NITROGEN-15 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY AS A PROBE OF HAPTEN–ANTIBODY INTERACTIONS

^{15}N-Enriched trinitrophenyl haptens binding to M315

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1. Introduction

One of the major contributions to the affinity of dinitrophenyl- and trinitrophenyl-binding antibodies is thought to arise from interactions between the protein and the nitro groups [1]. It was concluded from a thermodynamic study [2] on the myeloma protein M315 that hydrogen-bonding is involved in this interaction. A more direct investigation by resonance Raman spectroscopy on the same protein suggested, however, that the nitro group of dinitrophenyl haptens with different sidechains interact differently in the dinitrophenyl binding site [3]. ^{15}N NMR provides an alternative approach for the direct study of interactions between antibodies and the nitro groups of such haptens. We wish to report the first application of this method to the study of the binding of specifically enriched trinitrophenyl haptens (without large side chains) to the Fv fragment of the dinitro- and trinitro-phenyl-binding myeloma M315.

2. Materials and methods

2.1. *Preparation of the Fv fragment*

The Fv fragment of M315 was prepared by a modification of the method in [4]. IgA monomer was concentrated to 10 mg/cm^3 in a Diaflo ultrafiltration cell using a PM10 membrane, adjusted to pH 4.5 and then incubated with 1/50th (w/w) pepsin at 310 K for 3 h. The pH was lowered to 3.5 with 1 M acetic acid and the digestion continued for an additional 3 h at 310 K. Proteolysis was terminated by raising to pH 8.0 and the Fv fragment separated by gel filtration on Sephadex G-75 in 50 mM Tris–HCl at pH 8.0. The Fv was freeze-dried from an 0.1 M ammonium bicarbonate solution.

2.2. *Synthesis of haptens*

(i) Trinitroanilines: [2-^{15}N],4,6-Trinitroaniline was made from 2,4-dinitroaniline by reaction at 263 K with a stoichiometric quantity of 95% $K^{15}NO_3$ in conc. H_2SO_4 [5]. Starting with 2-nitroaniline and using 2 equiv. 95% $K^{15}NO_3$ gave [2,4-^{15}N],6-trinitroaniline under the same conditions, together with other reaction products from which it was separated by chromatography on 5% acetic acid-deactivated alumina eluted with $CHCl_3$.

(ii) Trinitrotoluenes: [2,4-^{15}N],6-Trinitrotoluene and [2-^{15}N],4,6-trinitrotoluene were prepared by reacting stoichiometric quantities of $K^{15}NO_3$ with 2-nitrotoluene and 2,4-dinitrotoluene for 7 h and 4 h, respectively. The reaction mixture in each case was poured onto ice and left overnight in the refrigerator to solidify. Recrystallisation from 96% ethanol gave long colourless needles, m.p. 78–80°C.

(iii) Trinitrophenols: [2-^{15}N],4,6-Trinitrophenol and [2,4-^{15}N],6-trinitrophenol were prepared by the same method as in (ii) except that reaction time was only 2 h. The product was recrystallised from dilute ethanol and gave yellow plates, m.p. 120–122°C.

The purity of all trinitro-compounds was checked by thin-layer chromatography and mass spectrometry.

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2.3. *NMR samples*

Protein samples were prepared by dissolving freeze-dried material in 10% ^2H_2O/90% H_2O at pH 6.9 containing 150 mM NaCl, 0.02% azide and 10 mM $MnCl_2$.

Hapten solutions in water were prepared in the same solvent as above. Trinitroaniline solutions in acetone–water mixtures had 10% total volume of ^2H_2O and the remainder of the water component as H_2O. These also were 10 mM in $MnCl_2$.

2.4. *^{15}N NMR*

A Bruker WH-180 wide-bore superconducting magnet operating at 18.24 MHz and 293 K was used with 25 mm tubes containing 14 cm^3 sample. The ^2H_2O was used for the field frequency lock, with chemical shifts given relative to an external reference of nitromethane, which was inserted coaxially into the larger 25 mm tube. A sweep width of 12 000 Hz, pulse width of 10 μs (30°C) and inter-pulse spacing of 0.335 s were mostly used. For the investigation of the solvent dependence of chemical shift a sweep width of 3000 Hz was employed.

In the determination of the effect of possible manganese chelation to free hapten, by using different concentrations of $MnCl_2$, the chemical shifts observed were corrected for changes in bulk susceptibility using the equation [6]:

$$\delta_{cor.} = \delta_{obs.} + 2/3\,(\chi_{ref.} - \chi_{sol.})$$

where $\chi_{ref.}$ and $\chi_{sol.}$ are the bulk susceptibilities of the reference and solution, respectively, and δ is the chemical shift.

Broad-band decoupling of protons did not improve the spectral resolution and was therefore not used because of the deleterious heating effects with protein samples. 5 000–33 000 scans were necessary, depending on hapten and concentration. Sample tubes were spun and contained a vortex plug.

2.5. *Other materials*

2-Nitroaniline, 2,4-dinitroaniline, 2-nitrophenol and 2,4-dinitrophenol were obtained from BDH, Poole, Dorset, 2-nitrotoluene and 2,4-dinitrotoluene and pepsin were obtained from Aldrich Chem., Gillingham, Dorset. ^2H_2O was purchased from Ryvan Chem., Southampton.

9 M 95% $H^{15}NO_3$ was bought from BOC, London and converted to solid $K^{15}NO_3$ by neutralisation with concentrated KOH followed by freeze-drying.

Sephadex G-75 was from Pharmacia Fine Chemicals, Uppsala.

3. Results and discussion

Because of the long relaxation times (T_1) of the nitro-group nitrogens [7], probably arising from the absence of nearby protons, it was found necessary to add a paramagnetic relaxation agent to the samples. Mn^{2+} was chosen because it is known from ESR studies [8] that it does not bind to the high affinity lanthanide binding site that exists on Fv close to the combining site [9]. However, there is a possibility of complex formation between Mn^{2+} and the hapten, as has been found for pyridines co-ordinated to metal through the ring nitrogen [10]. To determine any contribution of this to the chemical shift, samples of 2,4-enriched trinitrophenol were run at $Mn^{2+} \leqslant 40$ mM, or 4 times that used for the protein or hapten samples. After allowing for bulk susceptibility changes, no [Mn^{2+}] dependence of the chemical shift in the concentration range studied was found.

The ^{15}N NMR spectrum of [2,4-^{15}N],6-trinitroaniline shown in fig.1a consists of two resonances at −14.5 ppm and −16.8 ppm arising from the 2 and 4 nitro groups. The chemical shifts can be compared with a value of −18 ppm quoted for the peak distinguishable in the ^{14}N NMR spectrum of 2,4,6-trinitroaniline [11]. Assignment of the resonances to the 2 (−14.5 ppm) and 4 (−16.8 ppm) positions was made using the aniline enriched at only the 2 position (fig.1b). This was repeated for each hapten and it was found that the more downfield resonance always arises from the 2-nitro group (table 1) as predicted from studies on the effects of substitution on nitro compounds. In the series of nitroalkanes R-NO_2, increasingly electronegative R groups shift the nitrogen resonance to higher field [12]. Similar high field shifts are observed with NO_2, COR or CHO substituents in aromatic systems, with the suggestion from the variation with substituent position, that the shift is caused by inductive rather than conjugative effects [13]. With the activating groups O^-, NH_2 and CH_3 the 2-nitro group would therefore be expected to resonate at lower field than the 4-nitro group.

For the three haptens trinitrophenol, trinitrotoluene and trinitroaniline, the resonances are in fast exchange, enabling the changes in chemical shift of each resonance on binding to Fv to be determined;

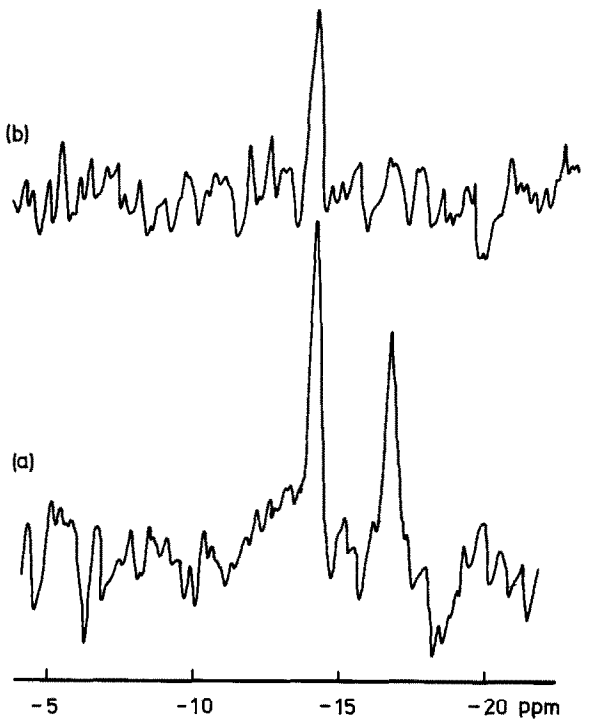

Fig.1. ^{15}NMR spectra of 95% enriched trinitroaniline haptens: (a) [2,4-^{15}N],6-trinitroaniline; (b) [2-^{15}N],4,6-trinitroaniline. Hapten was 1 mM.

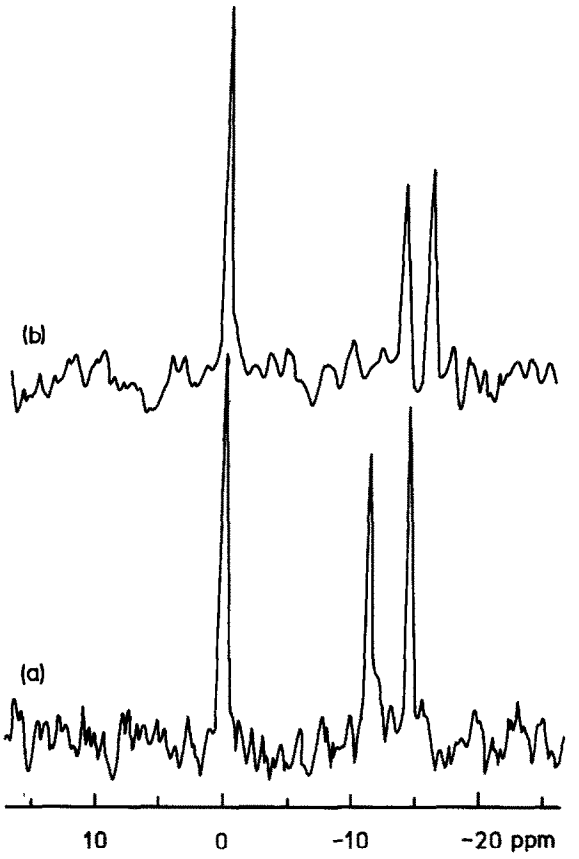

Fig.2. The effect of binding to M315 Fv fragment on the ^{15}N NMR spectrum of [2,4-^{15}N],6-trinitrophenol: (a) free hapten; (b) bound to Fv M315. 16 000 transients were accumulated for (a) and 33 000 for (b). The peak at 0 ppm is nitromethane external standard. Protein at 0.8 M and a hapten:protein ratio of 1:1 were used in (b).

these are summarised in table 1. Fig.2 shows the effect binding to M315 Fv has on the ^{15}N resonances of trinitrophenol. For all three haptens both resonances are shifted to higher field on binding. That this is not a non-specific effect was shown by addition of a comparable concentration of ovalbumin to a sample of trinitrophenol. This addition produced no change in the resonance positions. Because the haptens are symmetrical with respect to the 2-position, but with only one of the two *ortho* groups enriched with ^{15}N, the observed 2-group shift change must be multiplied by 2 to give the full effect sensed if both were enriched. The calculated shift changes are thus −4.2 to −6.6 ppm on the *ortho* and −1.5 to −1.8 ppm on the *para* groups. Though these are small changes compared with the total chemical shift range for nitrogen of ~800 ppm [14] they are a relatively large fraction of the 34 ppm range found for nitroaromatic compounds [13] and indicate a definite interaction between the nitro groups of the hapten and the Fv.

To determine the contribution to the hapten shift changes from a change in environment on transferring the hapten from aqueous solution to the protein interior, the chemical shifts of [2,4-^{15}N],6-trinitroaniline were recorded in solutions containing different percentages of acetone. For this solvent there is no significant effect on the chemical shift of either nitro group nitrogen (table 2). Given the large change in dielectric constant and in solvation properties on changing from water to acetone as solvent, it may be concluded that there is generally little solvent dependence in chemical shift for the nitrogen nuclei of these molecules. If this is so, the changes in the shift of the nitro groups on binding to the protein may be explained as being due to hydrogen-bonding. The reduced electron density of the nitrogen resulting

Table 1
Chemical shifts of the ^{15}N nitro group NMR resonances of trinitrophenyl haptens

Hapten	Chemical shift (ppm)		Shift change on binding to M315 Fv (ppm)	
	2-NO_2	4-NO_2	2-NO_2	4-NO_2
Trinitrotoluene	−12.7	−18.5	−3.3	−1.5
Trinitroaniline	−14.5	−16.8	−2.1	−1.6
Trinitrophenol	−11.4	−14.5	−3.0	−1.8

The assignments were made from haptens enriched at only the 2 position and at both 2 and 4 positions. A negative shift change indicates a shift to higher field

from the nitro group forming such a bond to the antibody could give rise to upfield shifts in the same way as does attaching more electonegative substituents to the benzene ring. In a ^{14}N NMR study of hydrogen-bond formation between solvent and several 5 and 6-membered ring nitrogen heterocycles [15] it was found that there are upfield and downfield shifts for nitrogen acting as a hydrogen-bond acceptor or donor, respectively. The upfield shift on hydrogen-bond formation was explained as being due to a decrease in the paramagnetic shielding arising from an increase in the $n-\pi$ excitation energy. Here, however, any effects on the nitrogen as a result of hydrogen bonding are attenuated by the intervening oxygen. It is therefore possible that the shifts arise from van der Waals interactions between the nitro groups and the protein. However, insufficient data are currently available to enable the effects of such an interaction to be estimated. Nevertheless the experimental shift changes can be used in a qualitative way for comparisons between the binding modes of different haptens.

The findings from Raman studies [3] are that different dinitrophenyl haptens do not bind identically and were attributed to modifications caused by the dinitrophenyl side chains. The shifts reported here for all three trinitrophenyl haptens are very similar. This could be due to the absence of a side chain which might otherwise modify the binding mode of the trinitrophenyl group to optimise the overall binding energy. It is pertinent to this that, where dinitrophenyl and trinitrophenyl haptens with the same bulky side chain have been studied, the nitrophenyl moieties bind similarly. Thus the shifts on the fluorine nuclei of the CF_3 group in γ-*N*-[2,6 dinitro-4-trifluoromethylphenyl]-aminobutyric acid and γ-*N*-[2-nitro-4-trifluoromethylphenyl]-aminobutyric acid on binding to M315 are identical [16]. The hapten-binding mode studied here is therefore most probably that for the trinitrophenyl moiety unperturbed by the presence of side chains.

Table 2
Solvent dependence of chemical shift

% Acetone	Chemical Shifts (ppm)	
	2-NO_2	4-NO_2
0	−14.5	−16.8
42	−14.2	−16.6
64	−14.5	−17.0
82	−14.2	−16.8

Chemical shifts of ^{15}N resonances of trinitroaniline in water/acetone mixtures. All solutions contained 10 mM Mn^{2+} and were 1 mM in hapten

It should be noted that it is not possible to separate the contribution from the individual 2 and 6 positions from the total *ortho* shift because of the fast exchange conditions; a much higher affinity hapten would be necessary for slow exchange conditions to hold at this low frequency and thereby give rise to 3 separate bound resonances. The strongest binding hapten studied here, trinitroaniline, has K_a 5×10^6 M^{-1}. Study of the corresponding dinitro haptens would help in deciding whether all three or only the nitro groups at positions 2 and 4 are involved in interactions with the antibody, if the assumption of the same mode of binding for dinitrophenyl and trinitrophenyl analogues can be made.

Whether used qualitatively for comparisons between different dinitrophenyl- and trinitrophenyl-binding antibodies or quantitatively to establish the strength of interaction, this method should be applicable to other antibodies with this specificity.

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