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Specificity of Interactions of Hapten Side Chains with the Combining Site of the Myeloma Protein MOPC 315

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The pK_a values of the three histidine residues in the Fv fragment (variable region of the heavy and light chains) of the mouse myeloma protein MOPC 315, measured by high-resolution n.m.r. (nuclear magnetic resonance), are 5.9, 6.9 and 8.2. The perturbation of the pK_a of one of the histidines (pK_a 6.9) on the addition of hapten and the narrow linewidth of its proton resonances suggests that it is at the edge of the combining site. References to the model of the Fv fragment [Padlan, Davies, Pecht, Givol & Wright (1976) *Cold Spring Harbor Symp. Quant. Biol.* **41**, in the press] allows assignment of the three histidine residues, histidine-102_H, histidine-97_L and histidine-44_L. The determination of the pK_a of the phosphorus group, by ^{31}P n.m.r., of a homologous series of Dnp- and Tnp- (di- and tri-nitrophenyl) haptens has located a positively charged residue. Molecular-model studies on the conformations of these haptens show that the residue is at the edge of the site. The model suggests that the positively charged residue is either arginine-95_L or lysine-52_H.

The specificity of the IgA‡ mouse myeloma protein MOPC 315 for ligands containing the dinitrophenyl group arises from the dinitrophenyl forming a complex with tryptophan-93_L. This interaction occurs within an 'aromatic box' of amino acids formed mainly by phenylalanine-34_H, tyrosine-34_L and tryptophan-93_L (Dower *et al.*, 1977). There is also the possibility of two hydrogen bonds (to tyrosine-34_L and asparagine-36_L) involving the nitro groups on the dinitrophenyl ring, which would provide an additional enthalpy contribution to the binding energy. The 'aromatic box' results in very large ring-current shifts in the n.m.r. resonances of the hapten. An analysis of these has shown that, irrespective of the hapten side chain, the dinitrophenyl ring is orientated almost identically in the protein in each case, despite differences of almost three orders of magnitude in the affinities of the haptens. The hapten side chain must therefore play a major role in determining the affinity for the myeloma protein. This has been noted for the binding of vitamin K_1OH to the Fab fragment from immunoglobulin G New (Poljak, 1975), in which half the total binding energy of the hapten was attributed to interactions involving the side chain.

‡ Abbreviations: IgA, immunoglobulin A; Fab fragment, *N*-terminal half of heavy chain and light chain; Fv fragment, variable region of heavy and light chain; Dnp, dinitrophenyl; Dnp, dinitrophenyl; Tnp, trinitrophenyl; e.s.r., electron spin resonance; n.m.r., nuclear magnetic resonance.

One of the advantages of the n.m.r. technique is that the titration of ionizable groups results in changes in chemical shifts which can be easily monitored. Thus the determination of individual histidine pK_a values is particularly easy by n.m.r. studies (Campbell *et al.*, 1975*a*,*b*). Often, however, the pK_a of a charged group may lie outside the stability range of a protein and other methods have to be used to probe for the neighbouring charges. A particularly attractive method is to measure the pK_a values of ligands containing phosphate or phosphonate groups, by using ^{31}P n.m.r. Changes in these pK_a values on binding may then give information on the nature of any charged residues in the vicinity of the phosphorus moiety.

The model of the binding site of protein MOPC 315 (Padlan *et al.*, 1976), constructed on the assumption of framework invariance in immunoglobulins, places several positively charged residues near the entrance, in particular arginine-95_L, lysine-52_H and histidine-102_H. One or more of these is postulated to account for the positive subsite of the combining site obtained in the kinetic mapping studies by Haselkorn *et al.* (1974). However, the n.m.r. studies should allow detailed discussion of the residues involved in any particular case. The present paper is concerned with measuring the pK_a values of the three histidine residues in the Fv fragment from protein MOPC 315 in the presence of a variety of different haptens. Several of the haptens contain phosphate and phosphonate groups, and, by using side

chains of different lengths, changes in their pK_a values on binding to the protein can be used to probe different areas of the combining site for positively and negatively charged groups. In particular, these phosphorus-containing haptens are expected to be good probes for arginine residues, since these can function as bivalent ligands for organic phosphate groups (Cotton *et al.*, 1973). Such interactions between phosphate and arginine have been reported for staphylococcal nuclease (Arnone *et al.*, 1971) and lactate dehydrogenase (Adams *et al.*, 1973). Marked effects on the pK_a values of the phosphoryl group of these haptens would result if such an interaction occurred with protein MOPC 315.

Materials and Methods

Preparation of hapten and protein solutions

Dnp-glycine, Dnp-L-aspartic acid and Dnp-ε-amino-L-lysine were obtained from BDH Chemicals, Poole, Dorset, U.K. Dinitrophenol was obtained from Fisons, Loughborough, Leics., U.K. Menadione and aminomethylphosphonic acid were obtained from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K. 2-Aminoethylphosphonic acid, 3-aminopropylphosphonic acid and *O*-phosphoryl-2-ethanolamine were obtained from Calbiochem, Hereford, U.K. Trinitrobenzenesulphonate was obtained from N.B.C. Chemicals, Cleveland, OH, U.S.A. 1-Fluoro-2,4-dinitrobenzene was obtained from Koch–Light Laboratories, Colnbrook, Bucks., U.K.

The preparations of Dnp-benzylamine (Haselkorn *et al.*, 1974) and *N*-(2,2,6,6-tetramethyl-4-aminopiperidinyl)-2,4-dinitrobenzene (Dwek *et al.*, 1975*a*) have been described previously.

Preparation of Dnp-N-methylphosphonate. Aminomethylphosphonic acid (1 mmol) and $NaHCO_3$ (4 mmol) were dissolved in 4 ml of water, and 1-fluoro-2,4-dinitrobenzene (0.9 mmol) was added with strong stirring. After 1 h of stirring at 37°C, the solution was made 1 mM in HCl and cooled to 0°C. The yellow crystals were filtered off, and the filtrate was extracted with ethyl acetate. The ethyl acetate was removed by rotary evaporation. The combined crystals were dissolved in acetone, precipitated with water and dried *in vacuo*.

Preparation of Tnp-N-methylphosphonate, Tnp-N-ethylphosphonate and Tnp-N-propylphosphonate. These were prepared similarly, by using 2,4,6-trinitrobenzenesulphonate. The pH was maintained at 8.0 with $NaHCO_3$.

Preparation of Dnp-N-ethyl phosphate. O-Phosphoryl-2-ethanolamine (1 g) was dissolved in 75 ml of 0.2 M-$NaHCO_3$. 1-Fluoro-2,4-dinitrobenzene (2.6 g) was dissolved in 25 ml of dioxan and the solution was added dropwise over 1 h. The reaction was considered to be complete when the solution became clear. An amount of $BaCl_2$ equivalent to both phosphate and carbonate was added and the precipitate washed with 1 M-$BaCl_2$ solution. The precipitate was then dissolved in 0.1 M-HCl, and the solution was extracted with ethyl acetate. The ethyl acetate solution was evaporated and the purity of the product checked by n.m.r. and t.l.c.

Preparation of Fv fragment of protein MOPC 315. This was prepared as described by Inbar *et al.* (1972). Solutions for n.m.r. were prepared by dissolving the freeze-dried Fv fragment and haptens in ^2H_2O (Ryvan Chemicals, Southampton, Hants., U.K.); they also contained 0.15 M-NaCl. The pH was adjusted with small volumes of dilute ^2HCl or NaO^2H.

N.m.r. measurements

Proton n.m.r. spectra at 270 MHz were recorded on a Bruker spectrometer modified in Oxford, as described in Dwek *et al.* (1975*a*).

^{31}P spectra were recorded at 129 MHz on an instrument constructed in this laboratory (Hoult & Richards, 1975), which is interfaced with a Nicolet BNC-12 computer. The spectrometer was stabilized with a ^2H-lock system; 500 transients were accumulated for 10 mM-free hapten, and 50000 transients for 0.5–1 mM-bound hapten. Samples were prepared by dissolving material in 0.15 M-NaCl in 20% ^2H_2O (Ryvan). All spectra were recorded at 20°C and chemical shifts were measured with 85% (v/v) phosphoric acid as an external standard.

Fluorescence measurements

These have been described in Dower *et al.* (1977). All measurements were taken at 25°C in a thermostatically controlled cell.

Model-building measurements

The construction of molecular models of the Dnp- and Tnp-phosphorus haptens used CRE (Cambridge Repetition Engineers, Ealing Scientific, Watford, Herts., U.K.) parts for the nuclear framework and Courtaulds hemispheres (Ealing Scientific) to represent the van der Waals distance of closest approach. All these models are to a scale of 2 cm = 0.1 nm.

Distance measurements are complicated by the possibility of motions about certain bonds for the longer haptens and so are given either for a particular conformation, usually extended chain, or else as contour diagrams showing the regions accessible to each phosphate group at a given distance from the aromatic moiety.

For the Tnp-haptens, the NHR group is assumed to be out of the trinitrophenyl plane, the two *ortho*-

nitro groups remaining co-planar, and is restricted to about 30° on each side of a perpendicular arrangement. Comparison of these Tnp-haptens is based on their extended-chain conformations, and shows their relative molecular volumes and the maximum separation at the phosphonate and trinitrophenyl ring moieties. The symmetry of the trinitrophenyl ring means that each conformation that places the side chain on one side of the aromatic trinitrophenyl ring has an equally allowed mirror-image conformation on the other.

In choosing favourable conformations for the Dnp-haptens, the NHR group is co-planar with the ring, enabling hydrogen-bond formation between the hydrogen of NHR and the oxygen of the 2-nitro group (Holden & Dickinson, 1969).

Despite the similarity between the Dnp- and Tnp-haptens, there is a difference in conformation, resulting from the second *ortho*-nitro group in the trinitrophenyl derivatives. Whereas Dnp-haptens can form intramolecular hydrogen bonds between the 2-nitro group and the proton of the NH group, the presence of the third nitro group in trinitrophenyl prevents this by forcing NH out of the plane of the ring. An analogous situation obtains for *N*-substituted 2- and 2,6-nitroanilines. With addition of the second *ortho*-nitro group, the NHR moiety is forced out of the benzene ring plane, which is shown by an abrupt increase in base strength over that expected for the planar molecule (Sykes, 1974). Further, the formation of the hydrogen bond for the Dnp-haptens prevents rotation about the bond Dnp-NH and also fixes the orientation of the next bond; this is in the plane of the dinitrophenyl ring, but pointing away from the ring. This has two consequences relating to the ability of these dinitrophenyl phosphorus groups to probe particular areas of the combining site. First, they no longer have mirror symmetry about a plane perpendicular to the ring plane, through atoms $C_{(1)}$ and $C_{(4)}$. Secondly, for two haptens, one a Dnp and one a Tnp, which both have the same number of bonds between ring and phosphorus, the phosphorus atom of the Tnp-hapten can probe different and more extensive areas, at a given separation from the aromatic ring, than can that of the Dnp-hapten.

Fig. 1 shows cross-sections of the areas that can be probed by the phosphate or phosphonate groups when the haptens are in their extended conformation. It is clear that the Dnp-NH-$[CH_2]_2$-OPO_3^{2-} can probe a much smaller area than can Tnp-NH-$[CH_2]_3$-PO_3^{2-} and that this area has lower symmetry than that for the Tnp-hapten, being largely confined to the side of the dinitrophenyl ring with no nitro group. If the NHR group is co-planar with the trinitrophenyl ring for Tnp-aminopropylphosphonate, with rotation of one of the *ortho*-nitro groups, the resulting contour diagram is identical with that obtained for Dnp-aminoethyl phosphate.

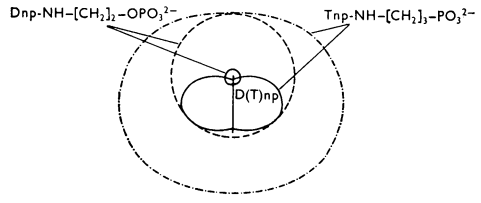

Fig. 1. *Cross-sections of the areas probed by the phosphorus moieties of the two haptens Dnp-NH-$[CH_2]_2$-OPO_3^{2-} and Tnp-NH-$[CH_2]_3$-PO_3^{2-} in their extended conformations*

Cross sections of the volumes probed by Dnp-NH-$[CH_2]_2$-OPO_3^{2-} and Tnp-NH-$[CH_2]_3$-PO_3^{2-} at 0.45 nm separation from the N atom of the NH group in a plane perpendicular to the axis through the $C_{(1)}$-NHR band. Full lines are *P* loci and broken lines the loci of the oxygen van der Waals radii. The Dnp-hapten can probe a much smaller area than can the Tnp-hapten. The contour diagrams are constructed from consideration of skeletal molecular models as described in the Materials and Methods section.

Results and Discussion

Effect of different haptens on the pK_a values of the three histidine residues in the Fv fragment

Sequence studies have shown that the Fv fragment has three histidine residues at positions 44 and 97 in the light chain (Dugan *et al.*, 1973) and at position 102 in the heavy chain (Francis *et al.*, 1974). Of these, histidines-97_L and -102_H are hypervariable (Wu & Kabat, 1970; Kabat & Wu, 1971), and are in or near to the combining site. This has been confirmed by the use of a Dnp-spin-labelled hapten, which broadens out the n.m.r. signals from those amino acids that are in or near to the combining site. In the presence of the spin-labelled hapten only one histidine resonance was observed, which was assigned to histidine-44_L (Dwek *et al.*, 1975*b*).

The chemical shifts of the resonances from histidine residues vary with the degree of ionization of the residue. Fig. 2(*a*) shows the aromatic regions of the 270 MHz proton n.m.r. spectra of the Fv fragment in the presence of Dnp-aminoethyl phosphate at several pH values. The histidine $C_{(2)}$ protons from the three residues are indicated. The changes in the n.m.r. spectrum with pH are most easily followed by the use of difference spectra, which involve the subtraction of spectra at different pH values. Difference spectra showing the $C_{(2)}$ and $C_{(4)}$ protons of the histidine residues are shown in Fig. 2(*b*). Fig. 2(*c*) shows the pH titration curves for the histidine $C_{(2)}$ protons in the Fv fragment alone and in the presence of two different haptens.

The resonances 1, 2 and 3 can be assigned to the $C_{(2)}$ protons of the histidine ring, since the resonance positions occur in the range characteristic of $C_{(2)}$ protons and the changes in chemical shift between the

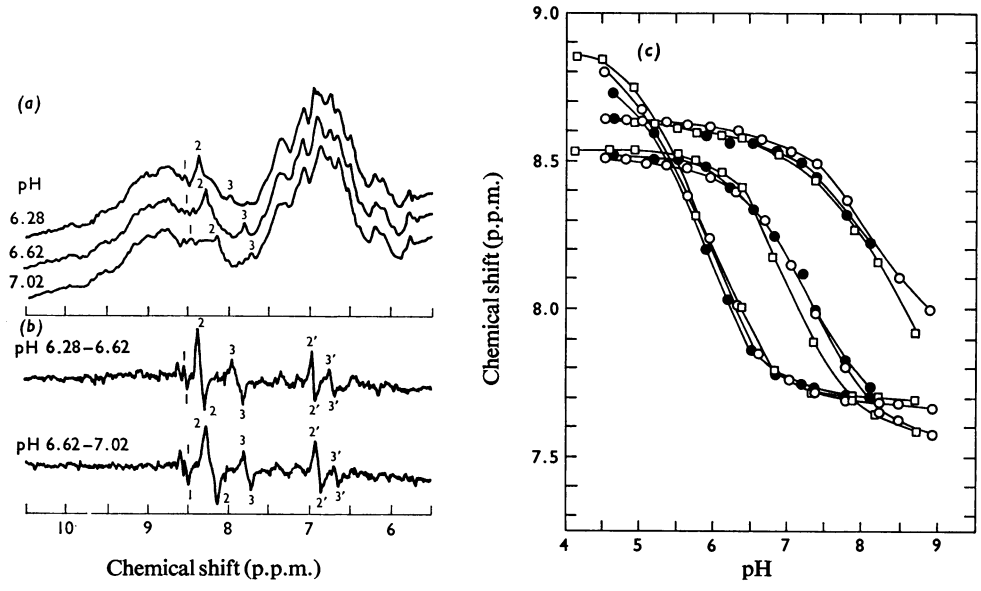

Fig. 2. *Titration of histidine residues in the Fv fragment from protein MOPC* 315 *at* 303 *K in the presence of* 0.15 M-*NaCl* (*a*) Proton spectrum at 270 MHz of the aromatic region of the Fv fragment in the presence of Dnp-aminoethyl phosphate at three different pH values. The numbers indicate the $C_{(2)}$ protons of the three titrating histidine residues. (*b*) Illustration of pH difference spectroscopy showing the $C_{(2)}$ and $C_{(4)}$ protons (primed numbers) of the titrating residues. Resonance 1′ is absent, since this does not change its position sufficiently over this pH range. (*c*) Titration of the histidine chemical shift versus pH for the Fv fragment alone (□), in the presence of Dnp-aminoethyl phosphate (○) and in the presence of Tnp-aminoethylphosphonate (●). Chemical shifts were measured by using the sodium salt of 3-(trimethylsilyl)propanesulphonic acid as an external standard.

protonated and unprotonated forms are approx. 1 p.p.m., which is characteristic of the behaviour observed for $C_{(2)}$ histidine protons (Shrager *et al.*, 1972). Similar arguments allow the assignment of resonances 2′ and 3′ to the $C_{(4)}$ histidine protons. The primed numbering has been assigned on the basis of the correspondence in the pK_a values, which are about 8.2 (resonance 1), in the range 6.9–7.4 (resonances 2 and 2′ depending on the hapten) and about 5.9 (resonances 3 and 3′) (Dwek *et al.*, 1975*a*).

The linewidths of the histidine resonances in fragment Fv provide further information about their environment. The histidine resonances 2 and 2′ (pK_a approx. 7.0) are always narrower than those from the other two histidine residues. This, and the fact that its pK_a value is almost identical with that from the imidazole group in model compounds, such as *N*-acetyl-L-histidine (Shrager *et al.*, 1972), suggests that this particular histidine residue is on the surface of the protein and readily accessible to the solvent. Conversely, the linewidth from resonances 3 and 3′ is always quite broad and the lower pK_a value (5.9) is consistent with this histidine residue being in a hydrophobic region of the protein. The acid extreme of the titration curve for the $C_{(2)}$ proton (resonance 3) is shifted downfield by about 0.3 p.p.m. relative to the other two histidine $C_{(2)}$ protons (Fig. 1*c*). This downfield shift could be a consequence of the ring current (Dower *et al.*, 1977) in a nearby aromatic residue. This aromatic residue could also account for the broad linewidth, since there would be significant dipolar interactions between the protons on it and the histidine protons.

The results of pH titrations of the three histidine resonances in fragment Fv alone and in the presence of a variety of haptens are summarized in Table 1. The data in Table 1 show that the pK_a values of two histidine residues, resonances 1 and 3 (pK_a values 8.2 and 5.9), are unperturbed by addition of hapten, whereas the pK_a value of the other histidine (approx. 6.9, resonance 2) is always increased, the trend being towards a slightly greater increase for charged haptens. The pK_a values for the second ionization of the phosphate and phosphonate haptens in the appropriate fragment Fv–hapten complexes can also be measured by ^{31}P n.m.r. (see below). These pK_a values are in the range 5.9–7.1 (see Table 2), so that, although the negative charge on these particular haptens varies between 1 and 2 over the titration range of the histidine residue (resonance 2), there are only minor increases in its pK_a value, which could

Table 1. *pK_a values of the histidine residues in Fv fragment from protein MOPC* 315 *in the presence of different haptens*
The pK_a values were obtained from 270 MHz proton-n.m.r. studies at T=303 K, in the presence of 0.15 M-NaCl. The numbers 1, 2 and 3 of the histidine residues refer to the resonances shown in Fig. 1. The binding constants were obtained from fluorescence titrations at pH 5.5 and T=303 K in the presence of 0.15 M-NaCl, except that for hapten 5, which is at pH 7.4 and is taken from Haselkorn *et al.* (1974).

Hapten	Histidine residue ... 3	Histidine pK_a values 2	1	K_D (μM)
* None	5.9	6.9	8.2	
1. Dnp-NH-CH_2-CH_2-OPO_3^{2-}	5.9	7.4	8.2	0.6
2. Dnp-NH-CH(-CO_2^-)-CH_2-CO_2^-	5.9	7.3	8.2	17.0
3. Dnp-NH-CH_2-PO_3^{2-}	5.9	7.3	8.2	5.0
4. Dnp-NH-[CH_2]$_4$-CH(NH_3^+)-CO_2^-	5.9	7.1	8.2	0.7
*5. Dnp-NH-CH_2-(benzene)	5.9	7.1	8.2	0.26
*6. Dnp-O^-	5.9	7.1	8.2	>10^2
*7. Dnp-NH-(2,2,6,6-tetramethylpiperidine)	5.9	7.2	8.2	4.0
8. Tnp-NH-CH_2-PO_3^{2-}	5.8	7.3	8.2	11.0
9. Tnp-NH-CH_2-CH_2-PO_3^{2-}	5.7	7.3	8.2	0.5
10. Tnp-NH-CH_2-CH_2-CH_2-PO_3^{2-}	5.9	7.2	8.2	0.4
11. (2-methyl-1,4-naphthoquinone)	Too broad to observe	7.0	8.2	6.0
12. Dnp-CH_2-CO_2^-	5.9	7.46	8.2	7.8

* From Dwek *et al.* (1975*b*).

arise from it being in the vicinity of the negatively charged group. The dimensions of the combining site are such (Sutton *et al.*, 1977) that the positions of the charged groups of the haptens would be at the entrance to the site. This suggests that the histidine residue (resonance 2, pK_a 6.9), which is on the surface of the protein and exposed to solvent, is also at the entrance of the combining site.

Use of the histidine data to refine the model of the Fv fragment

The model constructed by Padlan *et al.* (1976) has two histidine residues near the combining site and one at the back of the molecule, which is near to the $C_{(2)}$ domain in the Fab fragment. The model provides an assignment of all three histidine residues, since only one histidine-102_H occurs on the surface of the protein, at the very edge of the combining site on the H3 loop; this must be the residue with the pK_a value of 6.9. Histidine-97_L is further away (but within the spin-label broadening sphere; Dwek *et al.*, 1975*a*) from the combining site, in a partially hydrophobic environment, close to phenylalanine-94_L. Assignment of this to the residue with the pK_a value 5.9 enables an approximate positioning of histidine-97_L relative to phenylalanine-94_L. Although only one histidine protein is involved and the magnitude of the observed shift (0.3 p.p.m. downfield) is small, correlation of this shift with the ring-current effect at phenylalanine-94_L suggests a relative geometry where the histidine proton lies close to the plane of the aromatic ring of phenylalanine-94_L. The remaining histidine residue is the non-hypervariable histidine-44_L, which has the pK_a value of 8.2. This

assignment is also consistent with its position 1.8 nm from the combining site adjacent to an aspartic acid residue, which would raise its pK_a value.

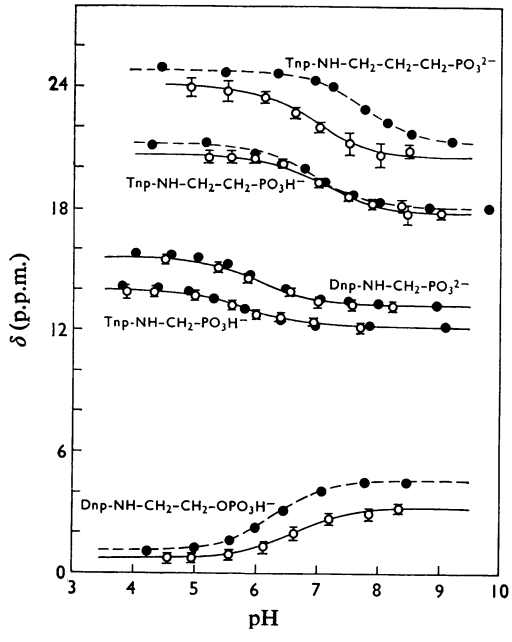

Fig. 3. *Titration of ^{31}P chemical shifts of phosphohaptens in the presence and absence of the Fv fragment* (293*K*, 0.15 M-*NaCl*)

The titration curves are calculated for the pK_a values listed in Table 2. ○, Titration curves for the bound haptens; ●, titration curves for the free haptens. The error bars represent the experimental uncertainty in the measurements in the Fv complex and arise from the broader resonance lines in the complexes. The chemical shifts are measured from 85% phosphoric acid as an external standard.

^{31}P magnetic resonance as a probe of electrostatic environment

The pK_a values of several phosphate and phosphonate haptens in the presence and absence of the Fv fragment have been measured from pH titrations (Fig. 3) of the ^{31}P chemical shifts. These pK_a values, together with the changes in chemical shift on binding to the Fv fragment, are given in Table 2. Of the Tnp-haptens, only the propylphosphonate has its pK_a significantly affected; the decrease of approx. 1 unit suggests the proximity of a positively charged group. Apart from Tnp-amino-

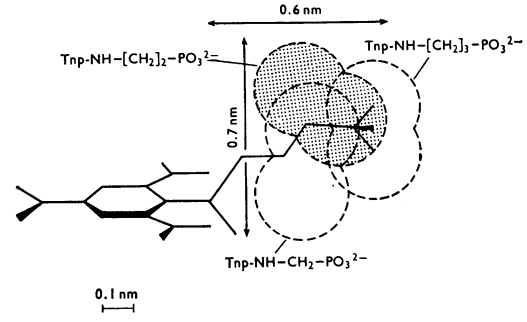

Fig. 4. *pK_a mapping in the combining site of the Fv fragment by ^{31}P magnetic resonance of phosphohaptens*

The areas probed by the phosphonate groupings of different Tnp-haptens in their extended conformations are shown. The circles represent the loci of oxygen radii for the three haptens. The lack of perturbation in the pK_a values of the two shorter haptens suggests that there are no charged groups within a cylinder of 0.7 nm±0.6 nm from the trinitrophenyl ring. (Note that the symmetry of the trinitrophenyl ring is such that there is an equally allowed mirror conformation on the other side of the trinitrophenyl ring.)

Table 2. *pK_a values of phosphonate and phosphate groups of phosphohaptens free and when bound to the Fv fragment from protein MOPC* 315

pK_a values were determined from ^{31}P n.m.r. titrations at 129 MHz. All measurements were made at T=293K in the presence of 0.15 M-NaCl. The errors are those obtained from analysis of the titration curves. The chemical shifts are the differences between the extremes of the titration curves of the ionizations for the free and bound haptens given in the Table.

Phosphohapten	pK_a free	pK_a bound to fragment Fv	Changes in chemical shift (p.p.m.) Acid extreme	Base extreme
Tnp-NH-CH_2-CH_2-CH_2-PO_3H^-	7.7	6.9±0.2	−0.7	−0.7
Tnp-NH-CH_2-CH_2-PO_3H^-	6.9	7.1±0.2	−0.5	−0.2
Tnp-NH-CH_2-PO_3H^-	5.7	5.9±0.2	0	0
Dnp-NH-CH_2-PO_3H^-	6.0	6.1±0.2	0	0
Dnp-NH-CH_2-CH_2-OPO_3H^-	6.3	6.6±0.1	+0.2	+1.3

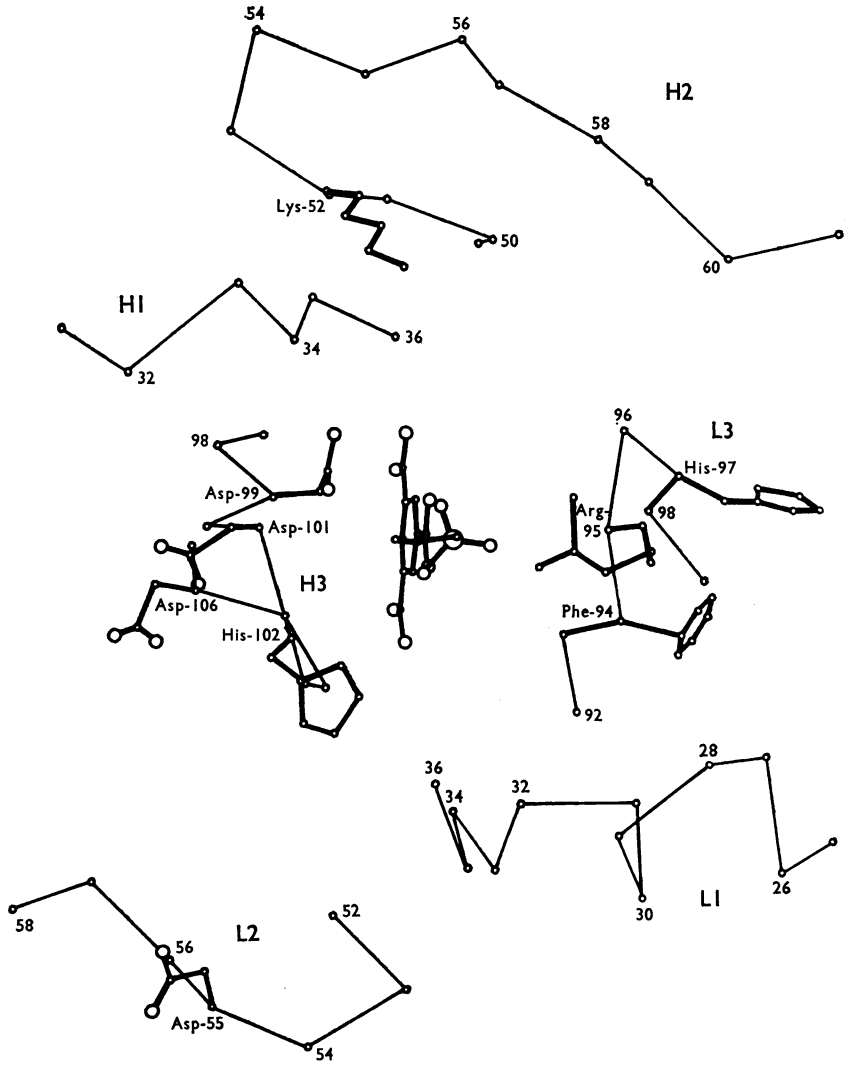

Fig. 5. *Combining site of protein MOPC* 315 *with Tnp-NH-*$[CH_2]_3$*-*PO_3^{2-} *in the site showing the location of the phosphorus group in the combining site*

The model is based on the co-ordinates of Padlan *et al.* (1976). L1, L2, L3, H1, H2 and H3 are the hypervariable regions of the light and heavy chains. The view is 10° rotation to the trinitrophenyl ring looking into the site. Note that the side-chain arginine-95_L is positioned to interact with the phosphonate group of the hapten. The positions of the two histidine residues are also clearly shown; histidine-102_H is at the edge of the combining site. The relative positioning of histidine-97_L and phenylalanine-94_L is based on the ring-current effects of phenylalanine-94_L.

propylphosphonate all the haptens show a slight increase in their pK_a values on binding to the Fv fragment. However, only for Dnp-aminoethyl phosphate is the increase outside the experimental uncertainty. This hapten is about 0.1 nm shorter than Tnp-aminopropylphosphonate and the increase in its pK_a value of 0.3 unit suggests that it experiences a negatively charged residue.

Conformational studies on the phosphoryl haptens

Since the pK_a values of neither of the shorter Tnp-haptens, or even Dnp-NH-CH_2-PO_3^{2-}, are affected by binding to the Fv fragment, the charged group must be further away from the tri- or di-nitrophenyl ring than is the phosphorus group. This suggests that the hapten side chains are in extended-chain conformations. Thus Fig. 4, which represents the side chains

in their extended conformations, is well chosen to describe the observed behaviour.

Although the extended length of the Tnp-methylphosphonate hapten is only 0.2nm shorter than the Tnp-propylphosphonate, its pK_a value is essentially unaltered within the experimental error. The unperturbed values of the shorter Dnp- and Tnp-haptens and the lack of changes in chemical shifts on binding to the Fv fragment (Table 2) suggest that there are no charged residues within a cylinder 0.6nm×0.7nm from the dinitrophenyl or trinitrophenyl ring (the radius of the phosphonate is 0.3nm and the length of the aminoethylphosphonate moiety is 0.6nm; see Fig. 4). These dimensions represent minimum values, since they do not take into account the distance over which a pK_a value will be sensitive to a neighbouring charge.

Fig. 1 shows that Tnp-aminopropylphosphonate can probe areas not accessible to Dnp-aminoethyl phosphate, thus providing a possible explanation for the differences in the pK_a changes on binding of the respective haptens to the Fv fragment.

Charge distribution in the combining site

Positioning the trinitrophenyl ring within the aromatic box of the combining site (Dower *et al.*, 1977), overlapping and parallel to the side chain of tryptophan-93_L with the 4-nitro group hydrogen-bonded to asparagine-36_L, places the positive charge at the entrance to the combining site. The model places four charged residues (asparagine-101_H, histidine-102_H, lysine-52_H and arginine-95_L) at the edge of the site. The small perturbation of the pK_a (6.9 to 7.2) of histidine-102_H suggests that it is not the perturbing positive charge. This leaves lysine-52_H and arginine-95_L as the only positively charged groups that can interact with the Tnp-aminoethylphosphonate.

The e.s.r. spin-label studies (Sutton *et al.*, 1977) have implicated arginine-95_L as a positively charged residue at the edge of the site.

Although lysine-52_H has been affinity-labelled by the Dnp-haptens Dnp-NH-$[CH_2]_n$-CH-NH-$COCH_2Br$ (Givol *et al.*, 1971), this does not accurately define the position of the lysine side chain, even though the model permits it to react with the reagent, without displacing the dinitrophenyl from the combining site. This point is further emphasized when it is noted that both *m*-nitrobenzene diazonium ion (Goetzel & Metzger, 1970) and Dnp-NH-$[CH_2]_2$-$COCH_2Br$ (Haimovich *et al.*, 1972), which are of entirely different length, label the same residue (tyrosine-34_L) on protein MOPC 315.

On the basis of the phosphorus pK_a mapping studies, it is therefore not possible to distinguish between the two possibilities for the positive charge. However, interaction with arginine-95_L of the five-membered nitroxide spin label, which is about the same length as Tnp-aminopropylphosphonate, suggests that this is the more likely of the two residues.

Note Added in Proof (Received 26 April 1977)

The chemical modifications of protein 315 by Klostergaard *et al.* (1977*a*,*b*) support the conclusion that arginine-95_L is the positively charged residue in the combining site.

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