A Multinuclear NMR Study of the Affinity Maturation of Anti-NP Mouse Monoclonal Antibodies: Comparison of Antibody Combining Sites of Primary Response Antibody N1G9 and Secondary Response Antibody 3B62†

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ABSTRACT: On the basis of multinuclear NMR data, the structures of the antibody combining sites of anti-4-hydroxy-3-nitrophenylacetyl (NP) antibodies were compared for N1G9, which is one of the primary response antibodies with low affinity for NP, and 3B62, which is one of the secondary response antibodies with high affinity for NP. It has been concluded, on the basis of the results of antibody engineering, that in most secondary response antibodies a Trp→Leu exchange at position 33 of the heavy chain is primarily responsible for the increased affinity for NP [Allen, D., Simon, T., Sablitzky, F., Rajewsky, K., & Cumano, A. (1988) *EMBO J. 7,* 1995–2001]. Although 3B62 exhibits one of the highest affinities for NP, it lacks the Trp→Leu exchange at position 33 of the heavy chain. A variety of stable isotope-labeled Fab analogues of N1G9 and 3B62 have been prepared. *Chain-specific* resonance assignments were made by recombination of the heavy chains and light chains of the Fab fragments. Binding experiments of a spin-labeled hapten and NOESY experiments have demonstrated that, compared with the environment of the antibody combining site of N1G9, the combination of mutations (including one codon deletion) and the particular D–J_H rearrangement in the heavy chain of 3B62 affords a more hydrophobic environment, which is formed by one Tyr residue originating from the light chain and two Tyr residues originating from the heavy chain. We conclude that the formation of the hydrophobic environment plays an important role and endows the secondary response antibody 3B62 with the high affinity for NP without the Trp→Leu exchange at position 33 of the heavy chain.

Immunoglobulin G (IgG),[1] which is an important member of the group of proteins that functions as antibodies, consists of two identical heavy chains and two identical light chains. The heavy chains are composed of four homologous units, V_H, C_H1, C_H2, and C_H3, whereas the light chains are divided into two homologous units, V_L and C_L. Papain digestion of the whole antibody molecule gives the Fab and Fc fragments.

One of the most important functions of immunoglobulin molecules is the specific recognition of the antigen. Antigen recognition is carried by the Fab fragment, which consists of the light chain (V_L and C_L) and the N-terminal half (V_H and C_H1) of the heavy chain. It is known that the antibody combining site is constructed from six hypervariable regions, three each from V_H (H1, H2, and H3) and V_L (L1, L2, and L3).

It is known that the average affinity of serum generally increases with time after immunization. This phenomenon is called affinity maturation of the immune response (Siskind & Benacerraf, 1969). To investigate the relationship between the antibody diversity and the progressive change in affinity, extensive sequence analyses of antibodies have been carried out by using several haptens, i.e., 4-hydroxy-3-nitrophenylacetyl (NP) (Bothwell et al., 1981, 1982; Cumano & Rajewsky, 1985, 1986), phenylarsonate (Wysocki et al., 1986), phosphorylcholine (Crews et al., 1981), and 2-phenyloxazolone (Berek & Milstein, 1987).

The primary immune response of C57BL/6 mice to NP coupled to T-cell-dependent carrier chicken IgG is dominated by a single type of λ1 light-chain-bearing antibodies (Jack et al., 1977; Mäkelä & Karjalainen, 1977) expressing the V186.2 V_H gene together with the DFL16.1 segment (Bothwell et al., 1981). It has been demonstrated that the variable regions of the primary response anti-NP antibodies carry few or no somatic mutations (Bothwell et al., 1981; Cumano & Rajewsky, 1985), and those of the secondary response anti-NP antibodies are somatically mutated and usually exhibit higher affinity for NP (Cumano & Rajewsky, 1986). By gene analysis of the variable regions of the secondary response of anti-NP antibodies, it has been shown that a Trp→Leu exchange at position 33 of the heavy chain is frequently observed in the secondary response antibodies (Allen et al., 1987). On the basis of the results of site-specific mutagenesis experiments, it has been concluded that the Trp→Leu exchange plays a crucial role in the affinity maturation of the immune response of anti-NP antibodies (Allen et al., 1988).

Anti-NP antibodies N1G9 and 3B62 are one of the primary response antibodies with low affinity and one of the secondary

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[1] Abbreviations: AmTEMPO, 4-amino-2,2,6,6-tetramethylpiperidine *N*-oxide; C_H1, C_H2, C_H3, constant domains of the heavy chain; C_L, constant domain of the light chain; FR1(H), FR2(H), FR3(H), FR4(H), framework regions in V_H; FR1(L), FR2(L), FR3(L), FR4(L), frame work regions in V_L; HPLC, high-performance liquid chromatography; Fab, antigen binding fragment composed of V_H, V_L, C_H1, and C_L; Fc, fragment composed of the C-terminal half of the heavy chain; HSQC, heteronuclear single quantum correlation; H1, H2, H3, hypervariable regions in V_H; IgG, immunoglobulin G; L1, L2, L3, hypervariable regions in V_L; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, two-dimensional NOE correlated spectroscopy; NP, 4-hydroxy-3-nitrophenylacetyl; NP-AmTEMPO, (4-hydroxy-3-nitrophenylacetyl)-2,2,6,6-tetramethylpiperidine *N*-oxide; V_H, the variable domain of the heavy chain; V_L, the variable domain of the light chain.

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response antibodies with high affinity, respectively. It is of great interest that 3B62 lacks the Trp→Leu exchange at position 33 of the heavy chain.

It has been reported that the combination of selective deuterium labeling and two-dimensional transferred nuclear Overhauser effect difference spectroscopy is effective for the analysis of antigen–Fab interactions (Anglister & Zilber, 1990). We recently performed a series of stable-isotope-aided NMR studies on an anti-dansyl Fv fragment in order to elucidate the static and dynamical structure of the antibody combining site (Kato et al., 1991; Takahashi et al., 1991a,b, 1992; Odaka et al., 1992).

In the present paper, we report an NMR study on the structures of the antibody combining sites of N1G9 and 3B62 by using Fab analogues selectively labeled with ^2H or ^{13}C. Chain-specific resonance assignments were made by recombination of the heavy chain and the light chain of each of these Fab fragments (Kato et al., 1991; Takahashi et al., 1991a). Binding experiments of a spin-labeled hapten and NOESY experiments were performed on a variety of Fab analogues of N1G9 and 3B62. On the basis of the NMR data obtained, we compare the structures of the antibody combining sites of N1G9 and 3B62.

MATERIALS AND METHODS

Materials. His-α,2′,4′-d_3 and Trp-2′,4′,5′,6′,7′-d_5 were prepared according to the procedures described in the literature (Matthews et al., 1977). Tyr-2′,3′,5′,6′-d_4 was prepared by enzymatic coupling of phenol-d_5 with pyruvate in the presence of CH_3COONH_4 (Nagasawa et al., 1981). Tyr-2′,6′-d_2 was prepared by treatment at 110 °C of Tyr-2′,3′,5′,6′-d_4 in 6 M HCl for 3 h. Phe-2′,3′,4′,5′,6′-d_5, L-[3′,5′-$^{13}C_2$]Tyr, and L-[2′-^{13}C]Trp were purchased from CIL. The isotope enrichment is 95% or higher for each of the amino acids. 4-Hydroxy-3-nitrophenylacetyl (NP) and 4-amino-2,2,6,6-tetramethylpiperidine *N*-oxide (AmTEMPO) were obtained from Sigma Chemical Co. (4-Hydroxy-3-nitrophenylacetyl)-2,2,6,6-tetramethylpiperidine *N*-oxide (NP-AmTEMPO) was synthesized according to the procedure of Chiniak & Polonski (1973). The purity of NP-AmTEMPO was checked by thin-layer chromatography. All other chemicals were of reagent grade and used without further purification.

Preparation of Selectively Isotope-Labeled Anti-NP Monoclonal Antibodies. The C57BL/6 mice hybridoma cell lines N1G9 and 3B62, which produce anti-NP IgG monoclonal antibodies, were kindly provided by Professor K. Rajewsky. Hybridoma cells were adapted to a medium (Nissui NYSF 404) containing 1% FCS and then grown in the medium containing stable-isotope-labeled amino acid(s) (Kato et al., 1991; Takahashi et al., 1991a). After cell growth, each cell supernatant was concentrated with a Minitan ultrafiltration system (Millipore) and then applied to an Affi-Gel protein A column (Bio-Rad).

Limited Proteolysis of IgG1. Fab fragments of the anti-NP monoclonal antibodies were prepared by papain digestion at pH 7.0 in 75 mM sodium phosphate buffer containing 75 mM NaCl, 2 mM EDTA, and 5 mM NaN_3. The antibody concentration was 5 mg/mL. The enzyme:substrate ratio (w/w) was 1:50, and L-cysteine–HCl·H_2O was added to the digestion buffer to a concentration of 2 mg/mL. The incubation time was 2 h. The reaction was stopped by adding *N*-ethylmaleimide to a final concentration of 30 mM, and then the reaction mixture was dialyzed against 20 mM Tris–HCl, pH 8.0. The digestion product was loaded onto a Pharmacia Mono Q column equilibrated with 20 mM Tris–HCl, pH 8.0, and then eluted with varying NaCl concentrations in the range of 0–400 mM. The purity of the Fab fragments obtained was checked by SDS–polyacrylamide gel electrophoresis. Recombination of the heavy and light chains of the Fab fragments was performed according to the procedure described previously (Kato et al., 1991; Takahashi et al., 1991a). For brevity, the Fab fragments derived from N1G9 and 3B62 will be designated as Fab(N1G9) and Fab(3B62), respectively.

Interaction of NP-Gly and NP-AmTEMPO with the Anti-NP Antibodies. Throughout the experiment we used NP-glycine (NP-Gly) as the hapten. The dissociation constants for NP-Gly and NP-AmTEMPO were determined by monitoring the quenching of the fluorescence of the anti-NP antibodies by the addition of haptens in 5 mM phosphate buffer, pH 8.0, containing 0.2 M NaCl (Eisen & McGuigan, 1971). The excitation wavelength was 285 nm, and emission was observed at 340 nm. Fluorescence measurements were performed with a Shimadzu RF-5000 spectrometer at 30 °C. The dissociation constants of N1G9 obtained for NP-Gly and NP-AmTEMPO were 1.8×10^{-5} and 1.5×10^{-6} M, respectively, and those of 3B62 were 4.5×10^{-7} and 1.4×10^{-5} M, respectively.

NMR Measurements. All NMR spectra were measured with a JEOL JNM-GSX 500 spectrometer operating at 500 MHz. A total of 256 and 64 blocks were acquired with data points of 1*K* for NOESY (Jeener et al., 1979) and ^1H–^{13}C HSQC (Bodenhausen & Ruben, 1980) experiments, respectively. The delay time for the ^1H–^{13}C HSQC experiment was set to 1.0 ms. The mixing time for the NOESY measurement was set to 90 ms.

All two-dimensional spectra were obtained in the pure absorption mode (States et al., 1982). Prior to 2D Fourier transformation, the acquired data were multiplied by a Gauss function in t_2 and by a shifted sine square function in t_1 and were zero-filled once along the t_1 direction.

For NMR measurements, the protein solutions were concentrated by ultrafiltration to a final volume of 0.45 mL with 5 mM phosphate buffer, pH 8.0, containing 0.2 M NaCl. All samples were prepared in D_2O. The final concentration of protein was typically 0.5–1.0 mM. The solvent resonance was suppressed by selective irradiation during the relaxation delay, which was taken to be 1.0–1.2 s. The probe temperature was 30 °C throughout the experiment.

RESULTS

On the basis of antibody sequence data, it has been indicated that a large number of Tyr and Trp residues exist in the hypervariable regions (Kabat et al., 1987; Mian et al., 1991). Mian et al. (1991) suggested that amphipathic amino acids such as Tyr and Trp residues play a significant role in antigen recognition. Both Fab(N1G9) and Fab(3B62) contain 21 Tyr residues, 11 in V_H, 3 in V_L, 3 in C_H1, and 4 in C_L, and 11 Trp residues, 4 in V_H, 3 in V_L, 2 in C_H1, and 2 in C_L. In the present multinuclear NMR study we used Tyr and Trp residues as probes for structural analysis of the antibody combining sites of N1G9 and 3B62.

Stable-isotope labeling is useful for the structural analysis of proteins by NMR in solution at the atomic level (Markley et al., 1986; Markley, 1989). Therefore, a variety of selectively labeled Fab analogues of the N1G9 and 3B62 antibodies was used. First, we established *chain-specific* assignments for all of the Tyr and Trp residues of Fab(N1G9) and Fab(3B62)

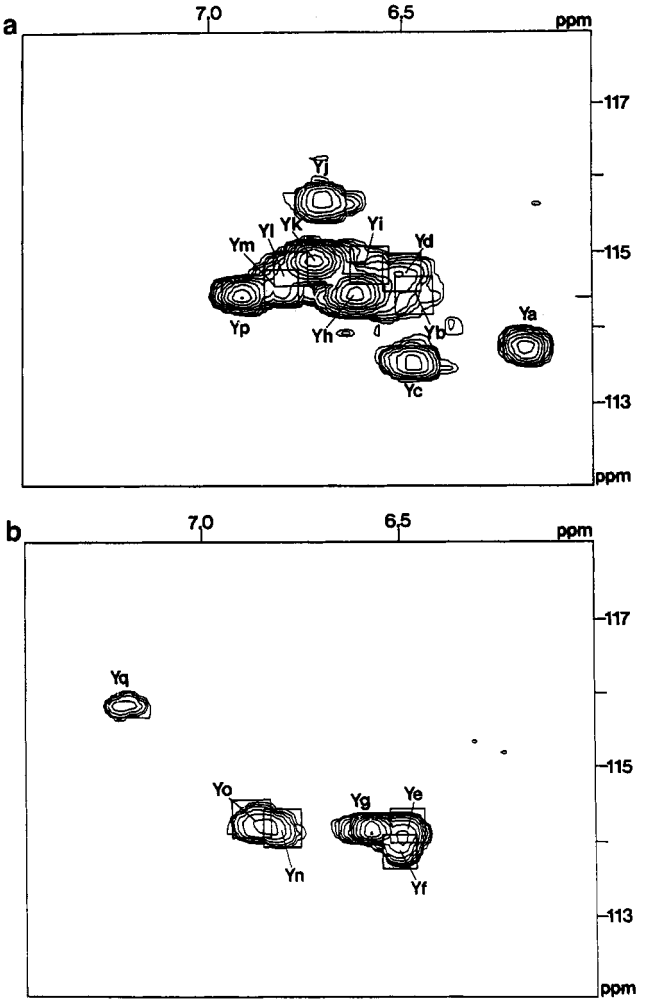

FIGURE 1: ^1H–^{13}C HSQC spectra of [Y]N1G9. The spectra of [Y]N1G9[H] and [Y]N1G9[L] are given in a and b, respectively. The protein samples were dissolved at a concentration of approximately 1.0 mM in 5 mM phosphate buffer containing 200 mM NaCl in D_2O at pH 8.0. The probe temperature was 30 °C.

by using recombination of the heavy chains and light chains of the Fab fragments along with stable-isotope-labeled Fab analogues. On the basis of the established *chain-specific* assignments, we compared the microenvironments of the antibody combining sites of N1G9 and 3B62 by means of binding experiments of a spin-labeled hapten and NOESY experiments performed on the stable-isotope-labeled Fab analogues.

For brevity, the Fab(N1G9) analogue selectively labeled with Phe-2′,3′,4′,5′,6′-d_5, Trp-2′,4′,5′,6′,7′-d_5, His-4′-d_1, and Tyr-2′,6′-d_2 will be designated as [^2H]N1G9. The Fab(N1G9) analogues labeled with [3′,5′-$^{13}C_2$]Tyr and [2′-^{13}C]Trp will be referred to as [Y]N1G9 and [W]N1G9, respectively. Similar abbreviations will be used for Fab(3B62), e.g., [^2H]-3B62, [Y]3B62, and [W]3B62.

Chain-Specific Assignments of the Aromatic Resonances of Tyr Residues of Fab(N1G9). To assign the 3′,5′ proton and carbon resonances of Tyr residues to either the heavy chain or the light chain, ^1H–^{13}C HSQC spectra of the recombined [Y]N1G9 fragments were measured. Recombined Fab(N1G9) fragments, in which the heavy (light) chains are exclusively labeled with [3′,5′-$^{13}C_2$]Tyr, will be designated as [Y]N1G9[H] ([Y]N1G9[L]). Figures 1a and 1b show the ^1H–^{13}C HSQC spectra of [Y]N1G9[H] and [Y]N1G9-[L] observed in the absence of NP-Gly, respectively. Eleven HSQC cross peaks for [Y]N1G9[H] and six HSQC cross peaks for [Y]N1G9[L] were detected separately. It was not possible to identify the remaining four cross peaks (three originating from the heavy chain and one originating from the light chain) due to the severe overlapping of the cross peaks. On the basis of the results of the recombinant experiments, cross peaks Ya, Yb, Yc, Yd, Yh, Yi, Yj, Yk, Yl, Ym, and Yp were assigned to the heavy chain and cross peaks Ye, Yf, Yg, Yn, Yo, and Yq to the light chain. The same procedure was followed for the *chain-specific* assignment of the Trp residues of Fab(N1G9) and the Tyr and Trp residues of Fab(3B62).

Effect of NP-AmTEMPO on the ^1H–^{13}C HSQC Spectra of the Fab(N1G9) and Fab(3B62) Analogues. It was previously shown that the use of a spin-labeled hapten is effective for the mapping of an antibody combining site (Dower & Dwek, 1979; Odaka et al., 1992). In this study, NP-AmTEMPO was used as a paramagnetic probe.

(i) Binding of NP-AmTEMPO to [Y]N1G9. Figures 2a and 2b show the ^1H–^{13}C HSQC spectra of [Y]N1G9[H] and [Y]N1G9[L] observed at a Fab(N1G9):NP-AmTEMPO molar ratio of 100:120. Cross peaks Ya, Yj, Ym, and Yp originating from the heavy chain and Yg originating from the light chain disappeared upon the addition of NP-AmTEMPO, see Figures 2a and 2b.

For determination of the binding site of NP-AmTEMPO in Fab(N1G9), L-ascorbic acid was added as a reductant to the [Y]N1G9[H]–NP-AmTEMPO ([Y]N1G9[L]–NP-AmTEMPO) solution in order to quench the effect of the electron spin of NP-AmTEMPO. In the ^1H–^{13}C HSQC spectra of [Y]N1G9[H]–NP-AmTEMPO ([Y]N1G9[L]–NP-AmTEMPO) observed in the presence of L-ascorbic acid, cross peaks Ya, Yj, Ym, Yp, and Yg reappeared (Figures 2c and 2d). The chemical shifts of these Tyr residues at the 3′,5′ protons are almost identical to those observed in the presence of NP-Gly. We therefore conclude that both NP-AmTEMPO and NP-Gly are bound in the antibody combining site of Fab(N1G9) in a similar manner.

(ii) Binding of NP-AmTEMPO to [W]N1G9. Figure 3a shows the ^1H–^{13}C HSQC spectrum of [W]N1G9 observed in the absence of the antigen. As Figure 3a shows, 11 peaks were observed in the ^1H–^{13}C HSQC spectrum. Thus, the cross peaks originating from all of the Trp residues were detectable. Furthermore, on the basis of the results of the recombination experiments, cross peaks Wa, Wd, Wg, Wh, and Wj were assigned to the light chain and the remaining peaks to the heavy chain.

Figures 3b and 3c show the ^1H–^{13}C HSQC spectra of the [W]N1G9–NP-AmTEMPO complex in the absence and presence of L-ascorbic acid, respectively. Cross peaks Wa and Wg originating from the light chain and cross peak We originating from the heavy chain disappeared upon the addition of NP-AmTEMPO.

(iii) Binding of NP-AmTEMPO to [Y]3B62 and [W]3B62. Similar binding experiments of NP-AmTEMPO were performed for [Y]3B62 and [W]3B62. Figures 4a and 4b show the ^1H–^{13}C HSQC spectra of the [Y]3B62–NP-AmTEMPO complex in the absence and presence of L-ascorbic acid, respectively. Comparison of Figures 4a and 4b clearly indicates that cross peaks Y1, Y3, Y4, Y10, and Y12 disappeared upon the addition of NP-AmTEMPO. The results of the recombination experiment indicate that cross peaks Y1, Y3, Y10, and Y12 originate from the heavy chain and cross peak Y4 from the light chain.

Figures 5a and 5b show the ^1H–^{13}C spectra of [W]3B62 observed in the presence of NP-AmTEMPO and in the

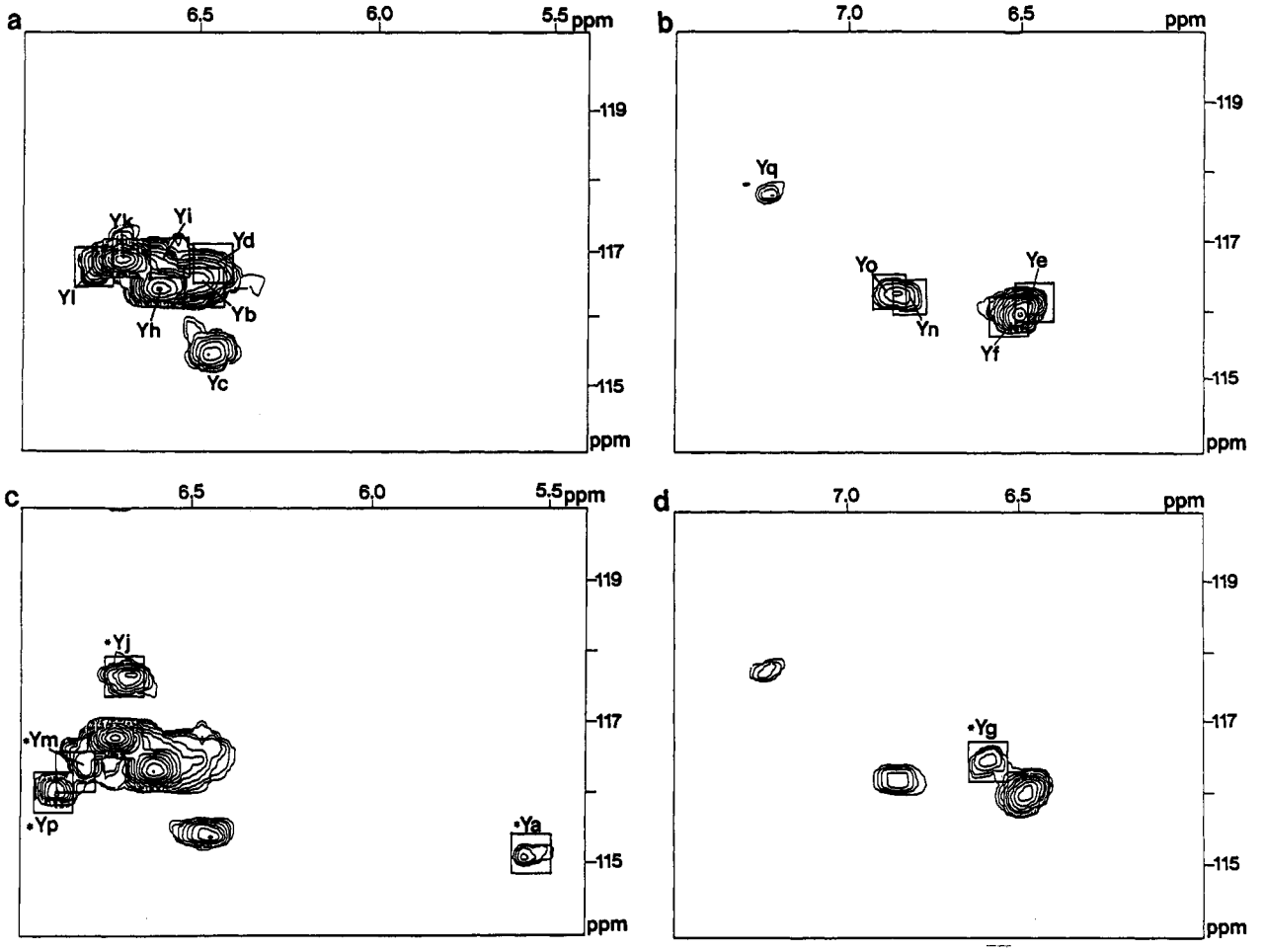

FIGURE 2: ^{1}H–^{13}C HSQC spectra of the [Y]N1G9–NP-AmTEMPO complex. The spectra of the [Y]N1G9[H]–NP-AmTEMPO complex and the [Y]N1G9[L]–NP-AmTEMPO complex are given in a and b, respectively. The spectra of the [Y]N1G9[H]–NP-AmTEMPO complex observed in the presence of L-ascorbic acid and the [Y]N1G9[L]–NP-AmTEMPO complex observed in the presence of L-ascorbic acid are given in c and d, respectively. In c and d, the cross peaks that reappeared upon the addition of L-ascorbic acid are indicated by asterisks.

presence of NP-AmTEMPO and L-ascorbic acid, respectively. The results of the NP-AmTEMPO binding experiment showed that the addition of NP-AmTEMPO reduces the intensities of cross peaks W1 and W6 originating from the light chain and cross peak W2 originating from the heavy chain.

NOESY Spectra of [^{2}H]N1G9 and [^{2}H]3B62 Observed in the Presence of NP-Gly. To detect the intermolecular NOEs between the Fab fragments and NP-Gly, we measured NOESY spectra of [^{2}H]N1G9 and [^{2}H]3B62 in the presence of NP-Gly.

Figure 6a shows the NOESY spectrum observed at a [^{2}H]-N1G9:NP-Gly molar ratio of 100:250. The chemical shifts of the 3′,5′ proton resonances of the Tyr residues of Fab-(N1G9) observed in the presence of NP-Gly were established by means of ^{1}H–^{13}C HSQC measurement of [Y]N1G9 observed in the presence of NP-Gly.

As Figure 6a shows, two intramolecular NOE cross peaks, Yp–Ya and Yq–Yo, were detected. We also observed a negative NOE cross peak, H6′–H5′, at the chemical shifts of the 5′ and 6′ protons of the NP ring in the free state. We conclude that cross peak H6′–H5′ is due to a transferred NOE. It appears that the exchange rate of NP-Gly between the free and bound states is in an appropriate range for the detection of transferred NOEs. It was also concluded that cross peak H6′–Ya in Figure 6a is due to a transferred NOE between the 6′ proton of the NP ring and Ya in the heavy chain. The above result indicates that one Tyr residue originating from the heavy chain exists in close spatial proximity to the NP ring of NP-Gly in the antibody combining site of Fab(N1G9).

We have performed similar analysis of Fab(3B62), which exhibits higher affinity for NP-Gly. In the case of 3B62, NP-Gly is slowly exchanging between the free and bound states. Therefore, it was possible to detect NOE cross peaks between Fab(3B62) and NP-Gly bound to Fab(3B62). Figure 6b shows the NOESY spectrum observed at a [^{2}H]3B62: NP-Gly molar ratio of 100:100. Intermolecular NOE cross peaks H6′–Y4, H5′–Y4, H6′–Y10, and H6′–Y12 were observed in addition to intramolecular NOE cross peaks H6′–H5′ and Y18–Y16. On the basis of the results of NOESY experiments using a series of mixing times, we confirmed that the cross peaks observed in the NOESY spectrum of [^{2}H]-3B62 in the presence of NP-Gly were not due to spin-diffusion. The chemical shifts for the bound NP-Gly were determined by means of an exchange NOE experiment, with a [^{2}H]3B62: NP-Gly molar ratio of 100:200. On the basis of the data obtained in these series of experiments, we conclude that two Tyr residues originating from the heavy chain and one Tyr residue originating from the light chain exist in close spatial proximity to the NP ring of NP-Gly in the antibody combining site of Fab(3B62).

Intermolecular NOEs between the NP ring of NP-Gly and the Trp residues of Fab(N1G9) and Fab(3B62) were not detectable in the ^{1}H–^{13}C HSCQ–NOESY spectra of [W]-N1G9 and [W]3B62 observed in the presence of NP-Gly. Figure 7 summarizes the results obtained by the NOESY experiments.

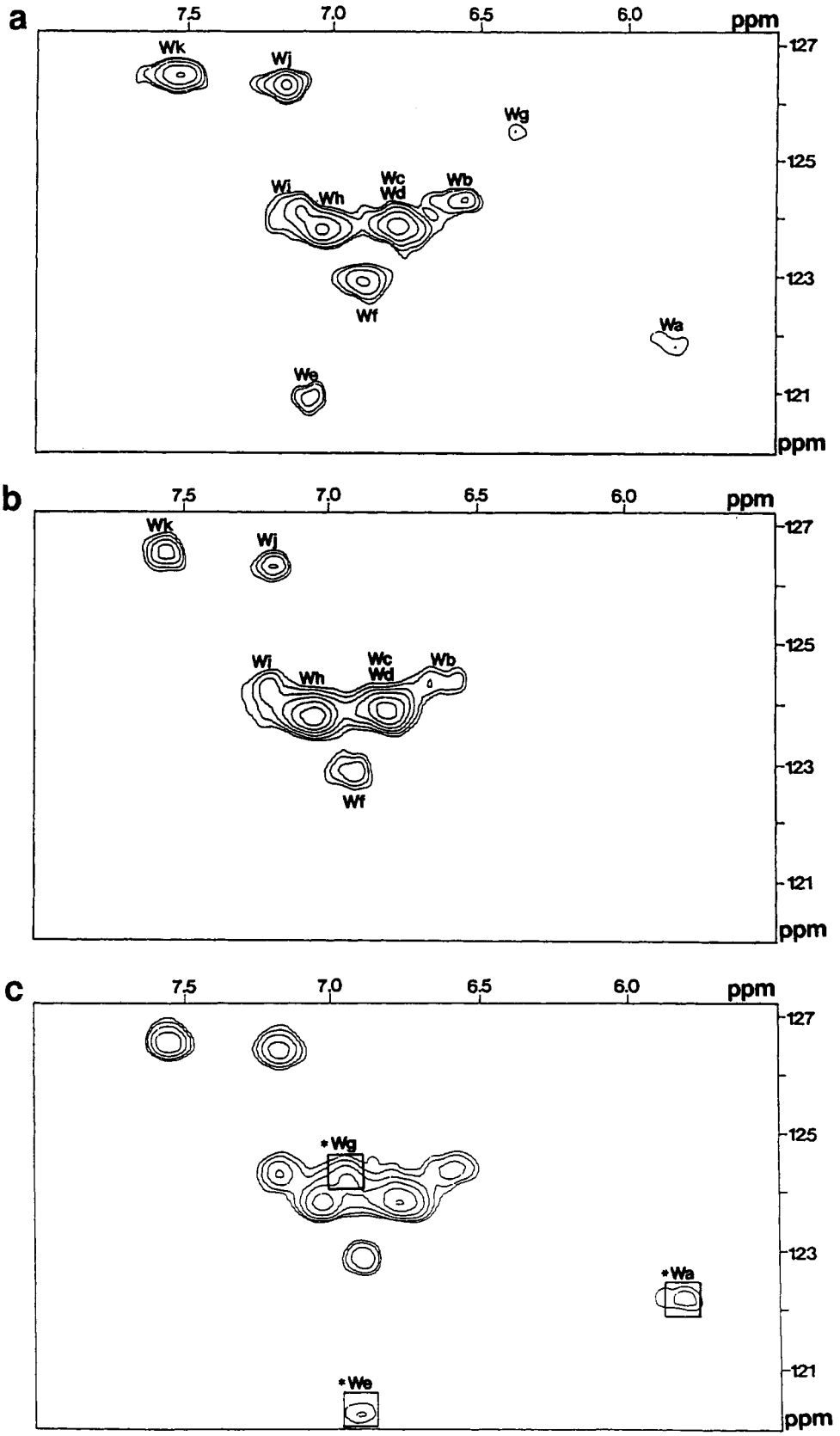

FIGURE 3: ^{1}H–^{13}C HSQC spectra of [W]N1G9. The spectra of [W]N1G9, the [W]N1G9–NP-AmTEMPO complex, and the [W]N1G9–NP-AmTEMPO complex observed in the presence of L-ascorbic acid are given in a, b, and c, respectively. In b and c, the cross peaks that reappeared upon the addition of L-ascorbic acid are indicated by asterisks.

Dependence upon the NaCl Concentration of the Dissociation Constants of N1G9 and 3B62 for NP-Gly. We investigated the effect of the NaCl concentration on the dissociation constants of N1G9 and 3B62 for NP-Gly. Figure 8 shows the dissociation constants of N1G9 and 3B62 obtained with various NaCl concentrations. As the NaCl concentration was increased, the dissociation constants of N1G9 and 3B62 for NP-Gly clearly increased. The dependence of the dissociation constant of N1G9 upon the NaCl concentration was similar to that of 3B62.

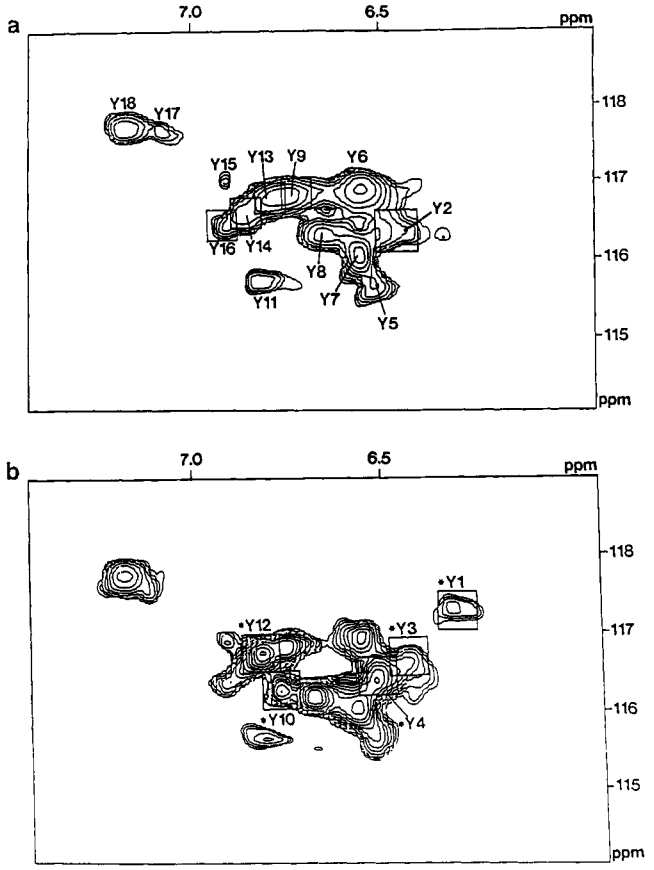

FIGURE 4: ^1H–^{13}C HSQC spectra of [Y]3B62. The spectra of the [Y]3B62–NP-AmTEMPO complex and the [Y]3B62–NP-AmTEMPO complex observed in the presence of L-ascorbic acid are given in a and b. In a and b, the cross peaks that reappeared upon the addition of L-ascorbic acid are indicated by asterisks.

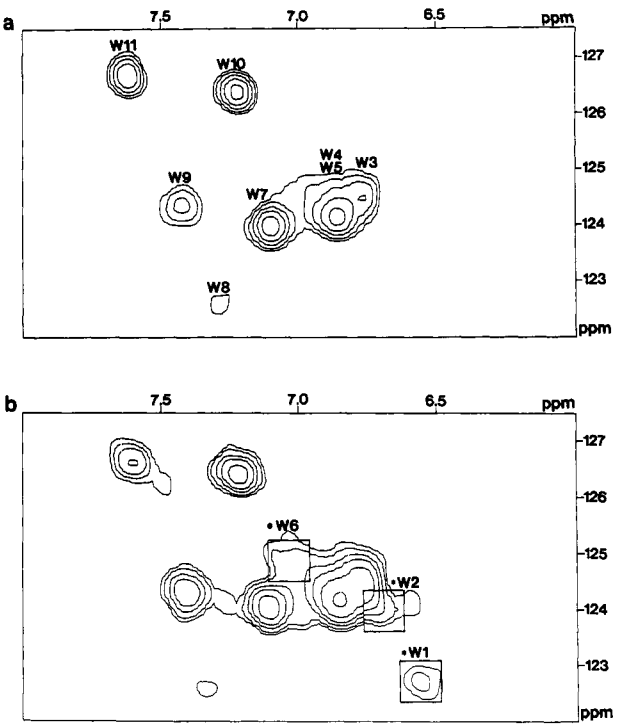

FIGURE 5: ^1H–^{13}C HSQC spectra of [W]3B62. The spectra of the [W]3B62–NP-AmTEMPO complex and the [W]3B62–NP-AmTEMPO complex observed in the presence of L-ascorbic acid are given in a and b, respectively. In a and b, the cross peaks that reappeared upon the addition of L-ascorbic acid are indicated by asterisks.

DISCUSSION

Interaction of NP-AmTEMPO with Anti-NP Monoclonal Antibodies N1G9 and 3B62. Figure 9 compares the amino acid sequences of the variable regions of anti-NP monoclonal antibodies N1G9 and 3B62, that were used in the present study (Cumano & Rajewsky, 1985, 1986). A typical primary response antibody, N1G9, which exhibits low affinity for NP, has no somatic mutations in the V186.2 V_H gene. A secondary response antibody, 3B62, which exhibit high affinity for NP, is heavily mutated in the V186.2 V_H gene (including one codon deletion in H2) and heterogeneous in the D–J_H region (H3). In the λ1 light chain, no somatic mutations are observed in N1G9, and only one somatic mutation is observed in 3B62 at position 55.

The results of the present NP-AmTEMPO binding experiments indicate that one Trp residue and four Tyr residues originating from the heavy chain and two Trp residues and one Tyr residue originating from the light chain exist within 15 Å of the paramagnetic center of the spin-labeled hapten in the antibody combining sites of both Fab(N1G9) and Fab(3B62).

In the light chains of both N1G9 and 3B62, one Trp residue (Trp-35L) and two Trp residues (Trp-91L and Trp-96L) are located at FR2(L) and L3, respectively (Figure 9).[2,3] The distribution of Trp residues in the amino acid sequences of the variable regions of N1G9 is identical to that in the case of 3B62. It is known, on the basis of X-ray crystallographic data, that each immunoglobulin domain possesses a Trp residue in its core region (Lesk & Chothia, 1982). The sequence data indicate that Trp-35L corresponds to this Trp residue. The V_L domains of N1G9 and 3B62 possess their Trp residues at the identical position of the sequences. This indicates that cross peaks Wa and Wg (N1G9) and cross peaks W1 and W6 (3B62), which disappeared upon the addition of NP-AmTEMPO, originate from Trp-91L and Trp-96L or *vice versa*. It has been shown, on the basis of X-ray crystallographic data, that there are *canonical structures* for L1, L2, L3, H1, and H2 (Chothia & Lesk, 1987; Chothia et al., 1989). A model built on the basis of the canonical structures also indicates that Trp-91L and Trp-96L exist in close spatial proximity to each other at the interface between V_H and V_L.

Furthermore, we have observed, using Fab(N1G9) analogues labeled with [^{15}N]Trp, that ^1H–^{15}N HSQC cross peaks originating from the amide groups of Trp-91L and Trp-96L, which can be assigned site-specifically by a double-labeling method (Kainosho & Tsuji, 1982), disappeared upon the addition of NP-AmTEMPO (T. Nakayama et al., manuscript in preparation). Therefore, we conclude that NP-AmTEMPO exists within 15 Å of Trp-91L and Trp-96L that belong to L3 in the antibody combining sites of N1G9 and 3B62.

Microenvironment of Antibody Combining Sites of Primary Response Antibody N1G9 and Secondary Response Antibody 3B62. Anti-NP antibodies have been studied extensively by means of variable region gene analysis and protein engineering (Cumano & Rajewsky, 1985, 1986; Allen et al., 1987, 1988). It has been demonstrated that most secondary response anti-NP antibodies exhibit higher affinity for NP than primary response antibodies and that 8 out of 13 secondary response antibodies are mutated at position 33 of the V186.2 V_H gene,

[2] The convention of Kabat et al. (1987) was followed for the numbering of V_H and V_L of the antibody. Amino acid residues in the heavy and light chains are denoted by H and L, respectively, e.g., Trp-33H and Trp-35L.

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

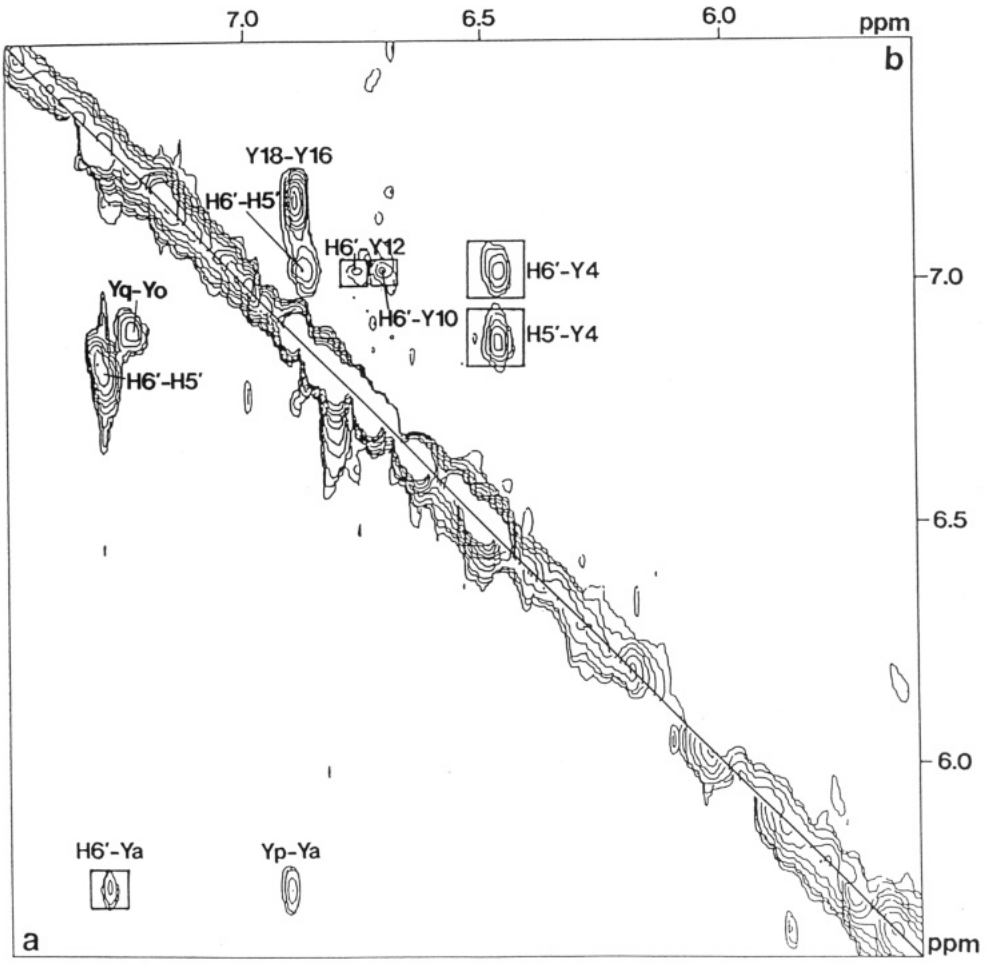

FIGURE 6: Aromatic regions of the NOESY spectra of [^{2}H]N1G9 and [^{2}H]3B62 observed in the presence of NP-Gly. The spectrum of [^{2}H]N1G9 is given in the lower left half (a), and the spectrum of [^{2}H]3B62 is given in the upper right half (b). In a and b, intermolecular NOE cross peaks are boxed. The mixing time used was 90 ms.

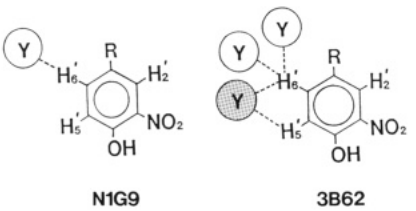

FIGURE 7: Schematic diagrams of the antibody combining sites of primary response antibody N1G9 and secondary response antibody 3B62. The open and shaded circles indicate the Tyr residues originating from the heavy chain and the light chain, respectively.

leading to the Trp→Leu exchange (Allen et al., 1987). On the basis of the results of site-specific mutagenesis at position 33 of the heavy chain of primary response antibody N1G9 along with gene analysis, it has been concluded that the Trp→Leu exchange at position 33 of the heavy chain plays a crucial role in gaining a high affinity in the affinity maturation of anti-NP antibodies (Allen et al., 1987).

Although secondary response antibody 3B62 exhibits the highest affinity for NP so far observed, it lacks the Trp→Leu exchange at position 33 of the heavy chain (Figure 9). The heavy chain of 3B62 carries 12 replacement mutations in the V186.2 V_H gene. The amino acid sequence of the light chain of 3B62 is identical to that of N1G9 except for a replacement at position 55. However, it has been suggested that the mutation at position 55 of the light chain is irrelevant as to the binding affinity of anti NP-antibodies (Allen et al., 1988). Therefore, it was not possible by gene analysis of the variable

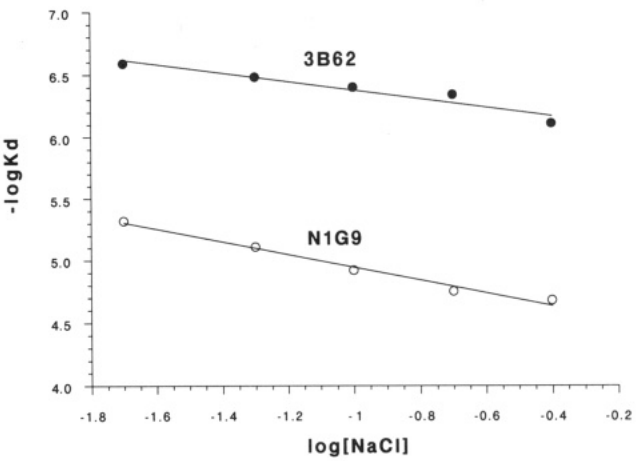

FIGURE 8: Dependence upon the NaCl concentration at 30 °C of the dissociation constants of N1G9 (○) and 3B62 (●) for NP-Gly.

regions to understand the mechanism for the high affinity of antibody 3B62 for NP.

On the basis of the results of NOESY experiments, it has been demonstrated that one Tyr residue originating from the heavy chain of Fab(N1G9) exists within 5 Å of the ring proton of NP and that two Tyr residues originating from the heavy chain of Fab(3B62) and one Tyr residue originating from the light chain of Fab(3B62) exist within 5 Å of the ring protons of NP (Figure 7). The results obtained by the NOESY experiments suggest that the antibody combining site of 3B62 is composed of more Tyr residues than that of N1G9. It

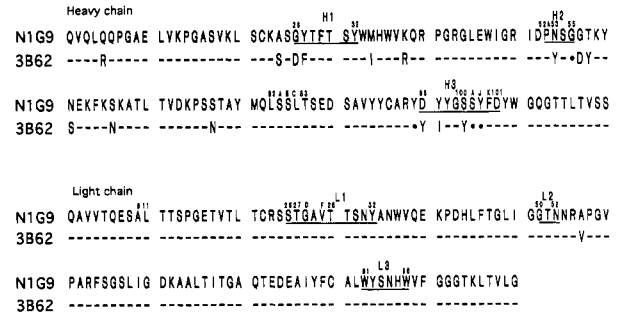

FIGURE 9: Comparison of the amino acid sequences of the variable domains of primary response antibody N1G9 and secondary response antibody 3B62. The amino acids belonging to the hypervariable regions are underlined.

should be noted that one Tyr residue originating from the light chain exists in close spatial proximity to NP in the antibody combining site of 3B62, although the sequence of the light chain of 3B62 is identical to that of N1G9, except for a mutation at position of 55, which is irrelevant as to the affinity.

It has been suggested that the positively charged groups exist in the antibody combining sites of anti-NP antibodies carrying the λ1 light chain (Azuma et al., 1987). To estimate the contribution of the electrostatic interaction in the antigen–antibody interaction, the dependence upon the ionic strength of the dissociation constants of N1G9 and 3B62 has been determined. As Figure 8 shows, in the cases of both N1G9 and 3B62, a high ionic strength leads to one-third lower affinity for NP. The results obtained in the present study indicate that the proportion of the electrostatic contribution to the interaction of N1G9 with NP is similar to that in the case of 3B62.

On the basis of the present NMR data, we conclude that the combination of mutations (including one codon deletion) and the particular D–J_H rearrangement in the heavy chain provide the antibody combining site of 3B62 with a more hydrophobic environment, which is formed from one Tyr residue originating from the light chain and two Tyr residues originating from the heavy chain. We conclude that this is the reason why secondary response antibody 3B62 exhibits high affinity for NP without the Trp→Leu exchange at position 33 of the heavy chain.

At the present stage, the molecular weight of the Fab fragment (M_r 50 000) hampers the site-specific assignment of all of the side-chain NMR signals originating from the Trp and Tyr residues of the anti-NP Fab fragments. To investigate in more detail the structures of the antibody combining sites of anti-NP antibodies and the mechanism of the affinity maturation of the immune response, an ^{15}N NMR study using ^{15}N-labeled anti-NP Fab analogues is now in progress in our laboratory.

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