Biophysical Chemistry, 38 (1990) 131–141
Elsevier

BIOCHE 01507

Quantitative reappraisal of general expressions for multivalent protein binding in subunit-exchange chromatography

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Received 5 September 1989
Revised manuscript received 19 December 1989
Accepted 9 May 1990

Protein binding; Subunit exchange; Affinity chromatography

Quantitative expressions have been derived for bivalent equilibria with immobilized ligand systems and for the equilibria for an immobilized protein whose self-association is modified by binding with a soluble ligand, as analyzed by affinity chromatography. These general expressions have been applied in a reexamination of multivalency in the affinity chromatography of antibodies, as reported by Eilat and Chaiken (Biochemistry 18 (1979) 790) and also to studies of neurophysin-peptide hormone interactions using glass matrices reported by Swaisgood and Chaiken (Biochemistry 25 (1986) 4148).

1. Introduction

Affinity chromatography is a useful analytical technique to determine quantitative properties of protein-protein and protein-ligand interactions [1–5]. Equilibrium dissociation constants are evaluated measuring mobile macromolecules on immobilized ligands, or vice versa [1–10]. Despite the utility of the technique, however, it has been limited to use in situations where the partitioning solute possessed only a single site for interaction with the affinity matrix. The limitations of such a treatment become readily apparent when considering a multivalent system, such as the interaction between a bivalent antibody and an immobilized antigen, when elution is affected by the small, monovalent antigen phosphorylcholine [10]. In this case, the established theory proved inadequate when the concentration of matrix affinity sites was sufficiently high to permit cross-linking of the matrix sites by the bivalent antibody [11].

In this study, we have reexamined: (i) The quantitative expressions for the bivalent equilibria with immobilized ligand systems, analyzed by affinity chromatography on a phosphorylcholine-Sepharose column, reported by Eilat and Chaiken [10]. (ii) The equilibria for an immobilized protein whose self-association is modulated by the binding of a soluble ligand, considering the interaction of a neurophysin-vasopressin system analyzed by affinity chromatography in which protein was immobilized on porous and nonporous glass beads, described by Swaisgood and Chaiken [12].

Because the interaction of the neurophysins and the peptide hormone vasopressin can be quantitated and evaluated with relative ease, it provides an excellent model for assessing the usefulness of analytical chromatography in characterizing macromolecular interactions. We propose general expressions for evaluating the dimerization constant, dissociation constant of monomer from the matrix site and dissociation of dimer from the matrix site.

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2. Theory: Equilibrium binding properties in quantitative affinity chromatography

2.1. Matrix acceptor-single ligand interaction

All solute-ligand and solute-matrix interactions are governed by the single intrinsic dissociation constants $K_{P/L}$ and $K_{P/X}$ (or $K_{P/LX}$) for ligand (L) and matrix site (X), respectively. The reacted site probability theory postulates that [13–19]:

$$f_{P/X}/(1-f_{P/X}) = (1+K_{P/L}[L])([P]_T/[P_0]_T)^{1/n}-1) \quad (1)$$

where $f_{P/X}$ denotes the probability that a protein site (P) has reacted with a matrix site in a system with a concentration of free ligand [L] and $(1-f_{P/X})$ the probability that protein has not reacted with the affinity matrix in which solute P possesses n sites for interaction with univalent ligand L or with sites (X) on the affinity matrix. $[P_0]_T$ is the total constituent concentration in the mobile phase. Eq. 1 may be converted to column chromatographic terminology by noting that $[P]_T/[P_0]_T = V_P/V_{P_0}$, where V_P is the elution volume of solute in the presence of any solute-matrix interaction, and V_{P_0} that in the absence of any solute-matrix interaction. For a system with the total concentration of matrix sites $[X]_T$, vastly in excess of $n[P]_T$, the total concentration of protein in the system, this concentration of matrix sites approximates the corresponding free concentration, [X] [1–3,20–22]; and accordingly the intrinsic site binding constant $K_{P/X}$ is given by $K_{P/X} = f_{P/X}/(1-f_{P/X})\,[X]_T$ [9] when $[X]_T \gg n[P]_T$ [1,3]. Thus,

$$K_{P/X}[X]_T/(1+K_{P/L}[L]) = ([P]_T/[P_0]_T^{1/n}-1) \quad (2)$$

where n is the number of binding sites the solute possesses for interaction either with univalent ligand L or with sites [X] on an affinity matrix. With $n = 1$, eq. 2 is essentially the expression used by Dunn and Chaiken [1,3] for the analysis of zonal affinity chromatography.

$$\frac{1}{\{([P]_T/[P_0]_T)-1\}} = \frac{1}{[X]_T/K_{X/P}} + \frac{[L]}{K_{L/P}[X]_T/K_{X/P}} = \frac{V_0 - V_m}{V - V_0} = \frac{\sigma_0}{(\sigma_p - \sigma_0)} \quad (3)$$

where V is the elution volume of solute in an affinity column, and V_0 and V_m the unretarded and mobile phase volumes, respectively. σ_p is the partition coefficient of solute protein, P, and σ_0 that of solute in the absence of any solute-matrix interactions.

2.2. Matrix acceptor-bivalent interaction

The generalized scheme in fig. 1 defines a set of bivalent ligand interactions, for a system in which an i-th-valent solute P reacts either with affinity matrix site [X] or with two ligands. The dependence of elution volume upon solute concentration is governed by the following expression:

$$\frac{1}{\{([P]_T/[P_0]_T)-1\}} = \frac{(K_{P_2/L}+[L]^2)}{\frac{K_{P_2/L}[X]_T}{K_{P_2/X}} + 2\frac{K_{P_2/L}}{K_{P_2/X}}[X]_T(K_{P_2/L}+[L])} = \frac{\left[1+2\frac{[L]}{K_{P_2/L}}+\left(\frac{[L]}{K_{P/L}}\right)^2\right]}{\left\{\left(\frac{[X]_T}{K_{P_2/X}}\right)^2 + \frac{2[X]_T}{K_{P_2/X}} + 2\frac{[X]_T[L]}{K_{P_2/X}K_{P_2/L}}\right\}} \quad (4)$$

This expression has been used by Chaiken [8] and Eilat and Chaiken [10] to evaluate bivalent antibody interaction parameters for ^{14}C-labeled immunoglobulin A ([^{14}C]IgA) monomer on a phosphorylcholine-Sepharose affinity column eluted with various concentrations of soluble phos-

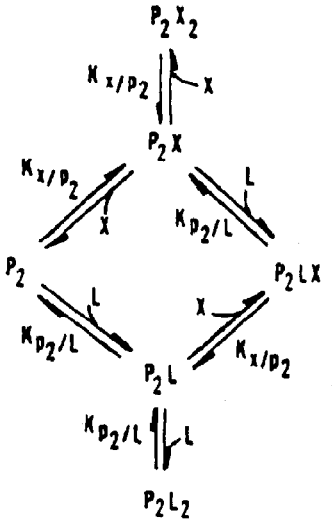

Fig. 1. Bivalent equilibria with immobilized protein systems (Eilat-Chaiken bivalent model).

$$K_{X/P_2} = \frac{[X][P_2]}{[XP_2]} = \frac{[P_2L][X]}{[P_2XL]} = \frac{[P_2X][X]}{[P_2X_2]}$$

$$K_{P_2/L} = \frac{[L][P_2]}{[P_2L]} = \frac{[P_2X][L]}{[P_2XL]} = \frac{[P_2L][L]}{[P_2L_2]}$$

P_2 and X represent bivalent protein and immobilized ligand, respectively. There are two ways of eluting matrix-bound proteins: (i) Elution by soluble ligand, which competes with matrix-bound ligand. (ii) Elution by soluble protein, which competes with matrix-bound protein.

phorylcholine [L]. This expression demonstrates the dependence of apparent binding affinity ($K_{P_2/X}$) on the various densities of immobilized ligand antigen. The enhancement of functional affinity by multivalency will depend on immobilized ligand density. All soluble ligand interactions are assumed to have identical affinities, as all are immobilized ligand interactions.

A similar expression applies for affinity chromatographic elution cases involving the possibility of bivalent ligand binding, namely, with IgA dimer. Each dimer binds to one ligand per monomer based on a simplified chromatographic relationship. These are

(i) $K_{P_2/L} = [L][P_2]/[P_2L] = [P_2L][L]/[P_2L_2] = [P_2X][L]/[P_2XL]$ and

(ii) $K_{X/P_2} = [X][P_2]/[XP_2] = [P_2L][X]/[P_2X] = [X][P_2X]/[X_2P_2]$. Then,

$$\frac{(V_0 - V_m)}{(V - V_0)} = \frac{\left[1 + \frac{[L]}{K_{P_2/L}} + \frac{[L]^2}{K_{P_2/L}}\right]}{\left[\left(\frac{[X]}{K_{X/P_2}}\right) + \left(\frac{[X]}{K_{X/P_2}}\right)^2 + \left(\frac{[X][L]}{K_{P_2/L}K_{X/P_2}}\right)\right]} \quad (5)$$

The equation describes quantitative affinity elution data for IgA dimer on phosphorylcholine-Sepharose to the monovalent binding model discussed in section 2.1.

2.3. General expression for matrix-n-th-valent interaction

Consider a partition equilibrium experiment in which P possesses n sites for interaction either with univalent ligand [L] or with site [X] on an affinity matrix.

$$\frac{1}{\{([P]_T/[P_0]_T) - 1\}} = \frac{1}{\left\{\frac{[X]_T K_{P/L} + K_{P/X}(K_{P/L} + [L])}{K_{P/X}(K_{P/L} + [L])}\right\}^n - 1} = \frac{(V_0 - V_m)}{(V - V_0)} \quad (6)$$

It should be noted that $K_{P/X}$ is the intrinsic dissociation constant for solute-matrix interaction, and $K_{P/L}$ is the dissociation constant with free ligand which might compete with either P or X. All soluble ligand interactions for sites are assumed to be identical as in the case of eq. 5, and $K_{P/X}$ and $K_{P/L}$ describe the n-th-valent interaction with the matrix.

2.4. Subunit-exchange chromatography of self-associating protein systems

Previous attempts to characterize interactions on the basis of subunit exchange chromatography [23,24] assume the concentration of matrix bound monomer available for the formation of immobilized polymers as being given by the analytical composition, an assumption that is not met in affinity chromatography with small ligands immobilized [16,25,26]. Quantitative expressions describing the behavior of a multivalent protein binding in subunit-exchange chromatography are derived in a form that is tractable from the viewpoint of characterizing the pertinent interaction.

2.4.1. n-subunit exchange chromatography and formulations for $[P_1]$, the concentration of monomer

Assuming protein dimer exchanges with protein matrix, the two pertinent interactions are as shown in fig. 2:

$$nP_1 \rightleftharpoons P_n,\ K_n = [P_n]/[P_1]^n$$

$$X + nP_1 \rightleftharpoons XP_n,\ K_{X/P_n} = [XP_n]/[X][P_1]^n$$

where K_n and K_{X/P_n} are the association equilibrium constants for the formation of soluble and immobilized single n-mers, respectively. The total concentration of $[P]_T$ and immobilized matrix $[X]_T$, may be expressed in terms of the corresponding total free monomer:

$$[P]_T = [P_1] + nK_n[P_1]^n + nK_{X/P_n}[X][P_1]^n \qquad (7)$$

Substitution of the total concentration in the mobile phase $[P_0]_T$

$$[P_0]_T = [P_1] + nK_n[P_1]^n \qquad (8a)$$

and the immobilized matrix concentration $[X]_T$

$$[X]_T = [X]\left(1 + K_{X/P}[P_1]^n\right) \qquad (8b)$$

into eq. 7 yields

$$[P]_T - [P_0]_T = \frac{nK_{X/P_n}[X]_T[P_1]^n}{\left(1 + K_{X/P_n}[P_1]^n\right)} \qquad (9)$$

This expression (eq. 9) represents the molar concentration of protein actually bound to matrix site

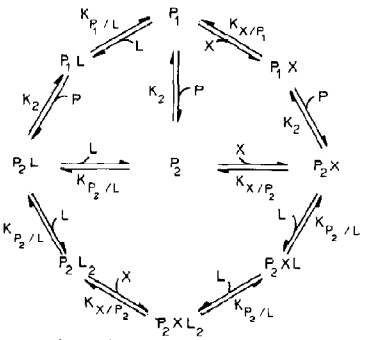

Fig. 2. Proposed model for an immobilized protein which undergoes self-association, modulated by ligand binding. In these equilibria X represents the molecule attached to the matrix, and P and L are the soluble ligands.

(1) Dimerization,

$K_2 = [P_1]^2/[P_2] = [P_1][P_1L]/[P_2L]$
$= [P_1][P_1X]/[P_2X]$

(2) Matrix binding affinity for dimer.

$K_{X/P_2} = [X][P_2]/[XP_2] = [X][P_2X]/[XP_2L]$

(3) Ligand binding affinity for dimer.

$K_{P_2/L} = [L][P_2]/[LP_2] = [L][P_2X]/[P_2XL]$
$= [L][P_2L]/[P_2L_2] = [L][P_2XL]/[P_2XL_2]$

(4) Matrix monomer interaction.

$K_{X/P_1} = [P_1][X]/[P_1X_1]$

(5) Monovalent interaction.

$K_{P_1/L} = [P_1][L]/[P_1L]$

X. The concentration of free monomer in the mobile phase can be evaluated from

$$[P_1] = \left\{ \frac{([P]_T - [P_0]_T)}{(n[X]_T[P]_T - [P_0]_T)K_{X/P_n}} \right\}^{1/n} \qquad (10)$$

Substitution of eq. 10 into eq. 9 and subsequent rearrangement then gives

$$[P_0]_T \left\{ \frac{n[X]_T - [P]_T + [P_0]_T}{[P]_T - [P_0]_T} \right\}^{1/n}$$
$$= \left(\frac{1}{K_{X/P_n}} \right)^{1/n} + \frac{nK_n}{K_{X/P_n}} \left[\frac{[P]_T - [P_0]_T}{n[X]_T - [P]_T + [P_0]_T} \right]^{1-1/n} \qquad (11)$$

A plot of $[P_0]_T\{(2[X]_T - [P]_T + [P_0]_T)/([P]_T - [P_0]_T)\}^{1/2}$ vs $\{([P]_T - [P_0]_T)/(2[X]_T - [P]_T -$

$[P_0]_T)\}^{1/2}$ is linear, with a slope of $2K_2/K_{X/P_2}$ and an ordinate intercept of $[1/K_{X/P_2}]^{1/2}$ when $n = 2$. The major problem to be confronted in this expression is the evaluation of the total effective concentration of matrix sites accessible to protein. This quantity may be defined operationally by extrapolation of results to infinite protein concentration in the liquid phase [25].

To solve the fourth-order equation to determine the concentration of monomer $[P_1]$, from eq. 7,

$$[P]_T = [P_1] + 2K_2[P_1]^2 + 2K_{X/P_2}[P_1]^2 \frac{[X]_T}{\left\{1 + K_{X/P_2}[P_1]^2\right\}} \quad (12)$$

Rearrangement of eq. 12 gives

$$2K_2K_{X/P_2}[P_1]^4 + K_{X/P_2}[P_1]^3 + \left(2K_{X/P_2}[X]_T - K_{X/P_2}[P]_T + 2K_2\right)[P_1]^2 + [P_1] - [P]_T = 0 \quad (13)$$

$[P_1]$ values were computed using the Newton-Raphson iteration method [31]. Only a single root will yield a value of $[P_1]$ which is real, positive and convergent, and within the range of 0.0–10 μM in this particular case.

2.4.2. Bivalent system with subunit-exchange affinity chromatography: Equilibria for an immobilized protein whose self-association is modulated by binding of a soluble ligand

We reexamined the data of Swaisgood and Chaiken [12] on the molecular interaction of a neurophysin-vasopressin system analyzed by high-pressure affinity chromatography using neurophysin immobilized on a CPG glass matrix. The expressions of Chiacone and Winzor [25] for determining the total concentration of matrix bound, $[X]_T$, are complex and we propose the use of eq. 16:

$$\sigma_p = \frac{[P_2X] + [P_2LX] + [P_1X] + [P_2XL_2]}{[P]_T}\sigma_0 + \sigma_0 \quad (14)$$

$$\frac{(V - V_0)}{(V_0 - V_m)} = \frac{[P_2X] + [P_2LX] + [P_1X] + [P_2XL_2]}{[P_2] + [P_2L] + [P_2L_2] + [P_1] + [P_1L]} = \frac{\left\{\frac{[P_2X]}{[P_2]} + \frac{[P_2LX]}{[P_2]} + \frac{[P_1X]}{[P_2]} + \frac{[P_2XL_2]}{[P_2]}\right\}}{\left\{1 + \frac{[P_2L]}{[P_2]} + \frac{[P_2L_2]}{[P_2]} + \frac{[P_1]}{[P_2]} + \frac{[P_1L]}{[P_2]}\right\}} \quad (15)$$

$$\frac{(V_0 - V_m)}{(V - V_0)} = \frac{\left\{1 + \frac{[L]}{K_{P_2/L}} + \left(\frac{[L]}{K_{P_2/L}}\right)^2 + \frac{K_2}{[P_1]} + \frac{K_2[L]}{K_{P_1/L}[P_1]}\right\}}{\left\{\frac{[X]}{K_{X/P_2}} + \frac{[X][L]}{K_{X/P_2}K_{P_2/L}} + \frac{K_2[X]}{[P_1]K_{X/P_1}} + \frac{[X][L]^2}{K_{X/P_2}K_{P_2/L}^2}\right\}} \quad (16)$$

Eq. 16 represents the bivalent binding affinity of a column whose dimerization reaction is modulated by binding of a small ligand [L].

2.4.2.1. (Case i) Elution of affinity column with protein: Concentration dependence of bovine neurophysin II (BNP-II)

When elutions are performed in the absence of competing soluble ligand, i.e. $[L]_T = 0$, then $Q = 1$ where $Q = 1 + ([L]/K_{P_2/L}) + ([L]/K_{P_2/L})^2$. Eq. 16 simplifies to

$$\left.\frac{V_0 - V_m}{V - V_0}\right|_{[L]=0} = \frac{[P_1]K_{X/P_1}K_{X/P_2} + K_2K_{X/P_1}K_{X/P_2}}{[X]\{K_{X/P_1}[P_1] + K_2K_{X/P_2}\}} \quad (17)$$

Substitution of eq. 8 into eq. 17 yields

$$\left.\frac{V_0 - V_m}{V - V_0}\right|_{[L]=0} = \frac{\{[P_1]K_{X/P_1}K_{X/P_2} + K_2K_{X/P_1}K_{X/P_2}\}\{1 + K_{X/P_2}[P_1]^2\}}{[X]_T\{K_{X/P_1}[P_1] + K_2K_{X/P_2}\}} \quad (18)$$

Eq. 18 shows that it is possible to obtain the dimerization constant, K_2, intrinsic dissociation constant of monomer from the matrix site, K_{X/P_1}, and dissociation of dimer from the matrix site K_{X/P_2}. On the basis of $([P]_T - [P_0]_T)$ as a function of $[P_1]$ distribution of BNP-II, the chromatographic interaction parameters of soluble BNP (II) on [BNP II] nonporous glass (NPG) were evaluated. The elution behavior of BNP II dependent upon the concentration of soluble protein was studied based on $[P_1]$. Eq. 18 was used to reevaluate and reanalyze fig. 6, in which the data of Swaisgood and Chaiken [12] are plotted. The concentration dependence of the elution volume observed for zonal chromatography of ^{125}I-BNP II with (BNP-II) NPG was regenerated from eq. 18 using the given values of K_{X/P_1}, K_{X/P_2}, and K_2 at $V_0[X]_T = 42.4$ nmol.

2.4.2.2. (Case ii) Elution of affinity column with [^{3}H][Arg^8]vasopressin (AVP)

It is noted from eq. 19 that the concentration of soluble ligand is not small relative to $K_{P_2/L}$. A significant concentration dependence would be expected. Swaisgood and Chaiken suggested that linear dependence is observed when $[L] \gg K_{X/L}$ at the initial concentration; however, the elution parameters from eq. 19 will not be linear.

$$[X]\frac{(V_0 - V_m)}{(V - V_0)} = \frac{\left(1 + \frac{K_2}{[P_1]}\right) + \left(\frac{1}{K_{P_2/L}} + \frac{K_2}{K_{P_2/L[P_1]}}\right)[L] + \left(\frac{[L]}{K_{P_2/L}}\right)^2}{\left(\frac{1}{K_{X/P_2}} + \frac{K_2}{[P_1]K_{X/P_1}}\right) + \left(\frac{[L]}{K_{X/P_2}K_{P_2/L}}\right) + \frac{1}{K_{X/P_2}}\left(\frac{[L]}{K_{P_2/L}}\right)^2}$$

$$= \frac{(1 + a) + (c + ae)[L] + c^2[L]^2}{(b + ad) + (bc)[L] + bc^2[L]^2} \quad (19)$$

where $a = K_2/[P_1]$, $b = 1/K_{X/P_2}$, $c = 1/K_{P_2/L}$, $d = 1/K_{X/P_1}$, $e = 1/K_{P_1/L}$ and $[X] = [X]_T/(1 + K_{X/P_2}[P_1]^2)$.

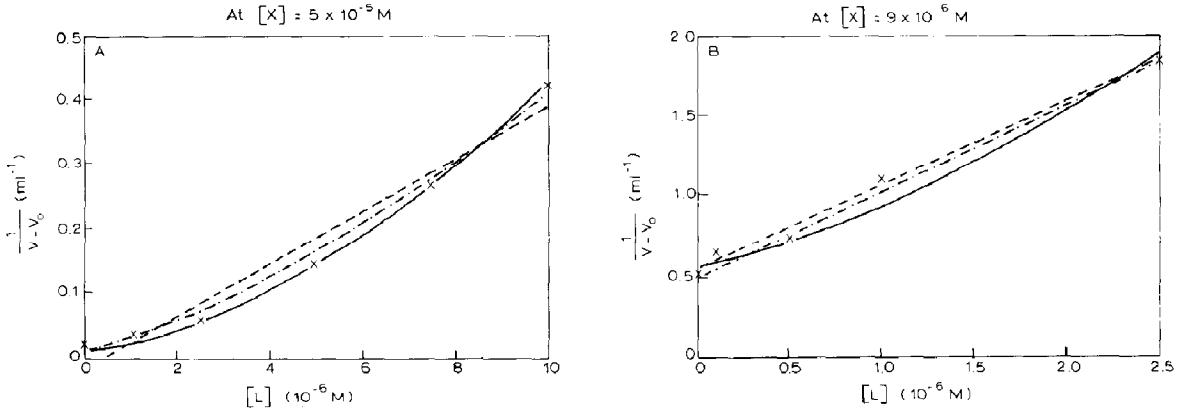

Fig. 3. Experimental data for the interaction between [^{14}C]IgA monomer and a phosphorylcholine-Sepharose affinity column eluted with various concentrations of soluble phosphorylcholine [L] [10]. Data were analyzed by linear least-squares analysis using an IMSL routine package. Numbers in parentheses represent values reported by Eilat and Chaiken [10]. [X] is the matrix site concentration. Chromatographic dissociation constants (M):

	Panel A (at $[X] = 5\times10^{-5}$ M)	Panel B (at $[X] = 9\times10^{-6}$ M)
Linear model	$K_L = -0.34\times10^{-6}$	$K_L = 1.04\times10^{-6}$ (1.2)
(- - - - - -)	$K_{X/L} = -0.17\times10^{-6}$	$K_{X/L} = 1.24\times10^{-6}$ (1.2)
Eilat and Chaiken model	$K'_L = 1.59\times10^{-6}$ (1.7)	$K'_L = 1.64\times10^{-6}$ (1.6)
(·—·—·)	$K'_{X/L} = 3.02\times10^{-6}$ (2.7)	$K'_{X/L} = 4.59\times10^{-6}$ (4.8)
Proposed model	$K_{P_2/L} = 2.06\times10^{-6}$	$K_{P_2/L} = 1.63\times10^{-6}$
(———)	$K_{X/P_2} = 3.60\times10^{-6}$	$K_{X/P_2} = 4.08\times10^{-6}$

Linear monovalent model of Dunn and Chaiken [1,3]:

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

Eilat-Chaiken bivalent model [10]:

$$\frac{1}{V-V_0} = \frac{\left\{1+2\frac{[L]}{K'_L}+\left(\frac{[L]}{K'_L}\right)^2\right\}}{(V_0-V_m)\left\{2\frac{[X]}{K_{X/L}}+\left(\frac{[X]}{K_{X/L}}\right)^2+2\frac{[L][X]}{K'_L K_{X/L}}\right\}}$$

Proposed model:

$$\frac{1}{V-V_0} = \frac{\left\{1+\frac{[L]}{K_{P_2/L}}+\left(\frac{[L]}{K_{P_2/L}}\right)^2\right\}}{(V_0-V_m)\left\{\frac{[X]}{K_{X/P_2}}+\left(\frac{[X]}{K_{X/P_2}}\right)^2+\frac{[X][L]}{K_{P_2/L}K_{X/P_2}}\right\}}$$

3. Computational procedures

In our treatment, the data on the elution profiles of zones of [^{14}C]IgA monomer on either a high- or low-density column of phosphorylcholine-Sepharose at ambient temperature [10] and those on the elution behavior of [^{3}H][Arg^8]vasopressin (AVP) and ^{125}I-bovine neurophysin self-association and neurophysin-peptide hormone interaction using glass matrices [12] were reanalyzed using nonlinear regression analysis, the Newton-Raphson method [31] and the general linear model procedure of the IMSL mathematical and statistical subroutine package.

4. Results and discussion

4.1. Evaluation of equations for bivalent interacting IgA monomer on affinity columns of varying phosphorylcholine density

The variation of $1/(V - V_0)$ with [L] from competitive elutions on the high-density column of 5×10^{-5} M or low-density column of 9×10^{-6} M phosphorylcholine-Sepharose is nonlinear, as shown in fig. 3A and B. This is consistent with

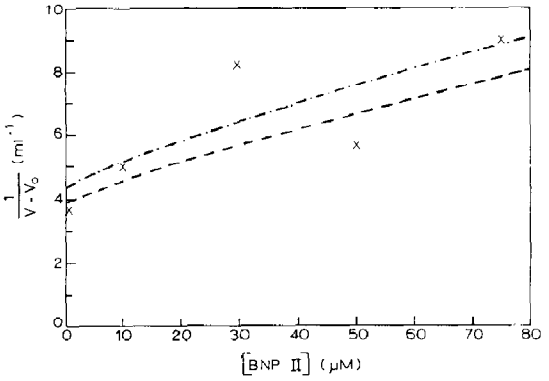

Fig. 4. Concentration dependence of elution volume observed for zonal chromatography of interaction between ^{125}I-bovine neurophysin II (^{125}I-BNP II) and immobilized bovine neurophysin II on nonporous glass beads ([BNP II]-NPG). Experimental data were analyzed by nonlinear least-squares method [31] using an IMSL/VAX subroutine package. Numbers in parentheses represent values reported by Swaisgood and Chaiken [12]. Values of chromatographic dissociation constant (μM) at $V_0[X]_T = 42.4$ nmol:

Swaisgood and Chaiken model [12] (- - - - -)	$K_{x/p} = 187$ (166)	$K_{p/p} = 164$ (172) (K_2)
Modified Swaisgood and Chaiken model (·—·—·)	$K_{x/p} = 187$	$K_{p/p} = 82$ (K_2)

Swaisgood and Chaiken model:

$$\frac{V_0 - V_m}{V - V_0} = \frac{K_{m/p}}{2[X]_T} + \left\{ \frac{K_{m/p}}{2}\left(1 + \frac{8[P]_T}{K_{P/P}}\right)^{1/2} + [P]_T \right\} \frac{1}{[X]_T}$$

Modified Swaisgood-Chaiken model:

$$\frac{V_0 - V_m}{V - V_0} = \frac{K_{m/p}}{2[X]_T} + \left\{ \frac{K_{m/p}}{2}\left(1 + \frac{4[P]_T}{K_{p/p}}\right)^{1/2} + [P]_T \right\} \frac{1}{[X]_T}$$

multivalent binding of IgA monomer to the matrix-bound ligand.

The elution data were fitted for [^{14}C]IgA monomer on a phosphorylcholine-Sepharose affinity column eluted with various concentrations of soluble phosphorylcholine, as three different models: a linear monovalent model [3] from eq. 3, the bivalent model of Eilat and Chaiken [10], from eq. 4, and our proposed model, based on eq. 5. It is clear from fig. 3A that the proposed model provides the best fit to the experimental data taken from the high-density column.

Fitting of these data makes it possible to compute the apparent binding affinity constant of IgA monomer on immobilized ligand ($K_{P_2/X}$), and that on ligand ($K_{P_2/L}$) for the proposed model, shown in fig. 1. These computed values were compared with those of Eilat and Chaiken [10] for K_L and $K_{X/L}$, which are the macroscopic dissociation constants for the interaction of protein with soluble and immobilized ligand, respectively. As seen from fig. 3, comparable values for the two models were of similar magnitude.

Fitting of the data for a low-density column, shown in fig. 3B, indicates that none of the models provides an exact fit to the experimental data, nor can any one model be judged best-fitting. Therefore, it is recommended to use data taken from a high-density column to evaluate the interaction parameters of a multivalent system. In both the model of Eilat and Chaiken [10] and our proposed model, the apparent dissociation constant for the interaction of IgA monomer with immobilized ligand is twice as high on the low-density column, indicating an increased probability of multivalent binding of IgA monomer under these conditions. Furthermore, the close agreement of values for $K_{X/L}$ and K_{X/P_2} indicates that the interaction of the antibody species with immobilized ligand is closely related to antibody recognition of soluble antigen or ligand and demonstrates that affinity chromatography is a useful technique for the direct measurement of microscopic binding parameters.

4.2. Evaluation of equations for multivalent neurophysin self-association and neurophysin-peptide hormone interaction

A comparison of the zonal elution data for ^{125}I-bovine neurophysin II (^{125}I-BNP II) binding to immobilized bovine neurophysin II-nonporous glass beads ([BNP II]-NPG) eluted with increasing concentrations of bovine neurophysin II is shown in fig. 4. Both the model of Swaisgood and Chaiken [12] and our proposed model take the following linear form:

$$\frac{(V_0 - V_m)}{(V - V_0)} = ax + b\sqrt{x} + c$$

where a, b and c are the proportionality coeffi-

cients and x is a variable quantity. As is obvious from fig. 4, neither model fits the data well, probably due to the linear expression from which they are derived. Computed values for the dissociation constant for immobilized neurophysin, $K_{X/P}$, as we evaluated them, were identical for the two models, 187 μM, compared to a value of 166 μM reported by Swaisgood and Chaiken [12].

The dimer dissociation constant, $K_{P/P}$, as evaluated by our method, was 164 μM for the model of Swaisgood and Chaiken, compared to their reported value of 172 μM, and 82 μM for our modified model. The discrepancy can be attributed to the method of evaluating the concentration of monomer.

In a multivalent system, the concentration of monomer evaluated from the quadratic expression takes the following form:

$$[P_1]^2 + \frac{K_{P/P}}{Q}\left\{1 + \frac{[L]}{K_{P/L}}\right\}[P_1] - \frac{K_{P/P}}{Q}[P]_T = 0$$

When $Q = 1$ in the absence of soluble ligand, then this expression gives the concentration of monomer

$$\frac{[P]_T}{[P_1]} = \frac{1}{2} + \frac{1}{2}\left(1 + \frac{4[P]_T}{K_{P/P}}\right)^{1/2}$$

when $[P]_T \ll K_{P/P}$ and $K_{X/P}$, the quantity in brackets in the lower expression becomes $2K_{X/P}/[X]_T$ rather than $K_{X/P}[X]_T$, as reported by Swaisgood and Chaiken [12].

As shown in fig. 5, the elution volume of ^{125}I-BNP II with [BNP II]-NPG as a function of BNP II concentration was analyzed based on eq. 18, representing an equilibria in immobilized neurophysin which undergoes self-association within the matrix. It is essential to determine the concentration of monomer $[P_1]$ from eq. 13 before solving eq. 18. The dimerization constant was found to be 160 μM, in reasonably good agreement with the value of 172 μM reported by Swaisgood and Chaiken [12]. The dissociation constant of the protein monomer of neurophysin immobilized at the matrix site, K_{X/P_1}, is 119 μM, and that of the dimer of neurophysin immobilized at the matrix site, K_{X/P_2}, is 3.0 μM. Consideration of K_{X/P_2} values obtained by this method led to the conclusion that the binding of dimer in immobilized neurophysin is much tighter when compared to binding of monomer.

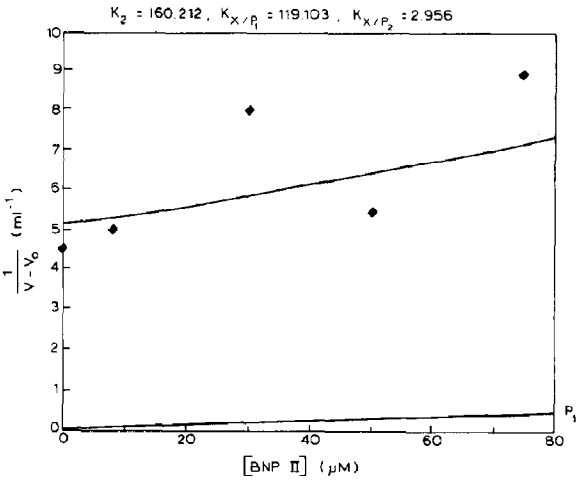

Fig. 5. Experimental data shown in fig. 4 were analyzed based on eq. 18, representing an equilibrium in immobilized neurophysin which undergoes self-association within the matrix. Distribution of the concentration of monomer is evaluated from the square-root relationship between $[P_1]$ and $([P]_T - [P_0]_T)$ (eq. 10).

$$[P_1] = \left\{\frac{([P]_T - [P_0]_T)}{([X]_T - [P]_T + [P_0]_T)K_{X/P_2}}\right\}^{1/2}$$

$$= \left\{\frac{1}{\frac{2[X]_T}{[P]_T - [P_0]_T} - 1}\right\}^{1/2}$$

$[P_1]$ as a function of the concentration of BNP-II is shown. The $[P_1]$ value obtained by iterative procedures is in the range of 1 μM. Values of chromatographically derived dissociation constants (in μM) for interactions of neurophysin-neurophysin immobilized on nonporous glass (NPG) at $V_0[X]_T = 42.4$ nmol:

	K_2	K_{X/P_1}	K_{X/P_2}
Proposed model	160	119	3.0
Swaisgood-Chaiken model	166	–	–

Results obtained from analytical chromatography by Whittaker and Allewell [27] under similar experimental conditions to those reported by Swaisgood and Chaiken [12] gave a range of 56–91 μM for dimerization of unliganded monomer, while Tellam and Winzor [28] and Nicolas et al. [29,30] reported values of 118–172 μM from sedimentation equilibrium measurements. Dimeriza-

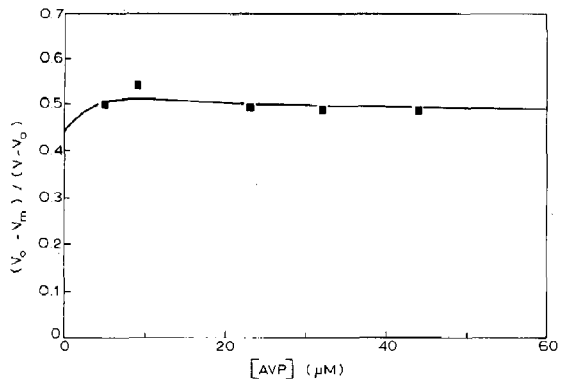

Fig. 6. Concentration dependence of the zonal elution chromatography of [^{3}H]AVP with [BNP II]-CPG. The data taken from Swaisgood and Chaiken [12] are plotted as a function of the concentration of AVP based on eq. 19 using non-linear regression analysis. Values of chromatographically derived dissociation constants (in μM) for interactions of [^{3}H]AVP-BNP II on controlled porous glass beads (CPG):

	$K_{P_1/L}$	$K_{P_2/L}$	K_{X/P_1}	K_{X/P_2}
Proposed Model	22.3	1.10	10.0	1.10
Swaisgood-Chaiken Model	$K_{P/L} = 11 \sim 75$	–	–	

tion of the unliganded monomer was 110 μM, as reported by Whittaker and Allewell [27]. The chromatographically derived equilibrium constants for our proposed model are in close agreement with those reported by Swaisgood and Chaiken [12], and reasonably close to the sedimentation equilibrium measurements [28], indicating that in chromatographic studies, matrix-bound neurophysin exhibits self-association to a much greater extent than does neurophysin in free solution.

Analysis of the interaction of vasopressin and neurophysin can be made from the chromatographic behavior of [^{3}H]arginine-vasopressin (AVP) on bovine neurophysin II or controlled porous glass beads (CPG), shown in fig. 6. For this purpose, we reexamined the concentration dependence of the elution volume for zonal chromatography of [^{3}H]AVP with [BNP II] CPG, applying non-linear regression analysis of eq. 19. The apparent dissociation constant of vasopressin from immobilized BNP II monomer, $K_{P_1/L}$ was found to be 22 μM, while that of neurophysin from the BNP II dimer, $K_{P_2/L}$ is 1.0 μM. Swaisgood and Chaiken [12] reported a range of values from 11 to 75 μM for the dissociation constant $K_{P/L}$. The apparent dissociation constant of immobilized neurophysin monomer, K_{X/P_1} was found to be 10 M and for immobilized dimer, 1 μM, of the same magnitude as $K_{P_2/L}$. These results suggest that the binding affinity of the hormone to dimer is much stronger than to monomer.

In summary, because the interaction of neurophysins and hormones can be quantitated and evaluated with relative ease, it provides an excellent model for exploring the usefulness of analytical affinity chromatography for characterizing macromolecular interactions. Using our proposed model, values for the self-association constant, K_n, dissociation constant of the protein monomer of immobilized protein-matrix, K_{X/P_1}, dissociation constant of n-mer from immobilized protein-matrix, K_{X/P_1}, and of ligand from immobilized protein, $K_{P_1/L}$ or $K_{P_2/L}$, are all obtainable under the same experimental conditions, provided the primary measurements are made with sufficient accuracy and precision.

Acknowledgements

This work was supported by NSF Grants DMB 83-12101(02). Portions of this paper have been presented at the joint meeting of ASBMB and ACS, San Francisco, California (1989).

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