_L, constant region of the light chain; C_H1, C_H2, and C_H3, constant regions of the heavy chain; CDR, complementarity determining region; DEPT, distortionless enhancement by polarization transfer; DNS-Lys, ϵ-dansyl-L-lysine; DTT, dithiothreitol; Fab, antigen binding fragment; Fab*, a three-domain fragment composed of V_H, V_L, and C_L; Fab[H], Fab in which only the heavy chain is labeled with [1-^{13}C]Met; Fab[L], Fab in which only the light chain is labeled with [1-^{13}C]Met; Fc, fragment composed of the C-terminal halves of the heavy chains; Fv, antigen binding fragment composed of V_H and V_L; IgG, immunoglobulin G; IgG[H], IgG in which only the heavy chain is labeled with [1-^{13}C]Met; IgG[L], IgG in which only the light chain is labeled with [1-^{13}C]Met; IgG2a(s), a short-chain mouse monoclonal antibody that lacks the entire C_H1 domain; [M]IgG, IgG in which all the Met residues are labeled with [1-^{13}C]Met; [M,X]IgG, IgG that is doubly labeled with [1-^{13}C]Met and ^{15}N-labeled amino acid X; NMR, nuclear magnetic resonance; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; V_H, variable region of the heavy chain; V_L, variable region of the light chain.

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be described in detail. The digestion products Fv, Fab*, Fab, and Fc have been used for *fragment-specific* and *domain-specific* resonance assignments. A method originally employed by Anglister et al. (1985) will be used for *chain-specific assignments* of resonances to either the heavy chain or the light chain. The double labeling method played a crucial role in the process of the spectral assignments. By combining the results of all of these experiments, site-specific assignments of all the carbonyl carbon ^{13}C resonances have been completed for the switch variant IgG1, IgG2a, IgG2a(s), and IgG2b antibodies.

We will briefly discuss the possibility of using ^{13}C chemical shift data for the analyses of domain–domain interactions and also the mode of interactions of the antibody with the antigen and with molecules such as staphylococcal protein A.

Materials and Methods

Materials. L-[1-^{13}C]Met was purchased from Isotec, Inc., Miamisburg, OH. L-[^{15}N]Ala, DL-[α-^{15}N]His·2HCl, 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. All other labeled amino acids were purchased from ICON Services Inc., Summit, NJ. The isotope enrichment is 95% or higher for each of these amino acids. Guanidinium chloride of biochemistry grade was obtained from Wako Pure Chemical Industries, Ltd. Clostripain and papain 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), which had been selected and cloned by using the fluorescence-activated cell sorter (Dangl et al., 1982; Dangl & Herzenberg, 1982), were kindly provided by Professor L. A. Herzenberg and Dr. V. T. Oi. The hybridoma cells were adopted to a serum-free medium (Nissui NYSF 404) and then grown in tissue culture flasks. For the preparation of antibodies, in which the Met residues are selectively labeled with ^{13}C at the carbonyl carbon, the unlabeled methionine in the medium was replaced by an equal amount of L-[1-^{13}C]Met. One liter of the medium contained 14.6 mg of L-[1-^{13}C]Met. For the double labeling of antibodies, one of unlabeled amino acids in the medium was replaced by the equal amount of the corresponding ^{15}N-labeled amino acid. For example, 56 mg of DL-[α-^{15}N]His was added to 1 L of the medium in place of the unlabeled His. For brevity, IgG proteins singly labeled with [1-^{13}C]Met will be designated as [M]IgG. An IgG that is doubly labeled with [1-^{13}C]Met and [α-^{15}N]His will be designated as [M,H]IgG. Similar notations will be used for all labeled antibodies and their proteolytic fragments. For example, [W,M]Fv denotes an Fv fragment that is doubly labeled with [1-^{13}C]Trp and [^{15}N]Met.

In incorporating ^{15}N-labeled amino acids except [α-^{15}N]Lys and [^{15}N]Thr, β-chloro-L-alanine was added to the medium to suppress the isotope dilution. For the preparation of [M,A]IgG, 16 mg of β-chloro-L-alanine and 50 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 double labeling of all other antibodies, β-chloro-L-alanine and L-alanine were added to the medium at concentrations of 16 and 50–100 mg/L, respectively. [^{15}N]Gly was used in place of unlabeled glycine for the double labeling by [^{15}N]Ser. An IgG2a(s) antibody that is singly labeled with [^{15}N]Met was also prepared in a similar way.

After growth, the cell supernatant was concentrated with a Millipore Minitan Ultrafiltration System and then applied to an Affigel protein A column (Bio-Rad). A typical yield was 20–40 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 at pH 7.0, 37 °C, in 75 mM sodium phosphate buffer which contains 75 mM NaCl, 2 mM EDTA, and 5 mM NaN_3. The protein concentration was 5 mg/mL for all of the antibodies. The enzyme:substrate ratios (w/w) were 1:50 and 1:25 for IgG2a and the rest of the antibodies, respectively. In the cases of the IgG1 and IgG2a antibodies, L-cysteine·HCl·H_2O was added to the digestion buffer at concentrations of 2 mg/mL and 2 μg/mL, respectively. The incubation times were 2 h (IgG1), 6 h (IgG2a), and 8 h (IgG2b).

The Fab* and Fv fragments were obtained by digestion of IgG2a(s) using clostripain. For the preparation of the Fv fragment, the IgG2a(s) protein was dissolved at a concentration of 5 mg/mL in a digestion buffer (100 mM Tris-HCl and 150 mM NaCl, pH 7.5) containing 5 mM DTT and incubated with clostripain at 37 °C for 4–7 h. The enzyme:substrate ratio was 1:(50–100). For the preparation of the Fab* fragment, IgG2a(s) 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 incubated at room temperature for 20 min in the dark. The reaction was stopped by adding 10 mM DTT, and the reaction mixture was dialyzed against the digestion buffer used for the Fv fragment. The concentration of the reduced and alkylated IgG2a(s) in the digestion buffer was 5 mg/mL. The Fab* fragment was obtained at a shorter incubation period of 0.5–1 h and an enzyme:substrate ratio of 1:100. With an increase in the incubation time and the enzyme:substrate ratio, the yield of the Fab* fragment decreased, with a concomitant increase in the yield of the Fv fragment.

Each of the digestion products of the IgG1, IgG2a, and IgG2a(s) antibodies was loaded onto a Pharmacia Mono Q culumn equilibrated with 20 mM Tris-HCl, pH 8.0, and eluted at varying NaCl concentrations in the range 0–400 mM. In the case of the IgG2b antibody, the digestion products were separated on a Pharmacia Superose 12 column in 10 mM sodium phosphate buffer, pH 7.2, containing 150 mM NaCl. Purity of each of the protein preparations were checked by SDS–polyacrylamide gel electrophoresis.

Recombination of Light and Heavy Chains. The heavy and light chains were isolated from each of the switch variant IgG1, IgG2a, IgG2a(s), and IgG2b antibodies that had specifically been labeled with [1-^{13}C]Met at the carbonyl carbon. The labeled heavy and light chains were recombined, respectively, with light and heavy chains isolated from the unlabeled preparations of the corresponding antibodies. Recombination was performed basically according to the procedure described by Björk and Tanford (1971a–c). Each of the antibodies 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 incubated at room temperature for 20 min in the dark. The reaction was stopped by adding 10 mM DTT, and the reaction mixture was dialyzed against 10 mM phosphate buffer, pH 7.2, containing 150 mM NaCl. The reduced and alkylated antibodies were denatured by dialyzing 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. Unlabeled heavy and light chains were prepared in the same way. For recombination, 6 M guanidinium chloride

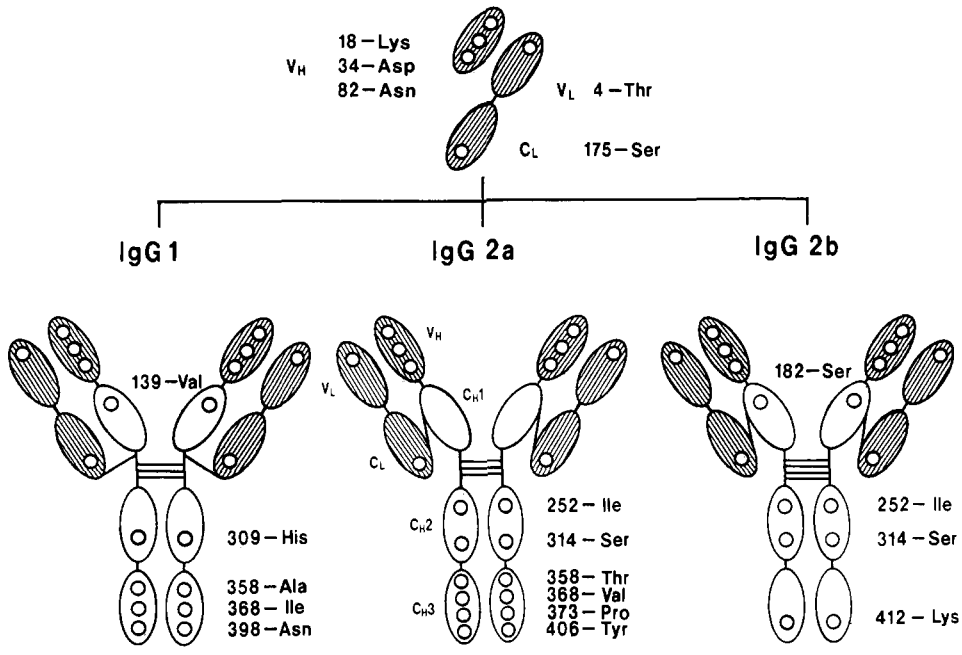

FIGURE 1: Schematic diagram of the distribution of all the Met residues in the switch variant anti-dansyl antibodies IgG1, IgG2a, and IgG2b. The Met residues are represented by the position numbers in the amino acid sequences, and the amino acid residue that is directly linked by a peptide bond to each of the Met residues is given by a three-letter code. For example, 18-Lys indicates a dipeptide sequence Met-18-Lys-19. The V_H, V_L, and C_L domains, which are identical in the amino acid sequences for all the proteins, are hatched and drawn separately on top of the figure.

solutions of labeled heavy (light) chains and of unlabeled light (heavy) chains were combined and dialyzed against 5 mM acetate buffer, pH 5.5. Buffer (100 mM phosphate) containing 1.5 M NaCl was gradually added to the dialysate over a period of 7–8 h, making the final concentration of 10 mM phosphate and 150 mM NaCl, pH 7.0. The recombined preparations of the switch variant antibodies were finally purified on a Pharmacia Superose 12 HPLC column equilibrated in 10 mM phosphate buffer, pH 7.2, containing 150 mM NaCl.

Limited proteolytic digestion of the recombined antibodies was performed as in the case of the native antibodies except that the concentration of cysteine was $0-{}^1/_{10}$ of that used for each of the corresponding native antibodies.

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. The final concentration of the proteins was in the range 0.1–0.4 mM. NMR measurements were made on Bruker AM 400 and JEOL JNM-GSX 400 spectrometers. ^{13}C NMR spectra were recorded at 100 MHz by using a Waltz 16 composite pulse decoupling sequence. 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 Gaussian window function prior to Fourier transformation. ^{15}N NMR spectra of [W,M]Fv, which is dissolved in 90% H_2O and 10% D_2O containing 5 mM phosphate, 0.2 M NaCl, and 3 mM NaN_3, pH 5.2, were measured by employing a DEPT sequence. An exponential window was applied to the acquired free induction decay prior to Fourier transformation. ^{13}C and ^{15}N chemical shifts are given in ppm from internal dioxane and external formamide, respectively. The probe temperature was 30 °C unless otherwise stated.

RESULTS

Basic Strategy for the Spectral Assignments for the Switch Variant Antibodies. The distribution of the Met residues in the amino acid sequences of the switch variant IgG1, IgG2a, and IgG2b antibodies used in the present experiment is schematically presented in Figure 1. We also use a short-chain antibody IgG2a(s), in which the entire C_H1 domain is deleted from the IgG2a antibody (Igarashi et al., 1990).

In principle, a most unambiguous method envisaged for the spectral assignments for the antibodies is the double labeling method. However, the double labeling method alone is obviously not quite sufficient for the assignments of all the Met resonances. This is especially true when the identical dipeptide Met-X, where X is one of 20 amino acids, appears more than twice in the polypeptide sequence(s) under consideration. In what follows, we will first briefly summarize the general strategy for the spectral assignments used in the present study.

The first step is to observe Met resonances for a variety of proteolytic fragments prepared from the IgG1, IgG2a, IgG2a(s), and IgG2b antibodies. We then compare these results with those obtained for the intact antibodies. We will show below that in most cases there is one-to-one correspondence between the Met resonances observed for the intact antibodies and their proteolytic fragments. If this is the case, the Met resonance observed for the intact antibodies can be assigned to the proteolytic fragments. This process will hereafter be referred to as *fragment-specific assignments*. When there is a significant difference in the chemical shift data for the intact proteins and their proteolytic fragments, observed results have to be interpreted with great care. In this situation we combine the fragment-specific assignment with other approaches, which will be described below.

One of other approaches is to assign the Met resonances to either the heavy chain or the light chain by using recombined proteins in which the heavy chain or the light chain is exclusively labeled by [1-^{13}C]Met. For this purpose we basically follow the procedure described by Anglister et al. (1985). In this experiment it should be confirmed that the resonances observed for the intact proteins are the sum of the two kinds of the corresponding recombined proteins. This step of the spectral assignments will be referred to as *chain-specific assignments*.

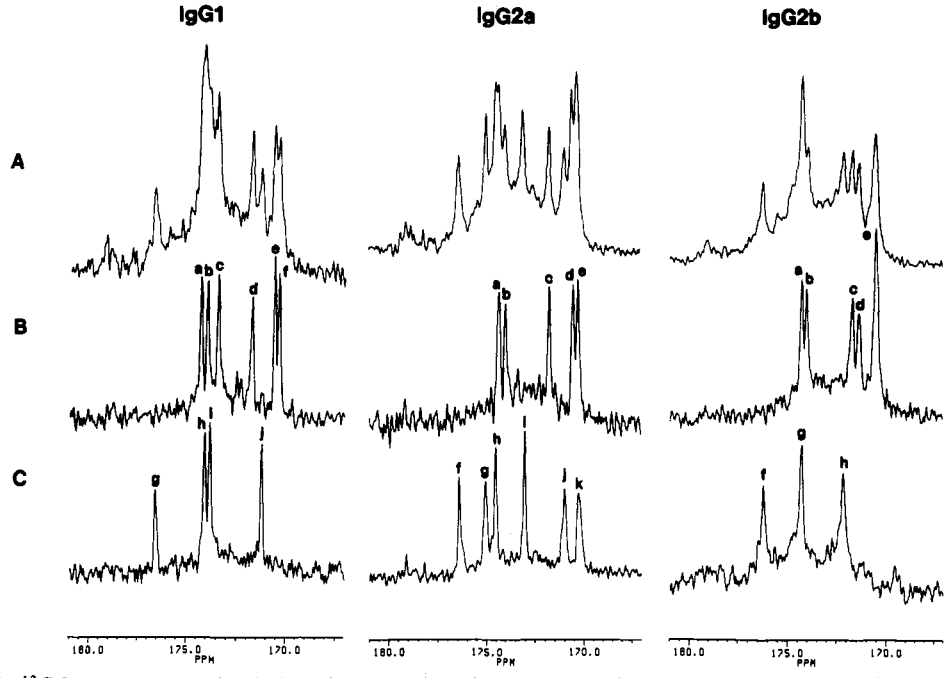

FIGURE 2: 100-MHz ^{13}C NMR spectra of switch variant anti-dansyl antibodies IgG1, IgG2a, and IgG2b. (A) [M]IgG; (B) [M]Fab; (C) [M]Fc. Protein concentrations and the pH of the sample solutions were in the range 0.1–0.4 mM and 7.1–7.5, respectively. The probe temperature was 30 °C. 32 000–180 000 transients were accumulated for the measurements. For each measurement, 32K data points were used with a spectral width of 24 000 Hz and a delay time of 0.3 s. In the spectra of the Fab and Fc fragments of the IgG1, IgG2a, and IgG2b antibodies, resonance lines were coded alphabetically starting from the signal at the lowest field for each of the Fab fragments.

In the present work, we are dealing with a set of switch variant antibodies, in which the amino acid sequences of the V_H, V_L, and C_L domains are identical. In addition, a two-domain Fv fragment and a three-domain Fab* fragment can be obtained from the IgG2a(s) antibody, which also belongs to the switch variant family. By fully taking advantage of this situation, we assign Met resonances for the Fab fragments to V_H, V_L, C_H1, and C_L domains. Assignment of resonances into either the variable region or the constant region is possible by comparing the spectrum observed for the Fv fragment with those for Fab* and Fab fragments. In this case, we have to be careful in confirming that resonances observed for different fragments correspond well to each other. By combining the results of *chain-specific assignments*, it is also possible to further assign resonances originating from the variable (constant) region to either V_H or V_L (either C_H1 or C_L). This step of spectral assignments will be referred to in the following as *domain-specific assignments*.

In all of the above approaches, we compare more than two spectra for the assignments. As pointed out above, relevant resonances have to be conserved, giving the identical chemical shifts, in order to establish an unambiguous assignment. When this is not the case, *site-specific assignments* have to be made by using the double labeling method. See below for the assignment of the Met-18H resonance. The double labeling method also becomes essential in differentiating more than two resonances originating from the same domain. It is also crucial to use the double labeling method for the assignments of resonances originating from the identical position in different, but homologous proteins as in the case of the Fc fragments prepared from the IgG1, IgG2a, and IgG2b antibodies. For example, the Met-358H resonances for the Fc(IgG1) and Fc(IgG2a) have to be assigned independently by the double labeling method (vide infra).

It is sometimes necessary to develop a method that is of limited use for a special situation. One of the examples is the assignment of Met-34H, which has been accomplished by a combination of ^{13}C and ^{15}N measurements (vide infra).

One of the most interesting applications of the present results is to use ^{13}C resonances for probing interactions involving immunoglobulin domains. Obviously, additivity does not hold for some of the resonances originating from interacting domains and fragments. In this case, we definitely have to have an independent way of assigning these resonances. The double labeling method is the most reliable and important one that is available at present in order to cope with this situation.

Fragment-Specific Resonance Assignments. The Fab and Fc fragments of the IgG1, IgG2a, and IgG2b antibodies were obtained by papain digestion. Limited digestion of the IgG2a(s) antibody by clostripain gave a three-domain Fab* fragment, which consists of V_H, V_L, and C_L. The Fv fragment comprising the V_H and V_L domains was also obtained by digestion of the IgG2a(s) antibody with clostripain.

Figure 2 compares the ^{13}C NMR spectra of the IgG1, IgG2a, and IgG2b antibodies with those of their digestion products Fab and Fc. In Figure 2 the observed resonances have been coded in alphabetical order starting from the lowest field resonance for each of the Fab fragments. Figure 3 shows ^{13}C spectra of the intact IgG2a(s) antibody and its proteolytic fragments Fv, Fab*, and Fc. It was confirmed that the Fc fragments derived from the IgG2a and IgG2a(s) antibodies give identical ^{13}C spectra. Therefore, the resonances for the Fc fragment derived from the IgG2a(s) antibody are coded in Figure 3 as in the case of the IgG2a antibody. See Figure 2. In Figure 3, all four Fv resonances coincide in chemical shift with resonances a, b, d, and e observed for the Fab* fragment and therefore are coded with the same letters.

Comparisons of the spectra shown in Figures 2 and 3 with the distribution of the Met residues illustrated in Figure 1 indicate that except in the case of resonance e for the Fab fragment of the IgG2b antibody, which is a superposition of two resonances, all other Met resonances that are expected from the amino acid sequences are separately observed for the Fv, Fab*, Fab, and Fc fragments. The ^{13}C spectra given in Figure 2 indicate that the carbonyl carbon resonances observed for the antibodies are simply a superposition of those for the

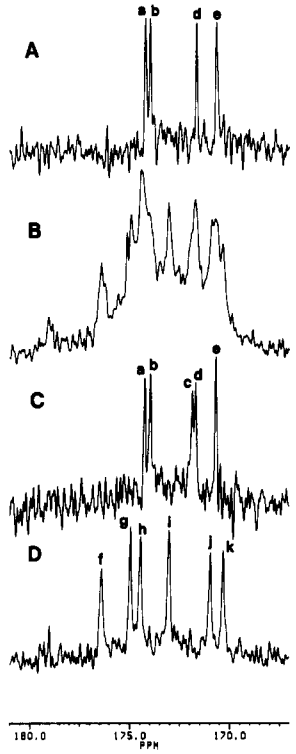

FIGURE 3: 100-MHz ^{13}C NMR spectra of the switch variant anti-dansyl antibody IgG2a(s). (A) [M]Fv; (B) [M]IgG2a(s); (C) [M]Fab*; (D) [M]Fc. Experimental conditions are as in Figure 2. Resonance lines are coded alphabetically starting from the signal at the lowest field for Fab*. Resonances a, b, d, and e observed in the Fv fragment correspond to each of those observed in the Fab* fragment.

corresponding Fab and Fc fragments. Figure 3 also indicates that the chemical shifts for the intact IgG2a(s) antibody coincide with those of the corresponding peaks for the Fv, Fab*, and Fc fragments. Thus, each of the ^{13}C resonances observed for the intact antibodies has been assigned to one of the corresponding proteolytic fragments.

Chain-Specific Resonance Assignments. In order to assign the Met resonances to either the heavy chain or the light chain, we basically followed the procedure described by Anglister et al. (1985). The labeled heavy (light) chain and unlabeled light (heavy) chain were separated from the labeled and unlabeled antibodies, respectively, by reduction and alkylation followed by treatment with guanidinium chloride. The separated labeled heavy (light) chain and unlabeled light (heavy) chain were recombined by removing guanidinium chloride by dialysis. Proteolytic digestion was performed for all of these recombined antibodies. Recombined antibodies, in which the heavy (light) chains are exclusively labeled with [1-^{13}C]Met, will be designated as IgG[H] (IgG[L]). Similar notations Fab[H] and Fab[L] will be used for the Fab fragment.

The spectra of the IgG1 and IgG2a(s) antibodies are compared in Figure 4 for the native and recombinant proteins. The spectra were also compared for the Fab, Fab[H], and Fab[L] fragments derived from the IgG1, IgG2a, and IgG2b antibodies (data not shown). All of these data show that the spectra of all the native proteins are simply a superposition of the two kinds of recombined proteins with either the heavy chain or the light chain exclusively labeled. The above result confirms that in all the recombined proteins the native structure has been restored after the process of recombination involving denaturation and renaturation. It should be noted that interchain disulfide bridges are reduced and alkylated in the recombined antibodies. This indicates that the presence

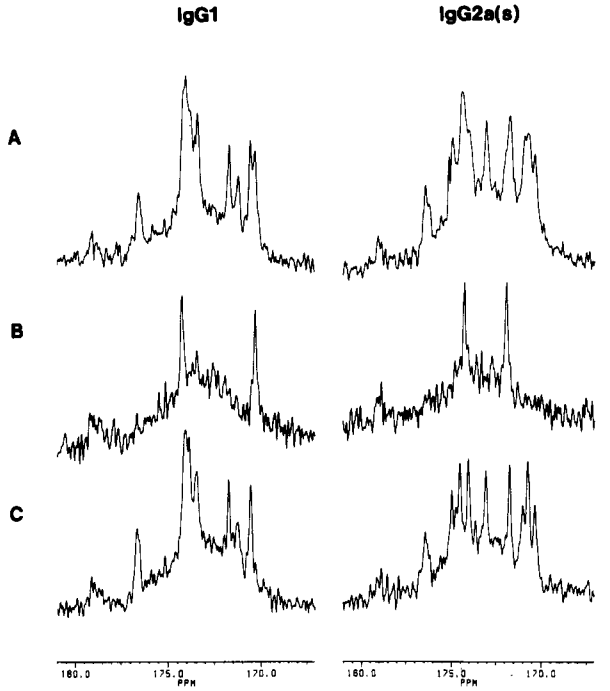

FIGURE 4: 100-MHz ^{13}C NMR spectra of the IgG1 and IgG2a(s) antibodies. The spectra of the IgG, IgG[L], and IgG[H] proteins are given in (A), (B), and (C), respectively. Experimental conditions are as in Figure 2.

Table I: Summary of Chain-Specific Assignments for IgG1, IgG2a, IgG2a(s), and IgG2b Antibodies

resonance[a]	IgG1[b,c]	IgG2a[c]	IgG2a(s)[b,c]	IgG2b[c]
a	L[d]	L	L	L
b	H[e]	H	H	H
c	H	H	L	H
d	H	H	H	H
e	H	L	H	H + L[f]
f	L			

[a] Figures 2 and 3. [b] The intact antibodies were used for the assignments. See Figure 4. [c] The Fab fragments were used for the assignments. [d] Light chain. [e] Heavy chain. [f] Two resonances superimposed.

or the absence of the interchain disulfide bridges does not influence the observed ^{13}C chemical shifts. Thus the observed resonances have been assigned to either the heavy chain or the light chain. The results of the *chain-specific assignments* accomplished in this way for the IgG1, IgG2a, IgG2a(s), and IgG2b antibodies are summarized in Table I.

Domain-Specific Resonance Assignments. The Met resonances observed for the Fv and Fab* fragments derived from the IgG2a(s) antibody are compared with those of a panel of the Fab fragments derived from the IgG1, IgG2a, and IgG2b antibodies. As Figure 3 shows, all of the resonances observed for the Fv fragment are conserved in the spectrum of the Fab* fragment. We therefore conclude that resonances a, b, d, and e observed for the Fab* fragment originate from either the V_H domain or the V_L domain. The Fab* fragment possesses five Met residues, i.e., three in V_H, one in V_L, and one in C_L. See Figure 1. This means that resonance c for Fab* originates from the C_L domain. Since there is only one Met residue in the C_L domain, resonance c can be assigned to Met-175L.[2-4]

[2] Sequence data of the V_H region of the switch variant antibodies used in the present study have been given by J. L. Dangl (Ph.D. Thesis, Stanford University, 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.

A comparison of Figure 2B and Figure 3A also shows that each of the four Fv resonances has its counterpart in the spectra of the Fab fragments examined, i.e., resonances a, b, d, and e (IgG1), resonances a, b, c, and d (IgG2a), and resonances a, b, c, and e (IgG2b). However, the chemical shift for resonance e for the Fv fragment is not quite identical with those for the Fab fragments derived from IgG1 (resonance e), IgG2a (resonance d), and IgG2b (resonance e) antibodies. Therefore, assignments for these resonances have been confirmed directly by the double labeling method (vide infra). The other three resonances show identical chemical shifts for the Fv, Fab* and Fab fragments. Table I indicates that resonance a commonly observed for the three Fab fragments (Figure 2B) originates from the light chain. As Figures 2B and 3A, C show, resonance a is conserved for the Fab, Fv, and Fab* fragments, indicating that it originates from the V_L domain. Since there is only one Met residue in the V_L domain, resonance a can be assigned to Met-4L. In the case of the Fab fragment derived from the IgG2b antibody, resonance a has independently been assigned by the double labeling experiment (Kato et al., 1989).

As Figure 1 shows, the Fv fragment possesses three Met residues in the V_H domain. We therefore conclude that resonances b, d, and e observed for the Fv fragment and corresponding resonances observed for the Fab and Fab* proteins (in Figures 2B and 3C, respectively) originate from the V_H domain. Site-specific assignments for these resonances will be made on the basis of the results of the double labeling method (vide infra).

All other resonances observed in Figures 2B and 3C presumably originate from the C_L and C_H1 domains. As described above, resonance c observed for the Fab* fragment has been assigned to Met-175L in the C_L domain. Table I indicates that resonances f (IgG1), e (IgG2a), and e (IgG2b, one of two overlapped peaks) originate from the light chain. We therefore assign these resonances to Met-175L in the C_L domain. These results also indicate that resonances c (IgG1) and d (IgG2b) are due to the C_H1 domain.

As described above, the Met-175L chemical shift is significantly different for the different proteins examined. The significance of the difference in the Met-175L chemical shifts observed for different antibodies will be discussed below.

Site-Specific Resonance Assignments. (i) Met Resonances in the Variable Regions. In the spectra of [M,T]Fv and [M,K]Fv, resonances a and e for Fv (Figure 3A) are split into doublets with a spin coupling constant of approximately 15 Hz, respectively (Kainosho & Tsuji, 1982; Kato et al., 1989). As illustrated in Figure 1, Met-4L and Met-18H are followed by Thr-5L and Lys-19H, respectively.[5] Therefore, resonances a and e for the Fv fragment (Figure 3A) can be unambiguously assigned to Met-4L and Met-18H, respectively. The result for the Met-4L resonance is consistent with that of the domain-specific assignment described above.

The above results indicate that resonances b and d for the Fv fragment are due to Met-34H and Met-82H, respectively, or vice versa. There is, however, little hope to apply the double labeling method for the assignment of these resonances, because Met-34H and Met-82H are followed by Asp and Asn, respectively. We therefore pay attention to the effect of the antigen binding on the chemical shifts of these peaks.

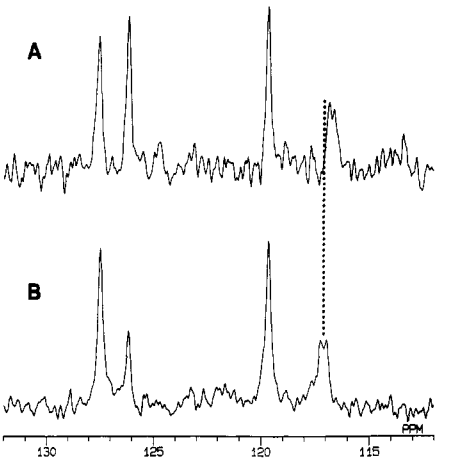

FIGURE 5: 50-MHz ^{15}N NMR spectra of [W,M]Fv observed with DEPT in the presence (A) and absence (B) of DNS-Lys. The flip angle of the last proton pulse in the DANTE sequence was set to $\pi/2$. The delay was set to $(2J_{NH})^{-1}$, i.e., 5.6 ms. The spectra were obtained with a spectral width of 12.5 kHz and 32K data points. The pulse delay time was 1.0 s. The sample solution was prepared as described in the text. Apparent intensity of the resonance observed at 126.2 ppm in the absence of the antigen is decreased due to an insufficient deuterium to proton exchange of the amide group. Note that only the Met-34H ^{15}N resonance, which is split into a doublet due to spin coupling with the ^{13}C of the carbonyl carbon of Trp-33H, gives a chemical shift that is different in the presence and absence of the antigen.

It was observed that resonance b in the ^{13}C spectrum of [M]Fv (Figure 3A) is significantly shifted downfield in the presence of DNS-Lys. No other peaks showed any significant shift upon addition of the antigen. Figure 5 gives ^{15}N spectra of [W,M]Fv observed in the absence and presence of DNS-Lys. Of the four Met residues that exist in the Fv fragment, Met-34H is the only one that is preceded by Trp. Therefore, the ^{15}N resonance that is split into a doublet can unambiguously be assigned to Met-34H. As Figure 5 shows, this is the only peak that is shifted in the presence of the antigen. Thus, a comparison of the ^{13}C and ^{15}N data led us to assign resonance b observed for [M]Fv to Met-34H. This result automatically leads to the assignment of resonance d to Met-82H.

As described above, each of resonances a, b, d, and e observed for the Fv fragment has its counterpart in the spectra of all the Fab fragments examined. Resonance e for the Fv fragment, which has been assigned to Met-18H, gives a chemical shift that is identical with that for the Fab*, but is not quite identical for each of the Fab fragments. We therefore first of all have to establish the assignments for these resonances using the double labeling method. A comparison of the spectra of [M]Fab and [M,K]Fab, both of which were prepared from the IgG1 antibody, indicated that resonance e is split into a doublet in the case of [M,K]Fab. As Figure 1 shows, Met-18H is the only one that is followed by Lys. Thus we are able to assign resonance e unambiguously to Met-18H. In a similar way, resonance d for the IgG2a protein has been assigned to Met-18H. In the case of the IgG2b antibody, one of two overlapping resonances observed as resonance e has been tentatively assigned to Met-18H previously (Kato et al., 1989).

By combining the results for the domain-specific assignments made for the Fab* and Fab fragments, we assign Met-4L, Met-18H, Met-34H, and Met-82H resonances for

[3] Met-4 in the light chain will be designated as Met-4L. Similar notations will be used for all Met residues in the light chain.

[4] 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.

[5] Met-18 in the heavy chain will be designated as Met-18H. Similar notations will be used for all Met residues in the heavy chain.

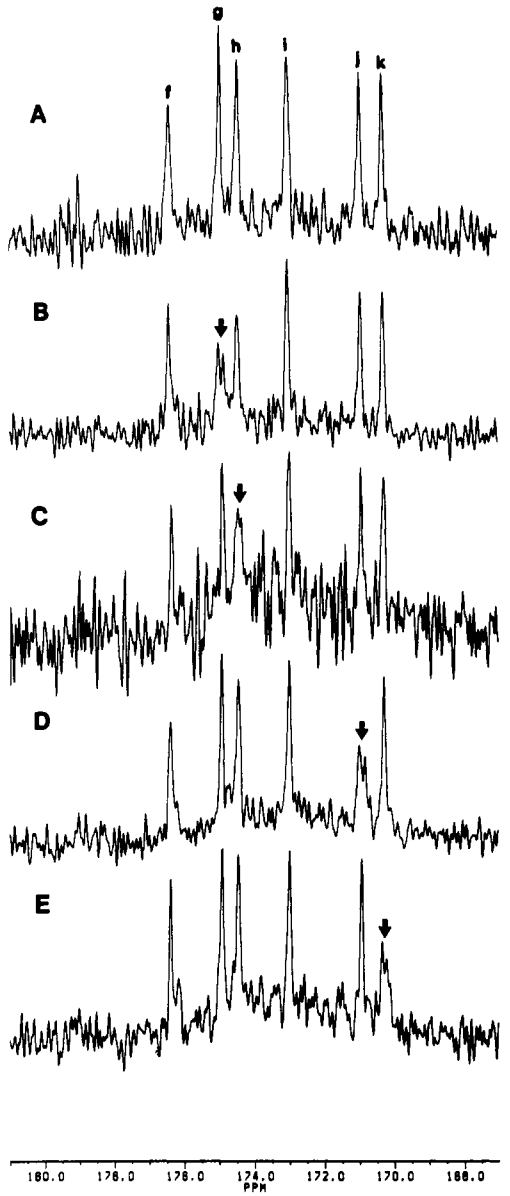

FIGURE 6: 100-MHz ^{13}C NMR spectra of the Fc fragment of the IgG2a(s) antibody. (A) [M]Fc; (B) [M,T]Fc; (C) [M,I]Fc; (D) [M,V]Fc; (E) [M,Y]Fc. Experimental conditions are as in Figure 2. Resonance lines split into doublets due to spin coupling with ^{15}N are identified by the arrows.

all the Fab* and Fab fragments.

(*ii*) *Met Resonances in the C_L Domain.* The switch variant antibodies examined in the present study share the identical light chain, which has two Met residues at positions 4 and 175. As described above, both of these resonances have been unambiguously assigned by combining the results of *fragment-specific, chain-specific, and domain-specific assignments.*

(*iii*) *Met Resonances in the C_H1 Domain.* As shown above, resonance c (IgG1) and resonance d (IgG2b) originate from the C_H1 domain. Figure 1 indicates that the IgG1 and IgG2b antibodies possess only one Met residue in the C_H1 domain at positions 139H and 182H, respectively. This means that resonance c (IgG1) and resonance d (IgG2b) are due to Met-139H and Met-182H, respectively. In the case of the IgG1 antibody, the above assignment has been confirmed by the double labeling experiment (data not shown).

(*iv*) *Met Resonances in the Fc Region.* The IgG1, IgG2a, and IgG2b antibodies used in the present study possess the heavy chain constant regions C_H1, C_H2, and C_H3 that have a significant degree of homology to each other. As a matter of fact, the IgG1 and IgG2a proteins share Met-358H and Met-368H in the C_H3 domain. It should particularly be noted that the Fc fragments derived from these antibodies do not give any resonances with identical chemical shifts. This clearly indicates that assignments on the basis of the homology of amino acid sequences have to be made with great care. We therefore made the assignments of all the Fc resonances independently using the double labeling method, and the results obtained will be summarized below.

Table II: Site-Specific Resonance Assignments for [1-^{13}C]Met-Labeled Switch Variant Anti-Dansyl Antibodies[a]

		subclass			
domain	residue no.	IgG1	IgG2a	IgG2a(s)	IgG2b
V_L	4	a	a	a[b]	a[b]
C_L	175	f	e	c	e
V_H	18	e[b]	d[b]	e[b]	e[b]
	34	b	b	b[b]	b
	82	d	c	d	c
C_H1	139	c[b]	–		–
	182	–	–		d
C_H2	252	–	h	h[b]	g[b]
	309	g[b]	–	–	–
	314	–	f	f[b]	f
C_H3	358	h[b]	g	g[b]	–
	368	j[b]	j	j[b]	–
	373	–	i	i	–
	398	i	–	–	–
	406	–	k	k[b]	–
	412	–	–	–	h[b]

[a] (–) indicates that at this position of each of the antibodies Met is replaced by other amino acid residues. It should also be noted that the IgG2a(s) antibody lacks the entire C_H1 domain. All resonances have been assigned by combinations of *fragment-specific, chain-specific, domain-specific, and site-specific assignments.* [b] Assignments confirmed by double labeling. See text.

(*1*) *IgG1 Antibody.* As shown in Figure 2, the Fc fragment obtained from [M]IgG1 gives four resonances, g–j. Double labeling with [α-^{15}N]His, [^{15}N]Ala, and [^{15}N]Ile resulted in a splitting of resonances g, h, and j, respectively. We therefore unambiguously assign resonances g, h, and j to Met-309H, Met-358H, and Met-368H, respectively. This result automatically leads to the assignment of resonance i to Met-398H.

(*2*) *IgG2a Antibody.* As described above, the Fc fragments derived from the IgG2a and IgG2a(s) antibodies gave identical ^{13}C spectra. Therefore, assignments were performed by using the Fc fragment derived from the IgG2a(s) antibody. Figure 6 shows that double labeling with [^{15}N]Thr, [^{15}N]Ile, [^{15}N]Val, and [^{15}N]Tyr results in a splitting of resonances g, h, j, and k, respectively. The ^{13}C spectrum of an Fc fragment obtained from [M,S]IgG2a(s) was also examined. Although efficiency of incorporation of [^{15}N]Ser was lower than 50%, it was possible to identify resonance f, which is split into doublet. On the basis of the sequence data of the dipeptides summarized in Figure 1, resonances f, g, h, j, and k can unambiguously be assigned to Met-314H, Met-358H, Met-252H, Met-368H, and Met-406H, respectively. This automatically leads to the assignment of resonance i to Met-373H.

(*3*) *IgG2b Antibody.* Resonance h has previously been assigned by the double labeling experiment to Met-412H (Kato et al., 1989). The spectrum of [M,I]IgG2b indicates that resonance g originates from Met-252H. This means that resonance f originates from Met-314H.

Summary of the Spectral Assignments. Table II summarizes the result of the completed spectral assignments for all the switch variant antibodies used in the present work.

Discussion

Observation and Spectral Assignments of the Carbonyl ^{13}C

Resonances of the Switch Variant Antibodies and Their Fragments. As shown above, the carbonyl ^{13}C resonances of antibodies are extremely narrow in line width for proteins with a molecular weight of 150K. This is presumably due to the fact that the carbonyl carbons are not in close spatial proximity to any protons, which can be a strong source of spin relaxation. For this reason, it was possible to observe ^{13}C resonances separately for the intact antibodies as well as for all other proteolytic fragments examined. Furthermore, the narrow ^{13}C line widths have made it possible to employ the double labeling method for the assignment of key ^{13}C resonances even in the case of the intact antibody (Kato et al., 1989). The same is true for the antibodies labeled with [1-^{13}C]Cys, [1-^{13}C]Trp, and [1-^{13}C]Tyr (to be submitted for publication).

It should be emphasized that a combined use of the double labeling method with a variety of proteolytic fragments of the native and recombined switch variant antibodies was essential in the spectral assignments reported in the present work. For example, in the case of the IgG2b antibody, the dipeptide Met-Ser appears three times; i.e., Met-175L (C_L), Met-182H (C_H1), and Met-314H (C_H2) are all followed by Ser. It would have been virtually impossible to assign all of these resonances solely on the basis of the results of double labeling experiments.

We will summarize in the following how we proceeded for the spectral assignment for the IgG2b antibody. As Figure 2 shows, correspondence of the observed ^{13}C resonances between the intact antibody and its proteolytic fragments Fab and Fc was straightforward. Assignments of the observed resonances to either the light chain or the heavy chain was established unambiguously by the recombination experiment. Thus, the light chain resonances originating from Met-4L and Met-175L can be identified. The site-specific assignment of the Met-4L resonance was made by the double labeling method. This has automatically led to the assignment of the Met-175L resonance. The Met-18H, Met-34H, and Met-82H resonances were assigned by a combination of different types of doubly labeled proteins. The Met-182H, which is the only Met residue in the C_H1 domain, was assigned by comparing spectra obtained with the Fv and Fab fragments. The Fc fragment of the IgG2b antibody contains three Met residues at positions 252H, 314H, and 412H. The Met-252H resonance was assigned by the double labeling experiment. The site-specific assignment of the Met-412H resonance has also been made by the double labeling method (Kato et al., 1989). These results have led to the assignment of the Met-314H resonance.

^{15}N Labeling of Antibodies. Isotope dilution is generally a most serious problem in labeling proteins with ^{15}N labeled amino acids. In the present experiments the isotope dilution was also significant except in the cases of [α-^{15}N]Lys and [^{15}N]Thr, both of which cannot become the substrate of transaminases. In the cases of all other ^{15}N labeled amino acids, a number of inhibitors have been tested. It was found that β-chloro-L-alanine is most effective in incorporating ^{15}N labeled amino acids. Thus, assignments of the methionyl carbonyl carbon resonances have been possible by the double labeling method using, in addition to [α-^{15}N]Lys and [^{15}N]Thr, ^{15}N-labeled Ala, His, Ile, Met, Ser, Tyr, and Val. In the case of the [^{15}N]Ala labeling, DL-*threo*-β-hydroxyaspartic acid was also added to the medium. See Materials and Methods. Typical examples of spectra of doubly labeled proteins are given in Figure 6. We have further confirmed in the process of assignments of tryptophanyl carbonyl carbon resonances of the switch variant antibodies that the double labeling is also possible using ^{15}N-labeled Gly, Leu, and Phe (to be submitted for publication). Incorporation of [^{15}N]Asp and [^{15}N]Pro has also been attempted so far without success.

Concluding Remarks. The aim of the present study is to understand how each of the immunoglobulin domains is assembled to express proper biological functions. An individual antibody molecule is obviously heterogeneous in the variable region, but it is significantly heterogeneous in the constant region as well due to the presence of different subclasses. In addition to the inherent complex multidomain structure, this heterogeneous nature has so far hampered a detailed structural study of the antibody molecule. Obviously, proper methods are needed along with proper systems for the elucidation of the structure–function relationship in the antibody molecule.

In the present study, we have shown using antibodies labeled with [1-^{13}C]Met at the carbonyl carbon that (1) it is possible to observe all the carbonyl carbon resonances separately and (2) site-specific assignments of all these resonances are possible. A most important feature of the present work is that site-specific assignments have been completed for all the methionyl carbonyl carbon resonances for a series of switch variant anti-dansyl monoclonal antibodies. The IgG1, IgG2a, IgG2a(s), and IgG2b antibodies used here all share identical V_H, V_L, and C_L domains. In the IgG2a(s) antibody, the C_H1 domain is entirely deleted from the IgG2a antibody (Igarashi et al., 1990). In addition, it is possible to use this short-chain IgG2a(s) antibody to prepare a three-domain Fab* fragment and a two-domain Fv fragment, which consist of V_H, V_L, and C_L and V_H and V_L, respectively. The methionyl carbonyl carbon resonances for the intact switch variant antibodies along with a variety of proteolytic fragments including Fab* and Fv fragments can therefore become an ideal probe for the interactions between variable–variable and variable–constant domains.

It has been shown that in most cases the ^{13}C spectra observed for the intact IgG antibodies are simply a superposition of those of their proteolytic fragments. This result presumably reflects the independent nature of the functional domains of the antibody as far as we are using methionyl carbon carbon resonances as a probe. The results of the present ^{13}C study have shown that even in the case of the switch variant IgG proteins, which share the identical V_L, V_H, and C_L domains, significant chemical shifts are observed for some of the ^{13}C resonances originating from the V_H and C_L domains. A most significant example is the Met-175L resonance observed for the Fab* and Fab fragments. A comparison of the spectra for the Fab* fragment (Figure 3C) and the Fab fragment derived from the IgG2a antibody (Figure 2B) shows that the Met-175L resonance is shifted downfield by 1.6 ppm upon deletion of the C_H1 domain. It is also observed that the Met-175L (C_L) resonance gives different chemical shifts for the different Fab fragments with homologous but different C_H1 domains. We suggest that the carbonyl carbon chemical shift data may be used to detect, for instance, the way in which information is transmitted through different domains upon antigen binding.

As shown above, the ^{13}C and ^{15}N chemical shifts for Met-34H are exclusively affected by the presence of antigen. See Figure 5. Met-34H is located in CDR1. No other Met residues in the switch variant antibodies exist in the CDR. Therefore, it is quite likely that the observed shift reflects the binding of DNS-Lys in the neighborhood of Met-34H. We have also observed effects of the antigen binding on the carbonyl carbon chemical shifts for the switch variant antibodies labeled with [1-^{13}C]Trp. The Trp-101H resonance originating from the CDR3 of the heavy chain shows a large shift upon

antigen binding (to be submitted for publication). This line of analyses using the ^{13}C chemical shift data of the unambiguously assigned carbonyl carbon resonances would be useful in mapping the antigen binding site. It should also be of interest to use, for example, the Met-314H resonance as a probe for the binding of staphylococcal protein A to the Fc region (Deisenhofer, 1981).

In order to proceed further, it is obviously necessary to look at different parts of the antibody molecule by using different types of ^{13}C labeling. Further analyses are under way in our laboratory using switch variant antibodies labeled with [1-^{13}C]Trp, [1-^{13}C]Tyr, and [1-^{13}C]Cys.

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SUPPLEMENTARY MATERIAL AVAILABLE

Eight figures showing additional ^{13}C NMR spectral data for singly and doubly labeled Fv, Fab, and Fc fragments used for the assignments in this work (10 pages). Ordering information is given on any current masthead page.

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Purification and Properties of Extracellular Signal-Regulated Kinase 1, an Insulin-Stimulated Microtubule-Associated Protein 2 Kinase†

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ABSTRACT: In rat 1 fibroblasts, insulin has little or no stimulatory effect on the activities of either MAP2 protein kinase or ribosomal protein S6 kinase. In contrast, in rat 1 cells that overexpress the normal human insulin receptor (rat 1 HIRc B; McClain et al. (1987) *J. Biol. Chem. 262*, 14663-14671), insulin activates both MAP2 and S6 kinase activities close to 5-fold. A MAP2 kinase has been purified from insulin-treated rat 1 HIRc B cells over 6300-fold by chromatography on Q-Sepharose, phenyl-Sepharose, S-Sepharose, phosphocellulose, QAE-Sepharose, UltrogelAcA54, DEAE-cellulose, and a second Q-Sepharose. Its specific activity is approximately 0.8-1 μmol·min^{-1}·mg^{-1} with MAP2 and 3 μmol·min^{-1}·mg^{-1} with myelin basic protein. The enzyme preparation contains one major band of M_r = 43 000 upon SDS-polyacrylamide gel electrophoresis, which is immunoblotted by antibodies to phosphotyrosine. A sequence from the 43-kDa band led to the isolation of a cDNA encoding the enzyme, which we have named ERK1 for extracellular signal-regulated kinase (Boulton et al. (1990) *Science 249*, 64-67).

Insulin exerts its effects on many cellular processes by regulating the phosphorylation state of serine and threonine residues of proteins. The phosphorylation of ribosomal protein S6 on serine residues is rapidly increased in many cell types by insulin (Smith et al., 1980), growth factors (Martin-Pérez et al., 1984), and transformation by viruses (Decker, 1981), largely as a result of the stimulation of ribosomal protein S6 kinases (Rosen, 1987; Erikson & Maller, 1986; Cobb, 1986; Gregory et al., 1989; Blenis & Erikson 1985; Jenö et al., 1989).

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