

THE CIRCULAR DICHROISM OF PHOSPHOCHOLINE-SPECIFIC MOUSE HYBRIDOMA AND MYELOMA PROTEINS: UNUSUAL PROPERTIES OF THE HYBRIDOMA PROTEIN 101.6G6

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Abstract—The circular dichroism (CD) spectra of five myeloma and six hybridoma proteins specific for phosphocholine were measured in the 250–310-nm range. The effect on the CD spectra of adding phosphocholine was also examined. The five myeloma proteins all had distinctive native spectra and, except for M603 and W3207, unique changes occurred on ligand binding. The hybridomas were chosen as pairs from each of the three known families of phosphocholine-specific immunoglobulins. Those from the T15 or M603 families resembled the appropriate prototype. However, the proteins from the M167 family were all distinctively different in their CD properties. In particular, the hybridoma protein 101.6G6 showed large CD changes on hapten binding and values for the association constant for phosphocholine of $1.1 \times 10^5 M^{-1}$ and of $5.8 \times 10^2 M^{-1}$ for acetylcholine were obtained by CD spectrophotometric titration. The CD properties of the proteins are interpreted in the light of the sequence data so far available, including the possible role of the D-segment.

INTRODUCTION

The processes by which the repertoire of antibody variable regions is generated are now known in outline (Tonogawa, 1983). However variation in sequence may not automatically mean variation in function, i.e. specificity for antigen (Cohn *et al.*, 1980; Kabat, 1981, 1982) and more detailed knowledge of the relationship of sequence to specificity is required. In particular, the role of the D-segment is not entirely clear. This gene segment and its junctions with the V_H - and J_H -segments form the third complementarity-determining region of the heavy chain (CDR3). The possibility exists that it may be a structural element rather than being directly involved in antigen binding (Potter, 1983).

Extensive sequence data [collected in Kabat *et al.* (1983)] and the X-ray crystallographic analysis of M603 Fab (Padlan *et al.*, 1976) make the phosphocholine-specific immunoglobulins very suitable for studies of structure and function. The original group of mouse myeloma proteins were assigned on the basis of their hapten-binding properties to three families, typified by T15, M603 and M167 respectively (Leon and Young, 1971). Subsequently, various hybridoma proteins specific for phosphocholine were assigned to the same families on the basis of idiotype analyses (Claffin *et al.*, 1981), hapten profiles (Andres *et al.*, 1981) and protein sequence analyses (Gearhart *et al.*, 1981). The hapten-binding studies indicated that the proteins of the T15 family are relatively constant in their functional properties, as are those of the M603 family except for some variation in binding of an antigen

from *Proteus morgani*. In contrast the proteins of the M167 family are quite diverse in their hapten-binding specificities. It may be possible to correlate the variations seen in the properties of proteins of the M167 family with differences in their amino-acid sequences.

Circular dichroism (CD) can provide additional information on both structural and functional aspects of antibodies. Earlier work on the phosphocholine-binding myeloma proteins (Morris *et al.*, 1974) showed that the Fv regions contribute strongly to the near-u.v. CD. Spectra in this region, 250–310 nm, arise chiefly from tyrosine and tryptophan chromophores. The structural differences among the three prototypic myeloma proteins gave rise to considerably different CD spectra in this region. In addition, functional differences were evident since addition of the hapten phosphocholine caused different changes in the spectra of the three proteins. In this paper, we report further CD studies of phosphocholine-specific myeloma and hybridoma proteins, particularly the unique hybridoma protein, 101.6G6. This protein, though assigned to the M167 family (Claffin *et al.*, 1981; Andres *et al.*, 1981) has an H-chain derived from the VII member (Clarke *et al.*, 1982) of the set of four T15 genes (Crews *et al.*, 1981), while the other proteins are all derived from the VI gene. It also has a different D-segment (Clarke *et al.*, 1983).

MATERIALS AND METHODS

Ascites fluids containing the M511, M603 and T15 proteins were given by Dr M. Potter, W3207 ascites fluid was given by Dr L. Hood and J558 ascites fluid

by Dr B. Clevinger. The tumor lines MOPC 167 and MOPC 104E were propagated in this laboratory. The phosphocholine-specific proteins were purified using the affinity medium of Chesebro and Metzger (1972) or a medium prepared by reacting *p*-aminophenylphosphocholine with an *N*-hydroxysuccinimide-activated agarose gel [Affi-Gel 10 (Bio-Rad Laboratories Ltd, Mississauga, Ontario)]. Neither the ascites fluids nor the purified proteins were reduced, and the predominant oligomeric form was the dimer in all cases. M104E and J558 were purified on the dextran affinity medium of Hiramoto *et al.* (1972). M315 was purified from ascites fluid (Litton Bionetics Inc.) with a ϵ -dinitrophenyl-lysine Affi-Gel 10 column. The hybridoma proteins have been previously described (Clafin *et al.*, 1981).

CD spectra were recorded on a Cary 61 instrument. The data are presented as mean residue molar ellipticities, $[\theta]_{\text{mrw}}$, assuming a mean residue wt of 110. Protein concentrations were estimated using the extinction coefficient, $A_{280}^{1\text{mg/ml}} = 1.35$, except for M104E where 1.60 (Leon *et al.*, 1970) was used. The samples were in 0.15 *M* NaCl, 0.01 *M* phosphate buffer, pH 7.3, except where noted.

RESULTS

Phosphocholine-specific myeloma proteins

In Fig. 1, the CD spectra of M511 and W3207 in the aromatic region are compared to the spectra of

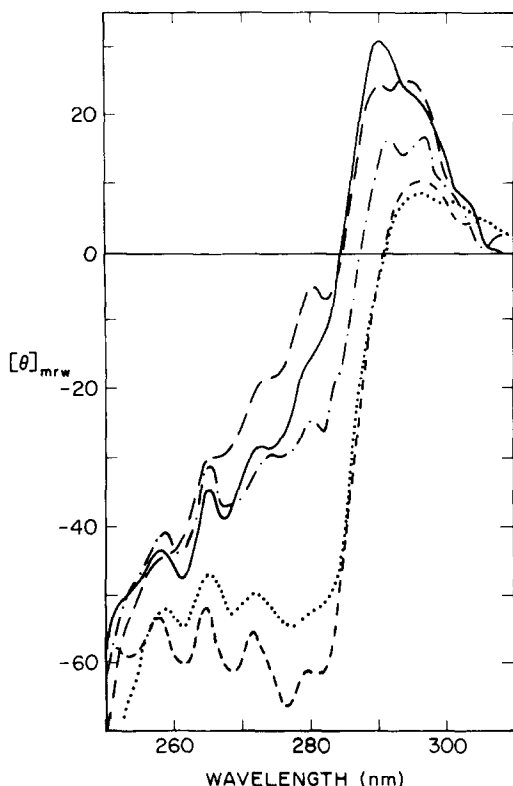


Fig. 1. CD spectra in the aromatic region of mouse myeloma proteins, all in the dimeric IgA form: T15 (—), W3207 (---), M603 (-·-·-), M511 (·····) and M167 (----).

M603, T15 and M167, the prototypes for the three phosphocholine families. The spectra were recorded with non-reduced samples, predominantly in the dimeric IgA form, unlike those reported previously for M603, T15 and M167 (Morris *et al.*, 1974). In the far-u.v. region, the M511 and W3207 proteins both had $[\theta]_{\text{mrw}}$ values at 217 nm of -6700 degrees \cdot cm²/dmole, greater than those of the other proteins which were between -4100 and -5600 degrees \cdot cm²/dmole (Morris *et al.*, 1974). It is apparent that M511 resembles M167 in its CD spectrum, as might be anticipated from its idiotype and overall sequence homology. W3207, though a member of the M603 family, has a CD spectrum more like that of T15. Clearly even the limited sequence differences within a family affect the structures of these proteins as reflected in the CD properties of their aromatic residues.

Addition of the ligand phosphocholine in site-saturating amounts caused changes in the CD spectra that were distinct for nearly all the proteins (Fig. 2). The exception is the W3207–M603 pair, which both showed similar positive shifts in ellipticity. The hapten profiles of M603 and W3207 are also similar, though their reactivities towards the *P. morganii* antigen are a little different (Andres *et al.*, 1981). In the case of M511, the spectral change produced by phosphocholine binding was greater than that of the other four proteins, particularly M167. Though related in many ways, M167 and M511 differ in hapten profile (Andres *et al.*, 1981) and the association

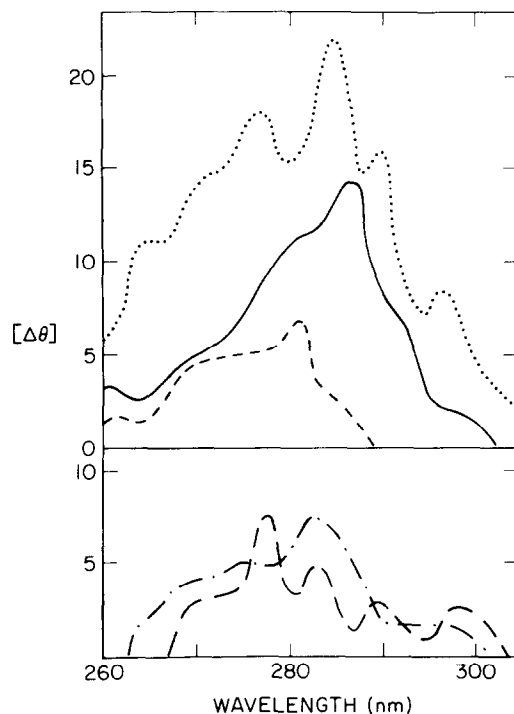


Fig. 2. CD difference spectra of the proteins of Fig. 1, calculated as θ (protein plus phosphocholine) $-\theta$ (native protein). T15 (—), W3207 (---), M603 (-·-·-), M511 (·····) and M167 (----). The errors are estimated as ± 1 degree \cdot cm²/dmole.

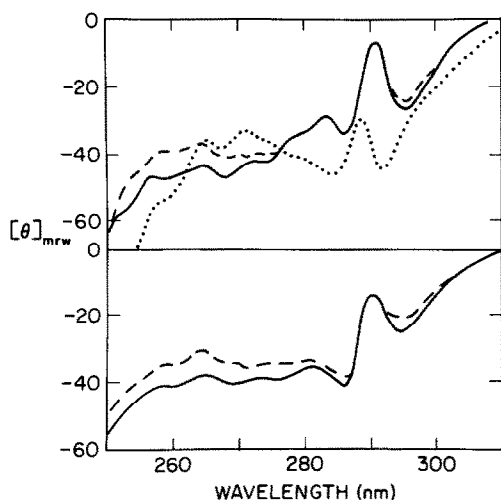


Fig. 3. CD spectra of dextran-specific myeloma proteins. Upper diagram, M104E (—), M104E plus nigerose (---) and M315 (···); lower diagram, J558 (—) and J558 plus nigerose (---).

constants for phosphocholine of M511 is 10 times lower than that of M167 (Metzger *et al.*, 1971).

Effect of isotype differences on the CD spectra

The phosphocholine-specific myeloma proteins above are all IgAs, whereas the hybridoma proteins specific for the same determinant are often IgMs. To allow comparison of the hybridoma proteins to the myeloma proteins, the effect of the class change on the CD spectrum was investigated. An almost ideal pair of myeloma proteins for this purpose are the dextran-specific proteins M104E, an IgM (Leon *et al.*, 1970), and J558, an IgA (Lundblad *et al.*, 1972). These two proteins differ only in two residues in their Fd regions and have identical L-chains. The sequence at residues 96H and 97H, the D-J_H junction, is Tyr-Asp in M104E and Arg-Tyr in J558 (Schilling *et al.*, 1980). The CD spectra of the two proteins (Fig. 3) are very similar in form, only a minor difference near 280 nm being apparent. Hence the class change from IgA to IgM has little effect when comparing CD spectra of mouse immunoglobulins.

When the specific hapten, nigerose, was added in site-saturating amounts, the CD spectra of M104E and J558 underwent very similar changes (Fig. 3); the slight difference around 280 nm is within experimental error. These small changes in the tyrosine region occurring with M104E and J558 on ligand binding contrast with the large changes, particularly in the tryptophan region, seen with M315 when this IgA binds dinitrophenyl haptens (Rockey and Freed, 1976). The CD spectrum of M315 resembles those of M104E and J558 (Fig. 3) possibly due to the homology of its λ_2 light chain to the λ_1 of M104E and J558 (Dugan *et al.*, 1973).

CD spectra of hybridoma proteins

Two hybridoma proteins from each of the three families of phosphocholine-binding proteins were

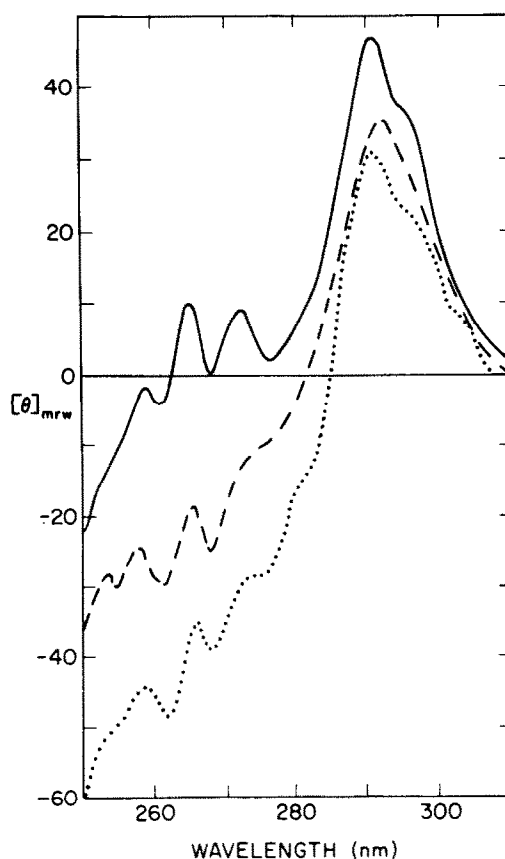


Fig. 4. CD spectra of hybridoma proteins of the T15 family. Protein 59.6C5.1 (—), 103.1C9.1 (---) and the myeloma protein T15 (···).

studied, four of the six being IgMs. It is worth noting that relatively little protein, approx. 1 mg, is needed to obtain a CD spectrum and to examine the effect of one hapten. The spectra are presented in Figs 4–6. Correct assignment of all these proteins to their respective families on the basis of the shape of their CD spectra would not have been possible. Proteins 59.6C5.1 (an IgG3) and 103.1C9.1 (an IgG2) might have been correctly assigned to the T15 family (Fig. 4) and 100.6G2.2 and 100.6F9.1 to the M603 family (Fig. 5), but the two proteins from the M167 family, 101.3C2.2 and 101.6G6 (Fig. 6), show a greater resemblance to T15 and M603 respectively. CD spectra do not therefore offer an accurate way of classifying hybridoma proteins into families.

Ligand effects on the CD spectra were examined for most of the proteins. The 59.6C5.1 protein behaved similarly to T15 and 100.6G2.2 and 100.6F9.1 to M603, as expected. The behavior of the M167-type proteins was markedly different (Fig. 6). 101.3C2.2 gave a difference spectrum resembling that of M511 in shape but of only about half the $\Delta\theta$ magnitude. The 101.6G6 protein gave an extremely large change described below.

Unique properties of the hybridoma protein 101.6G6

In its native CD spectrum (Fig. 6) 101.6G6 re-

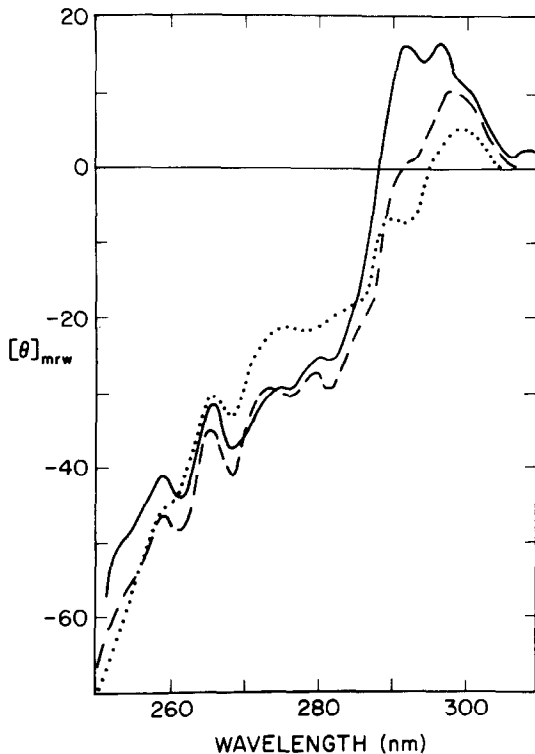


Fig. 5. CD spectra of hybridoma proteins of the M603 family. Protein 100.6G2.2 (···), 100.6F9.1 (---) and M603 (—).

sembles M603 more than M167. When phosphocholine is bound, a very large change occurs in the CD spectrum, while binding of acetylcholine causes a qualitatively similar change but of only half the $\Delta\theta$ magnitude. This contrasts with the behavior

of T15 where the two haptens caused very similar spectral changes (Morris *et al.*, 1974).

In these experiments, phosphocholine was added as the calcium salt, and calcium binding can cause changes in spectroscopic properties of immunoglobulins (Bhattacharjee and Glaudemans, 1983). Addition of calcium ion to the native 101.6G6 did not affect the CD spectrum and, when phosphocholine was added as the sodium salt to 101.6G6 in 20 mM EDTA or 5 mM CaCl_2 , the difference CD spectra and spectrophotometric titration curves (see below) were within experimental error. Hence calcium ions are not responsible for any part of the spectral differences and do not play a role in ligand binding.

The magnitude of the CD changes allowed the association constants for phosphocholine and acetylcholine to be measured by CD spectrophotometric titrations. Previous studies (Clarke *et al.*, 1983) using fluorescence spectroscopy had not given such data, as no changes occurred in the fluorescence properties of 101.6G6 when phosphocholine was bound. This is in surprising contrast to the effects seen in the CD. The titration data (Fig. 7) were recorded at 290 nm: similar results were obtained measuring at 265 nm. Scatchard plots of the titration data (Fig. 8) were obtained using the equation $r/c = nK - rK$, where r is the number of moles of ligand bound per mole of combining site, assuming a mol. wt of 75,000 per site, c is the concentration of free ligand and K is the association constant. For phosphocholine, K was $1.1 \pm 0.1 \times 10^5 M^{-1}$ and for acetylcholine $5.8 \times 10^3 M^{-1}$. The ratio of the two K -values lies between the ratios for M167 and T15 (or M603) obtained in inhibition experiments (Leon and Young, 1971).

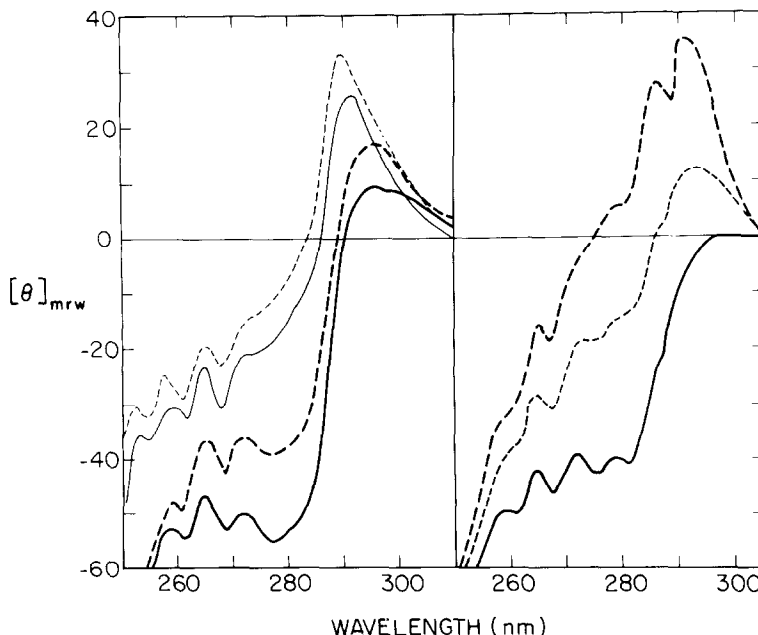


Fig. 6. CD spectra of hybridoma proteins of the M511 family. Left panel, protein 101.3C2.2 (—) and with phosphocholine added (---); M511 (—) and with phosphocholine added (---). Right panel, protein 101.6G6 (—) and with acetylcholine added (---) or phosphocholine added (···). Each ligand was added in saturating amounts.

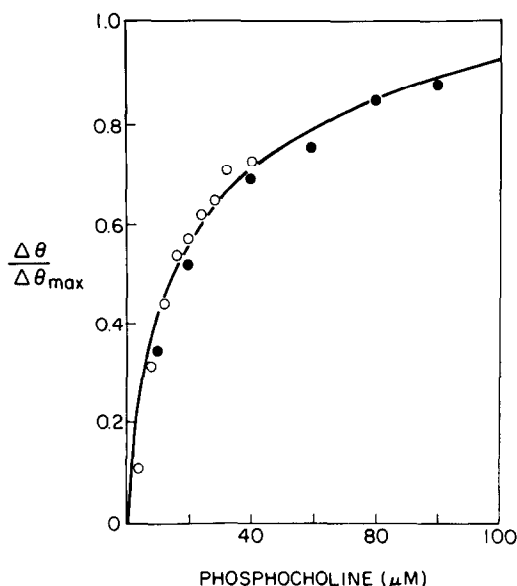


Fig. 7. CD spectrophotometric titrations of 101.6G6 with the sodium salt of phosphocholine in: (a) 5 mM CaCl_2 , 0.15 M NaCl, 0.02 M imidazole buffer, pH 7.2 (●); and (b) 0.15 M NaCl, 20 mM EDTA, pH 7.2 (○).

DISCUSSION

CD differences between related proteins in the aromatic region of their spectra can arise in two ways. Firstly, there may be some direct sequence differences involving tyrosine and tryptophan residues being replaced by other residues. Secondly, sequence differences of residues nearby tyrosine and tryptophan residues in the three-dimensional structure of the protein may affect their conformation or influence their electronic properties. To analyse the latter, extensive model building and interpretation of ligand binding would be necessary. Studies of this type have been undertaken for M315 (Padlan *et al.*, 1977), J539 (Feldman *et al.*, 1981), M167 (Coutre *et al.*, 1981) and M104E (Hovis, 1982). Comparison of the direct changes in sequence involving aromatic residues is more readily undertaken, but is much less complete.

Amino-acid sequence data for the five phosphocholine-specific myeloma proteins (Kabat *et al.*, 1983) and two of the hybridoma proteins (Clarke *et al.*, 1983) are summarized in Table 1 for those CDRs that show differences involving tyrosine or

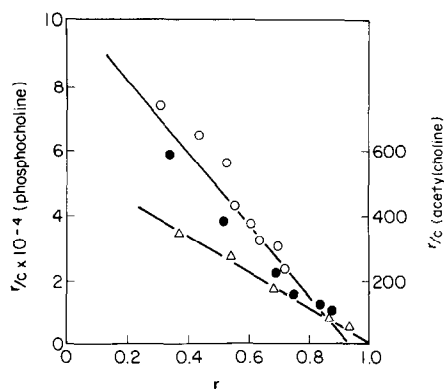


Fig. 8. Scatchard plot of the data from the CD spectrophotometric titrations of 101.6G6. Phosphocholine titrations in CaCl_2 (●) and EDTA (○) from Fig. 7, and an acetylcholine titration (Δ).

tryptophan residues. M603 is taken as the prototype since its structure is being determined by X-ray crystallography (Padlan *et al.*, 1976). The aromatic sequence differences occur mainly in the CDR1 of the L-chain and the CDR3 of the H-chain. The role of the CDR3 in antibodies has not been absolutely established. It has been suggested that it has a structural rather than antigen-binding function (Potter, 1983). It is probable that the variation in the native CD spectra among the proteins arises mainly from the differences in their CDR3 sequences.

The variation in the CD effects upon phosphocholine binding (Fig. 2) shows that the aromatic residues in the binding sites are not equivalent in properties. Either the tyrosine and tryptophan residues common to the five take up different conformations in each case, in either native or ligand-bound states or both, or additional aromatic residues are affected by binding. The source of such additional residues would predominantly be CDR3, which would imply that antigen binding affects this region.

The pair of dextran-specific myeloma proteins M104E and J558 differ also at the D-J_H junction, with Tyr-Asp in M104E becoming Arg-Tyr in J558, at residues 96 and 97 (Schilling *et al.*, 1980). No significant differences between the two proteins in the effect of binding the hapten nigerose were seen (Fig. 3), hence these tyrosines are not different in their CD properties. A recent model for the M104E site and its

Table 1. Sequence data for the CDRs that show differences in tyrosine and tryptophan residues

Protein	H-chain CDR3 ^a			L-chain (compared to M603)
	V _H	D	J _H	
M603	N	YYGST	WYF	Sequence not available ^b
W3207	N	YYKYDL	WYF	
100.6F9	N	YYDGSY	WYF	
T15	D	YYGSSY	WYF	27C L → Y; 32 F → Y; 55 E → Y; 92 H → Y
M167	D	ADYGDSYF	GYF	27D N → Y; 32 F → Y
M511	D	GDYGSSY	WYF	27D N → Y; 32 F → Y
101.6G6	A	NWDG	F	27D N → Y; 32 F → Y ^b

^aCDRs 1 and 2 have no differences in their aromatic residues.

^bSequence known only through CDR1 (Clarke *et al.*, 1983).

binding of nigerose (Hovis, 1982) includes a hydrogen bond from the hydroxyl of Tyr 96H to the hapten. Thus differences in the behavior of the two proteins might have been anticipated, but are not evident in these CD experiments.

Additional spectra and sequence data on hybridoma proteins should help with the analysis begun above. The findings with the present group showed that hybridoma proteins of the T15 and M603 families are generally consistent in their CD properties while the M167 family is a rather variable one. This confirms the results obtained by ligand profiling (Andres *et al.*, 1981). The four members of the M167 family studied here all differed in the shape of their native spectra and, more importantly, in the effect of hapten binding on their CD. The latter effects ranged from a small change in the case of M167, a larger one for 101.3C2.2, a still larger one for M511, which gave the greatest change of the group of myeloma proteins, to the very large change occurring with 101.6G6. Hence, the variation in the M167 family seen in the binding of analogs of phosphocholine (Andres *et al.*, 1981) is shown by the CD results to extend to the details of the interaction of the prototypic hapten, phosphocholine, with the combining sites. This family should therefore be particularly valuable for correlating the variation of function with structure, when the necessary sequence data are available and the M167 structural model (Coutre *et al.*, 1981) is refined.

The hybridoma protein 101.6G6 has been classified as a member of the M167 family on the basis of idiotypic and ligand profile (Andres *et al.*, 1981). Sequence work has shown that its L-chain is homologous to that of M167 (Clarke *et al.*, 1983). However, its H-chain is derived from the VII member of the T15 gene family (Clarke *et al.*, 1982) rather than the VI gene used by the other phosphocholine-binding immunoglobulins (Crews *et al.*, 1981). The H-chain of M47A is also derived from the VII gene and formed phosphocholine-binding immunoglobulins when combined *in vitro* with the 101.6G6 or M511 L-chains (Pierce *et al.*, 1984). Hence 101.6G6 can be considered as the prototype of a fourth family of phosphocholine-specific immunoglobulins.

The magnitude of the CD spectral changes seen with 101.6G6 is unusually large for a protein-ligand interaction. The different effects of acetylcholine and phosphocholine binding to 101.6G6 indicate different interactions around the quaternary ammonium moieties of the two ligands are occurring. This situation is analogous to previous results, obtained by resonance Raman spectroscopy, on the variation in behavior around the nitro groups of various dinitrophenyl ligands when bound to M315 and M460 (Kumar *et al.*, 1978). That an antibody combining site may adopt different conformations around apparently homologous regions of haptens is in accord with current views of the flexible nature of protein active sites in general.

The association constant obtained by CD spectrophotometric titration for 101.6G6 at $1 \times 10^5 M^{-1}$ is slightly below that of M167, $1.2 \times 10^5 M^{-1}$ at pH 8.0, but well above that of M511, $1.6 \times 10^4 M^{-1}$ (Metzger *et al.*, 1971). Hence the apparently large conformational change accompanying hapten binding does not reduce the effectiveness of 101.6G6 as a phosphocholine-binding protein, and evidently immunoglobulins derived from the VII gene can be as effective as those from the VI gene.

Several structural features may contribute to the large CD effects seen with 101.6G6. The differences between the 101.6G6 and other H-chain CDRs include an interesting difference in CDR1. In 101.6G6 the sequence Tyr-Tyr-Met-Ser occurs instead of the Phe-Tyr-Met-Glu sequence of the other proteins. The latter is characteristic of phosphocholine-binding immunoglobulins (Kabat *et al.*, 1976) and includes residues that contact the hapten (Padlan *et al.*, 1976). The additional tyrosine, a potential CD chromophore, in the CDR1 of 101.6G6 may be important for the protein's spectroscopic behavior. Secondly, the D-segment of 101.6G6 is considerably different in sequence and creates a much shorter CDR3 than those of the other proteins (Clarke *et al.*, 1983). It contains no tyrosine residues and one tryptophan, whereas the others have several tyrosines and except M167, one tryptophan. Finally, there may be differences in the overall association of a VII gene H-chain with a VK24 L-chain compared to the VI-VK24 system that necessitate greater site rearrangement upon ligand binding.

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