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The Binding of 2,4,6-Trinitrophenyl Derivatives to the Mouse Myeloma Immunoglobulin A Protein MOPC 315

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The binding of Tnp (2,4,6-trinitrophenyl) derivatives to the Fv fragment (variable region of heavy and light chains) of the mouse myeloma IgA protein MOPC 315 was investigated by 270 MHz proton nuclear magnetic resonance. Two of the haptens, Tnp-glycine and Tnp-L-aspartate, are in fast exchange with the Fv fragment, and the changes in chemical shifts for both protein and hapten resonances were determined by titrations. For the tightly binding hapten ε-*N*-Tnp-α-*N*-acetyl-L-lysine, which is in slow exchange with the Fv fragment, the changes in chemical shifts for the hapten H_3+H_5 resonances were determined by cross-saturation. By using these data and the known structure of the combining site of protein MOPC 315 [Dwek, Wain-Hobson, Dower, Gettins, Sutton, Perkins & Givol (1977), *Nature* (*London*) **266**, 31–37] the mode of binding of Tnp derivatives is deduced by ring-current calculations. The trinitrophenyl ring stacks with tryptophan-93_L (light chain) in the 'aromatic box' formed by tryptophan-93_L, tyrosine-34_L and phenylalanine-34_H (heavy chain). Further evidence for the stacking interaction with a tryptophan residue is provided by the similarity of the optical-difference spectra observed with Tnp-aminomethylphosphonate in the presence of either the Fab fragment (light chain and *N*-terminal half of heavy chain) of protein MOPC 315 or tryptophan. These data show that the modes of binding of all the Tnp derivatives are very similar, despite a 100-fold range in their affinities. It is also concluded that the modes of binding of Dnp (2,4-dinitrophenyl) and Tnp derivatives to protein MOPC 315 are very similar, and that the structural basis for this is that the aromatic box is large enought to allow the trinitrophenyl ring to stack with tryptophan-93_L while still forming hydrogen bonds to asparagine-36_L and tyrosine-34_L.

Comparison of immunoglobulin-sequence data (Wu & Kabat, 1970) with the results of X-ray-diffraction studies (Davies *et al.*, 1975) suggests that the variable domains of these proteins consist of regions of conserved three-dimensional structure (the immunoglobulin fold) to which are attached short sections of polypeptide chain (the hypervariable loops). It is these loops that form the antibody-combining site. By using such sequence homologies and structural similarities, it has been possible to build a model of the combining site of the mouse myeloma protein MOPC 315 (Padlan *et al.*, 1976), by fitting the sequence of this protein to the α-carbon co-ordinates of another mouse myeloma protein, McPC 603. This model has been refined by magnetic-resonance methods, to give a high-resolution structure of the combining site (Dwek *et al.*, 1977). The major feature of the combining site, an 'aromatic box' formed by tryptophan-93_L, phenylalanine-34_H and tyrosine-34_L, was investigated by using the binding of Dnp derivatives.

Tnp derivatives have also been shown to bind to protein MOPC 315 (Eisen *et al.*, 1968). In the present paper the binding of four Tnp derivatives to the Fv fragment of protein MOPC 315 is investigated, to compare the mode of binding of these haptens with that of the Dnp derivatives.

Abbreviations used: Dnp and Tnp, 2,4-di- and 2,4,6-trinitrophenyl; IgA, immunoglobulin A; Fv fragment, variable region of heavy and light chains; Fab fragment, light chain and *N*-terminal half of heavy chain; n.m.r., nuclear magnetic resonance; Pipes, 1,4-piperazinediethanesulphonic acid. Subscript H or L after residue numbers refers to the heavy and the light chain respectively.

Materials and Methods

Preparation of solutions of Fv fragment from protein MOPC 315

The Fv fragment of the mouse myeloma IgA protein MOPC 315 was prepared as described by Inbar *et al.* (1972). Samples for the n.m.r., fluorescence and absorption-difference-spectroscopy studies were prepared as described by Dower *et al.* (1977).

Synthesis of haptens

Tnp-L-aspartate. Aspartic acid (1.6g; BDH Biochemicals, Poole, Dorset, U.K.) was allowed to react with 4.2g of 2,4,6-trinitrobenzenesulphonic acid [Sigma (London) Chemical Co., Norbiton Station Yard, Kingston-upon-Thames, Surrey KT2 7BH, U.K.] in 10ml of water at room temperature (25°C) for 1h, in a flask silvered to exclude light. $NaHCO_3$ (2g) was added to maintain the pH at 7.

After 1h the reaction mixture was acidified with 1M-HCl, and extracted with ethyl acetate. The ethyl acetate was removed by evaporation under vacuum, and the yellow solid thus obtained was dissolved in the minimum volume of acetone. Water was added to the acetone solution until a slight turbidity appeared; the solution was then left to crystallize overnight. The crystals were separated by filtration and dried under vacuum.

The purity of the product was checked by t.l.c. in butan-1-ol/acetic acid/water (5:3:1, by vol.) on polyamide plates (BDH Biochemicals), in which it ran as one spot, with no ninhydrin-staining impurity co-chromatographing with aspartic acid (Found: C, 34.6; H, 2.4; N, 15.6. Calc. for $C_{10}H_8N_4O_{10}$: C, 35; H, 2.5; N, 16.5%).

Tnp-glycine. This was prepared similarly, from 1.2g of glycine (BDH Biochemicals) and 5.6g of 2,4,6-trinitrobenzenesulphonic acid.

ε-N-Tnp-α-N-acetyl-L-lysine. This was prepared similarly, from 0.47g of 2,4,6-trinitrobenzenesulphonic acid and 0.3g of α-*N*-acetyl-L-lysine.

Tnp-aminomethylphosphonate. This was prepared as described by Wain-Hobson *et al.* (1977).

Preparation of hapten and tryptophan solutions

The solutions were prepared as described by Dower *et al.* (1977).

Fluorescence studies

Fluorescence spectra were recorded on a Perkin–Elmer/Hitachi MPF-2A recording spectrofluorimeter, as described in Dwek *et al.* (1976).

Absorption-difference spectra

Absorption-difference spectra were recorded on a Cary 17 recording spectrophotometer, by using Hellma QS tandem cells.

High-resolution n.m.r. studies

270MHz proton n.m.r. spectra were recorded on a Bruker spectrometer as described in Dwek *et al.* (1976).

Analysis of n.m.r. data

Data from the model-compound studies were analysed as described in Dower *et al.* (1977).

Data for the hapten in the Fv-fragment-binding studies were analysed graphically according to:

$$\frac{\delta_{obs.}}{\Delta\delta_0[\text{protein}]_{total}} = \frac{1}{[\text{hapten}]_{total}} \quad (1)$$

(where $\Delta\delta_0$ is the maximum shift change and $\delta_{obs.}$ is the observed shift), which applies when [hapten], [protein] $\gg K_D$, such that when $[\text{hapten}]_{total} > [\text{protein}]_{total}$, $[\text{hapten}]_{bound} = [\text{protein}]_{total}$. This is illustrated in Fig. 5(*a*), which shows that when [hapten] is approximately equal to $[\text{protein}]_{total}$ the assumption breaks down and the plot of $\delta_{obs.}$ versus $[\text{hapten}]_{total}^{-1}$ becomes non-linear. However, by using either data in this region of the hapten-shift plot or protein-resonance-shift data and applying:

$$\bar{X}_B = \frac{\Delta\delta_{obs.}}{\Delta\delta_0} \quad (2)$$

the Scatchard equation:

$$\frac{\bar{X}_B}{[A_F]} = \frac{1}{K_D}(n - \bar{X}_B) \quad (3)$$

(where *A* is either hapten or protein, $\bar{X}_B$ is the fraction of *A* bound and *n* is number of sites) can be applied (Fig. 5*b*) to determine *n* and K_D (dissociation constant).

Results

Model-compound studies

The binding of Tnp-L-aspartate and Tnp-glycine to tryptophan was studied by 270MHz proton n.m.r., at pH6.8 and 303K, in the presence of 0.15M-NaCl. When hapten (40mM) was added to tryptophan (2mM), in both cases the resonances of the tryptophan were shifted upfield.

Shift titrations were performed by varying the Tnp-glycine concentration (0–46.5mM) against a fixed concentration of tryptophan (2mM), and varying the tryptophan concentration (0–20mM) against a fixed concentration of Tnp-glycine (2mM). It was not possible to fit the data of both titrations to binding in a simple binary complex. The simplest model consistent with the data is formation of a ternary complex, $(Trp)_2$–Tnp. Analysis of the data (Dower *et al.*, 1977) gave a dissociation constant of 57mM, and the shift ratios shown in Table 1.

From these data and the geometry of the ring-current field as predicted by the Johnson–Bovey equation (Johnson & Bovey, 1958), it follows that the Tnp-glycine and the tryptophan stack with the trinitrophenyl ring located over the centre of the tryptophan ring. This is consistent with the results of crystallographic analyses (Hansen, 1964).

Table 1. *Shift ratios in the complex of Tnp-glycine and* L-*tryptophan*

Measurements were made at 270 MHz and 303 K, in the presence of 0.15 M-NaCl.

Proton	Shift ratio
Tnp-glycine $H_{(3)}+H_{(5)}$	1
α-CH_2	0.44
L-Tryptophan $H_{(2)}$	0.45
$H_{(7)}$	0.64
$H_{(4)}$	0.59
$H_{(5)}$	0.54
$H_{(6)}$	0.54

Absorption-difference spectroscopy

The absorption-difference spectra for Tnp-aminomethylphosphonate with the Fab′ fragment of protein MOPC 315 and tryptophan are shown in Fig. 1. The similarity of these difference spectra indicates that the Tnp-aminomethylphosphonate interacts with a tryptophan residue in the combining site of the

Table 2. *Binding of 2,4,6-trinitrophenyl derivatives to the mouse myeloma protein MOPC* 315

All experiments were performed in the presence of 0.15 M-NaCl and 20 mM-Pipes at 25±2°C.

Hapten	K_D (μM)	pH	N.m.r. exchange properties
ε-*N*-Tnp-α-*N*-acetyl-L-lysine	0.2	6.95	Slow
Tnp-glycine	12	6.8	Fast
Tnp-L-aspartate	19	6.8	Fast
Tnp-aminomethylphosphonate	20	6.6	Fast

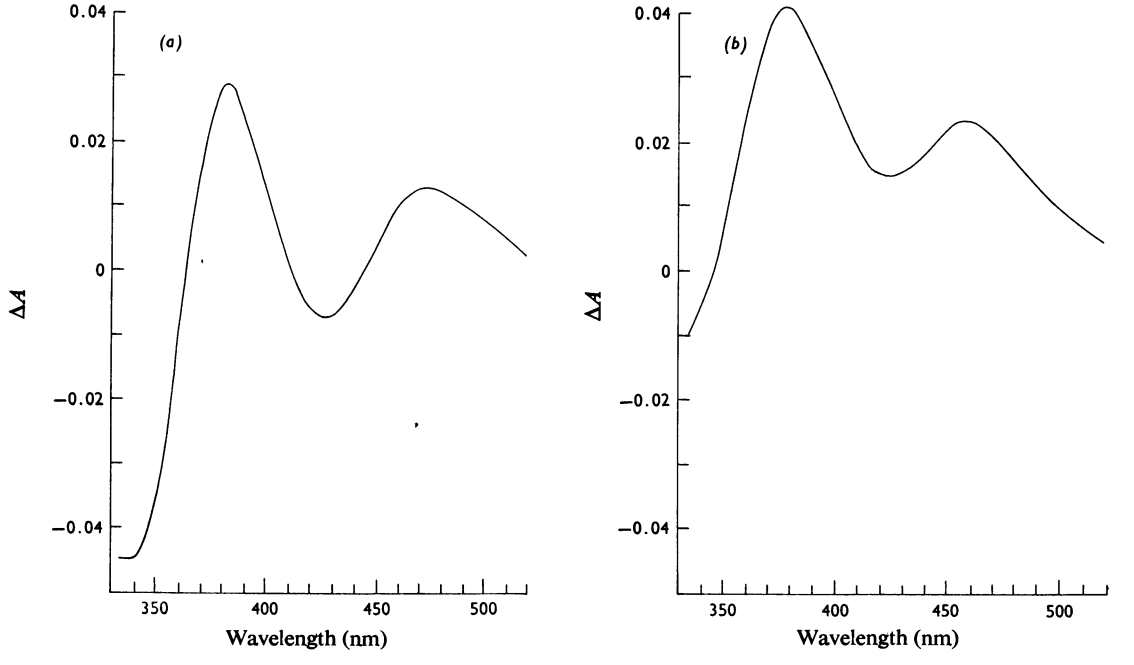

Fig. 1. *Absorption-difference spectra of Tnp-aminomethylphosphonate with* (*b*) L-*tryptophan and* (*a*) *protein MOPC* 315 *Fab′ fragment*

Conditions were as follows. (*a*) Spectrum obtained by subtracting the spectrum of 95 μM-Tnp-aminomethylphosphonate from that of the same concentration of Tnp-aminomethylphosphonate in the presence of 65 μM-protein MOPC 315 Fab′ fragment. All solutions were pH 7.4 in phosphate-buffered saline (0.15 M-NaCl/0.02 M-phosphate) (*b*) Spectrum obtained by subtracting the spectrum of 0.19 mM-Tnp-aminomethylphosphonate from that of the same concentration of Tnp-aminomethylphosphonate in the presence of 33 mM-L-tryptophan. All solutions were pH 6.0 and were unbuffered.

Fab' fragment of protein MOPC 315, in the same way as it interacts with tryptophan in solution (Little & Eisen, 1967). In conjunction with the results of the preceding section, this suggests that Tnp-aminomethylphosphonate stacks with a tryptophan residue in the combining site of the Fab' fragment of protein MOPC 315.

Fluorescence studies

The binding of four different haptens to the Fv fragment from protein MOPC 315 was studied by titrations using quenching of protein fluorescence. The dissociation constants thus determined are listed in Table 2. Also shown in Table 2 is the expected exchange-rate range (see below) for each hapten under the conditions of the n.m.r. studies of the hapten–Fv-fragment interactions (see below). These are based on the on-rate of $2.2\times10^8 s^{-1}\cdot M^{-1}$ determined for trinitrotoluene binding to the Fv fragment (Haselkorn *et al.*, 1974).

270 MHz n.m.r. studies

(*a*) *Difference spectroscopy.* The region 5.5–11 p.p.m. of the 270 MHz proton n.m.r. difference spectra of the four Tnp-haptens with that of Fv fragment from protein MOPC 315 is shown in Fig. 2, together with that for Dnp-L-aspartate. These spectra show that in the chemical shift range 5.5–7.5 p.p.m. the same resonances of the Fv fragment are perturbed by all the haptens. The differences between the spectra in the region 7.5–9.0 p.p.m. are due to the variation in the shifts of the three histidine H2 resonances with pH* (meter reading in ^2H_2O obtained with a pH-meter standardized with ^1H_2O standard buffers) (Wain-Hobson *et al.*, 1977). It is concluded that all these haptens interact similarly with the Fv fragment.

(*b*) *Shift titrations.* The difference spectra in Fig. 2 show the initial and final positions of resonances from the Fv fragment perturbed on hapten binding, and also the resonances of the bound hapten mole-

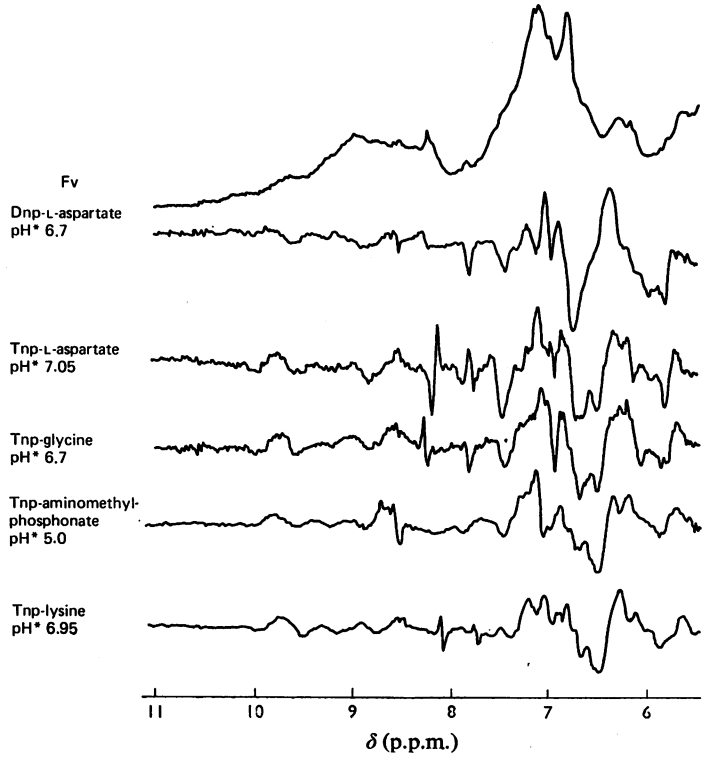

Fig. 2. *Downfield region of the 270 MHz proton n.m.r. difference spectra of protein MOPC 315 Fv fragment with several haptens*
All spectra were recorded in the presence of 0.15 M-NaCl, in ^2H_2O. Spectra are the average of 2048 transients. The intensities of the difference have been multiplied by 4 relative to the Fv spectrum, and the intensities were matched by using the Fv spectra from which each was generated.

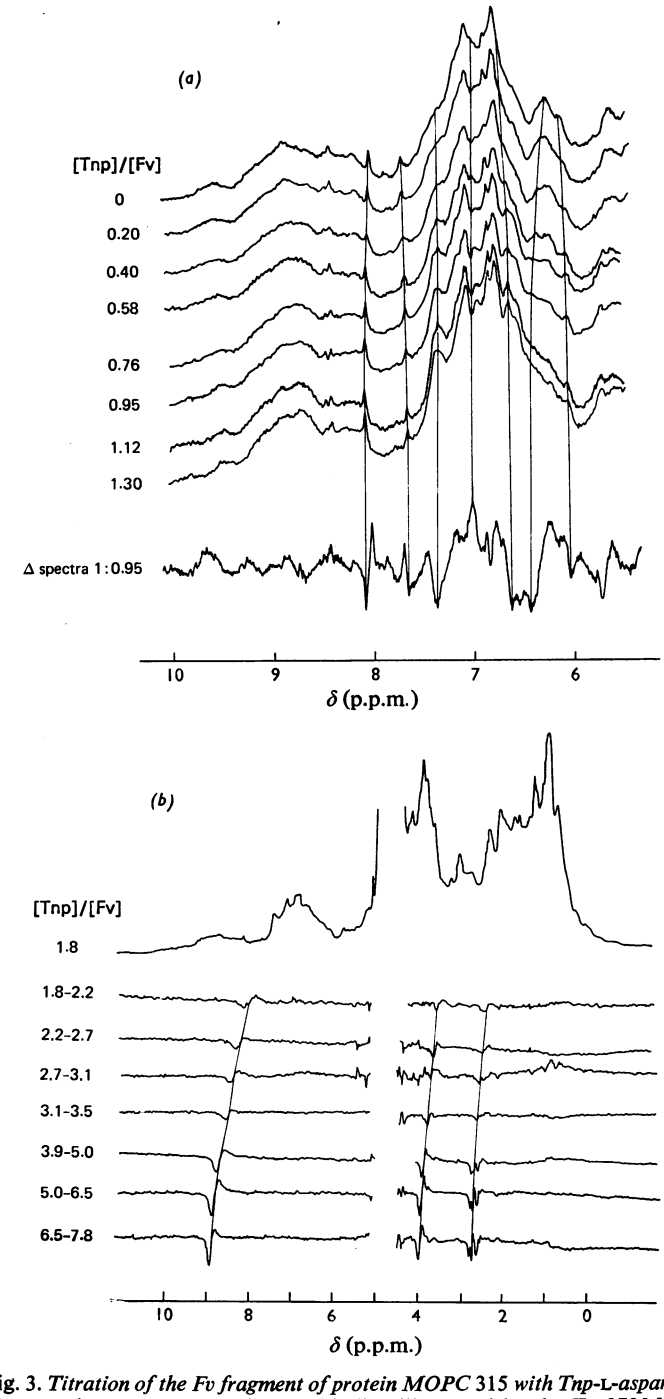

Fig. 3. *Titration of the Fv fragment of protein MOPC* 315 *with Tnp-*L*-aspartate*

(*a*) Shift changes of the protein resonances on hapten binding illustrated by the Fv 270MHz n.m.r. spectra and a difference (Δ) spectrum, showing the final positions of resonances perturbed. (*b*) Shift changes of hapten resonances, illustrated by serial differences taken from spectra of samples in which the concentration of hapten exceeded the concentration of the Fv fragment. The concentration of Fv was 1.3mM, the pH* was 7.05. All other conditions were as in Fig. 2. Chemical shift was measured from that of the sodium salt of 3-(trimethylsilyl)propanesulphonic acid as an external standard.

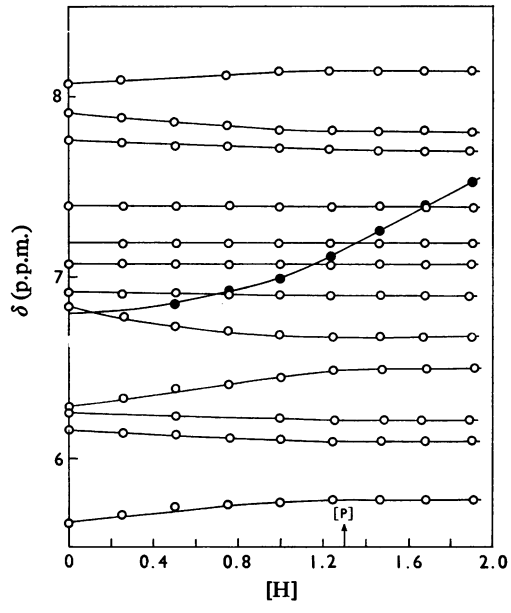

Fig. 4. *Changes in chemical shift in the aromatic region (5.5–8.5 p.p.m.) of protein and hapten resonances perturbed on addition of Tnp-L-aspartate to the Fv fragment of protein MOPC 315*

Protein concn. is indicated by [P]. The shift-change behaviour of the hapten $H_{(3)}+H_{(5)}$ resonance is indicated by –●–. Conditions are as given in Table 3.

cule. However, it is not possible, from such spectra, to assign a resonance in the spectrum of the Fv fragment–hapten complex to a resonance in the unperturbed hapten or protein spectra. Hence it is not possible to determine the shift change of such a resonance on hapten binding. To do this, titrations must be performed in which the chemical shift of a perturbed resonance changes continuously as a function of fraction of hapten (or protein) complexed. Such conditions are termed fast exchange and are given when $k_{off} \gg \Delta w_0$, where k_{off} is the rate of dissociation of hapten from protein and Δw_0 is the change in chemical shift (in Hz) of a resonance between bound and unbound environments.

Of the haptens listed in Table 2, Tnp-glycine, Tnp-aminomethylphosphonate and Tnp-L-aspartate are suitable for such titrations. Titrations were performed with Tnp-glycine and Tnp-L-aspartate.

The results for Tnp-L-aspartate are illustrated in Fig. 3, and the shift behaviour of resonances is shown in Fig. 4. Fig. 5(*a*) shows the use of a single-reciprocal plot of the hapten $H_{(3)}+H_{(5)}$ resonance chemical-shift behaviour (see Fig. 3*b*) to determine the shift change for this resonance (see above). Fig. 5(*b*) shows the shift data of the Fv-fragment resonance at 6.88 p.p.m. plotted according to the Scatchard equation; this shows that $n = 1$ and $K_D = 27\ \mu M$, in good agreement with the values determined by fluorescence studies. The results for both haptens are summarized in Table 3.

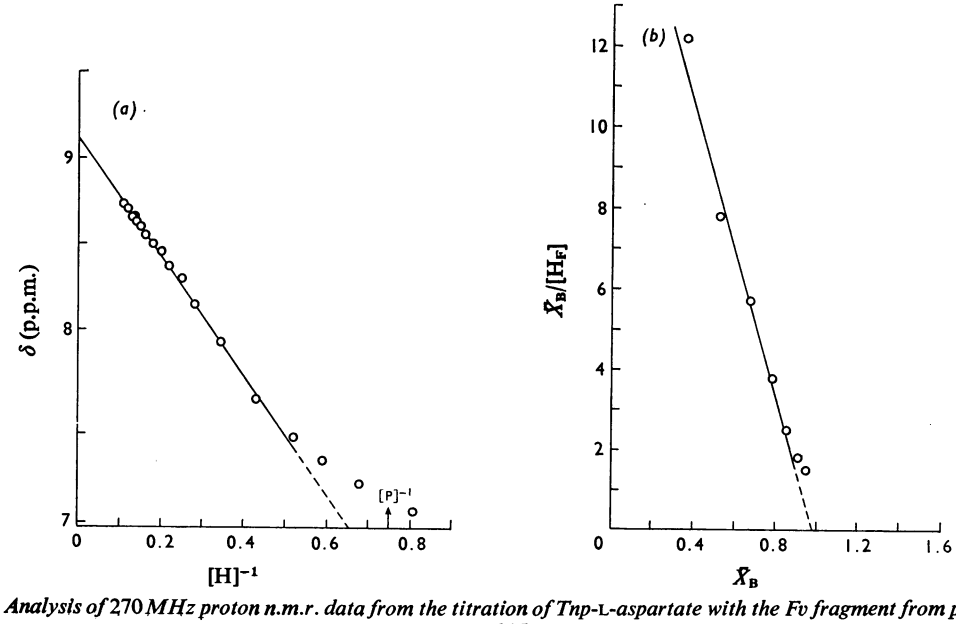

Fig. 5. *Analysis of 270 MHz proton n.m.r. data from the titration of Tnp-L-aspartate with the Fv fragment from protein MOPC 315*

(*a*) Single-reciprocal plot, as described in the Materials and Methods section, of the shift-change behaviour of the hapten $H_{(3)}+H_{(5)}$ resonance. (*b*) Scatchard plot obtained from the shift-change behaviour of the protein resonance at $\delta_0 = 6.88$ p.p.m. Conditions were as in Table 3.

Table 3. *Chemical shifts and changes in chemical shifts of resonances perturbed on binding of Tnp-glycine and Tnp-L-aspartate to the Fv fragment of protein MOPC* 315

Measurements were made at 270 MHz and 303 K, in the presence of 0.15 M-NaCl. Shifts were measured from that of the sodium salt of 3-(trimethylsilyl)propanesulphonic acid as an external standard. In the Tnp-glycine experiment the pH* was 6.7 and the concentration of the Fv fragment was 1.4 mM; in the Tnp-L-aspartate experiment the pH* was 7.05 and the concentration of Fv fragment was 1.3 mM. A negative sign indicates a downfield shift.

Hapten resonances	δ_0 (p.p.m.)	$\Delta\delta_0$ (p.p.m.) Tnp-Gly	$\Delta\delta_0$ (p.p.m.) Tnp-L-Asp	No. of protons
Tnp-L-Asp				
$H_{(3)}+H_{(5)}$	9.07	—	2.38	2
α-H	3.83	—	1.24	1
β-H	2.50	—	0.88	2
Tnp-Gly				
$H_{(3)}+H_{(5)}$	9.13	approx. 2.6	—	2
α-H	3.72	approx. 1.4	—	2
Protein resonances				
His-102_H H2	8.07	No shift	−0.07	1
	7.91	No shift	0.10	1
His-97_L H2	7.76 (7.87)	<0.05	0.06	1
	7.04	Broadens out in both		2
His-97_L H4	6.93 (7.01)	<0.05	<0.05	1
	6.93	−0.05	—	1–2
	6.88	0.15	0.2	1–2
	6.35	−0.17	−0.22	2
	6.31	0.15	0.05	2
	6.20	0.14	0.07	2
	5.65	—	−0.13	2
	7.23	Decreases in intensity in both		2
	7.43	Increases in intensity in both		2

Comparison of the data in Table 3 with those for Dnp-L-aspartate, Dnp-glycine and dinitrophenol (Dower *et al.*, 1977) shows that the same resonances in the protein spectrum are perturbed by Tnp-L-aspartate, Tnp-glycine and Dnp-haptens, and that the hapten aromatic proton resonances experience similar large shifts in all cases. It is concluded that the Tnp-haptens stack with tryptophan-93_L, in the aromatic box of the Fv-fragment-combining site (tryptophan-93L phenylalanine-34_H, tyrosine-34_L) (Dwek *et al.*, 1977).

(*c*) *Cross-saturation studies.* In conditions of slow exchange, when $k_{off} \ll \Delta w_0$, (see the preceding section), separate resonances are observed from free and bound hapten. This leads to the difficulties in determining Δw_0 discussed above. It is, however, easy to assign the free-hapten $H_{(3)}+H_{(5)}$ resonance, since this occurs downfield of the protein spectrum and has a characteristically narrow linewidth. This can be used to locate the bound-hapten resonance, by a cross-saturation experiment. This method uses the effect on the intensity of the free-hapten resonance of saturating irradiation of the bound hapten resonance(s). A decrease in the intensity of the free resonance will then be observed if $k_{off} \gg T_{1b}^{-1}$ where T_{1b}^{-1} is the spin-lattice relaxation rate of the bound hapten proton.

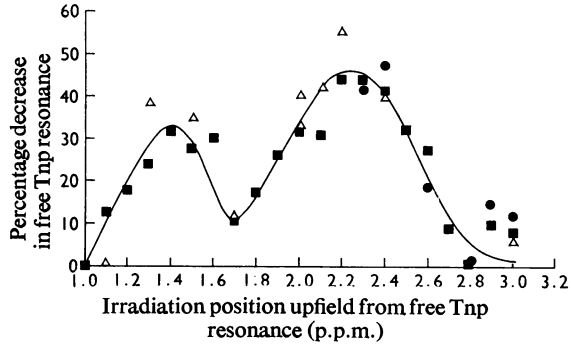

Fig. 6. *Dependence of the intensity of the free ε-N-Tnp-α-N-acetyl-L-lysine $H_{(3)}+H_{(5)}$ resonance on the frequency of selective irradiation of a sample containing the hapten in the presence of the Fv fragment of protein MOPC* 315

Sample conditions were the same as in Fig. 2, with 2 mM-Fv fragment and 4 mM-ε-*N*-Tnp-α-*N*-acetyl-L-lysine. The saturating irradiation was applied as a 0.8 s gated pulse of 50 dB intensity. (The three sets of symbols indicate three separate experiments.)

ε-*N*-Tnp-α-*N*-acetyl-L-lysine binds in slow exchange to the Fv fragment and can thus be studied by cross-saturation. Fig. 6 shows the effect on the inten-

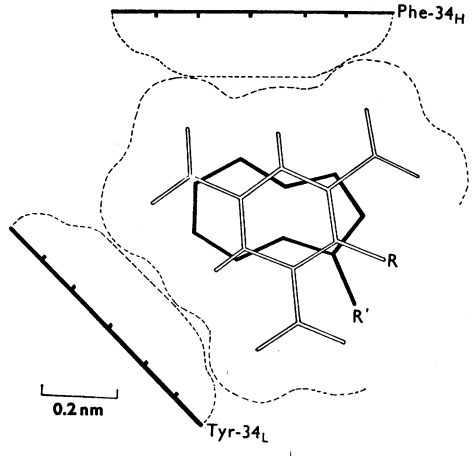

Fig. 7. *Mode of binding of Tnp derivatives to the Fv fragment of protein MOPC* 315

The Figure shows the orientation of the trinitrophenyl ring in the aromatic box of the combining site, determined by ring-current calculations, as described in the text. R is the hapten side chain; R′ is the link of tryptophan-93_L to the polypeptide chain. Phenylalanine-34_H and tyrosine-34_L are shown in cross-section (the section passing through C-1 and C-4). Carbon atom positions and the ring centre are indicated.

sity of the $H_{(3)}+H_{(5)}$ free-hapten resonance of irradiation at a series of frequences in the range 5.5–8.0 p.p.m., the two maxima are at 1.4 and 2.2 p.p.m. upfield of the free-hapten resonance chemical shift respectively and locate the chemical shifts of the bound-hapten $H_{(3)}$ and $H_{(5)}$ resonances.

Ring-current calculations

The data presented in the previous section were used to determine the mode of binding of the Tnp-haptens to the Fv fragment. This was accomplished by using the ring-current contour-map approach (Dower *et al.*, 1977) based on the Johnson–Bovey equation and assuming a plane-to-plane separation between tryptophan-93_L and the trinitrophenyl ring of 0.33 nm (Hansen, 1964). A complete redetermination of the geometry of the aromatic box was not attempted. Rather, the previously determined high-resolution structure of the Fv-fragment-combining site (Dwek *et al.*, 1977) was used as the basis for the calculations. The resulting structure is shown in Fig. 7 and the comparison of the shifts predicted by this structure with the experimental shifts is shown in Table 4.

The large shifts on the hapten are well predicted (Table 4) for Tnp-glycine and Tnp-L-aspartate. However, for ε-*N*-Tnp-α-*N*-acetyl-L-lysine one of the

Table 4. *Comparison of calculated and experimental ring-current shifts in the combining site of protein MOPC* 315 *on the addition of Tnp-haptens, based on the structure of Fig.* 7

All values are expressed as p.p.m.

	Shifts on hapten resonances: Contribution from residues				
Hapten resonance	Trp-93_L	Tyr-34_L	Phe-34_H	Total	Observed
$H_{(3)}$	0.94	1.52	—	2.4	2.4
$H_{(5)}$	0.6	1.6		2 2	

Shift on protein resonances caused by the trinitrophenyl ring	Calculated	Observed
Trp-93_L		
$H_{(2)}$	0.25	
$H_{(4)}$	0.33	
$H_{(5)}$	0.11	approx. 0.1
$H_{(6)}$	0.13	
$H_{(7)}$	0.44	
Phe-34_H		
$H_{(2)}$	−0.08	
$H_{(3)}$	−0.12	
$H_{(4)}$	−0.08	approx. −0.2
$H_{(5)}$	−0.10	approx. −0.2
$H_{(6)}$	−0.12	<−0.1
Tyr-34_L		
$H_{(2)}$	−0.12	<−0.1
$H_{(3)}$	−0.08	
$H_{(5)}$	−0.16	
$H_{(6)}$	−0.10	

shifts is too small by approx. 0.8 p.p.m.; however, this can be accommodated by moving tryptophan-34_L by approx. 0.05 nm to give a structure that is even more similar to that for Dnp (Dower *et al.*, 1977) than that of Fig. 7. The shifts on the protein are only poorly predicted, though of the right order of magnitude. This arises from two causes; firstly, because the shifts are so small (all <0.2 p.p.m.) that up to 50% (±0.1 p.p.m.) may arise from effects other than ring currents, and, secondly, because the ring-current properties of the trinitrophenyl ring system are poorly predicted by the Johnson–Bovey treatment, and no allowance can be made for the chemical-shift anisotropy of the nitro groups.

Discussion

In Dower *et al.* (1977) it was shown that the aromatic ring of Dnp-haptens bound to the Fv fragment of protein MOPC 315 is located in a box of aromatic amino acid residues, consisting of tryptophan-93_L, tyrosine-34_L and phenylalanine-34_H. It was also shown that the most important interaction between the hapten and the protein is the stacking of the hapten aromatic ring with the indole ring of tryptophan-93_L. The data described in the present paper show that this is also the major interaction between Tnp-haptens and the Fv fragment. Comparison of mode of binding of the Tnp-haptens (Fig. 7) with that of the Dnp-haptens (Dower *et al.*, 1977) shows that they are similar but not identical, since the trinitrophenyl ring is located approx. 0.05 nm further out of the site. However, the trinitrophenyl ring is located centrally over the indole ring of tryptophan-93_L, as indicated by the model-compound n.m.r. data and the red shift of the absorption spectrum. The shift data for the trinitrophenyl ring protons of the haptens Tnp-glycine, Tnp-L-aspartate and ε-*N*-Tnp-α-*N*-acetyl-L-lysine show that the trinitrophenyl ring of these haptens is bound very similarly in each case, as suggested by the proton n.m.r. difference spectra (Fig. 2). Thus the same interaction is maintained between tryptophan-93_L and the hapten aromatic ring, despite a range of 100-fold in the binding constant (Tables 3 and 5). This demonstrates that, although interactions between the hapten side chain and the Fv fragment make a significant contribution to the binding energy, the mode of binding of the whole hapten is determined by the mode of binding of the trinitrophenyl ring.

The similarity in the modes of binding of Tnp- and Dnp-haptens to protein MOPC 315 arises primarily because the aromatic box of the combining site is sufficiently large to allow the trinitrophenyl ring to stack effectively with tryptophan-93_L, despite the extra nitro group at position 6. Further, the high-resolution structure (Dwek *et al.*, 1977) shows that only tyrosine-34_L and aspartate-36_L are available for hydrogen-bonding interactions, and that both these are probably bonded to the nitro groups of the 2,4-dinitrophenyl ring; hence there is no hydrogen bond formed between the 6-nitro group of the trinitrophenyl ring and the protein. Thus from a structural viewpoint the 6-nitro group of 2,4,6-trinitrophenol has no strong interactions with the protein (either favourable or unfavourable). Therefore protein MOPC 315 discriminates poorly between Dnp and Tnp derivatives, and cannot be said to be more like an anti-Dnp antibody than an anti-Tnp antibody, or vice versa (Johnston *et al.*, 1974; Eisen *et al.*, 1970). This is supported by the dissociation constants given in Table 5, which are very similar for corresponding Dnp and Tnp derivatives. Finally, studies of the cross-reactivity of natural anti-Dnp and anti-Tnp antisera for Tnp and Dnp respectively (Little & Eisen, 1969) show that the properties of protein MOPC 315 in this respect lie well within the range found for such antisera, and also that for most of the antibodies in the antisera the stacking with a tryptophan residue demonstrated for protein MOPC 315 is a major interaction (Little & Eisen, 1967).

Table 5. *Comparison of dissociation constants from the Fv fragment of protein MOPC 315 of Dnp- and Tnp-haptens with identical side chains*

All compounds shown are of the form Tnp (Dnp)-R; in the lysine derivatives the di- or tri-nitrophenyl group is linked to the ε-amino group. The binding was measured by fluorescence quenching, and the conditions are as described in Wain-Hobson *et al.* (1977) or in Table 2.

	K_D (μM)	
Side chain	Dnp derivative	Tnp derivative
Aminomethylphosphonate	5.0	20
L-Aspartate	24	19
Glycine	12	8
L-Lysine	0.3	—
N-Acetyl-L-lysine	—	0.2

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