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Nonaromatic Amino Acids in the Combining Site Region of a Monoclonal Anti-Spin-Label Antibody†

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ABSTRACT: The nuclear magnetic resonance spectra of monoclonal Fab antibody fragments have been recorded in the absence and presence of the specific spin-label dinitrophenyl hapten. The difference spectra reveal the presence of about 50 amino acids in the region of the combining site. By selective deuteration and by use of double difference spectra, all the resonances in the spectral region −1 to 1.5 ppm have been identified. We have found that in the combining site region there are four or five valines, certainly three and possibly five threonines, three or four leucines, two or three isoleucines, and six or seven alanines. Selective deuteration of methionine and lysine reveals one methionine and two lysines in the difference spectra. All of these amino acids are estimated to be within 17 Å of the paramagnetic hapten. By using difference spectra involving low fractional occupancy of the combining site with the spin-label hapten, it is established that one threonine and one valine are very close to the paramagnetic hapten.

Much progress has been made in accounting for the diversity and specificity of antibodies through studies of amino acid sequences and immunoglobulin genetics. X-ray crystallographic studies have shown that the hypervariable loops are responsible for binding site structure and that the more highly conserved sequences in the variable region are responsible for forming the immunoglobulin fold, the structural motif upon which all variable regions are built (Wu & Kabat, 1970; Amzel & Poljak, 1979). The number of antibody–hapten complexes studied by X-ray crystallography has been far too small to enable one to predict combining site structure and specificity from the amino acid sequence. It is with this and related problems in mind that we have undertaken a nuclear magnetic resonance study of a monoclonal antibody and its interactions with the parmagnetic hapten that it is directed against.

Previous workers (Dower & Dwek, 1979) have shown that it is possible to use the differences between the NMR spectra of the antibody and the antibody–hapten complex to get information about the structure of the binding site. We have extended this concept by employing the broadening effect of the spin-label hapten and biosynthetic incorporation of deuterated amino acids to identify the aromatic amino acid present in the combining site region of our monoclonal AN02 antibody (Anglister et al., 1984a). We have also shown that hapten exchange is fast enough that titration of the binding site (Campbell et al., 1975) allows the determination of proton–spin-label distances for well-resolved resonances in the difference spectra (Anglister et al., 1984b).

In the present work, we have used these approaches to study the more complicated spectral region due to the nonaromatic amino acids. The complexity of the spectrum in this region makes it necessary to use double difference spectra. In this type of analysis, the normal difference spectra (without hapten minus with hapten) are calculated for Fab's that differ only in the incorporation of one selected deuterated amino acid. The two difference spectra are then subtracted, leaving only the contribution of the selected amino acid in the double difference spectrum. We have assigned a large number of the resonances in the difference spectrum by this technique and in some favorable cases have made estimates of the distances between the amino acids and the paramagnetic hapten. It is hoped that such information, together with the (as yet unknown) amino acid sequence, will lead to a useful model of the combining site. Our present work and previous work on this problem clearly indicate that there are significant structural changes upon hapten binding.

Materials and Methods

The synthesis of the spin-label hapten has been described (Balakrishnan et al., 1982). The chemical formula can also be found in Anglister et al. (1984a).

The origin, maintenance, and labeling of the AN02 cell line have been described previously (Anglister et al., 1984a). Fab fragments were prepared by standard procedures. The amino acids perdeuterated L-lysine, L-threonine, and L-isoleucine were purchased from Cambridge Isotopes Laboratories. Perdeuterated L-alanine, L-leucine, L-valine, and L-[*methyl*-$^2H_3$]methionine were purchased from MSD Isotopes. Medium labeled with deuterated alanine included 3–4 mM alanine. In all other cases, the amino acids were in the regular quantities for RPMI medium. NMR spectra were taken with a JEOL 500-MHz spectrometer. The concentration of Fab was in the range (1.3–3) × 10^{-4} M, and sample volumes were 550 μL. Free induction decays were collected in 8000 data points after

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60° pulses. Delays were 1 s using a sweep width of ±4000 Hz. A total of 10000 scans were taken per sample. No smoothing of the spectra by exponential multiplication was used.

Difference Spectra. Three types of difference spectra are calculated from the NMR spectra of the Fab fragment of the antibody and the NMR spectra of its complexes with the spin-labeled hapten (SL) and with dinitrophenylglycine (DNP-Gly); the individual spectra are designated Fab(0), Fab(SL), and Fab(DNP-Gly), respectively. The single difference spectra are (a) Fab(0) − Fab(SL), (b) Fab(DNP-Gly) − Fab(SL), and (c) Fab(0) − Fab(DNP-Gly). In the NMR spectrum of the Fab complex with the spin-labeled hapten, all the resonances of the protons that are within 17 Å of the unpaired electron are considerably broadened. Some of the resonances undergo changes in chemical shift upon binding of the hapten. These changes are usually accompanied by broadening of the resonances, since the major changes in chemical shift arise from protons that are in the binding site region and, therefore, in the range of the broadening caused by the unpaired electron. We have accumulated evidence that for most of the resonances the chemical shift changes are the same as those occurring upon DNP-Gly binding. Single difference spectra of type b therefore show only the resonances that are broadened by the spin-labeled hapten and appear as sharp positive peaks upon a base line that may be slightly distorted by broad negative features. Spectrum c shows only the resonances that undergo changes in chemical shift upon DNP-Gly binding; this is a complicated combination of sharp positive and negative features. Such spectra were used in the present work but are not shown. Spectrum a shows mainly the resonances that are broadened by the unpaired electron, but in addition shows the resonances which undergo changes in chemical shift that are not accompanied by broadening.

Double Difference Spectra. In this type of spectra, the difference spectra are calculated for two preparations of Fab that differ in the incorporation of one selected deuterated amino acid. The difference between two difference spectra of the same type (spectrum a, b, or c) will give difference spectra that show only the proton resonances of the amino acid that was selectively deuterated. The signal to noise ratio in the double difference spectrum is reduced by a factor of approximately $\sqrt{2}$.

Distance Calculations. The paramagnetic center to amino acid distance was calculated as previously described (Anglister et al., 1984b). These calculations are based on the peak heights in the difference spectra and the line width of the unbroadened line. This line width was taken from the Fab(0) − Fab(SL) spectrum and used to calculate a distance. Since the shape of the line being subtracted from the unbroadened line affects the apparent line width in the difference spectrum, the calculated distance was used to calculate the line width in the Fab(0) − Fab(SL) spectrum. If the calculated and observed line widths did not agree, an adjustment was made to the line width used in the distance calculation. The procedure was repeated until the calculated and observed line widths agreed.

Results

Figure 1a gives the difference spectrum Fab(DNP-Gly) − Fab(SL) in the spectral region −1.0 to +2.0 ppm. This spectrum reveals all the methyl resonances from all the aliphatic amino acids in the combining site region. These signals are assigned on the basis of double difference spectra of selectively deuterated Fab fragments as seen in Figure 1b–f. Some of the double difference signals due to Thr (Figure 1b), Leu (Figure 1d), and Val (Figure 1c) have particularly large

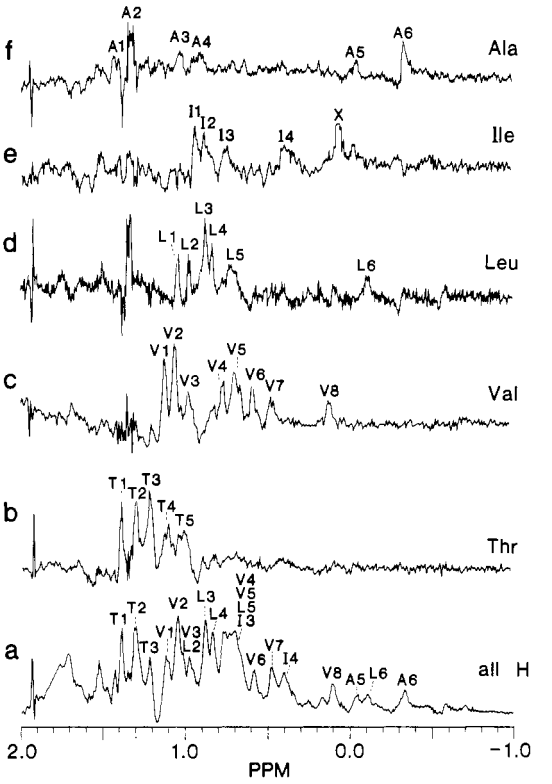

FIGURE 1: Proton magnetic resonance signals due to aliphatic amino acids in the Fab fragment of the monoclonal anti-spin-label antibody AN02. (a) Difference spectrum Fab(DNP-Gly) − Fab(SL), giving all aliphatic proton resonance signals in the region −1.0 to +2.0 ppm. (b) Double difference spectrum for threonine: [Fab(DNP-Gly) − Fab(SL)] − [Fab(DNP-Gly) − Fab(SL)]d(Thr). (c) Valine: [Fab(DNP-Gly) − Fab(SL)]d(Ala,Leu) − [Fab(DNP-Gly) − Fab(SL)]d(Ala,Val,Leu). (d) Leucine: [Fab(DNP-Gly) − Fab(SL)]d(Ala,Val) − [Fab(DNP-Gly) − Fab(SL)]d(Ala,Val,Leu). (e) Isoleucine: [Fab(DNP-Gly) − Fab(SL)] − [Fab(DNP-Gly) − Fab(SL)]d(Ile). Signal X is thought to be due to a contaminant. (f) Alanine: [Fab(DNP-Gly) − Fab(SL)]d(Val,Leu) − [Fab(DNP-Gly) − Fab(SL)](Ala,Val,Leu).

signal to noise ratios, which is probably due to a combination of their narrow intrinsic line widths and their proximity to the unpaired electron (Anglister et al., 1984b). On the basis of the number of signals and their relative intensities, we can identify five threonines, four to five valines, three to four leucines, two to three isoleucines (with two methyl signals per valine, leucine, and isoleucine), and six to seven alanines.

Figure 2a gives the difference spectrum Fab(0) − Fab(SL) in the same spectral region as Figure 1a. As seen in the double difference spectra in Figure 2b–e, some resonance signals undergo significant changes in chemical shift on hapten binding, and others do not. Negative features in the double difference spectra, such as those seen for leucine and isoleucine, arise when there are significant changes in chemical shift and no significant paramagnetic broadening. Most, but not all, of the threonine and valine methyl resonances do not undergo observable changes in chemical shift. On the other hand, almost all of the methyl resonances of isoleucine undergo changes in chemical shift. The changes in chemical shift on hapten binding are accompanied by various degrees of line broadening. For example, the negative features L7 and L8 and most of the isoleucine signals experience almost no broadening. The valine signal V7 undergoes a small chemical shift and some line broadening, leading to the unsymmetrical signal seen in the difference spectrum in Figure 2c.

Figure 3 shows the single difference spectra [Fab(DNP-Gly) − Fab(SL)]d(Val,Ala,Leu) and [Fab(0) − Fab(SL)]d(Val,-

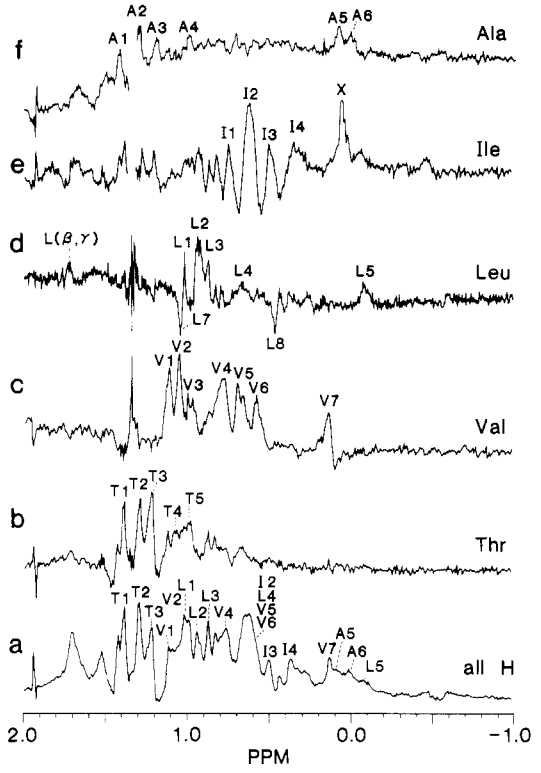

FIGURE 2: Proton resonance signals due to aliphatic amino acids derived from the difference spectra. (a) Fab(0) − Fab(SL). (b) [Fab(0) − Fab(SL)] − [Fab(0) − Fab(SL)]d(Thr). (c) [Fab(0) − Fab(SL)]d(Ala,Leu) − [Fab(0) − Fab(SL)]d(Ala,Val,Leu). (d) [Fab(0) − Fab(SL)]d(Ala,Val) − [Fab(0) − Fab(SL)]d(Ala,Val,Leu). (e) [Fab(0) − Fab(SL)] − [Fab(0) − Fab(SL)]d(Ile). Signal X is thought to be due to a contaminant. (f) [Fab(0) − Fab(SL)]d-(Val,Leu) − [Fab(SL)]d(Ala,Val,Leu).

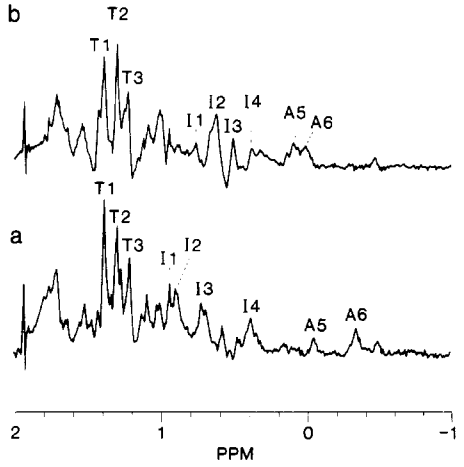

FIGURE 3: Difference spectra based on the simultaneous deuteration of three amino acids in the Fab fragment of AN02. (a) [Fab-(DNP-Gly) − Fab(SL)]d(Ala,Val,Leu); (b) [Fab(0) − Fab(SL)]d-(Ala,Val,Leu). The appearance of alanine in these difference spectra is thought to be due to the biosynthesis of this amino acid from protonated pyruvic acid.

Ala,Leu) [d(amino acid) means protein was grown with the indicated amino acids deuterated]. These spectra represent the only incident we have encountered where proton signals (due to alanine) appear in difference spectra where the corresponding amino acid was nominally perdeuterated. Presumably the protonated alanines giving these signals arose from biosynthesis of alanine from protonated precursors. The isoleucine peak amplitudes are relatively weak compared to the prominent threonine peaks in Figure 3a. The isoleucine peaks also appear to be relatively weak in the double difference

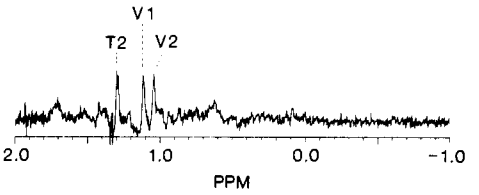

FIGURE 4: Proton methyl signals from a single threonine residue and a pair of methyl signals from a single valine residue, all of which are close to the paramagnetic hapten in the combining site region. The difference spectrum is Fab(0) − Fab(SL) for a 3.7% occupancy of the combining site with DNP-SL.

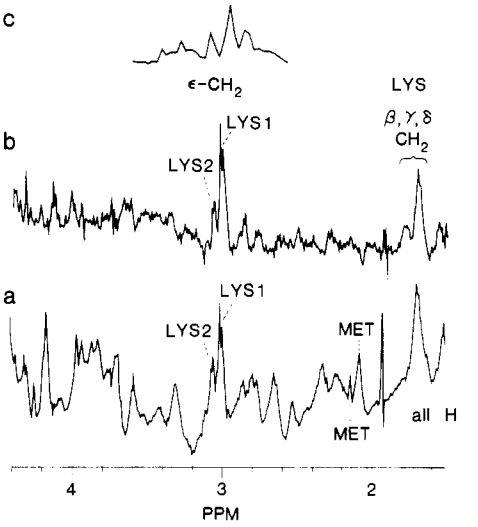

FIGURE 5: Proton resonance signals of AN02 in the region 1.5–4.4 ppm showing resonances due to lysine and methionine. (a) Fab(0) − Fab(SL). (b) [Fab(0) − Fab(SL)] − [Fab(0) − Fab(SL)]d(Lys). (c) The region from 2.9 to 3.1 ppm is given for 19% occupancy of the combining site with the spin-label hapten. Lysine is protonated.

spectrum in Figure 1e. This may be due to the fact that these residues are further removed from the paramagnetic center and/or have signals of larger intrinsic line width.

Figure 4 shows the single difference spectrum Fab(0) − Fab(SL) for 3.7% occupancy of the binding site. The two methyl signals V1 and V2 in Figure 4 have the same chemical shifts as V1 and V2 in the spectrum in Figure 2c. Moreover, these signals are gone when valine is deuterated, and the difference spectrum is calculated for the same fractional occupancy. The signal marked T2 has the same chemical shift as T2 in the spectrum in Figure 2b. This signal also appears when Ala, Val, and Leu are deuterated and difference spectra are calculated for the same fractional occupancy. These methyl groups are undoubtedly quite close to the nitroxide group of the hapten.

Figure 5a is the single difference spectrum Fab(0) − Fab-(SL) in the region 1.5–4.4 ppm. Note that signals due to methionine are identified in this spectrum. Figure 5b gives a double difference spectrum revealing signals due to lysine. Note that distinct resonances due to ϵ-CH_2 protons of two lysines are resolved, one lysine evidently being more distant from the hapten. The insert to Figure 5b shows the expanded spectrum of the ϵ-CH_2 of the lysines in a single difference spectrum where lysine is protonated and the binding site is 19% occupied with the spin-labeled hapten. This spectrum and similar difference spectra calculated for partial occupancies of the binding site are used to estimate distances.

Parts a and b of Figure 6 show experimental and theoretical values for the normalized difference spectra peak intensities, $J(f)$, for ϵ-CH_2 protons of lysine and for methionine CH_3

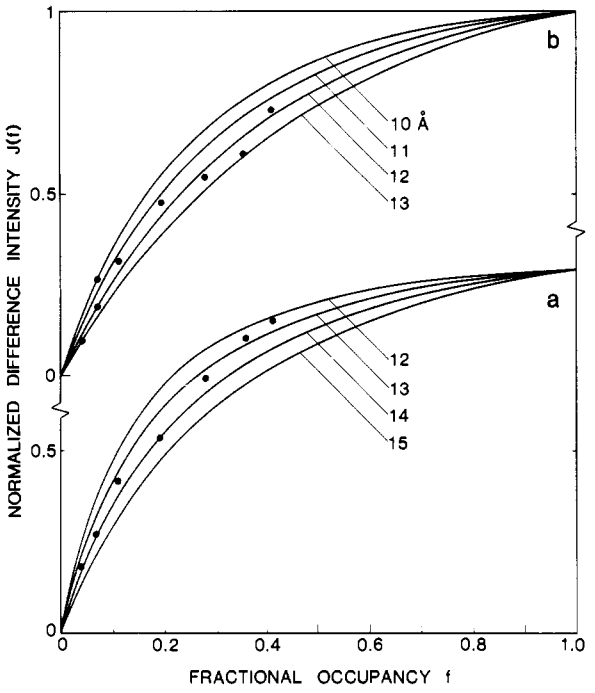

FIGURE 6: Experimental and theoretical values for the normalized difference spectra peak intensities $J(f)$. (a) Calculated values of $J(f)$ for the ϵ-CH_2 protons of lysine, assuming an intrinsic line width of 6.3 Hz. The best-fit distance of 13.5 Å corresponds to a theoretically predicted 5.45-Hz line width in the Fab(0) − Fab(SL) difference spectrum, where a line width of 5.4 Hz is actually observed. (b) $J(f)$ calculated for the methyl protons of methionine, assuming 20 Hz as the intrinsic line width. A distance of 11.5 Å gives a difference spectrum line width of 17 Hz, which agrees with the 17 Hz observed.

protons, respectively, as a function of the fractional occupation of the binding site, f. For the quantitative definition of $J(f)$ and its relation to distances, see Anglister et al. (1984). The data in Figure 6 indicate that the ϵ-CH_2 protons of lysine are (on the average) 13.5 ± 0.75 Å from the paramagnetic nitroxide group and the CH_3 protons of methionine are 12 ± 1 Å removed from the paramagnetic center. The ϵ-CH_2 of a second lysine is observed and estimated to be on the order of 16 Å from the paramagnetic center.

Discussion

In the present work, we have used NMR difference spectra, paramagnetic hapten line broadening of proton resonance signals, hapten-induced changes in chemical shifts, and selective deuteration to identify some 21–27 nonaromatic amino acids in the combining site region of the monoclonal anti-spin-label antibody AN02. These amino acids include four to five valines, three to five threonines, three to four leucines, two to three isoleucines, six to seven alanines, two lysines, and one methionine. From integration of the single difference spectra in the various preparations of Fab (fully protonated and partially deuterated), we conclude that the sum of the spectra of Thr, Val, Leu, Ile, and Ala accounts for the entire protonated spectrum in the region −1.0 to +1.5 ppm. Using quantitative arguments similar to those described previously, we can safely state that all of these amino acids are within 17 Å of the paramagnetic nitroxide group. Difference spectra involving low concentrations of spin-label hapten show that one threonine and one valine must be very close to the paramagnetic hapten.

A number of these nonaromatic amino acid proton signals undergo easily observable changes in chemical shift on hapten binding. It is likely that some of these changes in chemical shift are due to conformational changes. The changes in chemical shift due to the DNP spin-label appear to be nearly equal to those produced by DNP-Gly. This, of course, is very helpful for the interpretation of the difference spectra.

In previous work, we have identified 14 aromatic amino acids in the combining site region: 1 His, 3 Trp, 8–9 Tyr, and 2 Phe (Anglister et al., 1984a). Thus, altogether we have identified approximately 40 amino acids in the combining site region. Further work will be required to establish the presence or absence of the nine other amino acids. On the basis of the difference spectrum of the fully protonated Fab fragment, we estimate that we have identified about 75% of the amino acids in the combining site region. There is still some uncertainty about the number of alanines in the combining site region, since the deuteration of alanine in the present work is likely to be incomplete (due to conversion of protonated pyruvate to alanine). This problem can probably be circumvented by changes in the growth medium. It should be noted that all the amino acids we have selectively deuterated until now are essential amino acids except alanine and tyrosine.

It is anticipated that we can soon arrive at a useful working model for the combining site region when the heavy and light chains of the Fab fragment are sequenced, when the heavy and light chains are individually deuterated and recombined, and when a number of additional distance determinations are made. In work to be published elsewhere, we have found that DNP-Gly and a six-membered nitroxide ring system bind noncompetitively to AN02, and this should facilitate additional distance determinations, especially for short distances.

Registry No. Val, 72-18-4; Thr, 72-19-5; Leu, 61-90-5; Ile, 73-32-5; Ala, 56-41-7; Met, 63-68-3; Lys, 56-87-1.

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