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ANALYTICAL AFFINITY CHROMATOGRAPHY IN STUDIES OF MOLECULAR RECOGNITION IN BIOLOGY: A REVIEW

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SUMMARY

Measuring macromolecular and cellular interactions remains central to the study of recognition in biology and its application in biotechnology. Analytical affinity chromatography provides a versatile methodology to detect and quantitate such interactions. Both zonal and frontal elution approaches have been developed, essentially in parallel, for analytical affinity chromatography. A close quantitative relatedness of chromatographically obtained equilibrium constants and analogous constants determined fully in solution has been found for a growing number of proteins. This consistently observed correlation has formed the basis for extending theoretical treatments in order to evaluate not only monovalent molecular systems of varying types but also multivalently interacting macromolecules, including those which exhibit cooperativity. The potential to measure chemical rate constants by affinity chromatography also has been recognized, and experimental tests of the available theory are being made. As a micromethod, the quantitative use of affinity chromatography has important applicability for biochemical analysis of an increasing array of biologically active molecules being discovered and isolated but available in only relatively small amounts. Analytical affinity chromatography thus provides a means to use matrix—mobile interactant systems to study mechanisms of biomolecular interactions and therein to attain an understanding of such interactions which often is not easily achieved by solution methods alone.

INTRODUCTION

Molecular recognition forms the basis for biological processes from enzymatic conversions, the immune response and membrane transport to multimolecular organization to cellular and multicellular signaling to development. The underlying mechanisms which produce molecular recognition, including the interplay of structural framework, well defined and specific non-covalent contacts between molecular surfaces and segmental flexibility, continue to demand research inquiry. In this effort and the overall goal to characterize

the role of recognition in increasingly complex systems, detection and quantitation of macromolecular and cellular interactions, and the methods to do so, remain central to the study of recognition in biology and its application in biotechnology.

The use of affinity chromatography as an analytical tool to detect and measure molecular interactions is well suited to such demands [1—11]. Binding parameters for non-covalent interactions of small molecules, macromolecules and multimolecular assemblies can be measured from the degree of retardation of these substances on affinity matrices containing immobilized interactants. And since, for a given affinity matrix, competitors and other effectors can be varied, as can the structural nature of mobile interactants, binding specificity and dependence on solution conditions all can be evaluated. Both zonal and continuous (or broad zone) elution approaches have been developed, essentially in parallel, for analytical affinity chromatography. The zonal method has important applicability as a micromethod for biochemical analysis of a vast array of biologically active molecules being discovered and isolated but available in only relatively small amounts. Continuous elution, with associated frontal analysis, allows an explicit evaluation of interaction parameters at defined concentrations of mobile interactants when sufficient quantities of the latter can be used. During the development of analytical affinity chromatography, many protein—ligand and protein—protein interactions, including enzyme—cofactor, enzyme—inhibitor, antibody—antigen and hormone—carrier systems, have been examined successfully. Continued methods development, including that of analytical high-performance affinity chromatography, promises to stimulate more expanded usage for studies of macromolecular interactions.

The present paper presents basic theoretical considerations which have been developed for analytical affinity chromatography. A brief review is given

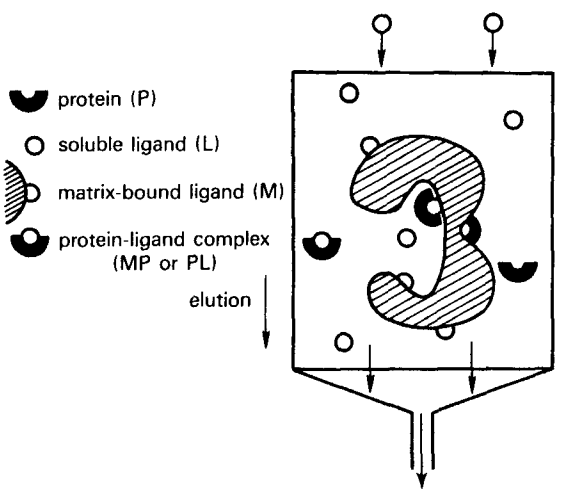


Fig. 1. Schematic diagram of competitive zonal elution affinity chromatography, depicting the interactions of mobile (P, L) and immobilized (M) interactants. Here, the elution buffer contains a soluble ligand (L) that competes with matrix-bound ligand (M) for binding to the same active site of the mobile interactant (depicted here as a protein, P). Adapted from ref. 3.

of some of the early experimental data which have helped verify the theory. Finally, a case study is summarized for the use of analytical affinity chromatography to define the multiple interactions of the neurohypophysial hormones and neurophysins, including ways the chromatographic approach has helped in defining molecular mechanisms of the biosynthetic pathways for these neuroendocrine peptides and proteins.

FORMULATIONS RELATING ELUTION VOLUMES AND EQUILIBRIUM BINDING PROPERTIES IN QUANTITATIVE AFFINITY CHROMATOGRAPHY

The zonal elution approach of analytical affinity chromatography was developed essentially in parallel with the continuous elution/frontal analysis approach (Figs. 1 and 2). The discussion given below summarizes formulations elaborated recently by Swaisgood and Chaiken [11] for both zonal and continuous elution methods. See Appendix for summarized definitions of terms.

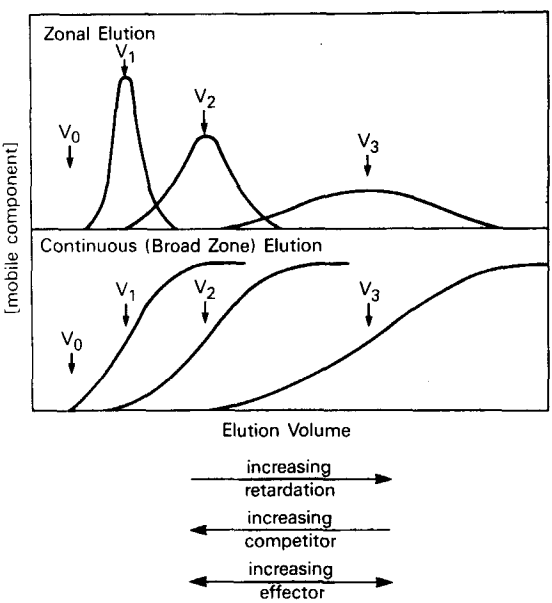


Fig. 2. Schematic representation of expected elution behavior in (top) zonal elution and (bottom) continuous elution quantitative affinity chromatography. Using the case of Fig. 1, zonal quantitative affinity chromatography is performed by introducing a small-volume aliquot of protein (mobile component) solution in the presence of varying concentration of soluble ligand to an affinity column of immobilized ligand (the column is pre-equilibrated with buffer with appropriate concentration of soluble ligand) and eluting with buffer containing soluble ligand (but not mobile component) until the zone of protein is detected in the eluate. The elution volume (V) of the protein zone and the peak width decreases as the concentration of soluble competing ligand increases. Continuous (broad zone) elution is performed as above except that a relatively large volume of the solution of protein (mobile component, with or without competing ligand) is introduced continuously to the affinity column until the eluted solution shows a plateau of [mobile component] equal to the concentration in the solution applied to the column. In both cases, values of V can be used to determine quantitative binding properties of protein to immobilized ligand as well as to soluble competing ligand. Elutions also can be carried out in the presence of effectors which either may enhance (increase V) or suppress (decrease V) binding of mobile and immobilized components; in such cases, binding properties of effectors also can be determined.

Monovalent systems

The generalized scheme in Fig. 1 defines a set of competing monovalent binding reactions

$$L + P \overset{K_{L/P}}{\rightleftharpoons} LP\ ; \quad M + P \overset{K_{M/P}}{\rightleftharpoons} MP \tag{1}$$

Using the revised notation established recently [11], P is the mobile interactant, M is the matrix-immobilized interactant, L is a soluble component that competes with M for binding to P; LP and MP are non-covalent complexes formed between mobile and immobilized species, respectively, and $K_{L/P}$ and $K_{M/P}$ are dissociation constants for LP and MP, respectively. By defining the partitioning of P in the presence of immobilized interactant by

$$\sigma_P = \frac{[MP]}{[P_\beta] + [LP_\beta]} + \sigma_{0,P} = \frac{[MP]\ \sigma_{0,P}}{[P_\alpha] + [LP_\alpha]} + \sigma_{0,P} \tag{2}$$

and the general relationships of chromatography as

$$V_i = V_m + \sigma_i V_s \tag{3}$$

and

$$V_{o,i} = V_m + \sigma_{o,i} V_s \tag{4}$$

substitution of the expressions for molecular equilibria from eqn. 1 yields

$$\frac{V_o - V_m}{V - V_o} = \frac{K_{M/P}}{[M]_T} + \frac{K_{M/P}\,[L]}{K_{L/P}\,[M]_T} + \left(1 + \frac{[L]}{K_{L/P}}\right) \frac{[P]}{[M]_T} \tag{5}$$

Here, V is elution volume of P and V_o, V_m and V_s are unretarded, mobile phase and stationary phase volumes, respectively; $[M]_T$, [P], [L], [MP] and [LP] are concentrations of total immobilized interactant, mobile interactant, mobile competitor, MP complex and LP complex, respectively; σ_P, $\sigma_{0,P}$ and σ_i are partition coefficients, respectively, of P at non-zero $[M]_T$, of P at $[M]_T = 0$ and of species i. If $[P] << [M]_T$, a condition almost always achievable for zonal elution, and if [P] is sufficiently small so that $[L] >> [LP]$, eqn. 5 becomes

$$\frac{1}{V - V_o} = \frac{K_{M/P}}{[M]_T\,(V_o - V_m)} + \frac{K_{M/P}\,[L]_T}{K_{L/P}\,[M]_T\,(V_o - V_m)} \tag{6}$$

Eqn. 6 was used initially [1, 3] to evaluate quantitative affinity chromatographic data, by relating the variation of V, the experimentally measured elution volume for mobile interactant, to the total concentrations of immobilized and soluble competing ligands, $[M]_T$ and $[L]_T$, respectively. The values for $[M]_T$, V_o and V_m are constants defined for a particular matrix and physical arrangement of the column. $[L]_T$ also is experimentally measurable. Thus, the dissociation constants $K_{M/P}$ and $K_{L/P}$ can be obtained directly from experimental chromatographic data. For a specific affinity matrix of fixed $[M]_T$, a series of elutions is carried out in which $[L]_T$ is varied. The elution volumes of zones of mobile interactant P are determined at the

various values of $[L]_T$. From a plot of $1/(V - V_o)$ or $(V_o - V_m)/(V - V_o)$ versus $[L]_T$, values for $K_{M/P}$ and $K_{L/P}$ can be calculated as defined by eqn. 6. Thus, $K_{L/P}$ can be derived from the ratio of slope/ordinate intercept and $K_{M/P}$ from the intercept directly. Values for $K_{L/P}$ and $K_{M/P}$ also can be calculated non-graphically by linear least-squares regression analysis of the elution data.

When elutions are performed in the absence of competing soluble ligand ($[L]_T = 0$), eqn. 6 simplifies to

$$\frac{V_o - V_m}{V - V_o} = \frac{K_{M/P}}{[M]_T} \text{ or } \frac{1}{V - V_o} = \frac{K_{M/P}}{[M]_T (V_o - V_m)} \tag{7}$$

Thus, $K_{M/P}$ can be calculated independently of competitive elution.

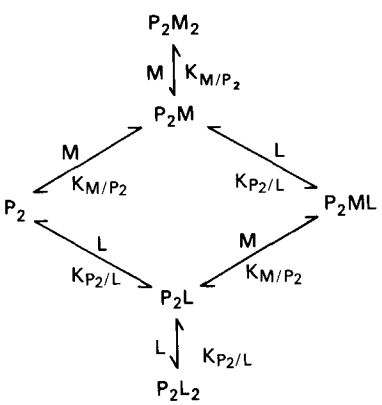
Eqns. 6 and 7 above are valid for quantitative purposes when the concentration of protein is small such that $[P]/[M]_T << 1$ and $[L]_T = [L]$. However, if this condition cannot be achieved experimentally, the last term in eqn. 5 cannot be neglected. Since the concentration of P is given by $[P] = [P]_T/(1 + [L]/K_{L/P})$, eqn. 5 becomes

$$\frac{V_o - V_m}{V - V_o} = \frac{K_{M/P} + [P]_T}{[M]_T} + \frac{K_{M/P}\,[L]}{K_{L/P}\,[M]_T} \tag{8}$$

Eqn. 8 is equivalent to an equation first derived by Nichol et al. [2] for this case. The value of $[P]_T$ is not definable (it changes continuously during elution) in zonal elution chromatography. Thus, when [P] is large, it is necessary to use the alternative procedure of continuous elution (or large zone) chromatography at constant $[P]_T$ and frontal analysis to evaluate the elution volume [2]. While continuous elution (or large zone) quantitative affinity chromatography is generally more time-consuming than zonal elution and can be impractical when the mobile interactant is available in only limited amounts, the method provides the benefit to evaluate chromatographic data when studies at high [P] are desirable or unavoidable.

Bivalent systems

Zonal elution chromatography also can be applied to evaluate bivalent binding systems, including the type in eqn. 9.



(9)

In a manner similar to that for monovalent binding systems and assuming all K_{M/P_2} and $K_{P_2/L}$ values are equal, this scheme leads [9, 10] to the formulation

$$\frac{1}{V - V_o} = \frac{1 + 2\left(\frac{[L]}{K_{P_2/L}}\right) + \left(\frac{[L]}{K_{P_2/L}}\right)^2}{(V_o - V_m)\left[2\left(\frac{[M]_T}{K_{M/P_2}}\right) + \left(\frac{[M]_T}{K_{M/P_2}}\right)^2 + 2\left(\frac{[L][M]_T}{K_{P_2/L}\,K_{M/P_2}}\right)\right]} \quad (10)$$

for sufficiently low concentrations of P_2. This expression allows microscopic dissociation constants, $K_{P_2/L}$ and K_{M/P_2}, to be evaluated for a bivalent binding species, P_2, by measuring V at varying [L]. However, unlike the case for monovalent binding systems, the variation at $1/(V - V_o)$ with [L] for bivalent systems is non-linear. Thus, the values of $K_{P_2/L}$ and K_{M/P_2} are derived from competitive elution data by non-linear least-squares regression analysis. Despite this difference, the experimental protocol for collecting data for a bivalent binding system is the same as for a monovalent system.

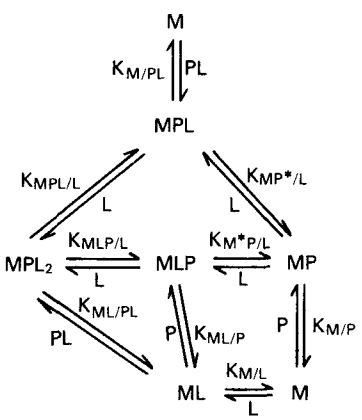
When zonal elutions are carried out without soluble ligand present ([L] = 0), eqn. 10 simplifies to

$$V - V_o = (V_o - V_m)\left[2\left(\frac{[M]_T}{K_{M/P_2}}\right) + \left(\frac{[M]_T}{K_{M/P_2}}\right)^2\right] \quad (11)$$

Thus, at [L] = 0, the value of K_{M/P_2} can be calculated directly from the data obtained in a single zonal elution.

Multiple equilibria with immobilized protein systems

Analytical affinity chromatography using a protein as the immobilized interactant offers the possibility to characterize cooperative protein—protein and protein—ligand interactions. Considering the specific case of neurophysin (M)—neurophysin (P) and neurophysin (M or P)—vasopressin (L) interactions [12—15], the equilibria occurring with immobilized P can be represented [10] as in eqn. 12.



(12)

Similar equilibria occur in the mobile phase when both P and L are present.

Protein self-association

For the system of eqn. 12, the elution volume of mobile protein on immobilized protein is a complicated function of the concentration of unbound ligand, L, involving all of the immobilized liganded protein dissociation constants. However, there are two experimentally accessible special cases [11].

Evaluation of self-association of unliganded protein by chromatography of the protein in the absence of ligand. When [L] = 0, only the equilibria defined by $K_{M/P}$ and $K_{P/P}$ need be considered. When $[P]_T << K_{M/P}$ and $K_{P/P}$ (zonal elution), the variation of V is as given in eqn. 7.

Evaluation of self-association of liganded protein by chromatography of protein in the presence of saturating ligand. When L is saturating, the only matrix-bound species present at significant concentrations are ML and MPL_2. Therefore, when $[P]_T << K_{ML/ML}$ and $K_{PL/PL}$ (zonal elution),

$$\frac{V_o - V_m}{V - V_o} = \frac{K_{ML/PL}}{[M]_T} \tag{13}$$

Protein—ligand interaction

When ligand is the mobile interactant with immobilized protein for the system of eqn. 12 and [P] = 0, zonal elution of L (at $[L]_T << [M]_T$) is analogous to that for elution of protein on immobilized ligand in the absence of soluble ligand (eqn. 7). Thus,

$$\frac{V_o - V_m}{V - V_o} = K_{M/L}/[M]_T \tag{14}$$

EXPERIMENTAL EXAMPLES OF ANALYTICAL AFFINITY CHROMATOGRAPHY TO MEASURE EQUILIBRIUM PARAMETERS FOR MONOVALENT AND BIVALENT INTERACTING COMPLEXES

Several protein—ligand and protein—protein interacting systems have been studied by zonal elution quantitative affinity chromatography during the development of this methodology. These include both monovalent (e.g. Staphylococcal nuclease—nucleotide, ribonuclease—nucleotide and glutamate dehydrogenase—drug) and bivalent (e.g. TEPC 15 immunoglobulin A bivalent monomer—phosphorylcholine) interacting complexes. Examples are shown in Figs. 3 and 4 for bovine pancreatic ribonuclease and immunoglobulin A monomer systems. Likewise, analyses using other elution approaches, including continuous elution, also have been made. Table I summarizes data obtained in several such studies. Importantly, the data show the correspondence of binding constants obtained by chromatographic and solution methods and therein emphasize the reliability of the analytical affinity chromatographic method to evaluate binding characteristics of macromolecular systems.

KINETICS OF THE INTERACTION BETWEEN SOLUBLE MOLECULES AND IMMOBILIZED SPECIES

In addition to equilibrium interaction constants which can be derived

TABLE I

COMPARISON OF DISSOCIATION CONSTANTS OBTAINED BY QUANTITATIVE AFFINITY CHROMATOGRAPHY WITH THOSE OBTAINED BY OTHER METHODS: A SELECTED LIST

Protein	Ligand[a]	Value chromatographic dissociation constant (M)		Dissociation constant by other methods		
		$K_{P/L}$[b]	$K_{M/P}$[c]	Value (M)	Method	Reference
Staphylococcal nuclease	pdTp	$2.5 \cdot 10^{-6}$		$2.5 \cdot 10^{-6}$	Equilibrium dialysis	3
				$5.9 \cdot 10^{-6}$	Kinetics as enzyme inhibitor	
	pdTpAP	$2.3 \cdot 10^{-6}$	$1.1 \cdot 10^{-6}$[d]	$2.5 \cdot 10^{-6}$	Kinetics as enzyme inhibitor	
	NPpdTp	$1.1 \cdot 10^{-5}$		$6.3 \cdot 10^{-6}$	Kinetics as enzyme inhibitor	
Bovine pancreatic ribonuclease	2′-CMP	$1.6 \cdot 10^{-5}$		$9.7 \cdot 10^{-6}$	Kinetics as enzyme inhibitor	5
	APpUp		$9.3 \cdot 10^{-6}$[d]	$1.7 \cdot 10^{-5}$	Kinetics as enzyme inhibitor	
Immunoglobulin A (TEPC 15), monovalent Fab[o]	Phosphoryl choline	$1.5—3.3 \cdot 10^{-6}$	$3.9—4.2 \cdot 10^{-6}$[e]	$3.0 \cdot 10^{-6}$	Equilibrium dialysis	9, 10
Immunoglobulin A (TEPC 15), bivalent monomer	Phosphoryl choline	$1.2—1.5 \cdot 10^{-6}$	$2.7—4.8 \cdot 10^{-6}$[e,f] $1.2 \cdot 10^{-6}$[e,g] $1.3 \cdot 10^{-7}$[e,h]	$2.0 \cdot 10^{-6}$	Equilibrium dialysis	9, 10
Bovine lactate dehydrogenase						
H_4 (heart)	NADH	$3.8 \cdot 10^{-7}$		$3.9 \cdot 10^{-7}$	Fluorescence titration	17
M_4 (muscle)	NADH	$1.1 \cdot 10^{-6}$		$2.0 \cdot 10^{-6}$	Quenching of protein fluorescence	
Rabbit muscle lactate dehydrogenase	NADH	$1.1 \cdot 10^{-5}$		$1.0 \cdot 10^{-5}$	Frontal gel filtration chromatography	18
Trypsin	N^{α}-Acetyl-Gly-Gly-Arg	$5.9 \cdot 10^{-4}$	$1.3 \cdot 10^{-4}$[i]	$4.7 \cdot 10^{-4}$[j]	Kinetics as enzyme inhibitor	4, 19

Trypsin	β-Amino-benzamidine	$1 \cdot 10^{-5}$	$1.2 \cdot 10^{-6}$	$8.2 \cdot 10^{-6}$	Kinetics as enzyme inhibitor	20
	Benzamidine	$1.6—3.9 \cdot 10^{-5}$		$1.8 \cdot 10^{-5}$	Kinetics as enzyme inhibitor	
α-Chymotrypsin	α-Chymotrypsin		$2.4 \cdot 10^{-5}$[k]	$2.4 \cdot 10^{-5}$	Ultracentrifugation	6
Bovine glutamate dehydrogenase	Perphenazine	$2 \cdot 10^{-6}$	$6 \cdot 10^{-6}$	$2.2 \cdot 10^{-5}$[l]	Kinetics as enzyme inhibitor	21
	Chlorpromazine	$4 \cdot 10^{-6}$		$3.2 \cdot 10^{-5}$[l]	Kinetics as enzyme inhibitor	
	Trifluperidol	$22 \cdot 10^{-6}$		$14 \cdot 10^{-5}$	Kinetics as enzyme inhibitor	
Rabbit muscle lactate dehydrogenase	Cibacron Blue		$0.3 \cdot 10^{-6}$[m]	$0.1—0.5 \cdot 10^{-6}$	Enzyme catalysis and spectrophotometric titration	22
	NADH	$3.4 \cdot 10^{-6}$		$0.5 \cdot 10^{-6}$	Enzyme catalysis and spectrophotometric titration	
Rabbit muscle lactate dehydrogenase	Cibacron Blue		ca. $0.5 \cdot 10^{-6}$[n]	$0.1—0.5 \cdot 10^{-6}$	Enzyme catalysis and spectrophotometric titration	23

[a] pdTp = Thymidine 3′,5′-diphosphate; pdTpAP = thymidine 3′-(*p*-aminophenylphosphate)-5′-phosphate; NPpdTp = thymidine 3′-phosphate-5′-(*p*-nitrophenylphosphate); 2′-CMP = cytidine 2′-monophosphate; APpUp = uridine 5′-(4-aminophenylphosphate)-2′,3′-phosphate.

[b] Dissociation constant for soluble ligand, as determined by competitive elution.

[c] Dissociation constant for immobilized ligand.

[d] For ligand immobilized to agarose through aminophenyl moiety.

[e] For ligand immobilized to agarose through a glycyl(azophenyl)tyrosyl arm on the phosphate moiety.

[f] Microscopic K_{M/P_2} determined using eqn. 10.

[g] Functional $K_{M/P}$ determined at $[M]_T = 9 \cdot 10^{-6}$ *M* using eqn. 6.

[h] Functional $K_{M/P}$ determined at $[M]_T = 5 \cdot 10^{-5}$ *M* using eqn. 6.

[i] For ligand immobilized to agarose through α-amino of Gly-Gly-Arg.

[j] Determined at pH 6.0, versus pH 6.2 and 6.0 for corresponding $K_{P/L}$ and $K_{M/P}$, respectively.

[k] For chymotrypsin immobilized at pH 8.

[l] Relative hierarchy of chromatographically derived dissociation constants is same as that for kinetically derived values; quantitative differences in kinetic versus chromatographic values observed consistently, suggesting contribution of matrix.

[m] Corrected for functional capacity.

[n] Value obtained after correcting for observed degree of bivalency.

[o] Papain fragment of TEPC 15 antibody, which contains phosphoryl choline binding site.

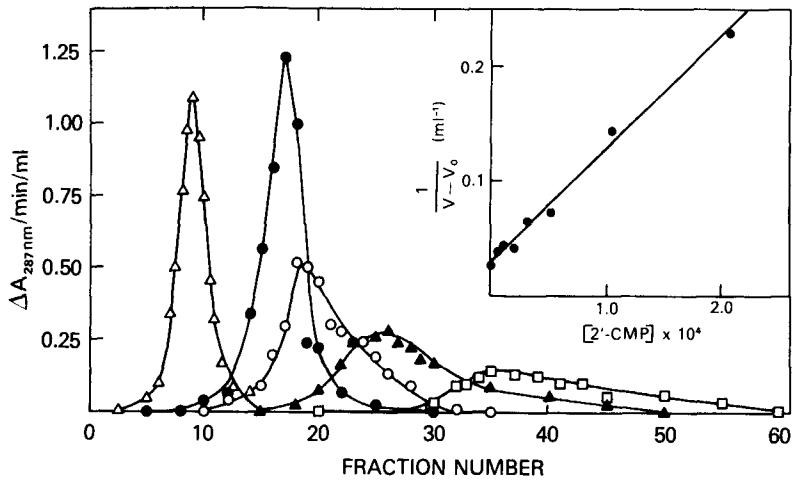


Fig. 3. Competitive elution profiles of 2.8 mg of RNase A on Sepharose—APpUp, in 0.4 *M* ammonium acetate, pH 5.2, and ambient temperature, containing various concentrations of cytidine 2′-monophosphate (2′-CMP) as follows: (▲) $2.1 \cdot 10^{-4}$ *M*; (○) $5.2 \cdot 10^{-5}$ *M*; (■) $3.2 \cdot 10^{-5}$ *M*; (△) $2.0 \cdot 10^{-5}$ *M*; (●) $5.7 \cdot 10^{-6}$ *M*. $\Delta A_{287\,nm}$/min/ml is enzymic activity against cytidine 2′,3′-monophosphate. Inset, plot of data according to eqn. 6, with the solid line representing the best fit calculated by least squares. For data obtained at $1.0 \cdot 10^{-4}$, $1.0 \cdot 10^{-5}$ and 0 *M* 2′-CMP, the derived points are included in the inset plot but the corresponding elution profiles are omitted from the main figure for clarity. Adapted from ref. 16.

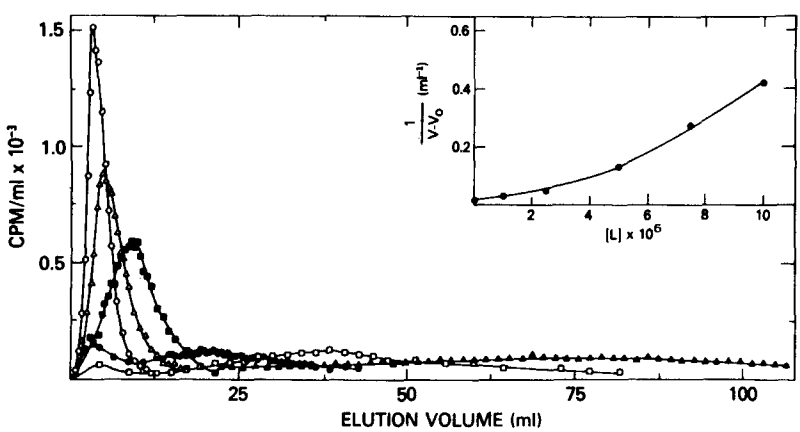


Fig. 4. Competitive zonal elution affinity chromatography for a bivalent binding system. Zones (100 μl) of [^{14}C]dIgA monomer (bivalent binding) were applied to high-density, $5 \cdot 10^{-5}$ *M* phosphorylcholine—Sepharose (25 × 7 mm) equilibrated in phosphate-buffered saline (with 1 mg/ml bovine serum albumin) and containing soluble phosphorylcholine at the following concentrations; 0 *M* (▲), $1 \cdot 10^{-6}$ *M* (□), $2.5 \cdot 10^{-6}$ *M* (●), $5.0 \cdot 10^{-6}$ *M* (■), $7.5 \cdot 10^{-6}$ *M* (△) and $1.0 \cdot 10^{-6}$ *M* (○). Elutions were carried out at room temperature with buffer containing the indicated amount of soluble competitive phosphorylcholine. Inset: elution data are plotted as $1/(V - V_0)$ versus [phosphorylcholine]. Dissociation constants, $K_{P/L}$ and $K_{M/P}$, were calculated from the non-linear plot using eqn. 10 and are shown in Table I. Taken from refs. 9, 10.

from the mean elution volume, the spreading of the zone for zonal elution chromatography also contains information related to the kinetics of association and dissociation of the mobile component—immobilized component interaction. There have been two theoretical treatments of such zone dispersion which have provided equations for evaluation of the association and dissociation rate constants, that of Denizot and Delaage [24] and that of Hethcote and DeLisi [25—28]. The kinetics of the dimerization of bovine neurophysin II (BNPII) and of its binding to the neuropeptide Arg^8-vasopressin (AVP) have been examined, using both of the above theoretical treatments, by analytical high-performance affinity chromatography with BNPII immobilized on non-porous glass beads and silica [11, 29, 30]. In the results obtained so far, the rate constants obtained by analytical affinity chromatography for both neurophysin dimer dissociation and AVP—neurophysin dissociation were smaller by several orders of magnitude than those observed for soluble dimers and hormone-neurophysin complexes, respectively. However, the relative rates of dissociation of AVP and BNPII from BNPII monomers were the same for the immobilized monomers and for monomers in solution.

CHARACTERIZATION OF THE MULTIPLE INTERACTIONS IN THE NEUROPHYSIN—NEUROHYPOPHYSIAL HORMONE SYSTEM: A CASE STUDY

Analytical affinity chromatography provides a flexible general approach to correlate interactions in multimolecular assemblies, including those for which different types of interactions modulate one another. There are no basic limitations on the size of molecular species which are immobilized or eluted as mobile interactants. Thus, a unified study can be made even when different interactions in the system involve molecules of sizes which obviate the use of other methods, such as equilibrium dialysis and ultracentrifugation. In addition, the chromatographic approach allows multiple interactions to be measured under similar experimental conditions (solvent, temperature, etc.), thus providing a unified quantitative description of a multimolecular system.

The study of multiple equilibria which occur in the above-mentioned complexes between the neuroendocrine peptide hormones oxytocin and vasopressin and the neurophysins, and in the related complexes of the biosynthetic precursors of these peptides and proteins, represents a useful case study of the application of analytical affinity chromatography [29—35]. Both peptide—protein and protein—protein interactions can be measured. Cooperative effects between these two types of interactions can be evaluated. And, the relationship between the cooperative properties of the hormone—neurophysin complexes and the molecular organization of hormone—neurophysin common biosynthetic precursors can be examined.

Oxytocin, vasopressin and the neurophysins are produced from common biosynthetic precursors both in the central nervous system and peripherally [13—15, 36—42]. In the major pathway quantitatively, the hypothalamo-neurohypophysial tract, the ultimate products of biosynthesis (Fig. 5) are a set of cooperatively interacting peptide—protein complexes, one for oxytocin

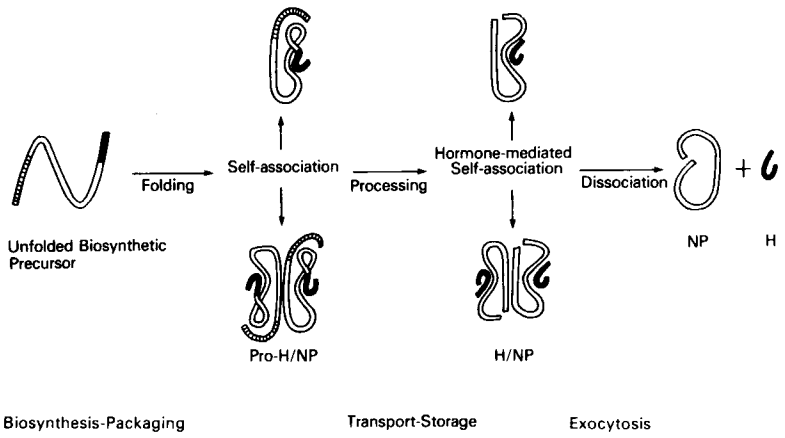


Fig. 5. Scheme depicting relationship of biosynthetic precursor structure to molecular events occurring in neurohypophysial hormone (H)/neurophysin (NP) biosynthesis. The filled and open lines denote hormone and neurophysin segments, respectively. The hatched line represents the C-terminal glycopeptide occurring in pro-AVP/BNPII. Folding of the precursors is visualized to lead to self-association through the NP demains of the precursors. The NP/NP and H/NP interaction surfaces are retained after enzymatic processing, the latter of which leads to formation of non-covalent complexes between H and NP as well as dimers in secretory granules until released exocytotically. Taken from ref. 35.

and its biosynthetically associated neurophysin and a second (produced in separate neurons) for vasopressin and its biosynthetically associated neurophysin. In the mature complexes, which act as storage forms of peptides and proteins produced in the neuroendocrine pathway, self-association of neurophysin and hormone—neurophysin interaction mutually modulate one another in a manner which likely is related to the molecular organization of the precursors from which they form (see Fig. 5). It has been possible to use the analytical affinity chromatographic method to characterize the mature complexes and to begin to correlate the properties of these forms to molecular properties of their biosynthetic precursors.

Characterization of mature neuropeptide hormone—neurophysin complexes

Two types of affinity matrices have been used in the examination of interactions in the neuropeptide hormone—neurophysin system. As shown in Fig. 6, top, elution of neurophysin on immobilized peptide ligand allows measurement of neurophysin—ligand interactions. Soluble hormone—neurophysin interactions as well as the effects of neurophysin self-association on ligand binding can be evaluated. Both immobilized vasopressin (Lys^8-linked) [43] and immobilized Met-Tyr-Phe (α-carboxyl-linked, the tripeptide is a neurophysin-binding analogue of the N-terminal sequence of vasopressin) [31] have been available for these studies. Elutions on immobilized neurophysin also can be performed (Fig. 6 bottom) allowing measurement of self-association and the effects of peptide—ligand binding on self-association.

Zonal elution of bovine NPII (vasopressin-associated) on Met-Tyr-Phe-aminoalkylagaroses shows behavior which fits a cooperative binding scheme distinct from strictly monovalent or bivalent models. Elution volumes do decrease generally as the concentration of competitive peptide (e.g. oxytocin

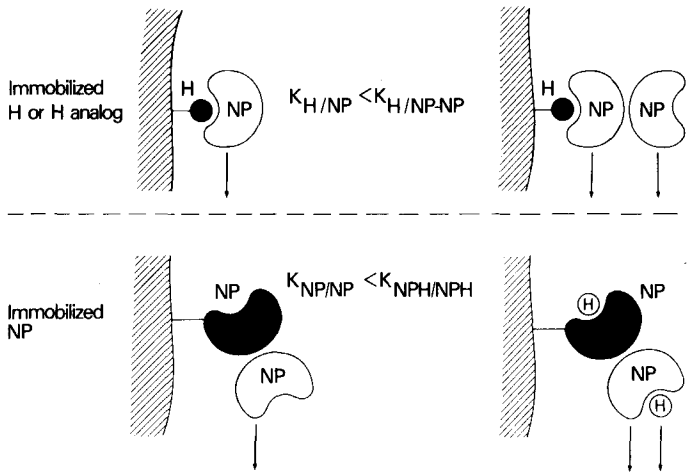


Fig. 6. Schematic diagram of quantitative affinity chromatographic analyses of interactions in the neurohypophysial hormone (H)/neurophysin (NP) system carried out with affinity matrices containing, top, immobilized peptide hormone or hormone analogue (H analogue) and, bottom, immobilized neurophysin. Affinity matrix preparations have been described [28–31, 42].

or vasopressin) is increased in the elution buffer [32]. However, when sets of competitive elution data are plotted as $1/(V - V_o)$ versus [L], curvilinearity at low [L] is observed in most cases. The reason for this behavior rests with the fact that neurophysin exists as a mixture of low-affinity monomer and high-affinity dimers, with the degree of dimerization increased by peptide–ligand binding. Thus, while elution of neurophysin at zero soluble ligand reflects affinity matrix binding of a mixture containing a significant amount of monomers, the more-retarded-than-expected elution in the presence of low concentrations of soluble peptide reflects elution of mixtures progressively more enriched in high-affinity dimers. Thus, elution volumes decrease more gradually with increasing but low soluble peptide concentrations than expected from the degree of competition of the soluble peptides with affinity matrix for binding to the mobile neurophysin [32]. Equilibrium binding constants can be calculated from these data at both [L] = 0 and at [L] $\neq$ 0, as given in Table IIa–d and below.

The higher peptide affinity of dimers than monomers can be expressed quite clearly as a strong dependence of neurophysin elution volume on the amount (i.e. concentration) of neurophysin in the initial zone. Thus, as shown in Fig. 7, increasing zonal protein concentrations leads to increased retardation. Values of $1/K_{M/P}$ vary sigmoidally with zonal concentration of neurophysin, allowing an estimation (see Table IIa–d) of immobilized tripeptide ligand affinities for neurophysin monomer (at lowest concentrations of mobile NP) and dimer (at high concentration of mobile NP).

Neurophysin dimerization has been measured by elution of neurophysin on immobilized neurophysin. As shown in the Fig. 8 inset, zonal elution of [^{125}I]BNPII in buffer (containing no soluble peptide ligand) shows a retardation displaced sufficiently from V_o to allow calculation of a dimerization

TABLE II

REPRESENTATIVE ZONAL ELUTION ANALYTICAL AFFINITY CHROMATOGRAPHIC DATA OBTAINED FOR NEUROPHYSIN SELF-ASSOCIATION AND NEUROPHYSIN—PEPTIDE INTERACTIONS USING IMMOBILIZED PEPTIDE AND NEUROPHYSIN AFFINITY MATRICES

Experiment	Mobile interactant	Amount in zone (μg)	Ligand immobilized on affinity matrix	$[M]_T$ (M)	Competitive ligand or effector	$K_{M/P}$*	$K_{M/P}$**	$K_{P/L}$**	Reference
a	[^{125}I]BNPII	<1	Met-Thy-Phe-aminobutyl	$3.0 \cdot 10^{-4}$	LVP (varying)	$4.8 \cdot 10^{-5}$	$4.7 \cdot 10^{-5}$	$6.6 \cdot 10^{-5}$	32
b		<1	Met-Tyr-Phe-aminohexyl	$5.9 \cdot 10^{-4}$	OT (varying)	$5.1 \cdot 10^{-5}$	$4.3 \cdot 10^{-5}$	$6.2 \cdot 10^{-5}$	32
c		41.5	Met-Tyr-Phe-aminobutyl	$3.0 \cdot 10^{-4}$	LVP (varying)	$1.4 \cdot 10^{-5}$	$8.9 \cdot 10^{-6}$	$1.4 \cdot 10^{-5}$	32
d		101	Met-Tyr-Phe-aminohexyl	$5.9 \cdot 10^{-5}$	OT (varying)	$6.1 \cdot 10^{-6}$	$7.0 \cdot 10^{-7}$	$1.8 \cdot 10^{-6}$	32
e	[^{125}I]BNPII	<1	BNPII	$1 \cdot 10^{-4}$	None	$1.4 \cdot 10^{-5}$			33
f		<1	BNPII	$1 \cdot 10^{-4}$	None	$1.6 \cdot 10^{-5}$			32
g					LVP ($1 \cdot 10^{-4}$ M)	$4.4 \cdot 10^{-7}$			33
h	[^{14}C-diAcet] BNPI	<1	BNPII	$7 \cdot 10^{-5}$	None	$7.7 \cdot 10^{-6}$			35
i					LVP ($1 \cdot 10^{-4}$ M)	$5.9 \cdot 10^{-8}$			35
j		<1	Met-Tyr-Phe	$8.5 \cdot 10^{-3}$	None	$1.2 \cdot 10^{-4}$			35
k	[^{14}C-diAcet, desHis 106] pro-OT/BNPI	<1	Met-Tyr-Phe	$8.5 \cdot 10^{-3}$	None	$5.3 \cdot 10^{-3}$			35
l		<1	BNPII	$7 \cdot 10^{-5}$	None	$5.9 \cdot 10^{-7}$			35
m					LVP ($1 \cdot 10^{-4}$ M)	$7.1 \cdot 10^{-8}$			35
n	[^{125}I]BNPII	2.5	BNPII	$1 \cdot 10^{-4}$	None	$1.3 \cdot 10^{-5}$			34
o	[^{125}I]BNPI	1	BNPII	$1 \cdot 10^{-4}$	None	$5.6 \cdot 10^{-6}$			34

*Calculated at [L] = 0 (with or without effector) with eqn. 12 or equivalent.
**Calculated at [L] ≠ 0 with eqn. 11 or equivalent.

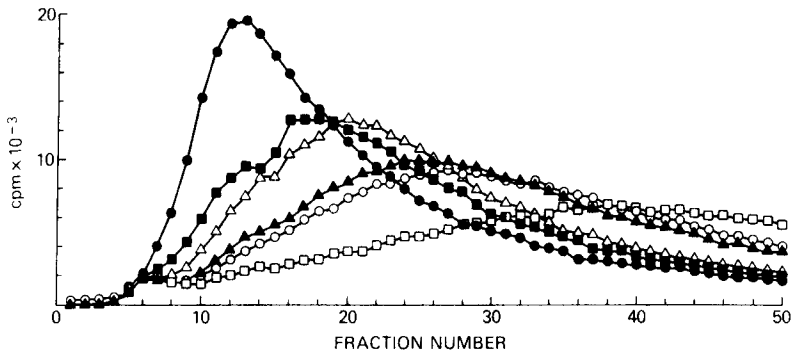


Fig. 7. Effect of concentration of applied bovine neurophysin II (BNPII) on zonal elution behavior on Met-Tyr-Phe-aminobutylagarose. Zones (100 μl) containing < 1 μg of [^{125}I]-BNPII and unlabeled BNPII (varying amounts as specified) were eluted in 0.4 *M* ammonium acetate, pH 5.7, in the absence of soluble competitive ligand. Each continuous profile represents a separate elution with the following amounts of added unlabeled BNPII per zone: 0 μg (•), 6.35 μg (▪), 10.3 μg (△), 20.7 μg (▲), 41.3 μg (○) and 82.6 μg (□). Elutions were carried out at room temperature. Taken from ref. 32.

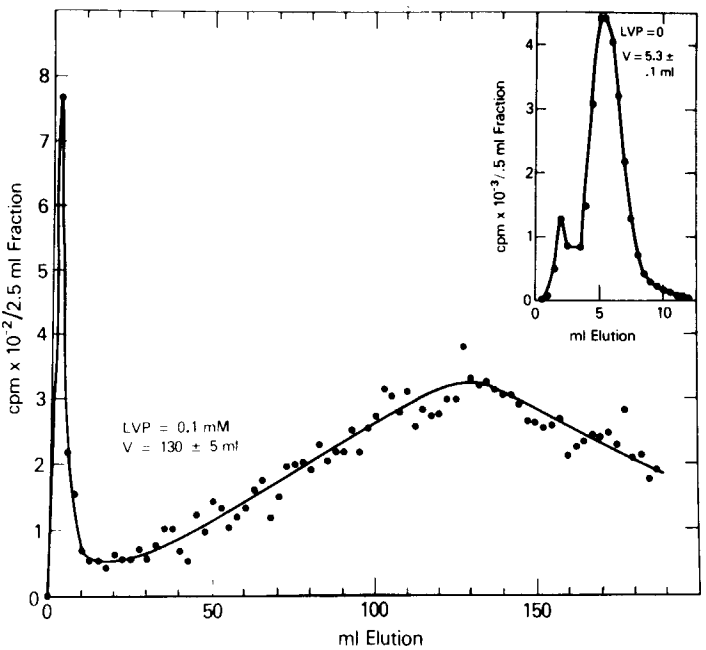


Fig. 8. Elution behavior of [^{125}I]BNPII on BNPII–Sepharose in the presence and absence of soluble peptide ligand, lysine-vasopressin (LVP). Zones (300 μl) containing ca. 1 μg of [^{125}I]BNPII were applied to BNPII–Sepharose (1.5 ml bed volume, $[M]_T = 1.4 \cdot 10^{-4}$ *M*) equilibrated with 0.4 *M* ammonium acetate, pH 5.7, containing 0.1 m*M* LVP (main diagram) or in the absence of added LVP (inset). The zonal elution volumes were used to calculate $K_{M/P}$ (dissociation constants for protein–protein interaction) using eqn. 7. These values are shown in Table II e and g and Table III. Taken from ref. 33.

constant as $K_{M/P}$ (see Table IIe and f). By including soluble peptide ligand in the elution buffer, dimerization dissociation constants also can be determined for partially or fully liganded neurophysin. The main elution profile

of Fig. 8 shows the case of close-to-saturating lysine vasopressin. For this liganded case, $K_{M/P}$ is much smaller (higher affinity) than that in buffer alone (see Table IIg). Thus, here as with the peptidyl affinity matrix, zonal elution of neurophysin can be used to measure the degree of modulation of binding affinities in the cooperative complexes between neurophysins and peptide hormones.

Analytical affinity chromatographic elutions on immobilized peptide ligand and neurophysin matrices allow calculation of a set of dissociation constants for self-association and peptide—ligand binding. These constants, compiled in Table III, fit with the cooperativity linkage diagram, in Fig. 9, which describes the interrelationship between non-covalent interactions which occur in mature neuropeptide hormone—neurophysin complexes. The relationship of binding parameters argues that, in the secretory granules in which these complexes are produced and stored, the polypeptide system is driven to the fully self-associated, fully liganded form.

The matrix systems used as described above to study the NPII—NPII self-association and NPII—peptide ligand interactions also can be used to evaluate binding characteristics of other native neurophysins (including formation of mixed hybrids), of sequence-modified neurophysins and of peptide hormone analogues. For example, the ability of different species of neurophysins to self-associate with one another can be evaluated directly by the degree of retardation on immobilized neurophysin or by the degree to which the species induces increased retardation of a labelled neurophysin on immobilized ligand. Such studies have demonstrated [34] the formation of mixed hybrids between

TABLE III

SUMMARY OF DISSOCIATION CONSTANTS FOR NEUROPHYSIN—NEUROPEPTIDE HORMONE COMPLEXES AND BIOSYNTHETIC PRECURSORS DETERMINED BY ANALYTICAL AFFINITY CHROMATOGRAPHY

Dissociation constant	Interaction process (immobilized component/mobile component)	Value of dissociation constant (M)	Reference
$K_{P/P}$	BNPII—[^{125}I]BNPII	$1.4 \cdot 10^{-5}$	33
		$1.6 \cdot 10^{-5}$	32
		$1.3 \cdot 10^{-5}$	34
	BNPII—[^{125}I]BNPI	$5.6 \cdot 10^{-6}$	34
	BNPII—[^{14}C-diAcet]BNPI	$7.7 \cdot 10^{-6}$	35
$K_{PL/PL}$	BNPII—[^{125}I]BNPII + LVP	$4.4 \cdot 10^{-7}$	33
	BNPII—[^{14}C-diAcet]BNPI + LVP	$5.9 \cdot 10^{-8}$	35
$K_{P/Pro}$	BNPII—[^{14}C-diAcet]Pro-OT/BNPI	$5.9 \cdot 10^{-7}$	35
$K_{PL/Pro}$	BNPII—[^{14}C-diAcet]Pro-OT/BNPI + LVP	$7.1 \cdot 10^{-8}$	35
$K_{P/L}$	Met-Tyr-Phe—[^{125}I]BNPII (low μg in Fig. 7; no competitor)	$0.5—1 \cdot 10^{-4}$	32
	Met-Tyr-Phe—[^{125}I]BNPI (no competitor)	$1.2 \cdot 10^{-4}$	34
$K_{PP/L}$	Met-Tyr-Phe—[^{125}I]BNPII (high μg in Fig. 7; no competitor)	$1 \cdot 10^{-5}$	32
$K_{PPL/L}$	Met-Tyr-Phe—[^{125}I]BNPII (high μg; with competitor)	$7 \cdot 10^{-7}$	32

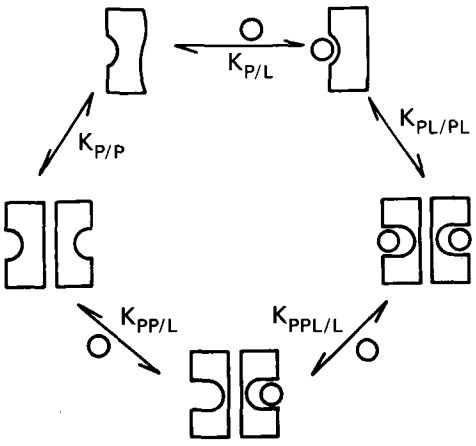


Fig. 9. Scheme of cooperative relationship between peptide—ligand (○) binding and protein self-association in the neurophysin—hormone system. $K_{P/L}$, $K_{PP/L}$, and $K_{PPL/L}$ are affinity constants of ligand for neurophysin monomer, unliganded dimer and singly liganded dimer, respectively. $K_{P/P}$ and $K_{PL/PL}$ are dissociation constants for self-association of, respectively, unliganded and liganded neurophysin monomers. The scheme denotes the relationship between intermolecular hormone binding and self-association occurring in mature hormone–neurophysin non-covalent complexes. A similar relationship pertains for precursor between intramolecular hormone domain—neurophysin domain interaction and precursor self-association. Adapted from ref. 15.

bovine NPI (oxytocin-associated in vivo) and bovine NPII (vasopressin-associated in vivo), with a $K_{NPII/NPI}$ of $5.6 \cdot 10^{-6}$ M versus $K_{NPII/NPII}$ of $1.3 \cdot 10^{-5}$—$1.6 \cdot 10^{-5}$ M for NPII homologous dimerization (see also Table IIe, f, n and o). These and related results have shown that the self-association surface is a common and homologous structural feature among native neurophysins of different sequence and species. In addition, affinity chromatographic behavior on both immobilized neurophysin and immobilized peptide ligand have been used to show that active-site photolabelled NPII has a greatly reduced ability to self-associate with immobilized NPII. The results with photolabelled neurophysin have led not only to the conclusion that the self-association surface is distorted in the derivative (even though the ligand binding site is occupied) but also to the general view that cooperativity between non-covalent peptide ligand binding and self-association surfaces can be used as a sensitive signal of molecular organization in native neurophysin.

Precursor interactions and molecular organization

The quantitative differences in self-association of liganded versus unliganded neurophysin allow predictions about the chromatographic behavior of biosynthetic precursors that can be used to gain insight into the molecular organization of these latter molecules. If the precursors fold into well defined conformations, it is likely that these would mimic the structure of liganded neurophysin. This seems especially likely with the oxytocin/neurophysin

precursor, which is composed of hormone and neurophysin domains with a tripeptide spacer linkage between them and a single His residue at the carboxyl terminus [35]. Thus, if folded precursors have accessible self-association surfaces, the precursor would be expected to self-associate and the affinity of this self-association would be close to that of liganded neurophysin.

This idea was tested by analytical affinity chromatography using [diacetimidyl-30,71, des His 106]pro-OT/NPI, a semisynthetic oxytocin/bovine neurophysin I precursor analogue which differs from the native precursor only in missing C-terminal His 106 (assumed unlikely to be important for self-association) and in having acetimidyl protecting groups at the two ϵ-NH_2 groups in the neurophysin domain [34]. The data of Fig. 10A and B show that diacetimidyl NPI can associate with immobilized NPII, with affinities in the presence and absence of hormone similar to the corresponding values for unprotected NPII (Table II h and i). The chromatographic retardation data for semisynthetic pro-OT/NPI (Fig. 10 and Table II l and m) show that the precursor can bind to immobilized neurophysin, with the latter in either the unliganded (Fig. 10A) or liganded (Fig. 10B) form. Most notable is that the quantitative degree of association of precursor to unliganded immobilized NPII is greater than that of unliganded NPII to unliganded immobilized NPII (Fig. 10A); and, the association of precursor to liganded immobilized NPII is closely related to that of liganded NPII to liganded immobilized NPII (Fig. 10B). The enhanced self-association potential of hormone/neurophysin precursor also was observed for pulse-labelled rat provasopressin/neurophysin by showing binding of the latter to unliganded immobilized NPII [32]. The affinity chromatographic results argue that the neuropeptide hormone/neurophysin precursors, with self-association properties similar to liganded neurophysin, fold into structures with a well defined intramolecular hormone domain—neurophysin domain contact mimicking the intermolecular hormone—neurophysin interaction in mature complexes (Fig. 5). In secretory granules, the biosynthetic precursors for oxytocin, vasopressin, and neurophysins likely form self-associated complexes; and it is in these self-associated forms that the precursors likely are processed enzymatically to produce mature neuropeptide hormones and neurophysins. Such a view helps define the molecular transitions which occur in the class of peptide secreting cells for neurohypophysial hormones and neurophysins.

The folding properties of neuropeptide/neurophysin precursors also can be established by analytical affinity chromatography. The proposed mechanism of biosynthetic origin (Fig. 5) predicts that the precursors, with eight potential disulfide bonds, fold spontaneously upon in vivo translation to form the native disulfide-bonded form. Given this prediction, pro-hormone/neurophysin, like proinsulin, chymotrypsinogen and other disulfide-containing precursors, should be able to maintain correct disulfides in disulfide interchange conditions. Neurophysin (with seven disulfides), like insulin and chymotrypsin, cannot maintain native disulfides under interchange conditions, indicating an inability to fold spontaneously [35, 44, 45]. Chromatographic analysis of semisynthetic pro-OT/NPI on immobilized NPII after treatment with reducing agent shows no decrease in self-association [34]. In contrast,

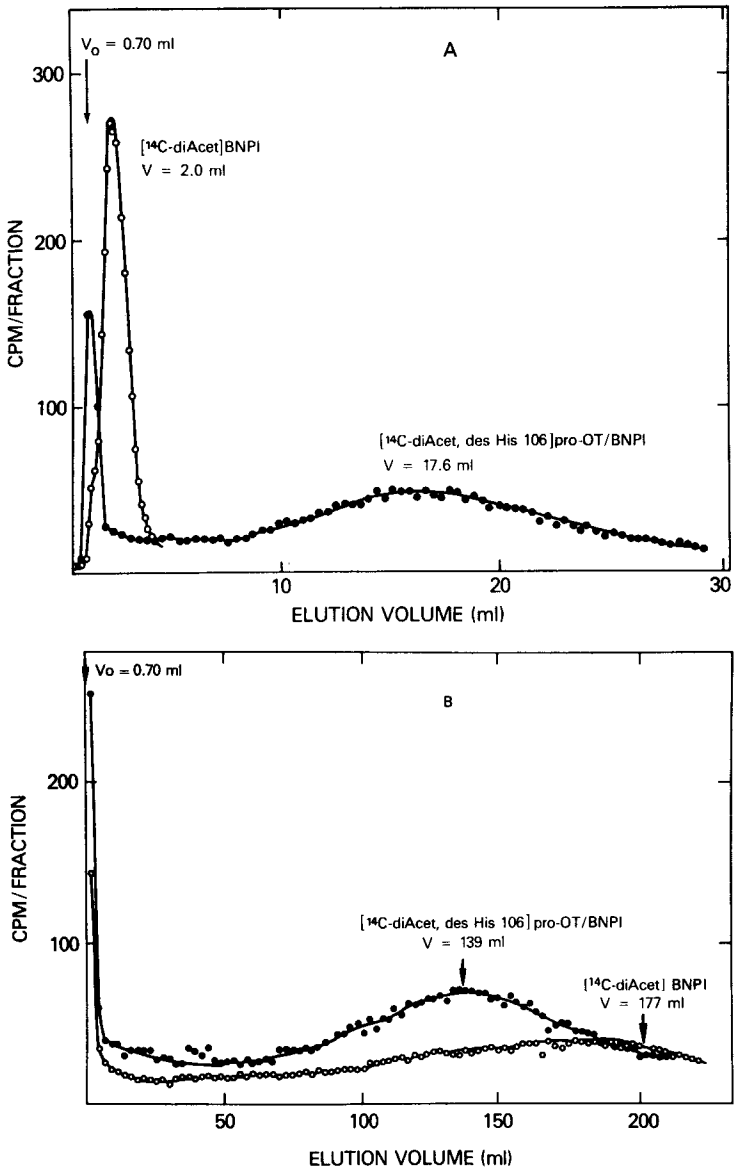


Fig. 10. Analytical affinity chromatography elutions of semisynthetic precursor on BNPII—Sepharose. Zones containing 1500—3000 cpm (< 0.5 μg) of [^{14}C-diAcet]BNPI and [^{14}C-diAcet,des His 106]pro-OT/BNPI were eluted on BNPII—Sepharose (70 nmol BNPII per ml of bed volume, 198 μl bed volume; prepared as before [32] with 0.4 *M* ammonium acetate containing 0.5% bovine serum albumin (pH 5.7) in the absence (A) and the presence (B) of 0.1 m*M* LVP. Other conditions were as follows: Fraction size: (A) 4 drops (157 μl) for BNPI and 10 drops (391 μl) for the precursor; (B) 60 drops (2.35 ml) for both elutions. Flow-rate: 5 ml/h; temperature: ambient. Taken from ref. 35.

neurophysin so treated loses its ability to bind peptide ligand. The results argue that, as shown in Fig. 5, hormone/neurophysin precursor folding ensues upon biosynthetic assembly, providing a form which self-associates upon packaging into secretory granules before enzymatic processing within granules.

CONCLUDING COMMENTS

Analytical affinity chromatography provides an effective methodology to measure quantitative properties of macromolecular interactions. A close quantitative relatedness of chromatographically obtained equilibrium constants and analogous constants determined fully in solution has been found for a growing number of proteins. This consistently observed correlation has formed the basis for extending the theoretical treatments to describe chromatographic behavior not only of monovalent molecular systems of varying types but also of multivalently interacting macromolecules, including those which exhibit cooperativity. Quantitative use of affinity chromatography as a micromethod offers a biochemical analysis tool to characterize biologically interesting molecules which are available in only relatively small amounts, a circumstance likely to become increasingly important given our rapidly expanding discovery of such species. The potential to measure chemical rate constants by affinity chromatography has been recognized, and experimental tests of the available theory are being made. While the relatedness of rate constants derived chromatographically to the interactions of macromolecules in their biologically meaningful environments needs more study, the potential value of being able to measure such kinetic parameters by the relatively simple experimental approach of zonal elution analytical affinity chromatography makes this evaluation worthwhile. The development of affinity chromatographic systems, including designing matrices and elution conditions, historically has been dependent on having at least a rudimentary understanding of the interaction characteristics (most often in solution) of the isolated molecules to be utilized as the affinity matrix—mobile interactant system. The development of analytical affinity chromatography now offers the opportunity to use matrix—mobile interactant systems to study mechanisms of biomolecular interactions and therein to obtain an understanding of such interactions which often is not easily obtained by solution approaches alone.

APPENDIX: GLOSSARY OF TERMS

L	Soluble interacting molecule
P	Mobile interactant
M	Immobilized interactant
$K_{I/J}$	Dissociation constant of binary complex of molecules I and J
σ_i	Equilibrium partition coefficient for molecule interacting with affinity matrix
$\sigma_{0,i}$	Equilibrium partition coefficient for a molecule unretarded on affinity matrix
α	Denotation of stationary phase
β	Denotation of mobile phase
V	Elution volume of mobile interactant
V_i	Elution volume of mobile interactant i
V_o	Elution volume of unretarded molecule
V_m	Volume of the mobile phase
V_s	Volume of the stationary phase (pore volume in the case of porous matrix)

$[M]$	Concentration of unliganded immobilized interactant
$[M]_T$	Total concentration of immobilized interactant
$[P]_T$	Total concentration of mobile interactant
$[P]_T^0$	Total initial concentration of a mobile interactant (in zonal elution, the initial concentration in the zone introduced to top of affinity column)
$[L]$	Concentration of soluble, free interacting molecule
$K_{IJ*/K}$	Dissociation constant of a ternary complex IJK, into IJ + K, in which dissociation of molecule K is from molecule J of the IJ complex

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