Quantitative high-performance affinity chromatography: Evaluation of use for analyzing peptide and protein interactions

ARTICLE *in* JOURNAL OF CHROMATOGRAPHY A · MAY 1986

Impact Factor: 4.17 · DOI: 10.1016/S0378-4347(00)80825-7 · Source: PubMed

3 AUTHORS, INCLUDING:

Giorgio Fassina
Independent Researcher
141 PUBLICATIONS **2,281** CITATIONS
SEE PROFILE

Harold E Swaisgood
North Carolina State University
208 PUBLICATIONS **4,999** CITATIONS
SEE PROFILE

Journal of Chromatography, 376 (1986) 87—93
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2965

QUANTITATIVE HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY: EVALUATION OF USE FOR ANALYZING PEPTIDE AND PROTEIN INTERACTIONS

GIORGIO FASSINA*, HAROLD E. SWAISGOOD* and IRWIN M. CHAIKEN

Molecular, Cellular and Nutritional Endocrinology Branch, National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20205 (U.S.A.)

SUMMARY

To examine analytical high-performance affinity chromatography as a microscale method for characterizing macromolecular interactions, the chromatographic behavior was evaluated of Arg^8-vasopressin on bovine neurophysin II covalently immobilized in its monomer form on several new high-flow and pressure-resisting affinity supports. Zonal elution of both tritiated and unlabeled peptide hormone and an extension of theoretical treatment of analytical affinity chromatography allowed determination of equilibrium dissociation constants of hormone binding to immobilized bovine neurophysin II. Microamounts of hormone, ranging from 0.05 to 15 μg, were eluted within 20—30 min, with a quantitative recovery of the amount injected. For zones containing more than 5 μg, continuous elution monitoring was possible by ultraviolet absorbance, providing greater speed and accuracy in data analysis. The values obtained for the equilibrium dissociation constants were in good agreement with those previously measured in solution. The above hormone—protein evaluation system has led to identification of several pressure-resistant affinity supports, including silica-, agarose- and glass-based matrices, which are appropriate for use with high-performance liquid chromatographic instrumentation for affinity chromatographic analysis of macromolecular interactions.

INTRODUCTION

The usefulness of affinity chromatography as an analytical technique to determine quantitative properties of molecular interactions has become widely recognized [1–10]. Equilibrium dissociation constants can be evaluated by measuring the retardation of the mobile macromolecules (or ligands) on

*Permanent address: Department of Food Science, North Carolina State University, Raleigh, NC 27695-7624, U.S.A.

immobilized ligands (or macromolecules). Given the advances in high-performance liquid chromatographic (HPLC) technology and its increasing use as a general biochemical tool, we have investigated whether this technology could be adopted for analytical high-performance affinity chromatography (HPAC). The neuroendocrine peptide–protein system of hormones and neurophysin, owing to its complex set of interrelated interaction equilibria [11], has been found to be a useful model to test the limits of analytical HPAC and the applicability of new affinity supports. On this basis, the protein bovine neurophysin II (BNP II) was immobilized on several high-flow, low-compressibility supports and the zonal elution behavior of the peptide hormone Arg^8-vasopressin (AVP) measured. For some of the supports tested, the degree of retardation of AVP conforms closely to that predicted by the affinity of hormone to BNP II in solution. The results help identify supports for which the immobilized neurophysin–mobile hormone interaction is biospecific and which therefore can be used analytically to measure properties of this and by extension other macromolecular interactions.

THEORETICAL

For the monovalent binding interaction,

$$M + L \underset{}{\overset{K_{M/L}}{\rightleftharpoons}} ML \tag{1}$$

where M is immobilized macromolecule, L is mobile ligand, ML is immobilized macromolecule–ligand complex, and $K_{M/L}$ is the equilibrium dissociation constant for the ML complex, the elution volume of L on M can be expressed for porous matrices [10] by

$$\frac{V_o - V_m}{V - V_o} = \frac{K_{M/L}}{[M]_T} + \frac{[L]}{[M]_T} \tag{2}$$

and for non-porous matrices [12] by

$$\frac{V_o}{V - V_o} = \frac{K_{M/L}}{[M]_T} + \frac{[L]}{[M]_T} \tag{3}$$

Here, V is the elution volume of the zone of L, V_o is the unretarded volume (determined with a non-interacting molecule of approximately the same size as L), and V_m is the outside (mobile phase) volume (determined with Dextran blue or other molecules large enough to be excluded from the pores). V_o is the sum of V_m and V_s, the stationary phase pore volume. In eqns. 2 and 3, when [L] is small relative to $[M]_T$, the $[L]/[M]_T$ term approaches zero and the quantity $1/(V - V_o)$ is independent of [L]; thus from eqn. 3,

$$\frac{V_o}{V - V_o} = \frac{K_{M/L}}{[M]_T} \tag{4}$$

On the other hand, at significantly large [L], $1/(V - V_o)$ varies with [L]. For zonal elution, [L] is not easily definable since it changes continuously during the elution. However, the extent of dependence of $1/(V - V_o)$ on [L] can be

approximated by the dependence of $1/(V - V_0)$ on $[L]_o$, the initial concentration of L in the zone applied to the top of the affinity column.

EXPERIMENTAL

Purification of bovine neurophysin II

Acetone powders of bovine pituitaries were extracted with dilute hydrochloric acid and the proteins fractionated by gel chromatography on Sephadex G-75. The fraction containing BNP II was purified further by ion-exchange chromatography on Trisacryl DEAE, as recently described [13]. Biologically active BNP II was obtained from the ion-exchange fractions by biospecific adsorption on a column of Met-Tyr-Phe-aminohexyl-Sepharose [14] equilibrated with 0.4 *M* ammonium acetate at pH 5.7 and elution with 0.2 *M* acetic acid.

Immobilization of neurophysin II on ACCELL-affinity medium

(a) A 500-mg amount of ACCELL-C_6-OSu (polyacrylamide-coated silica, 55—100 μm particle size, with a six-carbon spacer and a terminal carboxyl activated with N-hydroxysuccinimide, obtained from Waters Assoc., Milford, MA, U.S.A.) was washed quickly with 50 ml of deionized water on a sintered-glass funnel. The washed resin was suspended in 4 ml of a solution of 0.7 mg/ml of purified BNP II in coupling buffer at pH 8.0, containing 0.1 *M* sodium bicarbonate and 0.5 *M* sodium chloride. After shaking for 2 h at room temperature, excess liquid was decanted, 160 mg of glycinamide dissolved in 6 ml of the above coupling buffer were added and the suspension was shaken for 1 h at room temperature. The derivatized resin then was washed twice with 50 ml of coupling buffer and twice with 50 ml of water, and dried in vacuo. All of the solutions used in the derivatization procedures were sterilized by passing through a 0.22-μm filter. Product is denoted [BNP II]-C_6-ACCELL(G).

(b) A 758 mg amount of ACCELL-C_6-OSu was reacted with 0.9 mg of purified BNP II, dissolved in 4 ml of coupling buffer, according to the procedure described above. The residual reactive groups on the resin were end-capped with 4 ml of 0.1 *M* ethanolamine, pH 8.0, by shaking for 2 h at room temperature. Resin washing and drying were as above. Product is denoted [BNP II]-C_6-ACCELL(E).

Immobilization of neurophysin II on highly cross-linked agarose

A preparation of highly cross-linked agarose was provided by G. Lindgren (LKB, Bromma, Sweden). To obtain this matrix, a hot agarose solution of 5.6% concentration was emulsified in organic solvent in the presence of a suitable emulsifier [15]. The spherical agarose beads were sieved to approx. 40 · 60 μm (in the wet form). The beads were stabilized and then activated by the tresyl chloride method [16] to give a capacity of 1.0 mmol/g of dry gel (as measured by ^{1}H NMR after coupling with benzylamide).

Wet gel (3.85 g highly cross-linked agarose, tresyl-activated [15], particle size 50 μm,, obtained from LKB) were suspended in 10 ml of pH 8 coupling buffer containing 13.25 mg of purified BNP II. The suspension was incubated at room temperature and shaken for 24 h. After removing the supernatant, 20 ml of coupling buffer containing 144 mg of glycinamide were added and the

suspension was shaken for 3 h at room temperature. The derivatized gel, denoted [BNP II]HXL-agarose, was then washed twice with 30 ml of coupling buffer.

Immobilization of BNP II on controlled pore glass (CPG) and non-porous glass (NPG)

The preparation of these affinity supports has been described before [12].

Instrumentation for analytical high-performance affinity chromatography

[BNP II]-C_6-ACCELL(G) and -(E) supports were dry-packed into 150 mm × 3 mm I.D. and 50 mm × 4.6 mm I.D. stainless-steel HPLC columns, respectively. [BNP II]-HXL-agarose was slurry-packed into a 100 mm × 10 mm I.D. HPLC glass column. The packed columns were installed in an LKB high-performance liquid chromatograph, equipped with a Model 2150 HPLC pump (with ceramic heads), a Model 2151 variable-wavelength monitor and a Model 2211 fraction collector. 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

Chromatography of Arg^8-vasopressin on [BNP II]-C_6-ACCELL

To establish the applicability of [BNP II]-C_6-ACCELL for analytical HPAC, two columns were prepared with different amounts of immobilized BNP II and with different end-capping procedures. Zonal elution profiles of different concentrations of [^{3}H]AVP on [BNP II]-C_6-ACCELL(G) (end-capped with glycinamide, see preparation a in Experimental) are shown in Fig. 1. Each profile consisted of a major retarded peak, with retardation volume varying with μg hormone in the initial zone, and a relatively small, unretarded peak (of inactive component) which has been observed previously [12]. Amino acid analysis of [BNP II]-C_6-ACCELL(G) indicated the presence of 273.5 nmol of protein linked in the entire column volume, with a coupling yield of about 93%. The content of protein bound to the affinity support was intentionally kept low to allow elution of small zones of ligand in a reasonable time and to prevent covalent attachment of BNP II dimers to the matrix. Elution of Dextran blue showed that $V_o = V_m$ for both of the columns. Eqn. 3 indicates that a significant concentration dependence of elution volume is to be expected when the concentration of soluble ligand is large compared to the amount of immobilized binding macromolecule. Since the mobile phase concentration of AVP is not constant during zonal elution, the dependence of V on [L] was assessed by plotting $1/(V - V_o)$ as a function of initial amount of AVP in the 20-μl applied sample. The results are summarized in the inset of Fig. 1 for all of the elution profiles obtained. When the concentration of mobile interactant is negligible with respect to the total amount of immobilized interactant, no concentration dependence of the elution parameters should be observed (eqn. 4). On the other hand, when the injected amount of ligand is large enough, a decrease in elution volume with increasing [L] should occur, as predicted by

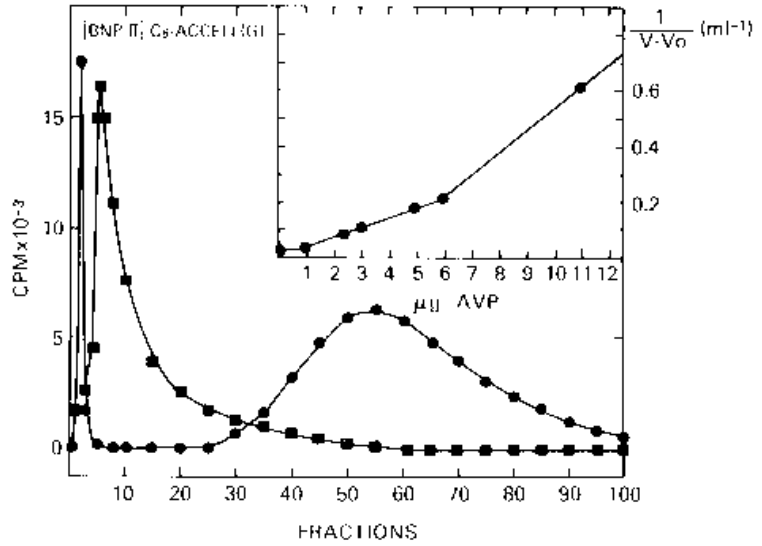

Fig. 1. Elution profiles of commercial [^{3}H]AVP on [BNP II]-C_6-ACCELL(G). The column (150 mm × 3 mm I.D.) was equilibrated and eluted with 0.4 *M* sodium acetate, pH 5.7, at room temperature, using a flow-rate of 0.6 ml/min. A 20-µl sample containing 0.1 µg (•) or 11 µg (▪) of [^{3}H]AVP was injected, with fractions of twelve drops (540 µl) collected directly into vials for scintillation counting. The elution profiles for both 0.1 and 11 µg have a break-through peak at 1.08 ml; for simplicity the peak is shown only for the 0.1-µg elution. Inset: dependence of the elution volume on [^{3}H]AVP concentration, expressed as $1/(V - V_o)$, versus the amount of [^{3}H]AVP in initial 20-µl zone. Note that the values of $1/(V - V_o)$ at lowest and highest µg AVP in inset correspond to • and ▪, respectively, of main figure.

TABLE I

EQUILIBRIUM DISSOCIATION CONSTANTS DETERMINED CHROMATOGRAPHICALLY FOR THE INTERACTION OF AVP WITH IMMOBILIZED NEUROPHYSIN

Literature values for interactions fully in solution range from 6.7 µ*M* (for binding of lysine vasopressin to soluble BNP II dimer from spectrophotometric titration analysis [17]) to 16–20 µ*M* (for binding of oxytocin to soluble BNP II from equilibrium dialysis measurements [18]) to 66 µ*M* (for binding of hormone to soluble BNP II, close to all monomers) from competitive elution quantitative HPAC of BNP II on Met-Tyr-Phe-aminobutyl-agarose [19]).

Method	$K_{M/L}$ (µ*M*)	Reference
[BNP II]-C_6-ACCELL(G)	10.3	This paper
[BNP II]-C_6-ACCELL(E)	10.4	This paper
[BNP II]-HXL-agarose	11.1	This paper
[BNP II]-NPG	74.9	12
[BNP II]-CPG	11.2	12

eqn. 3. Both of these characteristics are observed in the multiphasic variation of $1/(V - V_o)$ shown in the Fig. 1 inset. Using the experimentally determined value for $V_o[M]_T$, extrapolation of $1/(V - V_o)$ to [L] = 0 allows $K_{M/L}$ to be obtained. This value is given in Table I.

Zonal elution profiles also were obtained on [BNP II]-C_6-ACCELL(E) (end-capped with ethanolamine). The lower content of immobilized BNP II (40.5

nmol in the total column volume) permitted elution of zones containing ng quantities of hormone in less than 20 min. The validity of eqns. 3 and 4 was also verified, as illustrated by the biphasic variation of $1/(V - V_o)$. Again, a value for $K_{M/L}$ (Table 1) was calculated by extrapolation of $1/(V - V_o)$ to [L] = 0. With both this and the higher-capacity column (above), the recovery of eluted hormone was very high (i.e. 95% versus hormone applied) as determined by summing the cpm recovered in each fraction.

Chromatography of Arg^8-vasopressin on [BNP II]-HXL-agarose

With [BNP II]-HXL-agarose, the higher amount of immobilized BNP II (698.6 nmol in total column volume, yield 84%) allowed determination of the equilibrium constant $K_{M/L}$ by injecting UV-detectable amounts of hormone. Fig. 2 shows elution profiles of tritiated AVP monitored by either scintillation counting or UV absorbance. Determination of elution volumes with on-line UV monitoring was more accurate and rapid. The recovery of eluted hormone again was high (> 95%). No detectable difference between V_o and V_m was observed with this column. In the concentration range studied the variation of $1/(V - V_o)$ with amount of L conformed to eqn. 3. Extrapolation to [L] = 0 allowed an estimation of $K_{M/L}$ to be calculated, as given in Table I.

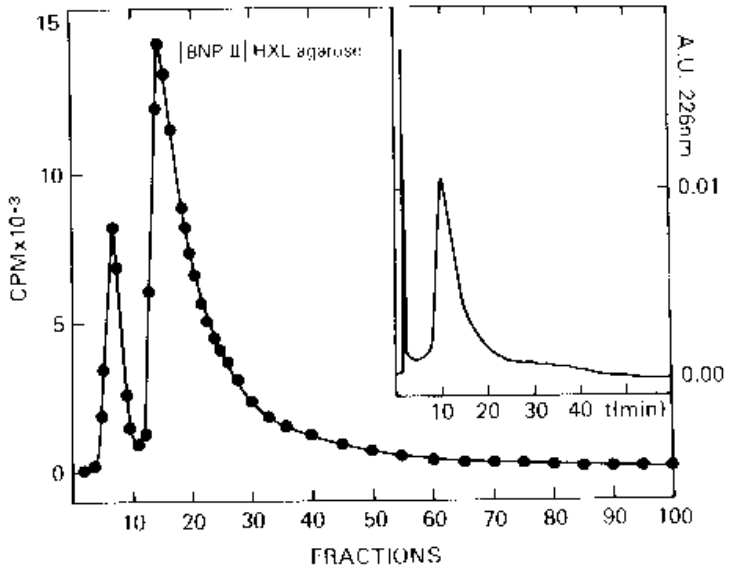

Fig. 2. Elution profiles for commercial [^{3}H]AVP on [BNP II]-HXL-agarose. The column (100 mm × 10 mm I.D.) was equilibrated and eluted with 0.4 *M* sodium acetate, pH 5.7, at room temperature using a flow-rate of 1.0 ml/min. Samples containing 10 μg of [^{3}H]AVP were injected. Fractions of eighteen drops (810 μl) collected directly into vials for scintillation counting. Inset: elution monitored on line by UV absorbance at 226 nm, 0.04 absorbance units (A.U.) full scale. V_o = 2.5 ml.

Zonal chromatography of [^{3}H]AVP on [BNP II]-NPG and -CPG

We previously have studied the zonal elution of tritiated AVP by analytical HPAC using BNP II immobilized on non-porous and porous glass beads [12]. Values of $K_{M/L}$ determined with these matrices are given in Table I for comparison with the values obtained with the matrices reported above.

DISCUSSION

In this paper we have identified affinity supports useful for analytical HPAC. The set of data obtained shows biospecificity of immobilized BNP II towards eluting hormone, with a behavior according to theory. HPAC with these supports allows measurements of reliable values of dissociation constants similar to those in solution. The ease of immobilization reaction, the high coupling yield and the dependable recovery of the soluble interactants injected without non-specific retardation make it possible to control the elution conditions of analytical HPAC experiments, permitting characterization of biomolecular interactions between macromolecules and ligands accessible only in limited amounts. Values of rate constants obtained from chromatography on [BNP II]-CPG and -NPG columns were several orders of magnitude different from those fully determined in solution. The meaning of these chromatographically derived rate constants needs to be clarified by further experiments. Nonetheless, based on the observed general reliability of equilibrium binding constants measured by analytical HPAC, study of biospecific interactions between immobilized BNP II and precursor forms of the hormone is now under way, as is the evaluation of several other affinity supports.

REFERENCES

1 B.M. Dunn and I.M. Chaiken, Proc. Natl. Acad. Sci. U.S.A., 71 (1974) 2382–2385.
2 L.W. Nichol, A.G. Ogsten, D.J. Winzor and W.H. Sawyer, Biochem. J., 143 (1974) 435–443.
3 B.M. Dunn and I.M. Chaiken, Biochemistry, 14 (1975) 2343–2349.
4 R.I. Brinkworth, C.J. Masters and D.J. Winzor, Biochem. J., 151 (1975) 631–636.
5 K.-I. Kasai and S.-I. Ishii, J. Biochem., 77 (1975) 221–264.
6 I.M. Chaiken and H.C. Taylor, J. Biol. Chem., 251 (1976) 2044–2048.
7 B.M. Dunn and W.A. Gilbert, Arch. Biochem. Biophys., 198 (1979) 533–540.
8 J. Donner, J.E. Somerville, J. Turner and B.M. Dunn, Biochemistry, 18 (1979) 3039–3045.
9 I.M. Chaiken, M. Wilchek and I. Parikh, Affinity Chromatography and Biological Recognition, Academic Press, Orlando, FL, 1983, p. 515.
10 I.M. Chaiken, J. Chromatogr., 376 (1986) 11.
11 I.M. Chaiken, D.M. Abercrombie, T. Kanmera and R.P. Sequeira, in M.T.W. Hearn (Editor), Peptide and Protein Reviews, Marcel Dekker, New York, 1983, p. 139.
12 H.E. Swaisgood and I.M. Chaiken, J. Chromatogr., 327 (1985) 193–204.
13 T. Kanmera and I.M. Chaiken, J. Biol. Chem., 26 (1985) 8474–8482.
14 I.M. Chaiken, Anal. Biochem., 97 (1979) 302–308.
15 S. Hjearten, Biochim. Biophys. Acta, 79 (1964) 393–396.
16 K. Nilsson and K. Mosbach, Biochem. Biophys. Res. Commun., 102 (1981) 449–457.
17 A.F. Pearlmutter and E.J. Dalton, Biochemistry, 19 (1980) 3550–3556.
18 P. Nicholas, P. Dessen, M. Camier and P. Cohen, FEBS Lett., 86 (1978) 188–192.
19 S. Angal and I.M. Chaiken, Biochemistry, 21 (1982) 1574–1580.