Structural Requirements of Peptide Hormone Binding for Peptide-
potentiated Self-association of Bovine Neurophysin II

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Site-specific, truncated, and sequence-simplified analogs of the hormone [Arg⁵]vasopressin were investigated for the relationship between their abilities to recognize immobilized bovine neurophysin and to promote neurophysin self-association. Peptide binding to neurophysin was measured quantitatively by analytical high performance affinity chromatography on immobilized bovine neurophysin II. Neurophysin self-association, measured as binding of soluble to immobilized neurophysin, was promoted (made higher affinity) by soluble peptide hormone and its analogs, with the effect of particular peptides being proportional to their binding affinities for neurophysin. Sequence-redesigned peptides able to recognize neurophysin, including dipeptide amides, were able to potentiate the self-association to the same extent as the natural hormone when tested at concentrations adjusted to effect equal degrees of saturation of neurophysin. The relationship between peptide affinity to neurophysin and the potentiation of self-association suggests that the latter is directly dependent on the former and can occur even with limited segments of hormone sequence. The data fit best to a model in which hormone binding and self-association surfaces of neurophysin are separate and linked through the neurophysin molecule to produce cooperativity (hormone-promoted self-association). Given that only limited structural elements of hormone are required for promoting self-association, the results fit less well with models in which cooperativity requires that hormone make dimer-stabilizing contacts with both self-associating subunits of neurophysin simultaneously.

Neurophysins are a class of small neuroendocrine proteins which form multimolecular noncovalent complexes with the neurophysypophilic peptide hormones oxytocin and vasopressin. In these complexes, neurophysin self-associates into at least dimers and binds hormone at 1:1 stoichiometry (1:2 under some conditions (1)). Many lines of data have shown that hormone binding and self-association mutually potentiate each other. However, the molecular mechanism which leads to the cooperativity between self-association and hormone binding remains poorly understood.

Hormone binding is associated with a marked protein conformational change (2). Evaluating such structural transitions has been carried out in several spectroscopic studies using the neurophysin Tyr-49. Proximity of the latter residue to the bound peptide hormone has been shown in many studies (3, 4). Using a des-1-8-neurophysin derivative produced by limited tryptic digestion (5, 6), recent NMR data (6) also placed Tyr-49 close to the intersubunit contact region. These NMR results suggested a model in which bound peptide is stabilized by supporting interactions from both subunits and thus participates directly in the self-association surface. This mechanism is distinguishable from models in which hormone binding and self-association surfaces are separate and the cooperative linkage between surfaces is through the neurophysin molecule. The latter has been assumed in previous studies on multimolecular interaction in our laboratory but not actually proven.

We were interested to investigate the question of the extent to which direct interaction versus through-molecule structural transition could account for cooperativity. Sequence redesign and analytical affinity chromatography, easily accomplished in the case of small peptide ligands, can provide a direct understanding of the residues involved in the binding process (7, 8). Previous work (9, 10) showed that immobilization of bovine neurophysin II (BNPII) on high performance affinity supports did not alter its hormone binding and self-association properties at least statistically, with thermodynamic parameters of the interaction process on the solid phase agreeing closely with values obtained in solution (10). Thus, several analogs and sequence-redesigned mutants of the native hormone were synthesized and their binding characteristics as well as their effect on self-association determined by analytical high performance liquid affinity chromatography (HPLAC) (11, 12) on BNPII columns. Results were evaluated with respect to models for the origin of structural linkage between neurophysin hormone binding and neurophysin self-association.

EXPERIMENTAL PROCEDURES AND RESULTS

Peptide and Protein Binding to Immobilized BNPII—Neurophysins are complex interactions with peptide hormones and hormone analogues as well as with neurophysin itself were examined

† The abbreviations used are: BNP, bovine neurophysin; NP, neurophysin; HPLAC, high performance liquid affinity chromatography; OT, oxytocin; AVP, [Arg⁵]vasopressin; RP-HPLC, reverse phase high performance liquid chromatography; Boc, t-butyloxycarbonyl; 4-CH₃Bzl, 4-methyl benzyl; Cl₂-Bzl, 2,6-dichlorobenzyl; Cl-Z, 2-chlorobenzylxoycarbonyl; Tos, tosyl; Pam, phenyl acetamidomethyl; Acet, acetimidyl; HOBT, 1-hydroxybenzotriazole; HXL-agarose, highly cross-linked agrose.

‡ Portions of this paper (including "Experimental Procedures," part of "Results," Tables I-III, and Figs. 1-5) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
Peptide-potentiated Self-association of Neurophysin

The present study was carried out to gain an improved insight into the structural linkage between hormone binding and self-association surfaces in neurophysin. When hormone binding affinity to immobilized NP was compared to the magnitude of hormone potentiation of NP binding to immobilized NP, a strong parallel was demonstrated in the two effects. Analytical HPLAC data with sequence-simplified peptides or dipeptide amides are indicative. Potentiation of self-association not only is present, but the affinities for neurophysin self-association with these peptides are similar to that with the native hormone, when both are tested at similar degrees of saturation. Residues 3-9 in the native hormone apparently are not required to potentiate the self-association properties of liganded neurophysin.

**DISCUSSION**

The present study was carried out to gain an improved insight into the structural linkage between hormone binding and self-association surfaces in neurophysin. When hormone binding affinity to immobilized NP was compared to the magnitude of hormone potentiation of NP binding to immobilized NP, a strong parallel was demonstrated in the two effects. Analytical HPLAC data with sequence-simplified peptides confirmed the major role of the two amino-terminal hormone residues, especially the α-amino and Tyr-2 side chain, in the immobilized NP binding process. Other structural elements which are mutable with only partial loss of binding are the phenylalanine in position 3 as well as the disulfide bridge. These latter are not expected to interact directly with NP but instead to help orient the first two residues for a stronger recognition.

Potentiation of self-association is strictly connected to peptide binding. Compared with native hormone, self-association is promoted to the same extent when sequence-simplified peptides or dipeptide amide are bound to neurophysin. From these data, it is concluded that residues 3-9 in the native hormone do not participate directly in the self-association process. Furthermore, since residue 1 can be substituted with methionine, phenylalanine, or leucine without eliminating ability to potentiate self-association, the side chain in the first residue is unlikely to be specifically involved in the self-association process. From our data we can establish that only the presence of the charged α-amino group and tyrosine 2 side chain, when properly oriented, are important for both binding and self-association. Both groups likely interact directly with the neurophysin surface, the first in an ionic interaction proposed with an NP glutamic acid residue (25) and the second in a hydrophobic interaction proposed with NP Tyr-49 (26).

There is a low but persistent likelihood that the backbone structures of hormone could provide an energetically dominant extension of the self-association surface, which would explain potentiation of self-association. However, potentiation by dipeptides rules out most hormone backbone elements


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**TABLE IV**

<table>
<thead>
<tr>
<th>Peptide in the mobile phase</th>
<th>NP peptide</th>
<th>Peptide concentration in self-association analysis</th>
<th>NP-NP self-association</th>
<th>$K_M$</th>
<th>$n_M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AVP</td>
<td>10.1</td>
<td>0.16</td>
<td>2.8 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>23.3</td>
<td>0.23</td>
<td>3.5 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>42.7</td>
<td>0.43</td>
<td>3.6 ± 0.1</td>
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<td></td>
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<tr>
<td>MYF-NH$_2$</td>
<td>79</td>
<td>0.80</td>
<td>3.8 ± 0.1</td>
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<td></td>
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<tr>
<td>FY-NH$_2$</td>
<td>50</td>
<td>0.50</td>
<td>3.7 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LY-NH$_2$</td>
<td>480</td>
<td>5.00</td>
<td>3.9 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG-NH$_2$</td>
<td>&gt;960</td>
<td>5.00</td>
<td>108.1 ± 1.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Data from Tables II and III.

* Arg(γ)-vasopressin.

* Amides of the following: MYF, Met-Tyr-Phe; FY, Phe-Tyr; LY, Leu-Tyr; GG, Gly-Gly.
as energetically critical. The interaction of main chain groups in the first two hormone residues with two neurophysin molecules cannot be excluded. However, except for this possibility, our data are more consistent with a different model, in which hormone binding leads to modification of neurophysin's tertiary structure so as to alter its recognition surface for self-association (Fig. 6). Previous data support this through-molecule model of structural linkage between hormone and neurophysin. Hormone binding is associated with marked changes in conformation, as judged by CD (27), NMR (28), and fluorescence (29) techniques. Furthermore, studies on semisynthetic precursors of neurophysins I (30) and II (31), in which the hormone is covalently linked to neurophysin through a Gly-Lys-Arg spacer, indicate that the interaction between neurophysin and hormone residues is energetically critical. The interaction of main chain groups through-molecule model of structural linkage between hormone and neurophysin's tertiary structure so as to alter its recognition surface in which hormone binding leads to modification of neurophysin's flexibility, our data are more consistent with a different model, to show a proximity of hormone residues to two neurophysin molecules. However, it is unlikely that this proximity is the sole root of cooperativity between hormone binding and self-association.

REFERENCES


Determination of planar geometry

Calculation of mean planar distribution constants by analytical NMR using eq. (1)

Precursor protein SYNTHESIS

Neurophysin peptides were synthesized by the solid-phase peptide synthesis technique as the Boc-aminoacyl-p-nitroanilide (PNA) resin CD 5-HO 5-CD (9). Peptide synthesis was performed on an Applied Biosystems gas-phase synthesizer using Merrifield's reagents. Deprotection and cleavage of the peptide from the resin were accomplished by treatment with a 40% trifluoroacetic acid (TFA)-anisole-water (50:49:1) mixture at 30°C in a nitrogen atmosphere for 1 h. The peptide was purified by gel chromatography on Bio-Gel P-4. A final purification step was performed by reverse-phase HPLC on a Vydac C18 column (Bondapak, 10 μm, 0.46×25 cm) using 50% acetonitrile in 0.1% trifluoroacetic acid. The peptide was recovered in > 95% yield.

Analysis of NMR

The properties of natural and synthetic peptides to neurophysin as well as self-association were evaluated by analytical NMR. NMR was recorded on a Bruker AMX 500 spectrometer using standard pulse sequences. The peptides were dissolved in D 2 O at concentrations ranging from 10 to 20 mg/ml. The peptides were studied with and without added glycine to a final concentration of 1 mg/ml. All data were collected at 25°C.

RESULTS

The data presented in Table I are a summary of the results obtained in this study. All data were obtained by NMR studies performed on a Bruker AMX 500 spectrometer using standard pulse sequences. The peptides were dissolved in D 2 O at concentrations ranging from 10 to 20 mg/ml. The peptides were studied with and without added glycine to a final concentration of 1 mg/ml. All data were collected at 25°C.

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Peptide-potentiated Self-association of Neurophysin

1. Introduction of the peptide-potentiated self-association of neurophysin was achieved by reduction of disulfide bonds with 2-mercaptoethanol and alkylation with methyl-
4-nitrobenzenesulfonyl fluoride (16). Briefly, purified neurophysin was dissolved at 50 mg/mL in a solution of 0.2 M sodium hydrogen carbonate, 2.5 M dithiothreitol, and 5% (v/v) acetic acid at pH 6.8. The solution was flushed with nitrogen and a 5-fold molar excess of 6-Mercaptohexaneperoxodisulfate was added under a nitrogen barrier. After incubation for 5 hours at 4°C, the solution was treated with 10% methanol and the 6-mercaptohexaneperoxodisulfate was removed by passage through an ion exchange column. The solution was treated with 5% acrylamide and the 6-mercaptohexaneperoxodisulfate was removed by reverse phase HPLC as described before.

Results

1. Binding Affinities of AVP and GnRH for Immobilized BMPI

Interaction of native and synthetic neurophysins with BMPI, as well as BMPI with itself, were measured by competitive binding to a biotinylated affinity matrix specifically designed in the context of human BMPI to allow evaluation of a wide range of affinities, from 10^{-9} to 10^{-11} M. From the linear variations of the extent of saturation, the dissociation constants, $K_d$, were calculated. Each column, without the column in the zinc and extraction of protein to the zinc column, was run in triplicate to evaluate the dissociation constants. For this calculation, the values of $K_d$, amount of active immobilized BMPI, and the amount of purified BMPI were calculated from the standard curves.

2. Elution profile of the zones of BMPI on [GNPII]HXL-agarose AVP 10.1

The column (100 ml) was equilibrated and eluted with 0.4 M sodium acetate, pH 5.1, at room temperature; after a flow rate of 1 ml/h, 180 ml of eluate was collected at 226 nm. The elution profile was obtained. 16-20 pmol of binding of OT to soluble BMPI from equilibrium dialysis studies (14) were calculated for the binding of BMPI to competitive binding with the peptides. The affinity of BMPI for OT was determined by competitive affinity chromatography (15), and 0.75 pmol for binding of insulin-like growth factors to soluble BMPI binder (16).

3. Peptide-potentiated self-association of neurophysin

A 4.7-fold increase in binding of the peptides to the BMPI resulted in a 4.7-fold increase in binding to the BMPI of neurophysin. The affinity of BMPI for OT was determined by competitive affinity chromatography (15), and 0.75 pmol for binding of insulin-like growth factors to soluble BMPI binder (16). The affinity of BMPI for OT was determined by competitive affinity chromatography (15), and 0.75 pmol for binding of insulin-like growth factors to soluble BMPI binder (16).

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TABLE II
Equilibrium Binding Constants of High Affinity Peptides on (BNP)3/Aggarose

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Kbp (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl AVP</td>
<td>0.1</td>
</tr>
<tr>
<td>AVP-1</td>
<td>0.1</td>
</tr>
<tr>
<td>AVP-2</td>
<td>0.1</td>
</tr>
<tr>
<td>AVP-3</td>
<td>0.1</td>
</tr>
</tbody>
</table>

TABLE III
Equilibrium Dissociation Constants of Low Affinity Peptides on (BNP)3/Aggarose

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Kdp (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl AVP</td>
<td>0.1</td>
</tr>
<tr>
<td>AVP-1</td>
<td>0.1</td>
</tr>
<tr>
<td>AVP-2</td>
<td>0.1</td>
</tr>
<tr>
<td>AVP-3</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Fig. 4. Effect of hormone binding on self-association. Peptide-potentiated self-association. Lines of equilibrium binding constants were eluted at a flow rate of 0.5 ml/min. Fractions of 5 drops (250 µl) were collected, mixed with scintillation fluid, and counted for 10 min. Values of Kbp calculated from Eq. 1. Values of Kdp calculated from Eq. 2. Values of Kbp and Kdp are reported in Table IV.