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#### ANNUALREPORT

#### GRANT # N00014-90-J-1407

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#### PRINCIPAL INVESTIGATOR: Harden M. McConnell

**INSTITUTE:** Stanford University

**<u>GRANT TITLE</u>**: Determination of the Structural Basis of Antibody Diversity Using NMR

PERIOD OF PERFORMANCE: 15 December 1989 - December 14 1990

<u>OBJECTIVE</u>: To use NMR to determine the composition, structure and kinetics of antibody combining sites.

#### ACCOMPLISHMENTS; (since July 1990)

(a) Conformational Heterogeneity

From the present work it is clear that the NMR of proteins such as antibodies can yield significant structural and kinetic information that is not available from x-ray crystallographic studies. As discussed in Theriault <u>et al.</u>, the AN02 Fab protein shows an H3,5 tyrosine proton resonance signal G that is strongly shifted upfield by 176 Hz on binding of a dinitrophenyl hapten, DNP-digly. The up-field signal is enoted G'. Signal G' has a number of properties that make it distinct from the doze is so signals arising from the other tyrosine residues in the combining site. For example, the line width of G' increases with increasing temperature, and increases in proportion to the increase in off-rate. Further G' is coupled by magnetization transfer to a second signal, G''. A detailed analysis of the data shows that on hapten binding signal G splits to give two signals, G' and G''. The data are accounted for by the kinetic scheme,



The tyrosine residue (probably Tyr 31L) takes on two conformations, denoted by  $(AbH)_1$ , and  $(AbH)_2$ , that interconvert when the binding site is empty. When hapten binds, the tyrosine in question becomes locked in one of two non-interconverting conformations.

(b) Chemical Shift Calculations

Conventional 2D NMR is not very helpful for signal assignments for Fab fragments. We have found that chemical shift assignments, especially for strongly shifted

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signals, are extremely useful. In our AN02 study, this has involved calculations based on the known crystal structure (Leahy & Fox, to be published). See Theriault <u>et al</u>. (manuscript attached).

#### (c) Confirmation of Spin Label Titration Method

In many of our earlier structural studies using NMR and spin label haptens, we have relied on the use of the "spin-label titration", to obtain spin-to-proton distances in the hapten-antibody complex. While this technique has a solid theoretical basis, it has never been verified experimentally. In the study by Theriault <u>et al</u>, this technique has been verified experimentally, using two tyrosine residues whose assignments can be made with absolute confidence (Signals A and H, Tyr 27H and Tyr 33H). The agreement between calculated (12.6-12.9,  $\sim$  7Å;) and observed (14.8, 8.9Å) distances is certainly good enough for us to have confidence in the method.

#### (d) Mutations

Previous NMR studies combined with techniques such as selective incorporation of deuteriated amino acids and spin label hapten titrations have provided us with detailed information about the amino acid composition and structure of the antigen binding site of the anti-DNP-spin label antibody AN02. In order to obtain amino acid sequence specific assignments of resonances in the aromatic region of the NMR spectrum of AN02, we have undertaken site specific mutagenesis experiments.

Using the cDNA expression vector pLAY designed by D.J. Leahy, we have successfully transfected AN02 into DHFR-CHO cells (DG44). The cells are grown on thymidine and hypoxhanthine deficient Ham's F-12 medium in roller bottles. Antibody yield from these cultures is 4-5 mg per liter of culture supernatant. Ongoing re-transfection experiments, in which transfected cells which are already producing a single amino acid mutant are transfected a second time with the same cDNA and a different selectible marker, show that it should be possible to increase the antibody yield to the 10 mg/l range. This is comparable to yields obtained from hybridomas.

At the present time, we have ten cell lines producing different single amino acid mutants of AN02 in which certain tyrosine or tryptophan residues have been replaced by phenylalanine or serine. The specific mutations are LY31S, LY31F, LY32F, LY34S, LY34F, LY36F, LW91F, HY50S, HW47F, and HW96F, A93E. We are also preparing cell lines which produce antibodies containing the mutations LY31C, LY32S, LY49S, LY49F, LY94S, LY94S, LY94F, HY33F, HY53F, HW47Y HW96F and HW96Y.

Our protocol for preparing antibody samples for NMR analysis usually involves feeding the cells a medium containing a mixture of deuteriated amino acids which are then selectively incorporated into the antibody produced by the cells. Thus, for example, we can obtain antibodies which contain perdueteriated tryptophan and phenylalanine and partially dueteriated (H-3,5) tyrosine. The aromatic region of the NMR spin label difference spectrum of this selectively deuteriated antibody shows only one peak for each tyrosine in the combining site (since there are no histidines in the binding region).

The H-3,5 tyrosine spectrum of the single amino acid mutants which we have examined thus far (LY31S, I Y34F, and HW47F) shows remarkable similarity to that of AN02. In the last two cases there is a resonance which is affected in a predictable manner by binding of the diamagnetic hapten DNP-diglycine, as is the case for AN02. The data obtained from these NMR studies is consistent with aromatic ring shift calculations done here for AN02 and with the tentative assignment of light chain tyrosine 31 as the resonance which shows a large shift upon binding DNP-diglycine. As more NMR data become available for these antibodies produced by site directed mutagenesis, it should be possible to make definite site specific assignments to all residues in the combining site of AN02.

#### SIGNIFICANCE:

The significance of this work is that a reliable method has now been developed to use NMR to study the structure of antibody combining sites. We believe this will be extremely useful to those groups seeking to engineer antibodies for specific specificities, or enzymatic activities.

#### WORK PLAN (next 12 months):

- (1) Using single site mutagenesis, complete all tyrosine proton signal assignments in AN02.
- (2) Screen for other haptens that bind to the same combining site, especialy peptides.
- (3) Analyze the kinetics of ANOX hapten binding in terms of hapten and antibody conformation.

INVENTIONS (Last 12 months)

#### None

PUBLICATIONS AND REPORTS (Last 12 months):

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"Specificities of germline antibodies," T.P. Theriault, G.S. Rule and H.M. McConnell. Proc. 10th Ettore Majorana/NATO Summer Institute on Protein Structure and Engineering, Erice, Sicily, Plenum Press (1989).

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# Structural and Kinetic Studies of the Fab Fragment of a Monoclonal Anti-Spin Label Antibody by NMR

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#### Summary

Nuclear magnetic resonance has been used to study the structure of the anti-spin label antibody AN02 combining site and the kinetic rates for the hapten-antibody reaction. The association reaction for the hapten dinitrophenyl-diglycine (DNP-digly) is diffusion limited. The activation enthalpy for association, 5.1 kcal/mol, is close to the activation enthalpy for diffusion in water. Several reliable resonance assignments have been made with the aide of the recently reported Structural data deduced from the NMR spectra crystal structure. compare favorably with the crystal structure in terms of the combining site amino acid composition, distances of tyrosine residues from the unpaired electron of the hapten, and residues in direct contact with the hapten. Evidence is presented that a single binding site region tyrosine residue can assume two distinct conformations on binding of DNP-digly.

The ANO2 antibody is an autoantibody. Dimerization of the Fab fragments is blocked by the hapten DNP-digly. The NMR spectra suggest that some of the amino acid residues involved in the binding of the DNP-hapten are also involved in the Fab dimerization.

†Abbreviations used: DNP-SL, 1-(N-2,2,6,6-tetramethylpiperidinyl 1-oxy 4-amino)-5-(N-2-aminoethyl amino)-2,4-dinitrobenzene ; DNP-digly, N-2,4-dinitrophenyl (bis) glycine; DNP-gly, N-2,4dinitrophenyl glycine; NOE, Nuclear Overhauser enhancement; NMT, Nuclear magnetization transfer; NOESY, 2 dimensional NOE; HOHAHA, Homonuclear Hartman Hahn

#### 1. Introduction

Nuclear magnetic resonance spectroscopy can be used to study the structure and dynamics of antibody combining sites (Anglister et.al., 1984*a*,*b*,*c*, 1985, 1987, 1990; Dwek et.al., 1977). Although the NMR spectra of the Fab portion of an antibody contain a large number of unresolved resonance signals, it is now clear that physical and chemical strategies can be combined with NMR to provide significant information on antibody combining site structure and composition, and antibody-hapten reaction kinetics. These strategies, enumerated below, are not inexpensive in terms of time and materials. However, this NMR approach to the study of antibody structure will be rewarding since antibody molecules share common structural motifs and the potential variety of such molecules is high (~10<sup>10</sup>) (Amzel and Poljak 1982, Chothia et.al., 1989, Kabat et.al., 1982).

The antibody AN02 is one of twelve monoclonal antidinitrophenyl spin label antibodies which have been characterized in terms of their cDNA sequences and binding constants (Leahy et.al., 1988). Strategies used in NMR studies of the AN02 combining site amino acid composition and structure have included the use of a paramagnetic spin label hapten to provide combining site region resonance spectra (difference spectroscopy), spin-label hapten titrations to estimate spin-to-proton distances, and the growth of antibody producing hybridomas on specifically deuterated amino acids to reduce the number of proton resonance signals (Anglister et.al., 1984*a,b,c*, 1985, 1987, Frey et.al., 1988). Selective amino acid deuteration is also useful for signal assignments through the sharpening of remaining proton signals. Further structural information and assignments have been obtained through magnetization transfer studies and two dimensional NMR techniques.

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A major motivation for our work is to relate the kinetics of antibody-antigen reactions to molecular structure. As discussed in the present paper, the antibody AN02 is one limiting case, in that the on rate constant for hapten binding is diffusion limited. Other antibodies directed against the same hapten have kinetic on rate constants which are much smaller suggesting the reactions are more complex than the hapten-AN02 reaction (Theriault and McConnell, unpublished data).

The present study of the Fab fragment of the monoclonal antidinitrophenyl spin label antibody AN02 took advantage of theoretical modelling before the crystal structure was determined (Brünger et.al. 1990, accompanying paper). Our first tentative resonance assignments were based on computer simulation model of the variable domains (models A1 and A2). Assignments made by interpreting the NMR data in terms of the crystal structure (model X1) are given in the present paper.

#### 2. Materials and Methods

Previous work has described the preparation of AN02 and synthesis of the following dinitrophenyl spin label (DNP-SL<sup>†</sup>) and dinitrophenyl diglycine (DNP-digly) haptens (Balakrishnan et.al., 1982).



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Also described previously are the NMR determination of the binding site amino acid composition (Anglister et.al., 1984a,c), the determination of distances to tyrosine residues by spin label titration (Anglister et.al., 1984b; Frey et.al., 1988), magnetization transfer experiments between hapten and tryptophan residues (Anglister et.al., 1987), and the cDNA sequencing of the antibody genes and determination of binding constants (Leahy et.al., 1988). A molecular model for ANO2 (model A2) was made as before (Anglister et.al., 1987 model A1) with a more refined minimization procedure. The ANO2 X-ray crystallographic structure (model X1) is described in the accompanying paper (Brünger et.al., 1990).

# (a) Determination of the kinetics of the hapten antibody reaction

In these studies the diamagnetic hapten DNP-digly was used. Spectra were recorded on a 500MHz GE spectrometer. The simple one step association reaction

$$H + Fab = HFab$$
(1)

was assumed. The H3 proton of the hapten is known to undergo a large chemical shift upon binding (Anglister et.al., 1987). In aqueous solution the resonance for this proton occurs at 9.15 ppm. When bound the resonance linewidth is lifetime broadened to greater than 100 Hz and lies in the range of 7-8 ppm at 37 °C. The exchange rate of the hapten from bound to free is 'slow' on the NMR timescale; thus the free hapten signal is Lorentzian in shape. The resonance linewidth for the signal of the free hapten is determined by the on rate of the hapten. The kinetic on and off rate constants can be calculated for a known equilibrium constant with the following equation (Jardetzky and Roberts, 1981) :

$$\pi \Delta v_{1/2} = \frac{P_b}{P_f} k_{off} + \frac{1}{T_2}$$
(2)

where  $\Delta v_{1/2}$  is the linewidth in Hertz,  $P_b$  and  $P_f$  are the fractions of bound and free hapten,  $k_{off}$  is the off rate constant for hapten dissociation, and  $T_2$ ' is the transverse relaxation time for the hapten proton in the absence of exchange.

#### (b) Calculation of chemical shifts

The chemical shielding for protons in the AN02 Fab fragment due to aromatic rings was estimated by placing a ring of current on each side of each aromatic ring atomic plane and calculating the magnetic field at the position of the protons due to the combined effect of these ring currents. The shielding  $\Delta\sigma(\text{ppm})$  for a proton due to a ring of current is (Waugh and Fessenden, 1956, Waugh, 1958, Johnson and Bovey, 1958, Haigh and Mallion 1979) :

$$\Delta \sigma = \frac{\mu \text{ine}^2}{24\pi^2 \text{m a}} \frac{1}{\left[(1+\rho)^2 + z^2\right]} \times \left( K(k) + \frac{1-\rho^2 - z^2}{(1-\rho)^2 + z^2} E(k) \right) \quad (3)$$

where *i* is an empirically determined current factor for the type of aromatic ring (Perkins et.al., 1977, Geissner-Prette et.al., 1969),  $\mu$  is the permittivity of a vacuum, *n* is the number of electrons in the current loop, *e* and *m* are the charge and mass of an electron, and  $\rho$  and *z* are the radial and elevational cylindrical coordinates in units of *a*, the ring radius. The coordinate system is chosen such that the origin is at the ring center and the *z* direction is perpendicular to the plane of the ring. K and E are complete elliptic integrals of the first and second kind with modulus :

$$k = \left(\frac{4\rho}{(1+\rho)^2 + z^2}\right)^{\frac{1}{2}}$$
(4)

Values for the parameters a and i are given in Table I. The separation of the current loop from the ring plane was taken as 0.638 Å (Perkins et.al., 1977). Each loop was given half of the aromatic electrons of the aromatic ring. The calculated chemical shift for a proton was determined by subtracting  $\Delta \sigma$  from the experimentally observed free peptide chemical shift value (Jardetzky and Roberts, 1981).

The chemical shielding due to a double bond was approximated in a similar manner by placing two rings of current on each side of the  $sp^2$  plane of the two doublely bonded nuclei. The current loops were separated from this plane by 0.638 Å. Each ring was given one electron. The ring radius, *a* was set equal to the separation of the nuclei and the value 2 was used for the current factor, *i*. Carboxyl groups and the nitro groups of the hapten were treated as having two partial double bonds between the central atom and the two

oxygen atoms. The ring geometry for the partial double bond was the same as for the full double bond. The  $\pi$  electrons were assumed to be equally distributed between the two partial double bonds.

#### (c) Determination of sequence specific resonance assignments

All spectra were recorded on a 500MHz GE spectrometer. Various combinations of selectively deuterated AN02 Fab preparations were probed for magnetization transfer between tyrosine HE protons and methyl groups of ali, hatic amino acids in both the presence and absence of hapten. For example, in studies of tyrosine to alanine magnetization transfers, the sample was prepared by growing cells in media in which the only amino acid with methyl protons was alanine and the only aromatic protons for amino acids in the variable regions were H $\varepsilon$  on tyrosine. (Histidine residues were not deuterated since they are only present in the constant region and do not appear in spin-label difference spectra.) To reduce the overlap of signals, double difference magnetization transfer spectra were obtained by subtracting the difference magnetization transfer spectra recorded with no hapten or diamagnetic hapten present from the difference magnetization transfer spectra recorded with spin label hapten present. Two dimensional spectra were recorded in hypercomplex mode for an AN02 Fab with perdeuterated phenylalanine and tryptophan and d-2,6 tyrosine. A NOESY spectrum (States et.al., 1982) was recorded with a 150ms mixing time. Α ΗΟΗΑΗΑ spectrum (Rance, M. and Cavanagh, J., 1990) was recorded with a 50ms isotropic mixing time. The X-ray crystal structure model X1 was modified by the addition of protons in the positions predicted by the usual bond angles and bond lengths for the connected atoms. The observed proton-proton magnetization transfers were interpreted in the context of this structure.

#### 3. Results

#### (a) Chemical kinetics

The kinetic association and dissociation rate constants were determined at temperatures in the 5 to 45 degree range from the observed free hapten linewidths using equation 2 and the known equilibrium constants. A plot of the observed linewidths verses the ratios of bound to free hapten yields a line with slope equal to the kinetic off rate constant. Figure 1 shows plots for an experiment with the hapten DNP-digly. The results are reported in Table II along with the appropriate binding constants and thermodynamic parameters. The calculated activation enthalpy for DNP-digly, 5.1 kcal/mol, is close to the value of 4.1 kcal/mol which is expected for a diffusion limited reaction in water (Pecht and Lancet, 1977).

#### (b) Conformational Heterogeneity

In studies with the haptens DNP-gly, DNP-digly, and reduced DNP-SL it was found that the HE proton resonance of a light chain tyrosine residue is markedly affected by the binding of these haptens. Addition of DNP-gly or reduced DNP-SL (Fig. 2) results in a large upfield shift of 176 Hz for tyrosine resonance G. That is, the signal G decreases in intensity and is replaced by a signal G' 176 Hz upfield. A similar upfield shift is observed in the presence of DNP-digly, but signal G' is broadened to more than one hundred Hertz in width at 37 degrees. The NMR spectra for AN02 Fab in the presence of DNP-digly at different temperatures are shown in Figure 3. The linewidth of signal G' at 6.08 ppm increases as the temperature is raised while other tyrosine resonances sharpen. This broadening of signal G' with temperature parallels the increasing off rate for the hapten. Magnetization transfer is observed at 5 degrees between the tyrosine signal G' and a resonance G" at 6.52 ppm.

In order to interpret the behavior of signals G' and G" we have first sought to determine whether the magnetization transfer results from cross relaxation or chemical exchange. To make this distinction, a steady state magnetization transfer buildup experiment was performed by saturating signal G" for various times and observing the magnetization at G'. In the case of NOE cross relaxation the magnetization at G' as a function of irradiation time is described by the equation (Solomon, 1955; Wüthrich, 1986)

$$\frac{d\langle G'\rangle}{dt} = \sigma \langle G''\rangle - \frac{\langle G'\rangle}{T_1}$$
 (5)

$$\sigma = \frac{h^2 \gamma^4}{20\pi} \frac{1}{r^6} \left( \frac{6\tau_c}{1 + 4(\omega_o \tau_c)^2} - \tau_c \right)$$
(6)

where  $\langle G' \rangle$  is the difference between the magnetization at signal G' and the equilibrium magnetization at G',  $\langle G'' \rangle$  is this difference in magnetization for signal G'', T<sub>1</sub> is the spin lattice relaxation time, and  $\tau_c$  is the correlation time. A theoretical estimate of 0.2 sec<sup>-1</sup> for  $\sigma$ was obtained using 15ns for  $\tau_c$  (Anglister et.al. 1984a) and a distance of 4Å. This distance is less than the closest approach to any light chain H $\epsilon$  proton from any other aromatic proton, as seen in the crystal structure model X1. (Resonance G' is known to arise from a light chain tyrosine. Also, the distance between two H $\epsilon$  protons on the same tyrosine is approximately 4.2Å.) With a 0.2 sec<sup>-1</sup> value of  $\sigma$ and a 400ms irradiation time, a reduction in the intensity of signal G' of 8% should be observed if the equilibrium magnetization of G' and G'' are the same. Experimentally we have observed a reduction of 45% for signal G' when G'' is saturated for 400ms. For chemical exchange,

$$\frac{d\langle G'\rangle}{dt} = k_{off}f\langle G''\rangle - k_{off}f'\langle G'\rangle - \frac{\langle G'\rangle}{T_1}$$
(7)

where f and f'' are the fractions of time the protons spend in states G' and G'' respectively. We find by using the known off rate for hapten dissociation at 5 °C,  $k_{off} = 16 \text{ sec}^{-1}$ , and relative populations, f = 0.1 and f' = 0.9, that the expected signal at G' is reduced by 42% when G'' is saturated for 400ms. These relative populations are in agreement with the integrated intensities of signals in the spectra in Figure 3. Further, using this ratio of G' to G'' together with the appropriate off rate for the various temperatures, the linewidth of signal G' in the spectra in Figure 3 can be modelled by equations governing two site exchange to within experimental error.

## (c) Dimerization of Fab fragments

The linewidths of tyrosine signals except resonance G are significantly smaller in the presence of the diamagnetic haptens (Frey et.al., 1988; Leahy 1988). In studies performed on the Fab of AN02 without hapten present we found the linewidths of the tyrosine resonances depend strongly on the temperature and Fab concentration. As the temperature is reduced from 37 degrees to 15 degrees the linewidths of the individual tyrosine He signals increase by different amounts. As the concentration is increased from  $50\mu M$ to 650µM the resonance linewidths again broaden by different amounts. In addition, the resonance position for signal G shifts with increasing concentration. Representative spectra are shown in Figure These data are consistent with a dimerization of the Fab 4. fragments involving the combining site region. Protons in the combining site region thus exchange between the free and dimerized

states of Fab. The shift in frequency for resonance G can be described under conditions of fast association-dissociation as follows

$$\mathbf{v} - \mathbf{v}_{\mathbf{F}} = \mathbf{f}_{\mathbf{D}} \times (\mathbf{v}_{\mathbf{D}} - \mathbf{v}_{\mathbf{F}}) \tag{8}$$

where  $v - v_F$  is the frequency shift,  $v_D - v_F$  is the frequency separation between the dimerized and free environments of the resonance G protons, and  $f_D$ , the fraction of protein in the dimerized state, is a function of the equilibrium association constant, K, and the initial concentration of Fab fragments. An estimate of the equilibrium constant for dimerization at 37 degrees,  $K = 2000 \ 1 \cdot m \ ol^{-1}$ , was obtained by fitting the frequency shift data and initial Fab concentration in the 50µM to 1.2mM range with this equation.

#### (d) Resonance Assignments

In order to compare the electron spin to HE proton distances obtained from NMR studies with those calculated from the crystallographic data, it is necessary to have sequence specific assignments for the proton resonances. A number of tryptophan and tyrosine resonances can be assigned with confidence based on a combination of NMR and the theoretical model or the crystal structure. We interpreted interresidue NMT, for instance tyrosine to tyrosine and tyrosine to alanine transfers, and the effect of deuterium substitution on linewidths in the context of both the theoretical model structures A1 and A2 and the crystal structure X1. Chemical shifts for protons shown in Table III have been estimated with equation 3 and atomic coordinates from the crystal structure. Table IV summarizes data used to make these tyrosine assignments. A brief discussion of the rationale for making assignments follows. Two tryptophan resonances can be assigned. In earlier studies NMT was observed from a light chain tryptophan H5 ring proton to the hapten DNP-digly H6 proton. NMT was also observed from one a heavy chain tryptophan ring proton to both the DNP-digly H6 and CH<sub>2</sub> protons. The model A1 structure was used to assign the light chain tryptophan to Trp 91 L. With some uncertainty the heavy chain tryptophan was assigned to Trp 47 H (Anglister et.al., 1987). The more refined model A2 leads us to assign this heavy chain tryptophan resonance to Trp 96 H. The crystal structure X1 confirms these assignments assuming DNP-digly and DNP-SL bind in a similar position. The distance seen in model X1 between H5 of Trp 91 L and H6 of DNP-SL is 4.2Å. Ring protons of Trp 96 H are within 5Å of both H6 of DNP-SL and the expected position for CH<sub>2</sub> protons.

From chain recombination studies (Anglister et.al., 1985) we know that tyrosine He resonances A, B, B', E and H are from heavy chain tyrosines while C, D, F, G and I belong to the light chain. Α representative HE tyrosine difference spectrum is shown in Figure 2. Magnetization transfer between A and H can be seen in both one (not shown) and two dimensional NOE experiments (Fig. 5a). Both the A and H signals also show NMT with a Val methyl group. Inspection of either model A1 or A2 shows a group of three tyrosine residues on the heavy chain which are close to each other. However, only two of these, Tyr 27 H and Tyr 33 H are close enough to a valine residue, Val 2 H, to show NMT. The crystal coordinates, model X1, show only one pair of tyrosines, 27 H and 33 H, whose HE protons are expected to be within the range of 5Å from each other where a strong magnetization transfer is likely to occur. These two residues are also within 5.5Å of one of the Val 2 H methyl groups. Chemical shift calculations yield a Tyr 33 H shifted to 6.35 ppm and a Tyr 27 H shifted to 7.07 ppm from the free tyrosine He resonance position of 6.86 ppm (Jardetzky and Roberts, 1981). Thus tyrosine resonance H,

at 6.34 ppm, is assigned to Tyr 33 H and resonance A, at 7.08 ppm, is assigned to Tyr 27 H.

Figure 5a shows NOESY crosspeaks between resonance E and methyl resonances labelled M1 and M2. One dimensional magnetization transfer studies on specifically deuterated Fab preparations reveal that resonances M1 and M2 are from valine methyl groups. The HOHAHA spectrum in Figure 5b shows that M1 and M2 are connected through a small number of bonds. Another NMT was observed between resonance E and an alanine methyl group. The only tyrosine that can satisfy these constraints is Tyr 102 H with Ala 101 H < 4Å distant and Val 2 H methyl groups at ~4.5Å and ~5.5Å. The calculated chemical shift for Tyr 102 H, 6.57 ppm, compares favorably with the observed shift for resonance E, 6.71 ppm.

The resonance labelled D' in Figure 6 is strongly affected by perdeuteration of methyl groups. The line narrowing is not seen in samples with protonated threonine and isoleucine. In earlier studies a small peak was observed at this position which was associated with the heavy chain. Distance determinations on the perdeuterated methyl sample indicate that this tyrosine is between 16Å and 20Å from the spin label. The most likely candidate for this resonance is Tyr 59 H. Shift calculations show that the Tyr 59 H He signal should appear near the free peptide tyrosine resonance position.

The resonances B and B' must arise from residues 50 and 53 H since these are the remaining choices for heavy chain tyrosines within 20Å of the unpaired electron. At least one of these tyrosines shows an NMT to alanine resonance A6 which is shifted upfield by 1.68 ppm from the free peptide resonance position of 1.4 ppm for alanine H $\beta$  protons (Fig. 5a). Model X1 shows that Tyr 50 H is within 5Å of Ala 34 H which is expected to be shifted upfield by 1.42 ppm. The specific assignment of the B and B' resonances is complicated by the overlap of their signals in spectra taken without hapten or with

either DNP-digly or DNP-gly. The signals are resolved in spectra with reduced DNP-SL (Fig. 2). The calculated chemical shifts favor the assignment of B to Tyr 50 H and B' to Tyr 53 H.

One light chain resonance can be assigned with confidence at this time. Resonance I protons show NMT to more than one leucine and a perhaps a weak transfer to an alanine. The crystal structure shows that only Tyr 36 L can satisfy these conditions : Leu 46 L is less than 4Å away and Leu 100K H is at ~5Å. Ala 101 H at ~6.5Å might account for the weak alanine transfer. The large decrease in linewidth upon methyl perdeuteration is consistent with this assignment (Fig. 6). Chemical shift calculations indicate that Tyr 36 L should have a resonance near the free peptide resonance position. However, a hydrogen bond is likely to occur between the hydroxyl of this tyrosine and the side chain amido group of Gln 88 L. The spacial arrangement of the side chains as seen in the model X1 crystal structure places the Gln 88 L amido oxygen very close to the Tyr 36 L aromatic plane. The distance between these two oxygens is 2.75 Å. The angle between the line joining the two oxygens and the line joining the tyrosine oxygen to the  $\zeta$  carbon is =115°. Hydrogen bonding to tyrosines can cause an upfield shift in the resonance position of He protons of 0.3 ppm (Jardetzky and Roberts, 1981).

#### 4. Discussion

(a) Chemical kinetics

The hapten DNP-digly binding to AN02 is close to the diffusion limit. The on-rate constant,  $k_{on}$ , at 35 degrees is  $7.1 \times 10^8$  l/mol sec. Further, the enthalpy of activation for the on reaction,  $\Delta H^+_{on}$ , is 5.1 kcal/mol. The expected activation enthalpy for a diffusion limited

reaction in water at this temperature is 4.1 kcal/mol (Pecht and Lancet, 1977). This indicates that any structural change in the protein on hapten binding must be small. The off-rate at 37 degrees,  $k_{off} = 460 \text{ sec}^{-1}$ , for DNP-digly was determined from the DNP-digly For comparison, Anglister et.al., 1984b, resonance spectrum. obtained a value of 500 sec<sup>-1</sup> for  $k_{off}$  for DNP-gly at 37 degrees by measuring the effects of hapten exchange on the protein resonance linewidths for tyrosine He signals A and H. It should not be concluded that the kinetics of the reaction between hapten and AN02 are rapid merely because of the simplicity of the hapten molecule. The kinetics of binding of DNP-digly to the closely related antibodies AN01 and AN03 are more than two orders of magnitude slower at 37 degrees (Theriault and McConnell, unpublished data). In studies of other hapten antibody reactions kinetic on rates much slower than the diffusion limit have also been observed (Pecht and Lancet, 1977).

## (b) Resonance assignments

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The assignments for the tryptophan and tyrosine resonance signals were made from a variety of data. Initially we used a combination of distances obtained from NMR spin label titrations, heavy and light chain recombinations of perdeuterated and specifically deuterated antibody preparations, and nuclear magnetization transfer experiments together with theoretical model A1 for the AN02 antibody variable region. These early studies revealed that the H5 ring proton of Trp 91 L was within 5Å of the bound DNP-digly H6 and CH<sub>2</sub> protons. A heavy chain tryptophan which could not be assigned with certainty was also seen to be within 5Å of the bound DNP-digly  $CH_2$  protons. The more refined model A2 lead us to assign Trp 96 H for this heavy chain tryptophan. The crystal structure model X1 confirms these assignments. In this

structure, the hapten DNP ring is sandwiched by stacking interactions with both Trp 91 L and Trp 96 H.

The tyrosine resonances assigned using the model A2 structure Some constraints could be placed on the remaining were A and H. The difficulty in assigning binding site resonance assignments. region tyrosine resonances based on model A2 and observed magnetization transfer data is partially due to the large number of tyrosine residues which occupy this region. This problem is complicated by the fact that the modelling of the variable domains was done without hapten or water present. During the energy minimization of the modelling procedure, residues on the periphery of the binding pocket move closer together to occupy this space. This further increases the density of tyrosine residues. Whether of not such a 'closed' conformation of the protein exists in the absence of the hapten is not known. However, as noted above, large structural rearrangements are unlikely in view of the fast on rate for the hapten.

The tyrosine H $\epsilon$  resonance assignments given in Tables III to VI are based on the interpretation of the NMR data in terms of the crystal structure. Resonances A and H are assigned to the same residues in both model A2 and X1. The heavy chain tyrosine resonances D' and E can be assigned with the help of model X1. The pair of tyrosine resonances B and B' can be confidently assigned to the pair Tyr 50 H and Tyr 53 H. Absolute assignment of this pair should be possible by observing tyrosine to alanine NMT in the presence of reduced DNP-SL.

The difficulty in assigning light chain tyrosine resonances is due to the large number of light chain tyrosine residues within the broadening range of the spin label. Signal I has been assigned to Tyr 36 L in the present work. Distances from the spin label to the tyrosines which give rise to resonances C, D, and F have been calculated previously (Anglister et.al. 1984b; Frey et.al. 1988). The

distance for resonance G could not be determined since this resonance is broadened by the rate of association and dissociation of the hapten at the temperature used in these previous studies. In the present work we have found that the broadening due to hapten exchange is significantly less at 5 degrees (Fig. 3). The observed linewidth of signal G in spectra recorded at 5 degrees in the presence of DNP-SL ( $\Delta v_{1/2}$  > 100 Hz, data not shown) has allowed us to place an upper limit of 12Å on the distance from the spin label to the tyrosine residue which gives rise to resonance G. Thus we believe Tyr 31 L, Tyr 32 L, Tyr 34 L, and Tyr 49 L which are close to the spin label, as seen in the crystal structure X1, account for the resonances C, D, F, and G which are observed as sharp lines in difference spectra (see Fig. 2). Additional resonances in the difference spectra in Figure 2C could arise from tyrosine residues which are more distant from the spin label (i.e. Tyr 71 L and Tyr 94 The production of sequence specific mutant antibodies should L). simplify the assignment of the remaining light chain resonances.

Assignment of the tyrosine resonances to specific amino acid residues allows one to compare directly the distances determined with spin label titrations and those expected from the coordinates of the crystal structure. This comparison is shown in Table VI. The distances calculated from the crystal structure model X1 coordinates represent an average of the distances r from the unpaired spin of the DNP-SL hapten to each of the two tyrosine He protons with a weighting of  $r^{-6}$ . The nitroxide nitrogen atomic position was used as the location for the unpaired electron (Humphries and McConnell, This average includes the flipping motion of the tyrosine 1982). aromatic rings and the distance dependence of the broadening effect of the unpaired electron. In general the distances compare favorably. The largest discrepancy is in the distances calculated for

Tyr 53 H. However, due to the overlap of signals B and B' the NMR distance calculations are more complicated for these tyrosines.

It must be noted that the method of distance determination using spin label titrations (Anglister et.al., 1984b, Frey et.al., 1988) has up until the present not been tested critically. Since signal assignments for the tyrosines giving signals A and H are virtually certain, the comparisons given in Table VI provide compelling evidence for the reliability of this method.

Although most of the light chain resonances have not been assigned, the distances seen in the crystal structure X1 for tyrosines 31, 32, and 34 are much closer than any seen in the NMR spin label titrations. Since the NMR data represent an average over all of the conformations of hapten and antibody which are present in solution, alternate binding configurations which preserve the crystal structure of the antibody but allow for motion of the hapten were considered. The distances shown in column five (model A3) of Table VI were calculated from the position which minimized the differences between the NMR and crystal derived distances for residues A, H, E, This best fit position is approximately 3.8Å from the position and I. of the nitroxide atom in the crystal structure model X1, thus it is beyond the expected range of error in atomic positions, taking into account the higher temperature factors for the spin label group This position (model A3) of the hapten preserves the stacking atoms. interactions with both Trp 91 L and Trp 96 H.

# (c) Protein conformational change

In earlier work with DNP-gly it was found that a tyrosine H $\epsilon$  resonance signal G undergoes a large chemical shift on hapten binding (Anglister et.al., 1985). This is also true for the reduced spin label hapten (N-O changed to N-0-H), and for DNP-digly. The large chemical shift of signal G is not accounted for by the calculated

protein proton shifts due to the ring currents of the dinitrophenyl group (Table V). The large shift must either be due to hydrogen bonding to a tyrosine hydroxyl or a structural change in the protein associated with hapten binding. The hydrogen bonding could be either directly to the hapten or induced by the hapten. The structural change may be very small. The stacking interactions of the hapten with Trp 91 L as well as Trp 96 H may induce a motion of one or both of the indole rings on hapten binding. The calculations indicate that tyrosine proton chemical shifts for Tyr 31, 32 and 34 L are very sensitive to the relative positions of these tryprophan rings. This is especially true for Tyr 31 L which is positioned in a A 0.7 Å relative 'herringbone' arrangement with Trp 91 L. translation of these two residues can cause as much as a 0.5 ppm calculated change in the chemical shift for Tyr 31 L. Further, it can be noted that the residues around tyrosine 94 L are disordered in the crystal structure X1. The interaction of the hapten and Trp 91 L may affect this region.

#### (d) Conformational heterogeneity

In the present study with DNP-digly the shifted proton resonance signal denoted G' broadens as the temperature is raised. (This broadening is not observed with DNP-gly or the reduced spin label hapten.) The increase in linewidth for signal G' parallels the increase in the off rate for the hapten. However the broadening cannot be simply due to association and dissociation of the hapten since the lifetime of the hapten-free binding site is short, of the order of microseconds in our experiments. That is, the combining site is occupied almost all of the time under the experimental conditions in Figure 3.

A broadening mechanism that can account for the apparent correlation between the off rate and resonance linewidth, as well as

the observed magnetization transfer between G' and G", assumes that a tyrosine residue can have two conformation in the protein. These conformations do not interconvert when the hapten is bound, but interconvert rapidly when the binding site is empty. Transitions between tyrosine conformations yielding signals G' and G" would then occur during the short lived hapten-free state of the protein. As discussed in Results, calculations show the resonance data can be modeled with this scheme to within the experimental error.

A structural model of the protein with bound DNP-digly was made to investigate the possible differences between binding to different haptens. This model was made by replacing DNP-SL in the X1 crystal structure with a model of DNP-digly. The DNP ring of DNP-digly was placed in the same location relative to the protein as the DNP ring of One significant difference between these structures is the DNP-SL. interaction between the hapten and the light chain residue Asp 50. In the crystal structure, X1, this residue is packed against the piperidine ring of the spin label hapten. However, with DNP-digly placed in the binding site the carboxyl group of Asp 50 L is in close proximity to the carboxyl group of one of the hapten glycine Both of these carboxyl groups are expected to be substituents. negatively charged at the experimental pH of 7.2. This unfavorable interaction may cause a reordering of binding site light chain residues and/or a change in the relative positioning of the hapten DNP ring with the protein compared to the case of DNP-SL.

# (e) Antibody dimerization

The chemical shift of resonance G is also sensitive to the concentration of Fab in the absence of hapten. The observation of a shift of resonance position clearly demonstrates a change in chemical environment as the concentration of Fab is increased. Other tyrosine resonance signals show a broadening in linewidth as the

concentration of Fab is increased. This broadening is not uniform for all of the observed signals. Changes in the solution viscosity alone cannot account for this effect since in the presence of the hapten DNP-digly the broadening with increasing concentration is much smaller and more uniform for all of the tyrosine resonances. These observations indicate that the Fab fragments dimerize in the absence of hapten. The line broadening for tyrosine resonances with increasing Fab concentration is only partially a reflection of the increase in molecular weight of the Fab complex. The inhibition of the line broadening by hapten and the shift of frequency for resonance G indicate the interaction is likely to involve the hapten binding site.

To estimate a binding constant for this reaction an analysis was made of the shift of resonance G in terms of a dimerization of Fab fragments. The observation that signal G shifts without extensive broadening requires that the exchange of magnetic environments is fast compared to the frequency separation between the free and dimerized signals. The resonant frequency for signal G is thus an average of the free and dimerized signal frequencies weighted by the fraction of Fab found in each state. An estimate of 2000 l/mol for the dimerization constant using equation 8.

# (f) Relationship to Germ line antibody structure

In a study reported elsewhere (Theriault et.al., 1989), we determined the light and heavy chain germ line gene sequences for AN02. The only significant difference in the complementarity determining loops occurs at position 31 L where the germ line serine is changed to a tyrosine in AN02. In the crystal structure this tyrosine forms a herringbone arrangement with Trp 91 L. Studies (Theriault and McConnell, unpublished) on a chimeric antibody which has the light chain germ line sequence and the heavy chain AN02 sequence reveal that the binding constant for the spin label hapten is the same as that of the native AN02 molecule, to within a factor of 2.

Since the germ line used for ANO2 as well as other antidinitrophenyl antibodies did not evolve to bind dinitrophenyl, it is interesting to consider the possible antigens that might have maintained these genes throughout evolution. The most likely candidate for constantly recurring antigens is the idiotypeantiidiotype network (Jerne, 1984). In this connection it is of interest that ANO2 is an autoantibody. The inhibition of dimerization of the Fab fragments by DNP-digly, and the effect of dimerization on a combining site region tyrosine resonance show that the DNP binding site overlaps with an antiidiotype binding site of ANO2. The DNP binding site is quite possibly involved in the idiotypeantiidiotype network.

#### Acknowledgements

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Table I. Parameters used for ring current shift calculations.

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	Six membered rings	Five membered ring
Ring radius a	1.39 Å	1.182 Å
Current factor i His		0.53
Tyr	0.94	
Phe	1.00	
Trp	1.04	0.56
DNP	1.00	

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(Perkins et.al., 1977)

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Table	II. K	inetic	and	thermodynamic	parameters	for	the	hapten-
AN02	reaction	ı.		·	-			-

• :

		DNP-digly		DNP-gly
Temp	Keq	kon	koff	Keq
С	×10 <sup>-6</sup>	×10 <sup>-8</sup>	(sec <sup>-1</sup> )	×10 <sup>-6</sup>
	(l/mol)	(l/mol sec)		(l/mol)
5	16	2.6	16	11
15	8.0	3.5	44	5.5
25	3.8	5.0	132	2.7
35	1.7	7.1	420	1.4
45	0.8	7.6	950	0.6
Parame	eter	DNP-digly	DNP-gly	
∆G 35	°C (kcal/mol	) -8.8	-8.7	
ΔH (k	cal/mol)	-13.3	-12.9	
ΔS (e.u	1.)	14.7	13.8	

Binding constants were determined by flourescence quenching and rate constants were determined by NMR. The thermodynamic values  $\Delta H$ , and  $\Delta S$  were calculated from Arrhenius plots.

Table III. Calculated and observed chemical shifts in ppm for binding region protons with hapten present.

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			С	alculated Shi	ifts	Observed	
Re	sidue	Atom	Ring	Carbonyl	Total	Shift	Signal
Tyr	36 L	He	-0.18	0.15	0.27 *	0.28	Ι
Туг	27 H	Ηε	-0.24	0.04	-0.20	-0.22	А
Туг	33 H	Ηε	0.36	0.15	0.51	0.51	Н
Tyr	50 H	Ηε	-0.31	0.13	-0.18	-0.05	В
Tyr	53 H	He	0.03	0.02	0.05	0.00	<b>B</b> '
Tyr	59 H	Ηε	-0.02	0.03	0.01	0.10	D'
Tyr	102 H	Ηε	0.20	0.10	0.30	0.15	E
Ala	34 H	Ηβ	1.16	0.27	1.43	1.68	A 6
Ala	101 H	Ηβ	0.57	0.07	0.64	0.39	
Val	2 H	Ηγ1	0.11	0.17	0.28	0.12	
Val	2 H	Ηγ2	0.56	0.13	0.69	0.66	
Not	curren	tly assi	gned:				
Tyr	31 L	, Ηε	0.49	0.15	0.64		
Tyr	32 L	, He	-0.19	0.22	0.03		
Tyr	34 L	, Ηε	-0.18	0.39	0.21		
Tyr	49 L	. Ηε	0.00	0.47	0.47		
Tyr	71 L	. Ηε	0.01	0.01	0.02		
Tyr	86 L	, Ηε	-0.09	0.18	0.09		
Tyr	87 L	. Ηε	0.02	0.31	0.33		
Туг	94 L	. He	-0.21	-0.04	-0.24		
Tyr	90 H	He	0.11	0.20	0.31		
						0.08	С
						0.06	D
						0.72	G
						0.12	F

\* Includes a 0.3 ppm shift due to hydrogen bonding.

Listed are the resonance and its assignment together with the IVMR and crystal derived distances to the in linewidths of some of the combining site region tyrosines in samples perdeuterated at methyl positions except tyrosine He protons. NMT denotes residues types connected by nuclear magnetization transfer. Weak transfers are indicated by a question mark. The last two columns report the residues which have readily observable nitroxide nitrogen. The chemical shift change upon hapten binding is listed under shift.  $\Delta\Delta v_{1/2}$  gives the decrease alanine. The numbers given in parenthesis in this column were obtained in the presence of hapten DNP-digly. Neighboring methyls are the number of methyl groups whose protons average less than 5Å from the indicated protons in the distance range expected for NMT and the calculated distances from the crystal structure.

				, ,	1	-				
Resonance	Residu	P	Distan NMR	cc (À) Crystal	Shifi(Hz)	א מאז <sub>ו/2</sub> (Hz)	leighboring Methyls	NMT	Crystal Residue	Distance
۲	27	Н	12.9	14.8	22.0	69 (01)	-	T yr Val	Y33H V 2H	3.5 5.6
8	50	н	12.3	13.1	<del>.</del> .		C	Ala	A 34H	4.7
в.	53	н	<7	12.9	·.5		0			
- D	59	н	16-20	22.9		-10.6 (-11.0)	Ś	Thr? llc lle	T57H 167H 71 169H 71 169H 72 L80H	ν. ν. ν. 4 .v. 4.
ш	102	н	13.7	13.6	8.0	-1.075 (58)	7	Ala Val Val	A 101H V 2H 7 V 2H 72	4 5.5 5.5
н	33	H	<٦	8.9	43.0	-2.64 (-1.35)	I	Туг Val	Ү <i>27</i> Н V 2H	3.5 5.5
L	36	L,	16	13.3	10	-7.42 (-13.1)	4	Leu Leu Ala?	L46L L100KH A101H	€ & & S. 5

Summary of data used to assign He tyrosine resonances to specific amino acid residues. Table IV.

			Ca	lculated Shif	fts*	Observed	
Resid	iue	Atom	Ring	Carbonyl	Total	Shift	Signal
Tyr	36 L	. He	-0.02	-0.03	-0.05	0.02	I
Tyr	27 H	He	0.01	0.01	0.02	0.04	Α
Tyr	33 H	Ηε	0.03	0.01	0.04	0.09	Н
Туг	50 H	ΙΗε	-0.02	0.00	-0.02	-0.04	В
Tyr	53 H	Ηε	0.01	0.00	0.01	-0.00	Β'
Tyr	59 H	Ηε	-0.00	-0.00	-0.00	0.00	D'
Tyr 1	02 H	Ι Ηε	0.01	0.01	0.02	0.02	Ε
Ala	34 H	ι Ηβ	0.10	0.10	0.20	0.37	<b>A</b> 6
DNPE	G	Н3	2.44	0.03	2.47	~1.8	
Not ci	urrent	ly assi	gned:				
Туг	31 L	. Ηε	0.06	0.04	0.10		
Tyr	32 L	. Ηε	0.03	0.01	0.04		
Tyr	34 L	. Ηε	-0.05	0.10	0.05		
Туг	49 L	. Ηε	-0.02	-0.01	-0.03		
Tyr	71 L	. Ηε	0.01	0.01	0.02		
Tyr	86 L	. Ηε	-0.00	-0.00	-0.00		
Tyr	87 L	. Ηε	-0.00	-0.00	-0.01		
Tyr	94 L	. Ηε	-0.02	-0.04	-0.06		
Tyr	90 H	ΙΗε	-0.00	-0.00	-0.00		
						0.03	С
						0.00	D
						0.35	G
						-0.04	F

Table V. Calculated and observed chemical shifts in ppm for binding region protons due to hapten binding.

\*Calculated shifts are based on the AN02 Fab crystal structure with hapten bound (model X1) and the assumption that the structure of the uncomplexed Fab molecule is identical. Table VI. Distances from tyrosine H $\epsilon$  protons to the unpaired spin determined by spin label titration and the calculated distances in the crystal structure X1.

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			]	Distance (Å)	
Re	sidue	Resonance	NMR	Crystal (X1)	Model A3
Tyr	36 L	I	16.0	13.3	15.9
Туг	27 H	А	12.9	14.8	12.6
Tyr	33 H	H	<7	8.9	7.4
Tyr	50 H	В	12.3	13.1	13.5
Tyr	53 H	B'	<7	13.0	10.6
Tyr	59 H	D'	16-20	22.8	23.0
Tyr	102 H	Ε	13.7	13.7	13.5
Ala	34 H	A 6	10-13	11.1	11.0
Not	current	ly assigned:			
Tyr	31 L			11.0	13.4
Tyr	32 L			5.7	9.2
Tyr	34 L			6.8	10.3
Tyr	49 L			6.9	9.1
Tyr	71 L			14.7	18.6
Tyr	86 L			22.4	25.3
Туг	87 L			22.7	25.6
Tyr	94 L			16.1	17.4
Tyr	90 H			27.3	28.6
		С	11.5		
		D	9.0		
		G	< 12		
		F	10.8		

Figure 1. The measured linewidth of the H3 free hapten proton versus the ratio of fraction bound to fraction free hapten. Studies were carried out over the temperature range of 5 to 45 degrees. The slope in each case is equal to the hapten off-rate constant  $k_{off}$  divided by  $\pi$ . (see text equation 2)

Figure 2. AN02 difference spectrum of the type AN02 Fab with reduced spin label hapten minus AN02 Fab with spin label. The sample was perdeuterated in the aromatic region except for tyrosine H $\epsilon$  protons. (A) Tyrosine spectrum of AN02 Fab saturated with reduced DNP-SL. (B) Tyrosine spectrum of AN02 Fab saturated with DNP-SL. (C) Difference spectrum A minus B.

Figure 3. Difference spectra of the type AN02/DNP-digly minus AN02/DNP-spin label at various temperatures. The linewidths of resonances G' and G" are related to the hapten off-rate. Inset are NMT studies between resonance G' and G". This transfer is only seen in the presence of DNP-digly at low temperature.

Figure 4. Tyrosine H $\epsilon$  spectra of AN02 Fab without hapten at various concentrations and temperatures. Broadening of the resonance lines can be seen in both the directions of increasing concentration and decreasing temperature. In both cases certain lines are affected more strongly. For example signal G, indicated by the arrows, is shifted 11 Hz to higher field at 37 degrees as the concentration increases. Also, at 37 degrees signal H is broadened twice as much as signal G.

Figure 5. Two-dimensional NMR spectra. (A) Sections of a NOESY spectrum of AN02 in the presence of DNP-digly showing aromatic and aromatic aliphatic crosspeaks. The crosspeaks between tyrosine signals A and H are consistent with one dimensional NMT studies (see text). Tyrosine resonance E has crosspeaks with two methyl region signals. One dimensional NMT studies indicate these signals are from valine methyl groups. (B) Section of a HOHAHA spectrum of the same sample in the aliphatic region. The crosspeak between the methyl signals indicate these protons are on the same residue.

Figure 6. He tyrosine difference spectra of AN02 Fab without hapten minus AN02 Fab with spin label. (a) The sample was prepared with perdeuterated tryptophan and phenylalanine and d2,6 tyrosine. (b) The sample contained the aromatic deuterations in (a) plus perdeuterated methyl groups on leucine, isoleucine, threonine, and valine.





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Bound Hapten/Free Hapten







(M4) .ono⊃



