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The Role of Nitro Groups in the Binding of Nitroaromatics to Protein MOPC 315

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Two series of dinitrophenyl haptens, in which chlorine replaces one or both nitro groups, were used to investigate, by a combination of high-resolution ^1H n.m.r. and fluoresence quenching, the presence of groups in the combining site of protein MOPC 315, which form hydrogen bonds to the aromatic-ring substituents of the hapten. The large differences in binding constants on successive replacement of nitro groups were shown to be due to specific hapten-substituent–protein interactions by (*a*) showing that there was little difference in the interaction between these haptens and 3-methylindole (a model for the residue tryptophan-93_L with which the hapten stacks in protein MOPC 315), (*b*) proving by ^1H n.m.r. that the mode of hapten binding is constant and (*c*) showing that the differences in K_d were consistent with the relative hydrogen-bonding capacities of chlorine and the nitro moiety. In this way it was established that each nitro group forms a hydrogen bond. Furthermore, from consideration of the ^1H n.m.r. chemical shifts of several dinitrophenyl haptens and their trinitrophenyl analogues, it was shown that there is no distortion of the *o*-nitro group on binding to the variable fragment of protein MOPC 315.

Extensive work has now been done to determine the structure, and thus explain the specificity, of a homogeneous antibody, the nitrophenyl-binding mouse myeloma protein MOPC 315 (Dower *et al.*, 1977). These studies relied heavily on high-resolution ^1H n.m.r. to provide the atomic details necessary to explain the specificity. It was shown that the nitroaromatic hapten binds in a hydrophobic pocket parallel to tryptophan-93_L, with which it interacts specifically and with the *p*- and one *o*-nitro group placed so that hydrogen-bonding is possible to asparagine-36_L and tyrosine-34_L respectively. The other major contact residue is phenylalanine-34_H, which forms another side of the pocket. The model of the hypervariable loops containing the combining site (Padlan *et al.*, 1976; Dower *et al.*, 1977) showed that there is another residue close enough to the nitrophenyl-binding site to be a potential hydrogen-bonding residue for trinitrophenyl haptens, namely tyrosine-33_H. However, it was shown that the mode of binding and affinity of these haptens is the same as for Dnp (Dower *et al.*, 1978) so that tyrosine-33_H does not form a hydrogen bond.

Abbreviations used: Dnp, 2,4-dinitrophenyl; Fv fragment, variable region of light and heavy chains; Tnp, 2,4,6-trinitrophenyl. Subscript H or L after residue numbers refers to the heavy and the light chains respectively.

The aims of the present paper are to investigate by ^1H n.m.r. whether or not the remaining two nitro groups are involved in hydrogen-bonding and also if binding results in a change in conformation of the *o*-nitro group. The latter was suggested by P. Carey (personal communication) on the strength of laser Raman experiments, which showed changes in frequency of nitro-group stretching modes on binding of certain dinitrophenyl haptens to Fv fragment and found to be consistent with the mode of hapten binding proposed on the basis of n.m.r. studies by Dower *et al.* (1977).

To probe the possible contribution to binding of hydrogen-bonding between the 2- and 4-nitro groups of Dnp and Tnp haptens and protein side chains, series of haptens were used in which one or two chlorine atoms replace one or both nitro groups. The choice of chlorine was dictated by the requirement to alter as few variables in the system as possible. Thus it has a high electron-withdrawing ability, as has a nitro group (Gould, 1959), so that binding contributions from the stacking with tryptophan-93_L (Dower *et al.*, 1978) should not be significantly affected. To confirm this, model studies were carried out between the anilines used and 3-methyl[1-^2H]indole in [^2H]chloroform. Most importantly, chlorine has a much lower ability than oxygen to form strong hydrogen bonds (Zumwalt &

Badger, 1939, 1940), so that any large changes in binding constant between Fv fragment and these haptens should be interpretable in terms of lower contributions from hydrogen-bonding. This is, however, dependent on ^1H n.m.r. showing that their mode of binding in the site is essentially unaltered. This shows how the n.m.r. results are used to complement the binding studies. It should be noted, however, that chlorine has a smaller van der Waals radius than the effective radius of a nitro group (0.31nm for chlorine, cf. 0.36nm for nitro group measured from the aromatic-ring carbon; Pauling, 1960), so that small differences in the position of the chlorinated haptens relative to dinitrophenyl haptens of up to 0.05nm may be expected. However, this will not affect the ability of the aromatic ring to stack with tryptophan-93_L in the combining site, nor should it prevent the chlorine from achieving van der Waals contact with tyrosine-34_L and asparagine-36_L, the two residues proposed as candidates for hydrogen-bond formation. It was considered preferable to use a substituent smaller than NO_2 rather than a larger one, such as CF_3 (Hardy & Richards, 1978), since the combining site is obviously large enough to accommodate the former without distortion, whereas this may not be the case for the latter.

The effect of distortion of the nitro group was also investigated by ^1H n.m.r. with a series of di- and tri-nitrophenyl haptens with different side chains and comparing the chemical shifts of the aromatic protons in the trinitrophenyl hapten with its dinitrophenyl analogue.

Materials and Methods

Preparation of solutions of Fv fragment of protein MOPC 315

Fv fragment was prepared as described by Inbar *et al.* (1972). Samples for n.m.r. were prepared by dissolving the freeze-dried protein in ^2H_2O (isotopic purity 99.8%; Ryvan Chemicals, Southampton, U.K.). pH* (pH uncorrected for ^2H isotope effect) was adjusted with dilute solutions of NaO^2H and ^2HCl. Samples for fluorescence were prepared in 50mM-Pipes (1,4-piperazinediethanesulphonic acid) in water at pH7.0. pH was adjusted with dilute HCl and NaOH. All solutions contained 0.15M-NaCl.

Preparation of haptens, 3-methyl[1-^2H]indole and solutions

2,4-Dinitroaniline, ε-Dnp-L-lysine, Dnp-glycine and Dnp-L-aspartate were from BDH Chemicals, Poole, Dorset, U.K. 2,4-Dichloroaniline, 2-chloro-4-nitroaniline, 4-chloro-2-nitroaniline and 3-methylindole were from Aldrich Chemical Co., Gillingham, Dorset, U.K. 2,4,6-Trinitroaniline was synthesized from 2,4-dinitroaniline (Holleman, 1930). *N*-α-Acetyl-ε-Tnp-L-lysine was prepared from 2,4,6-trinitrobenzenesulphonic acid and *N*-α-acetyl-L-lysine (Freedman, 1969). Tnp-glycine and Tnp-L-aspartate were prepared as described by Dower *et al.* (1978). 2-Chloro-4-nitrophenylglycine and 4-chloro-2-nitrophenylglycine were prepared from the corresponding aniline and bromoacetic acid (Passeron & Brieux, 1963). 2,4-Dichlorophenylglycine was prepared from the corresponding aniline and ethyl bromoacetate (Arct *et al.*, 1964). 3-Methyl[1-^2H]indole was prepared by recrystallizing 3-methylindole from ^2H_2O. This was used to prevent occultation of the aniline aromatic protons in n.m.r. titrations. Samples for n.m.r. for comparison of Dnp and Tnp analogues were prepared by dissolving the solid in [$^2H_6$]acetone or [$^2H_3$]methyl cyanide, with tetramethylsilane as internal standard. Samples for n.m.r. for investigation of stacking interactions between 3-methylindole and 2,4-substituted anilines were prepared in [^2H]chloroform, with tetramethylsilane as internal standard. Samples of haptens for n.m.r. for titration with Fv fragments were prepared in ^2H_2O containing 0.15M-NaCl. Hapten samples for fluorescence studies were made as described above for Fv fragment.

High-resolution n.m.r. studies

^1H n.m.r. spectra were recorded at 270MHz on a Bruker spectrometer as described by Dwek *et al.* (1975), operating in the Fourier-transform mode with a Bruker BNC-12 data system. Spectra in [^2H]chloroform were recorded at a probe temperature of 298K and those in [$^2H_3$]methyl cyanide, [$^2H_6$]acetone and ^2H_2O at 303K.

Fluorescence studies

Fluorescence spectra were recorded on a Perkin–Elmer/Hitachi MPF-2A spectrofluorimeter, as described by Dwek *et al.* (1976). Binding constants were obtained from the quenching of protein fluorescence by hapten (Inbar *et al.*, 1972; Haselkorn *et al.*, 1974). Studies were performed at 293K in Pipes buffer at pH7.0. A protein concentration of 1μM was used. Corrections for trivial quenching were made by determining the fluorescence quenching of a tryptophan solution in the same buffer for each hapten concentration considered. The tryptophan concentration was such as to give a similar initial fluorescence intensity to that of the protein solution. The decreased absorbance of the chlorinated haptens compared with dinitrophenyl compounds at the exciting wavelength used enabled concentrations of hapten up to 600μM for dichlorophenylglycine to be used, giving a sufficiently high fraction of sites occupied to estimate the K_d.

Results and Discussion

Binding of haptens to Fv fragment studied by protein fluorescence

The binding constants of the two monochloro- and one dichloro-substituted anilines and phenylglycines to Fv fragment were determined by protein fluorescence at pH 7.0 and are compared with those already determined for dinitroaniline and Dnp-glycine (Haselkorn *et al.*, 1974) respectively (Table 1). Although the exact changes down each series are not identical, the trend is significant and is the same for both. Thus replacement of either nitro group results in a fall in K_d by a factor of 13–20, corresponding to a decrease in ΔG of 6.4–7.5 kJ/mol. It is important to notice that the change in ΔG is comparable for both monochlorinated derivatives in each series. Therefore, if the changes are due to weakened hydrogen bonds, there must be two such bonds rather than one, since, especially for the glycine series, which has a charged side chain, it is not expected that the hapten orientations should differ by 120°. This would be necessary if the chlorine were to occupy the same position in the combining site for both haptens, as it would place a negative charge inside the hydrophobic pocket. Introduction of a second chlorine atom again results in a large fall in K_a for the aniline, though not quite so large as for the phenylglycine. This second decrease further supports the idea of two hydrogen bonds.

Interaction of 2,4-disubstituted anilines with 3-methylindole followed by ^1H n.m.r.

By determining the relative strengths of interactions between the anilines considered above and 3-methylindole it was possible to establish that the changes in K_d cannot be accounted for in terms of decreased interaction between the hapten and tryptophan-93$_L$, but are the result of specific interactions between the 2- and 4-substituents and groups on the protein. The absence of charged side chains on either of the components in the system, together with the use of a relatively non-polar solvent, chloroform (some of the haptens have very low solubility in the less polar carbon tetrachloride), ensured that any association observed was due to specific interaction rather than a more general hydrophobic effect. Despite the weak interaction that was found for each of the pairs considered, the high solubility of 3-methylindole enabled good estimates of K_d to be made for dinitroaniline. Fig. 1 shows a titration curve for this hapten at 270 MHz. Large upfield shifts were observed for the three aromatic protons of each aniline, which were interpreted as ring-current shifts (Johnson & Bovey, 1958) and therefore enabled a qualitative structure determination to be made involving parallel stacking of the two rings, with considerable overlap. The K_d values for the other haptens are extrapolated from the value for dinitroaniline (assuming that the complex has the same structure and thus the same chemical shift differences between the free and complexed forms), by considering the relative magnitudes of the observed shifts for a given proton under identical hapten and methylindole concentrations. This assumption is validated by the approximate invariance of the shift ratios. Table 2 summarizes the results.

The small differences in K_d between the 2,4-dinitro- and 2,4-dichloro-anilines binding to 3-methylindole thus establish that replacement of the nitro group by chlorine causes relatively small changes in the specific hapten–tryptophan interaction. Therefore the large changes in K_d seen on binding these haptens to Fv fragment must be due to a change in direct interaction between the substituents and the protein. In view of the changes in ΔG associated with successive substitutions being comparable with the differences in hydrogen-bond strength of nitro group and chlorine (Pauling, 1960) the interaction in question is probably hydrogen-bonding.

Table 1. *Binding constants (K_d) for 2,4-substituted anilines and phenylglycines at 293 K determined by fluorescence quenching*

A protein concentration of 1 μM was used. Titrations were performed at 20°C in 50 mM-Pipes at pH 7.0.

	Aniline (μM)	Phenylglycine (μM)
2,4-Dinitro-	3.5	5.0
2-Chloro-4-nitro-	40	83
4-Chloro-2-nitro-	69	68
2,4-Dichloro-	~500	230

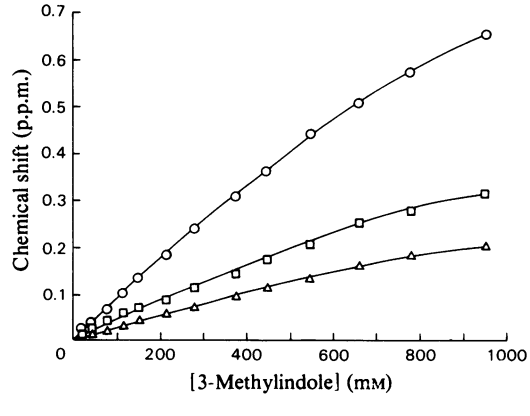

Fig. 1. *Titration of the chemical shift of the aromatic protons [$H_{(3)}$, △; $H_{(5)}$, □; $H_{(6)}$, ○] of 2,4-dinitroaniline (12 mM) with 3-methyl[1-^2H]indole in [^2H]chloroform at 298 K*

Effect of nitro-group rotation on the chemical shift of adjacent aromatic protons

It was suggested by P. Carey (personal communication), on the basis of laser Raman experiments, that there is a change in the conformation of the *o*-O–N–O···H–N group of certain Dnp haptens on binding to the Fv fragment. This could be due to a rotation of either the nitro group or the NH group out of the plane of the aromatic ring. Furthermore, from fitting the n.m.r. data obtained with other Dnp haptens to the model of the combining site (Dower *et al.*, 1977), it was considered likely that the *o*-nitro group was rotated about the C–N bond by about 30°.

Although the crystal structures of neither 2,4,6-trinitroaniline nor 2,4-dinitroaniline have been determined, the structures are known of several closely related compounds, i.e. 1,3,5-triamino-2,4,6-trinitrobenzene (Cady & Larsen, 1965) and 2,3,4,6-tetranitroaniline (Dickinson *et al.*, 1966). These show that for aminonitrobenzenes without large substituents adjacent to the nitro groups the molecules are planar because of strong intramolecular hydrogen-bonding. Introduction of bulky groups, as the 3-nitro group in 2,3,4,6-tetranitroaniline, results in rotation of one or more nitro groups out of the ring plane. Therefore both 2,4-di- and 2,4,6-tri-nitroanilines will be planar in solution, where intermolecular hydrogen-bonding will be at a minimum. A comparison of the chemical shifts of the $H_{(3)}$ protons of dinitroaniline and trinitroaniline will then give the effect that introduction of a third nitro group has on the electronic properties of the aromatic ring and thus on the shielding experienced by the $H_{(3)}$ proton.

If the chemical shifts of the $H_{(3)}$ protons of other substituted Dnp compounds are then determined, it is possible to predict the chemical shift of the same proton in the trinitro analogue, assuming that it is planar. Differences between the predicted and observed chemical shifts can then be interpreted in terms of distortion of the *o*-nitro group due to a

Table 2. *Chemical-shift changes associated with 3-methylindole–hapten interactions for 956 mM-3-methylindole and 20 mM-hapten in [^2H]chloroform at 298 K*

All spectra were recorded at 270 MHz as the average of 700 transients.

Aniline	$H_{(3)}$ (p.p.m.)	$H_{(5)}$ (p.p.m.)	$H_{(6)}$ (p.p.m.)	$H_{(6)}/H_{(5)}$	$H_{(5)}/H_{(3)}$	K_d (M)
2,4-Dinitro	0.25	0.31	0.65	2.1	1.3	3.0
2-Chloro-4-nitro	0.10	1.14	0.39	2.8	1.4	5.5
4-Chloro-2-nitro	0.09	*	0.40	2.1	2.3	5.4
2,4-Dichloro	*	*	0.22	2.2	1.0	9.8

* Peak obscured for higher concentrations of 3-methyl[1-^{2}H]indole.

Table 3. *^1H n.m.r. chemical shifts of $H_{(3)}$ in N-substituted and trinitroaniline compounds*

R represents the substituent in the aniline amino group. Spectra were recorded at 270 MHz and 303 K; 500 transients were collected for each spectrum. Tetramethylsilane was used as an internal standard. The difference in chemical shift for the Tnp derivative between that observed and predicted is also shown.

		Chemical shifts (p.p.m.)		Chemical shift differences (p.p.m.)	
Solvent	R	Dnp	Tnp	Dnp−Tnp	Tnp (predicted−calculated)
[$^2H_3$]Methyl cyanide	H	8.92	9.20	−0.28	
	CH_2CO_2H	8.99	8.97	+0.01	+0.29
	$CH(CO_2H)CH_2CO_2H$	8.98	8.97	+0.01	+0.29
	$[CH_2]_4CH(NH_2)CO_2H$	8.95	8.94*	+0.01	+0·29
[$^2H_6$]Acetone	H	8.94	9.25	−0.31	
	CH_2CO_2H	9.01	9.06	−0.05	+0.27
	$CH(CO_2H)CH_2CO_2H$	9.01	9.06	−0.05	+0.27
	$[CH_2]_4CH(NH_2)CO_2H$	†	9.02		

* α-*N*-Acetyl.

† Not sufficiently soluble.

bulky *N*-substituent. Table 3 presents the observed chemical shifts for four pairs of haptens in [$^2H_3$]-methyl cyanide and [$^3H_6$]acetone. There are two conclusions from these results. Firstly, for each solvent used the differences between the predicted and observed chemical shifts for the Tnp compound are identical irrespective of the side chains, which do vary considerably in their electrostatic nature. Secondly, the differences show little, if any, dependence on solvent. From this it can be concluded that there is a structural difference between planar trinitroaniline and *N*-substituted derivatives caused by the bulk of the substituent, which gives rise to an upfield shift on $H_{(3)}$. This difference must be a distortion of one *o*-nitro group, which would decrease the downfield shift experienced by $H_{(3)}$ in the trinitro derivatives (Jackman & Sternhell, 1969). Since only a weighted average of the shifts on $H_{(3)}$ and $H_{(5)}$ is seen, owing to the symmetry of the trinitro compounds and the existence of conditions for fast exchange (Gutowsky *et al.*, 1953), the magnitude of the upfield shift caused by nitro-group rotation is twice the observed difference, i.e. 2× 0.29p.p.m. = 0.58p.p.m. upfield. This result will be applied below when considering the shifts on $H_{(3)}$ for haptens with and without a 2-nitro group. Other things being equal the upfield shift on $H_{(3)}$ should be larger by about 0.6p.p.m. for the former if the 2-nitro group does distort on binding to Fv fragment.

Table 4. *Changes in chemical shift on binding to the Fv fragment from protein MOPC* 315 *for the aromatic protons of a variety of Dnp and chlorinated haptens on binding to Fv fragment at* 303 *K and pH** 6.9±0.1

Measurements were made at 270 MHz in the presence of 0.15 M-NaCl.

Hapten	Shift (p.p.m.) $H_{(3)}$	$H_{(5)}$	$H_{(6)}$
2,4-Dnp-glycine	1.21	2.20	1.70*
2-Chloro-4-nitrophenylglycine	1.91	2.35	1.52
4-Chloro-2-nitrophenylglycine	1.12	1.55	1.30
2,4-Dichlorophenylglycine	1.62	1.80	1.13
4-Chloro-2-nitroaniline†	1.42	1.96	1.70
2,4-Dnp-L-aspartate	1.68	2.31	1.30*
2,4-Dinitrophenol	1.25	2.00	1.15*

* From Dower *et al.* (1977).
† Approximate, owing to limited solubility.

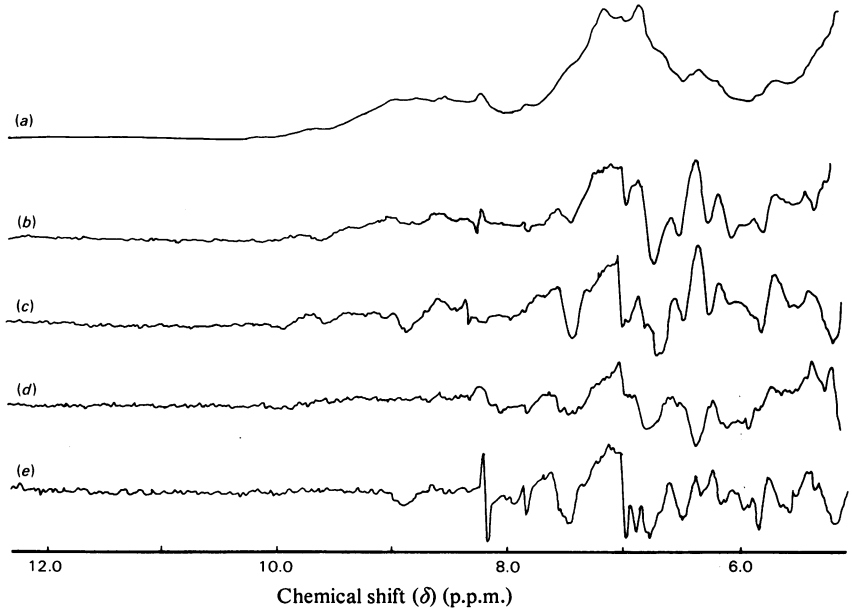

Fig. 2. *Aromatic region of protein MOPC* 315 *Fv fragment* ^1H *n.m.r. spectrum and difference spectra obtained with four haptens*

Spectra were recorded at pH*6.9, 303 K and 270 MHz in ^2H_2O containing 0.15 M-NaCl and difference spectra (*b*)–(*e*) obtained with chlorinated haptens at equimolar concentrations of hapten and protein (1.5 mM): (*b*) 2-chloro-4-nitrophenylglycine; (*c*) 4-chloro-2-nitrophenylglycine; (*d*) 2,4-dichlorophenylglycine; (*e*) 4-chloro-2-nitroaniline. Difference spectra were obtained by subtraction of the spectrum of protein plus hapten from that of protein alone (*a*). The intensities of the differences have been multiplied by 4 relative to the spectrum of Fv fragment.

High-resolution protein n.m.r. studies

(i) 4-Chloro-2-nitroaniline was titrated with Fv fragment at pH6.9. However, because of its very low solubility in water, it was necessary to prepare the concentrated hapten solution by adding the required quantity of aniline dissolved in [$^2H_6$]-acetone to ^2H_2O, which resulted in some precipitation of hapten. Although it was possible in this way to fully saturate the available Fv-fragment binding site, a sufficiently high concentration of free hapten could not be obtained to enable an accurate value of the chemical-shift differences between free and liganded species to be determined. An estimate is given in Table 4. This is unfortunate because of the simplicity of the hapten, being without any substituent on the amino group and therefore unaffected by charged groups on the protein. For this reason glycine derivatives of the three dinitrated anilines were used for titrating with Fv fragment.

(ii) The much greater solubility of dichloro- and chloronitro-phenylglycine derivatives in ^2H_2O allowed final concentrations greatly in excess of protein concentration (1.4–1.5mM) to be obtained quite easily, on titration of concentrated solutions into Fv fragment. Figs. 2(*a*)–2(*e*) show the aromatic-region difference spectra for these three haptens as well as for 4-chloro-2-nitroaniline at equivalence of hapten and protein concentrations. Spectra (*b*) and (*c*) are nearly identical, whereas (*d*) and (*e*) show features common for all four haptens, namely negative peaks at 8.8, 7.4, 6.7 and 6.2p.p.m. Because of the different chemical shifts of the hapten protons $H_{(3)}$, $H_{(5)}$ and $H_{(6)}$ for the haptens considered here, some dissimilarities are expected as these resonances will appear at different positions in the region of the difference spectrum and will therefore obscure some protein resonance differences. From this it can be seen that essentially the same combining site residues are perturbed for all four haptens. This provides the justification for interpreting the changes in K_d as being due to subtle differences in bonding possibilities rather than a different mode of hapten binding.

The fully bound chemical-shift differences were obtained from plots of $1/[\text{hapten}]_{\text{total}}$ versus chemical shift. At high concentrations of hapten the plot becomes linear with a slope equal to [Fv fragment] $\times \Delta\delta$, where $\Delta\delta$ is the fully bound chemical shift: knowing [Fv fragment], $\Delta\delta$ can be calculated. Fig. 3 shows the plots for the three aromatic protons of 2-chloro-4-nitrophenylglycine. The chemical shifts of the hapten peaks were obtained from consideration of both the cumulative and sequential difference spectra (Figs. 4 and 5 respectively). The concentration-dependence of hapten shifts in the absence of protein in the range 0–12mM is less than 4Hz and can therefore be ignored in the analysis.

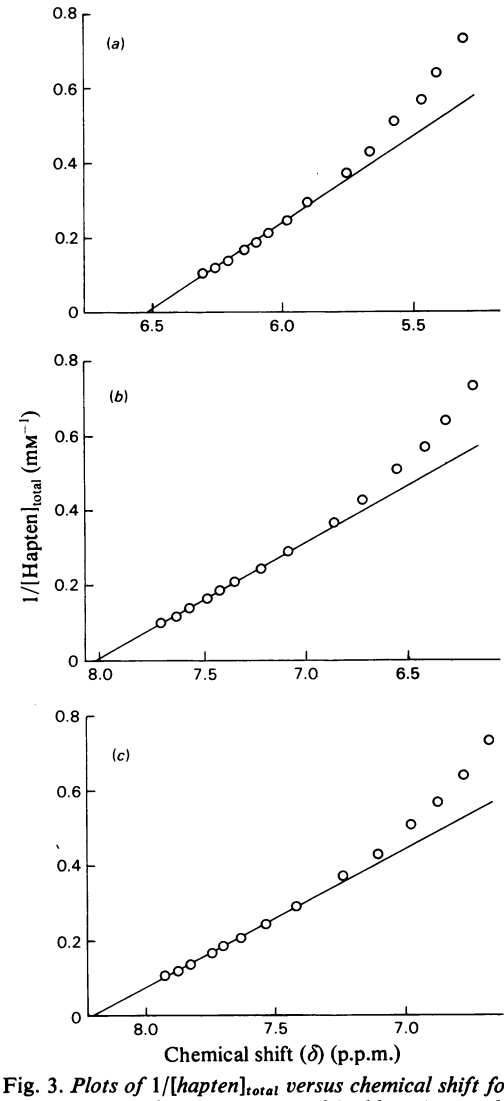

Fig. 3. *Plots of* $1/[\textit{hapten}]_{\textit{total}}$ *versus chemical shift for the three aromatic hapten protons of 2-chloro-4-nitrophenylglycine binding to Fv fragment at pH6.9 and 303K*
The slope equals [Fv fragment] $\times \Delta$, where Δ is the fully bound chemical shift. Plot (*a*) is for $H_{(6)}$, (*b*) for $H_{(5)}$ and (*c*) for $H_{(3)}$.

Table 4 provides a summary of fully bound chemical-shift differences for these haptens together with three dinitrophenyl haptens that have already been investigated (Dower *et al.*, 1978). Although there are variations in the shifts from one hapten to another, this is no more than those among the three dinitrophenyl haptens quoted, whose shifts could all be accommodated by relatively small movements of the hapten relative to the combining site. It was

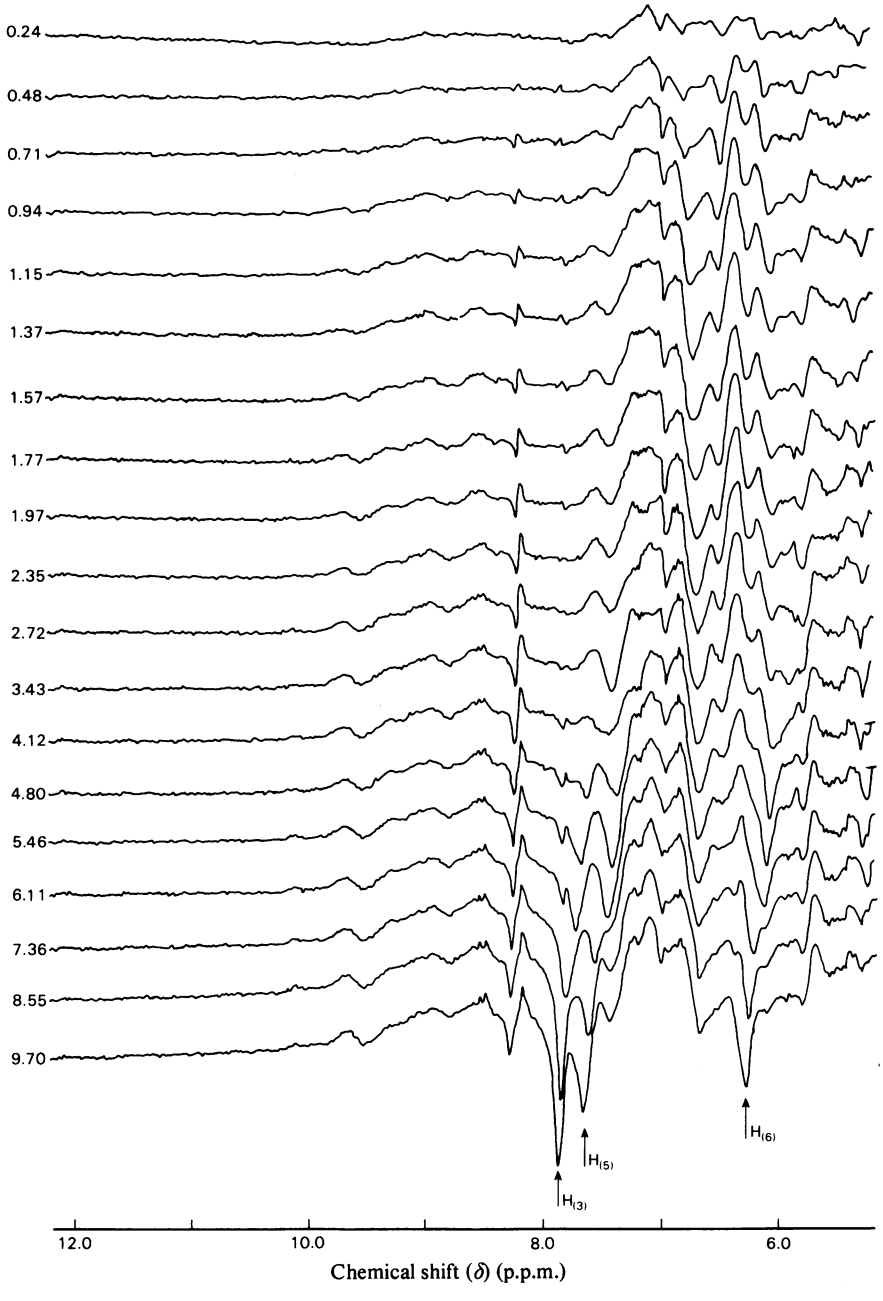

Fig. 4. *Cumulative ^1H n.m.r. difference spectra of the aromatic region of Fv fragment* (1.5mM) *on addition of* 2-*chloro*-4-*nitrophenylglycine*

Spectra were obtained by subtraction of the spectrum containing the concentration of hapten indicated from that of unbound Fv fragment. The three hapten aromatic protons are indicated at large excess. The values beside each spectrum give the hapten concentration (mM).

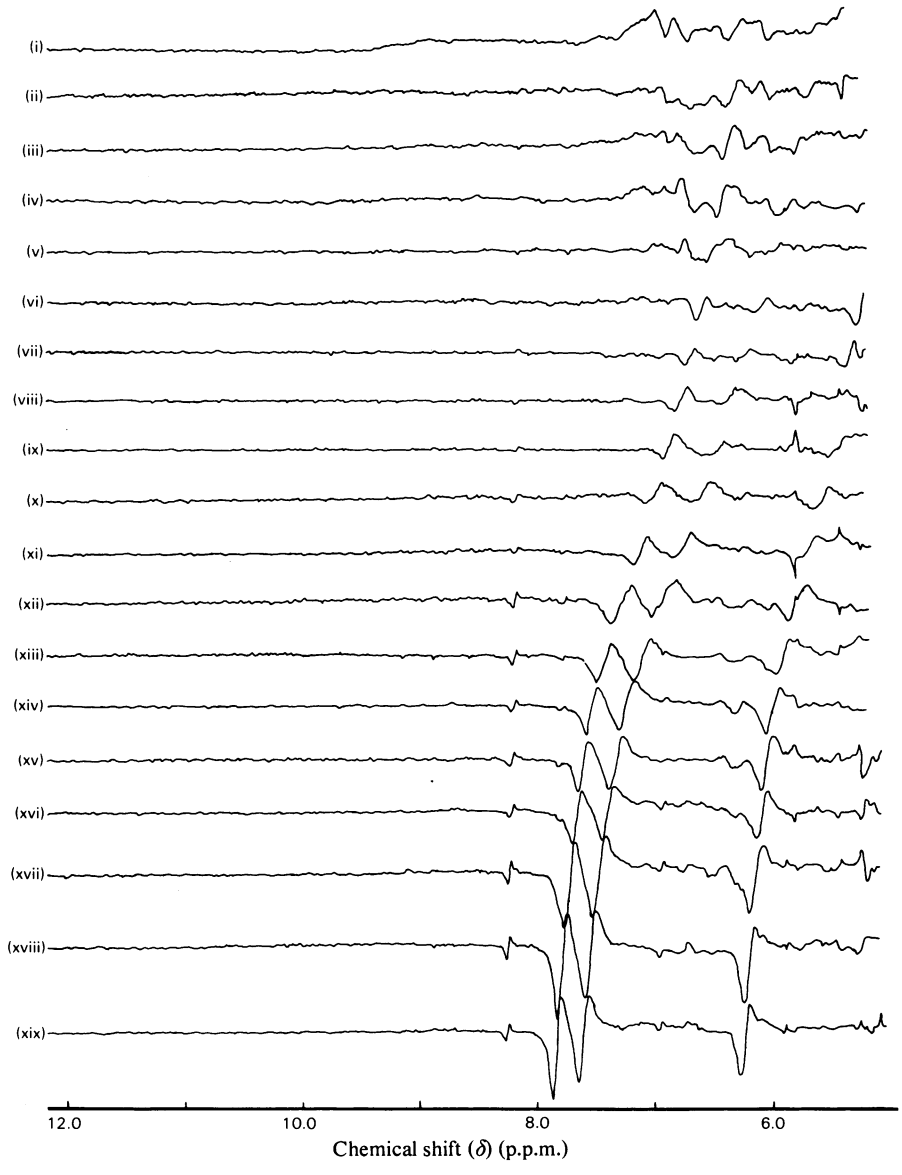

Fig. 5. *Sequential ^1H n.m.r. difference spectra of the aromatic region of Fv fragment* (1.5 mM) *on addition of* 2-*chloro*-4-*nitrophenylglycine*

These were obtained by subtraction of the spectrum of the mixture containing one more portion of hapten from the previous one. In the presence of excess hapten only the three hapten protons appear and can be seen to titrate quite clearly. The hapten concentrations relevant to each difference are (mM): (i) 0–0.24; (ii) 0.24–0.48; (iii) 0.48–0.71; (iv) 0.71–0.94; (v) 0.94–1.15; (vi) 1.15–1.37; (vii) 1.37–1.57; (viii) 1.57–1.77; (ix) 1.77–1.97; (x) 1.97–2.35; (xi) 2.35–2.72; (xii) 2.72–3.43; (xiii) 3.43–4.12; (xiv) 4.12–4.80; (xv) 4.80–5.46; (xvi) 5.46–6.11; (xvii) 6.11–7.36; (xviii) 7.36–8.55; (xix) 8.55–9.70.

mentioned above that, for the chlorine to remain in van der Waals contact with tyrosine-34_L, part of the hapten ring would be displaced downwards by up to 0.05 nm (Fig. 6). This will result in a larger shift on the $H_{(3)}$ proton. These differences in shift should therefore not be considered as important. Rather, the point to note is that the shifts remain very large and upfield, therefore requiring the hapten to

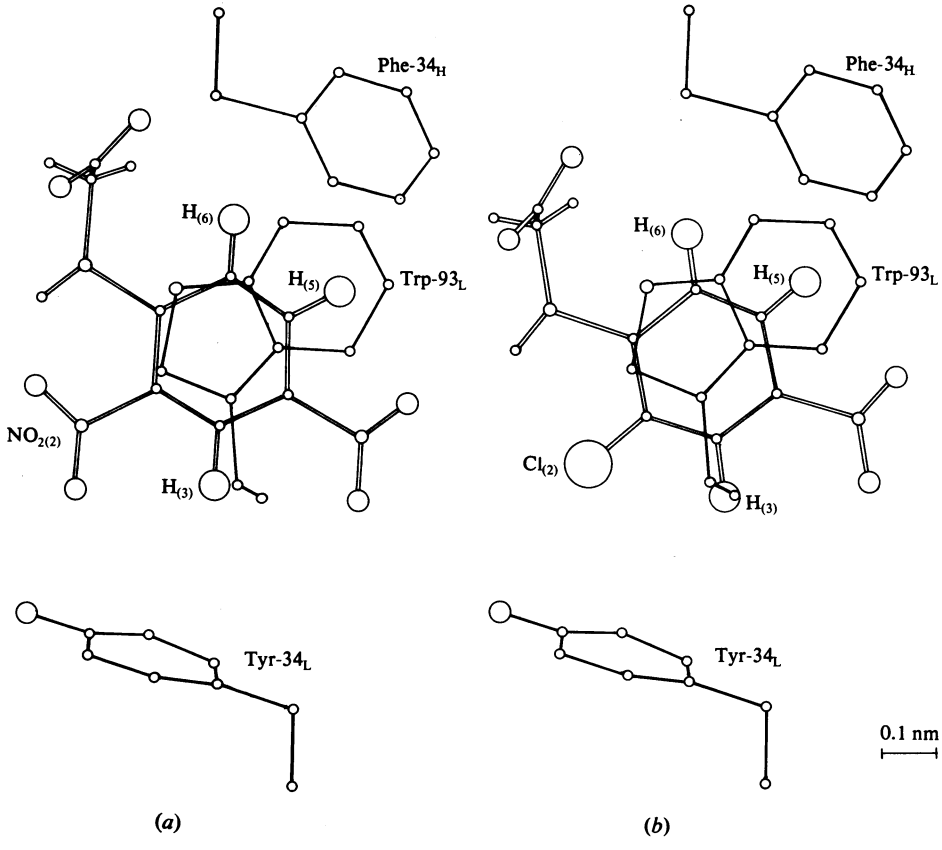

Fig. 6. *Three aromatic residues that constitute the main hydrophobic contact residues of the combining site with* (*a*) 2,4-*dinitrophenylglycine and* (*b*) 2-*chloro*-4-*nitrophenylglycine*

The effect of replacement of a nitro group by chlorine is to bring the centre of the hapten ring closer to tyrosine-34_L.

be in close contact with the aromatic residues of the combining site.

Table 4 shows that there is a significant trend in shifts on the $H_{(3)}$ proton with relevance to distortion of the 2-nitro group. It is, however, in the reverse direction to that expected for nitro group rotation. Thus the shifts for 2,4-nitro- and 4-chloro-2-nitrophenylglycines are smaller than those for 2,4-dichloro- and 2-chloro-4-nitrophenylglycines. The nitro group must therefore remain planar on binding to Fv fragment, and any change in conformation of the *o*-O–N–O$\cdots$H–N grouping for the haptens considered above must arise from rotation of the N–H out of the plane of the aromatic ring.

Conclusions

The high-resolution ^1H n.m.r. studies have shown that the mode of binding of chlorinated phenylglycines and anilines is the same as that for dinitrophenyl haptens and that the differences in binding constant must therefore be explained in terms of changes in hydrogen-bonding or stacking interactions. Model-compound studies between these haptens and 3-methylindole then eliminated changes in the strength of the specific hapten–tryptophan-93_L interaction as the major source of difference, leaving only hydrogen-bonding by the nitro groups as the important factor. This bonding is then weakened by chlorine replacing these nitro groups. The similarity of binding constants of the two pairs of monochlorinated haptens then established that there must be two hydrogen bonds rather than one. Furthermore, the shifts obtained from the high-resolution n.m.r. studies, when interpreted in the light of the results obtained from di- and tri-nitrophenyl model-compound studies, do not support the idea that the

2-nitro group distorts on binding to Fv fragment for the haptens considered.

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