The Binding Properties of Anti-Phosphorylcholine Mouse Myeloma Proteins as Measured by Protein Fluorescence

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SUMMARY

Ligand-induced enhancement of tryptophanyl fluorescence was evaluated in a series of mouse myeloma proteins with phosphorylcholine-binding activity. Phosphorylcholine-binding by the protein TEPC 15 and its Fab' fragment was studied. The enhancement in the Fab' preparation is produced by an increase in tryptophanyl quantum yield, which is sensitive to quenching in alkali but not in acid.

The Fab' preparation binds 0.9 mole of phosphorylcholine with an association constant of $5.5 \times 10^5$ M$^{-1}$. The enthalpy of binding is $-11$ Cal per mole and the entropy of binding is $-11$ cal per deg mole, indicating that association results from the enthalpy change. The binding affinity is insensitive to pH between pH 6 and 9, but is markedly decreased in acid.

The specific binding of small molecules by purified homogeneous antibody preparations (1, 2) and by certain human (3) and mouse (4-7) myeloma proteins provides a model for antibody binding to antigenic determinants. The nature of the binding affinity in these systems has generally been determined by equilibrium dialysis (4, 5, 7), and more conveniently by the ligand-induced quenching of intrinsic tryptophanyl fluorescence (4, 5, 7) in the case of chromophoric ligands. In this paper we report the ligand-induced enhancement of tryptophanyl fluorescence in several mouse myeloma proteins which bind phosphorylcholine. The increase in tryptophanyl emission may be employed to determine the binding stoichiometry, affinity, and homogeneity of these and other immunoglobulin systems behaving in this manner. This fluorescence change does not depend on energy transfer from fluorescent donors in the antibody to absorbing acceptor groups in the ligand. The method is applied to a study of the binding characteristics of TEPC 15 and its monovalent Fab' fragment.

MATERIALS AND METHODS

Mouse IgA myeloma proteins MOPC 167, MOPC 511, MOPC 603, TEPC 15, and HPDC 8, with activity toward phosphorylcholine, have been described by Potter and Leon (8) and Leon and Young (6). The mouse IgG myeloma protein ALPC 43 with activity toward phosphorylcholine has been isolated by Drs. Potter and Rudikoff, National Institutes of Health. All the myeloma proteins and the TEPC 15 Fab' were prepared and kindly provided by Dr. Rudikoff.

Phosphorylcholine-CaCl (reagent grade), iodoacetamide, and dithiothreitol were obtained from Calbiochem (La Jolla, California). The possibility of Ca$^{2+}$ effects on the studies reported here was eliminated by the inclusion of controls containing 0.005 M EDTA or 0.005 M CaCl$_2$. Disodium phosphoryl[methyl-3H]-choline (New England Nuclear Corp.) also produced identical results.

All chemicals were reagent grade.

Purification of Proteins—Mouse IgA myeloma proteins with activity toward phosphorylcholine were purified by Dr. Rudikoff as described by Chesebro and Metzger (9). Serum or ascites fluid from tumor-bearing animals was reduced with 0.005 M dithiothreitol and alkylated with 0.01 M iodoacetamide (recrystallized two times). The material was then passed through Sepharose-phosphorylcholine affinity columns equilibrated with 0.20 M borate buffer—0.16 M NaCl, pH 8.0. Columns were washed with this buffer until the absorbance at 280 nm of the effluent was less than 0.05, and protein was then specifically eluted with 0.002 M phosphorylcholine. Antibody-containing fractions were then dialyzed against the same buffer for removal of phosphorylcholine, concentrated, and frozen.

Fab' Fragments—Pepsin Fab' fragments were prepared as previously described (10). Purified protein was reduced again in order to complete reduction of the interheavy chain disulfide bonds with 0.01 M dithiothreitol, alkylated with 0.02 M iodoacetamide, and dialyzed against 0.1 M acetate buffer, pH 4.5. The protein was then digested with pepsin (1:100, enzyme to protein) for 4 hours. Fab' fragments were separated by passing the digestion mixture through a Sephadex G-100 column in the borate buffer followed by rechromatography on a Sephadex G-200 column.

Protein Concentration—The concentrations of the TEPC 15 and TEPC 15 Fab' solutions were determined by absorbance at 280 nm at neutral pH. The extinction coefficients were determined by differential refractometry (C. N. Wood Manufacturing Co.) of the protein solutions in water at 546 nm at 25° with the use of a refractive index increment of 1.89 $\times 10^{-4}$ ml per mg (11). The absorbances, $E_{280}$, were 14.0 for TEPC 15 and 14.6 for TEPC 15 Fab'.
determinations of nitrogen and agreed within 1% with the concentration determined on a weight basis.

**Tryptophanyl and Tyrosyl Contents**—The tryptophanyl and tyrosyl contents of the TEPC 15 and TEPC 15 Fab' proteins were determined spectrophotometrically in 6 M guanidine hydrochloride from absorption measurements at pH 6.5 and 12.5 (12). The contents are reported in Table I.

**Fluorescence Spectra**—Emission and excitation spectra were measured with the Turner model 210 spectrophotofluorometer which gives corrected spectra in quanta per unit bandwidth. Excitation was at 280 nm and at 295 nm. The absorbances of the protein solutions were less than 0.05 at the excitation wavelength. The quantum yield of tryptophanyl emission was calculated by comparing the absorbances, \( A \), at the exciting wavelength and the areas of the emission spectra of the protein and a standard of known quantum yield.

\[
Q = \frac{Q_{st}}{Q_{st}} \frac{(area)}{(area)_{st}} \frac{A_{st}}{A}
\]

The standard was a twice-recrystallized preparation of N-acetyl tryptophanamide. A quantum yield of 0.13 has been reported for this compound in aqueous solution at 25°C (13). The absorption of the protein residues (0.05 at 280 nm) was calculated from the protein absorbance, the tryptophanyl and tyrosyl contents indicated in Table I, and the molar extinction coefficients of the tryptophanyl and tyrosyl residues of 5000 and 1200 (14), respectively, at 280 nm.

**Determination of Binding by Fluorescence Enhancement**—The enhancement of tryptophanyl emission provides a rapid method of determining the binding of phosphorylcholine by TEPC 15 and its Fab' subunit. The protein solution (1.5 ml), with concentration less than 1.5 \( \mu \text{g} \), was excited at 295 nm (band width, 4 nm). The emission at 330 nm (band width, 10 nm) was followed in a Perkin-Elmer model MPF3 spectrofluorometer equipped with a temperature-controlled cell compartment. Dust was removed from the solutions by centrifugation. The ligand was added in small volumes to the fluorometric cell with magnetic stirring, employing an Agla syringe with a small Teflon nozzle. The total volume of phosphorylcholine added in the entire titration was about 100 \( \mu \text{l} \). Fluorescence standards were included to monitor any drift of the instrument. The total enhancement in emission was determined by adding small volumes of 0.01 M or several crystals of phosphorylcholine until no further increase in emission occurred. After corrections for dilution, the fraction of binding sites occupied was determined from the ratio of fluorescence increase to maximum increase (saturation). The concentration of free ligand was calculated by subtracting the small amount of bound ligand from the concentration of added ligand.

The stoichiometry of binding for TEPC 15 Fab' was determined from the fluorescence data at protein concentrations of 2.3 \( \times \) \( 10^{-5} \text{ M} \) and 2.3 \( \mu \text{M} \), essentially following the method of Halfman and Nishida (15). At the higher protein concentration, small corrections for changes in midcell absorbance at the exciting wavelength (295 nm), employing the allogiography of one-half of the absorbance, were included (16). The moles of ligand bound per mole of protein, \( \bar{\nu} \), was calculated from the values of added ligand per mole of protein (\( C/P \)) which give the same value of \( \Delta F/\Delta F_{\text{max}} \) at protein concentrations \( P_a \) and \( P_b \), using the relation of Halfman and Nishida (15)

\[
\bar{\nu} = \frac{P_a(C/P)_{b} - P_b(C/P)_{b}}{P_a - P_b}
\]

For TEPC 15 Fab' there is a linear dependence of \( \Delta F/\Delta F_{\text{max}} \) on \( \bar{\nu} \). Extrapolation of \( \bar{\nu} \) to \( \Delta F = \Delta F_{\text{max}} \) gave a value of \( \bar{\nu}_{\text{max}} = 0.9 \) (see "Results"). For all of the other studies with this fragment, \( \bar{\nu} \) for phosphorylcholine binding by TEPC 15 Fab' obtained from this graph was used. TEPC 15 was considered to have 1.9 sites as measured by equilibrium dialysis (9).

### RESULTS

**Tryptophanyl Fluorescence Enhancement**

**Intensity**—The mouse IgA myeloma proteins TEPC 15, HOPC 8, MOPC 167, MOPC 511, McPC 603, and the IgG protein ALPC 43, all of which have activity toward phosphorylcholine (6, 7), were examined for ligand-induced enhancement of phosphorylcholine fluorescence. MOPC 315 (IgA with activity toward dinitrophenyl derivatives) (4) and MOPC 173 (IgG), both of which have no phosphorylcholine-binding activity, were included as controls. The emission maxima and the percentage increase in tryptophanyl fluorescence with excitation at 280 nm are given in Table II. Ligand-induced enhancement of tryptophanyl fluorescence, associated with small blue shifts of emission maxima of about 1 nm, was present for all of the proteins with phosphorylcholine-binding activity. The percentage increases in emission ranged from 10 to 25%. The MOPC 315 and MOPC 173 controls showed no change in tryptophanyl emission.

**Emission Spectra**—In order to investigate further the nature of the ligand-induced enhancement of tryptophanyl fluorescence, the binding of phosphorylcholine by TEPC 15 Fab' (henceforth referred to as Fab'), where the number of tryptophanyl residues per binding site is reduced from 12 to 7 or 8, was studied. The emission spectra in the absence and presence of excess phosphorylcholine are depicted in Fig. 1. With excitation at 295

| Protein | Type | Emission maximum | Enhancement of tryptophanyl fluorescence (excitation at 280 nm) |
|---------|------|-----------------|---------------------------------------------------------------|
| TEPC 15 | IgA  | 332             | 25                                                            |
| TEPC 15 | Fab' | 332             | 30                                                            |
| HOPC 8  | IgA  | 332             | 30                                                            |
| McPC 603| IgA  | 333             | 10                                                            |
| MOPC 167| IgA  | 333             | 12                                                            |
| MOPC 511| IgA  | 334             | 13                                                            |
| MOPC 43 | IgG  | 334             | 10                                                            |
| MOPC 315| IgG  | 334             | 0                                                             |
| MOPC 173| IgG  | 334             | 0                                                             |

Table I

| Protein | Assumed molecular weight | Binding sites | Tryptophanyl residues | Tyrosyl residues |
|---------|--------------------------|---------------|-----------------------|-----------------|
| TEPC 15 | \( 1.5 \times 10^{5} \mu \text{g} \) | 1.09          | 24                    | 48              |
| TEPC 15 Fab' | \( 0.5 \times 10^{5} \mu \text{g} \) | 0.9           | 7.4                   | 21              |

^a Reference 5.  
^b Reference 9.  
^c Reference 10.
nm, where tyrosyl residues do not absorb significantly, the emission comes only from tryptophanyl residues. In the presence of excess phosphorylcholine, tryptophanyl emission increased 28% to give a quantum yield of 0.09, and the emission maximum was 2 nm blue-shifted to 329 nm. When Fab' with and without phosphorylcholine is excited at 280 nm, where tyrosyl residues absorb about 40% of the exciting radiation, the wave length dependence of the emission curve corresponds to that obtained with excitation at 295 nm. Therefore, tyrosyl emission is not observed and is quenched by other groups in the protein. In addition, the wave length dependence of the excitation spectra of the protein and complex is virtually identical and corresponds to the absorption spectrum of tryptophan, indicating that there is no significant energy transfer from tryosyl to tryptophanyl residues in the univalent antibody or in the complex.

**pH Dependence**—The effect of high and low pH on the tryptophanyl emission of Fab' in the absence and presence of excess phosphorylcholine is illustrated in Fig. 2. Solutions were titrated from neutral pH by adding either acid or alkali. The fluorescence of the fragment alone is quenched in acid by 40% and in alkali by 65% with midpoints near pH 4.4 and 9.9, respectively. There is no change in the wave length of the emission maximum from pH 3.0 to 11.0, which suggests that no major structural change occurs in this pH range. Above pH 11, time-dependent changes in fluorescence indicate that the protein is no longer stable. The alkali quenching transition is seen with most native proteins and usually represents the quenching of tryptophanyl fluorescence by ionized tyrosyl residues through radiation-less energy transfer. The rather strong quenching observed in acid is presumably due to quenching by neighboring protonated carboxyl groups, since antibodies appear to be relatively stable in dilute acid solutions (17). The fluorescence curve was largely reversible when the pH was increased from 3 to 7.

The fluorescence curve of Fab' with phosphorylcholine closely resembles that of the Fab' alone with the same two transitions and midpoints. However, the acid titrations of the protein and complex reveal a constant difference in emission intensity, whereas the alkaline titrations show a continuously decreasing difference which disappears above pH 11. This indicates that the ligand is still bound at the lowest pH of the fluorescence curve and until pH 11. The binding in acid was demonstrated directly by adding phosphorylcholine to a solution of Fab' at pH 3.0 (Fig. 2). The fluorescence increased as expected, but much larger amounts of ligand were necessary to produce the same increase as was found at neutral pH. The data gave a linear Scatchard plot with a binding constant of $0.9 \times 10^{-3}$ M$^{-1}$ (see below).

**Binding of Phosphorylcholine**

**Stoichiometry**—The phosphorylcholine-induced fluorescence enhancement of TEPC 15 Fab' may be used to determine the stoichiometry of binding. The number of binding sites was determined from the fluorescence enhancement curves, obtained with two solutions differing 10-fold in protein concentration (Fig. 2), using Equation 1. A plot of the fraction of maximal change in fluorescence, $\Delta F / \Delta F_{\text{max}}$, as a function of the number of moles of ligand bound per mole of protein, $n$, is shown in the inset of Fig. 3. Within experimental error, the fractional fluorescence change is a linear function of $n$, justifying its use as an index of ligand binding. Extrapolation to $\Delta F = \Delta F_{\text{max}}$ gives 0.9 mole of phosphorylcholine bound per mole of Fab'.

**Affinity**—The binding constants for TEPC 15 and its Fab' fragment were determined in 0.02 M Tris (pH 8.0)-0.15 M NaCl, at 25°C, from the fluorescence enhancement curves as described under “Materials and Methods.” The result for Fab' was $5.5 \times 10^5$ M$^{-1}$, in accord with that determined by equilibrium dialysis (7). The binding constant for TEPC 15 was identical within experimental errors with that found for its Fab' fragment.

**pH Dependence**—The pH dependence of phosphorylcholine binding to Fab' from pH 5 to 9 in 0.01 M acetate-0.01 M Tris-0.15 M NaCl at 25°C is depicted in Fig. 4. The binding constant is relatively insensitive to pH between pH 6 and 9, but it is reduced by more than 50% at pH 5 and at pH 9. At pH 3.0 in 0.01 M citrate, 0.15 M NaCl, the binding affinity is reduced about 100-fold from that at neutral pH, yielding a value of $6.0 \times 10^5$ M$^{-1}$.

**Salt Concentration**—The dependence of binding on NaCl and NaI concentration in 0.01 M Tris, pH 8.0, was also investigated.
FIG. 3. The fraction of maximal increase, ΔF/ΔF_{max}, of TEPC 15 Fab' tryptophanyl emission at 330 nm as a function of added phosphorylcholine. Protein concentrations were 2.3 × 10^{-4} M (●–●) and 2.3 μM (■–■) in 0.02 M Tris (pH 8.0)-0.15 M NaCl. The inset shows ΔF/ΔF_{max} as a function of the moles of phosphorylcholine bound per mole of protein, υ, calculated as described under "Materials and Methods" (O--O).

FIG. 4. Scatchard plots of TEPC 15 Fab' binding of phosphorylcholine at several pH values, in 0.01 M acetate-0.01 M Tris-0.15 M NaCl. The protein concentration was 1.5 μM, pH 5.0, ●–●; pH 6.0, ○–●; pH 7.0, ■–■; pH 9.0, □–□. The binding constant in 0.01 M citrate (pH 3.0)-0.15 M NaCl (not shown) was found to be 6 × 10^6.

The Scatchard plots are shown in Fig. 5. The binding constant is relatively insensitive to ionic strength above that contributed by the buffer, since there is only a small decrease (about 15%) between 0 and 0.50 M NaCl. In the presence of 0.50 M NaI, the affinity was approximately 50% smaller than in 0.50 M NaCl. It should be noted that iodide is a collisional quencher of tryptophan emission (18), and Fab' emission is reduced by 12% in 0.50 M NaI.

Temperature Dependence—The temperature dependence of phosphorylcholine binding by Fab' was also examined at pH 8.0, in 0.02 M Tris-0.15 M NaCl, in order to evaluate the thermodynamic binding constants. The results (Fig. 6) show that the binding constant increases about 4-fold as the temperature is lowered from 40° to 15° C. A plot of log K versus 1/T is linear (Fig. 6, inset). From the dependence of K on temperature,

\[ \frac{\partial \log K}{\partial (1/T)} = -\Delta H/2.3R, \]

a value of −11 Cal per mole was obtained for ΔH of binding. Substitution into the equation,

\[ \Delta S = (\Delta H/T) + 2.3R \log K, \]

gives \( \Delta S = -11 \) cal per deg mole. Binding occurs, therefore, because of the large decrease in the enthalpy of reaction.

DISCUSSION

Phosphorylcholine Binding—The ligand-induced enhancement of tryptophanyl fluorescence in the mouse myeloma proteins with phosphorylcholine activity provides a convenient, rapid method for studies of ligand binding. The applicability of this method was demonstrated by measuring the binding properties of TEPC 15 Fab' under a variety of conditions. The stoichiometry of binding was determined from the fluorescence data at two widely different protein concentrations and was found to be 0.9 mole of ligand per mole of protein (Fig. 3). The fluorescence response was proportional to the extent of ligand binding. The resulting
Scatchard plots under varying conditions were linear, demonstrating homogeneity of binding sites. The Scatchard plot for phosphorylcholine binding to TEPC 15 was likewise linear, with virtually the same binding constant as for its Fab' fragment. As in other hapten-binding studies (19), this indicates the absence of interactions between the two sites and rules out any large ligand-induced structural change in the Fe portion of TEPC 15.

Phosphorylcholine binding to Fab' was independent of pH between 6.0 and 9.0 (Fig. 4). It is unlikely that electrostatic interactions between the antibody and ligand contribute significantly to the free energy of binding, since large changes in ionic strength had very little effect on affinity at pH 8.0 (Fig. 5). However, the binding affinity decreased by a factor of 2 at pH 5.0 and by 100 at pH 3.0. The binding of protons by the carboxylate groups titrated in this region (17) increases the positive charge on the protein. The greater net charge at acid pH values could lead to minor displacements of neighboring groups and impairment of some of the loci of the binding site. With the loss of binding loci, the affinity would decrease. It is also possible that protonation of the secondary acid of phosphorylcholine (pK of the ligand analogue, phosphorylethanolamine, is 5.6 at this ionic strength (20)) may eliminate a functional group on the ligand needed for binding.

The enthalpy and entropy of the binding reaction were obtained from the temperature dependence of the binding constant (Fig. 6). The enthalpy was -11 Cal per mole and the entropy was -11 Cal per deg mole. These values are similar to those reported for rabbit antibodies to 2,4-dinitroaniline (21). At 25°, the enthalpy (-11 Cal per mole) favors binding while the entropy effects decrease the negative free energy by 3 Cal per mole, resulting in a ΔG of -8 Cal per mole. Since phosphorylcholine has little or no hydrophobic character to account for the affinity, it is possible that hydrogen bonding is responsible for its high enthalpy of binding. The phosphate group offers a very good site as a hydrogen bond acceptor.

Fluorescence Enhancement—Since the tryptophanyl quantum yields of TEPC 15 (0.06) and TEPC 15 Fab' (0.07) are approximately equal, the increased fluorescence enhancement with excitation at 295 nm from 18% for TEPC 15 to 25% for the Fab' is fully accounted for if the fluorescence enhancement is confined to the Fab portions of the TEPC 15 protein, and it is independent of the Fe portion of the molecule.

The small blue shift of the emission maximum (2 nm) for Fab' with phosphorylcholine binding suggests that the microenvironment of the affected tryptophanyl residue(s) becomes slightly more nonpolar (22). Although this may represent decreased exposure to solvent by direct ligand shielding, other changes in aromatic chromophoric properties of the antibody leave open the possibility of a ligand-induced change in protein conformation. Further studies on this problem will be presented elsewhere.

The acid quenching curves of Fab' fluorescence (Fig. 2) indicate that the groups (presumably carboxyl) responsible for the quenching have no effect on the emission behavior of the tryptophanyl residue(s) whose quantum yield(s) increase on ligand binding. Contrariwise, the alkali quenching of tryptophanyl emission by energy transfer to the numerous tyrosyl phenolate ions is a much longer range effect than that due to carboxyl quenching. Consequently, the difference curve in alkali becomes increasingly smaller as tryptophanyl intensity decreases, and the percentage increase with binding does not increase as in acid but remains constant.

Ligand-induced enhancement of intrinsic tryptophanyl fluorescence is present in all of the mouse myeloma proteins examined which have activity toward phosphorylcholine (Table II). Although this finding might suggest a common structural relationship among these proteins which is independent of their individual immunoglobulin type, specificity spectrum (6), idiotype (23), and binding affinity (7), the origin of the fluorescence changes may well be different in each case since the magnitude of the observed enhancement varies from 10 to 25%. Recently, ligand-induced enhancement of tryptophanyl fluorescence in two myeloma proteins possessing anti-β1,6-galactan activity has also been found (24). It is unclear at present whether this finding reflects the presence of tryptophanyl residue(s) in the active sites of these molecules, or ligand-induced modification of protein conformation, or both effects.

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