same nature as protein thermal denaturation.

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Interdependence of Neurophysin Self-Association and Neuropeptide Hormone Binding As Expressed by Quantitative Affinity Chromatography†

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ABSTRACT: The reciprocal modulation of neurophysin self-association and noncovalent peptide–protein interaction between neurophysin and the hormones oxytocin and vasopressin has been assessed by quantitative affinity chromatography. Competitive elutions of radiolabeled bovine neurophysin II (NPII) from the affinity matrices Met-Tyr-Phe-ω-(aminohexyl)- [and (aminobutyl)-] agarose were performed with increasing concentrations of either of the soluble ligands oxytocin or lysine-vasopressin. Also, the dependence of NPII retardation by the same adsorbents on the concentration of applied protein was investigated in the absence of soluble ligand. The affinity constant of NPII for the immobilized peptide increased markedly with increasing amounts of applied protein and with the addition of small amounts of soluble ligand, the latter being more pronounced at higher protein concentrations. The affinity constant of the protein for the soluble ligand showed a smaller increase. The variation of $1/(V - V_0)$ (where V = the NPII elution volume and V_0 = the elution volume of noninteracting control protein) with soluble ligand concentration was linear except near [ligand] = 0. The quantitative affinity chromatographic results on the tripeptidyl affinity columns are consistent with the view that NPII exists in a monomer ⇌ dimer equilibrium, with the dimer exhibiting a stronger interaction with both neuropeptide and tripeptide analogues. The data also indicate that the self-associated protein dimer itself exhibits cooperativity, that is, stronger binding of the immobilized ligand at one site when a second site is occupied with a molecule of the soluble ligand than when no soluble ligand is bound. The deduction from the above of ligand-induced dimerization is evident also in the increased retardation of NPII on neurophysin–Sepharose when the eluting buffer contains soluble peptide hormone.

Neurophysins, a class of disulfide-rich acidic proteins, are synthesized in the hypothalamus and then transported via axons to the posterior pituitary, in neurosecretory granules, as parts of noncovalent complexes with the peptide hormones oxytocin (OXT)[1] and VP (Seif & Robinson, 1978; Acher, 1979; Breslow, 1979). It has become apparent from biosynthesis studies (Sachs et al., 1969; Russell et al., 1980; Nicolas et al., 1980b; Schmale & Richter, 1981; Chaiken et al., 1981) that the origin of neurophysins and the associated peptide hormones is coordinate and involves precursor molecules which

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[1] Abbreviations: NPI and -II, bovine neurophysins I and II; Nps, *p*-nitrophenylsulfenyl; OXT, oxytocin; LVP, 8-lysine-vasopressin; VP, vasopressin; Met-Tyr-Phe-AH-A and Met-Tyr-Phe-AB-A, Met-Tyr-Phe immobilized through the α-carboxyl to (aminohexylamino)- and (aminobutylamino)agarose, respectively.

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may be common for both protein and hormone. The general characteristics of the noncovalent interaction of mature hormones and neurophysins indicate the presence of one major peptide binding site in each protein molecule (Breslow, 1979). However, nonlinear Scatchard plots of binding data (Pliska & Sachs, 1974; Chaiken et al., 1975; Hope et al., 1975; Pearlmutter & Dalton, 1980a) and the presence of a secondary peptide binding site (Nicolas et al., 1976, 1978b, 1980a) have been observed, suggesting complexities in the protein–peptide interactions. Several correlations of peptide binding behavior with independently observed variation of molecular weight (Chaiken et al., 1975; Nicolas et al., 1980b; Tellam & Winzor, 1980) have suggested that protein–protein and peptide–protein interactions may affect one another reciprocally. Such observations have stimulated substantial interest in the description of the fundamental properties of the protein–hormone interaction and of the relationship of these properties to the biosynthetic origin and storage of the complexes in the hypothalamo–neurohypophyseal system.

Previous studies (Dunn & Chaiken, 1974, 1975; Nichol et al., 1974; Lowe et al., 1974; Chaiken & Taylor, 1975; Kasai & Ishii, 1975; Brodelius & Mosbach, 1975; Eilat & Chaiken, 1979; Chaiken, 1979a) have shown that quantitative affinity chromatography can be useful in characterizing noncovalent intermolecular interactions. Affinity adsorbents suitable for such study of the neurophysin–neuropeptide system have been reported, with immobilization of either LVP by coupling through the ϵ-amino group of lysine-8 (Robinson et al., 1976) or Met-Tyr-Phe by coupling through the α-carboxyl function (Chaiken, 1979b). Both matrices allow biospecific interaction with neurophysins by providing a free α-amino and sequence-specific amino-terminal tripeptidyl unit found to be important for peptide ligand binding from solution studies (Breslow et al., 1971; Griffin et al., 1973). We report here the use of the tripeptidyl affinity matrix to detect and quantitate several characteristics both of neurophysin–peptide interactions and of the interdependence of these with neurophysin–neurophysin self-association.

Materials and Methods

Materials. Acetone powders of bovine pituitaries were obtained from Pel-Freeze (Rogers, AR). L-Tyr-L-Phe and Nps-Met (as the dicyclohexylammonium salt) were purchased from Sigma Chemical Co. (St. Louis, MO). Synthetic hormones OXT and LVP were obtained from Calbiochem-Behring Corp. (La Jolla, CA). ω-(Aminobutyl)- and ω-(aminohexyl)agaroses were from Miles Biochemicals (Elkhart, IN). Cyanogen bromide activated Sepharose 4B, DEAE-Sephadex A-50, and Sephadexes G-75 and G-10 were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Bolton–Hunter reagent (monoiodo) was from New England Nuclear (Boston, MA). [^{3}H]Ribonuclease A was kindly donated by Dr. Akira Komoriya in this laboratory. All other chemicals were of reagent grade.

Preparation of Native and Radiolabeled Neurophysins. NPI and NPII were isolated from acetone-dried bovine posterior pituitary glands essentially as cited previously (Fischer et al., 1977). Briefly, acid extraction of dried pituitaries was followed by separation of the neurophysins from their companion hormones by Sephadex G-75 fractionation in 1 M formic acid. Chromatography on DEAE-Sephadex A-50 resulted in the resolution of the two major neurophysins (I and II). These were purified further on Met-Tyr-Phe-containing affinity matrices as described before (Chaiken, 1979b). The purified NPII was homogeneous by electrophoresis on polyacrylamide gels and did not differ substantially in amino acid composition from that determined by Wuu & Crumm (1976). In contrast with results on LVP–Sepharose (Robinson et al., 1976), we did not observe tailing of the acid-eluted peaks on the tripeptidyl matrix. This finding may reflect at least in part the higher rate of dissociation of the (immobilized Met-Tyr-Phe)–neurophysin complex relative to that of the immobilized hormone–neurophysin complex. Such a difference of tripeptide vs. hormone dissociation rate constants has been observed for soluble neurophysin complexes (Pearlmutter & Dalton, 1980a).

NPII was labeled with *N*-succinimidyl 3-(4-hydroxy-5-[^{125}I]iodophenyl)propionate by using the method of Bolton & Hunter (1973). This labeling procedure was chosen since it leads to modification of α-amino-terminal and side-chain ϵ-amino groups of proteins while avoiding modification of the functionally involved tyrosine residue at position 49. The latter residue has been shown to be at or close to the ligand binding region (Griffin et al., 1973; Lord & Breslow, 1980; Abercrombie et al., 1982). On the other hand, considerable modification of the available amino groups permits retention of the ligand binding activity of the neurophysins (Fischer et al., 1977). The ^{125}I-labeled NPII was separated from low molecular weight material by gel-permeation chromatography on Sephadex G-10 in 0.4 M ammonium acetate, pH 5.7, repurified by affinity chromatography, and lyophilized in the presence of added crystalline bovine serum albumin (5 mg). The dried, labeled protein was dissolved in 10 mM phosphate buffer, pH 7.4 (1 mL), to produce a stock solution (specific activity approximately 2.5 μCi/nmol of NPII) which was stored at −20 °C until required. Greater than 90% of the radiolabeled, protein-sized material was found to retain the ability to bind to the affinity column. Some reduction in this percentage was evident upon prolonged storage; therefore, preparations were used before such degeneration became significantly disturbing for affinity chromatographic analysis.

Preparation of Affinity Adsorbents. Adsorbents for analytical chromatography were prepared by coupling Nps-L-Met to L-Tyr-L-Phe followed by condensation of the resultant Nps-Met-Tyr-Phe with ω-(aminohexyl)- or ω-(aminobutyl)-agaroses in the presence of equimolar amounts of dicyclohexylcarbodiimide and *N*-hydroxysuccinimide and subsequent removal of the Nps group (Chaiken, 1979b). The matrices thus produced, viz., Met-Tyr-Phe-AH-A and Met-Tyr-Phe-AB-A, were analyzed for bound peptide content by amino acid composition after 6 N HCl hydrolysis in vacuo at 110 °C for 24 h (Spackman et al., 1958) and were found to contain 0.59 and 0.30 μmol/mL peptide per mL of bed volume of settled gel, respectively.

The NPII–Sepharose matrix was made by coupling NPII to commercial cyanogen bromide activated Sepharose 4B (Pharmacia, Inc.) according to the protocol specified by Pharmacia (protocol sheet 01-900-2-1153-02). The washed product contained about 1 mg of bound protein/mL of bed volume of settled gel. This was estimated by subtracting the amount of protein remaining in solution from the amount of soluble protein added initially, with the amounts determined by the absorbance at 280 nm.

Affinity adsorbents (1–2.5 mL) were packed into Bio-Rad Econo columns (internal diameter 7 mm) and equilibrated in 0.4 M ammonium acetate, pH 5.7. Column elutions were run at room temperature and under gravity flow (20–30 mL/h). Void volumes, V_m, were determined by elutions of blue dextran (10 mg/mL) in 2 M KCl. Total penetrable volumes, V_0, were estimated from elutions of [^{3}H]ribonuclease A, a noninteracting control protein. Fractions containing 0.5 or 1.0 mL were collected and assayed for radioactivity or optical density.

Bound proteins were eluted with 0.2 M acetic acid.

Quantitative Affinity Chromatography. The basic approach in this technique consists of the elution of small zones of protein on affinity columns by using either buffer alone or buffer containing varying amounts of soluble competing ligand (Dunn & Chaiken, 1975). Essentially two types of experiments were performed. (1) Zones of ^{125}I-labeled NPII (10-μL stock solution containing less than 1 μg of labeled protein + 90 μL of 0.4 M acetate buffer) were eluted with 0.4 M acetate buffer on Met-Tyr-Phe-AB-A and Met-Tyr-Phe-AH-A in the absence and presence of varying amounts of OXT and LVP. The procedure was repeated by using zones containing added unlabeled NPII (40.5 and 100 μg on the butyl and hexyl matrices, respectively). (2) Zones of ^{125}I-labeled NPII supplemented with increasing amounts of unlabeled neurophysin were eluted on the same columns by using buffer only. The small amounts (about 50 μg) of carrier bovine serum albumin present in the zones of labeled neurophysin were judged to have no significant effect on the affinity chromatographic behavior of the neurophysins. This was evident from the observation of similar behavior in several elutions in which carrier was omitted (D. M. Abercrombie and I. M. Chaiken, unpublished experiments).

Protein and hormone contents of stock solutions used to make solutions for chromatographic analyses were determined by amino acid analysis after 6 N HCl hydrolysis at 110 °C in vacuo for 24 h. The concentration of ^{125}I-labeled NPII was estimated as an upper limit from the amount of neurophysin initially reacted with K^{14}CNO and the typical yield of protein in subsequent gel filtration and affinity chromatography separation steps.

Interaction of Neurophysin with NPII–Sepharose. A zone of ^{125}I-labeled NPII was eluted on the NPII–Sepharose column with 0.4 M acetate buffer to evaluate the self-association of neurophysin itself. Elution of an identical zone was attempted in the presence of 30 μM LVP. Column parameters V_0 and V_m were estimated as for the peptide affinity columns.

Results

Competitive Elutions and Analysis of Peptide Binding Parameters. Well-separated profiles were obtained when zones of ^{125}I-labeled NPII were eluted on the peptidyl affinity adsorbents by using varying amounts of the soluble competitors OXT and LVP. A representative set of elution profiles is shown in Figure 1 for the elution on the butyl matrix with varying concentrations of soluble LVP. As expected, elution volumes decrease and peaks sharpen as competitor concentrations increase. The comparatively insignificant peak around fraction 6 is independent of hormone concentration and likely is due to inactivation of ^{125}I-labeled NPII derived between preparation and use of the labeled protein. The elution volume, V, was determined visually by triangulation for the major peak in each profile.

Data such as those in Figure 1 were used to determine dissociation constants of ^{125}I-labeled NPII for immobilized and soluble ligands. For this analysis, experimentally derived values of V (elution volumes of NPII on the affinity matrices), V_0 (elution volume in the absence of retardation), V_m (elution volume of impenetrable blue dextran), [$\bar{L}$] (concentration of the immobilized ligand), and [L] (concentration of the soluble ligand) were determined as defined above and under Materials and Methods and used in eq 1 as defined by Dunn & Chaiken (1974). Plots of $1/(V - V_0)$ vs. [L] are shown in Figure 2

$$\frac{1}{V - V_0} = \frac{K_L}{(V_0 - V_m)[\bar{L}]} + \frac{K_L[L]}{K_L(V_0 - V_m)[L]} \quad (1)$$

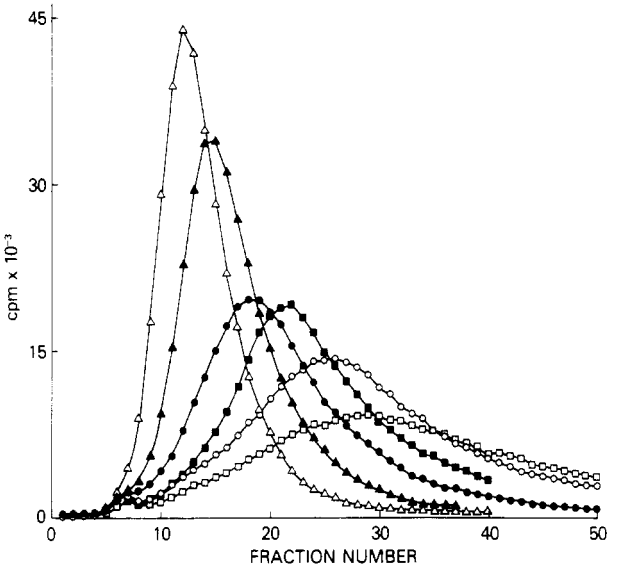

FIGURE 1: Neurophysin competitive zonal elution chromatography. Zones (100 μL) containing ^{125}I-labeled NPII (340 000 cpm) and unlabeled NPII (41.5 μg) were eluted on the affinity adsorbent Met-Tyr-Phe-AB-A (2.5-mL bed volume) in the presence of varying amounts of the soluble competing ligand LVP. Each continuous profile represents an experiment at the following LVP concentration (μM): (△) 64.2; (▲) 42.8; (●) 27.9; (■) 17.1; (○) 9.3; (□) 0. Other operating conditions were as follows: room temperature, gravity flow, 0.5 mL/fraction, and 0.4 M ammonium acetate buffer, pH 5.7. See text for other details.

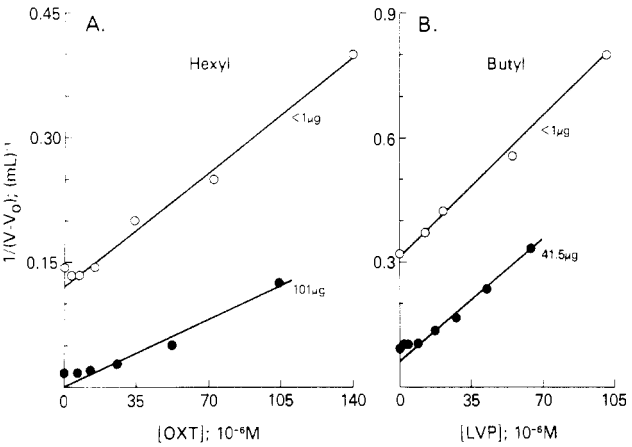

FIGURE 2: Linearized plots of NPII competitive zonal elution data. Elution volumes (V) from the elution series in Figure 1 and three other such series of profiles are represented in terms of $1/(V - V_0)$ vs. [L] as defined by eq 1. Data in (A) are for elution on Met-Tyr-Phe-AH-A, while those in (B) are for Met-Tyr-Phe-AB-A. Open circles are for elutions at low total amounts of protein in zones, while solid circles are for elutions at high total amounts of protein [<1 μg of labeled protein plus unlabeled protein in the amount of 101 μg (A) or 41.5 μg (B)]. Elution volumes (V_0) of a noninteracting protein (ribonuclease A) were determined as described in the text. Straight lines drawn through the data are best fits as defined by linear regression analysis of all data except both those at [L] = 0 and those, for values of [L] near 0, which deviate from linearity.

for the elution series of Figure 1 and for several other series not shown. All plots were linear except at or near [L] = 0. The deviations from linearity were in the direction of greater K_L [greater value of $1/(V - V_0)$] and were particularly apparent at higher protein concentrations. From linear regression analysis of each set of $1/(V - V_0)$ vs. [L] data (excluding those points, at or near [L] = 0, which deviate from linearity), eq 1 allows calculation of dissociation constants for the interaction of NPII with immobilized ligand (K_L) and with soluble hormone (K_L). These values, defined as "K_L and K_L calculated

Table I: Chromatographically Derived Dissociation Constants for Neurophysin and Its Peptide Ligands[a]

amount of neurophysin used per elution (μg)	K_L^- calcd from V at [L] = 0 (M)	K_L^- and K_L calcd from V at [L] ≠ 0 (M)		soluble ligand	spacer
		K_L^-	K_L		
<1	4.8×10^{-5}	4.7×10^{-5}	6.6×10^{-5}	LVP	butyl
<1	5.1×10^{-5}	4.3×10^{-5}	6.2×10^{-5}	OXT	hexyl
41.5	1.4×10^{-5}	8.9×10^{-6}	1.4×10^{-5}	LVP	butyl
101	6.1×10^{-6}	7.0×10^{-7}	1.8×10^{-6}	OXT	hexyl

[a] Numerical values for the dissociation constants were obtained by using eq 1. K_L^- at [L] = 0 is obtained by algebraic calculation from the value of $1/(V - V_0)$ at [L] = 0 by using the simplified form of eq 1 (second term = 0); K_L and K_L^- values at [L] ≠ 0 were obtained by linear regression analyses for $1/(V - V_0)$ values at [L] ≠ 0 as defined in the legend to Figure 2.

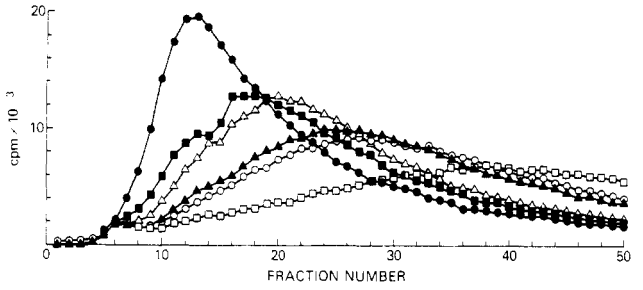

FIGURE 3: Effect of concentration of applied neurophysin on elution behavior. Zones (100 μL) containing ^{125}I-labeled NPII (370000 cpm) and unlabeled NPII (varying amounts as specified) were eluted on Met-Tyr-Phe-AB-A (2.5 mL) in 0.4 M ammonium acetate buffer, pH 5.7, in the absence of soluble ligand. Chromatography was carried out as described in the text, and fractions (0.5 mL) were assayed for radioactivity. Each continuous profile represents a separate elution. Amounts (in micrograms) of added unlabeled NPII per zone were as follows: (●) 0; (■) 6.25; (△) 10.3; (▲) 20.65; (○) 41.3; (□) 82.6.

at [L] ≠ 0", are presented in Table I. Also presented in Table I are values of K_L calculated from the elution volumes at [L] = 0 by using the appropriate simplification of eq 1.

The following observations can be made from examination of the dissociation constants given in Table I. (1) The value of K_L at [L] = 0 is greater than the corresponding value at [L] ≠ 0, with the difference being greater at higher protein concentrations. (2) K_L values decrease with increasing protein concentration, with the decrease more marked in the presence of soluble ligand. (3) K_L decreases similarly with increasing protein concentrations, but to a lesser extent. (4) All of the above trends are qualitatively independent of the nature of the soluble ligand, the length of the spacer, and the density of the ligand immobilized on the matrix.

Effect of Concentration of Applied Protein on Elution Behavior. When zones (100 μL) containing constant amounts of ^{125}I-labeled NPII and increasing amounts of unlabeled NPII were chromatographed on the two tripeptidyl affinity adsorbents, dramatic increases in the elution volumes were observed. This is shown in Figure 3 for the results with the butyl matrix. Minor breaks in the smoothness of some of the elution profiles were not repeatable and therefore were considered to be due to mechanical error. The existence of the small, consistently observed peak at about fraction 6 is due to a small amount of inactive labeled protein.

The greater retardation of labeled protein by both peptidyl affinity matrices in the presence of increasing amounts of unlabeled protein may be expressed as an increase in the

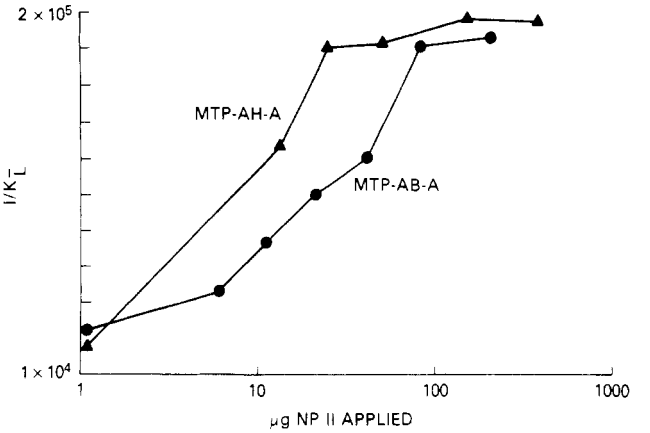

FIGURE 4: Affinity of neurophysin for immobilized ligands as a function of protein concentration. Elution volumes (V) obtained with Met-Tyr-Phe-AB-A (from Figure 3) and with Met-Tyr-Phe-AH-A (not shown) were substituted into eq 1 to yield association constants ($1/K_L$) of NPII for the immobilized ligands. Details are as described in the legend to Figure 3 and the text.

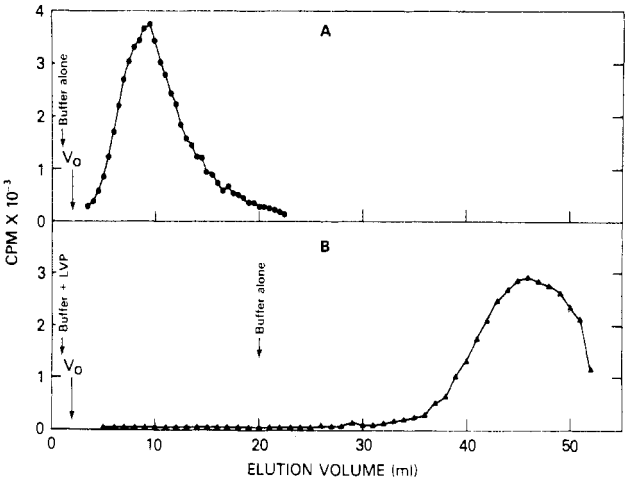

FIGURE 5: Neurophysin self-association in the presence and absence of soluble ligand. (A) Elution of a zone of ^{125}I-labeled NPII (100 μL containing <1 μg of protein) on a column of NPII–Sepharose (1 mL of settled gel) which had been equilibrated in 0.4 M ammonium acetate buffer, pH 5.7. (B) Elution of ^{125}I-labeled NPII as in (A) but with buffer containing 30 μM LVP. After 20 mL, the elution was continued with 0.4 M ammonium acetate alone. Other chromatographic conditions were as described for Figures 1 and 3.

affinity constant ($1/K_L$) of the immobilized ligand for the eluted neurophysin. Values of K_L were calculated for each elution profile in Figure 3 from eq 1 for [L] = 0, assuming the interaction of labeled and unlabeled NPII with the immobilized ligand to be the same. These values are plotted in Figure 4 as $1/K_L$ vs. the amount of protein applied. As shown, both the hexyl and butyl adsorbents were found to exhibit similar affinities for NPII at the lowest and the highest protein amounts used, indicating an effective equivalence of the matrices with four- and six-carbon spacer links at these extremes. However, over the intervening range of the amount of NPII applied, the affinity of the hexyl matrix was always greater than that of the butyl matrix. This behavior can be rationalized on the basis of the view that the 2-fold greater immobilized ligand density of the former matrix should have a greater concentrating effect on the zones of protein, therefore leading to a greater degree of self-association and therein a greater apparent affinity constant (see Discussion).

Hormone-Dependent Neurophysin Self-Association. The retardation of ^{125}I-labeled NPII by the matrix NPII–Sepharose

is shown in Figure 5A. Comparison of the elution volume (V = 8.7 mL) with that of a control elution with [^{3}H]ribonuclease A (V_0 = 2 mL, not shown), using experimentally obtained values of [$\bar{L}$] and V_m, allows an estimate to be made for the affinity of ^{125}I-labeled NPII for immobilized NPII. The association constant ($=1/K_{\bar{L}}$) value so calculated, using eq 1 simplified for [L] = 0, was 6.1×10^4 M^{-1}. As shown in Figure 5B, a repetition of this experiment in the presence of soluble LVP showed that no elution of the labeled protein could be affected with 20 mL of the ligand-containing buffer. Labeled protein could be eluted only after eliminating the hormone from the eluting buffer. These results directly demonstrate a ligand-facilitated protein self-association. As discussed below, this same phenomenon can be deduced from the earlier zonal-competitive elution experiments (Figure 2, Table I) carried out with varying amounts of neurophysin.

Discussion

The last few years have witnessed an explosion in the discovery of hormonally active peptides, particularly in the central nervous system, which has stimulated interest in the biosynthesis and biochemistry of small peptides in general. One set of well-characterized brain peptides which provides a convenient model for the study of peptide–protein interactions is that of oxytocin and vasopressin (Kamm et al., 1928; duVigneaud, 1952; Acher et al., 1958). These chemically related nonapeptides and the noncovalently associated companion neurophysins are biosynthesized coordinately, transported in neurosecretory granules, and stored in the neurohypophysis until released into the circulation upon appropriate stimulation. Beyond the apparent simplicity of the neurophysin–hormone interacting system (stability, ease of purification, and known sequence of the protein; knowledge of the chemical groups of the hormone which interact with the protein and their thermodynamic contributions) lie several unsolved mechanistic aspects. The identities of the amino acid residues of the protein sequence responsible for binding hormone have not been mapped completely (McCormick, 1979). All the neurophysins sequenced to date show a very high degree of homology and internal duplication of a large segment, suggesting the possibility of two active sites. Indeed, although most binding measurements have demonstrated the stoichiometry of binding to be one hormone per polypeptide, Nicolas et al. (1976, 1980b) have noted consistently the binding of a second molecule of LVP, albeit with an apparently lower affinity than that of the first LVP. Furthermore, a second molecule of OXT may bind in the presence of 1.4 M LiCl, the latter of which lowers the affinity of both strong and weak peptide binding sites (Nicolas et al., 1976, 1978b). In contrast, Bothner-By et al. (1980) have been unable to confirm either of these observations of monomer bivalency, and Abercrombie et al. (1982) have observed only one binding site for a neuropeptide analogue photoaffinity labeling reagent. The understanding of the peptide–neurophysin interaction is further complicated by the existence of a concentration-dependent dimerization of neurophysin (Breslow et al., 1971; Nicolas et al., 1976), with the dimer proposed to have a greater affinity for the peptide ligand than the monomer (Nicolas et al., 1976). While Pearlmutter & Dalton (1980a) have determined the hormone association rate constants for the two major sites of the dimer to be equivalent, their data and those of others (Hope et al., 1975; Nicolas et al., 1978a) can be fit to a model involving positive cooperativity in the binding of hormone to the two dimer binding sites. The degree of this cooperativity as reported by Nicolas et al. (1978a) is somewhat greater than that reported by the other two studies. Also, negative cooperativity has been reported for the binding of some small analogue peptides, including Met-Tyr-Phe amide, by mononitrated neurophysin dimers (Pearlmutter & Dalton, 1980a). Briefly, then, an increasingly detailed view has been developing concerning the nature of the interaction of neurophysins and associated peptides.

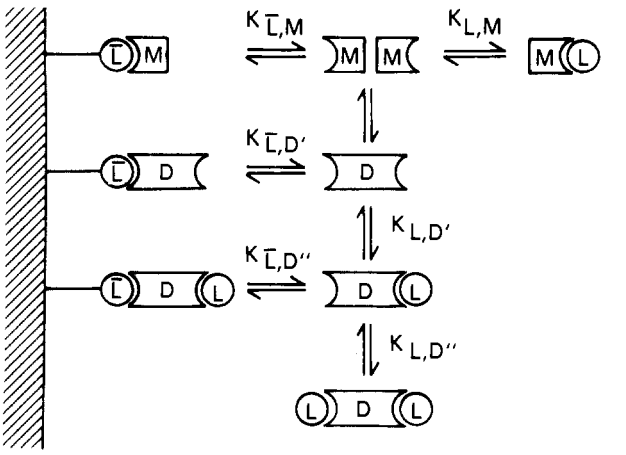

FIGURE 6: Macromolecular interactions taking place in the affinity chromatography of neurophysin on immobilized Met-Tyr-Phe with and without competitive elution with soluble hormone. Interactions are denoted for immobilized Met-Tyr-Phe ($\bar{L}$) and soluble competing hormones (L) for monomeric (M) and dimeric (D) protein species.

In the present paper, we have attempted to use affinity chromatographic behavior to analyze the peptide–protein and protein–protein binding phenomena involved in the mature neurophysin–neuropeptide system. The results obtained can be explained in a unified manner by the model presented in Figure 6. Here, ligand binding is viewed as being mediated both by a protein monomer–dimer equilibrium, where the dimer has a higher affinity for peptide ligands, and by cooperativity of the dimer.

The ways in which the present chromatographic data relate to the model in Figure 6 are reviewed below. At the extremely low concentrations of protein (<1 μM) used for the elution of NPII in the absence of hormone (Figure 4), most if not all of the NPII likely exists in the monomeric state. Since the concentration of immobilized ligand is relatively high (0.3 or 0.59 mM), the probability of monomer-immobilized ligand association is much higher than that of the monomer–monomer collision required for dimer formation. As the protein content of the zone is increased (Figures 3 and 4), a greater potential exists for dimerization. This, combined with the initial concentrating effect of the affinity matrix, probably is sufficient to lead to virtually total dimerization as indicated by the plateau value of $1/K_{\bar{L}}$ at high neurophysin content in Figure 4. Since the profiles plateau at similar values, it may be concluded that the optimum affinities of NPII dimers for both matrices are equal. That an influence of the adsorbent exists is evident, however, from the differences in the profiles for the two matrices. At any given concentration of applied protein, the hexyl matrix is able to induce dimerization to a greater extent than the butyl matrix. This ability can be attributed to the higher ligand density of the former matrix, with the increased density increasing the local concentration of protein. With the assumption that the $1/K_{\bar{L}}$ values at low and high protein concentration in Figure 4 correspond to monomer and dimer binding, respectively, these data further allow estimation of the affinity constants of the immobilized Met-Tyr-Phe for the monomer ($1/K_{\bar{L},M}$) and dimer ($1/K_{\bar{L},D'}$), viz., $\sim 2 \times 10^4$ and $(1-2) \times 10^5$ M^{-1}, respectively. That the dimer has a

5–10-fold greater affinity for peptide ligands than the monomer is consistent with previously reported data (Nicolas et al., 1978a; Pearlmutter & Dalton, 1980b). Interestingly, aggregation to higher order complexes has not been observed for the neurophysins under solution conditions like those used in the present chromatographic study. We verified this for the neurophysin preparations used here by molecular exclusion fractionation analysis. While higher order aggregates may well form at the very high concentrations of protein found in vivo, it seems reasonable to confine the present consideration to monomers and dimers.

The introduction of soluble hormones results in additional effects on the elution behavior of neurophysins on tripeptidyl affinity matrices (Table I). Again, the affinity constant of NPII for the immobilized ligand increases with increasing protein content. Furthermore, the highest value derived from the $1/K_L$ value at $[L] \neq 0$, viz., 1.4×10^6 M^{-1}, is about 8-fold greater than the optimum value of the affinity constant in the absence of soluble ligand, viz., 1.8×10^5 M^{-1} (Figure 4). This observation can best be explained by assuming that the two different moieties containing dimer, that is, dimer alone (D) and the dimer complex with one molecule of bound ligand (DL), have different affinities for the matrix. The higher affinity, since it occurs in the presence of soluble ligand, must be assigned to the binding of the DL species to a molecule of immobilized ligand, thereby forming the binary complex $\bar{L}DL$.

Discontinuous deviations from linearity are observed near $[L] = 0$ in the $1/(V - V_0)$ vs. [L] plots for both tripeptidyl affinity matrices (Figure 2). Considering that the tripeptide concentration of the butyl matrix is half that of the hexyl and that the two matrices differ in the spacer arm size, it is unlikely that the consistently observed deviations are due to differences in monovalent and bivalent binding of the eluting protein to the matrix-bound peptide. Indeed, the absence of the predicted continuous curvilinearity (Eilat & Chaiken, 1979) in the $1/(V - V_0)$ vs. [L] plots (Figure 2) argues against the probability of any significant degree of bivalent binding of the dimer to the matrix, that is, formation of an $\bar{L}D\bar{L}$ complex. Indeed, it may be argued that, at low concentrations of soluble hormone and relatively high concentrations of protein, the effects of ligand-induced dimerization and positive cooperativity have not been fully expressed. Therefore, the affinity of the protein for the matrix is lower under these conditions than it is with high ligand concentration. In contrast, at the lowest concentrations of protein, where about 1% of the NPII would be expected to be present as a dimer as calculated from $K_a = 5.8 \times 10^3$ M^{-1} (Nicolas et al., 1978a), the advent of soluble ligand is insufficient to shift the equilibrium in favor of the dimer to a significant extent. Thus, at the lowest protein concentrations, the dissociation constants in the presence of ligand are similar to those calculated from elutions in the absence of soluble ligand (Table I). Again, the complete absence of continuous curvilinearity in these plots argues against bivalent binding of the monomer on these matrices, under the conditions used. However, since affinity chromatographic behavior is reflective of only a limited steric arrangement of immobilized ligands, the chromatographic evaluation cannot unambiguously exclude the possibility of bivalency of the monomer per se.

Examination of the values of K_L in Table I indicates that arguments analogous to those pertaining to the dependence of K_L on self-association may be invoked for K_L. The value of K_L at low protein concentrations is similar to that reported for the neurophysin monomer (Pearlmutter & Dalton, 1980b; Nicolas et al., 1976), while that of K_L at the highest protein concentration is comparable to literature values for the dimer (Pearlmutter & Dalton, 1980a; Glasel et al., 1976).

Elution of ^{125}I-labeled NPII on the NPII–Sepharose matrix in the absence of hormone (Figure 5A) has enabled the calculation of the monomer–monomer association constant as $1/K_L$ by using eq 1. This value, viz., 6.1×10^4 M^{-1}, is approximately 1 order of magnitude greater than that previously reported (Nicolas et al., 1976). With the assumption that iodination has no significant effect on dimerization, this chromatographic result implies that immobilization may afford greater stability to the dimer. Elution of the labeled NPII from NPII–Sepharose was not feasible in the presence of soluble LVP and occurred only when LVP was eliminated in the washing buffer (Figure 5B). From the elution of labeled protein in the presence of LVP, a lower limit for the elution volume, V, may be set at 45 mL (the elution volume in Figure 5B). Consequently, the protein association constant in the presence of ligand (using eq 1) would be greater than 3×10^5 M^{-1}. This value gives a lower limit to the extent of increase in neurophysin self-association promoted by peptide hormone. However, the actual increase could be much greater, since it is likely that the eventual elution volume under conditions of continuous LVP–buffer elution would far exceed 45 mL.

In summation, the technique of quantitative affinity chromatography has made possible the analysis of various binding phenomena in the neurophysin–neuropeptide system by one unified experimental technique. Ligand-induced self-association of neurophysin has been observed. In addition, differences in equilibrium constants have been defined for the interaction of monomer and dimer with immobilized as well as soluble ligand, and cooperative binding of immobilized ligands to neurophysin dimers has been shown. As reviewed above, phenomena analogous to these have been described previously for neurophysin–peptide complexes in solution, although this in general has required combining observations from several types of measurements (e.g., equilibrium sedimentation, equilibrium dialysis, and stopped-flow kinetics). The special appeal of the affinity chromatographic approach is not only its basic simplicity but also its ability to provide analysis of the various protein–protein and protein–peptide interactions in this system either simultaneously or under closely related conditions.

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