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Magnetic Resonance Studies of the Binding Site Interactions between Phosphorylcholine and Specific Mouse Myeloma Immunoglobulin†

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ABSTRACT: The interaction of phosphorylcholine-binding mouse myeloma protein M603 and the isotopically substituted hapten phosphoryl[*methyl*-^{13}C]choline has been investigated using ^{13}C and ^{31}P nuclear magnetic resonance (NMR) spectroscopy. Upon binding to antibody, upfield shifts of 0.7 and 1.5 ppm are observed for the hapten ^{13}C and ^{31}P resonances, respectively, and both spectra are in the "slow" exchange limit. Linewidth analysis indicates some immobilization of the phosphate group but essentially unrestricted methyl group rotation for the bound hapten. Hapten–antibody dissociation rate constants of 10 and 38 s^{-1} are calculated from ^{13}C and ^{31}P NMR spectra, respectively, suggesting the possibility of differential dissociation rates for the two opposing ends of the phosphorylcholine molecule. The NMR data are entirely consistent with the known x-ray structure of the M603 Fab′–phosphorylcholine complex (Segal, D. M., Padlan, E. A., Cohen, G. H., Rudikoff, S., Potter, M., and Davies, D. R. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4298).

Antibodies, the primary molecules of the immune response, are responsible for the specific recognition of antigen and also for other, biologically important, effector functions which include initiation of the complement cascade, release of histamine from mast cells, and activation of the differentiation of B lymphocytes into antibody-producing plasma cells (Metzger, 1974). X-ray crystallographic techniques have recently revealed the three-dimensional structures of some antibodies and their Fab fragments (Davies et al., 1975; Poljak, 1975) and correlation of antibody structure with various biological functions has attracted interest (Yasmeen et al., 1976; Hurst et al., 1974). A precise knowledge of the molecular details (both structural and dynamic) of the interactions between antigen and antibody is central to a thorough understanding of antibody specificity and the relationship between the structure of an antibody and its function. Various physical techniques have been employed to elucidate such information including circular dichroism (Rockey et al., 1972), chemical modification (Grossberg et al., 1974), and magnetic resonance (Dwek et al., 1976). We have initiated a systematic study of the correlation between antibody structure and hapten binding properties of a group of myeloma proteins which have specificity for phosphorylcholine and related substances (Potter, 1972). The study of these antibodies offers several advantages: (i) the immunoglobulins can easily be obtained as homogeneous proteins in relatively large (gram) quantities; (ii) extensive amino acid sequence data are available for many of them (Barstad et al., 1974; Hood et al., 1975); (iii) the three-dimensional structure of the Fab′ fragment of a typical member of this group, M603, has recently been reported (Segal et al., 1974). This knowledge allows the results of binding experiments to be interpreted in terms of known and inferrable molecular structures.

Magnetic resonance affords a physical technique for studying hapten–antibody interactions which is particularly well suited to phosphorylcholine as one can observe events occurring at either end of the hapten using the signals from ^{31}P (natural abundance) in the phosphoryl group and ^{13}C (enriched) in the trimethylammonium group without significant interference from protein signals. We report here the results

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of such a study of the interaction between phosphorylcholine and M603. The main goals of this work are (i) to characterize the specific spectral changes (chemical shift and line width) of the ^{13}C and ^{31}P hapten resonances caused by binding to protein M603; (ii) to rationalize these observations in terms of known binding-site interactions including the dynamics of this hapten–antibody interaction. We hope this particular study can serve as a basis for subsequent studies of the interactions between phosphorylcholine and other phosphorylcholine binding myeloma proteins of somewhat different structure and specificity.

Materials and Methods

Protein Purification. Plasmacytoma McPC 603 was obtained from the Salk Institute, San Diego, Calif., and maintained by serial, subcutaneous transplantation in BALB/c mice. For production of large quantities of immunoglobulin, the tumor was converted to the ascites form in CDF_1 mice (Cumberland Farms, Tenn.). The ascites, obtained from tapping of the tumor-swollen mice, was subjected to mild reduction and alkylation and phosphorylcholine-binding IgA protein M603 immunospecifically isolated by passage through a Sepharose–phosphorylcholine column (Chesebro and Metzger, 1972). All experiments described in this report were carried out in 0.02 M borate, 0.15 M NaCl, 1 mM EDTA, pH 8.0 buffer.

M603 Fab′, for use in NMR[1] experiments, was prepared as described for MOPC 315 (Inbar et al., 1971). The digestion mixture was again applied to the Sepharose–phosphorylcholine column and Fab′ immunospecifically eluted. Fab′ prepared in this manner was judged essentially homogeneous by sodium dodecyl sulfate–acrylamide gel electrophoresis (Fairbanks et al., 1971).

Synthesis of Phosphoryl[methyl-^{13}C]choline. [*methyl*-^{13}C]Iodide (90.8 atom % ^{13}C) was obtained from Thompson Packard Inc. [*methyl*-^{13}C]Choline iodide was first prepared by the direct combination of dimethylaminoethanol and [*methyl*-^{13}C]iodide (Chesebro and Metzger, 1972) and was subsequently phosphorylated (Baer, 1952) to yield the product.

Hapten Binding Assays. Phosphoryl[*methyl*-^{14}C]choline was obtained from New England Nuclear. Equilibrium dialysis was performed in Lucite cells with 2-ml compartments using reduced and alkylated monomeric IgA at concentrations of 1–2 mg/ml. Protein concentration was determined by absorbance at 280 nm using $E_{1cm}^{0.1\%} = 1.36$ and a molecular weight of 150 000. The data were plotted by the method of Scatchard (1949) and a least-squares fit was used to obtain the best line.

NMR Experiments. NMR spectra were obtained on a Varian XL-100-15 spectrometer interfaced with a Varian 620i computer and operating in the Fourier transform mode. Both ^{13}C spectra (obtained at 25.2 MHz) and ^{31}P spectra (40.5 MHz) were proton noise decoupled and obtained at the normal probe temperature of 30 ± 2 °C. A deuterium field-frequency lock was provided by means of a capillary insert containing D_2O. The data were accumulated using a 90° pulse and an acquisition time of 0.3–0.4 s. A sweep width of 1000 Hz was commonly used. Generally at least 100 000 transients were accumulated per spectrum.

NMR Sample Preparation. After affinity purification, protein solutions were extensively dialyzed to remove bound hapten. They were then concentrated by ultrafiltration in an Amicon cell using a PM-10 membrane. NMR samples consisted of 2–3 ml of 3–4 mM M603 Fab′ or reduced and alkylated 7S monomer prepared in this manner. Small aliquots of a concentrated (0.2 M) phosphorylcholine stock solution were added to the protein sample to achieve the desired hapten concentration. To prevent the formation of small amounts of turbidity which were occasionally observed after 24 h, sodium azide to 0.02% was added to NMR samples.

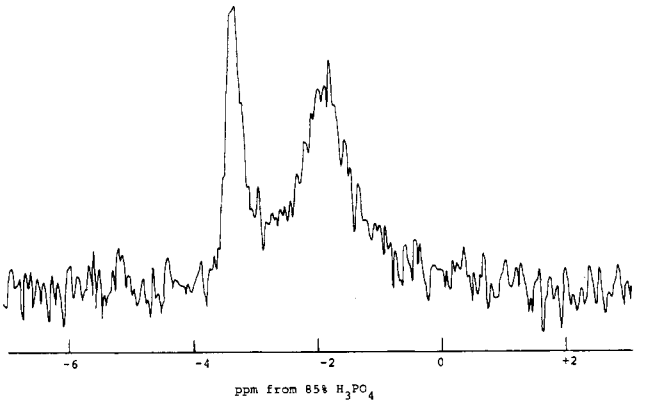

FIGURE 1: ^{31}P NMR spectrum of 3.7 mM M603 Fab′ and 4.7 mM phosphorylcholine at pH 8.0. An acquisition time of 0.4 s and a pulse width of 105 μs were used. The spectrum represents the accumulation of 113 000 transients.

Results

Hapten Binding Constants. Scatchard plots of binding of phosphorylcholine to reduced, alkylated M603 were linear and extrapolated to 1.9 binding sites per IgA monomer. Mild reduction and alkylation of immunoglobulins have been previously demonstrated to have no effect on their binding properties (Sher and Tarikas, 1971). For M603 and phosphorylcholine this technique gave association constants of 8.2×10^5 (at 4 °C) and 1.0×10^5 M^{-1} (at 30 °C). These constants are in good agreement with those reported previously (Rudikoff et al., 1972; Metzger et al., 1971; Potter, 1972) under somewhat ill-defined experimental conditions. For interpretation of the NMR results, however, we wished to have association constants obtained under conditions identical with respect to buffer and temperature with those employed in the NMR measurements. Although the protein concentrations were significantly higher in the NMR experiments than in the equilibrium dialysis measurements, previous work has shown the hapten–antibody association constant of phosphorylcholine specific myeloma proteins to be independent of immunoglobulin concentration (Sher and Tarikas, 1971).

NMR Results. ^{31}P magnetic resonance may be used to monitor the state of ionization of phosphate esters (Crutchfield et al., 1967; Moon and Richards, 1973) and our results in this case show that monoprotonation of dianion to the monoanion occurs with a pK of 5.4 and results in an upfield shift of 3.8 ppm. At pH ≥7 phosphorylcholine exists almost exclusively as the dianion, the ionization state of the hapten which is recognized by the antibody under physiological conditions.

At pH 8.0 in the absence of immunoglobulin, the ^{31}P NMR spectrum of phosphorylcholine is a single sharp line ($\Delta\nu_{1/2}$ = 3 Hz) which occurs at −3.36 ppm from an external reference of 85% H_3PO_4. Figure 1 shows a typical ^{31}P spectrum for solutions containing both phosphorylcholine and M603. Binding of hapten to antibody leads to an upfield shift of 1.5 ppm and is accompanied by appreciable line broadening. That both the

[1] Abbreviations used: NMR, nuclear magnetic resonance; L and H, light and heavy chains, respectively.

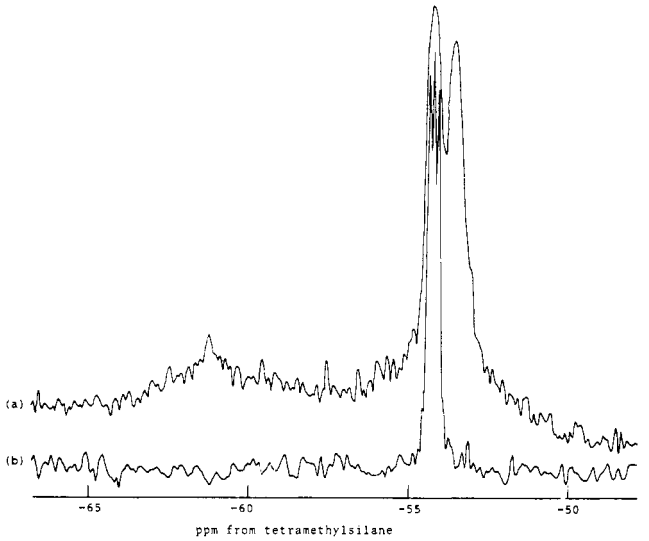

FIGURE 2: (a) ^{13}C NMR spectrum of a twofold excess of phosphoryl-[*methyl*-^{13}C]choline over M603 Fab′ at pH 8.0. Fab′ concentration was 3.2 mM. An acquisition time of 0.3 s and a pulse width of 35 μs were used. Transients (227 000) were collected to obtain the spectrum. The spectral baseline is formed by the natural abundance ^{13}C spectrum of the protein. (b) ^{13}C NMR spectrum of phosphoryl[*methyl*-^{13}C]choline at pH 8.0.

observed change in chemical shift as well as line-broadening effects were due to specific interactions of the hapten with the immunoglobulin was verified by control experiments with nonspecific proteins such as bovine serum albumin in which cases neither line width nor chemical shift was significantly affected. Even at high protein concentrations, nonspecific binding of phosphorylcholine to regions of M603 Fab′ other than the antigen binding site is considered unlikely since crystals of the protein soaked with phosphorylcholine have been shown to exhibit only one binding site per Fab′ monomer (Rudikoff et al., 1972). Fab′ fragments were generally used for the NMR experiments primarily because they lead to a lower sample viscosity and allow one to obtain higher effective binding site concentrations. When the complete M603 protein was used, spectra were observed which were identical with those resulting from the use of Fab′ fragments.

The proton-noise decoupled ^{13}C spectrum of phosphoryl-[*methyl*-^{13}C]choline in solution consists of a 1:1:1 triplet ($J_{^{13}C-^{14}N}$ = 4 Hz) which occurs at −54.3 ppm from tetramethylsilane. Binding of hapten to M603 Fab′ results in a small upfield shift (0.7 ppm) with little concomitant line broadening as shown in Figure 2. On binding of hapten to antibody, the ^{13}C–^{14}N splitting is lost.

For both nuclei, the spectra of hapten in the presence of antibody consist of two signals, one for the hapten free in solution and one for the hapten bound to antibody.

Discussion

General. The three-dimensional structure of M603 Fab′ has been determined to 3.1-Å resolution (Segal et al., 1974) and provides a structural basis for subsequent discussion. In this study, the phosphorylcholine molecule was shown to bind in a wedge-shaped cavity lined exclusively by residues from the hypervariable regions of the heavy (H) and light (L) chains. The choline end of the hapten binds in the interior of this cavity and is in close van der Waals contact with mainly hydrophobic protein side chains, whereas the phosphate group binds more toward the exterior of the cavity. Specific hydrogen bonds are formed between the phosphate oxygens and Tyr-33 (H) and Arg-52 (H); further interaction of an ionic type may arise from the nearby Lys-54 (H). A carboxylate anion (Glu-35) (H)) likely interacts ionically with the positively charged quaternary nitrogen of the hapten. Thus hydrogen bonds and ionic and hydrophobic interactions play specific roles in stabilizing the antibody–hapten complex.

The potential usefulness of phosphorylcholine arises from the possibilities of observing chemical shifts as well as relaxation phenomena (T_1 and T_2) independently for both ends of the hapten. Knowledge of chemical shifts for the bound hapten can reveal information about the local environment of the nucleus being observed. Analysis of spin-lattice (T_1) and spin-spin (T_2) relaxation rates may shed light on dynamic processes such as rates for association and dissociation and the mobilities of different regions of the hapten molecule when bound to antibody.

Chemical Shift and Environment. The ionization state of phosphate esters can be sensitively monitored by ^{31}P NMR spectroscopy and an upfield shift of 3.5–4.5 ppm is commonly observed for the addition of one proton (Crutchfield et al., 1967; Lee and Chan, 1971; Gorenstein and Myrwicz, 1973; Moon and Richards, 1973) which agrees well with the shift of 3.8 ppm upfield on monoprotonation of phosphorylcholine. On binding to M603, the ^{31}P hapten resonance moves upfield by 1.5 ppm which we suggest is due to partial protonation of the phosphate group by the formation, between amino acid residues in the immunoglobulin and the hapten, of hydrogen bonds (from Tyr-33 (H) and Arg-52 (H)) that partially neutralize the negative charge on the phosphate group.

This interpretation parallels that of Gorenstein and Myrwicz (1973) for the binding of cytidine 3′-monophosphate to ribonuclease A in which the ^{31}P signal experienced a chemical shift of 0.3 ppm which was interpreted to be the result of partial neutralization of the negative charge on the phosphate as a consequence of interaction with the enzyme. Similar conclusions had previously been reached from proton NMR studies (Meadows and Jardetzky, 1968; Meadows et al., 1969) as well as from x-ray diffraction studies of the complex between ribonuclease A and cytidine 3′-monophosphate (Richards and Wyckoff, 1971).

Many factors (Stothers, 1972) could account for the observed ^{13}C shift on binding of 0.7 ppm upfield. The most likely possibility is that the effective charge on the carbon nucleus is reduced when the trimethylammonium group interacts with negatively charged residues in the hapten binding pocket of the immunoglobulin (for example, Glu-35 (H)).

Hapten may have a substantially different conformation when bound to antibody than when free in solution which provides another potential contribution to changes in chemical shift of hapten on binding. The "eclipsed" conformation would bring the nitrogen and phosphorus atoms into such close spatial proximity that severe steric crowding would result. In solution, therefore, phosphorylcholine probably exists predominately in a substantially "trans" conformation even though such a conformation would minimize favorable intramolecular ionic interactions between cationic nitrogen and anionic oxygen atoms. Phosphorylcholine when bound by M603 Fab′ has been revealed by diffraction studies of crystals to have a conformation possibly slightly more eclipsed than one might expect for phosphorylcholine free in solution. Such a change in conformation might cause a small downfield shift in the ^{31}P resonance on binding due to a linear diamagnetic field effect arising from the closer proximity of the cationic quaternary nitrogen. Since we observe an upfield shift for the ^{31}P nucleus on binding, such an effect, if present, is nevertheless dominated

by the upfield shift contribution we have attributed to charge neutralization by partial protonation of and hydrogen bond formation to the phosphate group by antibody residues in the binding pocket.

Though effects other than charge neutralization could undoubtedly contribute to both the observed ^{31}P and ^{13}C shifts, the importance of interactions between charges on the hapten and on the immunoglobulin has been previously suggested not only by x-ray diffraction studies but also by binding studies. For example, the inability of the phosphorylcholine analogues phosphorylethanolamine and choline to compete effectively for hapten binding sites on M603 (Leon and Young, 1971) provides additional evidence for the importance of both the quaternary cationic nitrogen and the negative phosphate group to the hapten–antibody interaction.

Relaxation-Dynamic Behavior. The observation of two distinct peaks in both the ^{13}C and ^{31}P NMR spectra which correspond to phosphorylcholine free in solution and bound to antibody implies that the hapten exchanges between these two environments at a rate which is slow on the NMR time scale. If τ_B is the mean lifetime of the hapten–antibody complex and Δ_{AB} is the chemical shift difference between the two environments, the condition for such "slow" exchange is $\tau_B > (2^{1/2})(2\pi\Delta_{AB})^{-1}$ (Pople et al., 1959). Thus for a chemical-shift difference between free and bound hapten of 60 Hz, the condition for slow exchange is that the rate constant for dissociation of the hapten–antibody complex, $k_{off} = \tau_B^{-1}$, be less than 133 s^{-1}.

Though the hapten is in "slow" exchange between solution and antibody, such that two separate peaks are observed rather than a single composite peak, the situation is not one in which the mean lifetime of the hapten–antibody complex is so long that the observed spectrum is not to some degree influenced by the exchange process. Broadening of the line widths results from this exchange and the magnitude of this exchange broadening allows one to estimate τ_B. The spectra of both Figures 1 and 2 manifest some exchange broadening allowing lifetimes for both the phosphoryl and trimethylammonium ends of the bound hapten to be measured. Apparent inequalities between these two values may reflect possibilities for dissociation of one end of the hapten molecule from its binding pocket on the antibody to an essentially solution-like environment while the other end remains bound. Values of $k_{off} = \tau_B^{-1}$ estimated in this way can be used in conjunction with the independently determined equilibrium association constant of 10^5 M^{-1} (at 30 °C) to obtain values for k_{on}, the rate constant for hapten–antibody association. From analysis of the ^{31}P and ^{13}C spectra, k_{off} rates of 38 ± 15 (^{31}P) and 10 ± 4 s^{-1} (^{13}C) were determined. This allows one to calculate an approximate value for k_{on} of 1–4 × 10^6 M^{-1} s^{-1}. The observed rate constants fall within the range of values measured independently for a variety of hapten–antibody systems (Haselkorn et al., 1974; Pecht, 1974). In the work of Haselkorn et al. (1974) large differences in k_{on} were observed for haptens with only slightly differing structures, leading these authors to conclude that antibody–hapten association is more complex than a simple diffusion-controlled process. In fact, association rate constants more than three orders of magnitude lower than the theoretically limiting value of 1–2 × 10^9 M^{-1} s^{-1} (Froese and Sehon, 1965) have been observed for simple antibody–hapten associations (Pecht, 1974). These discrepancies between observed and diffusion-controlled rates have been attributed to effects ranging from steric and electrostatic repulsion (Day et al., 1963; Pecht et al., 1972) to a two-step encounter mechanism (Haselkorn et al., 1974).

The absence of splitting of the ^{13}C resonance by coupling to the ^{14}N nucleus ($J_{^{14}N-^{13}C}$ = 4 Hz) for the hapten bound to antibody requires that the dissociation rate for the antibody–hapten complex (A·H) be fast compared with the coupling constant, that is, $k_{off} > 4$ s^{-1}. Similarly, the absence of splitting of the ^{13}C resonance for the free hapten which is in equilibrium with bound hapten requires that the association rate of antibody (A) and hapten (H) be faster than the coupling constant, that is, $k_{on}[A] > 4$ s^{-1}. As $K_{assoc} = k_{on}/k_{off} = [A{\cdot}H]/[A][H]$, whence $k_{on}[A] = k_{off}([A{\cdot}H]/[H])$, equal concentrations of free hapten (H) and bound hapten (A·H) (as in Figure 2a) will lead to $k_{on}[A] = k_{off}$. Line width measurements show that $k_{off} \sim 10$ s^{-1} (which is indeed larger than $J_{^{14}N-^{13}C} = 4$ s^{-1}) which accounts for the absence of splitting in both the free and bound hapten signals in Figure 2a.

The value of k_{off} for the ^{31}P nucleus exceeds that of the ^{13}C nucleus by a factor of almost four. While this is not a large difference and is subject to some uncertainty, it may reflect real differences in the microscopic dissociation rates of different regions of the hapten, suggesting that the phosphoryl end may move between its binding pocket and a solution-like environment faster than does the quaternary ammonium end of the hapten. This view is consistent with the known x-ray structure of M603 (Segal et al., 1974) in which the trimethylammonium end of the bound hapten lies deep in the binding cleft while the phosphate end lies nearer to the surface. This could well allow dissociation of the hapten phosphoryl group without appreciable disruption of binding-site interactions to the trimethylammonium region. On the other hand, dissociation of the trimethylammonium region prior to phosphate group dissociation is rendered unlikely by the topology of the binding site.

In the limit of slow exchange, the observed line width depends on T_2 and on the mean lifetime, τ, of the species:

$$\pi\Delta\nu_{1/2} = \frac{1}{T_{2_{obsd}}} = \frac{1}{T_2} + \frac{1}{\tau} \tag{1}$$

For the Fab′–hapten complex, this may be expressed as (Hull et al., 1976)

$$(\pi\Delta\nu_{1/2})_{A\cdot H} = \frac{1}{T_{2_{A\cdot H}}} + \frac{1}{\tau_{A\cdot H}} = \frac{1}{T_{2_{A\cdot H}}} + k_{off} \tag{2}$$

and for the free hapten

$$(\pi\Delta\nu_{1/2})_H = \frac{1}{T_{2_H}} + \frac{1}{\tau_H} = \frac{1}{T_{2_H}} + k_{on}[A] \tag{3a}$$

$$= \frac{1}{T_{2_H}} + k_{off}\frac{[A\cdot H]}{[H]} \tag{3b}$$

These relationships allow one to estimate not only the rates for hapten–antibody association and dissociation but also T_2 for the bound and free hapten. For the ^{13}C nucleus of the trimethylammonium group at equal concentrations of free and bound hapten, the line widths are nearly equal, leading to the conclusion that T_2 for this nucleus is essentially the same in the bound and free form, quite probably because the major dipolar relaxation process depends on the free rotation of the methyl group about the N–C bond; this rotation is apparently not restricted when hapten is bound to antibody. In contrast, the line widths of the ^{31}P signals suggest different values of T_2 for the free (~0.1 s) and antibody-bound (~0.01–0.02 s) phosphate groups. The shorter value of T_2 for the bound form indicates that the phosphate region of the hapten is significantly less mobile when hapten is bound to antibody than when free in solution. It is interesting to compare these results with

the proton NMR studies of the binding of tetramethylammonium to rabbit antibodies carried out by Burgen et al. (1967). These authors observed a direct relationship between the free energy of complex formation and the energy barrier to methyl group rotation (manifested in an increased nuclear relaxation rate). In contrast to this example, the binding energy of phosphorylcholine to M603 does not depend on the rotational immobilization of the hapten methyl groups.

Conclusions

The concurrent use of ^{13}C and ^{31}P magnetic resonance allows one to probe the environments of the two ends of the phosphorylcholine molecule as it binds to a homogeneous, specific antibody. The evidence supports a picture of the hapten in the M603 binding pocket in which the trimethylammonium region is stabilized by ionic interactions with an anionic side chain(s) which neutralizes to some degree the positive charge of the ammonium nitrogen and causes the small upfield ^{13}C shift. Although the choline end is in tight van der Waals contact with the binding pocket, the methyl groups are free to rotate about the C–N bond. The phosphate oxygens of the hapten are partially protonated due to hydrogen bonds formed with amino acid side chains within the binding pocket and the phosphate group is significantly immobilized. This view of the bound complex is entirely consistent with the known x-ray structure.

Studies of the dynamics of exchange indicate that the phosphate end of the bound hapten may exchange more rapidly with a solution-like environment than does the trimethylammonium region.

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