INVESTIGATION OF HAPTEN–ANTIBODY INTERACTIONS IN McPC603 BY ^{1}H AND ^{31}P NMR SPECTROSCOPY

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1. Introduction

With the determination of the three dimensional structures of two Fab fragments [1,2] by X-ray crystallography and of the Dnp binding site of an Fv fragment by NMR [3], our understanding of the molecular basis of antibody–antigen recognition has been considerably advanced. It is now realised that there is a high degree of complementarity between the different functional moieties of the hapten and the three dimensional distribution of amino acids in the combining site, all of which come from the small percentage of residues designated as hypervariable [4].

Although the structure of the combining site of the phosphoryl choline-binding antibody McPC603 has been largely determined by X-ray crystallography [5], questions which remain unanswered are the importance of interactions between the phosphate moiety and the side chains of Arg 52_H and Tyr 33_H and whether the hapten is bound as the mono- or dianion. This paper is concerned with the use of NMR spectroscopy to answer this by unambiguously identifying the phosphorus species involved in binding and to do this in solution, thereby providing dynamic as well as structural information. In addition, the crystal structure is used as a means of interpreting high resolution ^{1}H NMR difference spectra obtained on addition of hapten. This is important for the extension of NMR to the solving of unknown protein structures, by obtaining at least empirical agreement between the predicted and observed effects of aromatic and other perturbing residues.

2. Materials and methods

2.1. *Preparation of protein 603 Fab' solutions*

The Fab' fragment of the mouse myeloma IgA protein McPC603 was prepared as described for MOPC315 [6]. Samples for all NMR studies were prepared by dissolving the freeze-dried protein in $^{2}H_2O$ (Ryvan Chemicals, Hedge End, Southampton; 99.8% $^{2}H_2O$). pH* (pH uncorrected for the deuterium isotope effect) was adjusted with dilute solutions of $NaO^{2}H$ and ^{2}HCl. All solutions contained 0.15 M NaCl.

2.2. *Preparation of hapten solutions*

Phosphoryl choline chloride was obtained as the calcium salt from Sigma Chemical Co. Concentrated stock solutions for use in ^{1}H NMR hapten titrations were made by dissolving the phosphoryl choline in $^{2}H_2O$ containing M603 at a concentration equal to that of the main sample, to avoid dilution effects. Solutions for ^{31}P NMR pH titrations were made by dissolving sufficient M603 in a solution of hapten in $^{2}H_2O$ to give the desired concentrations.

2.3. *^{1}H NMR studies*

Spectra were recorded at 270°MHz on a Bruker spectrometer as described [7], operating in the Fourier transform mode with a Bruker BNC-12 data system. The probe temperature was 303°K. 2000 transients were accumulated for each spectrum. Chemical shifts are quoted relative to the sodium salt of 3-(trimethylsilyl)-propane sulphonic acid as external standard.

2.4. *^{31}P NMR studies*

Spectra were recorded on an instrument constructed in this laboratory [8] which is interfaced with a Nicolet BNC-12 computer. The spectrometer was stabilised with a ^{2}H-lock system. The probe temperature was 295°K. 4000 transients were accumulated for each spectrum, with an acquisition time of 0.5 s and a repetition rate of 0.5 s^{-1}. Chemical shifts were measured relative to 44% phosphoric acid as external standard.

3. Results and discussion

3.1. *The role of the phosphate group in stabilising hapten binding*

The characteristics of the ^{1}H and ^{31}P NMR spectra of the phosphoryl choline hapten were determined for use in interpreting the spectra obtained on binding to M603. The ^{1}H NMR spectra showed three resonances; two due to the CH_2 groups and one from the NMe_3 group. Their chemical shifts titrate with pH and have acid and base extremes of: NMe_3 3.22–3.21 ppm; CH_2 α to NMe_3 3.66–3.58 ppm; CH_2 β to NMe_3 4.29–4.16 ppm. A pK_a^* of 5.5 ± 0.1 was obtained. The ^{31}P NMR spectrum consisted of a single resonance, which showed a titration range from −3.4 ppm at high pH to +0.4 ppm at low pH, with a pK_a^* of 5.60 ± 0.05. This compares with a pK_a of 5.4 [9] given for phosphorylcholine in H_2O.

Two ^{31}P pH titrations were performed on samples of M603 containing, respectively, excess phosphoryl-choline and excess M603. The results are shown in fig.1. With excess phosphoryl choline at high pH, two resonances are observed at −3.40 ppm and −1.96 ppm, corresponding to the free and bound dianion respectively, which merge at pH* 5.8 and cannot be resolved below this pH*. The starting positions at high pH* are in agreement with those of Goetze and Richards [9]. When M603 is in excess over hapten only one resonance is observed, whose chemical shift at pH* 8.0 again corresponds to phosphoryl choline bound as the dianion. This resonance titrates with a pK_a^* of 5.07 ± 0.10 assuming a reduced titration range of 3.5 ppm. A plot of pH* versus $\log_{10}$ PC^{2-}/PC^{-} then gives a slope of 0.99. If however the normal titration range of 3.84 ppm is used, the plot remains linear, but with a gradient of only 0.93 and gives a

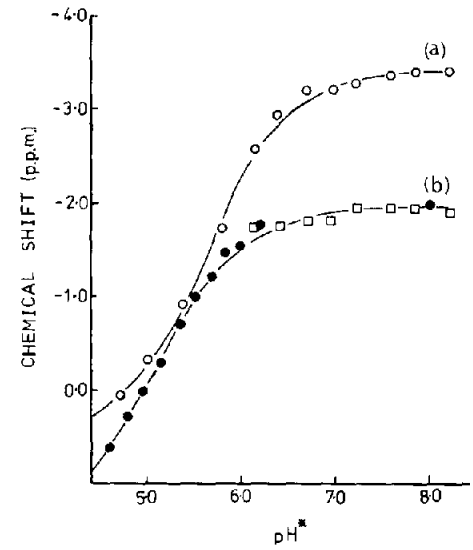

Fig.1. pH* titration of phosphoryl choline and M603 by ^{31}P NMR. (a) Represents free hapten. (b) Represents hapten bound to M603. Points (○) and (□) were obtained from a sample containing 2 mM M603 and 4 mM phosphoryl choline and points marked (●) from a solution 1.5 mM in M603 and 1 mM in hapten. Sample volumes were 0.8–0.9 cm^{-3}.

pK_a^* of 4.97. Such a reduced titration range can be understood on the basis of partial charge neutralisation of the phosphate moiety by hydrogen bond formation to Arg 52_H and Tyr 33_H, which would reduce the change in electronic environment of the phosphorus nucleus on ionisation of the monoanion.

The observation that the bound phosphoryl choline resonance titrates without loss of peak height shows that both the mono- and dianions bind to M603 and that they must do so with quite similar binding constants. If the binding of the monoanion were extremely weak the bound resonance would not titrate and would instead diminish in intensity as the concentration of phosphoryl choline dianion was reduced, on lowering the pH*. Titration rather than diminution of a non-titrating resonance is also consistent with the small magnitude of the change in pK_a^* on binding to Fab'.

Using the values of 5.55 and 5.07 for the pK_a^* values of phosphoryl choline free and bound, respectively, and a binding constant of 2×10^5 M^{-1} [10] for the dianion to M603, one obtains a value of

6.6×10^4 M^{-1} for the binding of the monoanion. This corresponds to a difference in binding energies of the two species of only 2.8 $kJ.mol^{-1}$, or 9% of the total for the dianion, showing that, although there is probably a definite requirement for some negative charge for tight binding, one unit is sufficient. It is important to realise that NMR is the only technique which can identify the monoanion as the phosphoryl choline species binding at low pH*.

3.2. ^1H *Hapten difference spectra*

Studies by high resolution ^1H NMR on hapten binding to M603 were made both to corroborate the conclusions of ^{31}P regarding the phosphate group and to investigate the rest of the binding site, to which ^{31}P shifts are insensitive.

Two titrations of hapten into M603 were performed at different pH* values; one well above and one at the pK_a^* of free phosphoryl choline. Very few protons are perturbed (fig.2), providing another instance of no major conformational changes occurring on hapten binding to antibody fragments [3]. An estimate of the number of protons affected was made using a histidine C(2) proton as an internal marker, whose resonance peak was taken to correspond to one proton: this is the only titratable histidine in the Fab′ fragment and has a pK_a^* of 7.4. By this method, a value of 6–10 aromatic and 11–14 aliphatic protons was thus

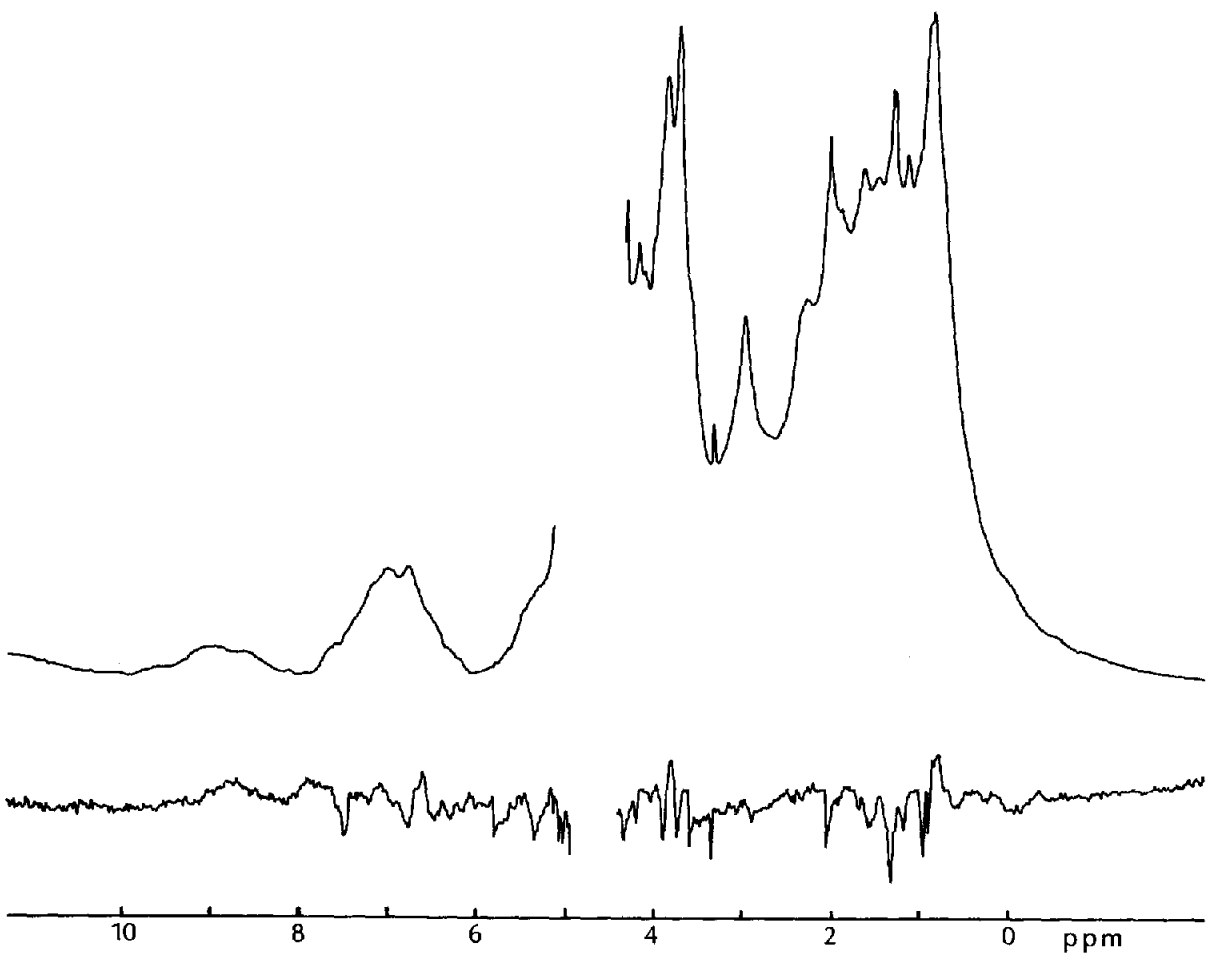

Fig.2. ^1H 270 MHz. NMR spectrum of 1.5 mM M603 (upper) at pH* 7.5 and the difference spectrum (lower) obtained by subtracting the spectrum after addition of 1.5 mM phosphoryl choline. The lower spectrum has a vertical scale 4 × that of the upper.

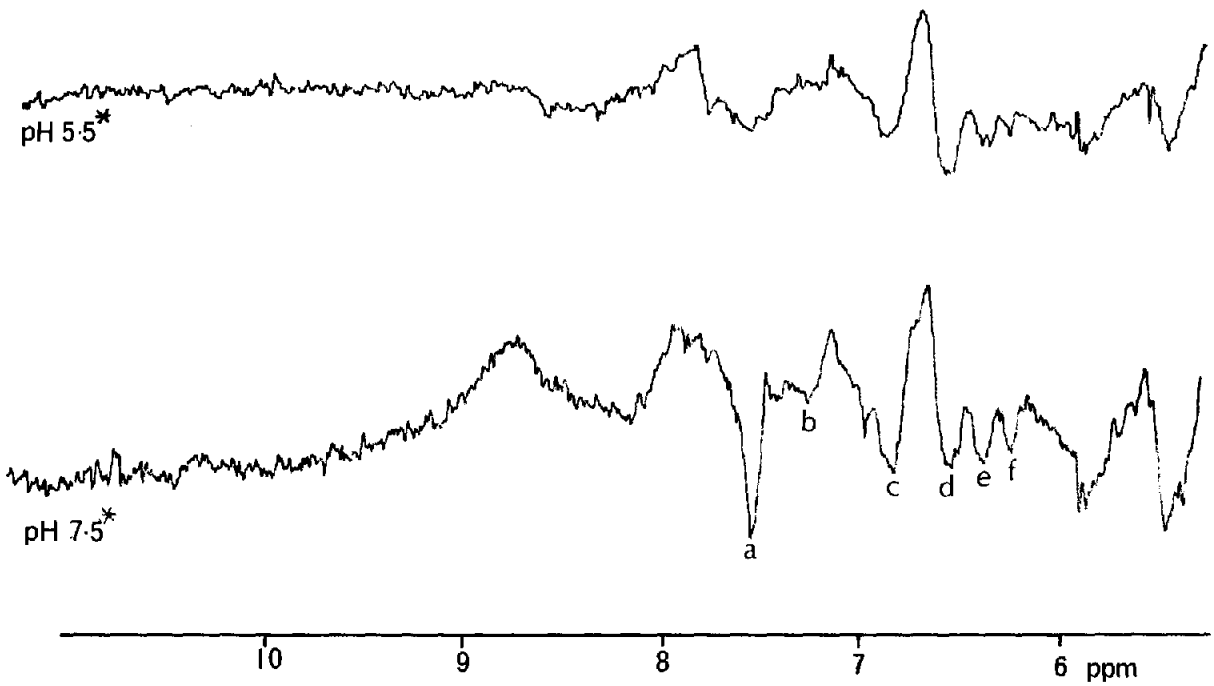

Fig.3. ^{1}H 270 MHz NMR aromatic difference spectra at two pH* values, formed by subtraction of the spectrum containing hapten at 1:1 concentration from that without hapten. The letters in the lower spectrum correspond to resonances referred to in table 1. The aromatic region is shown.

obtained for a fragment containing about 4000 protons.

Figure 3 shows the difference spectra obtained at 1:1 concentrations of hapten and protein at both pH* values. These are very similar to one another and show that the interactions between hapten and antibody at these pH* values are essentially the same; consistent with the ^{31}P result that, even at pH* 5.5, M603 is still binding the dianion because of the reduction in pK_a^*.

Unfortunately the proton resonances perturbed by hapten and the two CH_2 resonances of the hapten itself are in 'slow' exchange [11] between free and bound environments, so that it is not possible to determine the changes in chemical shift for these resonances on binding but only to indicate final positions. However, the NMe_3 resonance does titrate downfield as excess phosphoryl choline is added, showing it to be in 'fast' exchange [11]. The chemical shift change on binding is small – only 0.09 ppm upfield – which is due to it being an average of 9 different shifts, some of which will probably be significantly larger than 0.09 ppm. This value is useful in that it puts a lower limit on the perturbations of other protons in the difference spectra and also gives an upper limit to the rate of exchange between the antibody and solution (k_{off}). $k_{off} = \tau_B^{-1}$ estimated from the conditions for slow exchange has a value of $\ll 152\ s^{-1}$, while k_{on}, derived from $K_a = k_{on}/k_{off}$ and using the value of $2 \times 10^5\ M^{-1}$ for K_a, $\ll 3 \times 10^7\ M^{-1}\ s^{-1}$. These agree with the more definite values of $10\ s^{-1}$ and $1–4 \times 10^6\ M^{-1}\ s^{-1}$ respectively, obtained by Goetze and Richards [9] from independent parameters.

Because the chemical shift changes on binding are not known, any assignment of resonances in the difference spectra must be quite tentative. However, the crystal structure [12] shows that both Trp $104a_H$ and Tyr 33_H are hapten contact residues and should be perturbed by hapten, the tyrosine probably very strongly because of hydrogen bonding with the phosphate group. In view of this and the approximate

Table 1
Possible assignment of resonances appearing in the negative (bound) part of the difference spectrum shown in fig.3, at pH* 7.5

Resonance	Chemical shift (ppm)	Possible assignment	Random coil positions (ppm)
a b	7.50 7.25	Trp $104a_H$	7.19–7.68
c d	6.79 6.52	*m*-Tyr 33_H	7.19
e f	6.33 6.19	*o*-Tyr 33_H	6.84

correspondance between the expected number of aromatic protons affected and that calculated from the difference spectra, an assignment is suggested in table 1. The chemical shifts of the resonances attributed to Tyr 33_H are significantly upfield from random coil positions [13]. Inspection of the amino acid sequence of the heavy chain [14] shows that there is a phenylalanine residue adjacent to Tyr 33_H which could give rise to upfield shifts on the tyrosine due to ring current shifts [15,16].

To determine exactly the titration ranges of the perturbed protons it will be necessary to use a hapten which binds weakly enough for fast exchange conditions to be satisfied. This should be possible using phosphate esters of phosphoryl choline. Further investigation of these haptens by ^{31}P NMR will enable the transition from neutral species to monoanion to be studied and thus enable the binding energy contribution of the first negative charge to be determined in the same way as described above for the second.

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