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Onset of neurophysin self-association upon neurophysin/neuropeptide hormone precursor biosynthesis

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The potential of the common biosynthetic precursor of neurophysin and neuropeptide hormones to self-associate has been assessed by quantitative affinity chromatographic analysis. The precursor form, with the hormone sequence in the amino terminal region and assumed able to interact intramolecularly with the hormone binding site of the neurophysin domain of the folded precursor, exhibits an affinity for neurophysin-agarose which is intermediate between those of unliganded neurophysin and non-covalently hormone-liganded neurophysin. The results lead to a prediction that neurophysin self-association is established upon precursor synthesis and prior to limited proteolysis of the precursor to release mature neurophysin and hormone components. Such self-association could play a role in packaging of the precursor into secretory granules and in regulating subsequent precursor processing events within the granules.

Neuropeptide hormone *Neurophysin* *Prohormone association* *Hormone–protein interaction* *Quantitative affinity chromatography*

1. INTRODUCTION

Neurophysins and the neuropeptide hormones, oxytocin and vasopressin, interact with neurosecretory granules to form non-covalent complexes, with the latter serving for storage of the neuropolypeptides in the posterior pituitary after axonal transport from the hypothalamus [1,2]. In the granules of a particular peptide secretory neuron, only one of the two major NP's is present and is associated with only one of the two hormones. This is a consequence of biosynthesis of the protein-peptide molecular pairs in single-chain common precursors [3,4] and the production of only one such precursor type by a particular neuron. In vitro, the neurophysin–neuropeptide hormone system is characterized by a functional interdependence of non-covalent hormone binding and NP self-association [2,5]. Thus, NP self-association, which can occur in the absence of peptide hormone, is enhanced by binding of hormone or hormone analogue. The resultant mutual enhancement of self-association and hormone binding may be an important element in the neurosecretory storage of the protein–neuropep-

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Abbreviations: BNP-II, bovine neurophysin II (vasopressin-associated); NP, neurophysin; LVP and AVP, lysine and arginine vasopressin; OT, oxytocin; pro-NP/AVP, pulse-labelled biosynthetic precursor for AVP and AVP-associated NP; pro-NP/OT, pulse-labelled biosynthetic precursor for OT and OT-associated NP; K_L^{NP}, dissociation constant of unliganded, soluble NP for unliganded, immobilized NP; K_L^{NPL} dissociation constant of liganded, soluble NP for liganded, immobilized NP; K_L^{proNP}, dissociation constant of rat pro-NP/harmone for unliganded, immobilized NP

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tide complexes, for example to help concentrate precursor in the secretory granule forming in the Golgi, to regulate precursor processing within the granule or to regulate intragranular osmotic pressure [6].

It was proposed previously that the hormone binding surface on the neurophysin molecule is established upon folding of the neurophysin-hormone common precursor [2,7]. This is suggested not only by the fact that the hormone sequence is contained in the NP precursor [3,4] but also by the observation that NP disulfide formation, and therefore NP folding as a whole, must occur before proteolytic processing of the precursor [8,9]. It is thus reasonable to propose that the self-association surface on NP is also formed upon precursor folding. If this surface is accessible, folded precursor molecules would self-associate, with an affinity modulated by the intramolecular interaction between NP and peptide hormone domains.

In order to examine the self-association potential of NP precursors, we used quantitative affinity chromatography [10,11]. In [12–14], we found that the presence of a functioning self-association surface in NP's and NP derivatives could be measured with NP–Sepharose, by following either the degree of retardation of radiolabelled NP on this matrix or the extent of interference with this retardation by added unlabelled protein. The method is appropriate for analysis of the micro amounts of available NP-hormone precursors.

2. MATERIALS AND METHODS

BNP-II was isolated and fractionated as in [12,13], with all of the protein samples purified by affinity chromatography before use. [^{125}I]BNP-II was prepared as in [12]. LVP was from Calbiochem. Rat pro-NP/AVP and pro-NP/OT were obtained as either [^{3}H]Cys- or [^{35}S]Cys-containing species by pulse labelling as in [15], with fractionation by gel filtration and immunoprecipitation before use.

[BNP-II]–Sepharose preparation and affinity chromatographic procedures were similar to those in [12,13]. For elutions with [^{125}I]BNP-II, about 1 μg amounts of protein were applied to the affinity column. For elutions of [^{3}H]pro-NP/AVP and -NP/OT and [^{35}S]pro-NP/AVP, very small amounts (less than 10 ng) were applied to the affinity column.

Other details of materials and methods are cited in the text.

3. RESULTS AND DISCUSSION

The data of fig.1 show the behaviour of both unliganded and liganded neurophysin on [BNP-II]–Sepharose. As expected from [12,13], [^{125}I]BNP-II is retarded significantly when eluted in buffer without added hormone (see fig.1 inset for unliganded NP). The elution volume, V = 5.3 ± 0.1 ml, reflects a dissociation constant (K_L^{NP}) of unliganded BNP-II for unliganded immobilized BNP-II of $1.4 \times 10^{-5} \pm 0.1 \times 10^{-5}$ M,

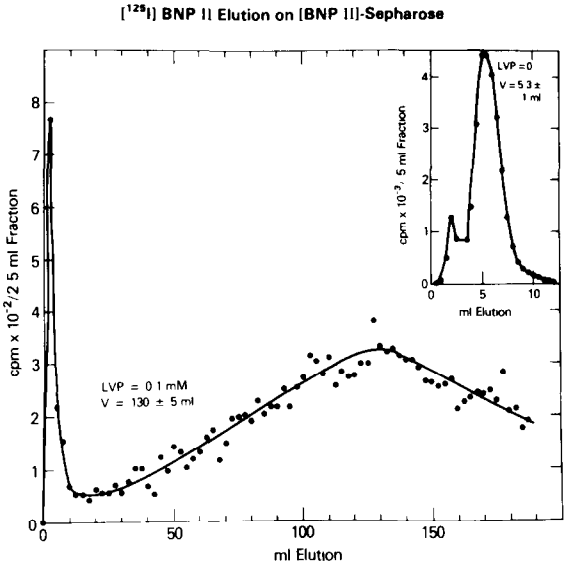

Fig.1. Elution of [^{125}I]BNP-II on [BNP-II]–Sepharose (1.5 ml bed volume, prepared as in [17,25]) in the presence and absence of neuropeptide ligand. Main figure: elution of ~1 μg [^{125}I]BNP-II (containing ~16000 cpm, iodinated by amino group modification as in [12]) in the presence of 0.1 mM lysine vasopressin (LVP, Sigma), in 0.4 M ammonium acetate (pH 5.7) containing 1% bovine serum albumin (BSA fraction V, Pentex). Eluted radiolabel was followed with a Beckman Gamma 4000 counter. Inset: elution of ~1 μg [^{125}I]BNP-II in 0.4 M ammonium acetate (pH 5.7) containing 1% BSA. Radiolabelled protein was applied to the affinity column in 0.3 ml in both cases. Values of elution volumes (V) were determined by triangulation of the elution profiles.

as calculated from the expression [10,11]:

$$K_{\bar{L}} = (V_o - V_m)\,[\bar{L}]/(V - V_o) \quad (1)$$

where: V_o (unretarded elution volume, measured with [^{3}H]RNase-A) = 1.3 ml; V_m (excluded volume, measured with dextran blue) = 0.5 ml; and $[\bar{L}]$ (immobilized protein concentration, measured by amino acid analysis) = 0.07 μmol/ml bed volume. Eq.1 is based on a model of 1:1 interactions of soluble and immobilized molecules. In the case of unliganded NP elution in fig.1, eq.1 holds since the concentration of soluble NP is sufficiently small that self-association of soluble NP monomers can be ignored.

In the presence of 0.1 mM LVP in the elution buffer, BNP-II retardation is much stronger, with $V = 130 \pm 5$ ml (fig.1). From eq.1, apparent $K_L^{NPL} = 4.4 \times 10^{-7} \pm 0.2 \times 10^{-7}$ M. Since the immobilized BNP-II is also capable of binding peptide ligands [16], the data of fig.1 indicate that liganded NP self-association is at least 30-times stronger than that of unliganded NP. That the differential is greater is considered likely on two bases: (i) The LVP concentration in the eluting buffer in fig.1 (1×10^{-4} M) approaches but does not achieve full saturation of NP (K_d for BNP-II is 6.6×10^{-5} M at pH 5.7 for LVP under the chromatographic conditions [12]); (ii) The concentration of soluble NP in the initial zone of applied protein is in a range (10^{-6}–10^{-7} M) at which significant self-association of soluble, liganded monomer can occur. While dilution will ensue during elution, partial self-association in the initial zone would decrease the magnitude of observed retardation of soluble protein and thus would also reduce the observed increase in affinity of liganded *vs* unliganded protein for immobilized NP. Based on the above, the apparent value of K_L^{NPL} must be considered an upper limit.

When rat [^{3}H]pro-NP/AVP is eluted on [BNP-II]–Sepharose, the elution profile shown in fig.2 is obtained. Here, retardation of the major portion of the rat pro-NP/AVP preparation, $V = 21.5 \pm 1.5$ ml, is greater than that for [^{125}I]BNP-II alone (fig.1). The unretarded peak in fig.2 is assumed to be non-specific or inactive protein present in the pro-NP/AVP preparation. From the elution volume of the retarded pro-NP/AVP and eq.1, $K_L^{proNP} = 2.8 \times 10^{-6} \pm 0.2 \times 10^{-6}$ M, compared to $K_L^{NPL} = 1.4 \times 10^{-5} \pm 0.1 \times 10^{-5}$ M and

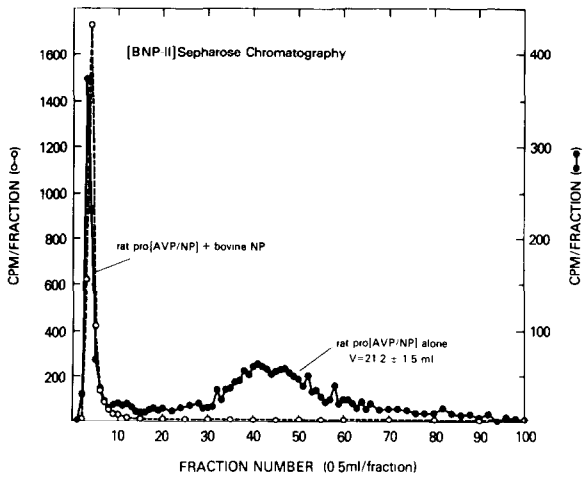

Fig.2. Elution of rat [^{3}H]pro-NP/AVP on [BNP-II]–Sepharose (column as in fig.1 legend) with 0.4 M ammonium acetate buffer (pH 5.7) containing 1% BSA. The precursor protein was prepared as a [^{3}H]Cys-containing species, by pulse labelling and subsequent isolation by gel filtration and immunoprecipitation [15]. It was applied to the affinity column in a zone of 0.44 ml of 0.4 M ammonium acetate (pH 5.7) containing either 1.1 mg (by wt) BSA (●——●) or 1.1 mg (by wt) of an approximately equimolar mixture of BNP-I and -II (○---○). Elution of radiolabelled protein was followed by scintillation counting with a Nuclear Chicago Mark III Counter. The elution volume (V) was determined by triangulation of the elution profile.

apparent $K_L^{NPL} = 4.4 \times 10^{-7} \pm 0.2 \times 10^{-7}$ M. That the retardation of pro-NP/AVP is specific is indicated, also in fig.2, by the observation that addition of a relatively large amount of bovine neurophysin (a mixture of BNP-I and -II) to the zone of [^{3}H]pro-NP/AVP before applying to [BNP-II]–Sepharose leads to elution of all labelled protein without retardation.

In other precursor experiments not shown in fig.2, the elution profile of rat [^{3}H]pro-NP/OT also showed significant (greater than unliganded BNP-II) retardation of most of this precursor preparation on [BNP-II]–Sepharose. In addition, elution of [^{35}S]pro-NP/AVP gave results essentially as shown in fig.2. Interestingly, a previous study [15] also showed retardation of pro-NP/hormone species on immobilized NP, although in that case, elution of precursors was not observed upon prolonged elution with 0.1 M ammonium acetate (pH 5.7) and could be accomplished only with a Triton-formic acid mixture.

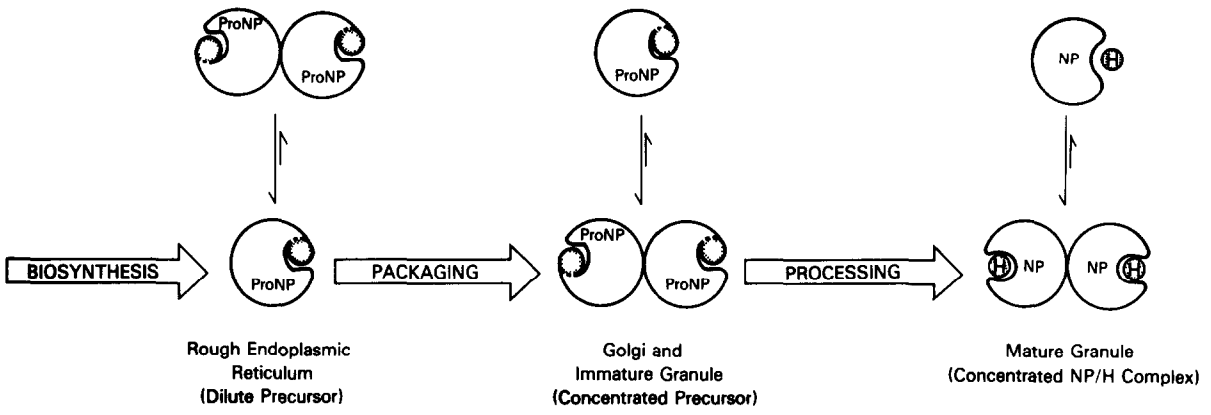

Fig.3. Schematic diagram depicting the onset of NP self-association upon biosynthesis of pro-NP/hormone precursors and their packaging into neurosecretory granules. Subsequent proteolytic processing within the granules is viewed as leading to mature NP–hormone non-covalent complexes which can also self-associate to an extent which is enhanced by hormone binding. (Figure adapted from [17].)

The results of fig.2 argue that pro-NP/AVP not only can associate with mature NP but that this association is stronger than that expected for mature, unliganded NP with itself (fig.1). Given the stronger association of liganded *vs* unliganded NP as seen quantitatively in fig.1, it would appear that pro-NP/hormone precursor behaves in protein association more like the liganded (active site occupied) form. Since the present data describe only pro-NP/hormone association with unliganded NP as the immobilized species, it is reasonable to propose that pro-NP/hormone self-association likely is stronger, approaching perhaps more closely the degree of self-association affinity of liganded NP reflected in fig.1.

In view of the present data, a simplified model for the onset of NP self-association is proposed as shown in fig.3. According to this model, the NP-hormone common precursor self-associates upon precursor folding and before proteolytic processing to non-covalent NP–hormone complexes. The surface for ultimate intermolecular neurophysin–hormone contact is also shown to form first in the folded precursor, as an intramolecular contact between two domains [7]. In this regard, the formation of protein–peptide hormone non-covalent complexes from folded precursors is analogous to the case for bovine pancreatic ribonuclease A; in the latter [18], S-peptide and S-protein 'domains' in ribonuclease A form an intramolecular contact which is retained as an intermolecular contact upon limited proteolysis to ribonuclease S. The observation of NP/hormone precursor self-association lends credence to the possibility that protein self-association is a driving force in the condensation and packaging of precursor molecules into secretory granules and has an effect on subsequent intragranular processing [2,19].

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