Effect of Neurophysin on Enzymatic Maturation of Oxytocin from Its Precursor*

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We examined the extent to which rates of enzymatic conversion of the oxytocin biosynthetic precursor to mature peptide are modulated by intramolecular and intermolecular assembly of precursor and polypeptide intermediates. The biosynthesized precursor contains hormone and neurophysin sequences linked by a Gly-Lys-Arg sequence and undergoes enzymatic processing reactions which include endoproteolytic cleavage at the Lys-Arg dibasic sequence, carboxypeptidase B-like exopeptidase cleavage, and enzymatic amidation. We evaluated the effect of neurophysin on such processing reactions using semisynthetic precursors of oxytocin/bovine neurophysin I and synthetic oxytocinyl precursor intermediates as substrates. Neurophysin I at high concentration (0.7 mM) reduced the rates of carboxypeptidase B-like conversion of oxytocinyl-Gly-Lys-Lys-Arg to oxytocinyl-Gly and the enzymatic amidation of oxytocinyl-Gly to mature (C-terminal amidated) oxytocin. The dependence of rate suppression on the concentrations of peptide substrate and neurophysin I suggested that suppression is due to intermolecular formation of hormone–neurophysin complexes which are aggregated at least to dimers. An analogous intramolecular neurophysin effect was found for endoproteolytic processing of semisynthetic precursors. Endoproteinase Lys-C cleaved the Lys11–Arg12 peptide bond in a native-like semisynthetic precursor at a significantly slower rate than it did an assembly-deficient precursor analogue. The difference in semisynthetic precursor endoproteolysis rates is most substantial at the high concentrations at which the native-like precursor would form dimers but the assembly-deficient analogue would not. The native-like semisynthetic precursor was more stable than the assembly-deficient precursor analogue to tryptic digestion. The concentration-dependent effects of neurophysin, both intramolecularly as a precursor domain and intermolecularly as an interacting protein, are likely to occur in the secretory granules in which the biosynthetic precursors are packaged. The molecular organization of both hormone/neurophysin precursors and the noncovalently complexed hormone/neurophysin intermediates can be expected to play a role in modulating enzymatic processing reactions that lead to mature neurohypophysial hormones.

The neurohypophysial nonapeptide hormones oxytocin and vasopressin are synthesized as parts of common precursor proteins with neurophysins I and II, respectively. The complete amino acid sequences of the precursors, as deduced by sequencing cloned complementary DNA (1, 2), have the general form, hormone-Gly-Lys-Arg-neurophysin-carboxyl-terminal extension. Production of biologically active oxytocin from its precursor occurs in secretory granules during axonal transport by post-translational enzymatic processing reactions that include endoproteolytic cleavage at the Lys-Arg sequence between the hormone and neurophysin sequence, exoproteolytic removal of the terminal basic residues (Arg and Lys) of hormone intermediates by a carboxypeptidase B-like enzyme and perhaps an aminopeptidase, and amidation at sites marked by a C-terminal Gly. Processing enzymes which account for all of these conversion reactions have been detected in secretory granules. Clamagirand et al. (3) reported a dibasic endopeptidase activity which cleaves on the carboxyl side of the dibasic Lys-Arg sequence of a synthetic oxytocinyl-neurophysin fragment. Parish et al. (4) reported the purification of a dibasic endopeptidase which cleaves pro-opiomelanocortin, proinsulin, and proarginine vasopressin/neurophysin II. A secretory granule-associated carboxypeptidase B-like enzyme has been reported (5–8) and recently purified and cloned (9). And, Bradbury et al. (10) and Eipper et al. (11) have detected and purified an amidating enzyme which converts peptides terminating in X-Gly-COOH sequences to X-amides. Characterization of the reactions and specificities of the range of processing enzymes identified in vitro has been accomplished mainly with small molecular weight synthetic peptide substrates. As a result, current understanding of the molecular mechanisms underlying neuropeptide precursor processing as it would occur in secretory granules, including the impact of precursor folding and multimolecular assembly, is still rudimentary.

The precursors of bovine oxytocin and vasopressin, pro-oxytocin/BNPI1 and provasopressin/BNPII, respectively, fold into compact, well-defined conformations upon biosynthesis. Study of the molecular properties of a semisynthetic oxytocin precursor, denoted pro-O/BNPI, and its analogues has shown that the precursor folds so that the hormone and  

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1 The abbreviations used are: BNPI and BNPII, bovine neurophysins I and II, respectively; pro-O/BNPI, semisynthetically derived precursor of oxytocin and bovine neurophysin I in which the ε-amino groups of Lys6 and Lys3 are acetylated and His3 is missing; [Nα- Ac,Ala]pro-O/BNPI, semisynthetic analogue of pro-O/BNPI containing an acetylated Nα-amino and Ala instead of Tyr at position 2; [dIAc]BNPII, bovine neurophysin I (full sequence 1–93) in which ε-amino groups of the 2 lysine residues at positions 18 and 59 are acetylated; OT-GKR, oxytocinyl-Gly-Lys-Arg; OT-GK, oxytocinyl-Gly-Lys; OT-G, oxytocinyl-Gly; RP-HPLC, reverse-phase high performance liquid chromatography; HPLC, high performance liquid chromatography; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Boc-, N-butyxycarbonyl-.
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Neurophysin domains interact intramolecularly and the entire molecule dimerizes (12, 13). Mature hormones and neurophysins are present in granules at concentrations of 60–70 mM and thus are likely to be present predominantly as dimers or larger aggregates (14). Similarly, precursors most likely assemble into self-associated forms upon signal peptide cleavage and intragranular packaging (13). Thus, enzymatic processing of precursors occurs on hormone substrates that almost certainly interact with neurophysins in self-associated complexes. The impact of such molecular interactions on the enzymatic processing reactions which form oxytocin and vasopressin has not been evaluated in depth.

In this study, we have examined the effects of the hormone-neurophysin interaction on enzymatic processing using both semisynthetic oxytocinyl precursors and synthetic oxytocin intermediates as substrates. We compared endoproteolysis rates with trypsin and endoproteinase Lys-C for semisynthetic precursors in which the neurophysin and hormone domains interact to different extents. Carboxypeptidase B and amidation rates were compared for oxytocinyl peptides alone and in the presence of neurophysin.

**MATERIALS AND METHODS**

**Synthesis of Oxytocinyl-Gly-Lys-Arg (OT-GKR)**—OT-GKR was synthesized, analogously as before (12), by conventional solid-phase peptide synthesis using Boc-Ag (tosh)-phenylacetic acid methyl ester (Vega Biotechnologies, Inc., Tucson, AZ). After stepwise coupling of Boc-amino acids using the DCC method and, for Asn and Gin, the dichololhexylocarbodiimidide-hydrobenzotriazole active ester method, the protected peptide was treated with hydrogen fluoride at 0°C for 1 h. Formation of the disulfide bridge between 4-carboxyl residues 1 and 6 was carried out with potassium ferricyanide. The crude product was purified by gel filtration on Sephadex G-25 (1.09 cm width at half-height). Consecutive linear gradients, from 0.1% trifluoroacetic acid in 5% acetonitrile at zero min and 36% acetonitrile at 36 min, were stored at -20°C in the presence of 50% ethylene glycol and 1.0 mg/ml bovine serum albumin. The particular preparation of peptide-glycine α-amidating monooxygenase B used consisted of approximately equal amounts of 38- and 43-kilodalton proteins having the same amino-terminal amino acid sequence (18).

**Preparation of Oxytocinyl-Gly (OT-G)**—OT-G was prepared from OT-GKR by treatment with porcine pancreatic carboxypeptidase B (Sigma). OT-GKR (0.5 mg by weight) was dissolved in 180 μl of 0.2 M sodium hydrogen carbonate, pH 8.3, and incubated with 20 μl of carboxypeptidase B solution (5.6 μg of protein/ml in 0.1 M NaCl) at 37°C for 30 min. The reaction was monitored, and the OT-G product was purified by RP-HPLC on Zorbax CN (0.46 x 25 cm) with elution by three consecutive linear gradients, from 95% to 30% TEAP (67 mM phosphoric acid/triethanolamine buffer, 3.0), 5% acetonitrile at zero time to 95% TEAP, 15% acetonitrile at 15 min from the latter to 30% TEAP, 70% acetonitrile at 50 min from the former, was approximately 1:15,000. After incubation at 37°C for 45 min (Beckman LS-65, 25°C rotator) to remove Percoll. The liquid layer (0.5 ml) of secretory granules (above the semisolid Percoll layer) was withdrawn using a Pasteur pipette. After sonication, fractions were stored at -70°C until used for carboxypeptidase B analysis.

**Purification of Amidation Enzyme**—The purification of peptide-glycine α-amidating monooxygenase was performed on a 40-kilodalton mass for the former, as described above for preparation of OT-G. Enzyme purifications were monitored by UV absorbance at 226 nm.

**Isolation of Neurosecretory Granules**—Fresh bovine neurointermediate pituitaries were isolated from Treuth and Sons (Catonsville, MD). Isolation of neurosecretory granules was carried out, at 0°C on ice, unless stated otherwise, following the method of Russell (16). The pituitaries (12 pieces) were minced and homogenized in 20 ml of 0.25 sucrose containing 10 mM HEPES, pH 7.5, and triturated in a microcentrifuge at 5°C. The supernatant was centrifuged at 26,000 x g for 15 min (Sorvall RC2B, SS-34 rotor) to obtain the crude secretory granule pellet. Each pellet was suspended in 0.25 M sucrose (1.0 mg/ml) for 45 min (Beckman LS-65, 4°C centrifuge, T150-rotor). Six fractions of 50 drops (2 ml) each were collected by puncturing the bottom of the tube, and 8 ml of 0.25 M sucrose solution were added to each fraction. All fractions were then centrifuged at 100,000 x g for 45 min (Beckman L-55, 24°C centrifuge, T50-rotor). The samples were stored at -70°C until used for carboxypeptidase B analysis.

**Amidation of Oxytocinyl Peptides**—The amidation of oxytocinyl peptides was performed by fragment coupling of [diAcet]-BNPI with either [N,N'-dicyclohexylcarbodiimide-hydrobenzotriazole active ester method, (12)]. The protected semisynthetic intermediates were purified by RP-HPLC on Zorbax CN (0.46 x 25 cm width at half-height). Consecutive linear gradients, from 0.1% trifluoroacetic acid in 5% acetonitrile at zero min and 36% acetonitrile at 36 min, were stored at -20°C in the presence of 50% ethylene glycol and 1.0 mg/ml bovine serum albumin. The particular preparation of peptide-glycine α-amidating monooxygenase B used consisted of approximately equal amounts of 38- and 43-kilodalton proteins having the same amino-terminal amino acid sequence (18). The matrix (272 nmol of BNPI/ml-bed volume) was packed in an Omnim column (Pierce Chemical Co., 0.66 x 7.6 cm). Peptides were dissolved at 1 mg/ml in 0.4 M ammonium acetate at pH 5.7. Different amounts (10, 7.5, 2.5, and 1 μl) of each peptide sample were eluted on the affinity matrix with the same buffer at a flow rate of 1 ml/min. Elution profiles were monitored by UV absorbance at 226 nm.

**Analytical Affinity Chromatography of Oxytocin**—The binding affinities of OT-GKR, OT-G, and OT-G for neurophysin were measured by analytical HPLAC using bovine neurophysin II immobilized on highly cross-linked agarose (15). The matrix (272 nmol of BNPI/ml-bed volume) was packed in an Omnim column (Pierce Chemical Co., 0.66 x 7.6 cm). Peptides were dissolved at 1 mg/ml in 0.4 M ammonium acetate at pH 5.7. Different amounts (10, 7.5, 2.5, and 1 μl) of each peptide sample were eluted on the affinity matrix with the same buffer at a flow rate of 1 ml/min. Elution profiles were monitored by UV absorbance at 226 nm.

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was calculated from peak areas. In controls, S-protein was substituted for BNPI in OT-G reaction mixtures.

Site-specific Cleavage of Semisynthetic Precursors by Endoproteinase Lys-C—Semisynthetic precursors Pro"-OT/BNPI and \( \text{N}^{2-}\text{Ac,Ala}^{2} \text{pro}^{-}\text{OT/BNPI} \) (5.5 nmol) were dissolved in 4.2 µl of 150 mM MES, pH 5.5, and incubated at 37 °C with 4.2 µl of endoproteinase Lys-C (Boehringer Mannheim) dissolved (at 38.5 ng/µl) in the same buffer. The final concentration of precursors was 700 µM; the ratio of precursor to enzyme was about 400:1 by weight. The amount of endoprotease Lys-C was chosen to slow the reactions enough to follow their time courses. After incubation for 15, 30, 45, and 60 min, 1.5 µl of each sample were withdrawn, mixed with 30 µl of 10% acetic acid, and frozen until analysis. Samples were analyzed by RF-HPLC on Zorbax CN (0.46 × 25 cm) using an elution by consecutive linear gradients of 90% TEAP, 10% acetonitrile at zero time to 75% TEAP, 25% acetonitrile at 20 min, to 72% TEAP, 28% acetonitrile at 50 min, and then to 40% TEAP, 60% acetonitrile at 55 min. The elution profiles were monitored by UV absorbance at 210 nm.

Limited proteolysis reactions also were carried out using diluted precursors (5.9 nmol of proteins dissolved in 420 µl of 10 mM Na-H₂PO₄/NaOH buffer, pH 5.7), which were incubated with 4.2 µl of endoproteinase Lys-C solution (38.5 ng/µl). The final precursor concentration was 14 µM.

Degradation of Semisynthetic Precursors by Trypsin—Semisynthetic Pro"-OT/BNPI and \( \text{N}^{2-}\text{Ac,Ala}^{2} \text{pro}^{-}\text{OT/BNPI} \) (4.9 nmol each) were dissolved in 58 µl of 150 mM MES, pH 5.5, and incubated at 37 °C with 5.3 µl (0.55 µg in water) of 1-tosylamido-2-phenylthyl chloromethyl ketone-treated trypsin (Millipore Corp.). The final concentration of precursor was 57 µM, and the ratio of precursors to enzyme was about 100:1 by weight. After 5 and 30 min and 1, 2, and 4 h, 9 µl aliquots of each reaction were withdrawn, mixed with 30 µl of 10% acetic acid, and stored frozen until analysis. Samples were analyzed by RF-HPLC on Zorbax CN (0.46 × 28 cm) with elution of 10% acetic acid, and stored frozen until analysis. Samples were monitored by absorbance at 226 nm, and elution volumes \( V \) were determined from peak positions by triangulation. \( V_0 \) was determined with ribonuclease S-protein.

RESULTS

Binding of Synthetic Oxytocin Precursor Intermediates to Neurophysin—An initial hypothesis in this study was that the synthetic oxytocin precursor intermediates OT-GKR, OT-GK, and OT-G are likely to bind to neurophysin noncovalently during processing and that these interactions could affect processing reactions by carboxypeptidase B-like and amidation enzymes. In order to confirm the expectation of neurophysin binding by oxytocinyl substrates, extents of neurophysin interaction were measured for OT-GKR and OT-G by analytical HPLC on BNPII-Sepharose 4B, which was cross-linked to Sepharose 4B as used previously (15) to measure hormone-neurophysin binding. Dissociation constants \( (K_{d, BNPII}) \) for mobile peptide (L)-immobilized neurophysin (M) complexes were calculated using the equation:

\[
V_0/V - V_c = (K_{d, BNPIII}) + ([L]/[M])_T,
\]

where \( V_c \) is experimental elution volume of a small volume zone of L, \( V_0 = \) elution volume of an unretarded molecule (here S-protein), \([L]_T = \) total immobilized BNPII concentration (measured by amino acid analysis of matrix), and \([L] \) = concentration of L in mobile phase. The quantity [L] varies continuously during zonal elution and cannot be determined. Nonetheless, \( K_{d, BNPII} \) can be obtained from the ordinate intercept \( ([L]_T = 0) \) of the plot of \( 1/(V_c - V_0) \) versus \([L]_T \). \([L]_T \) is the initial concentration of [L] in the injected zone. For the column used in this study, the value of \( V_0/[M]_T \) was 7 × 10⁻⁷ mol. Zonal elutions were carried out with 10, 7.5, 5, 2.5, and 1 µl of each peptide, with the data shown in Fig. 1. The values of \( K_{d, BNPII} \) for oxytocin, OT-GKR, OT-GK, and OT-G binding to immobilized BNPII were calculated to be 6.7 × 10⁻⁴, 3.9 × 10⁻⁴, and 8.8 × 10⁻⁵ M, respectively. The results show that the processing intermediates of the family OT-GKR, OT-GK, and OT-G have affinities for neurophysin similar to that of mature hormone and thus almost certainly are bound to neurophysin noncovalently in secretory granules during enzymatic processing.

Effect of Neurophysin on Carboxypeptidase B-like Processing of OT-GKR—We previously reported the action of a carboxypeptidase B-like activity in bovine posterior pituitary secretory granule lysate on OT-GKR (8). We found that rates of conversion of OT-GKR to OT-GK and OT-G could be determined by monitoring product and substrate chromatographically on Zorbax CN (Fig. 2). Using this method of analysis, we measured rates of conversion of 0.71 mM OT-GKR incubated with lysate in the presence of 0, 0.18, 6.35, 0.71, and 1.42 mM BNPI. The elution profiles obtained in the absence of neurophysin are shown in Fig. 2, and the data for all neurophysin concentrations are shown in Fig. 3. A suppression of reaction rate was observed at all neurophysin concentrations except the lowest, 0.18 mM neurophysin I, at which a slight but reproducible acceleration was observed. A slight acceleration also was observed with 71 µM OT-GKR and 71 µM BNPI versus 71 µM OT-GKR alone (data not shown). The acceleration of carboxypeptidase B-like activity also was produced with S-protein, a polypeptide of almost the same molecular weight as BNPI but with no known binding site for oxytocin, and thus was concluded to be nonspecific. However, rate suppression was not detected at any concentration of S-protein. These results argue strongly that the BNPI suppression of carboxypeptidase B-like conversion of OT-GKR is specific and depends on neurophysin binding to substrate. Available affinity data show that the \( K_e \) values for oxytocin-BNPI binding and dimerization of oxytocin-bound bovine neurophysin are 10⁻⁵ and 10⁻⁴ M, respectively. Thus, it is most likely that, at the concentrations at which BNPI affects rate suppression, BNPI and oxytocin-containing peptides exist as noncovalent complexes that are predominantly dimerized. Thus, rate suppression appears to be correlated with formation of substrate-neurophysin dimers or higher aggregates.

![Affinity chromatographic analysis of interactions of oxytocin processing intermediate peptides and oxytocin (OT) with neurophysin by zonal elution on immobilized bovine neurophysin II.](image-url)
Neurophysin Effect on Enzymatic Maturation of Oxytocin

Fig. 2. Enzymatic conversion of OT-GKR by secretory granule carboxypeptidase B-like activity. OT-GKR (71 nmol) was dissolved in 90 µl of 0.2 M sodium acetate, pH 5.5, containing 1 mM CoCl₂ and incubated with 10 µl of granule lysate at 37 °C. At 1, 3, and 9 h, 15 µl of sample were withdrawn, mixed with 35 µl of TEAP, and stored at -70 °C until analyzed. In the right-most elution profile, the chromatographed sample was a mixture of a 7-µl aliquot of the 6-h reaction and 4.3 nmol of authentic oxytocin. Sample analyses were by elution on Zorbax CN as described under "Materials and Methods."

Effect of Neurophysin on Amidation of OT-G—Given the above, the effect of neurophysin on enzymatic conversion of OT-G to mature, COOH-terminally amidated oxytocin was evaluated. OT-G and mature oxytocin are distinguishable chromatographically as shown in Fig. 2. Oxytocinoic acid, which contains a free α-carboxyl instead of an amide group at Gly², elutes at the position of OT-G in the RP-HPLC system.

When OT-G was incubated with purified peptidyl-glycine α-amidating monooxygenase B, significant conversion to oxytocin was detected only in alkaline buffers, with an optimal pH of about 8.5 (Fig. 4). No amidation conversion of OT-G to oxytocin was observed at the reported internal pH of 5.7 for mature secretory granules (19, 20), although peptidyl-glycine α-amidating monooxygenase B is active at that pH based on the observed conversion of D-Tyr-Glu-Gly (18). When 70 μM OT-G was incubated with purified peptidyl-glycine α-amidating monooxygenase B (molar ratio of enzyme to substrate was 1:15,000) in 150 mM TES buffer, pH 8.5, for 8 h, 8% conversion was observed; no other degradation products were detected. In these amidation reactions with OT-G, conversion levels were kept relatively low by using low enzyme amounts. This condition was set up purposely in order to keep the reaction rates in the linear range. Higher levels of conversion could be observed by adding more enzyme. Repeat experiments were performed routinely to confirm the pattern of enzyme activity variation observed with the low conversion levels used.

To evaluate the effect of neurophysin on amidation, the amidation assays were performed in the presence of 70 and 700 μM BNPI at pH 8.0, 8.5, and 9.0. Addition of 700 μM BNPI suppressed the amidation rate; but, again, at lower concentrations of BNPI (70 μM), rate enhancement was observed (Fig. 4). When the same reactions were done in the presence of 70 and 700 μM S-protein instead of BNPI, rate suppression was not seen at either concentration and only rate enhancement was observed, of a magnitude similar to that found with BNPI. Again, results argue that suppression of amidation of OT-G by high concentration of BNPI is specific, whereas the low concentration enhancement is not. The concentration dependence of rate suppression suggests a correlation of this rate effect with formation of self-associated substrate-neurophysin complexes. Noncovalent hormone-neurophysin binding and, to a lesser extent, neurophysin self-association are weakened as the pH is raised above 6 (21). However, both interactions do occur at pH values at least as...
high as 8 and also can be expected to potentiate each other by positive cooperativity.

Site-specific Cleavage of Semisynthetic Precursors by Endoproteinase Lys-C—The neurophysin effect on enzymatic processing of peptide intermediates leads to the postulate that endoproteolytic processing of oxytocinyl and vasopressinyl precursors themselves might be affected by their assembly properties. To test this idea, the susceptibility of pro'-OT/BNPI and \([N^\prime\prime-\text{Ac,Ala}^2]\text{pro}'-\text{OT/BNPI}\) to endoproteinase Lys-C was evaluated. Pro'-OT/BNPI folds with oxytocin and neurophysin domains interacting intramolecularly and, as a consequence, self-associates with relatively high affinity into precursor dimers and possibly higher order aggregates (13). In contrast, in the \(\alpha\)-acetyl-Ala\(^2\) variant, the acetylated, Ala\(^2\) hormone domain does not interact with the neurophysin domain, and, thus, precursor derivatives self-associate with only low affinity (13).

Endoproteinase Lys-C cleaves only on the carbonyl side of lysyl residues. Since the 2 lysyl residues in the neurophysin domains of semisynthetic precursors are acetimidated, the enzyme can cleave only at the lysine in the linker region between hormone and neurophysin domains. Both pro'-OT/BNPI and \([N^\prime\prime-\text{Ac,Ala}^2]\text{pro}'-\text{OT/BNPI}\) were treated with endoproteinase Lys-C. Enzymatic degradation was carried out at pH 5.5, close to the pH of mature secretory granules (19, 20), in order to maintain the interaction between the hormone and neurophysin domains in pro'-OT/BNPI (22, 23). When either precursor (700 \(\mu\text{M}\) was incubated with enzyme, peaks corresponding to OT-GK, \([N^\prime\prime-\text{Ac,Ala}^2]\text{OT-GK}\), and arginyl-[diAcet]BNPI were detected by 15 min (Fig. 5). Under these conditions, the degradation of pro'-OT/BNPI was much slower than that of \([N^\prime\prime-\text{Ac,Ala}^2]\text{pro}'-\text{OT/BNPI}\). When lower concentrations (14 \(\mu\text{M}\) of each precursor were incubated with enzyme, the degradation rate of pro'-OT/BNPI was much greater than that of \([N^\prime\prime-\text{Ac,Ala}^2]\text{pro}'-\text{OT/BNPI}\). Thus, the endoproteinase reaction rate of pro'-OT/BNPI, but not of \([N^\prime\prime-\text{Ac,Ala}^2]\text{pro}'-\text{OT/BNPI}\), was dependent on substrate concentration. At both concentrations used here (14 and 700 \(\mu\text{M}\)), the hormone domain of pro'-OT/BNPI is expected to bind to the neurophysin domain in folded precursor, as judged by analytical affinity chromatography and CD measurement (12, 13). Thus, precursor folding does not interfere with endoproteolysis between the two domains and conceivably could enhance this degradation. But, when the concentration of precursor is sufficiently high, cleavage of pro'-OT/BNPI is suppressed, apparently due to self-association of the precursor to dimer or higher aggregated forms. The differential degradation rates of pro'-OT/BNPI and the \(N^\prime\prime\)-Ac,Ala\(^2\) species at low concentration likely reflect the effects of intra- and intermolecular conformation (see “Discussion”).

Degradation of Semisynthetic Precursors by Trypsin—Enzymatic degradation rates of pro'-OT/BNPI and \([N^\prime\prime-\text{Ac,Ala}^2]\text{pro}'-\text{OT/BNPI}\) also were measured with trypsin at pH 5.5. In principle, trypsin is capable of hydrolysis at peptide bonds containing the carbonyl groups of Lys\(^{22}\), Arg\(^{22}\), and 4 arginyl residues in the neurophysin domain. The 2 lysyl residues in the neurophysin domain are acetimidated and protected from trypsin cleavage. Under the conditions used in this study, peaks corresponding to [diAcet]BNPI and either OT-GKR or \([N^\prime\prime-\text{Ac,Ala}^2]\text{OT-GKR}\) were detected early in the trypsin reaction, showing that cleavage at the Arg\(^{22}\)-Ala\(^2\) bond proceeded rapidly and was virtually complete by 5 min. The further degradation of [diAcet]BNPI was only about 15% complete for both precursors after 5 min. The results suggest that the dibasic residue linker region in precursor is the most susceptible to trypsin, even with the hormone domain of pro'-OT/BNPI bound to the neurophysin domain. Cleavage be-

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**Fig. 4.** Effect of neurophysin on enzymatic amidation of OT-G. Enzymatic reactions and RP-HPLC analyses were carried out as described under “Materials and Methods.” A, elution profile of pH 8.5 reaction mixture after 8 h of reaction; B, conversion of OT-G to oxytocin as a function of pH at 70 \(\mu\text{M}\) initial OT-G and varying concentration of BNPI (0, 70, and 700 \(\mu\text{M}\)).

**Fig. 5.** Limited proteolysis of semisynthetic precursors by endoproteinase Lys-C. For reactions at 700 \(\mu\text{M}\), 5.9 nmol each of pro'-OT/BNPI and \([N^\prime\prime-\text{Ac,Ala}^2]\text{pro}'-\text{OT/BNPI}\) (pro'-AA/BNPI) were dissolved in 4.2 \(\mu\text{l}\) of 150 mM MES/NaOH, pH 5.5, and incubated with an added 4.2 \(\mu\text{l}\) of endoproteinase Lys C (38.5 ng/\(\mu\text{l}\) in MES) at 37°C. For reactions of 14 \(\mu\text{M}\), each precursor was dissolved in 100 \(\mu\text{l}\) of NaOH, pH 5.7, and then treated with 4.2 \(\mu\text{l}\) of enzyme solution (the same as at 700 \(\mu\text{M}\)). After 15, 30, 45, and 60 min, 1.5 or 150 \(\mu\text{l}\) of 700 and 14 \(\mu\text{M}\) reactions, respectively, were withdrawn, mixed with 40 \(\mu\text{l}\) of 10% acetic acid, and stored at -70°C until analyzed by elution on Zorbax CN as described under “Materials and Methods.” Relative amounts of degradation were determined from peak areas.
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![Diagram](https://example.com/diagram.png)

**FIG. 6.** Proteolytic cleavage of semisynthetic precursors by trypsin. Pro'-OT/BNPI (●) and [N\(^{\text{Ac}}\)-Ac,Ala\(^{2}\)]pro'-OT/BNPI (●) at 77 mM final concentration, were reacted with 1% (by weight) trypsin. Reaction products were assayed by RP-HPLC as described under "Materials and Methods." After a 5-min reaction at 37 °C, peptide bond hydrolysis between Arg\(^{2}\) and Alas\(^{2}\) was complete by disappearance of intact substrate. The amount of [diAcet]BNPI, the first product formed and subsequently hydrolyzed to des-1-8-[diAcet]-BNPI, was determined with time of reaction. The peak positions of both [diAcet]BNPI and the des-1-8 derivative were determined using standard [diAcet]BNPI (12, 13) and the tryptic product formed from it analogously as before (24). The relative amount of [diAcet]BNPI remaining was determined from peak areas as the percentage ratio [diAcet]BNPI (100)/([diAcet]BNPI + des-1-8-[diAcet]BNPI).

The results of trypsin reaction provided a useful insight into the stabilization of neurophysin in intermediate complexes by following the time course of degradation of [diAcet]BNPI by trypsin. This degradation is expected to be due mainly to cleavage at the Arg\(^{2}\)-Gln\(^{8}\) bond of [diAcet]BNPI to produce des-1-8-[diAcet]BNPI since this cleavage is the first step of degradation of unproteolyzed neurophysin by trypsin (24). Only small amounts of other degradation products were detected in 4 h. The degradation of [diAcet]BNPI to des-1-8-[diAcet]BNPI proceeded slower in the mixture with [N\(^{\text{Ac}}\)-Ac,Ala\(^{2}\)]OT-GKR than in that with OT-GKR (Fig. 6). These results suggest that formation of the complex of [diAcet]BNPI and OT-GKR protects the noncovalently bound hormone and neurophysin components from further endoproteolysis. [N\(^{\text{Ac}}\)-Ac,Ala\(^{2}\)]OT-GKR does not bind to [diAcet]BNPI.

**DISCUSSION**

In this study, we have made several observations showing that enzymatic processing of oxytocin/neurophysin precursor and precursor intermediates is modulated by folding and multimolecular assembly. As shown in Figs. 3 and 4, both carboxypeptidase B-like and amidation enzyme reactions were suppressed by relatively high concentrations of neurophysin. Since OT-GKR binds to neurophysin with an affinity constant of \(K_a = 2.6 \times 10^5 \text{ M}^{-1}\) (this study) and the complex of OT-GKR and neurophysin dimerizes with a constant of \(K_a = 1.7 \times 10^7 \text{ M}^{-1}\) (12), the OT-GKR-neurophysin complex can be expected to dimerize readily when formed. At 0.71 mM OT-GKR and 0.18, 0.35, 0.71, and 1.42 mM neurophysin I, 25, 49, 99, and 100%, respectively, OT-GKR can be estimated to be present as dimer. The suppression effect on carboxypeptidase B-like and amidation reactions can be correlated with the formation of aggregates of monomer complex, presumably mostly dimer, but perhaps tetramer or higher order aggregation states as well. Tetramers of hormone-neurophysin complexes have been suggested from NMR (25), analytical ultracentrifuge (26), and x-ray diffraction (27) analyses.

Corresponding to these results with noncovalent hormone-neurophysin intermediate complexes, rate suppression by neurophysin domain was observed for precursor endoproteolysis. This can be seen in the variation of endoproteolysis Lys-C cleavage rates at different precursor concentrations. When semisynthetic precursors at the relatively low concentration of 14 \(\mu\)M were treated with enzyme, cleavage at Lys\(^{11}\) proceeded faster for pro'-OT/BNPI than for [N\(^{\text{Ac}}\)-Ac,Ala\(^{2}\)]pro'-OT/BNPI. Since the hormone domain of pro'-OT/BNPI can bind to the neurophysin domain at concentrations at least as low as 1 \(\mu\)M as judged by CD and affinity chromatographic analysis (12, 13), folding of the close-to-native precursor per se, including the intramolecular domain-domain interaction, does not interfere with endoproteolytic cleavage at Lys\(^{11}\). Monomer folding in pro'-OT/BNPI even appears to cause a rate increase. Whether this latter effect might be related to the acceleration of carboxypeptidase B-like and amidation reactions in the presence of low concentrations of neurophysin I is not known at present.

In contrast, endoproteolysis of pro'-OT/BNPI proceeded significantly more slowly than that of [N\(^{\text{Ac}}\)-Ac,Ala\(^{2}\)]pro'-OT/BNPI at higher concentration, namely 700 \(\mu\)M. The cleavage rate for [N\(^{\text{Ac}}\)-Ac,Ala\(^{2}\)]pro'-OT/BNPI is relatively unchanged at the higher concentration. It is very likely that reduction in rate for pro'-OT/BNPI is due to aggregation (self-association) of pro'-OT/BNPI. Association of pro'-OT/BNPI to liganded neurophysin has been measured to have \(K_a = 10^7 \text{ M}^{-1}\). Thus, the precursor can be assumed to be effectively all dimer (or, again, possibly larger aggregates) at 700 \(\mu\)M.

Currently available data argue rather convincingly that oxytocinyl precursor and intermediate complexes exist mainly as dimers or higher order aggregates at the very high concentrations that exist in granules and thus that these self-assembled forms, and not monomers, are the predominant intragranular forms that exist during enzymatic processing (Fig. 7). The data presented here strongly suggest that enzymatic conversion of precursors to mature peptides is slowed substantially by this multimolecular assembly. However, two somewhat different possibilities for how molecular organization slows processing rates cannot be distinguished at present. The fully aggregated precursors and intermediates may be low rate, but nonetheless the major substrates of enzymatic conversion in granules. Alternatively, fully assembled precursors may be converted at only extremely low rates or not at all, with degradation occurring predominantly with a relatively small pool of precursors and peptides which are dissociated...
to monomers. The latter seems less likely since concentration conditions which should lead to virtually full (>95%) self-association lead to observed rate decreases in the range of only 50–70%. The above arguments notwithstanding, the current results confirm the view that the rates of enzymatic conversion of oxytocinyl precursor to mature hormone likely are controlled by well-ordered folding and assembly events which occur in secretory granules.

Judging from ongoing semisynthesis experiments, the vasopressinyl precursor also appears to fold and to self-associate. Thus, the neurophysin effect observed in this work for oxytocinyl precursor and intermediates is likely to be a general factor in modulating the rates of maturation of both neurohypophysial peptide hormones. In addition, multimolecular assembly of the precursors and intermediate complexes is likely to protect hormones and neurophysins from excessive enzymatic degradation, as seen in the precursor trypsin digestion experiment in this study. The actual biological role of processing rate modulation by the molecular organization of precursors, intermediates, and products remains to be clarified.

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