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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND STUDIES OF NEUROPHYSIN—NEUROHYPOPHYSIAL HORMONE PATHWAYS

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SUMMARY

High-performance liquid chromatography (HPLC) is being used extensively to characterize active polypeptides, precursor processing mechanisms, and cooperative peptide—protein noncovalent complexes in neuroendocrine pathways for neurohypophysial peptide hormones, oxytocin and vasopressin, and the hormone-associated proteins, neurophysins. Reversed-phase and ion-exchange HPLC polypeptide mapping have been used to detect the hormones, associated proteins, and other molecular forms containing these. This mapping has provided a means not only to isolate these molecules when present in micro amounts but also ultimately to identify anatomical sites which contain the neurophysin/hormone molecular pathways and to define the relatedness of polypeptide forms contained in different pathways. Reversed-phase HPLC also has provided a means to study proteolytic precursor processing, both to isolate synthetic and semisynthetic polypeptides prepared for use as substrates in processing reactions and eventually to study the polypeptides and intermediates produced by these reactions. Finally, bioaffinity HPLC is being evaluated as a separatory and analytical tool. The latter includes its use to characterize the noncovalent peptide—protein and protein—protein interactions which occur among the molecular forms of the neurophysin/hormone pathways. These experiments typify the impact of HPLC for both analytical and preparative separations in studies of biologically active peptides and proteins.

INTRODUCTION

The neurohypophysial peptide hormones, oxytocin and vasopressin, and the associated small proteins, neurophysins, comprise molecular components of pathways which produce neuroendocrine-active polypeptides for delivery to target sites [1—10]. In the classical, central nervous system neuronal pathway,

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the hormones are made biosynthetically in cell bodies within the hypothalamus, each with one of the two major neurophysins as parts of a single-chain hormone/neurophysin biosynthetic precursor. Folded precursors are packaged in neurosecretory granules and translocated axonally to the neurohypophysis, with concomitant proteolytic processing to generate hormone—neurophysin noncovalent complexes. The complexes remain in the granules at the nerve terminals, as storage forms, until exocytotic release into the circulation, and the hormones subsequently are delivered to receptors at peripheral targets. The scheme of Fig. 1 gives an overall view of the molecular events, leading from biosynthetic precursors to processed polypeptides, which occur in such a pathway. The occurrence of neurophysin/hormone pathways in anatomical sites other than neurons leading from hypothalamus to neurohypophysis, and the physiological meaning of these other pathways, are receiving increased attention. In addition, the molecular mechanisms defining neurophysin/hormone pathways, including structural transitions leading from biosynthetic precursors to biologically active peptides, noncovalent interactions between polypeptide components, and the relatedness of these mechanisms among different pathways, continue to be much-studied subjects.

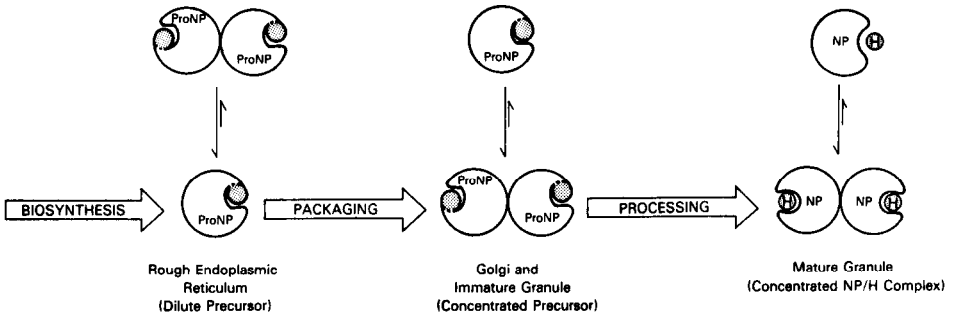

Fig. 1. Schematic diagram depicting the molecular events occurring in neurophysin/neurohypophysial hormone (NP/H) pathways. Overall processes of biosynthesis, packaging into neurosecretory granules, and proteolytic processing of neurophysin/hormone precursors are shown. Precursors are viewed as folded to form intramolecular interactions between hormone and neurophysin domains, with these molecules able to self-associate. The latter interaction may modulate the concentrations of hormones within granules as well as precursor processing events. Proteolytic processing within granules leads to mature hormone—neurophysin noncovalent complexes which also self-associate to an extent which is enhanced by hormone binding. The molecular events viewed to occur in such pathways are based largely on characterization of the most abundant case, hypothalamo-neurohypophysial neurons. The degree of similarity of this scheme to molecular mechanisms in other hormone/neurophysin pathways is not yet fully established. (Reproduced from ref. 11, with permission.)

MOLECULAR COMPONENTS OF MULTIPLE NEUROENDOCRINE PATHWAYS

Recent use of reversed-phase (RP-HPLC) and ion-exchange HPLC for protein and peptide mapping has allowed micro-detection and -isolation of neurophysins, hormones, and molecularly related species. For example, RP-HPLC has provided a valuable tool for detecting neurophysins and differentiating their isoforms, as shown in Fig. 2 for fractionation of bovine

posterior pituitary extracts on a C_8 column. Here, at least two major neurophysin II (vasopressin-associated) and four major neurophysin I (oxytocin-associated) species have been detected. The pattern of isoforms seen in these maps is relatively similar from preparation to preparation (see Fig. 2). Similar isoform separation has been accomplished with C_3 and C_{18} reversed-phase matrices as well as a Pharmacia Mono Q ion-exchange column. All of the above major RP-HPLC isoforms, and several of the minor ones eluting in between, are active, as judged by their ability to bind to peptide ligand affinity columns. The assignment of species as bovine I versus II has been made by comparing amino acid compositions with those expected from the known bovine neurophysin sequences [13, 14]. However, the precise structural differences between species within each of the neurophysin I and II families remain to be established fully. In this regard, limited proteolysis followed by RP-HPLC peptide mapping and analysis of isolated peptide fragments is being used. The data obtained so far suggest that at least some of the microheterogeneity within the neurophysin I family is due to proteolytic truncation at the carboxyl terminus; such truncation perhaps ensues upon prolonged storage of the neurophysins in secretory granules.

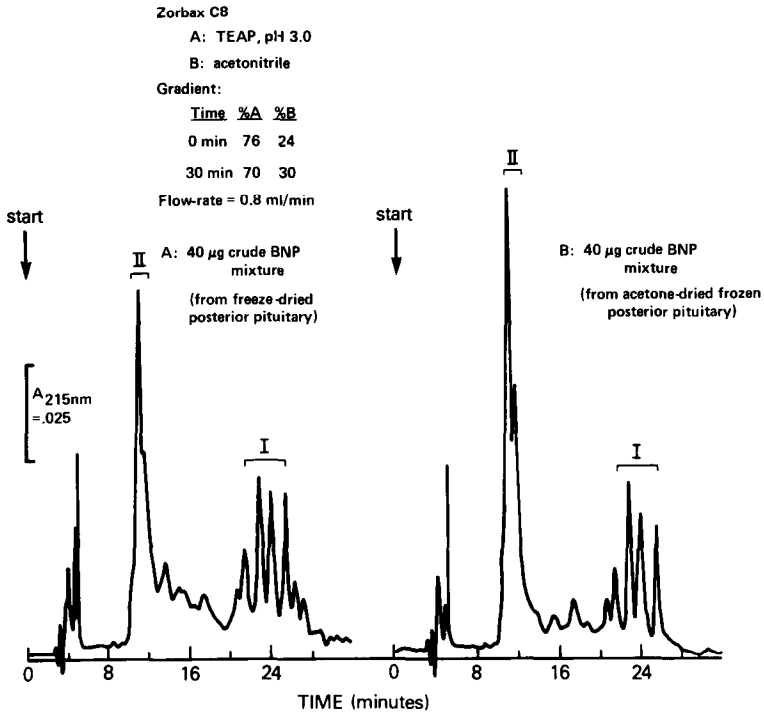

Fig. 2. Reversed-phase HPLC separation of bovine neurophysin (BNP) isoforms. Crude neurophysins were obtained by conventional methods of acid extraction of either (A) freeze-dried posterior pituitaries or (B) acetone-dried frozen posterior pituitaries, salting out of extracts with sodium chloride, and fractionation of dissolved precipitates on Sephadex G-75 in 1 *M* formic acid [12]. Neurophysin fractions from the gel exclusion separations were dissolved in triethylammonium phosphate (TEAP), pH 3.0, and separated on an analytical Zorbax C8 column (Dupont, No. 6119, 25 × 0.46 cm, 6 μm particle size) as denoted. Designations of peaks as neurophysin I- and II-related are based on comparisons of amino acid analyses of individual peaks.

Overall, our recent experience with C_8 and other matrices, along with previous reports with C_{18} [15, 16], emphasize the value of HPLC for detection of neurophysins and neurohypophysial hormones from posterior pituitary extracts. Given the particularly high resolving power of reversed-phase separations such as for the C_8 matrix in Fig. 2, it is encouraging that the proteins obtained from the C_8 fractionation remain functionally active after exposure to the solvents used.

RP-HPLC polypeptide mapping has been used to examine extracts obtained from ovary and peripheral nerve tissue as a means to demonstrate the presence in these anatomical sites of hormone- and neurophysin-related molecular species [17, 18]. For example, RP-HPLC fractionation of bovine ovary extract and analysis of eluted fractions by radioimmunoassay with oxytocin and bovine neurophysin I antibodies has yielded elution profiles such as shown in Fig. 3. The correspondence of elution positions of major immunoreactive species with autentic oxytocin and bovine neurophysin I strongly suggests that substantial amounts of intact or close-to-intact forms of the hormone/neurophysin pair are present in the ovary. This observation agrees with previous reports of oxytocin in the ovary [21, 22]. A developing view from data such as these is that oxytocin and its associated neurophysin are derived by a pathway independent of sources in peripheral circulation. This view is supported by the finding, using an analysis similar to that in Fig. 2, that far more neurophysin I

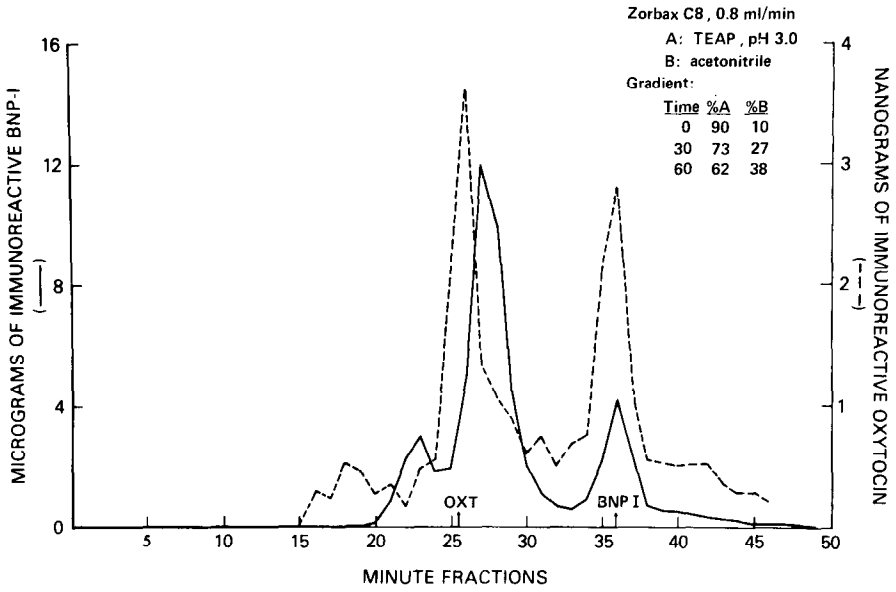

Fig. 3. Reversed-phase HPLC identification of oxytocin and oxytocin-associated neurophysin (neurophysin I) in extracts from bovine ovary tissue. Acid extracts of fresh bovine ovaries were fractionated using a C-18 Sep Pak (Waters), with adsorption in 0.1 *M* acetic acid and elution with ethanol—acetic acid—water (90:4:6, v/v) [19]. The latter fraction (from 0.4 g ovary tissue), after solvent removal, was fractionated on an analytical Zorbax C8 column (No. 6119) as denoted in the figure. Dried fractions were assayed for competition either with [^{125}I]bovine neurophysin I for binding to anti-bovine neurophysin I or with [^{125}I]oxytocin for binding to anti-oxytocin. Radioimmunoassays were carried out by methods similar to those described before [20].

than II is present in ovary extracts and that these relatively large amounts are unlikely to be derived by uptake from the circulation. Also, chromatograms such as that in Fig. 3 show the presence of hormonal immunoreactivity in a peak eluting close to neurophysin, a peak which may represent a larger, precursor-like form of oxytocin (based on elution characteristics of semi-synthetic oxytocin—neurophysin precursor [23]). Such data as the above indicate that a neurophysin/hormone pathway separate from the classical pathway of hypothalamus-to-neurohypophysis generates ovarian oxytocin. One view currently being examined is that the biosynthesis-processing pathway may occur within the ovary itself.

PATHWAYS OF PRECURSOR PROCESSING

While the structure of hormone/neurophysin precursor (see Fig. 4) now has been defined through studies by in vitro translation [24], pulse-labelling [7], and, ultimately, molecular cloning [25], the proteolytic mechanisms by which such precursors are converted to active peptide forms are yet to be defined rigorously. A major constraint has been the lack of readily available precursor in amounts suitable for use in assays for isolating processing enzymes and studying enzymic reactions with the natural substrate or intermediate.

An approach currently being used to alleviate this constraint is the chemical synthesis of sequences which represent likely substrate species. One sequence which has been synthesized chemically is the dodecapeptide, oxytocin-Gly-Lys-Arg (Fig. 4). This peptide represents a possible processing intermediate, derived by proteolytic cleavage at the dibasic sequence, which itself would be converted further to mature oxytocin by a combination of carboxypeptidase and α-amidating activities. As with solid phase synthesized peptides in general, reversed-phase HPLC has been effective in purifying oxytocin-Gly-Lys-Arg from closely related peptides which contaminate the crude synthetic product.

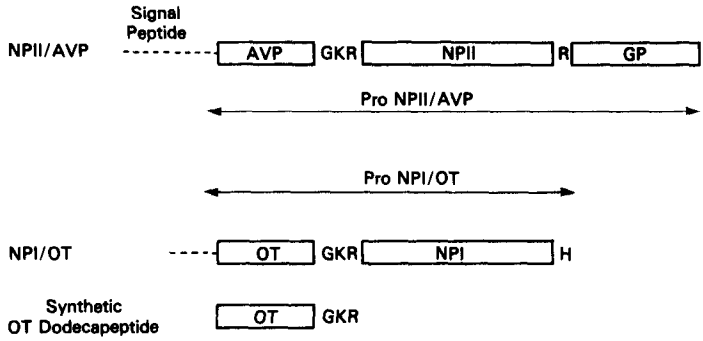

Fig. 4. Schematic diagram of the structure of bovine neurophysin/neurohypophysial hormone biosynthetic precursors [7, 24, 25]. The precursor structures were deduced from nucleotide sequences of cloned c-DNA's. The in vivo-occurring "pro" forms [7] are shown, in addition to the presence of N-terminal signal peptide regions which are contained in in vitro translation products [24, 25]. The synthetic oxytocin dodecapeptide, made as a substrate to detect and characterize processing reactions in the hormone—neurophysin spacer region [26], also is shown. Abbreviations: NPI = neurophysin I; NPII = neurophysin II; AVP = arginine vasopressin; OT = oxytocin; GKR = Gly-Lys-Arg; GP = glycopeptide. (Reproduced from ref. 26, with permission.)

Beyond purification, reversed-phase HPLC mapping is being used as an analytical tool to follow the proteolytic degradation of this peptide by neurosecretory granule extracts [26].

A second tactic in examining molecular properties and processing of precursors has been to prepare semisynthetic precursors by recombining synthetic hormone and mature neurophysin pieces. Recent data [23] have shown that this can be accomplished by chemical coupling of the above-defined oxytocin-Gly-Lys-Arg (as an active ester) with bovine neurophysin I (blocked at ϵ-amino groups). The semisynthetic product, which is distinguishable from native precursor only in its lack of the C-terminal His extension (see Fig. 4), has been isolated from coupling reactions by reversed-phase HPLC using a C_{18} column. It has affinity chromatographic properties, including lack of binding to immobilized peptide ligand but significant retardation via self-association on immobilized neurophysin, consistent with its identity as a precursor-like molecule. These findings suggest the promise to produce and isolate molecules suitable for detection and ultimate isolation of processing enzymes and determination of structural features of precursors which control the processing reactions.

MECHANISMS AND FUNCTION OF COOPERATIVE HORMONE—NEUROPHYSIN INTERACTIONS

Noncovalent neurophysin—hormone complexes produced by precursor proteolysis exhibit cooperative interactions, including neurophysin dimerization, higher hormone binding affinity of dimers versus monomers, and higher hormonal affinity of singly liganded than unliganded dimers. These interrelationships are shown in Fig. 5. Recent data have shown that the biosynthetic

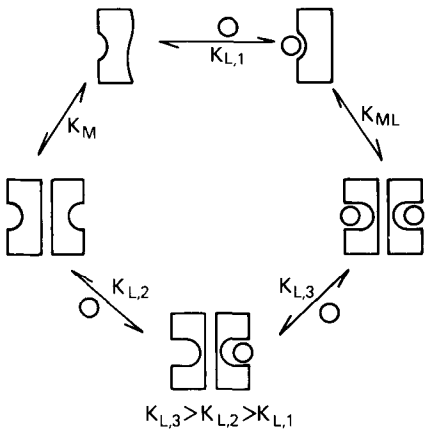

Fig. 5. Scheme of cooperative relationship between peptide ligand (○) binding and protein self-association in the neurophysin/hormone system. $K_{L,1}$, $K_{L,2}$, and $K_{L,3}$ are affinity constants of ligand for neurophysin monomer, unliganded dimer, and singly liganded dimer, respectively. K_M and K_{ML} are affinity constants for self-association of, respectively, unliganded and liganded neurophysin monomers. The scheme denotes the relationship between intermolecular hormone binding and self-association occurring in mature hormone—neurophysin noncovalent complexes. A similar relationship pertains for precursor between intramolecular hormone domain—neurophysin domain interaction and precursor self-association. (Reproduced from ref. 10, with permission.)

precursors self-associate and that the degree of self-association is enhanced over that expected by unliganded monomers [11, 23]. These and other data suggest that the precursor molecules effect hormone—neurophysin binding as an intramolecular domain—domain interaction and, in addition, self-association as an intermolecular interaction enhanced by the intramolecular hormonal interaction. Thus, the noncovalent interactions of the mature neurophysin—hormone complexes can be thought of as vestigial expressions of binding processes that initially are features of biosynthetic precursor structure. In any case, any cogent description of the structural transitions leading from precursors to active peptides in neurophysin/hormone pathways will be aided by an understanding of the inter- and intramolecular interactions which occur at different stages of the pathway.

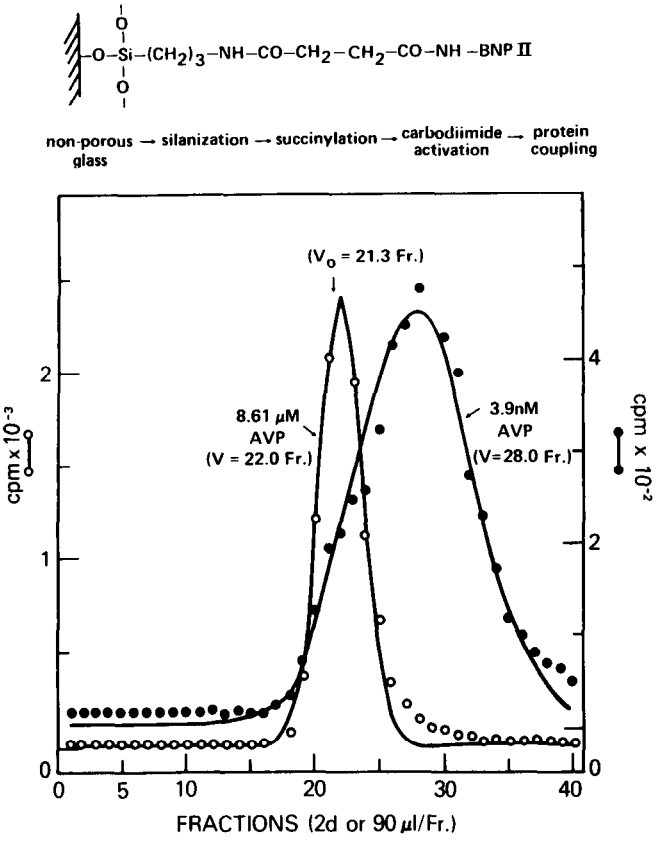

Fig. 6. Analytical bioaffinity HPLC of vasopressin on glass-immobilized bovine neurophysin II. Top: schematic structure of bovine neurophysin immobilized onto non-porous glass. Protein was immobilized via amino groups using a reaction scheme, outlined in the figure, that was developed previously [29]. Bottom: bioaffinity HPLC zonal elutions of tritiated arginine vasopressin on neurophysin—glass matrix in 0.4 *M* ammonium acetate, pH 5.7. The matrix was packed into an empty analytical 25 × 0.46 cm steel column and incorporated into a Varian 5000 liquid chromatograph. [^{3}H]Vasopressin elution, at 0.2 ml/min, was monitored by scintillation counting of collected fractions (2 drops or 90 μl each). Elution volumes, *V*, were calculated by computer-assisted fit of elution data to Gaussian distributions. Elution profiles are shown for two concentrations of vasopressin, 8.61 μ*M* and 3.9 n*M*, which represent the initial concentrations of tritiated peptide in the zone injected onto the affinity column.

We have carried out studies to analyze surface recognition processes in the neurophysin/hormone system by analytical affinity chromatography, and therein have become interested in the use of bioaffinity HPLC. Previous experiments have shown that agarose-immobilized peptides and neurophysins can be used to define quantitative relationships between self-association and hormone binding equilibrium constants [27, 28]. Such analytical chromatography in principle should benefit from the improved instrumentational features afforded by bioaffinity HPLC, including reproducibility in repetitive elution experiments, detection accuracy, possible use of narrow-bore or microbore miniaturizing configurations, and use of non-porous matrices. Recently, glass-immobilized neurophysin has been prepared and tested for use as a tool for quantitative bioaffinity HPLC. As shown in Fig. 6, neurophysin immobilized on non-porous glass beads is functionally active, such that vasopressin is retarded specifically. Interestingly, in this initial work the degree of retardation of vasopressin has been found to be dependent on the amount of hormone in the initial zone, a behavior expected if the amount of mobile interactant is significant compared to the amount of immobilized interactant [30]. Chromatographic data such as in Fig. 6 are being evaluated to calculate both rate and equilibrium constants for the vaspopressive—neurophysin interaction. In general, tests with glass-immobilized neurophysins are being used to help evaluate the use of bioaffinity HPLC as both an analytical and, ultimately, preparative tool.

CONCLUDING COMMENTS

The continuing development of HPLC has led to progressively greater impact of this methodology in separations of peptides and proteins. In many cases, a high-performance method has replaced an existing conventional chromatographic method mainly because the HPLC alternative could accomplish the separation better (more conveniently, with improved resolution, etc.). But, new capability not feasible with conventional separations also has marked the evolution of HPLC. In this regard, application of HPLC to studies of neurophysin/neurohypophysial hormone pathways perhaps is typical. The sensitivity and separatory power of reversed-phase HPLC has had important benefits both in detecting neurophysin isoforms not normally separated in conventional liquid chromatographic preparations from classical anatomical sites (e.g. the neurohypophysis) and in isolating peptide and protein species present in micro amounts (often difficult to separate preparatively by conventional chromatography) in such non-classical sites as the ovary and peripheral nerves. The increased resolving power and ability to fractionate and recover micro quantities by reversed-phase HPLC also have been beneficial in purification and analysis of synthetic precursor fragments and close-to-intact semisynthetic precursors. In the case of bioaffinity HPLC, the advantages over conventional affinity chromatography are as yet not fully defined. But, results so far suggest that analytical and high-sensitivity preparative affinity chromatographic separations both will benefit from application of HPLC technology.

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