Contributions by Ionic and Steric Features of Ligands to Their Binding with Phosphorylcholine-Specific Immunoglobulin IgA H-8 as Determined by Fluorescence Spectroscopy†

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ABSTRACT: The murine myeloma IgA H-8 Fab′ fragment which exhibits a binding specificity for phosphorylcholine was assayed for its ability to bind with a number of charged ligands. Monitoring of the ligand-induced changes of protein fluorescence provided a fast and accurate method of determining the equilibrium binding constants. The binding data along with fluorescence spectral properties of the protein permitted an assessment of the relative importance of some binding parameters as well as an evaluation of certain ionic and steric contributions made by ligands exhibiting significant binding affinity for the antibody fragment. Among the conclusions reached is that the dielectric of the binding site microenvironment is important in determining the strength of binding and that hydrophobic groups surrounding a quaternary cationic ligand are important in creating an appropriate binding site of low dielectric value.

One of the most important and interesting areas in molecular biology concerns mutual attractions of complementary molecules resulting in noncovalent associations and the myriad of cascading events which such associations trigger.

A system that has received considerable attention is the antigen–antibody system involving the choline or phosphorylcholine (PC)[1] hapten. Both serum pool and homogeneous murine myeloma antibodies of anti-PC specificity have been studied (Cohn, 1967; Marlow et al., 1969a; Potter & Leon, 1968; Potter & Lieberman, 1970). Valuable insight into the binding phenomena was obtained from experiments that employed induced antibody pools (Marlow et al., 1969a), but the use of heterogeneous antibody was an obvious shortcoming since the conclusions obtained applied to the "average" antibody. The identification of homogeneous immunoglobulins (myeloma proteins) with anti-phosphorylcholine specificity (Cohn, 1967; Potter, 1977) has provided a powerful tool for studying not only the composition and structure of antibody molecules but also some aspects of antigen–antibody recognition and binding phenomena as well (Glaudemans et al., 1977).

Thirteen murine anti-PC myeloma antibodies have been described and studied to varying degrees thus far (Potter, 1977). Complete or partial amino acid sequences have been reported for at least nine of these proteins (Barstad et al., 1974; Kabat et al., 1976), and a complete three-dimensional structure of one immunoglobulin, M603, has been determined to a 3.1-Å resolution from X-ray diffraction data (Segal et al., 1974a,b). From crystallographic studies with the phosphorylcholine ligand present in the binding site, the identities and relative locations of the amino acid side chains that form the combining site are known (Padlan et al., 1976). This information permits some assessment as to the details of the noncovalent association between the ligand and the antibody, and these have been summarized as follows (see Figure 1). "Electrostatic interactions, hydrogen bonding, and van der Waals forces contribute to the interaction between phosphorylcholine and the M603 protein. The positively charged trimethylammonium moiety of the hapten interacts with the acid groups of Glu-35H and Glu-59H. The negatively charged phosphate group interacts with the positively charged guanidinium of Arg-52H and possibly also with the side chain of Lys-54H. In addition, one oxygen of the phosphate is hydrogen bonded to the phenolic OH of Tyr-33H and another to the guanidinium of Arg-52H. There are extensive van der Waals contacts between hapten and the side groups of Tyr-33H and Trp-104aH and an as yet unidentified residue at position 96 of the light chain" (Padlan et al., 1976).

This summary has relevance to our study in that even though we have used H-8 instead of M603, the important hapten-contacting residues (Tyr-33H, Glu-35H, Arg-52H, Lys-54H, and Glu-59H) are the same in both proteins. Moreover, of the three hypervariable (hv) loops of the heavy chain, the first and second have exactly the same number of amino acid residues and thus probably the identical conformation (Potter et al., 1977). The third heavy chain hv loop, while having one amino acid less than M603, has the contact residue (Trp-104aH) in the exact same position as in M603 since it is only two residues removed from the framework of the variable region.

A great deal of insight into the binding of phosphorylcholine to antibody has been accumulated (Grossberg et al., 1974; Goetze & Richards, 1977, 1978; Edelhoch, 1973; Pollet et al., 1974). In spite of all of the evidence about the nature of the antibody combining site, results of our experiments suggest that the model for, and description of, hapten binding is far from complete. For example, according to Padlan et al. (1976) there appears to be no reason why phosphorylethanolamine should not bind almost as well to M603 as does PC itself. Yet as Leon & Young (1971) previously reported, and as we have confirmed (unpublished experiments), phosphorylethanolamine has very little affinity for any of the anti-PC myeloma proteins tested. In addition, from both X-ray crystallographic (Segal et al., 1974a,b; Padlan et al., 1976) and NMR studies (Goetze & Richards, 1977, 1978) the conclusion was reached that "the choline end is in tight van der Waals contact with the binding

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[1] Abbreviations used: PC, phosphorylcholine; NMR, nuclear magnetic resonance. Myeloma proteins derived from plasmacytomas are designated by the first letter followed by the number; i.e., H-8 is the protein produced by plasmacytoma HOPC-8, etc.

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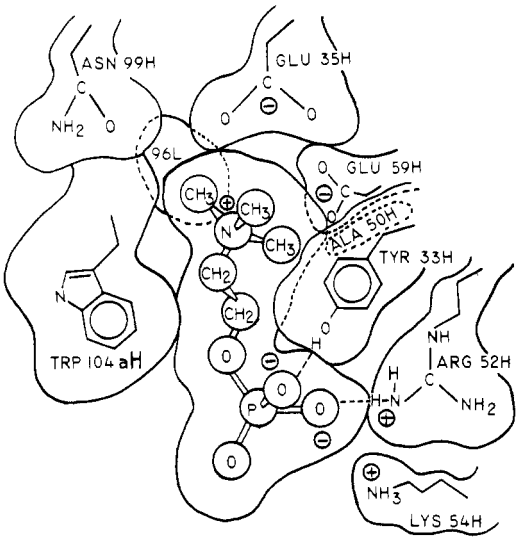

FIGURE 1: Amino acid side chains forming the phosphorylcholine combining site of the IgA myeloma antibody M603, according to Padlan et al. (1976).

pocket . . ." (Goetze & Richards, 1977) and that the residues Tyr-33H, Trp-104aH, and unidentified residue 96L "define the width of the binding cavity and discourage accommodation of much structural variation in the complementary region of the hapten" (Goetze & Richards, 1978). Or as was stated by Padlan et al. (1976), "The cross-sectional size and shape of the cavity are further delineated by backbone atoms in L3 and H3, and its maximum depth is limited by the highly conserved Trp-47H and Phe-105H". If these conclusions are correct, the antibody combining site must be much more flexible than has been previously implied, because our data will show that the binding pocket can accommodate a number of different ligands of substantially different size, shape, and electronic nature with only modest changes in affinity from choline itself.

Results of our binding assays permit an assessment of the apparent lack of specificity of the antibody, as manifested by its ability to bind a variety of compounds. Also the apparent flexibility of the antibody combining site as manifested by the ability to bind compounds of varying molecular *size* will be discussed. Finally, we will describe some of the apparent steric and electronic requirements that ligands must possess if they are to bind in the combining site where phosphorylcholine and "choline-like" compounds bind.

Materials and Methods

Immunoglobulin H-8 was isolated from the ascites fluid of BALB/c mice bearing the plasmacytoma HOPC-8 (Potter, 1972). The antibody was precipitated from pooled fluid by addition of ammonium sulfate and then isolated in pure form by affinity chromatography (Chesebro & Metzger, 1972). Monovalent Fab′ antibody fragments were obtained according to the procedure described by Rudikoff et al. (1972). H-8 Fab′ was dialyzed extensively against 0.05 M citrate buffer, pH 7.2, containing 0.85% NaCl, and the concentration of protein was adjusted to an absorbance at 280 nm of 0.05 OD (a protein concentration of 7.2×10^{-7} M).

Due to the alkaline nature of many of the ligands tested, the pH of the concentrated ligand solution was adjusted to pH 7.2 prior to dilution to the appropriate concentration for use in the fluorescence titration binding assays. Amino compounds other than quaternary ammonium may exist either protonated, thus possessing a positive charge, or nonprotonated, thus being electrically neutral. Knowing the pK_a of the amine species, we determined the relative amounts of charged and neutral species at pH 7.2. Only the concentration of the charged species was considered in the calculation of equilibrium constants.

All binding assays were performed by the method of ligand-induced fluorescence alteration described elsewhere (Pollet & Edelhoch, 1973; Jolley & Glaudemans, 1974). H-8 was chosen for this study because it was found to exhibit substantial changes of fluorescence intensity upon binding a variety of ligands [see also Pollet & Edelhoch (1973)]. This characteristic allows for a more rapid and accurate assessment of binding data in determining equilibrium constants and also permits the measurement of affinity constants, which are substantially below the cutoff limit for the equilibrium dialysis method. The H-8 Fab′ concentration was 7.2×10^{-7} M in 0.05 M citrate buffer, pH 7.2, containing 0.85% NaCl, and titrations were carried out at 25 °C. The protein solution was excited at 295 nm, and fluorescence was monitored at 333 nm.

Most of the ligands tested for binding to H-8 are available commercially. A few were synthesized in our laboratory, and these will be so noted in Results and Discussion.

Proton magnetic resonance spectra were obtained at 25 °C on a Varian XL-100 spectrometer at 100 MHz. About 25 mg of Fab′ was dissolved in D_2O and lyophilized. This process was repeated several times to exchange most of the bound H_2O for D_2O. Aliquots of choline chloride in D_2O were then added to the protein solution. Spectra were obtained by using a pulsed Fourier transform mode and a minimum of 50 000 accumulations. D_2O provided an internal field frequency lock, and tetramethylsilane was the external standard.

Results and Discussion

Our interest in the binding of choline and similar analogues to specific protein was stimulated by the discussion of Padlan et al. (1976) of the binding of phosphorylcholine to the homogeneous immunoglobulin M603. Little reference was made to the importance of the three methyl groups around the quaternary ammonium nitrogen. It is obvious that these methyl groups must be essential to the binding because if they are absent the ligand becomes phosphorylethanolamine, a molecule whose affinity for M603 is known to be negligible (Leon & Young, 1971). Also, since ethanolamine is positively charged at physiological pH, it seemed that the importance of the ionic attraction between the protein's glutamic acid residues and the hapten's quaternary ammonium was overemphasized. The conclusion that the methyl groups of the trimethylammonium formed important van der Waals interactions that dominated the binding attraction seemed unlikely, and experimental results confirmed that the presence and stereoconfiguration of the methyl groups surrounding the charged nitrogen did not contribute in a major fashion to van der Waals binding energy. Analogues were obtained in which the charged nitrogen was replaced by a neutral carbon. Thus, phosphorylcholine was compared with 3,3-dimethylbutanol phosphate and betaine with 3,3-dimethylbutyric acid (Table I). 3,3-Dimethylbutanol was found to be very poorly soluble, but even under conditions where the aqueous solution containing the protein was saturated with the ligand no binding was detected, either directly or by inhibition of phosphorylcholine binding, whereas choline binds reasonably well. The steric arrangement of the three methyl groups surrounding either the charged nitrogen or betaine or the neutral carbon of 3,3-dimethylbutyrate is approximately equivalent. The only significant difference is that betaine bears a positive charge, whereas the other does not (Table I). The binding data showed that whereas the betaine bound the antibody with a K_a of 3.6

Table I: Association Equilibrium Constants, Free Energy of Binding, and Observed Maximum Fluorescence Change of H-8 Fab′ upon Binding Ligands Possessing a Charged Quaternary Ammonium and Analogues not Possessing a Quaternary Ammonium Group

ligand	K_{eq} (M^{-1})	ΔG (kcal/mol)	ΔF_{max} (%)
$H_3C^+N(CH_3)_2CH_2CH_2OH$ (choline)	2.7×10^3	−4.67	+28.2
$H_3CC(CH_3)_2CH_2CH_2OH$ (3,3-dimethylbutanol)	nbd[a]		0
$H_3C^+N(CH_3)_2CH_2COO^-$ (betaine)	3.6×10^3	−4.84	+22.4
$H_3CC(CH_3)_2CH_2COO^-$ (3,3-dimethylbutyrate)	nbd		0
$H_3C^+N(CH_3)_2CH_2CH_2OP(=O)(O^-)_2$ (phosphorylcholine)	6.8×10^5	−7.93	+35.4
$H_3CC(CH_3)_2CH_2CH_2OP(=O)(O^-)_2$ (3,3-dimethylbutanol phosphate)	*b*		quenching
Ph_4As^+ (tetraphenylarsonium)	1.2×10^3	−4.19	−33.2
Ph_4B^- (tetraphenylboride)	nbd		0

[a] nbd = no binding detected. [b] See text for explanation.

$\times 10^3$ M^{-1}, the 3,3-dimethylbutyric acid showed no detectable binding and was unable to inhibit PC binding.

Results of the binding assay with 3,3-dimethylbutanol phosphate[2] as the ligand were ambiguous. Upon addition of increasing amounts of 3,3-dimethylbutanol phosphate a substantial quenching of protein fluorescence was observed (in contrast to the fluorescence enhancement observed upon binding phosphorylcholine). In addition, data points did not provide a characteristic saturation curve. Titration of H-8 Fab′ with phosphorylcholine in the presence of 0.1 M 3,3-dimethylbutanol phosphate resulted in a much lower fluorescence enhancement than expected, suggesting that the binding of PC was being inhibited in a *noncompetitive* manner. One possible explanation of this behavior is that the 3,3-dimethylbutanol phosphate possesses sufficient detergent-like properties so as to partially denature the protein, thus preventing or inhibiting binding of PC. (It is also possible that dimethylbutanol phosphate binds weakly at the PC binding site of the protein, but, due to protein denaturing, accurate binding assessment was obscured.) Nevertheless, binding assays for betaine and 3,3-dimethylbutyric acid clearly suggested that the methyl groups of the PC ligand per se are not directly involved in binding and that any van der Waals attractions contributed to binding of the PC ligand are probably minor contributions to the overall binding affinity. [In an earlier study where similar ligands were tested for their ability to bind with heterogeneous serum antibody of anti-choline specificity, the neutral ligand 3,3-dimethylbutanol was reported to bind with 100-fold less affinity than choline (2.5×10^4 M^{-1} = K_{eq} of choline, 2.2×10^2 M^{-1} = K_{eq} of dimethylbutanol; Marlow et al., 1969b).] Thus, in the case of the homogeneous H-8 Fab′, the ionic attraction between negatively charged groups of the protein's combining site and the positively charged portion of the ligand appears to be essential for significant binding to occur. The results of our binding assays, therefore, support the conclusion, also reached by Grossberg et al. (1974), that electrostatic attractions are important for ligand binding to occur. Not surprisingly, we found that not only should the ligand be charged but also it should be positively charged if it is to bind in the same combining site as choline. Thus, while positively charged tetraphenylarsonium binds H-8 nearly as well as choline itself (Table I), negatively charged tetraphenylboride showed no detectable binding. The stereochemistry of these two molecules should be quite similar.

For the interaction between charged species, in aqueous environments, the distance of charge separation is important, but equally important is the dielectric value and the pH in *the immediate vicinity* of each species. When these last two parameters are considered, some problems become immediately evident. The microenvironment of areas in the combining site of a protein may vary tremendously with respect to dielectric constant and pH. There is, as yet, no easy way to determine these parameters at the very site where a specific ligand can bind. The importance of such parameters is obvious in a noncovalent association such as the interaction of choline and H-8 Fab′.

Table II: Association Equilibrium Constants, Free Energy of Binding, and Observed Maximum Fluorescence Change of H-8 Fab′ upon Binding Ammonium Ions Bearing Increasing Degrees of Methyl Substitution

ligand	K_{eq} (M^{-1})	ΔG (kcal/mol)	ΔF_{max} (%)
NH_4^+	nbd[a]		0
$(CH_3)NH_3^+$	nbd		0
$(CH_3)_2NH_2^+$	4.0×10^1	−2.18	+7.0
$(CH_3)_3NH^+$	3.5×10^2	−3.46	+9.7
$(CH_3)_4N^+$	5.8×10^3	−5.12	+25.1

[a] nbd = no binding detected.

X-ray crystallographic data on M603 (Padlan et al., 1976) show two glutamate carboxylate groups in close proximity (~5 Å) to the positively charged quaternary ammonium headgroup of the bound PC (Figure 1). However, the pH of this microenvironment is not known, and the ionization states of these carboxylates under physiological conditions cannot be unequivocally stated. However, our data on choline and 3,3-dimethylbutanol do support the interpretation that it is important to have a positive charge on the ligand situated in the deep part of the binding cavity.

The dielectric nature of the microenvironment of the binding site may differ substantially from that of the bulk solvent. Protein microenvironments may vary from a nonpolar dielectric of about 2 to a polar dielectric of about 78 (Vinogradov & Linnel, 1971). A relatively small change in the dielectric of the combining pocket of the protein may be reflected in a rather substantial change in the observed equilibrium constant of association between the protein and ligand, and we propose this as a possible explanation for the observed binding phenomena between H-8 Fab′ and the ligands listed in Table II; binding studies showed the ammonium ion to have little affinity for the protein's combining site. As the hydrogens of the ammonium ion are replaced by methyl groups (monomethyl, dimethyl, trimethyl, and tetramethyl), the ammonium ion shows progressively more affinity for the combining site, and the tetramethylammonium ion has a greater affinity than does choline itself (5.8×10^3 and 2.7×10^3 M^{-1}, respectively). Replacement of the hydrogens on the ammonium ion by methyl groups not only allows for increased van der Waals contacts between the combining site of the protein and the ligand but also could provide a mechanism whereby more water is excluded from the vincinity of Glu-35H and Glu-59H

[2] 3,3-Dimethylbutanol phosphate was prepared by phosphorylation of 3,3-dimethylbutanol. Purity of the compound was determined by elemental analysis.

Table III: Association Equilibrium Constants, Free Energy of Binding, and Observed Maximum Fluorescence Change of H-8 Fab' upon Binding Primary Amines and Symmetrical Quaternary Ammonium Ligands Bearing Hydrophobic or Hydrophilic Substituents

ligand	K_{eq} (M^{-1})	ΔG (kcal/mol)	ΔF_{max} (%)
$H_3CCH_2NH_3^+$ (ethylamine)	10[a]		0
$H_3CC(CH_3)_2NH_3^+$ (1,1-dimethylethylamine)	1.4×10^3	−4.28	+24.3
$HOH_2CC(CH_2OH)_2NH_3^+$ [tris(hydroxymethyl)aminomethane]	nbd		0
$(H_3CCH_2)_4N^+$ (tetraethylammonium)	2.8×10^3	−4.69	+22.6
$(HOCH_2CH_2)_4N^+$ [tetrakis(hydroxyethyl)ammonium]	2.7×10^1[b]	−1.95	
$(H_3CCH_2CH_2)_4N^+$ (tetrapropylammonium)	2.5×10^2	−3.20	+22.8

[a] At sufficiently high concentrations (0.1 M) of ethylamine, some inhibition of choline binding was detected. An accurate assessment of the K_{eq} was not possible, however. [b] Determined by inhibition of choline binding.

in the combining site, both of which have been implicated in ligand binding (Padlan et al., 1976). As we have previously discussed, increased surface area available for van der Waals contacts is probably not terribly important. The contribution to binding would be twofold if more bound water could be displaced from the combining site: (a) lowering the dielectric of that microenvironment and providing for a stronger ionic attraction and (b) returning structured water molecules to the bulk solvent, causing an entropy change favorable to binding. The concepts described above could qualitatively answer the question as to why phosphorylethanolamine and other aliphatic amines, for example, may be unable to bind in the choline binding site of H-8 since in the absence of sufficiently bulky hydrophobic groups surrounding the charge enough water is not displaced from the combining site.

The nature of the groups surrounding the charged nitrogen or quaternary ammonium should be hydrophobic and not hydrophilic if significant binding is to occur. The mere presence of bulky groups around the charged nitrogen may facilitate binding, but hydrophobic groups appear to be much better than hydrophilic groups at promoting binding. Binding data on some representative ligands is shown in Table III. Ethylamine showed only very slight affinity for the choline combining site. At high concentrations of ethylamine (0.1 M) some inhibition of choline binding was observed, but the data suggested an affinity constant for ethylamine of less than 10 M^{-1}. When the two hydrogens on the C-1 of ethylamine were replaced by methyls to give 1,1-dimethylethylamine (*tert*-butylamine), the affinity for the choline binding site on H-8 increased dramatically (Table III). Minimizing the importance of van der Waals contacts receives support from the observation that hydrophilic groups surrounding the positively charged nitrogen are less effective than hydrophobic groups in promoting binding, even though the opportunity for van der Waals contacts is unchanged in comparison to the situation in which hydrophobic groups surround the charge. Thus, when one hydrogen on each of the three methyls of 1,1-dimethylethylamine is replaced by a hydroxyl group to give tris(hydroxymethyl)aminomethane, the binding affinity drops by as much as three orders of magnitude from 1.4×10^3 M^{-1} to a state in which no binding was detected with the tris ligand (Table III). The same effect was observed, although not dramatically, with the quaternary ammonium compounds, tetraethylammonium, tetrakis(hydroxyethyl)ammonium,[3] and tetrapropylammonium (Table III). When a hydrogen on each of the four methyls of tetraethylammonium was replaced by a hydroxyl group, the binding affinity dropped by about two orders of magnitude from 2.8×10^3 to 2.7×10^1 M^{-1}. Some of this decrease in affinity could a priori be steric in nature in that the hydroxyl group is larger than a hydrogen, but it is not larger than a methyl, and as is evident from the binding affinity of tetrapropylammonium, the presence of a hydroxyl simply reduced the binding affinity regardless of the steric factors involved. Also, tetraphenylarsonium and -phosphonium bind to H-8 (see below).

[3] Prepared by nucleophilic addition of triethanolamine to bromoethanol.

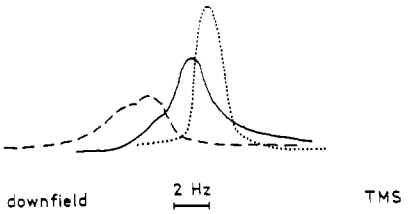

FIGURE 2: Tracings of three proton magnetic resonance spectra of the trimethylammonium protons of choline in the presence of increasing amounts of H-8 Fab' binding protein. The dotted line represents the spectrum of the trimethyl ammonium protons of free choline. The solid line represents the spectrum of the trimethylammonium protons of choline in the presence of H-8 Fab' in a ratio of 3 mol of choline/1 mol of H-8 Fab'. The dashed line represents the spectrum of the trimethyl protons of choline in the presence of H-8 at a ratio of 1 mol of choline/20 mol of H-8 Fab'. The scale indicates the peak widths as well as the degree of downfield shift that the methyl protons experience upon being bound by the protein.

Table IV: Association Equilibrium Constants, Free Energy of Binding, and Observed Maximum Fluorescence Change of H-8 Fab' upon Binding Symmetrical *N*-Alkyl-Substituted Ammonium Ions and Maximum Molecular Radius of the Ions

ligand[a]	max radius (Å)	K_{eq} (M^{-1})	ΔG (kcal/mol)	ΔF_{max} (%)
tetramethylammonium	3.0	5.8×10^3	−5.12	+25.1
tetraethylammonium	4.4	2.8×10^3	−4.69	+22.6
tetrapropylammonium	5.6	2.5×10^2	−3.26	+22.8
tetrabutylammonium	6.8	4.4×10^2	−3.60	+43.5
tetrapentylammonium	8.0	1.8×10^2	−3.07	+50.9
tetrahexylammonium	9.2	2.5×10^2	−3.26	+25.0

[a] All ammonium salts were chlorides.

The protein's apparent ability to accommodate charged ligands substantially larger than tetramethylammonium was also of particular interest, especially in view of the proposed limitations of the combining site area as discussed in some earlier investigations (Padlan et al., 1976; Goetz & Richards, 1977, 1978). If the protein binds tightly to a small ligand such as tetramethylammonium (volume 113 Å^3), how is it that the combining site can also accept the much larger ligand tetrahexylammonium (volume 3260 Å^3), with only a modest change in binding affinity? X-ray crystallographic and NMR data have suggested that amino acid residues of the protein combining site are in close van der Waals contact with the methyl groups surrounding the charged nitrogen (Padlan et al., 1976; Goetz & Richards, 1977, 1978). Results from our own NMR experiments likewise suggested that the choline methyl groups of the bound ligand are in an environment much more restrictive to free rotation than when the ligand is free in solution. Figure 2 shows tracings of the choline methyl

Table V: Association Equilibrium Constants, Free Energy of Binding, and Observed Maximum Fluorescence Change of H-8 Fab' upon Binding Aromatic Cations

ligand	K_{eq} (M^{-1})	ΔG (kcal/mol)	ΔF_{max} (%)
trimethylphenylammonium	1.6×10^3	−4.36	−12.2
2,4,6-trimethylpyridinium	2.5×10^3	−4.62	−55.7
tetraphenylarsonium	1.2×10^3	−4.19	−33.2
benzyltriphenylphosphonium	9.6×10^2	−4.06	−27.9

proton resonance signals obtained in the presence and absence of Fab'. Significant characteristics of the spectra are the downfield shift as the ratio of protein/ligand increases and the peak broadening. These characteristics indicated decreased methyl proton mobility, a phenomenon very likely to occur when the ligand becomes tightly bound to protein (Burgen et al., 1967). Table IV lists the binding equilibrium constants of a series of symmetrical quaternary ammonium compounds of increasing size, along with the maximum molecular radius. The most likely conclusion is that the antibody combining site can possess a great deal of flexibility in order to accommodate ligands of such different sizes. The Stokes radius of tetrahexylammonium suggests that its molecular volume could be nearly 30 times that of the tetramethylammonium ligand. Of course, with such a flexible molecule as tetrahexylammonium the aliphatic groups would probably arrange themselves in such a way that the hydrophobic chains would be in close proximity to one another, forming an asymmetric molecule in which the charged nitrogen is on one side of the molecule, while the hydrophobic chains are folded back on one another into a much less polar region. Molecular models show that this sort of arrangement would expose the charged nitrogen much more and provide for much closer contact with charged amino acid residues in the combining site. The molecular volume would also be substantially reduced as opposed to the situation in which the hexyl side chains were maximally and symmetrically extended with the charged nitrogen directly in the center.

Two ligands which are much more defined in their steric conformation are tetraphenylarsonium and benzyltriphenylphosphonium (Table V). Due to the rigidity of the aromatic rings, the molecular volumes of these ligands are much easier to calculate. Thus, they occupy 6–7 times the volume of tetramethylammonium, but their binding affinities are not a great deal less than that of tetramethylammonium. Compare 1.2×10^3 M^{-1} for tetraphenylarsonium and 9.6×10^2 M^{-1} for benzyltriphenylphosphonium with 5.8×10^3 M^{-1} for tetramethylammonium. Again, we conclude that the H-8 antibody is capable of substantial flexibility in the region of its combining site for choline. The extent of this flexibility undoubtedly plays a significant role in determining the binding specificity and affinity of the protein. Such flexibility would appear not to be confined to the H-8 antibody, however. Marlow et al. (1969a,b) reported similar results for their binding assays of heterogeneous serum antibody to the choline determinant for a wide variety of compounds.

In addition to the properties of H-8 Fab' described above, several other interesting observations have been made concerning the binding of various ligands. One has to do with the blue shift in the fluorescence maximum of H-8 upon binding ligand, and another concerns the fluorescence enhancement observed upon binding some ligands while binding of other ligands causes quenching of protein fluorescence.

A small but definite blue shift of about 3 nm of the emission spectrum of H-8 Fab' was observed upon binding any of several ligands tested containing aliphatic groups. Excitation of the protein at 295 nm establishes a condition in which only tryptophan fluorescence is observed, and a blue shift in tryptophan fluorescence as a result of some kind of perturbation is indicative of the tryptophan's microenvironment becoming more hydrophobic (Van Duuren, 1963; Cowgill, 1967; Weinryb & Steiner, 1971). Such a blue shift was clearly evident when either tetramethyl- or trimethylammonium ligands were tested. However, when dimethylammonium was tested, a blue shift of only about 1 nm was observed. Such results suggested that the trimethyl- and tetramethylammonium ions were more effective at inducing a hydrophobic environment near some tryptophan residue(s) than was dimethylammonium. The location(s) of the tryptophan(s) affected is not known, although it is certainly tempting to speculate that Trp-104aH (Figure 1) would be a likely candidate, because the spectral shift results suggest that a tryptophan that is in a hydrophilic region (such as on the exposed surface of the protein) becomes part of a relatively more hydrophobic region upon binding ligand.

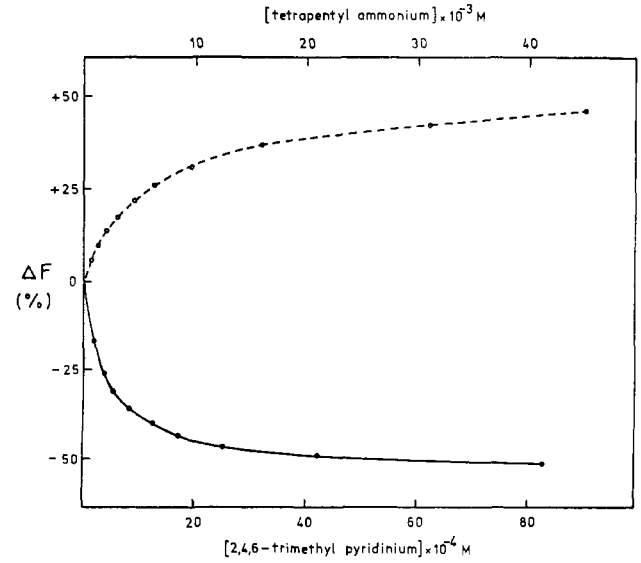

FIGURE 3: Plot showing the saturation kinetics of H-8 Fab' in the presence of increasing amounts of two different ligands, tetrapentylammonium and 2,4,6-trimethylpyridinium. The change in H-8 Fab' fluorescence (F) is shown in response to increasing amounts of ligand added.

Figure 3 shows the extremes of fluorescence enhancement (tetrapentylammonium, +50.9%) and fluorescence quenching (2,4,6-trimethylpyridinium, −55.7%) observed in the course of binding assays on numerous ligands. All ligands tested which did not contain aromatic groups caused fluorescence enhancement, whereas those that possessed aromatic groups quenched the protein's fluorescence upon binding. Results from several studies suggest that radiationless exchange of excitation energy among different tryptophans within a protein appears not to be significant (Steiner et al., 1964; Steinberg, 1971). Thus, changes in protein fluorescence upon ligand binding are probably indicative of localized perturbations at the binding site. Our results tend to support such a conclusion. Qualitatively, not only did an increased hydrophobic environment result in a blue shift in tryptophan fluorescence, as discussed above, but also it probably resulted in an increased quantum yield, i.e., enhanced fluorescence (Steiner et al., 1964; Cowgill, 1967; Weinryb & Steiner, 1971). Both the blue shift and the enhanced fluorescence were observed when the protein bound to ligands containing aliphatic groups. Thus, these observations are consistent with the proposal that the combining site becomes less polar upon ligand binding and pro-

motes a higher binding affinity due to ionic attractions. Although the increased hydrophobicity of the combining site may contribute to enhanced fluorescence upon ligand binding, other mechanisms may of course be operating which also provide for enhanced fluorescence.

A possible mechanism of fluorescence quenching by aromatic cationic ligands could be energy loss from excited tryptophan residues to aromatic systems, and especially aromatic cations, by charge transfer (Foster, 1969). The indole aromatic ring system of tryptophan is known to be a good electron donor in its excited state (Hopkins & Lumry, 1972; Feitelson, 1971). The increased quenching could also result directly from an increased efficiency of charge transfer due to the closer proximity of the bound ligand to the tryptophan fluorophore (see Figure 1).

This study is not intended to be a rigorous quantitative evaluation of the absorption and fluorescent phenomena descriptive of ligand–protein binding association interactions. It is, rather, an attempt to elucidate some of the contributing factors important in binding affinity differences observed among the various ligands of H-8. In general terms, the conclusions we have made would very likely apply to any number of similar situations in which ionic attractions would likely contribute the major force of interaction. That is, the strength of any ionic attraction is determined by the charge separation distance, charge delocalization, and dielectric of the charge microenvironment. The steric and electronic nature of the charged groups, including their relative degree of complementarity, will determine the specificity and hence the strength of the interaction. The more closely protruding groups of a molecule match with cavities of the other (hydrophobic with hydrophobic and polar with polar) and the more confined and closer the approach achieved by groups of opposite charge, the more competing solvent molecules will be excluded from the combining site and the stronger will be the interaction.

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