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Molecular Studies of Subspecificity Differences among Phosphorylcholine-Binding Mouse Myeloma Antibodies Using ^{31}P Nuclear Magnetic Resonance†

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ABSTRACT: We have determined the pH dependencies of the binding affinities of the mouse myeloma immunoglobulins M603, W3207, and M167 for the haptens phosphorylcholine (PC) and L-α-glycerophosphorylcholine (GPC). These affinities are generally maximal near neutral pH with the exception of the binding of PC by M167 which is strongest at pH 5.5. These data have helped to clarify the nature and relative importance of the ionic interactions between hapten and antibody. ^{31}P nuclear magnetic resonance (NMR) techniques were used to probe the influence of pH on the microenvironment of the phosphate group of several haptens when these were bound to M603, W3207, and M167. The phosphate subsites of M603 and W3207 are both electropositive and also show other similarities; that of M167 has a net electronegative character. The two hydrogen bonds known to be formed between M603 and the phosphate oxygens of PC are also involved in binding GPC and are essentially unaffected by pH in the region 3–9. Studies with the hapten 3-trimethylamino-1-propanol phosphate (TMAPP) show that the binding cavity of M167 is substantially wider than those of M603 and W3207. These results lead to a detailed molecular model of the pH dependent binding of PC and related haptens to these three antibodies; they further indicate the roles of various amino acid residues in defining the differing ligand specificities of these antibodies.

A group of mouse myeloma immunoglobulins which specifically bind phosphorylcholine, PC[1] (Potter, 1972; Rudikoff et al., 1972; Potter & Lieberman, 1970), serves as a convenient system for studying structure–function relationships between similar, homogeneous antibodies. In this group, the primary structures of the heavy chains and of portions of the light chains from six different proteins are now known (Hood et al., 1975, 1976). The three-dimensional structure of the Fab′ fragment of one of these proteins, M603, as well as its complex with PC (Segal et al., 1974) has allowed direct visualization of the interactions between hapten and specific residues in the antigen combining site of the immunoglobulin which define the antibody specificity.

The high degree of sequence homology among the heavy chain residues of immunoglobulins specific for phosphorylcholine in both mouse and man (Padlan et al., 1976; Riesen et al., 1976) and the observation that most of the residues which interact with hapten in M603 are located in the heavy chain have allowed some rationalization of the molecular origins of the varying binding properties of these proteins (Padlan et al., 1976; Goetze & Richards, 1977a). These facts also suggest that a single general structure may define PC specificity in several immunoglobulins and this work is an attempt to understand how, or if, this structure can serve as an example of the ability of the immune response to create a binding site optimally complimentary to a class of antigenic determinants. We have, therefore, examined the detailed molecular interactions responsible for the high affinity of these proteins for PC and related ligands and how these affinities are modulated by changes in a small number of crucial amino acids within this group of immunoglobulins. Such information may yield insight into the question of how immunoglobulins with subtly differing binding specificities can be created with a minimum number of changes in amino acids (mutations) and, thereby, help us to understand the origins of the broad diversity and exquisite specificity of the immune response.

In this work we have used ^{31}P NMR to probe the molecular details of the environment of the phosphate group of several haptens when bound to three of these myeloma proteins (M603, W3207, and M167). Several factors proved fortuitous for these experiments: (i) the phosphate binding sites in these proteins appear (by analogy to M603) to be formed exclusively by residues from the heavy chains whose complete sequences are known; (ii) the various known subspecificities of these proteins (Leon & Young, 1971) most likely result from differences in the phosphate binding subsites; (iii) ^{31}P NMR is an especially useful technique for not only can one use ^{31}P chemical shift information to probe the microenvironment of

† Contribution No. 5655 from the Church Laboratory of Chemical Biology, Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125. *Received August 30, 1977.* This work was supported by the President's Fund of the California Institute of Technology and the National Institutes of Health (Grant GM-16424).

[1] Abbreviations used: NMR, nuclear magnetic resonance; PC, phosphorylcholine; GPC, L-α-glycerophosphorylcholine; NPPC, *p*-nitrophenylphosphorylcholine; TMAPP, 3-trimethylamino-1-propanol phosphate.

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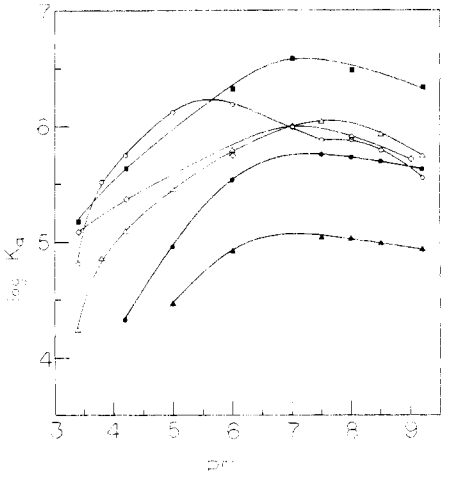


FIGURE 1: The pH dependence of the binding constants of M603, W3207, and M167 for PC and GPC as measured by equilibrium dialysis at 5 °C. The curves represent M603-PC (●), M603-GPC (▲), W3207-PC (■), W3207-GPC (◇), M167-PC (○), and M167-GPC (△). Each data point represents the result of a Scatchard plot of 10 points (PC) or an inhibition curve of a similar number of points (GPC). The binding constants were estimated to be accurate to ±10% for PC and ±15% for GPC. All experiments were performed in borate–cacodylate-buffered saline except those of pH 8 which were done in borate-buffered saline. The PC affinity of M603 at pH 8 is 5.4×10^5 M^{-1} or 34% lower than the value previously reported (Goetze & Richards, 1977b). This previous value had been obtained using protein from the M603 tumor line obtained from a different source than that used in the current experiments.

the phosphorus but one can also study such chemical shift behavior as a function of pH and thereby learn about the electrostatic nature of groups in the phosphorus microenvironment by the way they perturb the pK of the phosphate group of bound haptens. M603 was chosen because its three-dimensional structure is known (Segal et al., 1974); W3207 has the same light chain type as M603, exhibits a similar pattern of binding specificity, and has the highest known affinity for PC of the mouse myeloma proteins (Goetze & Richards, 1977a); M167 has a different ligand specificity which is characterized by an unusually high affinity for choline.

Recent studies have shown that a large portion of the binding energy for the interaction between haptens and PC-specific antibodies arises from ionic attractions between groups of opposite charge (Grossberg et al., 1974; Krausz et al., 1976). However, little information is available on the pH dependence of the binding affinities to allow one to assess the relative importance of the various charged residues of the protein to the total binding interaction. Though some data on T15 are available, notably the observation that the PC affinity of T15 drops sharply below pH 5 (Pollet & Edelhoch, 1973), one has not been able to ascribe this effect unambiguously to protonation of the phosphate group of the hapten or to protonation of carboxylate groups on the protein which are known to interact strongly with bound hapten (Grossberg et al., 1974). In order to resolve such ambiguities we have studied the pH dependence of the binding affinities of these proteins both for PC and for L-α-glycerophosphorylcholine (GPC).

Materials and Methods

Haptens. Phosphorylcholine (PC) and L-α-glycerophosphorylcholine (GPC) were purchased from Sigma and phosphoryl[*methyl*-^{14}C]choline from New England Nuclear. *p*-Nitrophenylphosphorylcholine (NPPC) was synthesized as previously described (Chesebro & Metzger, 1972). 3-Trimethylamino-1-propanol phosphate (TMAPP) was synthesized in complete analogy to PC (Chesebro & Metzger, 1972; Baer, 1952) except that 3-dimethylamino-1-propanol (Aldrich) was substituted for dimethylaminoethanol.

Protein Purification. Ascites fluid was mildly reduced, alkylated, and filtered through a Sepharose-PC column (Chesebro & Metzger, 1972). PC-specific protein was immunospecifically eluted by washing the column with 1 mM PC in borate-buffered saline (pH 8).

Hapten Binding Affinities. Affinities for PC at varying pHs were determined by equilibrium dialysis as previously described (Goetze & Richards, 1977b). Experiments were carried out in borate–cacodylate-buffered saline (0.02 M borate, 0.02 M cacodylate, 0.13 M NaCl, 1 mM EDTA, 0.02% NaN_3) and care was taken to preequilibrate all samples and stock solutions to the desired pH. All binding affinities were measured at 5 ± 1 °C.

Affinities for all other haptens were determined indirectly by inhibition of labeled PC binding (Karush, 1956; Michaelides & Eisen, 1974).

NMR Experiments. A Varian XL-100-15 spectrometer interfaced with a Varian 620i computer was used. Spectra were obtained at 40.5 MHz at a probe temperature of 25 ± 1 °C and were proton-noise decoupled. A capillary insert containing D_2O provided the field-frequency lock. A 90° pulse with an acquisition time of 0.4 s and a sweep width of 1000 Hz were used to accumulate the data.

NMR Titration. NMR samples consisted of immunospecifically purified Fab′ fragments (Inbar et al., 1971) which had been extensively dialyzed to remove bound hapten. Protein solutions were concentrated to 3–5 mM by ultrafiltration in an Amicon apparatus and the desired hapten concentration was achieved by the addition of a small volume of a concentrated stock solution.

Titration experiments were started at the high pH extreme by having previously dialyzed the protein sample against borate–cacodylate buffered saline of the appropriate pH. Samples were titrated toward low pH by the addition of 5 N HCl. With care, it was possible to avoid protein precipitation above pH 3. The pH of each sample was measured immediately after each NMR spectrum was obtained.

Amino Acid Numbering. The numbering system of heavy chain amino acids is that for M603 (Rudikoff & Potter, 1974).

Results

pH Dependence of Binding Constants for PC and GPC. Figure 1 shows the binding affinities of M603, W3207, and M167 for the haptens PC and GPC at 5 °C. Affinities were measured over the pH range 3.4 to 9.2 except in the case of M603 where, at pH <4, the affinities were too low to allow accurate measurements.

The curves for M603 and W3207 are qualitatively similar. Affinities for both PC and GPC reach a maximum at pH 7.0–7.5. Raising or lowering the pH from neutrality causes the affinities to decrease appreciably but the amount of this decrease as the pH is lowered is significantly different for M603 and for W3207. For example, the affinity for PC of M603 drops 47-fold from pH 7.3 to pH 4 whereas that of W3207 drops 12-fold. Both proteins bind PC with a higher affinity than GPC over the entire pH range studied, although these affinities converge near pH 3.

The pH dependence of GPC binding to M167 is similar to that for GPC binding to M603 and W3207. In contrast, the pH maximum for PC binding to M167 is lowered to pH ~5.5

TABLE I: Subspecificities of Three PC-Binding Myeloma Proteins.[a]

Hapten	M603	W3207	M167
GPC	5.0	3.9	0.8[b]
NPPC	6.8	5.6	0.7
TMAPP	13	18	2

[a] Represented as K_a(PC)/K_a (hapten). Binding experiments were performed by equilibrium dialysis in borate-buffered saline at 5 °C. [b] From Leon & Young (1971).

and one observes two distinct ionizations as the pH is raised above 5.5. Because of this, the affinity of M167 is actually greater for GPC than for PC in the region pH >7.

Table I lists the affinities at pH 8 of these three proteins for the haptens GPC, NPPC, and TMAPP relative to their affinities for PC.

NMR Titrations. At neutral pH, the ^{31}P signal of PC (Goetze & Richards, 1977a,b), and also the other haptens, obeys the condition of slow exchange on the NMR time scale, $k_{off} < 2\pi\Delta_{AB}/\sqrt{2}$ (Pople et al., 1959; where k_{off} is the dissociation rate constant and Δ_{AB} is the chemical shift difference between free and bound hapten), so that one observes separate peaks for the hapten free in solution and bound to antibody.

However, binding affinities and, hence, the dissociation rate constant vary with pH as do the differences in chemical shift of the various haptens (Δ_{AB}) when they are free in solution as compared with when they are bound to the antibody. Accordingly, the NMR spectra vary from the slow exchange limit as a function of pH. In order to minimize interference from free hapten signal under conditions of slow exchange, especially in regions where the positions of the signal for bound and free hapten were very close, and also to maximize the contribution to the observed chemical shift from the bound hapten under conditions of fast exchange, the NMR experiments were generally carried out under conditions where antibody was in excess; in this way almost all hapten present was bound to antibody. The line width of the observed peak for bound hapten ($\Delta\nu_{1/2}$ = 20–30 Hz) generally remained constant as the pH was lowered to pH ~4 indicating that conditions of fast exchange had not yet been reached. With M603, which has a lower affinity for hapten (and therefore a higher k_{off}), experiments involving hapten binding approach the fast exchange limit at slightly higher pH values.

These observations were confirmed by carrying out some titration experiments under conditions of excess hapten. When exchange was slow, separate signals were observed for free and bound hapten; the position of the signal for bound hapten was identical with that previously observed when antibody was in excess.

The binding affinities for haptens decrease as the pH is raised above neutrality; this decrease is, however, sufficiently small so that all spectra continued to reflect slow exchange in this region.

Below pH ~4, the observed signal often narrowed appreciably indicating fast exchange. The NMR titrations were carried out at 25 °C and the binding affinities are not known accurately at this temperature. As a result, accurate determination of the chemical shift for the bound hapten was not possible. However, by assuming that the affinities at 25 °C are about 25% those at 5 °C (Goetze & Richards, 1977a), one could establish an approximate position for the chemical shift of bound hapten, although these data (below pH 4) are considerably less accurate than those obtained under conditions of slow exchange.

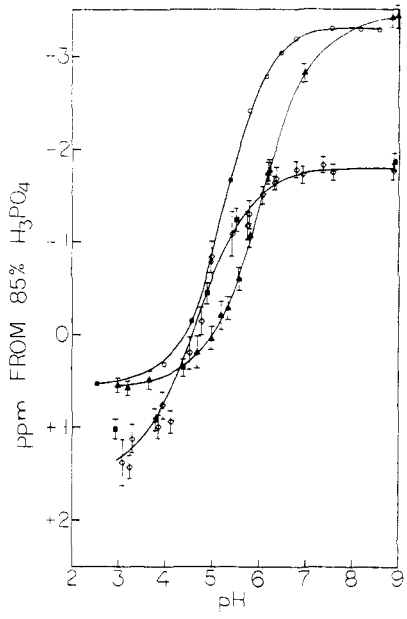


FIGURE 2: ^{31}P NMR titration curves of PC free in solution (○) and bound to M603 (■), W3207 (◇), and M167 (▲). Upfield shifts correspond to an increase in offset (ppm). Spectra of the bound hapten were obtained under conditions of protein excess. Offsets of the free hapten were measured in separate experiments using protein-free samples. Errors were estimated from the quality of the spectra. Further details are given in the text.

Figure 2 illustrates the titration behavior of the ^{31}P resonance of PC both free and bound to M603 and W3207. Upon addition of a single proton to the free, dianionic hapten, the ^{31}P signal moves upfield 3.9 ppm and titrates with a pK_2 of 5.3 ± 0.1. Titration of the hapten bound either to M603 or W3207 occurs at a decreased pK of 4.7 ± 0.2. Within experimental error, PC bound either to M603 or W3207 experiences the same chemical shift over the range pH 4–9, although a small difference may exist at the lower pH limit. The total shift caused by protonation of PC bound to W3207 is ~ 3.5 ppm which is about 0.4 ppm less than for protonation of the free hapten. At pH >7.5 the position for the signal of PC bound to W3207 is shifted upfield by 1.5 ppm relative to the signal for the free hapten; at pH <2 the analogous difference extrapolates to 1.0–1.3 ppm upfield.

Figure 2 also shows the titration results for PC bound to M167. In this case, the bound PC titrates with an *increased* pK of 6.0 ± 0.1 and shows only minimal changes in chemical shift on binding at the two pH extremes.

Figure 3A illustrates the pH dependence of the ^{31}P resonance of the hapten GPC bound to M603 or W3207. At pH >6, the signal for GPC bound to W3207 occurs 1.5 ppm upfield of the position of the free hapten which, itself, does not titrate in the region pH 2–9 (pK_a < 1.0). Below pH 6, the position of the bound peak shifts gradually downfield. The pH behavior of GPC when bound to M603 is similar to that when bound to W3207 but the chemical shift for the hapten bound to M603 is systematically shifted 0.3 ppm further downfield over the pH range studied.

Figure 3 also illustrates the pH behaviour of GPC and NPPC when bound to M167. In the region pH >7, these haptens experience a *downfield* shift of 0.7 ppm when they bind to antibody. As the pH is decreased, the resonance of the bound haptens moves gradually upfield toward the position of the free hapten. For either GPC or NPPC, the difference in chemical shift for the hapten when free in solution and when

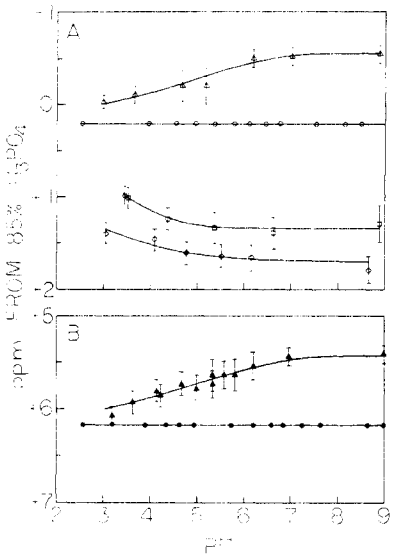


FIGURE 3: pH dependence of the ^{31}P resonance of the diester haptens GPC and NPPC. (A) pH dependence of GPC free in solution (○) and bound to M603 (□), W3207 (◇), M167 (△). (B) pH dependence of NPPC free in solution (●) and bound to M167 (▲).

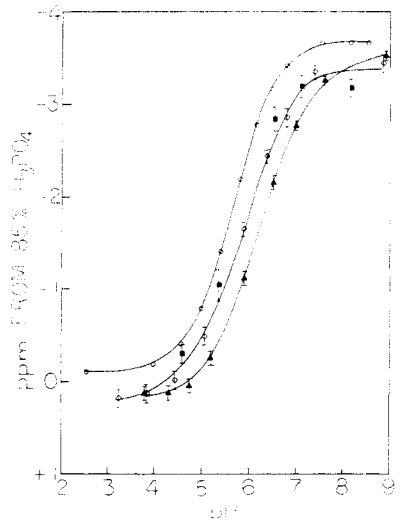


FIGURE 4: ^{31}P NMR titration curves of TMAPP free in solution (○) and bound to M603 (■), W3207 (◇), and M167 (▲). For clarity, the data for TMAPP bound to M603 and W3207 have been fitted to a single line, although small differences may exist between them.

TABLE II: pKs of Free and Protein-Bound Haptens.

Hapten	pK	pK when bound to W3207	ΔpK	pK when bound to M167	ΔpK
PC	5.3 ± 0.1	4.7 ± 0.2	−0.6	6.0 ± 0.1	+0.7
TMAPP	5.6 ± 0.1	5.9 ± 0.1	+0.3	6.3 ± 0.1	+0.7

bound to M167 is identical at any given pH over the pH range observed.

The ^{31}P signal of TMAPP titrates with a pK of 5.6 ± 0.1 and a total chemical shift of 3.6 ppm upfield on addition of a single proton. Figure 4 shows that binding TMAPP to M603 or to W3207 causes only a slight perturbation in the magnetic environment of the ^{31}P nucleus of this hapten. Both the pKs of the bound and free hapten (summarized in Table II) and the chemical shift behavior of the bound and free hapten with changing pH are very similar.

Figure 4 also shows the pH dependence of TMAPP bound to M167. In this case, the pK of the bound hapten is raised to 6.3 ± 0.1 and the changes in chemical shift on binding virtually parallel the behavior of PC bound to M167.

Discussion

pH Dependence of Binding Constants. The data in Figure 1 illustrate that, though individual differences in the affinity of M603, W3207, and M167 for PC and GPC do exist, there are strong similarities between M603 and W3207. Accordingly, we shall first examine possible origins of the similar behavior of M603 and W3207 and then discuss explanations for the affinities of M167.

At pH 7, the phosphate group of PC exists as a dianion and acquires a proton with a pK of 5.3 ± 0.1. In contrast the phosphate region of GPC remains a monoanion. Thus the larger affinity of W3207 for PC than for GPC most likely arises from an additional favorable ionic interaction between the protein and the dianionic phosphate of PC; this advantage is lost after protonation of PC below pH 4.7 where the affinities for the two haptens converge. One can even explain the differences from this source on a semiquantitative basis. The observed ratio of the affinity for PC to GPC at pH 7.3 is 3.9. If one assumes that this represents the maximum stabilization due to interaction of the protein with a dianionic as contrasted to a monoanionic phosphate, then the ratio of the affinity for PC relative to that for GPC at any pH correlates with the degree of ionization of PC bound to W3207 as monitored by ^{31}P NMR (Figure 2). A similar situation is observed in the case of M603.

Since GPC does not itself change its state of ionization between pH 2 and 10, the dramatic decrease in the *absolute* affinity of W3207 and M603 for GPC as the pH is lowered from pH 7.3 to 3.4 must reflect ionization changes in the protein, most likely residues with carboxylic acid groups such as Glu-35H and Glu-59H which interact with the positively charged quaternary nitrogen of the hapten (Padlan et al., 1976; Segal et al., 1974). The crucial importance of such an interaction has been verified by chemical modification experiments on a related protein, H8 (Grossberg et al., 1974), and by the observed inability of any compound lacking the quaternary nitrogen to bind effectively to these proteins (Leon & Young, 1971; Krausz et al., 1976). The decrease in absolute affinity for PC as the pH is lowered reflects both protonation of the protein carboxylates and monoprotonation of the phosphate group of the hapten. Quantitatively, protonation of the phosphate group of PC accounts for ~35% of the decrease in affinity of W3207 observed on lowering the pH from 7.3 to 3.0.

We observe a decrease in the affinity for PC and GPC on raising the pH above 7.3 in contrast to the results of a previous study on binding by T15 using fluorescence techniques (Pollet & Edelhoch, 1973). For all three proteins we have studied (W3207, M167, M603), the ratio of the affinity for PC relative to that for GPC remains constant in this pH region indicating that both haptens are equally affected. We shall subsequently discuss the hydrogen bonds between antibody and hapten; as the NMR evidence suggests that they are not altered in this pH range, we cannot ascribe this decrease in affinity to ionization of Arg-52H which is the cationic residue in closest proximity to bound PC.

Padlan et al. (1976) have suggested that the presence of Asp-100aH in M167 and M511 may account for the unique binding specificities of these two proteins. This residue lies between the positive nitrogen and negative phosphate of the hapten and may interact with both. Such an interaction would

enhance binding of choline but simultaneously depress binding of PC and such effects have been observed (Goetze & Richards, 1977a; Leon & Young, 1971). The presence of Asp-100aH can also explain our binding data for M167 (Figure 1). The increase in PC affinity from pH 3.4 to 5.5 is due to ionization of Glu-35H, Glu-59H, and Asp-100aH; all three of these residues, when ionized, help to stabilize the positive charge on the quaternary nitrogen of the hapten. The 2.3-fold drop in affinity from pH 5.5 to 7.5 results from the ionization of the phosphate of PC which occurs with a pK of 6.0 when this hapten is bound to M167 (Figure 2). The additional negative charge on the phosphate is repelled by the negative charge on Asp-100aH and the affinity for PC is accordingly reduced. The further decrease in affinity at pH >8 is probably caused by the ionization of another, presently unknown residue(s). The results for binding of GPC to M167, though qualitatively similar to those observed for binding to M603 and W3207, also agree with this model. Since GPC does not itself ionize in the pH range in question, any repulsion between the monoanionic phosphate and Asp-100aH should depend only on the state of ionization of Asp-100aH. This effect is therefore included in the ascending line of the curve from pH 3.4 to 7.3 and is not observed as a separate titration (in contrast to the case when PC is bound).

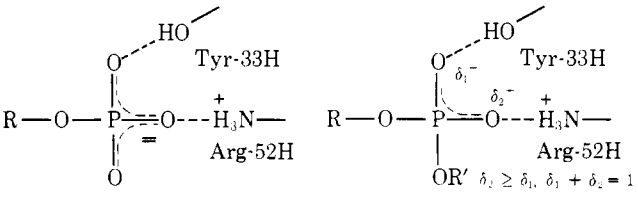
M167 shows another difference from M603 and W3207 in that, with M167, GPC and monoprotonated PC do not bind with equal affinity. At pH 3.4, where bound PC is essentially monoprotonated as determined by ^{31}P NMR (Figure 2), the affinity of M167 for PC is 3.8 times greater than that for GPC. Accordingly, some unfavorable steric interactions may exist between residues in the binding pocket of M167 and the glycerol group of GPC.

NMR Titrations of Haptens Bound to M603 and W3207. At pH ≥7.5, the ^{31}P resonance of PC bound to M603 or to W3207 occurs 1.5 ppm upfield of that of the free hapten (Figure 2). We have attributed this shift to the formation of two specific hydrogen bonds from Tyr-33H and Arg-52H of the antibody binding pocket to the phosphate oxygens of bound hapten (Goetze & Richards, 1977b). This chemical shift change experienced by hapten on binding remains constant in the region pH 7.5–9 indicating that Arg-52H does not ionize in this range and therefore cannot be the cause of the observed decrease in binding affinity at the high pH limit. The similarity in the titration curve for PC bound to M603 or to W3207 suggests that the specific interactions between the phosphate region of the hapten and the phosphate-binding subsite of W3207 are essentially identical with those observed in the M603–PC complex for which the three-dimensional structure is known (Segal et al., 1974).

The pK of PC is lowered from 5.3 in solution to 4.7 when bound to M603 and W3207. This decrease results from the anionic charge-stabilizing influence of the positive residues which are located in the phosphate subsite: Arg-52H which forms a hydrogen bond with bound hapten and, to a lesser degree, the more distant Lys-54H.

The similarity in chemical shift change upon binding at pH ≥7.5 observed for GPC (Figure 3A) and PC (Figure 2) suggests that the hydrogen bonds which exist between antibody and bound PC also exist between antibody and bound GPC. Because of the topography of the binding site, this implies that the choline portions of both haptens likewise bind in an identical manner and, furthermore, that, for bound GPC, the phosphate oxygen esterified to the glycerol residue corresponds to the oxygen atom which is seen to be uncoordinated to any protein residue in the crystal structure of the M603–PC complex (Segal et al., 1974).

CHART I



The fact that GPC and monoanionic PC bind with equal affinity to both M603 and W3207 suggests that, at low pH, both these haptens are involved in identical hydrogen bond interactions. The postulated charge distribution of the phosphate group of these two haptens in the binding pocket of M603 and W3207 is schematically illustrated in Chart I.

As the pH is lowered below 6, the ^{31}P resonance of bound GPC shifts slightly downfield. This shift is not caused by titration of groups directly involved in hydrogen bonding to bound hapten but may reflect a small conformational rearrangement within the binding pocket. Any such change, even if very small, could influence the ^{31}P chemical shift which is extremely sensitive to changes in the OPO angle (Blackburn et al., 1971; Gorenstein & Kar, 1975).

NMR Titrations of Haptens Bound to M167. Figure 2 shows that PC when bound to M167 has its second pK_a *raised* to 6.0 from 5.3 when in solution. This surprising result suggests that the phosphate subsite of M167 has a net negative character which might be caused by the presence of Asp-100aH, the only anionic group in close proximity to the phosphate (Padlan et al., 1976). In analogy to the known structure of M603, one would expect that Arg-52H and, to a lesser degree, Lys-54H and perhaps Arg-58aH might all be positioned close enough to the phosphate group of the hapten to perturb its ionization. The fact that the presence of a single anionic residue, Asp-100aH, can apparently outweigh the possible influence of several cationic groups on the phosphate ionization, leads us to suggest that the architecture of the phosphate-binding subsite of M167 is appreciably different from that of M603 and W3207. Indeed, M167 has several potentially important substitutions in this region relative to M603 (for example, Asp-56H→His, Thr-58aH→Arg, Trp-104aH→Gly) and these could so alter the structure of the phosphate subsite that Arg-52H, Lys-54H, and Arg-58aH are moved sufficiently far from the bound phosphate that the hydrogen bonding and electrostatic interactions characteristic of the M603–PC complex are not possible with M167. However, in spite of the negative character of the phosphate subsite of M167, a *net* favorable thermodynamic interaction between protein and the phosphate group of the hapten must exist because, at pH 8, M167 exhibits an affinity for PC that is ten times that for choline (Leon & Young, 1971). Thus the situation may be one in which the favorable energetic consequences of a hydrogen bond between Tyr-33H and hapten outweigh the repulsive interactions between the negatively charged phosphate and a negative binding subsite which has been created by the intrusion of Asp-100aH into, and the ejection of Arg-52H from, the immediate phosphate subsite.

In spite of the large difference in the absolute chemical shift between the signals of the free haptens, GPC and NPPC, both haptens experience changes in chemical shifts on binding which are identical at any given pH over the entire pH range studied (Figure 3). This implies that the binding site of M167 perturbs the phosphorus environment of both haptens to a very similar

degree and that the phosphate group of both haptens has essentially identical interactions with binding site residues. The antibody interaction with the PC portion of the haptens is thus not significantly affected by such diverse substituents attached to the phosphate as glycerol or *p*-nitrophenyl. Moreover, as shown in Table I, each of the three proteins exhibits similar affinities for both GPC and NPPC which suggests that there is little, if any, *specific* interaction between the antibodies and the glycerol or *p*-nitrophenyl substituents.

However, with M167, the phosphate environment of PC differs appreciably from that for the diester haptens (GPC and NPPC) since these two classes of haptens experience significantly different changes in the chemical shift of their ^{31}P resonances on binding. Possibly Asp-100aH affects the phosphate group of these two groups of haptens differently, or there may be a *nonspecific* steric crowding of the glycerol and *p*-nitrophenyl substituents of GPC and NPPC. The present evidence does not discriminate between these, or other possibilities. However, affinity labeling experiments using the hapten, *p*-diazoniumphenylphosphorylcholine (Chesebro et al., 1973) have shown extensive differences in the labeling pattern between many of the PC-binding myeloma proteins; these differences suggest that the outer edges of the binding pocket, with which the diazonium group presumably reacts, have appreciably different structures. Thus, whereas M603 and W3207 can freely accommodate the bulky glycerol or *p*-nitrophenyl substituents, binding to M167 may introduce some nonspecific steric repulsion. With decreasing pH, there is a decrease in the downfield shift of bound GPC and NPPC; this suggests that there may be reduction in this nonspecific repulsion with decreasing pH.

Binding of TMAPP. The requirements on the binding sites of M603, W3207, and M167 for the optimal relationship between the phosphate and trimethylammonium groups of the hapten were probed by studying the binding of TMAPP in which these groups are separated by three methylene groups rather than two as in PC itself. Table I shows that TMAPP binds to M167 with an affinity nearly equal to that of PC but binds much less well to M603 and W3207.

Figure 4 shows that the ^{31}P chemical shifts of TMAPP bound to M603 and W3207 vary only slightly from the chemical shifts of the free hapten. Moreover, the titration behavior of bound TMAPP is completely different from that of bound PC. For example, the p*K* of TMAPP bound to W3207 is slightly higher than that of the free hapten whereas the p*K* of PC bound to W3207 is lower by 0.6 p*K* unit. Thus the ionic influence of W3207 is quite different on the phosphate of bound TMAPP than it is on the phosphate of bound PC. Moreover, the 1.5 ppm upfield shift attributed to hydrogen bonding between protein and bound PC and GPC is not observed with bound TMAPP. All these observations suggest that the phosphate group of TMAPP is not able to interact correctly with the residues of the phosphate subsite in W3207 and M603. The structure of the M603–PC complex shows extensive van der Waals contacts of the methyl and methylene groups of the hapten with Tyr-33H, Trp-104aH, and residue 96 of the light chain (Padlan et al., 1976; Segal et al., 1974). These residues define the width of the binding cavity and discourage accommodation of much structural variation in the complementary region of the hapten. PC has been shown to bind to M603 with the trimethylammonium portion of the hapten projecting into the binding cavity. Therefore, TMAPP probably also binds to M603 and W3207 in such a fashion as to preserve the important interactions between the quaternary nitrogen and acidic, anionic groups on the antibody. Such an orientation of a hapten with three methylene groups forces the phosphate out of the binding pocket past the phosphate-binding subsite. This destroys the energetically favorable interactions of the phosphate group with antibody residues and probably accounts for the 13–18-fold drop in affinity for TMAPP relative to PC.

In contrast to the difference between PC and TMAPP interacting with M603 and W3207, there are many parallels between PC and TMAPP binding to M167. Thus, the ^{31}P NMR titration curve of TMAPP bound to M167 (Figure 4) bears virtually the same relationship to that of free TMAPP as the titration curve of PC bound to M167 does to that of free PC (Figure 2). In both cases the p*K* change on binding is + 0.7 pH unit and the lack of an appreciable change in chemical shift on binding at both high and low pH limits observed for TMAPP is very similar to the behavior of PC. These parallels suggest that, in contrast to M603 and W3207, the binding pocket of M167 is able to accommodate TMAPP so that this larger hapten is able to bind with its phosphate group located in an essentially normal position within the phosphate subsite of the protein (as is the case also for PC and GPC). This accommodation may be allowed by the substitution Trp-104aH→Gly which creates a wider binding cavity thereby making it possible for the extra methylene group of TMAPP to "buckle out" into this extra space. In this way the net N–P distance in bound TMAPP can remain close to that for bound PC. Such a distortion has the advantage of retaining the favorable interactions of both the phosphate and trimethylammonium groups of the hapten with the complementary antibody subsites so that the net affinity for TMAPP is only lower by a factor of two than that for PC.

Conclusion

These results show that these three proteins which, because of similar thermodynamic affinities may at first glance seem to have essentially similar interactions with PC, can be seen, in fact, to interact in importantly different ways with hapten when the molecular details of these interactions are probed. Does the presence of these various immunoglobulins with PC specificity in the BALB/c mouse increase the Darwinian fitness of the host or do these antibodies represent a case of immunological overkill? Though studies such as the present ones cannot provide an unambiguous answer to such a general question, the findings of this work, together with the already known differing specificities of these proteins for bacterial antigens (Potter & Leon, 1968; Potter, 1972), support the argument that these antibodies, because of their subtly differing interactions with PC type antigens, may more successfully protect their host against a wider range of antigenic challenges than would be possible with a more limited diversity of anti-PC immunoglobulins.

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Complete Covalent Structure of Human β-Thromboglobulin†

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ABSTRACT: The complete primary structure of the platelet-specific protein human β-thromboglobulin has been determined. β-Thromboglobulin consists of identical subunits of 81 amino acids, each with a molecular weight of 8851. The amino acid sequence of the β-thromboglobulin subunit is: Gly-Lys-Glu-Glu-Ser-Leu-Asp-Ser-Asp-Leu-Tyr-Ala-Glu-Leu-Arg-Cys-Met-Cys-Ile-Lys-Thr-Thr-Ser-Gly-Ile-His-Pro-Lys-Asn-Ile-Gln-Ser-Leu-Glu-Val-Ile-Gly-Lys-Gly-Thr-His-Cys-Asn-Gln-Val-Glu-Val-Ile-Ala-Thr-Leu-Lys-Asp-Gly-Arg-Lys-Ile-Cys-Leu-Asp-Pro-Asp-Ala-Pro-Arg-Ile-Lys-Lys-Ile-Val-Gln-Lys-Lys-Leu-Ala-Gly-Asp-Glu-Ser-Ala-Asp. Disulfide bridges link half-cystine-16 to half-cystine-42, and half-cystine-18 to half-cystine-58. The amino acid sequence of β-thromboglobulin shows a marked homology with that of platelet factor 4. When the sequences are aligned for maximum homology, 42 of the 81 residues of β-thromboglobulin are identical with those of platelet factor 4, including the position of the four half-cystines.

The compound β-thromboglobulin (βTG)[1] is a small protein with a molecular weight of approximately 36 000 which is a significant component of the protein secretion from platelets in the release reaction induced by thrombin and other agents (Moore et al., 1975). Many of the contents of platelets are thought not to be of megakaryocytic origin, but to be absorbed from the plasma by the circulating platelet (Adelson et al., 1961). However, there is strong evidence that some clearly defined proteins including βTG (Moore et al., 1975), the anti-heparin activity known as platelet factor 4 (PF4) (Broekman et al., 1975; Niewiarowski et al., 1976), and possibly the platelet growth factor (Ross et al., 1974) are specific for platelets. Both PF4 (Walsh, 1976) and platelet growth factor (Ross & Glomset, 1976) may play a role as important mediators of the role of platelets in the thrombotic process. Radioimmunoassays which may provide methods for measurement of platelet activation both in vivo and in vitro have required detailed characterization of the platelet specific proteins. The complete amino acid sequence of human PF4 has been determined (Deuel et al., 1977; Hermodson et al., 1977; Morgan et al., 1978a). This paper describes the complete amino acid sequence and disulfide bond arrangement of βTG which exhibits a striking and unexpected homology with PF4.

Experimental Procedure

β-Thromboglobulin was prepared from fresh washed platelets as described previously (Moore & Pepper, 1977).

Trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone was from Worthington, carboxypeptidase Y from Pierce Chemical Co., and staphylococcal protease from Miles Laboratories. Column chromatography, *S*-^{14}C-labeled carboxymethylation and citraconylation of proteins, enzymatic digestions, and amino acid analyses were performed according to standard procedures which have been described in detail elsewhere (Morgan et al., 1978a). Performic acid oxidation was performed according to Hirs (1967).

Estimation of sulfhydryl groups was performed with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959). For production

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[1] Abbreviations used: βTG, β-thromboglobulin; PF4, platelet factor 4; Theed, *N,N,N′,N′*-tetrakis(2-hydroxyethyl)ethylenediamine; Quadrol (Wyandotte Chemicals Corp.), *N,N,N′,N′*-tetrakis(2-hydroxypropyl)-ethylenediamine; SCM, *S*-carboxymethyl; Pth, phenylthiohydantoin.

0006-2960/78/0417-1739$01.00/0