Analytical High-Performance Affinity Chromatography: Evaluation by Studies of Neurophysin Self-Association and Neurophysin–Peptide Hormone Interaction Using Glass Matrices

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ABSTRACT: Bovine neurophysin II (BNP II) was covalently immobilized on both nonporous and porous (200-nm pore diameter) glass beads and incorporated in a high-performance liquid chromatograph to evaluate analytical high-performance affinity chromatography as a microscale method for characterizing biomolecular interactions. By extension of the theoretical treatment of analytical affinity chromatography, both the self-association of neurophysin and its binding of the peptide hormone vasopressin were characterized by using a single chromatographic column containing immobilized neurophysin predominantly in the monomer form. Both [^{3}H][Arg^8]vasopressin (AVP) and ^{125}I-BNP II were rapidly eluted (<25 min). The relatively symmetrical elution peaks obtained allowed calculation of both equilibrium dissociation constants and kinetic dissociation rate constants. The dissociation constant measured chromatographically for the AVP-immobilized neurophysin complex, $K_{M/L}$ = 11 μM with porous glass beads and 75 μM with nonporous glass (NPG) beads, was in reasonable agreement with those previously obtained by curve fitting of Scatchard plots (16–20 μM) and from binding to [BNP II]Sepharose (50 μM). The values obtained are larger than that for dissociation of AVP from BNP II dimer, by a factor consistent with the intended nature of immobilized BNP II as monomers. Chromatography of BNP II on the [BNP II]NPG gave a dimer dissociation constant of 166 μM, a value in excellent agreement with that derived from equilibrium sedimentation studies (172 μM). In contrast to the agreement of chromatographic equilibrium binding constants with those measured in solution, the dissociation rate, k_{-3}, determined from the variance of the affinity chromatographic elution profile with nonporous beads, was several orders of magnitude smaller than the solution counterpart. This latter difference may reflect the probability of rebinding to contiguous sites immobilized on a surface, a feature which would be related to that for contiguous sites on a membrane.

Biomolecular recognition is widely used, in affinity chromatography, to separate biologically active molecules (Cuatrecasas et al., 1968; Porath & Kristiansen, 1975; Chaiken et al., 1983b; Dean et al., 1985). Analytically, the potential to use affinity chromatography as a quantitative technique has been demonstrated (Dunn & Chaiken, 1974, 1975; Nichol et al., 1974; Kasai & Ishii, 1975). By the latter methodology, it is possible to determine quantitative properties of molecular interactions, such as equilibrium dissociation constants, by measuring the retardation of mobile macromolecules on immobilized small ligands and the retardation of both small and large mobile molecules on immobilized macromolecules (Dunn & Chaiken, 1975; Brinkworth et al., 1975; Kasai & Ishii, 1975, 1978; Chaiken & Taylor, 1976; Smith et al., 1978; Dunn & Gilbert, 1979; Danner et al., 1979; Eilat & Chaiken, 1979; Lagercrantz et al., 1979; Angal & Chaiken, 1982; Liu et al., 1983). In addition, recent theoretical treatments have suggested that kinetic properties of "on-rates" and "off-rates" also may be obtained for the interaction between the immobilized molecule and molecules in solution having specific recognition sites for the immobilized molecule (Denizot & Delaage, 1975; DeLisi et al., 1982; Hethcote & DeLisi, 1982a,b, 1983).

Quantitative affinity chromatographic studies generally have been carried out by relatively simple conventional chromatographic methodology. However, the analytical precision of the growing HPLC[1] technology and the increasing availability of affinity matrices for use in HPLC make HPAC a potentially valuable technology for analytical affinity chromatography. The neuroendocrine peptide–protein system, composed of neurohypophysial hormones oxytocin and vasopressin and the hormone-binding protein neurophysin, was chosen to test the potential of analytical HPAC. This molecular system exhibits a multiple set of peptide–protein and protein–protein interactions [for recent reviews, see Breslow (1979), Cohen et al. (1979), and Chaiken et al. (1983a)]. The interacting peptide and protein domains display recognition both as parts of intact biosynthetic precursors and after precursor processing, with the quantitative properties of these interactions changing during the biosynthetic process. This molecular system has proven useful in developing and using analytical affinity chromatography methods to characterize molecular recognition and its role in cellular pathways.

In this report, the methodology is developed for, and the molecular interactions of the neurophysin–vasopressin system are analyzed by, analytical high-performance affinity chromatography using the protein immobilized on both porous and nonporous glass derivatives. In addition, the theory of analytical affinity chromatography is extended to systems undergoing self-association. The results suggest that analytical affinity chromatography, particularly using nonporous glass

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[1] Abbreviations: HPLC, high-performance liquid chromatography; HPAC, high-performance affinity chromatography; CPG, controlled-pore glass; NPG, nonporous glass; AVP, [Arg^8]vasopressin; BNP II, bovine neurophysin II.

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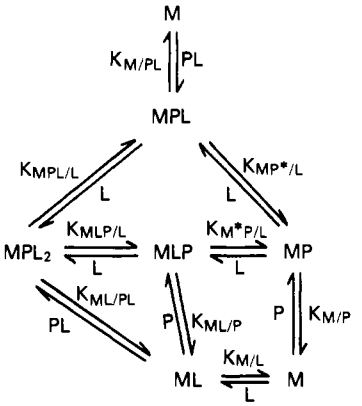

FIGURE 1: Equilibria for an immobilized protein (such as neurophysin) whose self-association is modulated by binding a soluble ligand (such as peptide hormone). In these equilibria M represents the molecule attached to the matrix, and P and L are the soluble protein and ligand, respectively. The subscripts for the dissociation constants give the products of the dissociation. Where ambiguity arises, the asterisk (*) indicates from which molecule the ligand is dissociating.

bead derivatives, may be a powerful tool for microscale characterization of biomolecular interactions.

THEORY

The model in Figure 1 describes the set of interactions for an immobilized protein for which self-association is affected by small ligand binding, when both soluble ligand and protein are present. We have chosen the convention of using M to denote the immobilized molecule and subscripts with the dissociation constants to denote the products of that dissociation. Also, P and L denote the soluble protein and ligand, respectively.[2] Thus, for example, for the specific case of immobilized neurophysin, $K_{M/PL}$ represents the dissociation of the liganded monomer, PL, from the immobilized, unliganded monomer.

For the interactions in Figure 1 at equilibrium, the partitioning of the soluble protein is given by

$$\sigma_p = ([MP] + [MLP] + [MPL] + [MPL_2] + [P^\alpha] + [PL^\alpha] + 2[P_2^\alpha] + 2[PPL^\alpha] + 2[PLP^\alpha] + 2[P_2L_2^\alpha])/([P^\beta] + [PL^\beta] + 2[P_2^\beta] + 2[PPL^\beta] + 2[PLP^\beta] + 2[P_2L_2^\beta]) \quad (1)$$

where the superscripts α and β with the soluble components indicate their presence in the stationary and mobile phases, respectively. If the partition coefficient in the absence of immobilized protein is defined as $\sigma_{0,p}$, then the partition coefficient given by eq 1 becomes

$$\sigma_p = \frac{([MP] + [MLP] + [MPL] + [MPL_2])\sigma_{0,p}}{[P^\alpha]_T} + \sigma_{0,p} \quad (2)$$

where $[P^\alpha]_T$ is the total concentration of soluble protein in the stationary phase. Using the general expression in chromatography $V = V_m + \sigma_p V_s$ and noting that $V_0 = V_m + \sigma_{0,p}V_s$, we obtain from eq 2

$$V - V_0 = \frac{([MP] + [MLP] + [MPL] + [MPL_2])(V_0 - V_m)}{[P^\alpha]_T} \quad (3)$$

[2] Because of the expansion in complexity of molecular interactions we expect to be treatable in quantitative affinity chromatography, we have adopted a new general notation, to replace that previously used (Chaiken, 1979; Abercrombie & Chaiken, 1975), which can be adapted for multiple interacting systems as well as simple ones.

In these expressions V is the observed elution volume, V_m is the mobile phase volume, V_s is the stationary phase volume, and V_0 is the elution volume for an unretarded molecule.

If nonspecific partitioning does not occur and equilibrium is achieved, then $[P^\alpha]_T = [P^\beta]_T$. Substitution of the equilibria expressions gives

$$\frac{V - V_0}{V_0 - V_m} = \frac{\frac{[M][P]}{K_{M/P}}\left(1 + \frac{[L]}{K_{M^*P/L}} + \frac{[L]}{K_{MP^*/L}} + \frac{[L]^2}{K_{MPL/L}K_{MP^*/L}}\right)}{[P]_T} \quad (4)$$

for the elution expression and

$$[M]_T = [M]\left(1 + \frac{[L]}{K_{M/L}}\right) + \frac{[M][P]}{K_{M/P}}\left(1 + \frac{[L]}{K_{M^*P/L}} + \frac{[L]}{K_{MP^*/L}} + \frac{[L]^2}{K_{MPL/L}K_{MP^*/L}}\right) \quad (5)$$

for the total concentration of immobilized protein. In eq 4 and 5, the dissociation constants are as defined by the equilibria in Figure 1. The subscripts for the dissociation constants designate the products of the dissociation. Where ambiguity arises, the asterisk (*) indicates from which molecule the ligand is dissociated. Defining the last term in brackets in eq 4 and 5 as Q, the expression for the elution volume parameter can be rearranged, after combination of the two equations, to give

$$\frac{V_0 - V_m}{V - V_0}[M]_T = \frac{K_{M/P}}{Q}\frac{[P]_T}{[P]} + \frac{K_{M/P}[L][P]_T}{K_{M/L}Q[P]} + [P]_T \quad (6)$$

By use of the conservation of mass principle, an expression for the concentration of unliganded protein monomer can be obtained, viz.

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

The solution of this quadratic expression yields

$$[P] = \frac{K_{P/P}}{4Q}\left(1 + \frac{[L]}{K_{P/L}}\right)\left[\left[1 + \frac{8Q[P]_T}{K_{P/P}\left(1 + \frac{[L]}{K_{P/L}}\right)^2}\right]^{1/2} - 1\right] \quad (7)$$

Both eq 6 and eq 7 are complicated functions of unbound ligand concentration and all of the immobilized liganded protein dissociation constants, so it is unlikely that individual constants can be extracted from chromatographic data. However, there are two special cases of interest that are experimentally accessible.

(1) Chromatography of Protein in the Absence of Soluble Ligand. When [L] = 0, Q = 1, so eq 6 and 7 can be simplified to give respectively

$$\frac{(V_0 - V_m)[M]_T}{V - V_0} = K_{M/P}\frac{[P]_T}{[P]} + [P]_T \quad (6a)$$

and

$$[P] = \frac{K_{P/P}}{4}\left[\left(1 + \frac{8[P]_T}{K_{P/P}}\right)^{1/2} - 1\right] \quad (7a)$$

Equations 6a and 7a can be combined to give

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

This result is further simplified when $[P]_T \ll K_{P/P}$ and $K_{M/P}$. Since the quantity in brackets reduces to $K_{M/P}/2$, we obtain

$$\frac{V_0 - V_m}{V - V_0} = \frac{K_{M/P}}{[M]_T} \qquad [P]_T \ll K_{P/P} \text{ and } K_{M/P} \quad (9)$$

(2) Chromatography of Protein in the Presence of Saturating Ligand. When L is saturating, the only species present at significant concentrations are ML and MPL_2. Combination of equations analogous to eq 4 and 7 yields

$$\frac{V_0 - V_m}{V - V_0} = \frac{K_{ML/PL}}{2[M]_T} + \left[\frac{K_{ML/PL}}{2}\left(1 + \frac{8[P]_T}{K_{PL/PL}}\right)^{1/2} + [P]_T\right]\frac{1}{[M]_T} \quad (10)$$

When $[P]_T \ll K_{ML/PL}$ and $K_{PL/PL}$, this becomes

$$\frac{V_0 - V_m}{V - V_0} = \frac{K_{ML/PL}}{[M]_T} \qquad [P]_T \ll K_{PL/PL} \text{ and } K_{ML/PL} \quad (11)$$

Equations 9 and 11 show that it is possible to obtain the equilibrium dissociation constants for the interaction of unliganded or fully liganded monomers with immobilized monomer.

Similar expressions also are obtained for elution chromatography using nonporous beads as the support. In this case the equilibrium partition coefficient is defined analogously to that for porous matrices except that the void volume replaces the stationary phase (pore) volume in the relationship, i.e.

$$\sigma \equiv Q_s/(V_0C) \quad (12)$$

where Q_s is the amount of solute adsorbed to the matrix, V_0 is the unretarded elution volume, and C is the solute concentration in the mobile phase. The resulting expressions are identical with those for porous matrices except that $V_0/(V - V_0)$ replaces $(V_0 - V_m)/(V - V_0)$.

Materials and Methods

Purification of Neurophysin II (BNP II). Acetone powders of bovine pituitaries were extracted with dilute HCl and the proteins fractionated by gel chromatography on Sephadex G-75, and the fraction containing BNP II was obtained by ion-exchange chromatography on DEAE-Sephadex A-50 (Fischer et al., 1977). BNP II was purified further by preparative reverse-phase HPLC (Chaiken et al., 1984a,b) on octadecyl silica (Zorbax ODS, Du Pont). For the latter, protein was injected into a 0.9 × 25 cm column and equilibrated with 67 mM triethylamine phosphate buffer (pH 3.0) containing 23% acetonitrile, and BNP II was eluted with a 60-min 23–25% acetonitrile gradient and a flow rate of 3.2 mL/min. Finally, biologically active BNP II was obtained from the HPLC fraction by biospecific adsorption on a column of [(Met-Tyr-Phe-amino)hexyl]-Sepharose (Chaiken, 1979), equilibrated with 0.4 M ammonium acetate at pH 5.7, and elution with 0.2 M acetic acid.

Immobilization of Neurophysin II. Protein was covalently immobilized on 200-nm pore diameter controlled-pore glass (CPG-2000, obtained from Electro-Nucleonics, Fairfield, NJ) and on nonporous glass beads (type 1-W, 75-150-μm diameter, from Sigma Chemical Co., St. Louis, MO). BNP II was attached to thionyl chloride activated succinamidopropyl-CPG, which was prepared, as described by DuVal et al. (1984), with a 1:100 dilution of reactive succinamidopropyl sites in unreactive glyceropropyl sites (Figure 2). All solutions used in these procedures were filter-sterilized by using 0.2-μm Millipore filters. A 6-mL solution of BNP II (0.5 mg/mL) in 0.05 M sodium phosphate, pH 7.0, was recirculated through 2.5 g of the activated glass in a fixed-bed column for 3 h at room temperature. Following immobilization, the beads were washed in the fixed-bed reactor with, successively, 500 mL of 1 M NaCl in the phosphate buffer (pH 7.0) and 200 mL of 0.4 M sodium acetate (pH 5.7). The CPG-immobilized BNP II was stored in the 0.05 M phosphate buffer (pH 7.0) at 4 °C.

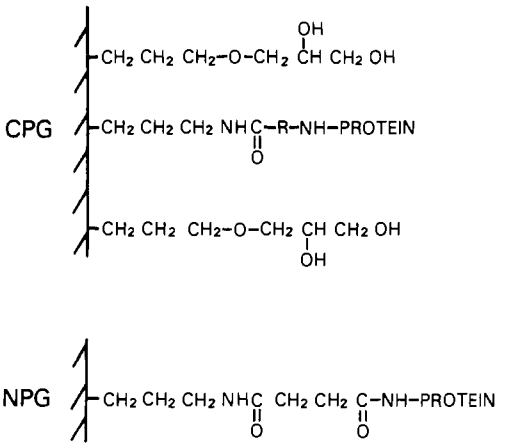

FIGURE 2: Illustration of the glass derivatives used for immobilization of neurophysin. Succinamidopropyl derivatives of both controlled pore and nonporous glass, CPG and NPG, respectively, were prepared. In the case of porous glass, thionyl chloride activated succinamidopropyl sites were dispersed (1:100) among glycerolpropyl groups (DuVal et al., 1984). With nonporous glass, the protein was attached to the succinamidopropyl surface following activation of the carboxyl groups with water-soluble carbodiimide.

BNP II was immobilized on nonporous glass beads derivatized by an aqueous silanization–succinylation procedure (Janolino & Swaisgood, 1982), again using filter-sterilized solutions in all steps. Nonporous glass beads (10 mL) were acid-cleaned and reacted with 20% (v/v) aqueous (γ-aminopropyl)triethoxysilane, adjusted to pH 8 with HCl, at 70 °C for 3 h. After the excess liquid was decanted, further polymerization was induced by drying overnight at 100 °C. The γ-aminopropyl-glass beads were succinylated at 4 °C by addition of 10 mL of distilled water, followed by 10 g of solid succinic anhydride. The reaction was stirred by bubbling nitrogen gas, and the pH was maintained at about 6 with 40% NaOH. After reaction, the beads were thoroughly washed with distilled water and dried by lyophilization. For immobilization of BNP II on the above succinamidopropyl-glass beads (5-mL settled volume), the simultaneous activation–immobilization procedure (Janolino & Swaisgood, 1982) was used. The matrix carboxyl groups were activated with 0.01 M 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) by dissolving 11.54 mg of EDC (solid) in 3.0 mL of 0.047 M phosphate buffer (pH 7.0). To this mixture in a column reactor was added immediately 3.9 mg of BNP II in 3.0 mL of the same buffer, and the immobilization reaction (at an effective concentration of BNP II of 0.35 mg/mL) proceeded with recirculation overnight at 4 °C. After immobilization, the beads were washed with 500 mL of phosphate buffer adjusted to 1 M NaCl, rinsed with the phosphate buffer, and

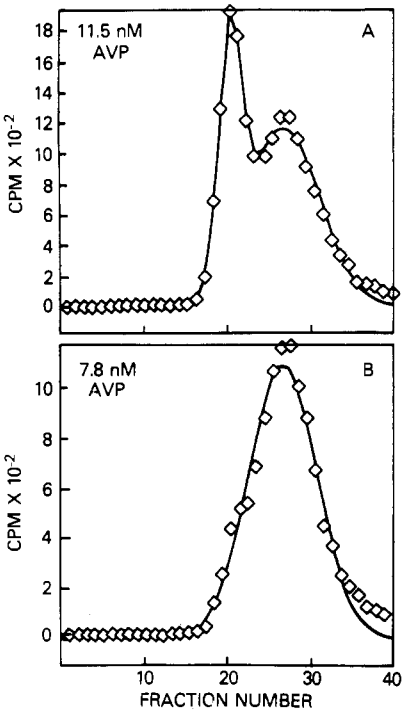

FIGURE 3: Zonal elution profiles for [^{3}H][Arg^8]vasopressin obtained by chromatography with [BNP II]NPG. The column (25 × 0.46 cm i.d.) was equilibrated and eluted at room temperature and a flow rate of 0.2 mL/min with 0.4 M ammonium acetate, pH 5.7. A 200-μL sample was injected and 2-drop fractions (90 μL) were collected, starting at 0.5 min, directly in scintillation vials for counting. The solid lines represent computer fits to Gaussian equations. (A) Elution profile for 11.5 nM commercial [^{3}H]AVP. (B) Elution profile for 7.8 nM affinity-purified [^{3}H]AVP.

stored in 0.4 M ammonium acetate, pH 5.7, at 4 °C.

Analytical High-Performance Affinity Chromatography. Both the porous and nonporous glass beads, with BNP II immobilized, were packed in 0.46 × 25 cm stainless steel HPLC columns. A slurry was introduced into the top of the column, and the beads were packed with vibration while a flow rate of 0.8 mL/min was maintained. For chromatography, the columns were placed in a Varian 5000 HPLC system with exit tubing leading directly to a fraction collector capable of collecting directly in scintillation vials. Samples of 200 μL were injected with a standard sample loop and 2-drop (nonporous beads) or 6-drop (porous beads) fractions were collected. Zero time was taken as the time when half the sample had entered the column. All solutions for chromatographic elutions were filter-sterilized and degassed under vacuum prior to use. When not in use, the columns were stored at 4 °C in the elution buffer containing 0.02% sodium azide.

RESULTS

Analytical Affinity Chromatography of [Arg^8]Vasopressin on [BNP II]NPG. When [^{3}H][Arg^8]vasopressin (AVP) was eluted on [BNP II]NPG, chromatographic profiles were obtained as shown in Figure 3. Panel A in this figure represents the elution for commercial [^{3}H]AVP. The data indicate the presence of expected bound component in addition to a labeled component which does not bind to the stationary phase. The elution volume for the unbound component was identical with that for [^{3}H]ribonuclease and thus represents the elution volume for an unretarded molecule, V_0. This component was removed by chromatography of microquantities on [BNP II]CPG (Swaisgood & Chaiken, 1985). An elution profile for the purified [^{3}H]AVP is shown in Figure 3B. Storage of the [BNP II]CPG-purified [^{3}H]AVP was accompanied by progressive inactivation with the resulting reappearance of material in the unretarded elution volume (Swaisgood & Chaiken, 1985).

Table I: Reproducibility of Unretarded Elution Volumes, V_0, for [BNP II]NPG and [BNP II]CPG Packed in Stainless Steel Columns (0.46 × 25 cm) and Incorporated into a High-Performance Liquid Chromatograph

unretarded molecule used as elution volume marker	V_0 (mL)[a] for nonporous bead column	V_0 (mL)[a] for porous bead column
nonbinding [^{3}H]AVP fraction—internal analysis	1.887 (9)	4.300 (17)
	1.855 (10)	4.496 (18)
	1.852 (11)	4.430 (20)
	1.860 (12)	4.420 (26)
	1.880 (13)	
purified nonbinding [^{3}H]AVP fraction	1.922 (17)	4.390 (8)
	1.917 (22)	4.239 (21)
	1.832 (31)	4.442 (25)
	1.720 (35)	
[^{3}H]ribonuclease	1.747 (27)	4.091 (5)
mean ± SD	1.847 ± 0.067	4.351 ± 0.133

[a] The number in parentheses is the chromatogram run number from a total of 47 chromatographic runs with the nonporous bead column and 39 chromatographic runs with the porous bead column. The data illustrate the reproducibility throughout the use of the columns.

Since the elution profiles of [^{3}H]AVP on [BNP II)NPG were relatively symmetric, the elution position, V, and the elution profile variance, W_e, were determined by fitting to Gaussian curves, as illustrated in Figure 3. The constancy of elution volumes of unretarded molecules obtained over the entire period of this study (Table I) illustrates the reproducibility of elution behavior, the stability of the supports, and the dependability of the Gaussian curve-fitting method for assigning the elution position.

Chromatographic Evaluation of Equilibrium Constants for [^{3}H]AVP and ^{125}I-BNP II Binding to [BNP II]NPG. By use of an approach similar to that described under Theory for relating elution parameters to the equilibrium dissociation constant for BNP II dimerization, a relationship for chromatography of AVP on nonporous beads can be derived (Swaisgood & Chaiken, 1985), viz.

$$\frac{V_0}{V - V_0} = \frac{K_{M/L}}{[M]_T} + \frac{[L]}{[M]_T} \qquad (13)$$

where $K_{M/L}$ is the dissociation constant for the vasopressin-immobilized neurophysin interaction and [L] is the total concentration of AVP in the mobile phase. The relationship is identical with that derived by Nichol et al. (1974) (their eq 8). It is noted from this expression that when the concentration of soluble ligand is not small relative to $K_{M/L}$, a significant concentration dependence would be expected. However, in the zonal elution approach used here, the mobile phase concentration of AVP is neither constant nor equal to the initial concentration injected. Nevertheless, the data obtained for chromatography of AVP, shown in Figure 4, indicate that the elution parameter $1/(V - V_0)$ was linearly related to the initial concentration of AVP injected over the concentration range studied. More importantly, data can be obtained at very low concentrations of AVP and accurately extrapolated to [L] = 0 to give $K_{M/L}$ when V_0 and $[M]_T$ are known. The amount of BNP II in the column, $[M]_T$, was determined by amino acid analysis; the resulting value for $K_{M/L}$ is listed in Table II.

On the basis of the known dimerization of BNP II, the chromatographic behavior of soluble BNP II on [BNP II]NPG was evaluated. A typical elution profile for soluble ^{125}I-BNP II is shown in Figure 5. The elution profiles for varying concentrations of soluble BNP II were observed to be de-

Table II: Chromatographically Derived Thermodynamic and Kinetic Constants for Interactions of Neurophysin–Vasopressin System

method	$K_{M/L}$ (μM)	$K_{M/P}$ (μM)	k_{-3} (s^{-1}) for AVP binding: H–D method[a]	k_{-3} (s^{-1}) for AVP binding: D–D method[b]	k_{-3} (s^{-1}) for BNP binding: H–D method[a]	k_{-3} (s^{-1}) for BNP binding: D–D method[b]
[BNP II]NPG chromatography[c]	74.9	166	0.013 ± 0.003	0.017 ± 0.009	0.024 ± 0.006	0.09 ± 0.06
[BNP II]CPG chromatography[d]	11.2		0.0016 ± 0.0002	0.0018 ± 0.0003		
lit. values for soluble components	6.7–50[e]	56–172[f]	11[g]		12[h]	

[a] Calculated by using the method of Hethcote and DeLisi (1982a,b). [b] Calculated by using the method of Denizot and Delaage (1985). [c] Total BNP II immobilized in the column determined by amino acid analysis was 41.4 ± 2.9 nmol by using seven amino acids for the calculation. [d] Total BNP II immobilized in the column determined by amino acid analysis was 58 ± 4 nmol by using seven amino acids for the calculation. [e] Values obtained: 16–20 μM for binding of oxytocin to soluble BNP II from equilibrium dialysis studies (Nicolas et al., 1978; Tellam & Winzor, 1980); 50 μM for binding of hormone to soluble BNP II from competitive binding quantitative affinity chromatography (Angal & Chaiken, 1982); and 6.7 μM for binding of [Lys[8]]vasopressin to soluble BNP II dimer (Pearlmutter & Dalton, 1980a). [f] Values obtained for dimerization of unligand monomer under conditions similar to those used here: by analytical gel chromatography, 56–91 μM (Whittaker & Allewell, 1984; and by sedimentation equilibrium, 118–172 μM (Tellam & Winzor, 1980; Nicolas et al., 1976, 1980). [g] Value for dissociation of [Lys[8]]vasopressin from singly liganded BNP II dimer (Pearlmutter & Dalton, 1980a); the value for [Lys[8]]vasopressin from doubly liganded BNP II dimer is 6 s^{-1} (Pearlmutter & Dalton, 1980a). [h] Value for dissociation of BNP I dimer (Pearlmutter, 1979); the k_{-3} value for oxytocin binding to BNP II monomer, a value also relevant to compare, is 2 s^{-1} (Pearlmutter & Dalton, 1980b).

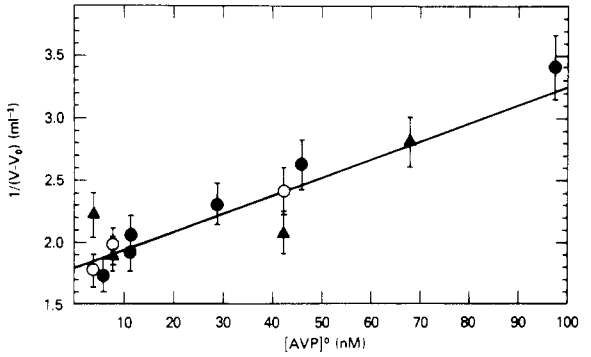

FIGURE 4: Concentration dependence of the elution volume observed for zonal chromatography of [^{3}H][Arg[8]]vasopressin with [BNP II]NPG. The data are plotted as a function of the concentration of AVP in the applied 200-μL zone, which represents an approximation to eq 13. The line is generated by the function $Y = 1.808 + 0.015[AVP]^0$ with a correlation coefficient of 0.93. Symbols: ●, data obtained with commercial [^{3}H]AVP; ○ and ▲, data obtained in two separate experimental series with affinity-purified [^{3}H]AVP.

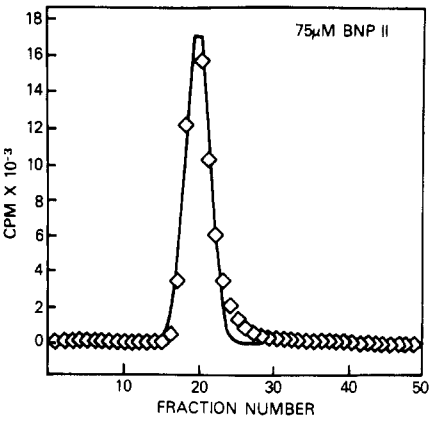

FIGURE 5: Zonal elution profile for ^{125}I-BNP II, at an initial BNP II concentration of 75 μM, obtained by chromatography with [BNP II]NPG. The column (25 × 0.46 cm i.d.) was equilibrated and eluted at room temperature and a flow rate of 0.2 mL/min with 0.4 M ammonium acetate, pH 5.7. A 200-μL sample containing 7.5 pmol of ^{125}I-BNP II and 15 nmol of BNP II was injected, and 2-drop fractions (90 μL) were collected directly in scintillation vials for counting. The line represents a computer fit to a Gaussian equation from which both the peak position and the variance were obtained.

pendent upon the concentration of soluble protein (Figure 6). Since the total concentration of soluble BNP II in the zone is not constant, due to spreading, and also is not known, it is not possible to accurately determine the form of the concentration dependence. However, as for the case of AVP chromatography, plotting $1/(V - V_0)$ as a function of the concentration of BNP II applied to the initial zone permitted accurate extrapolation to zero protein concentrations. Thus the dimer equilibrium dissociation constant, $K_{M/P}$, could be calculated by using eq 9; the value obtained is listed in Table II. The sensitivity of this method is best illustrated by noting that when the concentration dependence in Figure 6 is ignored, the mean and standard deviation for the value of $K_{M/P}$ are 253 ± 96 μM. The kinetic rate constant for the dissociation also was determined, as described below for AVP binding, and is listed in Table II.

Chromatographic Evaluation of Rate Constants for [^{3}H]AVP and ^{125}I-BNP II Binding to [BNP II]NPG. Several methods have been proposed for using the degree of elution zone spreading to calculate kinetic rate constants for dissociation of the soluble molecule from the immobilized molecular complex. Denizot and Delaage (1975) used a random-walk approach to derive eq 14 for calculation of the dissociation rate constant from affinity chromatographic behavior. Here E_{t_0}

$$k_{-3} = \frac{2E_{t_0}^2(E_{t'} - E_{t_0})}{\sigma'^2 E_{t_0}^2 - \sigma_0^2 E_{t'}^2} \qquad (14)$$

and $E_{t'}$ are the elution times for unretarded and binding

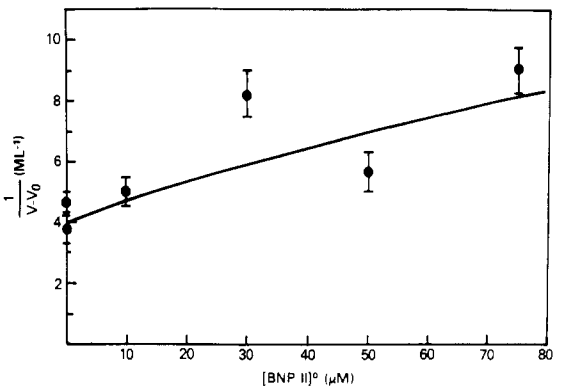

FIGURE 6: Concentration dependence of the elution volume observed for zonal chromatography of ^{125}I-BNP II with [BNP II]NPG. The data are plotted as a function of the concentration of BNP II applied in the zone, which is an approximation of eq 8. The line was generated from eq 8 by using $K_{M/P}$ = 166 μM, $K_{P/P}$ = 172 μM, and $V_0[M]_T$ = 42.4 nmol.

molecules, respectively, and σ_0 and σ' are the standard deviations of the elution profiles for unretarded and binding molecules, respectively. More recently, Hethcote and DeLisi (1982a,b, 1983) used a nonequilibrium model of affinity chromatography to derive the relationship

$$k_{-3} = 2F(V - V_0)/W_e \qquad (15)$$

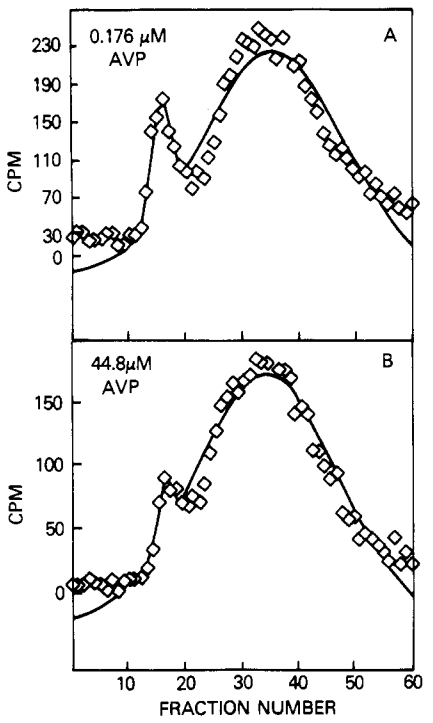

FIGURE 7: Zonal elution profile for [^{3}H][Arg^8]vasopressin obtained by chromatography with [BNP II]CPG. The column (25 × 0.46 cm i.d.) was equilibrated and eluted at room temperature and a flow rate of 0.2 mL/min with 0.4 M sodium acetate, pH 5.7. A 200-μL sample was injected, and 6-drop fractions (0.27 mL) were collected, starting at 0.5 min, directly in scintillation vials for counting. The line represents a computer fit to Gaussian equations. (A) Elution of 0.176 μM AVP (0.9 pmol of [^{3}H]AVP and 34 pmol of AVP in 200 μL). (B) Elution of 44.8 μM AVP (0.6 pmol of [^{3}H]AVP and 8.96 nmol of AVP in 200 μL).

Here, F is the flow rate, V and V_0 are the mean elution volumes for binding and unretarded molecules, respectively, and W_e is the variance of the elution profile.

On the basis of the above, the data for elution of [^{3}H]AVP and ^{125}I-BNP II]NPG were used to calculate values for k_{-3}. Elution profiles were evaluated by fitting to a Gaussian equation, illustrated by the fits in Figures 3 and 5, and the rate constants calculated by using both eq 14 and eq 15. The resulting constants are listed in Table II.

Analytical Affinity Chromatography with [BNP II]CPG. Chromatography of [^{3}H]AVP also was performed with [BNP II]CPG, yielding elution profiles such as those shown in Figure 7. These profiles are very similar to those observed for chromatography on nonporous bead matrices except that the elution boundaries exhibit greater spreading. Comparison of the profiles in Figure 7, representing a more than 300-fold variation of AVP concentration in the zone applied to the column, reveals only a weak effect of concentration on the elution position in the concentration range used. As with the nonporous bead column, the presence of a labeled nonbinding component in the [^{3}H]AVP preparation served as an internal marker for the unretarded elution volume, V_0 (Figure 7). The reproducibility of the measurements of V or V_0 over a 6-month period is illustrated by the data in Table I. For the [BNP II]CPG case, variation of the elution parameters as a function of the concentration of AVP injected is shown in Figure 8. From these data, the equilibrium dissociation constant, $K_{M/L}$, was determined by using a relationship similar to that given by eq 13. For the case of porous beads, $V_0 - V_m$ represents the stationary phase or pore volume. Using the amount of BNP II immobilized as determined by amino acid analysis to define $[M]_T$ gives the value for $K_{M/L}$ listed in Table II.

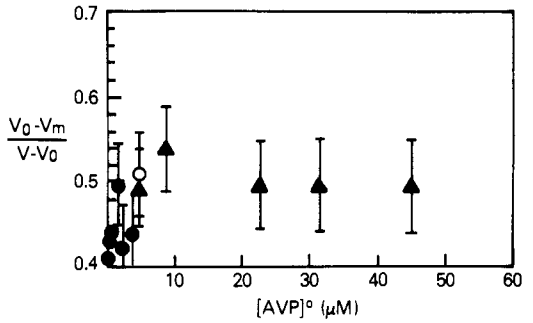

FIGURE 8: Concentration dependence of the elution volume observed for zonal chromatography of [^{3}H]AVP with [BNP II]CPG. The data are plotted as a function of the concentration of AVP in the applied zone, which represents an approximation to an equation analogous to eq 13. The symbols ●, ▲, and ○ represent data for three independent sets of experiments using different [^{3}H]AVP preparations.

The apparent kinetic rate constant for dissociation also was calculated from the elution profile by using eq 14 and 15. The resulting constants are given in Table II.

Discussion

The neurophysins and their associated peptide hormones, vasopressin and oxytocin, represent a well-characterized system of protein–protein and protein–peptide interactions (Chaiken et al., 1983a). The neurophysins exhibit hormone-enhanced self-association as well as hormone binding that is enhanced by self-association (Breslow et al., 1971; Chaiken et al., 1975; Nicolas et al., 1976, 1980; Pearlmutter & Dalton, 1680; Tellam & Winzor, 1980; Angal & Chaiken, 1982). The interactions of the neurophysins and hormones have been evaluated previously by analytical affinity chromatography on nonrigid, agarose-based affinity matrices (Angal & Chaiken, 1982; Abercrombie et al., 1984; Kanmera & Chaiken, 1985). Consequently, this neuroendocrine peptide–protein system provides an excellent model for evaluating the usefulness of analytical high-performance affinity chromatography as a methodology for characterizing macromolecular interactions.

In the present study, analysis of the hormone–neurophysin interaction could be made from the chromatographic behavior of [^{3}H]AVP on [BNP II]NPG and [BNP II]CPG. The use of immobilized BNP II limits the binding process measured to that of peptide ligand largely to immobilized protein monomer.[3] The concentration of [^{3}H]AVP was varied over a sufficiently wide range that the concentration dependence predicted theoretically (eq 13) was observed on the NPG matrix (Figure 4). In contrast, no clearcut concentration dependence was observed with the CPG matrix (Figure 8), suggesting that for this matrix $[L]/[M]_T$ is negligible. Elution on both matrices led to calculated values for $K_{M/L}$, by extrapolating to [L] = 0 for [BNP II]NPG and by averaging

[3] On the basis of the dimer association constant reported by Whittaker and Allewell (1984) of 1.1 × 10^4 M^{-1} for BNP II in 0.1 M phosphate buffer, pH 5.6, and the expected similarity of (or small decrease in) such values at pH 7 (Whittaker & Allewell, 1984; Tellam & Winzor, 1980; Nicolas et al., 1980), 74% and 72% of the BNP II molecules would be expected to be monomers in the immobilization solutions for CPG and NPG, respectively. Given the relatively low concentrations of dimers in the reaction solutions and the fact that both monomeric partners of the dimer would have to be covalently attached for protein to remain immobilized as dimers after washing, the probability is low for dimer immobilization. The effect of the presence of a small amount of immobilized dimer would be to slightly decrease $K_{M/L}$, since dimers bind hormone more strongly than do monomers.

the values for all elutions with [BNP II]CPG. There is an appreciable difference in the dissociation constant obtained with NPG vs. CPG matrices, for reasons that are not yet apparent; the contribution of a low but finite concentration of immobilized dimers in the case of [BNP II]CPG cannot be ruled out.[3] The differences found in $K_{M/P}$ for [BNP II]-NPG vs. [BNP II]CPG also could reflect observed differences in chromatographically measured rates of dissociation of AVP from immobilized BNP II (see below) if equilibrium between mobile and stationary phases were not established. Furthermore, rate constants should affect only the zone dispersion and not the elution position (Hethcote & DeLisi, 1983), and the elution profiles were Gaussian and unaffected by flow rate (unpublished data), indicating local equilibrium was achieved. In any case, all of these values (11–75 μM) are in reasonable agreement with those previously obtained for dissociation of hormone from the monomer, $K_{P/L}$. Curve fitting of Scatchard plots of [^{3}H]oxytocin binding in 0.1 M sodium acetate, pH 5.6, gave $K_{P/L}$ = 16–20 μM (Nicolas et al., 1978; Tellam & Winzor, 1980), and quantitative affinity chromatography using tripeptidyl-agarose yielded a value of 50 μM (Angal & Chaiken, 1982). The dissociation constants obtained with [BNP II]CPG and [BNP II]NPG are larger than that estimated for the dimer, $K_{P/L}$ = 6.7 μM, from curve fitting of spectrophotometric measurements of binding to nitrated BNP II (Pearlmutter & Dalton, 1980a). Previous quantitative study of vasopressin binding to BNP II using affinity chromatography on compressible gel matrices has shown that the dissociation constants can be determined by competitive elution of ^{125}I-BNP II from Met-Tyr-Phe-agarose columns with varying [Lys^8]vasopressin concentrations (Angal & Chaiken, 1982). In these latter elutions, the BNP II concentration was sufficiently small that the term in the chromatographic expression containing [BNP II] would be negligible, although both monomer and dimer theoretically could be present in the particular BNP II solution used.

High-performance analytical affinity chromatography on [BNP II]glass matrices also has been found to be applicable to measuring the BNP II dimerization process. In the case of ^{125}I-BNP II chromatography on [BNP II]NPG, the elution volume in the absence of hormone is predicted theoretically, eq 8, to vary nonlinearly with the protein concentration (except when $[P]_T \ll K_{P/P}$ and $K_{M/P}$). Although the data are not sufficiently precise to unequivocally distinguish the form of the dependence and the concentration of BNP II applied was plotted rather than that in the mobile phase, the line generated from the theoretical relationship was consistent with the experimental values obtained (Figure 7). More importantly, the dissociation constant for the dimer, $K_{M/P}$, calculated at zonal [BNP II] extrapolated to zero, agrees very well with that obtained by sedimentation equilibrium studies (Nicolas et al., 1976, 1978, 1980; Tellam & Winzor, 1980) and by analytical gel filtration chromatography (Whittaker & Allewell, 1984). Interestingly, the $K_{M/P}$ determined by quantitative affinity chromatography using [BNP II]Sepharose was more than 10-fold less (Chaiken et al., 1983c). The reason for the difference in chromatographic $K_{M/P}$ values determined by both NPG- and Sepharose-based matrices is not clear. Nonetheless, it is clear from the data for both [^{3}H]AVP and ^{125}I-BNP II elution that, in addition to being considerably more rapid (a single elution requires less than 25 min), analytical affinity chromatography using [BNP II]NPG and [BNP II]CPG allows the dependable analysis of equilibrium properties for the binding process for both peptide–protein and protein–protein interactions.

In contrast to the usefulness of analytical HPAC for measuring equilibrium constants, that for measuring rates of interaction is as yet uncertain. The rate constants for dissociation of [^{3}H]AVP and ^{125}I-BNP II from [BNP II]NPG and [BNP II]CPG were calculated from spreading of the eluting boundary by using the theoretical relationships derived by Denizot and Delaage (1975) and Hethcote and DeLisi (1982a,b). The apparent rates of dissociation from the porous beads were 10-fold smaller than those for the nonporous beads. The test for validity of comparing chemical kinetics with mass-transfer kinetics, $k_{-3}(1 + [M]_T/K_{M/L})^2/([M]_T/K_{M/L}) \ll k_{-1}$, where k_{-1} is the mass-transfer dissociation rate (Hethcote & DeLisi, 1983), indicates that for the CPG system mass-transfer rates are slow relative to the chemical off-rate. Hence, the data obtained with [BNP II]CPG do not reflect the chemical dissociation rates. However, use of [BNP II]-NPG should optimize the potential for measuring the dissociation rate constant by analytical affinity chromatography; both immobilization of the macromolecular partner of the interaction and use of nonporous beads have been suggested as independent methods for obtaining this constant (Hethcote & DeLisi, 1983). Nonetheless, the dissociation rate constants obtained with [BNP II]NPG are still several orders of magnitude smaller than those obtained fully in solution. Since the chromatographically derived equilibrium constants are close to those determined fully in solution, the association rate constants describing the (mobile peptide or neurophysin)–immobilized neurophysin interaction also would be expected to be correspondingly smaller. The significantly slower rate constants observed here chromatographically vs. those measured in solution may result from limitations of mass transfer in an unstirred solvent layer on the surface of the affinity matrix; this feature would affect both association and dissociation rate constants equally. Alternatively, the methods used for calculation of affinity chromatographic rate constants may not fully account for diffusion effects on zone dispersion. As opposed to the above, it is possible that intrinsic dissociation rates for independent recognition sites in solution would not be the same as those for multiple sites on a contiguous surface such as an affinity matrix. In their theoretical treatment of ligand binding to cell receptors, DeLisi and Wiegel (1981) showed that the dissociation rate from the cell could be substantially reduced relative to that for solubilized receptor as a result of the probability of rebinding to another receptor on the same cell. If this were so in the current affinity chromatography case, a decrease in the chemical association constant roughly equivalent to that in the dissociation constant would have to occur; such a decrease, caused by independent but as yet undefinable factors, cannot be ruled out. In any case, while the meaning of rate constants determined by affinity chromatography is difficult to interpret fully from the present data, it is apparent that use of nonporous beads eliminates a major contribution to band broadening by removing the pore mass-transfer term and thus could be further explored as a method for determining kinetic constants.

In summary, the present studies have demonstrated the potential of analytical high-performance affinity chromatography on glass-based affinity matrices to characterize biospecfic molecular interactions. We have defined this rapid, experimentally straightforward technique both theoretically and experimentally for characterizing macromolecular self-association as well as interactions of macromolecules with peptides. This single technique yields similar molecular parameters to those derived from both sedimentation equilibrium and equilibrium dialysis studies. Importantly, values for the

dimer equilibrium dissociation constant, $K_{M/P}$, and the constant for dissociation of AVP from the monomer, $K_{M/L}$, are obtainable directly and under the same experimental conditions by analytical affinity chromatography, whereas the measurement of binding parameters for different interaction processes otherwise requires different experimental methods (e.g., sedimentation equilibrium, equilibrium dialysis) for different sized interactants and often, as with spectroscopy, depends on measurement of a property that results from binding instead of a direct observation of binding. As high-performance liquid chromatographic technology continues to develop, the use of this technology with suitable affinity matrices offers the opportunity to use analytical HPAC as a flexible and simple tool for measuring macromolecular and small biomolecular interactions—over a wide range of affinity, molecular size, and concentrations of interacting species.

Registry No. AVP, 113-79-1; vasopressin, 11000-17-2.

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