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MOLECULAR, THERMODYNAMIC, AND BIOLOGICAL ASPECTS OF RECOGNITION AND FUNCTION IN NEUROPHYSIN-HORMONE SYSTEMS: A MODEL SYSTEM FOR THE ANALYSIS OF PROTEIN–PEPTIDE INTERACTIONS

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I. Introduction

The peptide hormones oxytocin and vasopressin are synthesized principally within the hypothalamus, each as part of a common precursor with its associated carrier protein neurophysin. The major processing of the precursors into hormone and neurophysin occurs subsequent to their packaging into neurosecretory granules (1), the

latter transporting hormones and protein along the nerve axons to the posterior pituitary. Within the granules the processed hormones and neurophysins interact to form noncovalent complexes, which are dissociated by dilution upon secretion into the blood. The events are summarized in Fig. 1.

The hormone-neurophysin system has been extensively investigated from different perspectives. Particular progress has been made in delineating the broad details of precursor processing and elucidating factors involved in hormone–neurophysin recognition. With respect to the latter, the neurophysin–hormone system provides a particularly useful model in which to analyze the thermodynamics of formation of individual noncovalent bonds between peptide and protein and a potential opportunity to relate factors involved in intermolecular complex formation to the process of protein folding itself.

A number of reviews on the neurophysin–hormone system have appeared earlier (2–5). This chapter selectively covers material previously reviewed and more generally describes recent investigations, with particular emphasis on structure–function relationships in the neurophysins and the analysis and implications of bonding thermodynamics in this system.

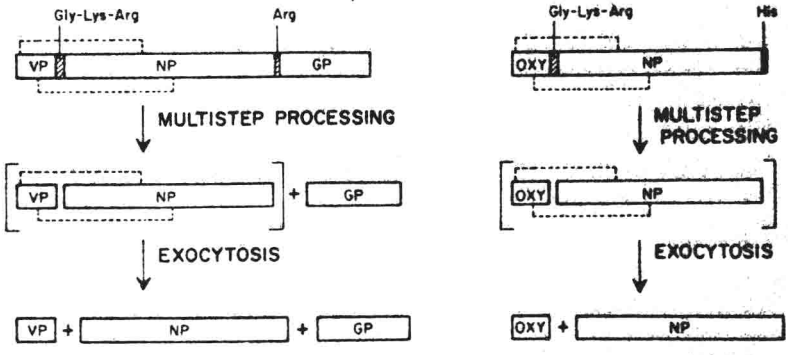


Figure 1. General structures of the vasopressin and oxytocin precursors, after cleavage of signal peptide, and delineation of the path from precursor to free plasma hormone. VP, vasopressin sequence or hormone; OXY, oxytocin sequence or hormone; NP, neurophysin; GP, glycopeptide (copeptin). Dashed lines indicate noncovalent bonds.

II. Primary Structures of Components of the Neurophysin–Hormone System: Relationship to Gene Structure and Evolution

A. MAMMALIAN SYSTEMS: OXYTOCIN, VASOPRESSIN, AND THEIR ASSOCIATED NEUROPHYSINS

The structure of oxytocin and its relationship to vasopressin are shown in Fig. 2. Each hormone is biologically compartmentalized with the neurophysin with which it shares a common precursor. Accordingly, there are two principal neurophysins in each mammalian species, one associated with each hormone; minor neurophysins, representing proteolytic modifications of the principal neurophysins, also are found [e.g. (5)]. The neurophysins were historically numbered according to their relative electrophoretic mobilities, a nomenclature that presents problems when neurophysins from different species are compared (2). The more useful but cumbersome nomenclature is oxytocin-associated neurophysin and vasopressin-associated neurophysin, respectively.

The sequences of neurophysins from a large number of species

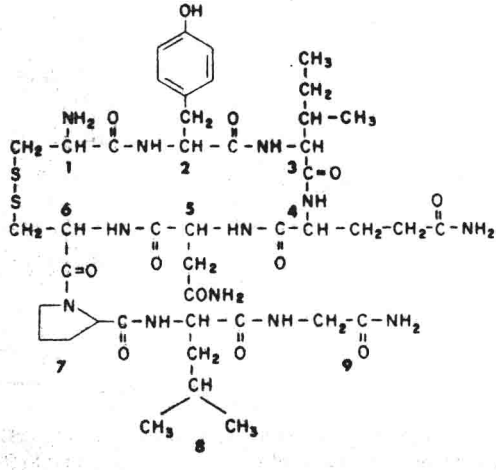
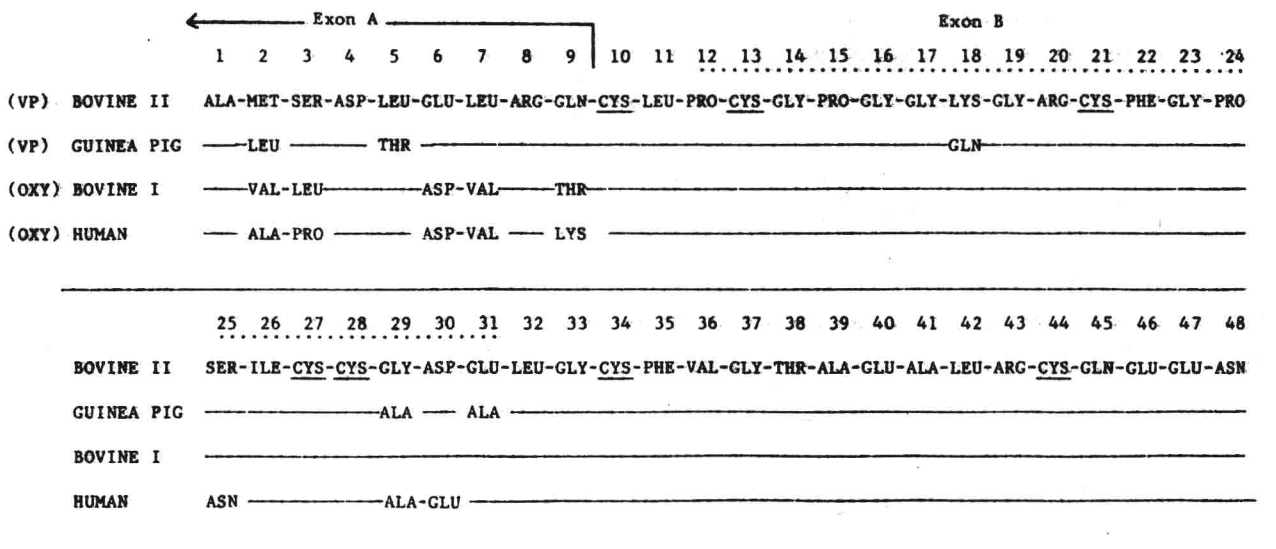


Figure 2. Structure of oxytocin. The structure of vasopressin is identical with the exception of the presence of Phe in position 3 and Arg or Lys in position 8 (arginine vasopressin and lysine vasopressin, respectively).

have been determined by either Edman degradation or cDNA methodology [e.g. (6–12)]. Figure 3 compares the primary structures of bovine neurophysin-II (vasopressin associated) and bovine neurophysin-I (oxytocin associated) with that of guinea pig vasopressin-associated neurophysin and human oxytocin-associated neurophysin. (The guinea pig and human neurophysins were in part selected for comparison here for structural reasons to be discussed later.) The high degree of homology between the two bovine neurophysins indicates that they arose by gene duplication of a common evolutionary precursor (13). This homology is strongly preserved when neurophysins from different species are compared (Fig. 3), a central region of each neurophysin, comprising residues 10–76, being almost invariant in all neurophysins [e.g. (5)]. This conserved region is encoded by a single exon, exon B, of the neurophysin gene, as also indicated in Fig. 3 (14) and probably plays a central role in neurophysin function. Two internally duplicated segments, residues 12–31 and 60–77 (13) also fall largely within the conserved region encoded by exon B (Fig. 3). These segments show ~60% homology to each other, indicating that the primordial neurophysin gene itself arose from a partial gene duplication that extended an initially smaller structure (13).

The less conserved regions of neurophysin structure, residues 1–9 and 77–ca. 95 are encoded by different exons, A and C, respectively (Fig. 3). These regions are not as invariant as that encoded by exon B, but nonetheless contain individual residues, such as Asp-4 and Arg-8 that are strictly conserved. Additionally, they have evolved so that stronger homologies occur between neurophysins with the same hormone association from different species than between the two neurophysins of a single species (5, 13, 15), as shown in Fig. 3. Specific residues within these less conserved regions (e.g., residues 6, 76–78, and 82) in fact show such a high degree of evolutionary conservation within each neurophysin class that they can be used as markers of neurophysin hormone association (5). The designations MSEL- and VLDV-neurophysin are also occasionally used to signify vasopressin-associated and oxytocin-associated neurophysin, respectively (15), and were derived from an early but now somewhat less useful (5) analysis of sequence relationships between the two classes of neurophysins in the amino-terminal region.



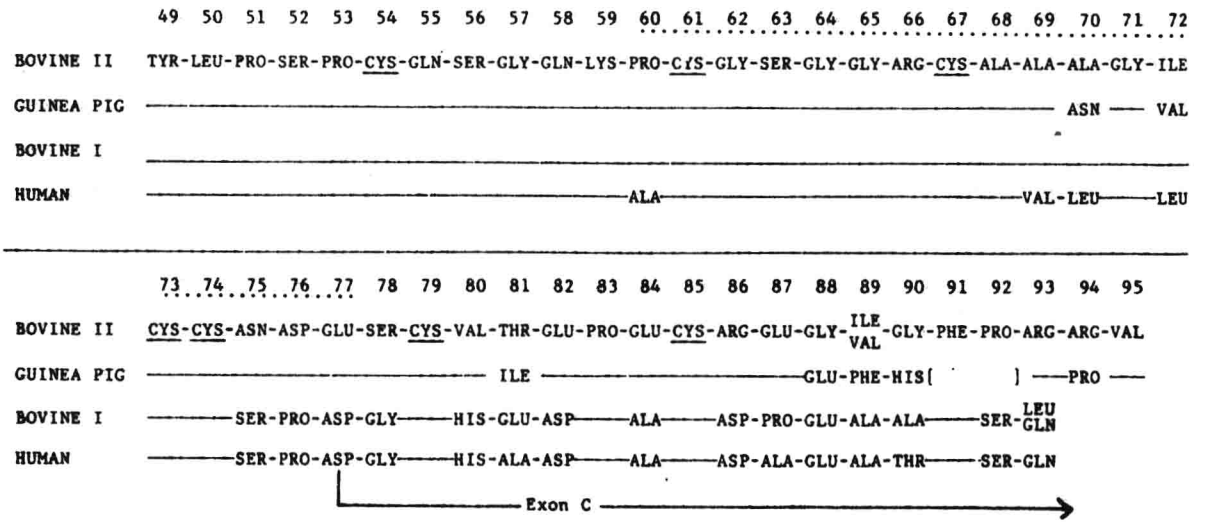


Figure 3. Structures of bovine neurophysin-II (9, 117) and -I (6, 10), guinea pig vasopressin-associated neurophysin (7) and human oxytocin-associated neurophysin (8). The biological hormone association of each bovine neurophysin is also given. The complete sequence is shown only for bovine neurophysin-II. Individual residues in the other sequences are shown where they differ from bovine neurophysin-II. Dotted lines delineate internally duplicated segments. The gene exons encoding the different regions of the protein are also shown.

B. STRUCTURES OF THE OXYTOCIN AND VASOPRESSIN PRECURSORS

The hormones and neurophysins are organized within the *pro* form of their precursors as shown in Fig. 1. The hormone amino terminus is the amino terminus of the *pro* form of the precursor, a fact shown below to have critical implications for precursor folding. Analysis of the DNA encoding the hormone-neurophysin precursor (14, 16, 17) also indicates that, as expected, there is a hydrophobic signal peptide sequence (not shown) that precedes the hormone sequence in the initial product of translation and which is cleaved as the newly synthesized protein crosses the endoplasmic reticulum. In vasopressin precursors, there is also a 39 residue glycopeptide (copeptin) that follows the neurophysin sequence (Figs. 1 and 4) after an intervening Arg residue (17–19). Copeptin shows a high degree of evolutionary conservation (19) and may have a separate biological function (see the following section). The signal peptide and hormone sequences, together with the first nine neurophysin residues are encoded by exon A of the precursor gene, while the glycopeptide is encoded by the same exon (exon C) that encodes the last ~20 residues of neurophysin (14).

C. EVOLUTIONARY CONSERVATION OF HORMONE AND NEUROPHYSINS EXTENDS TO LOWER SPECIES

In nonmammalian species, the hormones oxytocin and vasopressin are replaced by related hormones, with closely related structures, such as mesotocin and vasotocin, respectively (20). The mesotocin and vasotocin precursor sequences in the toad have been deduced by cDNA sequencing (12) and show strong homology with the respective sequences of the mammalian oxytocin and vasopressin precursors. The most unusual substitutions are in the mesotocin precursor, which shows inserts of two additional residues within the neurophysin sequence when aligned for maximal homology with mammalian neurophysins. A single Met can be shown to be inserted

```
 1                5                  10                 15                 20
Ala-Asn-Asp-Arg-Ser-Asn-Ala-Thr-Leu-Leu-Asp-Gly-Pro-Ser-Gly-Ala-Leu-Leu-Leu-Arg

21               25                 30                 35                 39
Leu-Val-Gln-Leu-Ala-Gly-Ala-Pro-Glu-Pro-Ala-Glu-Pro-Ala-Gln-Pro-Gly-Val-Tyr
```

Figure 4. Structure of bovine copeptin (19).

between mammalian neurophysin residues 5 and 6, and a single Asn is inserted between residues 63 and 64 (comparison not shown). The latter insertion is also seen in the mesotocin-associated neurophysin recently sequenced from the ostrich (11).

The high degree of homology among different neurophysins and the homology between internally duplicated segments extends to identities in the encoding nucleotide sequences when different neurophysins within a species are compared. This phenomenon has been attributed to gene conversion (14, 21). Another phenomenon potentially attributable to gene conversion is the occasional parallel evolution of the same residue in both oxytocin- and vasopressin-associated neurophysins. For example, both human neurophysins contain Ala in position 60, while in all other neurophysins this residue is Pro (21).

As implied, the structures of the genes encoding the oxytocin and vasopressin precursors have been determined (14). The two genes are located proximal to each other on complementary DNA strands (22). No evidence is found within the vasopressin gene structure, or that of the gene encoding ACTH (adrenocorticotropic hormone) (23) to support the thesis (24) that the vasopressin- and ACTH-precursors share a common polypeptide precursor.

III. Processing of Neurophysin Precursors

Removal of the signal peptide from the initial product of translation of the oxytocin and vasopressin genes yields the "*pro*" forms of the precursors shown in Fig. 1. The signal peptide cleavage step has not been uniquely characterized in this system. However, it is significant that folding of the precursors must occur subsequently to signal peptide cleavage, since such folding is dependent on a protonated α-NH_2 at hormone residue 1. Hormone and neurophysin segments interact within the *pro* form of the precursor similarly to their intermolecular interactions within the processed hormone–neurophysin complexes, interactions critically dependent on the hormone α-NH_3^+ group (see below). Processing of the *pro* form of the precursors to the mature hormones and neurophysin is in turn mediated by a series of proteolytic enzymes in reactions that are probably modulated by protein conformation. Interactions within the precursors and the self-association properties of precursors and

complexes (see below) appear to decrease the rate of processing and may otherwise alter processing pathways (25).

Enzymatic processing begins with endoproteolytic cleavage at the carboxyl terminus of the Lys-11-Arg-12 dipeptide sequence that separates hormone and neurophysin domains within the precursor (Fig. 1). This is followed by carboxypeptidase B-like exoproteolytic cleavage of Arg-12 and Lys-11 and the subsequent enzymatic amidation of hormone Gly-9 to form the mature hormone. The carboxyl-terminal histidine of the oxytocin precursor and the carboxyl-terminal glycopeptide of the vasopressin precursor, and its preceding arginine, are also excised in most, but not all, species. These steps are described further in the following sections.

A. ENDOPROTEASE AND EXOPROTEASE ACTION ON THE LYS-ARG DOUBLET

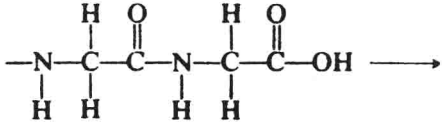
Clamagirand et al. (26, 27) have recently reported purification of an endoprotease from bovine secretory granules that cleaves after the basic amino acid doublet. For substrates, their studies employed synthetic 20-residue peptides with sequences analogous to that of the precursor amino terminus, encompassing the hormone sequence through residue 8 of neurophysin. The purified protease is a 58-kDa protein that behaves as a metalloenzyme with a possible thiol group at the active site rather than as a serine protease or an aspartyl protease. It requires a basic dipeptide for its action; replacement of one of the basic residues by a neutral amino acid or a D-amino acid prevents cleavage. A similar endoprotease has been isolated from bovine corpus luteum (28) and is believed responsible for processing of the oxytocin-associated neurophysin precursor that is synthesized in the ovary. Alternatively, Parish et al. (29) have reported the isolation of an endoprotease from bovine neural lobe secretory vesicles, capable of cleavage at the paired basic residues in proopiomelanocortin, proinsulin, and the arginine vasopressin precursor. The enzyme is similar in its action and properties to proopiomelanocortin converting enzyme derived from bovine intermediate lobe secretory vesicles (30) and is a glycoprotein of $M_r \sim 70{,}000$. It behaves neither as a thiol protease, a serine protease, or a metalloenzyme, but is inhibited by pepstatin A, an aspartyl protease inhibitor. The enzyme therefore appears to be significantly different from that reported by Clamagirand et al. (26, 27) although both were isolated from neural

lobe neurosecretory granules. It remains to be determined which is more important *in vivo*.

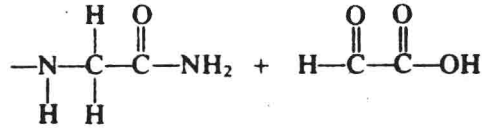
Once the precursor is cleaved at Arg-12 (Fig. 1), a carboxypeptidase B-like enzyme excises Arg-12 and Lys-11. Hook and Loh (31) reported the isolation of this enzyme from the anterior, intermediate, and neural lobes of rat pituitary. Kanmera and Chaiken (32) also reported the isolation of a crude preparation of carboxypeptidase B-like enzyme from bovine posterior pituitary. The enzyme preparations cleave the carboxyl terminal Lys-Lys-Arg resídues from adrenocorticotropin fragment 1–17 in anterior and intermediate lobe granules, and -Lys-Arg residues from [Arg-8] vasopressin-Gly-Lys-Arg in the posterior lobe (31). Enzyme inhibitor studies indicate that the pituitary carboxypeptidase B is similar but nonidentical to pancreatic carboxypeptidase (33, 34).

B. SYNTHESIS OF THE MATURE HORMONES

The central step in the processing of hormone peptides to mature oxytocin and vasopressin is the amidation of Gly-9 (e.g., Fig. 2). The product of carboxypeptidase activity contains the nonapeptide hormone sequence followed by Gly-10; the amide of Gly-9 is supplied by cleavage between the α nitrogen and α carbon of Gly-10 as shown below. Bradbury et al. (35) were the first to report the isolation of an enzyme from porcine pituitary, responsible for the conversion of peptides containing a C-terminal glycine to the corresponding des-glycine peptide amides. They proposed a reaction



$-Gly_9-Gly_{10}$



$-Gly_9-NH_2$ + Glyoxalate

mechanism involving dehydrogenation of the C-terminal glycine and spontaneous hydrolysis of the resulting imino linkage. Subsequently, Eipper et al. (36) and Murthy et al. (37) reported the isolation of enzymes involved in the α-amidation of glycine-extended peptides. These were identified in the secretory granules of rat and bovine anterior, intermediate, and neural pituitary lobes, purified from bovine neurointermediate pituitary granules, and named peptidylglycine α-amidating monooxygenases (PAMs). Multiple forms of the enzyme have been found, differing in apparent molecular weight and charge. The enzymes require molecular oxygen, are stimulated by ascorbate and Cu^{2+} ion, and inhibited by EDTA (ethylenediaminetetracetic acid), 2-mercaptoethanol, and diethyldithiocarbamate. A carboxyl-terminal glycine is needed for substrate processing (36). Based on the requirement for molecular oxygen and ascorbate, the authors postulated a reaction mechanism similar to the one involved in monooxygenases such as dopamine β-hydroxylase.

C. PROCESSING AT THE PRECURSOR CARBOXYL TERMINUS

Cleavage of the C-terminal glycopeptide from the vasopressin precursor probably occurs subsequent to the endoproteolytic cleavage of the hormone-Gly-Lys-Arg sequence. This inference has been drawn from the fact that, in the guinea pig (38), a significant fraction of neurophysin can be isolated that remains covalently attached to copeptin, but from which the hormone-Gly-Lys-Arg sequence has been cleaved. This is also true for vasotocin-associated neurophysin in the frog and related species (39), where copeptin cleavage may not occur at all. The enzyme responsible for copeptin cleavage in vasopressin precursors has not been identified. However, a carboxypeptidase B-like enzyme has been isolated (40), which appears responsible for cleavage of the carboxyl-terminal His of the bovine oxytocin precursor (Fig. 1). Depending on the mechanism by which copeptin is cleaved, the enzyme might also cleave a terminal Arg resulting from copeptin cleavage from the vasopressin precursor. The relationship of this carboxypeptidase to that involved in cleaving the Lys-11-Arg-12 sequence (see above) is uncertain. In addition, the role of the chymotrypsin-like enzyme reported by North et al. (41) in C-terminal precursor processing is unclear. Chauvet et al. (38a) have suggested that interactions between the copeptin and neurophysin domains might modulate carboxyl-terminal processing;