Distances of Tyrosine Residues from a Spin-Label Hapten in the Combining Site of a Specific Monoclonal Antibody†

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ABSTRACT: The nuclear magnetic resonance spectra of an Fab fragment of a monoclonal antibody specifically directed against a nitroxide spin-label hapten have been recorded at different concentrations of the hapten. The hybridoma producing this antibody was grown on deuterated phenylalanine, tryptophan, and 3,5-dideuteriotyrosine or 2,6-dideuteriotyrosine. Difference spectra—without hapten minus with hapten—were calculated for each concentration of hapten. The difference spectra reveal five well-resolved singlet proton resonance signals from tyrosine deuterated in the 3,5-positions (H 2,6 Tyr) and nine from tyrosine deuterated in the 2,6-positions (H 3,5 Tyr). The measured intensities of these signals as a function of combining site occupation have been interpreted in terms of a theory involving intrinsic line widths (T_2), the hapten off-rate (k), and distances to the paramagnetic center. Good agreement with theory is found for all of the isolated proton signals. The best estimate of k is 350 s^{-1}; distances in the range 13 to <9 Å are calculated. Extension of this analysis to other amino acids is discussed.

Paramagnetic probes bound to a macromolecule broaden the nuclear resonance signals of nearby nuclei in a way that is related to the distance between the nuclei and the probe (Carrington & McLachlan, 1967; Sternlicht et al., 1965a,b; Jardetzky & Roberts, 1981). When the resonance lines corresponding to the nuclei studied are well resolved and the broadening can be measured, distances can be accurately determined. The distances are usually in the range 15–20 Å (Wien et al., 1973). For smaller distances the broadening is so large that the resonance signals cannot be detected. Campbell et al. (1975) suggested a method to measure these shorter distances by varying the fractional occupancy of binding sites for paramagnetic probes. Semiquantitative results were obtained by titrating lysozyme with Gd^{3+}.

In the present paper we give a detailed analysis of broadening effects, which enables accurate determinations of distances smaller than 15 Å. The method is used to determine distances of tyrosine residues from the combining site of a monoclonal antibody (ANO2) that is specifically directed against a spin-labeled hapten. Our study employs a hybridoma grown on selected deuterated amino acids, and combinations of amino acids, so as to produce monoclonal antibody with resonance signals that come from unique protons in single amino acids. This approach was used previously to determine which amino acids are in the combining site region of this AN02 antibody (Anglister et al., 1984).

Materials and Methods

The synthesis of the spin-label hapten has been described (Balakrishnan et al., 1982).

The origin, maintenance, and labeling of the AN02 cell line have been described previously (Anglister et al., 1984)). Fab fragments were prepared by standard procedures. The equilibrium stability constant for the binding of the spin-label hapten is 4×10^6 mol/L at room temperature in PBS.[1] The stability constant of DNP-Gly is 2×10^6 mol/L under the same conditions.

Amino acids L-tryptophan-*2′,4′,5′,6′,7-d*$_5$, L-(4-hydroxyphenyl-3,5-*d*$_2$)alanine (tyrosine), L-(4-hydroxyphenyl-*2,6-d*$_2$)alanine-*2-d*$_1$ (tyrosine), and L-phenyl-*d*$_5$-alanine-*3,3-d*$_2$ were purchased from MSD Isotopes.

NMR spectra were taken on a JEOL 500-MHz spectrometer. Free induction decays were collected in 800 data points after 60° pulses. Delays were 1 s when a sweep width of ±4000 Hz was used; 10 000 scans were taken per sample. No smoothing of the spectra by exponential multiplication was used.

Theory

Theoretical Background: Difference Spectra. In our previous work we have used isotopic difference spectra to determine which aromatic amino acids are in the combining site region of AN02. In the present work we use difference spectra to obtain information on the distances of these amino acids from the combining site—specifically distances from the paramagnetic electron on the spin-label nitroxide group. Under conditions of fast hapten chemical exchange between the combining sites in a solution of Fab (f), the transverse relaxation time T_2 of an amino acid proton is given by the formula

$$\frac{1}{T_2} = \frac{f}{T_{2M}} + \frac{1-f}{T_{2N}} \tag{1}$$

Here f is the fraction of the time the combining site is occupied by the hapten. The transverse nuclear relaxation times T_{2M} and T_{2N} apply to the solutions Fab(1) and Fab(0), that is, solutions in which the combining site is always occuped or always empty, respectively. The relaxation rate T_{2M}^{-1} itself is a sum of two terms:

$$\frac{1}{T_{2M}} = \frac{1}{T_{2M'}} + \frac{1}{T_{2N}} \tag{2}$$

where $(T_{2M'})^{-1}$ is the enhancement of the nuclear relaxation rate due to the hapten. The Solomon–Bloembergen dipolar enhancement of the transferse relaxation due to the unpaired electron spin is

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[1] Abbreviations: Fab, antigen binding fragment of the antibody; DNP-Gly, (2,4-dinitrophenyl)glycine; PBS, phosphate-buffered saline, pH 7.2; H 2,6 Tyr, tyrosine deuterated in the 3,5-positions; H 3,5 Tyr, tyrosine deuterated in the 2,6-positions.

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$$(T_{2M}')^{-1} = \frac{S(S+1)\gamma_I^2 g^2 \beta^2}{15}\left[4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2}\right]\left\langle\frac{1}{r^6}\right\rangle \quad (3)$$

Here the broken brackets designates an average over high-frequency intramolecular motions. The correlation time τ_c due to molecular tumbling for Fab fragments has been estimated to be 1.5×10^{-8} s (Anglister et al., 1984). The other symbols have their usual significance; r is the distance between the odd electron and the nucleus. The second term in the square brackets is then totally negligible. Insertion of the fundamental constants in eq 3 leads to

$$T_{2M}' = \langle (r/30.1)^6 \rangle \quad (4)$$

Here r is given in angstroms.

We assume for simplicity that each proton resonance line shape is Lorentzian with intensity $I(\omega)$.

$$I(\omega) = \frac{1}{\pi}\frac{T_2}{1+(\omega-\omega_0)^2 T_2^2} \quad (5)$$

Under conditions of fast exchange, at the resonance frequency ($\omega = \omega_0$), the Lorentzian peak height is

$$I(f,\omega_0) = (1/\pi)(T_{2M}T_{2N})/(f\Delta T_2 + T_{2M}) \quad (6)$$

where $\Delta T_2 = T_{2N} - T_{2M}$. Under conditions of arbitrary exchange rate, the general expression for the peak intensity is

$$I(k,f,\omega_0) = \frac{1}{\pi}\frac{[T_{2M}(1-f) + \Delta T_2(1-f)^2 + kT_{2N}T_{2M}]}{[(1-f) + \Delta T_2 kf + T_{2M}k]} \quad (7)$$

Here k is the off-rate for hapten dissociation. Equation 7 reduces to eq 6 when k is sufficiently large. In the slow exchange limit, where $k = 0$, we have

$$I(0,f,\omega_0) = (1/\pi)[T_{2M} + \Delta T_2(1-f)] \quad (8)$$

In comparing these theoretical expressions with experimental data, it is convenient to calculate normalized intensities $J(f)$.

$$J(f) = \frac{[I(k,0,\omega_0) - I(k,f,\omega_0)]}{[I(k,0,\omega_0) - I(k,1,\omega_0)]} \quad (9)$$

As will be seen later, chemical shifts due to hapten binding are also significant and can sometimes be dominant in affecting difference spectra. Relevant formulas allowing for simultaneous effects of chemical shifts and paramagnetic broadening are not given here explicitly but can be found in the literature (Carrington & McLachlan, 1967).

Results

Under Theory it is clear that distance determinations are best carried out if the off-rate k can be measured independently. In the present work the selective partial deuteration makes it possible to estimate k directly from the spectra. The logic behind this determination is the following. We assume that the signals labeled H in the spectra of Figure 1 arise from the same pair of H 3,5 Tyr protons and that the chemical shift of these protons is changed upon binding of DNP-Gly. Signal H in Figure 1b should thus have a position and line width depending on the concentration of DNP-gly and the exchange rate, providing the exchange rate is comparable or large compared to the chemical shift difference (Gutowsky et al., 1953; McConnell, 1958). By numerical calculation we find that the position and line width of signal H in Figure 1b is accounted for to within the experimental error with $k = 500$ s^{-1}. A similar analysis for line A yields the same value of k (see Table I). This value of k applies of course to DNP-Gly.

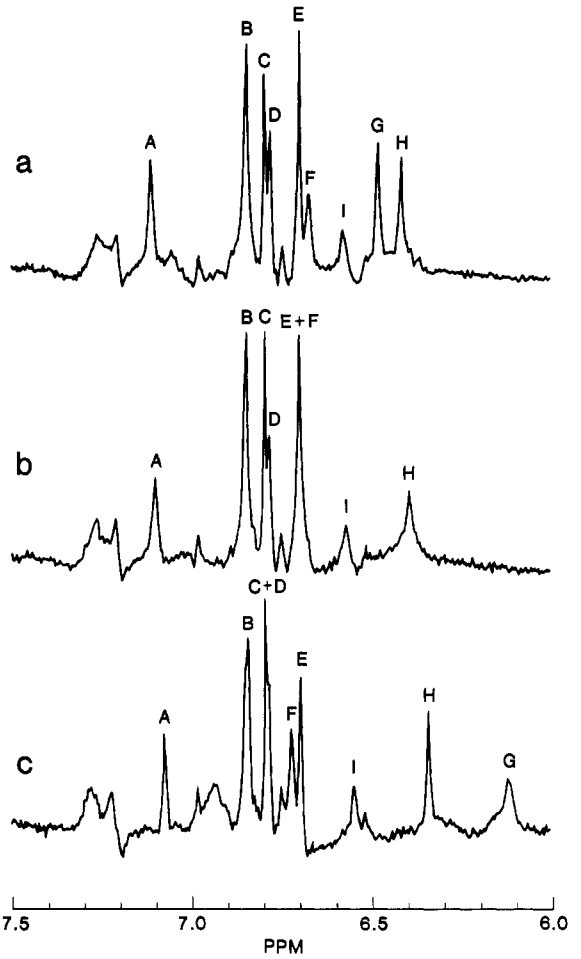

FIGURE 1: Proton resonance difference spectra for H 3,5 Tyr. The spectra are the differences between the proton spectra of Fab solutions of various concentrations of DNP-Gly minus the spectrum Fab(1) in which the combining site is completely occupied by the spin-label hapten. The combining site occupations by DNP-Gly are 0%, 31%, and 100% in (a), (b), and (c), respectively. The Fab fragment contains deuterated tryptophan and phenylalanine in addition to the partially deuterated tyrosine. Proton signals A and H are used to estimate the off-rate k for DNP-Gly (see text and Table I).

Table I: Determination of DNP-Gly Off-Rate from Chemical Shifts and Line Broadening of H 3,5 Tyr[a]

resonance line	off-rate k	change in chemical shift (Hz)	line width $\Delta\nu_{1/2}$ (Hz)
		−5.80 (obsd)	8.80 (obsd)
A	600	−6.00	8.50
	500	−5.75	8.80
	400	−5.75	9.15
		−12.15 (obsd)	12.45 (obsd)
	600	−12.00	11.25
H	500	−11.75	12.50
	400	−11.50	13.50

[a] Observed and calculated results refer to a solution of Fab in which 31% of the combining sites are occupied ($f = 0.31$). Positive change in chemical shift is toward lower field.

The stability constant of DNP-Gly at 37 °C is 1.3×10^6, and the calculated k_{on} is therefore 6.5×10^8 M s^{-1}. As discussed below we have taken $k = 350$ s^{-1} for the spin-label hapten. Spectra for H 2,6 Tyr are shown in Figure 2. These spectra were not used to estimate the exchange rate since the necessary lines are not resolved in the partially occupied case (see Figure 2b).

We now consider spectra that depend on exchange rate, distance-dependent line broadening, and chemical shifts. For

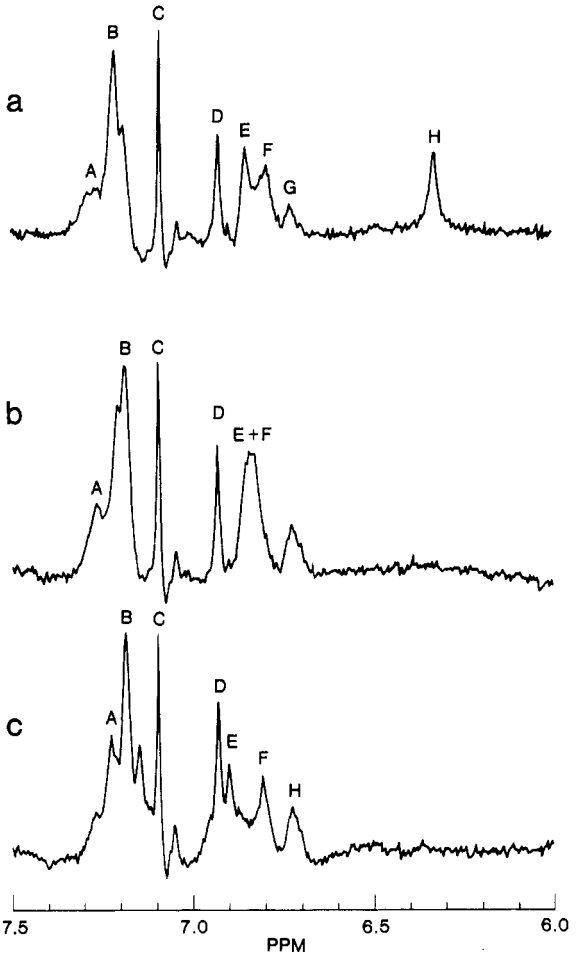

FIGURE 2: Proton resonance difference spectra for H 2,6 Tyr. The spectra are the differences between the proton spectra of Fab solutions of various concentrations of DNP-Gly minus the spectrum Fab(1) in which the combining site is completely occupied by the spin-label hapten. The combinding site occupations by DNP-Gly are 0%, 31%, and 100% in (a), (b), and (c), respectively. The Fab fragment contains deuterated tryptophan and phenylalanine in addition to the partially deuterated tyrosine.

this purpose we give plots of experimental values of $J(f)$ in Figure 3. The data in Figure 3a can be considered first. When it is assumed that change in the chemical shift induced by DNP-Gly is the same as that induced by the DNP group in the spin-label hapten, then calculated values of $J(f)$ for signal H in H 2,6 Tyr do not depend significantly on the distance r. Evidence for the equality of these two chemical shifts will be presented in detail elsewhere. These calculated values of $J(f)$ also do not depend strongly on the chemical shift difference itself, as long as it is large. Under these circumstances the $J(f)$ data also provide an estimate of k for the spin-label hapten itself. The data in Figure 3a (and comparable data in Figure 3b for line G in H 3,5 Tyr) yield $k = 350$ s^{-1}. This value is used in the subsequent calculations of the distances r.

Typical plots comparing experimental and theoretical values of $J(f)$ are given in Figures 4 and 5 by using $k = 350$ s^{-1}, and the best estimated values of r are also given. The numerical data used, and values of r, are given in Table II. Table II also gives values of $\Delta\nu_{1/2} = 1/(\pi T_{2N})$ and the changes in chemical shifts which are required for the theoretical calculations.

Discussion

In the present work we have shown that it is possible to (a) obtain chemical exchange rates for a spin-label hapten from proton resonance signals in a specific Fab combining site and (b) obtain distances of eight tyrosine residues from the paramagnetic center (nitroxide group) of the hapten. In terms of the use of nuclear resonance spectra to study combining site composition and structure, our approach is novel in that the monoclonal antibody was raised against this spin-label hapten, complete and partial amino acid deuteration was used to simplify the spectra, and various hapten concentrations were employed to facilitate distance determinations.

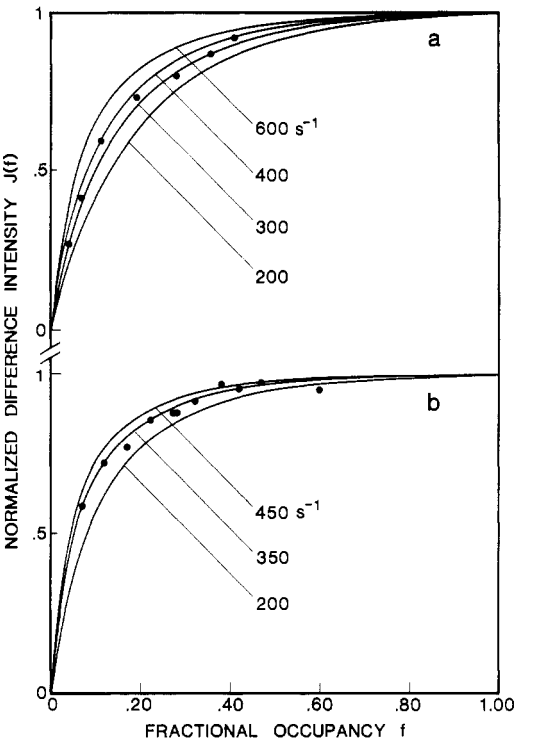

FIGURE 3: Experimental and theoretical values for the normalized difference spectra peak intensities $J(f)$ for proton signals H (upper part a) from H 2,6 Tyr and signal G (lower part b) from H 3,5 Tyr. These data are used to calculate the off-rate k for the spin-label hapten. Solid lines are from theoretical calculations using the indicated off-rates k and changes in chemical shift and line widths given in Table II. Since calculated values of $J(f)$ are virtually independent of the spin-label–proton distance for these large chemical shift differences, values of r were arbitrarily taken to 7 Å.

Table II: Calculated Distances and Relevant Data

	chemical shift[a] (Hz)	line width $\Delta\nu_{1/2}$ (Hz)	distance, r (Å)
H 3,5 Tyr			
A	−18.65	7.6	14 ± 1.5
B (2 pairs)	0	8.7[b]	≤9.0[b]
C	0	5.2	11 ± 2
D	7	7	12 ± 2
E	3	5	13 ± 1.5
F	25	10.5	≤10
G	−200	6.3	?
H	−38.65	7.2	<12
H 2,6 Tyr			
A	?		
B	overlap		
C	2.5	5	12 ± 1
D	0	8.5	13 ± 1
E	23.5	10	>11
F	7.5	14	12 ± 1
G			"far"
H	195	13	?

[a] Positive change in chemical shift is toward lower field. [b] This line may be inhomogeneous because it arises from two pairs of protons. If the width is assumed to be 5 Hz, the calculated distance is 11 Å.

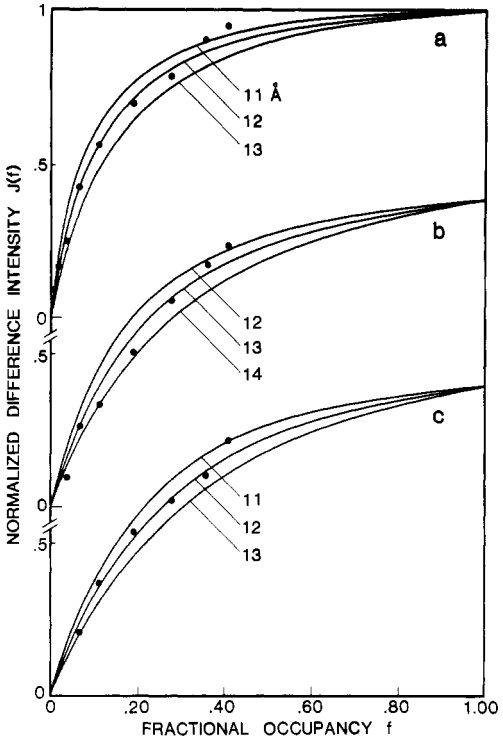

FIGURE 4: Experimental and theoretical values for the normalized difference spectra peak intensities $J(f)$ for selected proton signals from H 2,6 Tyr. Data are from line C in Figure 2 (a, upper), line D (b, middle), and line F (c, lower). Solid lines are from theoretical calculations for the indicated distances, and $k = 350\ s^{-1}$. Line widths, changes in chemical shift, and best values of the distance are given in Table II.

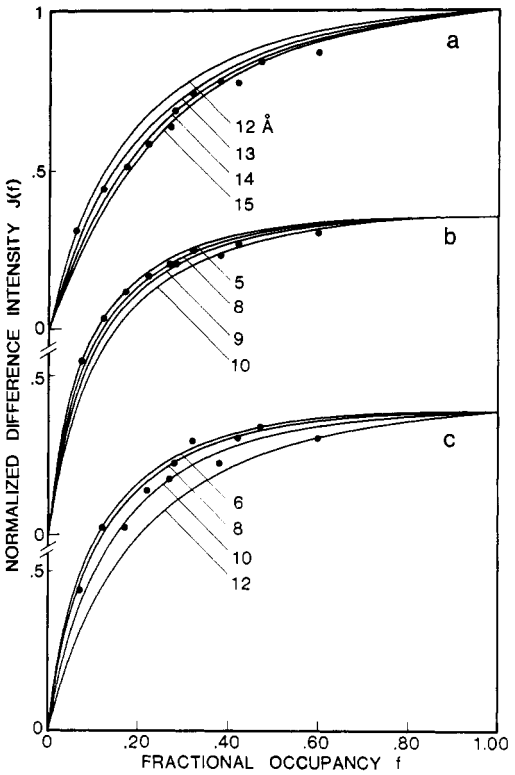

FIGURE 5: Experimental and theoretical values for the normalized difference spectra peak intensities $J(f)$ for selected proton signals from H 3,5 Tyr. Data are from line A in Figure 1 (a, upper), line B (b, middle), and line F (c, lower). Solid lines are from theoretical calculations for the indicated distances, and $k = 350\ s^{-1}$. Line widths, changes in chemical shift, and best values of the distance are given in Table II.

In general, the determination of the distance of any given amino acid proton from the paramagnetic center requires a line-shape analysis that depends on the intrinsic line width and line shape (assumed Lorentzian), the change in chemical shift produced by hapten binding, and the chemical exchange rate. The concordance between experimental data and theoretical calculations exhibited in Figures 3–5 is good. At the present time we believe that most of the discrepancy between observed and calculated data is due simply to overlap of resonance signals. This can be a rather subtle effect, involving the overlap of spin-label-broadened proton resonance signals that is not apparent in the spectra shown in Figure 1.

It will be seen from Table II that our calculated distances of tyrosine residues from the paramagnetic center cluster in the range 10–13 Å. A number of effects may contribute to this apparent clustering. From a purely statistical point of view there is 20% more volume in a spherical shell between 10 and 13 Å than there is in a sphere of radius 10 Å. However, we have only identified two tyrosine residues at distances less than 10 Å (signal B for H 3,5 Tyr). There are weak tyrosine signals in different spectra corresponding to greater distances. Tyrosine residues giving rise to signals G and H from H 3,5 Tyr and signal H from H 2,6 Tyr may also be within 10 Å of the paramagnetic center. It is entirely possible that the concentration of tyrosine residues at distances less than 10 Å is indeed less than that in the 10–13-Å shell. The structure of the nitroxide ring itself suggests to us that it may be surrounded by aliphatic residues. Preliminary data support this conjecture.

One pair of protons in H 3,5 Tyr (Figure 1) and one pair of protons in H 2,6 Tyr (Figure 2) give rise to signals G and H, respectively, that undergo large changes in chemical shift upon binding of DNP-Gly. These large shifts, ~200 Hz, are in opposite directions. The starting chemical shift of signal H due to H 2,6 Tyr is unusual and is shifted toward a normal position on DNP-Gly binding. The line widths of both signals, G of H 3,5 Tyr and H of H 2,6 Tyr, are both increased on DNP-Gly binding. This (or these) tyrosine residue(s) must occupy some unique position in the structure. No distance information on these protons can be obtained because of the large changes in chemical shift.

It is clear that the use of partially deuterated amino acids to remove spin–spin splittings and simplify spectra greatly facilitates studies of this type.

Registry No. Tyr, 60-18-4.

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