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Expression of Multivalency in the Affinity Chromatography of Antibodies†

Dan Eilat and Irwin M. Chaiken*

Appendix: Derivation and Evaluation of Equations for Independent Bivalent Interacting Systems in Quantitative Affinity Chromatography

I. M. Chaiken,* D. Eilat, and W. M. McCormick

ABSTRACT: The expression of multivalency in the interaction of antibody with immobilized antigen was evaluated by quantitative affinity chromatography. Zones of radioisotopically labeled bivalent immunoglobulin A monomer derived from the myeloma protein TEPC 15 were eluted from columns of phosphorylcholine–Sepharose both in the absence and presence of competing soluble phosphorylcholine. At sufficient immobilized phosphorylcholine concentration, the variation of elution volume of bivalent monomer with soluble ligand was found to deviate from that observed for the univalent binding of the corresponding Fab fragment. In addition, the apparent binding affinity of the bivalent monomer increased with immobilized antigen density. Use of equations relating the variation of elution volume with free ligand concentration for a bivalent binding protein allowed calculation of microscopic single-site binding parameters for the bivalent monomeric antibody to both immobilized and soluble phosphorylcholine. The chromatographic data not only demonstrate the effect of multivalency on apparent binding affinity but also offer a relatively simple means to measure microscopic dissociation constants for proteins participating in bivalent interactions with their ligands.

The enhancement of antibody–antigen binding affinity upon going from a monovalent antigen to a polyvalent one is now well documented (Karush, 1976). The term "intrinsic affinity" usually refers to the microscopic equilibrium association constant for the binding of a single antibody combining site to a single antigenic determinant. The affinity of a multivalent interaction is usually termed "avidity" or "functional affinity". Several investigators have tried to evaluate the factor by which the intrinsic affinity will increase to give the functional affinity of a multivalent complex. Crothers & Metzger (1972) and Schumaker et al. (1973) developed theoretical equations in order to estimate this factor. Hornick & Karush (1972) and Gopalakrishnan & Karush (1974) approached the problem experimentally by comparing the association constants of anti-dinitrophenyl or anti-lactoside antibody obtained by equilibrium dialysis with monofunctional ligands to the constants obtained by antibody mediated neutralization of ϕX 174 phage, to which these chemical groups have been multiply conjugated. These workers observed a dramatic increase in phage neutralization upon going from a monovalent Fab fragment through a bivalent IgG antibody to a decavalent IgM molecule. They did not emphasize, however, the crucial dependence of the enhancement factor on the density of the repeating antigenic determinants on the surface to which these groups were chemically attached. Another difficulty inherent in these studies was the comparison of data obtained by a binding technique, equilibrium dialysis, with those obtained by a phage neutralization technique, a biological phenomenon which is related to but not identical with primary binding.

We have approached the problem of assessing the quantitative expression of multivalency by using the recently developed method of quantitative affinity chromatography (Dunn & Chaiken, 1974, 1975; Chaiken & Taylor, 1976). This technique has been used to measure the binding affinity of proteins to their ligands by elution of zones of protein (in both the presence and absence of soluble ligand) on an insoluble matrix to which a ligand is covalently attached. Using this approach, we have measured the functional affinity of a bivalent antibody (TEPC 15) for its immobilized antigen (phosphorylcholine) as it varies with immobilized antigen density. We have compared the values for this functional affinity to the "intrinsic affinity" of a monovalent fragment derived from the same antibody. A mathematical expression was developed which can be used to calculate the microscopic intrinsic affinity of antibody–antigen systems from data obtained by quantitative affinity chromatography.

Experimental Section

Antibody and Derivatives. The phosphorylcholine binding IgA myeloma protein of the BALB/c plasmacytoma TEPC 15 was purchased from Litton Bionetics Inc., Kensington, MD. It was shown to be pure by polyacrylamide gel electrophoresis and immunoelectrophoresis against class specific antisera. Elimination of polymerized products was achieved by reduction and alkylation of the protein according to Miller & Metzger (1965). This procedure was also used to obtain radioactively labeled IgA monomer by using [1-^{14}C]iodoacetamide (57 mCi/mmol, Amersham, England) in the alkylation reaction. The resulting specific activity of the protein was 1400 dpm/μg.

IgA Fab fragments from TEPC 15 IgA monomer were prepared by papain digestion essentially according to Porter

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(1959). The reaction was carried out at 37 °C for 1 h at a 1:20 enzyme to myeloma protein ratio. Fab fragments were separated from the mixture by preparative zone electrophoresis in agar (Logan, W. J., to be submitted for publication). A small amount of undigested protein was removed by Sephadex G-100 gel filtration. Radioactively labeled Fab fragments were obtained by reductive methylation of the intact protein prior to digestion, using a modification of the procedure described by Rice & Means (1971). The monomeric IgA (2 mg) was dissolved in 2 mL of 0.2 M borate buffer, pH 8.3, and 40 mCi of sodium [^{3}H]borohydride (10 Ci/mmol, New England Nuclear) was added. Formaldehyde (0.4% solution, 20 μL) was then added and the mixture was left to react for 10 min on ice. Excess label was removed by filtration on Sephadex G-25 and the protein was dialyzed against PBS.[1] The resulting specific activity of the tritiated protein was 10^6 dpm/μg. About 90% of the tritium label was found to be associated with the Fab fragments.

Equilibrium Dialysis. Experiments were carried out as generally described before (Chaiken & Sanchez, 1972) using phosphoryl[*methyl*-^{14}C]choline (50 mCi/mmol, Amersham). The protein compartments contained 70 μL of 0.45 A_{280nm} of IgA monomer or 0.5 A_{280nm} of IgA Fab. (No attempt was made to correlate the protein concentration of the two species since this would not change the resulting affinity constants.) The ligand compartments (other side of membrane) contained 70 μL of increasing concentrations of phosphorylcholine. Following equilibration (24 h at ambient temperature), 50 μL of solution was withdrawn from each compartment and counted in 5 mL of Aquasol.

Preparation of Affinity Matrices. Preparation of phosphorylcholine–Sepharose was done according to Chesebro & Metzger (1972). Low substitution and high substitution matrices were prepared by reacting a fixed amount (10 mL wet pack volume) of CNBr-activated Sepharose 4B with either 3.5 or 35 μmol of glycyltyrosine. The substituted resins were then reacted respectively with 2.5 and 5 μmol of *p*-diazonium phenylphosphorylcholine (obtained from Dr. S. Rudikoff) and washed extensively as previously described. Phosphate content of the final gels, determined by the Fiske–SubbaRow method (1925) after acid hydrolysis (Bartlett, 1959), was 2.3×10^{-4} M for the low substitution matrix and 1.0×10^{-3} M for the high substitution matrix.

Capacity Measurements. IgA monomer (2 mg) containing a tracer amount of radioactive antibody was chromatographed at room temperature on a column of 0.1 mL bed volume of high substitution matrix in PBS containing 1 mg/mL of BSA. The column was washed with buffer until the unbound protein was eluted. The antibody subsequently eluted with buffer containing 10^{-3} M phosphorylcholine was taken to be the effective capacity, defined below as $[\overline{LM}]$. For the more dilute matrix, capacity was determined with a 1-mL bed volume column at 7 °C, by elution of saturating amounts of IgA monomer in PBS–BSA and measurement of the retarded protein. By the above procedures, the capacities were determined to be 9×10^{-6} and 5×10^{-5} M for the low and high substitution matrices, respectively.

Analytical Affinity Chromatography. Elutions of IgA monomer and IgA Fab were performed using columns of 1 mL bed volume (7×25 mm) of phosphorylcholine–Sepharose in Bio-Rad plastic microcolumns at room temperature. For a particular elution, the column was equilibrated with PBS buffer containing 1 mg/mL of BSA and the appropriate concentration of free phosphorylcholine. A 100-μL zone of protein was applied which contained 25 μg of [^{14}C]IgA monomer, 6 μg of [^{3}H]IgA monomer, or 0.04 μg of [^{3}H]IgA Fab in equilibration buffer. Elution then proceeded with the equilibration buffer. The eluate was collected, in fractions of 0.1–0.5 mL, and analyzed for the position of protein elution by radioisotope counting of aliquots in 5 mL of Aquasol (New England Nuclear), using a Nuclear Chicago Mark III scintillation counter. Elution volume, V, defined as the peak position of eluted protein, was determined by triangulation of the elution profile. Variation of V with free phosphorylcholine concentration, [L], was evaluated according to the equation, derived previously from consideration of the behavior of a monovalently interacting system in chromatography (Dunn & Chaiken, 1974), of

$$\frac{1}{V - V_0} = \frac{1}{(V_0 - V_m)[\overline{LM}]/K_{\overline{LM}}} + \frac{[L]}{K_L(V_0 - V_m)[\overline{LM}]/K_{\overline{LM}}} \quad (1)$$

In this relationship, V_0 = volume at which protein elutes in the absence of interaction; V_m = void volume of column (defined by Dextran Blue elution); $[\overline{LM}]$ = concentration of immobilized ligand (determined as capacity); $K_{\overline{LM}}$ = dissociation constant for interaction of protein with immobilized ligand; K_L = dissociation constant for interaction of protein with free ligand; and V and [L] are as defined above.

For affinity chromatographic elution cases involving the possibility of bivalent ligand binding, namely, with IgA monomer, elution data were evaluated by a second relationship (see Appendix) analogous to eq 1, namely

$$\frac{1}{V - V_0} = \frac{1 + 2\frac{[L]}{K_L'} + \left(\frac{[L]}{K_L'}\right)^2}{(V_0 - V_m)\left[2\frac{[\overline{LM}]}{K_{\overline{LM}'}} + \left(\frac{[\overline{LM}]}{K_{\overline{LM}'}}\right)^2 + 2\frac{[L][\overline{LM}]}{K_L'K_{\overline{LM}'}}\right]} \quad (2)$$

In eq 2, V, V_0, V_m, [L], and $[\overline{LM}]$ are as defined above. K_L' and $K_{\overline{LM}'}$ are microscopic dissociation constants for the interaction of protein with soluble and immobilized ligand, respectively; the primes are used to distinguish values of these parameters obtained by eq 2 from those obtained by eq 1.

Results

Retardation of IgA Monomer on Affinity Columns of Variant Phosphorylcholine Density. The chromatographies of both [^{14}C]IgA monomer and [^{3}H]IgA monomer were carried out on two different phosphorylcholine–Sepharose columns, one at $[\overline{LM}] = 5 \times 10^{-5}$ M (column 1) and the second at $[\overline{LM}] = 9 \times 10^{-6}$ M (column 2), in buffer containing no added soluble phosphorylcholine. The values of V for these elutions (shown for [^{14}C]IgA monomer as the most retarded elution profiles in Figures 1 and 2) were used to calculate values of $K_{\overline{LM}}$ from eq 3 which is a simplification of eq 1 under

$$\frac{1}{V - V_0} = \frac{1}{(V_0 - V_m)\frac{[\overline{LM}]}{K_{\overline{LM}}}} \quad (3)$$

conditions of [L] = 0. As noted in the Experimental Section, the $[\overline{LM}]$ values used for these calculations, and all others in

[1] Abbreviations used: PBS, phosphate-buffered saline (pH 7.4); BSA, bovine serum albumin.

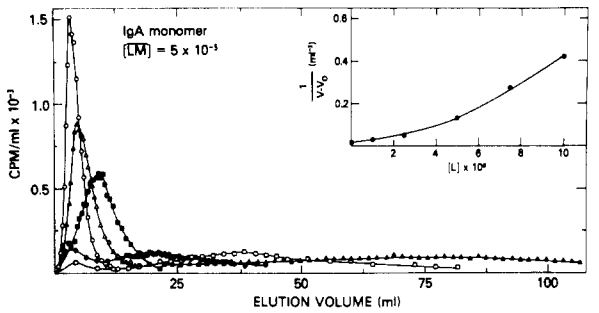

FIGURE 1: Elution profiles of zones of [^{14}C]IgA monomer on high-density (column 1) phosphorylcholine–Sepharose, at ambient temperature, in PBS containing 1 mg/mL of BSA and the following molar concentrations of free phosphorylcholine: 0 (▲), 1×10^{-6} (□), 2.5×10^{-6} (●), 5×10^{-6} (■), 7.5×10^{-6} (△), and 1×10^{-5} (○). The small void volume peak observable in elution profiles at low phosphorylcholine concentration (see, for example, 2.5×10^{-6} M data) is due to the presence of a contamination of nonbinding but isotopically labeled protein in the IgA monomer preparation. Inset: plot of variation of V with [L] according to eq 1. The solid line is drawn by hand through the experimental points.

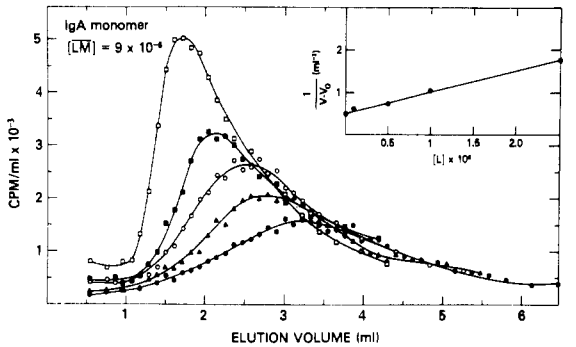

FIGURE 2: Elution profiles of zones of [^{14}C]IgA monomer on low-density (column 2) phosphorylcholine–Sepharose, at ambient temperature, in PBS containing 1 mg/mL of BSA and the following molar concentrations of free phosphorylcholine: 0 (●), 1×10^{-7} (▲), 5×10^{-7} (○), 1×10^{-6} (■), and 2.5×10^{-6} (□). Inset: plot of variation of V with [L] according to eq 1. The solid line is the linear least-squares best fit of eq 1 to the experimental points.

this work, were the capacities (in molar concentrations) of the appropriate matrices for IgA monomer. The resultant $K_{\overline{LM}}$ values, listed in Table I, indicate that there is apparent tighter binding of IgA monomer to high density than to low density immobilized ligand.

Competitive Elution of IgA Monomer from Affinity Columns. [^{14}C]IgA monomer elution was studied by competition with various concentrations of soluble phosphorylcholine ([L]) on both columns 1 and 2. The resulting elution profiles are shown in Figure 1 for column 1 and Figure 2 for column 2. For column 2, the lower density matrix, the variation of $1/(V - V_0)$ with [L] (according to eq 1) is linear, as shown in the inset of Figure 2. From these data, the parameter K_L was calculated as the ratio of intercept/slope of the $1/(V - V_0)$ vs. [L] plot. This value is given in Table I.

The variation of $1/(V - V_0)$ with [L] from competitive elutions on the higher density column 1 is nonlinear (inset, Figure 1). This latter behavior is consistent with multivalent binding of the IgA monomer to the matrix-bound ligand and prevents evaluation of K_L using eq 1.

Analytical Elutions of IgA Fab from Affinity Columns. When monovalent [^{3}H]IgA Fab (prepared from the [^{3}H]IgA monomer) was eluted from columns 1 and 2 with varying amounts of competitive soluble phosphorylcholine, variations of V with [L] were noted as shown in Figure 3. In contrast

Table I: Affinity Chromatography Derived Apparent Dissociation Constants for Bivalent IgA Monomer and Monovalent IgA Fab to Free and Sepharose-Bound Phosphorylcholine, Calculated from Monovalent Model[a]

	col 1: $[\overline{LM}] = 5 \times 10^{-5}$		col 2: $[\overline{LM}] = 9 \times 10^{-6}$	
antibody species	$K_{\overline{LM}}$[b] (M)	K_L[c] (M)	$K_{\overline{LM}}$[b] (M)	K_L[c] (M)
[^{14}C]IgA monomer	1.3×10^{-7}	[d]	1.2×10^{-6}	1.2×10^{-6}
[^{3}H]IgA monomer	1.9×10^{-7}		1.8×10^{-6}	
[^{3}H]IgA Fab	4.2×10^{-6}	3.3×10^{-6}	3.9×10^{-6}	1.5×10^{-6}

[a] Constants are reliable to ±50%, as evaluated previously (Dunn & Chaiken, 1975). [b] Calculated from elution volume for elutions with no competitive ligand using eq 3. [c] Calculated from $1/(V - V_0)$ vs. [L] plots as shown in Figures 2 and 3 using eq 1. [d] Not calculable due to nonlinearity of $1/(V - V_0)$ vs. [L] plot (Figure 1, inset).

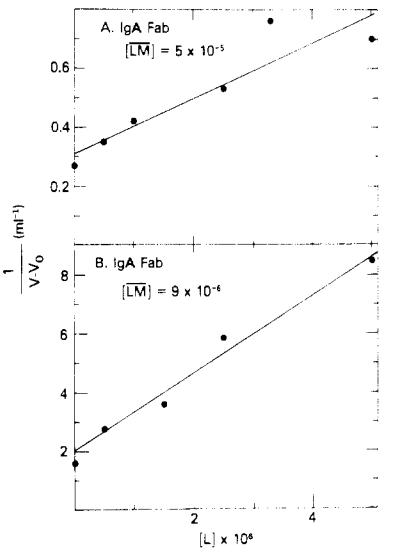

FIGURE 3: Elution behavior of [^{3}H]IgA Fab on high-density (A) and low-density (B) phosphorylcholine–Sepharose, plotted as the variation of V with [L] according to eq 1. Chromatographic conditions were as in the legend to Figure 1. The solid lines are the linear least-squares best fits of eq 1 to the experimental data.

to the results for the bivalent IgA monomers, the $K_{\overline{LM}}$ values obtained with IgA Fab at [L] = 0 (Table I) were found to be similar for the two variant-density affinity columns tested. In addition, plots of $1/(V - V_0)$ vs. [L] for both columns are linear (Figure 3). From these plots, K_L values were obtained as listed in Table I.

Analysis of Elution Data Using a Bivalent Binding Model. In view of the above results showing the effect of bivalent binding on the calculated apparent equilibrium constants, the data for IgA monomer elutions on phosphorylcholine–Sepharose were analyzed using eq 2. Curve fitting of the variation of V with [L] for [^{14}C]IgA monomer for both high- and low-density ligand matrices yielded K_L' and $K_{\overline{LM}}'$ values listed in Table II. Additionally, the values of V at [L] = 0 for elutions of both [^{3}H]IgA monomer and [^{14}C]IgA monomer were used to obtain an independent estimate of the parameter $K_{\overline{LM}}'$ by eq 4 (a simplification of eq 2 for [L] = 0).

$$\frac{1}{V - V_0} = \frac{1}{(V_0 - V_m)\left[2\frac{[\overline{LM}]}{K_{\overline{LM}}'} + \left(\frac{[\overline{LM}]}{K_{\overline{LM}}'}\right)^2\right]} \quad (4)$$

Measurement of Microscopic Affinity Constants by Equilibrium Dialysis. The binding of [^{14}C]phosphorylcholine in solution was measured for both the bivalent IgA monomer

Table II: Affinity Chromatography Derived Dissociation Constants for Bivalent IgA Monomer to Soluble and Sepharose-Bound Phosphorylcholine, Calculated from Bivalent Model[a]

antibody species	col 1: $[\overline{LM}] = 5 \times 10^{-5}$ $K_{\overline{LM}}'$	K_L'	col 2: $[\overline{LM}] = 9 \times 10^{-6}$ $K_{\overline{LM}}'$	K_L'
[^{14}C] IgA monomer	2.7[b]	1.7[b]	4.8[b]	1.6[b]
	2.7[c]		4.7[c]	
[^{3}H] IgA monomer	3.3[c]		6.3[c]	

[a] Dissociation constants (in units of M × 10^6) are reliable to ±40%. [b] Calculated from competitive elution data (Figures 1 and 2) using eq 2. [c] Calculated from V at [L] = 0, using eq 4.

and the monovalent IgA Fab fragment by equilibrium dialysis in PBS. The data are plotted as shown in Figure 4, with the dissociation constants calculated from the slopes. The expected similar values of microscopic K_d for the two species can be compared with the dissociation constants, in Tables I and II, derived from the affinity chromatographic analyses (see Discussion).

Discussion

Antibodies and their antigens provide a convenient system for studying multivalent interactions in biological reactions. This is due to the fact that immunoglobulins are produced as monomers (IgG) or oligomers (IgA, IgM) and that each monomer contains two independent combining sites having equivalent intrinsic affinity. This multivalency of antibodies, together with their extensive molecular heterogeneity and the multivalent nature of many natural antigens, increases the efficiency of the immune system far beyond the individual binding capacity of a single antibody site. In fact the majority of immunological phenomena, including antigen recognition by cell surface receptors, antigen precipitation and elimination, neutralization of infectious agents, and pathogenic autoimmune complexes, may depend in large part on the energetic contribution of multivalency. On the other hand, this property of antibodies and antigens often complicates the study of specificity of an immune reaction, because the intrinsic affinity for the chemical groups bound may be too low to measure by conventional methods. For example, a chicken IgM antibody described by Hornick & Karush (1972), which had no detectable binding for dinitrophenyllysine on equilibrium dialysis, was able to neutralize dinitrophenylated phage with a binding constant greater than 10^9 M^{-1}. A quantitative assessment of the energetic contribution by multivalency could therefore be very instrumental in understanding immunologic interactions and, in some cases, in revealing the true antigenic determinants. The analysis of antibody–antigen interaction by the method of quantitative affinity chromatography is a step toward this goal.

The results of the present study show the effects of antibody bivalency at two levels, the shape of $1/(V - V_0)$ vs. [L] plots and the magnitude of calculated $K_{\overline{LM}}$ values. As shown for the elution of IgA monomer on high-density phosphorylcholine–Sepharose (column 1), the variation of $1/(V - V_0)$ with [L] is curvilinear (see Figure 1), indicating deviation from eq 1 and the monovalent model defined previously. This is in contrast to the behavior for IgA Fab with the high-density matrix, for which a linear $1/(V - V_0)$ vs. [L] plot is obtained (Figure 3), in accord with the expected monovalent character of this species.

The effect of antibody bivalency on the $K_{\overline{LM}}$ values calculated for IgA monomer from [L] = 0 elution is evident in

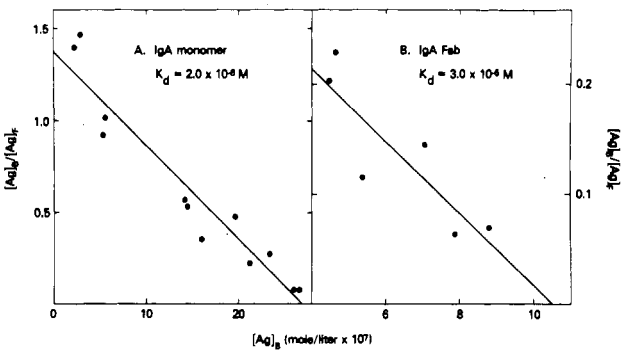

FIGURE 4: Equilibrium dialysis data for binding of [^{14}C]phosphorylcholine to both TEPC 15 IgA monomer and its Fab fragment, plotted according to Scatchard (1949). The solid lines are from linear least-squares regression analyses of the experimental points. The K_d values described by the slope of the best fit lines are shown.

the data of Table I. When these parameters are obtained for the bivalent monomer using the monovalent model-derived eq 3, the $K_{\overline{LM}}$ values are smaller than those obtained for the monovalent Fab. Further, the discrepancy in $K_{\overline{LM}}$ values is greater for the high-density antigen matrix than for the low-density matrix, again as expected from the increased probability of bivalent binding in the former case. Of note, even though the $1/(V - V_0)$ vs. [L] plot for IgA monomer on low-density matrix is apparently linear (Figure 2), the lower $K_{\overline{LM}}$ value than that for IgA Fab on this same matrix shows that some bivalent binding probably occurs in this case also.

In view of the deviation from monovalent behavior, the estimation of microscopic binding constants for such systems as bivalent IgA monomer in affinity chromatography requires the use of equations derived from an explicitly bivalent model. This calculation was carried out for IgA monomer on both antigen matrices used in this study (see Table II) with eq 2 and 4. The $K_{\overline{LM}}'$ values therein obtained agree with the $K_{\overline{LM}}$ values for IgA Fab (Table I) as well as with the K_d values obtained independently by equilibrium dialysis (Figure 4). The agreement of K_d obtained for soluble ligand by equilibrium dialysis with $K_{\overline{LM}}$ values for immobilized ligand by affinity chromatography indicates that the interaction of antibody species with immobilized ligand is closely related to antibody recognition of soluble antigen and thus that the chromatographic approach is useful for direct measurement of microscopic binding parameters. This same conclusion is apparent in the similarity of chromatographic K_L values to those for $K_{\overline{LM}}$ (for IgA Fab) and $K_{\overline{LM}}'$ (for IgA monomer). The results show that the immobilized phosphorylcholine used in this study is recognized, without major perturbation, by the TEPC 15 combining site. That the microscopic $K_{\overline{LM}}$ values were consistently slightly higher than the K_L values may connote some minor impedence of antibody binding to immobilized ligand. However, the differences in these parameters are only minimally outside the range of variation (generally ± 50%) expected from experimental error.

The success of the described results suggests several general uses of quantitative affinity chromatography for bivalent antibodies. Firstly, this approach offers a means to delineate the dependence of apparent binding affinity ($K_{\overline{LM}}$) on the density of immobilized antigen. Our chromatographic $K_{\overline{LM}}$ data demonstrate that the enhancement of functional affinity by multivalency will depend on immobilized ligand density. Measuring $K_{\overline{LM}}$ by elution at zero free ligand concentration on a series of antigen matrices representing a range of ligand

densities would allow the evaluation of functional affinity at each density using eq 3. Secondly, by manipulating antigen substitution to affinity matrices and by using equations appropriate for the bivalent model, one can deduce microscopic single-site antibody affinities. This should be achievable even when the binding interaction is too weak, in the absence of multivalent attachment, to be measured by other methods. The chromatographic approach has the advantages of being applicable for use with protein available in only small amounts (especially if radioisotopically labeled), for systems involving large antigens if they can be successfully immobilized, and for antigens that are not available with radioisotopic label. Thirdly, and as a technical corollary of the method, the information derived from quantitative affinity chromatography can be used to devise more efficient schemes for purification of bivalent antibodies on an antigen affinity matrix. The observation of competitive elution can provide a test for the biospecificity of a particular affinity chromatographic system. Also, knowledge of the approximate intrinsic affinity of an antibody to its antigen should enable one to estimate the optimal immobilized antigen concentration that would provide adequate retention and convenient elution.

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Appendix

The behavior of single site protein–ligand interactions in zonal elution affinity chromatography has been formulated previously (Dunn & Chaiken, 1974). It has been shown that this formulation adequately describes the single site cases of

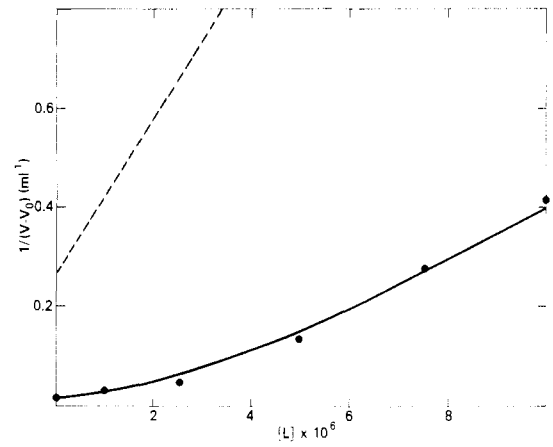
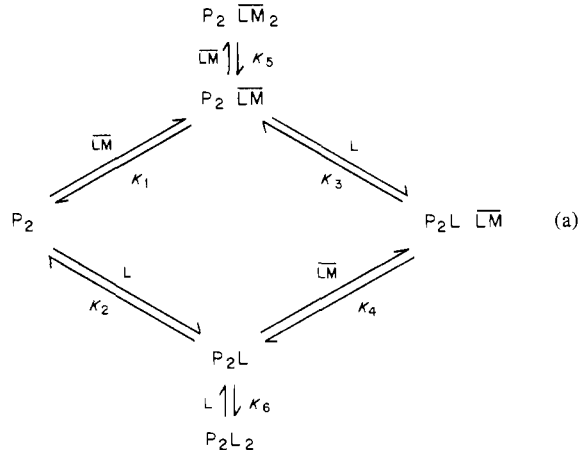


FIGURE A1: Fit of quantitative affinity chromatographic elution data for IgA monomer on phosphorylcholine–Sepharose to the bivalent binding model-derived eq f. The experimental data, in solid circles, are those of Figure 1, inset (see main paper), for IgA monomer competitive elution on the high-density phosphorylcholine–Sepharose matrix. The solid line is the fit of the data to eq e; this curve defines the parameters K_L' and $K_{\overline{LM}}'$ of 1.7×10^{-6} M and 2.7×10^{-6} M, respectively. For comparison, the broken line defines the progression expected for a system behaving monovalently (according to eq 1 of the main paper) with the above microscopic binding constants for soluble and immobilized ligand. The curvilinearity of the solid vs. the broken line and smaller Y intercept of the former (denoting a smaller $K_{\overline{LM}}$ value) are evident.

both staphylococcal nuclease (Dunn & Chaiken, 1974, 1975) and bovine pancreatic ribonuclease (Chaiken & Taylor, 1976) but not the bivalent antibody case (main paper). The latter system may be represented by the general reaction scheme shown in reaction a. Here, P_2 represents bivalent protein, L



(a)

represents soluble ligand, $\overline{LM}$ represents immobilized ligand, and the overbar denotes an immobilized species. In addition, based on the need to distinguish the various binding possibilities from a statistical point of view (Klotz, 1953; Rodbard & Bertino, 1973), P_2L represents either $-P_2-L$ or $L-P_2-$, both of which are presumed equal members of the protein pool containing one bound ligand ("P_2L pool"). Likewise $P_2\,\overline{LM}$ is either $-P_2-\overline{LM}$ or $\overline{LM}-P_2-$; and $P_2\,L\,\overline{LM}$ is either $L-P_2-\overline{LM}$ or $\overline{LM}-P_2-L$. Given the above, K_1 through K_6 are definable as the individual microscopic dissociation constants. Two major assumptions for this scheme have been invoked in order to obtain a simplified chromatographic relationship. These are (1) that $K_2 = K_3 = K_6$ and (2) that $K_1 = K_4 = K_5$.

Thus, all soluble ligand interactions are assumed to have identical affinities, as are all immobilized ligand interactions; the respective microscopic dissociation constants thus are defined simply as K_L' and $K_{\overline{LM}}'$. Given this scheme and the above assumptions, an equation describing the chromatographic elution behavior of bivalent protein on multivalent immobilized ligand matrix is derived, analogously to the previous derivation for the univalent case (Dunn & Chaiken, 1974), as follows.

For liquid chromatography,

$$D = (V - V_m)/V_S \quad \text{(b)}$$

where D is the chromatographic distribution coefficient for the gel, V = elution volume under interacting conditions, V_m = mobile phase volume (outside gel), and V_s = stationary phase volume (inside gel). Also, for the case wherein soluble species can move freely between the inside and outside of the gel,

$$D = ([P_2] + [P_2L \text{ pool}] + [P_2L_2] + [P_2\overline{LM} \text{ pool}] + [P_2\overline{LM}_2] + [P_2L\ \overline{LM} \text{ pool}])/([P_2] + [P_2L \text{ pool}] + [P_2L_2]) \quad \text{(c)}$$

By (1) equating the expressions of D from eq b and c, (2) using the definition

$$D_0 = (V_0 - V_m)/V_S \quad \text{(d)}$$

where V_0 = elution volume of protein when no interaction occurs, and (3) substituting the definitions of K_L' and $K_{\overline{LM}}'$ in the original scheme, one obtains eq e.

$$V = (V_0 - V_m)\left[\left[1 + 2\frac{[L]}{K_L'} + \left(\frac{[L]}{K_L'}\right)^2 + 2\frac{[\overline{LM}]}{K_{\overline{LM}}'} + \left(\frac{[\overline{LM}]}{K_{\overline{LM}}'}\right)^2 + 2\frac{[L][\overline{LM}]}{K_L'K_{\overline{LM}}'}\right] \Big/ \left[1 + 2\frac{[L]}{K_L'} + \left(\frac{[L]}{K_L'}\right)^2\right]\right] + V_m \quad \text{(e)}$$

Rearrangement of eq e leads directly to eq 2 of the main paper; and when [L] = 0, eq 4 of the main paper is obtained. In addition, it should be noted that, when all bivalent interactions are ignored, eq e reduces to the formulation derived earlier for the univalent case, namely, eq 1.

Equation e, or more directly the rearranged eq 2 of the main paper, has been found to adequately describe the data for the competitive elution of IgA monomer on phosphorylcholine–Sepharose. The fitted curve obtained for the high concentration affinity matrix is shown in Figure A1. For comparison, the straight line is shown in Figure A1 that would be obtained if the protein bound only monovalently but with the same microscopic affinities for bound and free ligand.

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Purification and Characterization of RNA Polymerase II Resistant to α-Amanitin from the Mushroom *Agaricus bisporus*†

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ABSTRACT: The DNA-dependent RNA polymerase II or B (ribonucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) from the mushroom *Agaricus bisporus* has been purified to apparent homogeneity. The purification procedures involve precipitation with polyethylenimine, selective elution of RNA polymerase II from the polyethylenimine precipitate, ammonium sulfate fractionation, DEAE-cellulose chromatography, CM-cellulose chromatography, and exclusion chromatography on Bio-Gel A-1.5M. With this procedure 11 mg of RNA polymerase II is recovered from 1.5 kg of mushroom tissue. RNA polymerase II from *Agaricus bisporus* has 12 subunits with the following molecular weights: 182000, 140000, 89000, 69000, 53000, 41000, 37000, 31000, 29000, 25000, 19000, and 16500. Purified RNA polymerase II from *Agaricus bisporous* was half-maximally inhibited by the mushroom toxin α-amanitin at a concentration of 6.5 μg/mL (7×10^{-6} M), which is 650-fold more resistant than mammalian RNA polymerases II. The apparent K_i for the α-amanitin–RNA polymerase complex was estimated to be 12×10^{-6} M. The activity of purified RNA polymerase II from the mushroom was quite typical of other eukaryotic RNA polymerases II with regard to template preference, salt optima, and divalent metal cation optima.

The cytoxin, α-amanitin, is a bicyclic octapeptide occurring in high concentrations in deadly poisonous mushrooms of the genera *Amanita* (Wieland & Wieland, 1972), *Galerina* (Tyler & Smith, 1963), *Conocybe* (Brady et al., 1974), and *Lepiota* (Gérault & Girre, 1975). In addition, Faulstich & Cochet-Meilhac (1976), using very sensitive bioassays, suggest that most mushrooms may contain at least very low concentrations of the amatoxins. The primary cytopathogenicity of the amatoxins is the inhibition of RNA synthesis (Stirpe

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