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Application of ^{13}C NMR spectroscopy to paratope mapping for larger antigen-Fab complexes

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Abstract

For the purpose of engineering the antibody combining site, mapping residues that are involved in antigen binding provide us with valuable information. By use of ^{13}C NMR spectroscopy with selectively ^{13}C-labeled Fv fragments, we have established a general strategy to identify the residues that are perturbed upon binding of small antigen (hapten) molecules [(1990) Biochemistry 30, 6604–6610]. In the present paper, we demonstrate that this strategy can be extended to molecular structural analyses of the complexes of an Fab fragment and a larger antigen molecule such as *Pseudomonas aeruginosa* exotoxin A with a molecular mass of 67 kDa.

Key words: Antigen-binding site; Antigen–antibody interaction; Fab; ^{13}C NMR; Immunoglobulin G; Mouse; Exotoxin A (*Pseudomonas aeruginosa*)

1. Introduction

Since hybridoma technology was established, monoclonal antibodies have been widely used as immunochemical tools, diagnostic reagents, and therapeutics. Recently, genetically engineered antibodies have been produced in order to improve specific reactivity with target antigens, to reduce antigenicity for human therapy, and to control the expression of effector functions [1]. The antigen combining site in the antibody molecule is particularly attractive as a target for antibody engineering including the design of catalytic antibodies. With the knowledge of the structure of the antigen combining site, one could make an improved antibody with a higher binding affinity by a conventional method such as site-directed mutagenesis. Hence, the mapping of residues involved in *paratope* would be crucial.

X-ray crystallography is a powerful method in identifying the residues involved in the antigen binding on the basis of three-dimensional structural data (for a review, see Padlan, [2]). However, it is not always possible to prepare the crystal of antigen–antibody complexes with high quality. NMR spectroscopy is an alternative way to obtain structural information on ligand–protein interactions. However, the size of IgG (M_r ~150 K) or its Fab fragment (M_r ~50 K) hampers detailed structural analyses by traditional NMR spectroscopy. To cope with this problem, Anglister and coworkers have developed strategies for ^1H NMR spectral analyses using selective deuteration of Fab fragments (for a review, see Anglister, [3]). We have developed a variety of heteronuclear NMR techniques for the investigation of affinity maturation by use of Fab fragments [4]. In these studies, however, the problem of site-specific resonance assignments remained to be solved.

Recently, by use of Fv fragments (M_r ~25 K) labeled with ^{13}C and/or ^{15}N, it has become possible to identify residues involved in the antigen binding using ^1H-detected heteronuclear NMR spectroscopy [5–9]. However, almost all of the materials used so far as antigens in these NMR studies were limited to small molecules such as haptens or peptides. In addition, the large amount of stable Fv fragments that is needed for the NMR analysis has not always been available. Taking into account of all the above points, it is obviously necessary to establish an NMR strategy on the basis of unambiguous spectral assignments to deal with the complexes of Fab with a larger antigen.

For the spectral analyses of large antigen–Fab complexes with molecular mass more than 100 kDa, only possible way envisaged is the use of carbonyl ^{13}C resonances as spectroscopic probes. In the previous papers, we have demonstrated that ^{13}C NMR methods on the basis of unambiguous assignmets of carbonyl ^{13}C resonances by a ^{13}C–^{15}N double labeling technique [10] pro-

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Abbreviations: CDR, complementarity-determining region; C_H1, the first domain of the constant region of the heavy chain; C_L, constant region of the light chain; DNS-Lys, *Nε*-dansyl-L-lysine; Ex-A, *Pseudomonas aeruginosa* exotoxin A; Fab, antigen binding fragment composed of V_H, V_L, C_H1, and C_L; [X]Fab, Fab labeled with [1-^{13}C]X; [X,Z]Fab, Fab that is labeled with [1-^{13}C]X and [α-^{15}N]Z; Fv, antigen binding fragment composed of V_H and V_L; IgG, immunoglobulin G; NMR, nuclear magnetic resonance; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; V_H, variable region of the heavy chain; V_L, variable region of the light chain.

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vide us with detailed structural information from large proteins [11–15]. We have also revealed that the chemical shift of the carbonyl ^{13}C resonances sensitively reflects microenvironmental changes in Fv fragments induced by hapten binding and, therefore, can be a useful tool for the mapping of antigen binding sites [12, 13]. In the present study, we apply this strategy to much larger systems, i.e. hapten–Fab and protein antigen–Fab complexes. Applicability of the strategy to molecular structural analyses of a complex of *Pseudomonas aeruginosa* exotoxin A (Ex-A) with a molecular mass of 67 kDa and an anti-Ex-A Fab fragment will be examined.

2. Materials and methods

2.1. Materials

L-[1-^{13}C]Met was purchased from Isotec, Inc., USA. L-[α-^{15}N]His was from Isocommerz GmbH, Germany. All other ^{13}C- or ^{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. β-Chloro-L-alanine, Nε-dansyl-L-Lys (DNS-Lys), and papain were purchased from Sigma. Ex-A produced by and purified from *Pseudomonas aeruginosa* was purchased from Swiss Serum and Vaccine Institute Berne, Switzerland.

2.2. Cell lines

Switch variant cell lines 27–44, 27–13.6, and 27–35.8, which had been selected and cloned by using a fluorescence-activated cell sortor [16, 17], were kindly provided by Professor L.A. Herzenberg and Dr. V.T. Oi. These cell lines produce mouse monoclonal anti-dansyl IgG1(κ), IgG2a(κ), and IgG2b(κ), respectively, with the V_H, V_L, and C_L domains that are common in the amino acid sequences. We have established a cell line Ex-3C7, which produces mouse monoclonal anti-Ex-A IgG1(κ) [18]. This monoclonal antibody has been demonstrated to recognize the amino acid residues 241 to 297 in domain Ia and II of Ex-A, to inhibit incorporation of Ex-A into target cells, to neutralize strongly cytotoxicity in cell culture [18–20]. It has also been shown that the Ex-3C7 antibody does not inhibit the enzymatic activity of ADP-ribosyltransferase but are protective against pseudomonal infection caused by Ex-A-producing strains in experimental mouse models [18–20]. The amino acid sequences of the V_H and V_L domains were determined on the basis of DNA sequence data according to the procedure described in the literature [21].

Hereafter, the Fab fragments that are derived from cell lines 27–44, 27–13.6, 27–35.8, and Ex-3C7 will be referred to as anti-dansyl Fab(γ1), anti-dansyl Fab(γ2a), anti-dansyl Fab(γ2b), and anti-Ex-A Fab, respectively.

2.3. Preparation of isotopically labeled Fab fragments

Isotopically labeled Fab fragments were prepared as described previously [12]. Hybridoma cells were grown in a serum-free medium (Nissui NYSF-404) that contains an appropriate [1-^{13}C]amino acid (and [α-^{15}N]amino acid). For example, IgG selectively labeled with ^{13}C and ^{15}N at the methionine carbonyl carbon and the histidine amide nitrogen, respectively, was prepared by cultivating cells in the medium where unlabeled L-Met and L-His had been replaced respectively by an equal amount of L-[1-^{13}C]Met and a twice amount of DL-[α-^{15}N]His in the presence of 100 mg/ml of L-Ala and 16 mg/ml of β-chloro-L-alanine. After cells were grown, the supernatant was concentrated with an ultrafiltration system (Millipore Minitan) and applied to a protein A affinity column (Bio-Rad). Typically 10–20 mg of purified IgG proteins were obtained per liter of the cell culture. The purified antibody (5 mg/ml) was digested by papain at pH 7.0, 37°C, in 75 mM sodium phosphate buffer containing 75 mM NaCl, 2 mM EDTA, and 5 mM NaN_3. The enzyme/substrate ratios (w/w) were 1:25, 1:50, and 1:25 for IgG1, IgG2a, and IgG2b, respectively. In the case of 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, 6h, and 8h for IgG1, IgG2a, and IgG2b, respectively. The reaction was terminated by addition of 30 mM of *N*-ethylmaleimide. The digestion products were loaded onto a Pharmacia Mono Q FPLC column equilibrated with 20 mM Tris-HCl, pH 8.0, and Fab fragments were separately eluted from other products by increasing the NaCl concentration up to 400 mM. Purity of the Fab preparations was checked by SDS-PAGE. The size of an antigen-Fab complex was determined by use of a Pharmacia Superose 6 gel filtration column.

In the present paper, an Fab analog selectively labeled with [1-^{13}C]Met is designated using the one-letter abbreviation for the amino acid as [M]Fab. An Fab analog that is doubly labeled with [1-^{13}C]Met and [α-^{15}N]His will be designated as [M,H]Fab. Similar notations will be used for all labeled Fab analogs.

2.4. NMR measurements

For NMR measurements, the Fab solutions were concentrated to a final volume of 2 ml in 5 mM sodium phosphate buffer, pH 7.4, containing 200 mM NaCl and 3 mM NaN_3 in ^2H_2O. The final concentration of the Fab was 0.2–0.7 mM. NMR measurements were made on a Bruker AM 400 spectrometer. ^{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 32 K data points, a spectral width of 24,000 Hz, and a repetition period of 1 s. The free induction decay was multiplied by a Gaussian window function prior to Fourier transformation. Chemical shifts are given in ppm from internal dioxane. The probe temperature was 30°C.

3. Results and discussion

In the previous paper, we have mapped the binding site of DNS-Lys in an anti-dansyl Fv fragment using carbonyl ^{13}C resonances from Cys, His, Met, Trp, and Tyr as spectroscopic probes [13]. On the basis of the chemical shift data obtained, we have concluded that the CDR3 loop in the V_H domain is significantly perturbed upon addition of DNS-Lys and, therefore, is primarily responsible for the binding of DNS-Lys [13]. This conclusion was confirmed by stable-isotope-assisted NOESY experiments [22]. We are extending the ^{13}C NMR strategy on the basis of chemical shift data of the carbonyl carbon to the analyses of the interactions between DNS-Lys and anti-dansyl Fab(γ1), Fab(γ2a), and Fab(γ2b) fragments. The anti-dansyl Fab fragments used in the present study possess the V_H and V_L domains composed of the amino acid sequences identical with those of the anti-dansyl Fv fragments.

Fig. 1A shows the ^{13}C NMR spectrum of the anti-dansyl [W]Fab(γ2b) fragment. The site-specific assignments for each of the Trp resonances have been reported in a previous paper [13]. Upon addition of DNS-Lys, resonances originating from Trp-101H and Trp-105H, which are located in and at the end of the CDR3 in the V_H domain, respectively, exclusively showed large chemical shift changes of more than 0.1 ppm (Fig. 1B)*. These

*The numbering system used in the present paper for the constant region is based on human myeloma protein Eu for the heavy chain [32] and mouse myeloma protein MOPC21 for the light chain [26]. The convention of Kabat et al. [26] has been followed for the numbering of the V_H and V_L regions. The notations H and L following the residue numbers mean the heavy and light chains, respectively, e.g. Trp-101H means Trp-101 in the *heavy* chain.

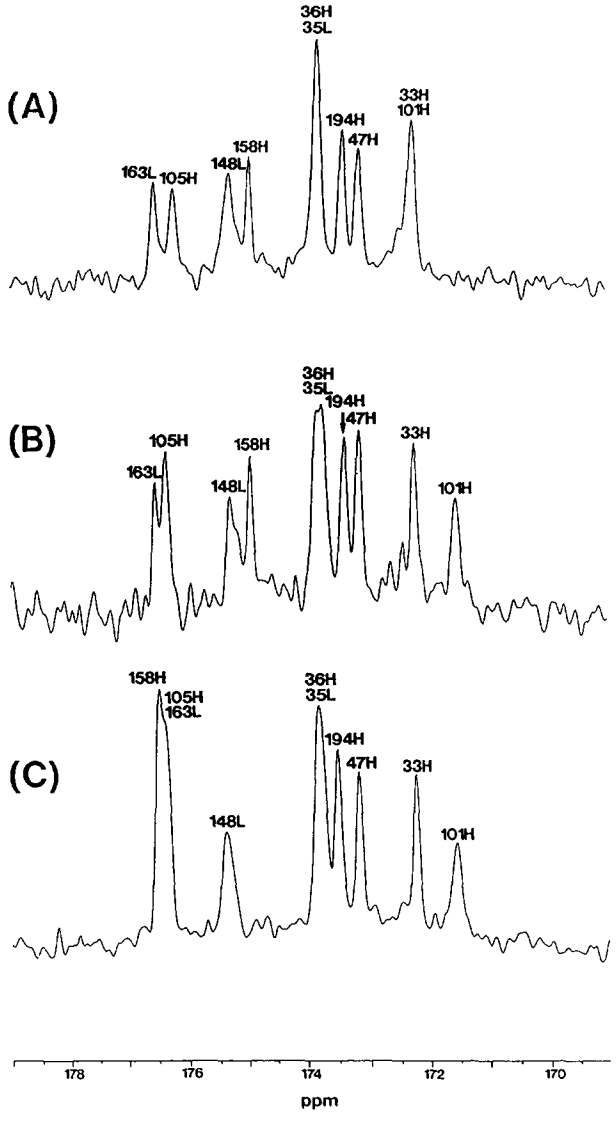

Fig. 1. 100-MHz ^{13}C NMR spectra of (A) anti-dansyl [W]Fab(γ2b) in the absence of DNS-Lys, (B) anti-dansyl [W]Fab(γ2b) in the presence of DNS-Lys, and (C) anti-dansyl [W]Fab(γ2a) in the presence of DNS-Lys. Protein concentrations and the pH of the sample solutions were 0.4–0.7 mM and 7.4, respectively. The probe temperature was 30°C. 51,000–70,000 transients were accumulated for each spectrum, where 32 K data points and a spectral width of 24,000 Hz were used with a repetetion period of 1 s. In the spectra (B) and (C), the molar ratio of Fab/DNS-Lys was 1 : 1. Assignments of Trp-36H and Trp-47H may be reversed because both of these Trp residues are followed by Val [13].

spectral changes were also observed in the case of the [W]Fv [13], [W]Fab(γ2a) (Fig. 1C), and [W]Fab(γ1) fragments (data not shown). On the basis of these observations, we suggest that the binding mode of DNS-Lys is unaffected by the existence of the constant domains, and also by the difference between the subclasses. This result was confirmed by use of Met resonances as spectroscopic probes (data not shown) and is quite consistent with the fluorescence spectral data [23] and also with the fact that dissociation constants of DNS-Lys are identical for the IgG1, IgG2a, and IgG2b antibodies and the Fv fragment [23–25]. As demonstrated above, we have successfully established the NMR strategy to identify the residues in the Fab fragments that are specifically perturbed upon hapten binding.

Next, we attempted to extend this method toward much larger systems. Here we use a complex of Ex-A and anti-Ex-A Fab as an example. Fig. 2 shows the amino acid sequences of the V_H and V_L domains of the anti-Ex-A Fab. For the spectral assignments of an Fab that is newly dealt with, we compare the spectrum with that of homologous Fab whose resonances have already been assigned. Fig. 3A and B compare the ^{13}C NMR spectra of the anti-dansyl [M]Fab(γ1) and anti-Ex-A [M]Fab fragments. Both of these two Fab fragments are of the IgG1(κ) isotype. In a previous paper, we have completed the site specific assignments of the Met resonances originating from the anti-dansyl Fab(γ1) as shown in Fig. 3A [12]. Anti-Ex-A Fab possesses five Met residues, i.e. Met-20H and Met-80H (V_H), Met-33L (V_L), Met-139H (C_H1), and Met-175L (C_L). See Fig. 2 and Kabat et al. [26]. The resonances originating from the constant region, i.e. Met-139H in the C_H1 domain and Met-175L in the C_L domain, have their counterparts in the spectrum of anti-

V_H

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1                                                                   20
Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala Ser Vla Arg Met

21                                  31  32  33  34  35
Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asn Tyr Asn Ile His Trp Val Lys Gln Lys
                                                CDR1
41                                50  51  52  52A 53  54  55  56  57  58  59
Ala Gly Gln Gly Leu Glu Leu Ile Gly Tyr Thr Asn Pro Tyr Asn Asp Val Ser Lys Tyr
                                                    CDR2
60  61  62  63  64  65
Asn Glu Lys Phe Lys Gly Lys Ala Thr Leu Thr Ser Asp Lys Ser Ser Ser Thr Val Tyr

80      82  82A 82B 82C 83                                              95  96
Met Glu Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys Ala Arg Phe Lys

97  98  99  100 100A100X101 102
Asp Gly Tyr Ser Ser Phe Ala Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ala
        CDR3
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V_L

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1
Asp Ile Val Leu Thr Gln Ser Pro Ala Ser Leu Ala Val Ser Leu Gly Gln Arg Ala Thr

        23  24  25  26  27  27A 27B 27C 27D 28  29  30  31  32  33  34  35
Ile Ser Cys Arg Ala Ser Lys Ser Val Ser Thr Ser Gly Tyr Ser Tyr Met His Trp Tyr
                                        CDR1
37                                              49  50  51  52  53  54  55  56
Gln Gln Lys Pro Gly Gln Pro Pro Arg Leu Leu Ile Tyr Leu Ala Ser Asn Leu Glu Ser
                                                                    CDR2
57                  62                              71
Gly Val Pro Ala Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Asn Ile His

77                                          88  89  90  91  92  93  94  95  96
Pro Val Glu Glu Glu Asp Ala Ala Asn Tyr His Cys Gln His Ser Arg Asp Leu Pro Leu
                                                                        CDR3
97  98                                      108
Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu Lys Arg
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Fig. 2. The amino acid sequences of the V_H and V_L domains of the anti-Ex-A Fab used in the present study.

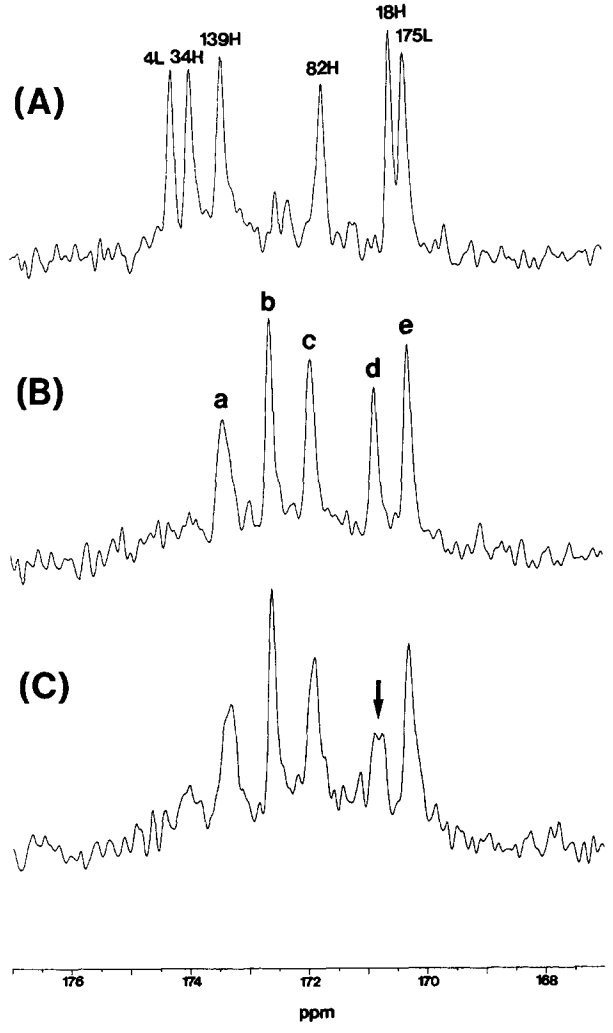

Fig. 3. 100-MHz ^{13}C NMR spectra of (A) anti-dansyl [M]Fab(γ1), (B) anti-Ex-A [M]Fab, and (C) anti-Ex-A [M,H]Fab. Protein concentrations were 0.2–0.4 mM. 51,000–198,000 transients were accumulated. Other spectral conditions were as in Fig. 1. In the spectrum (C), the Met-33L resonance, which is split into a doublet due to spin coupling with ^{15}N, is indicated by the arrow.

Ex-A [M]Fab, i.e. resonances a and e, respectively, as shown Fig. 3B. Thus, we were able to identify the three resonances originating from the variable region of the anti-Ex-A [M]Fab, i.e. b, c, and d.

Site-specific assignments of the resonances originating from the variable region of the anti-Ex-A [M]Fab were made by the ^{13}C–^{15}N double labeling method [10–12]. Fig. 3C shows a ^{13}C NMR spectrum of the anti-Ex-A [M,H]Fab fragment, where resonance d is split into doublet due to $^1J_{CN}$ spin coupling. Among the five Met residues existing in the anti-Ex-A [M]Fab, only Met-33L in the V_L domain is directly followed by His as shown in Fig. 2. Therefore, resonance d was unambiguously assigned to Met-33L in the V_L domain.

Upon addition of Ex-A, the Met-33L resonance was shifted downfield by 0.2 ppm (Fig. 4). Gel-filtration HPLC analysis demonstrated that the anti-Ex-A Fab and Ex-A form a 1:1 complex with a molecular mass of 120 kDa under the solution condition employed for the present NMR measurement. On the basis of these observations, we conclude that a microenvironment surrounding Met-33L located in the CDR2 in the V_L domain is significantly perturbed upon binding to Ex-A, while those of the other Met residues are little affected. Line broadening was observed for Met-33L beyond what had been expected from the increase of the molecular size. An interesting possibilty is that a microenvironment surrounding Met-33L in the complex fluctuates between multiple states. Specific spectral changes induced by Ex-A binding were also observed in the ^{13}C NMR spectra obtained by use of the anti-Ex-A [W]Fab and anti-Ex-A [F]Fab (data not shown).

Quantitative interpretation of the structural changes on the basis of the chemical shift and line width data is quite difficult at the present stage. However, it should be emphasized that, in many systems, larger chemical shift changes are observed in the region that has been indicated by X-ray crystallography to be responsible for ligand binding [27–31]. The ^{13}C NMR approach employed in the present study will open up a new way for the molecular structural analyses of larger systems in-

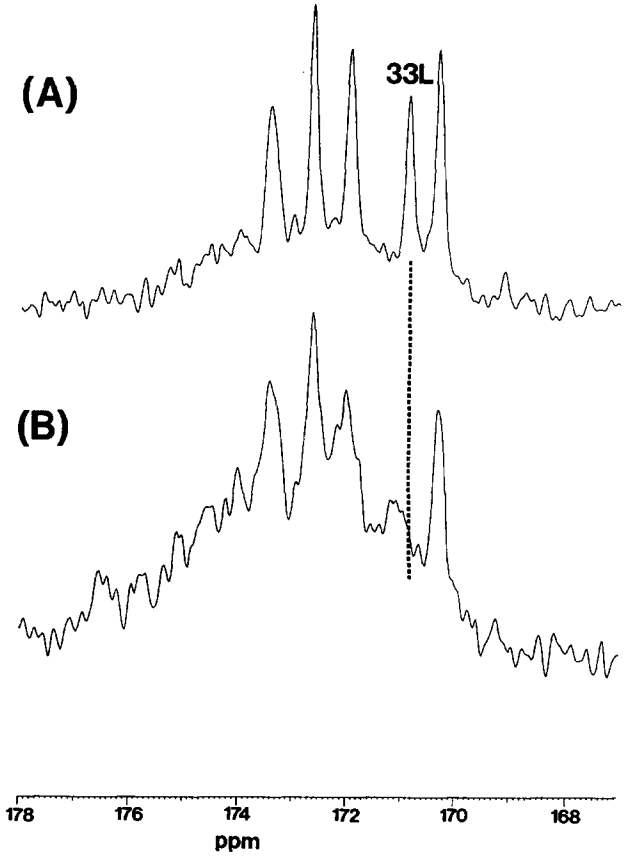

Fig. 4. 100-MHz ^{13}C NMR spectra of anti-Ex-A [M]Fab (A) in the absence and (B) in the presence of Ex-A. 198,000–275,000 transients were accumulated. In the spectrum (B), the molar ratio of Fab/Ex-A was 1:1. Other spectral conditions were as in Fig. 1.

cluding Fab fragmnets such as idiotype–anti-idiotype antibody interactions and Fc-rheumatoid factor interactions.

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