Evidence for Conservation of the Vasopressin/Oxytocin Superfamily in Annelida*

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Annetocin is a structurally and functionally oxytocin-related peptide isolated from the earthworm Eisenia fetida. We present the characterization of the annetocin cDNA. Sequence analyses of the deduced precursor polypeptide revealed that the annetocin precursor is composed of three segments: a signal peptide, an annetocin sequence flanked by a Gly C-terminal amidation signal and a Lys-Arg dibasic processing site, and a neurophysin domain, similar to other oxytocin family precursors. The proannetocin showed 37.4–45.8% amino acid homology to other prohormones. In the neurophysin domain, 14 cysteines and amino acid residues essential for association of a neurophysin with a vasopressin/oxytocin superfamily peptide were conserved, suggesting that the Eisenia neurophysin can bind to annetocin. Furthermore, in situ hybridization experiments demonstrated that the annetocin gene is expressed exclusively in neurons of the central nervous system predicted to be involved in regulation of reproductive behavior. These findings confirm that annetocin is a member of the vasopressin/oxytocin superfamily. This is the first identification of the cDNA encoding the precursor of an invertebrate oxytocin-related peptide and also the first report of the identification of an annelid vasopressin/oxytocin-related precursor.

The cyclic nonapeptides vasopressin (VP)† and oxytocin (OT) and their structurally related peptides are well known as neurohypophysial hormones involved in osmoregulation and reproduction in all vertebrates (1–4). They are classified into the VP and OT families based on the amino acid residue present at position 8: the VP family peptides contain a basic amino acid, and the OT family peptides contain a neutral amino acid at this position (1–4). Both the VP and OT family peptides are present in all vertebrate species except the cyclostomes, which have only the VP-related peptide vasotocin (2–4). The difference in the polarity of this amino acid residue is believed to enable the VP and OT peptides to interact with the respective receptor.

The structural organization of the precursor polypeptides of neurohypophysial hormones is highly conserved in all vertebrates (2–4). The prohormones are also structurally divided into the same two classes. The mammalian VP family precursors are composed of four regions: a signal peptide, a nonapeptide, a neurophysin, and a copeptin domain. The architecture of non-mammalian precursors is quite similar, except that the copeptin is not generated due to the absence of post-translational cleavage in the precursor. Thus, a non-mammalian neurophysin contains a C-terminal extended domain (2–4). The OT family precursors are organized similarly, but they completely lack copeptin(-like) domains, except for the isotocin precursor of the white sucker fish, which also contains an extension of the C-terminal region (2–4). Phylogenetic studies of the primary sequence of hormones, the structural organizations of the hormone precursor, and gene structure (5–10) led to the hypothesis that the VP and OT families separately evolved from a common ancestral gene via gene duplication (2, 4). In addition, only vasotocin is present in the lowest vertebrate cyclostomes (9), suggesting that a duplication of the ancestral gene might have occurred after the evolutionary process of the Agnata (2, 4, 11).

The VP/OT superfamily peptides have also been characterized from invertebrates, including insects (12), molluscs (13–16), and Annelida (17, 18), as well as vertebrates (Table I) (4). The peptides from invertebrates are all amidated at the C-terminus and share five residues, namely Cys1, Asn5, Cys6, Pro7, and Gly9 (Table I) (4). Annetocin has been isolated from the lumbricid earthworm Eisenia fetida. The primary sequence Cys-Phe-Val-Arg-Asn-Cys-Pro-Thr-Gly-NH2 is homologous to sequences of OT-related peptides (18), and injection of annetocin into the earthworm and leech results in induction of egg-laying behavior (19). The similarity of not only the primary structure but also the reproductive function implies that annetocin is a member of the OT family. However, whether annetocin belongs to the superfamily remains to be concluded since the organized structure of an annetocin precursor polypeptide has not been characterized. In invertebrates, the structure of the Lymnaea Lys-conopressin precursor has been identified, demonstrating that the typical architecture of the precursor of the VP/OT superfamily peptide is also highly conserved in molluscs (20). This is the only identification of the invertebrate VP/OT superfamily precursor, and thus, the phylogenetic relationship of the VP/OT superfamily among invertebrates has not been understood. To determine the feature of the precursor polypeptide of the annelid VP/OT-related peptide and to investigate the molecular evolution or divergence of the VP/OT superfamily in invertebrates, we isolated and characterized the annetocin precursor polypeptide cDNA. In this report, we present indisputable evidence for the existence of the VP/OT superfamily in Annelida. The annetocin precursor, very much like other precursors of the OT family, consists of the typical three segments, i.e. a signal peptide, annetocin, and a neurophysin-like domain with 14 cysteine residues positioned identically to those of other neurophysins. The proannetocin

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The nucleotide sequence reported in this paper has been submitted to the DDBJ/GenBankBEI Data Bank with accession number AB014478.

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‡ The abbreviations used are: VP, vasopressin; OT, oxytocin; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; DIG, digoxigenin; PBS, phosphate-buffered saline.
displays amino acid sequence identities between 37.4 and 45.8%. A comparative study of amino acid sequences of prohormones also revealed the presence of amino acid residues crucial for interaction of a neurophysin with a hormone peptide in the *Eisenia* neurophysin domain. Furthermore, in situ hybridization directly detected expression of the annetocin gene in neurons of the subesophageal ganglion, which is known to be a central nervous tissue of the earthworm, confirming the specific synthesis of annetocin in the central nervous system as a neuropeptide. To the best of our knowledge, this is the first report on the precursor structure of the invertebrate OT-related peptide and also the first identification of cDNA encoding the VP/OT-related peptide from Annelida, the most primitive species from which a VP/OT-related peptide has ever been isolated.

### EXPERIMENTAL PROCEDURES

**Animals**—*E. foetida* lumbricid earthworms were purchased from a fishing/bait store and kept in wet compost at 25°C.

**Oligonucleotides**—All nucleotides were ordered from Sawady Technology.

**Total RNA Preparation**—Frozen partial earthworm heads (1 g) were pulverized by grinding under liquid nitrogen. The ground tissues were dissolved in 10 ml of TRizol reagent (Life Technologies, Inc.), and total RNA was extracted according to the manufacturer’s protocol.

**3’-RACE**—All PCR amplifications were carried out in a reaction mixture containing Taq polymerase (EX Taq polymerase (Takara Shuss) or *Taq* DNA polymerase (TOYOBO)) and 200 μM dNTP in a thermal cycler (Perkin-Elmer GeneAmp PCR System 2400). First-strand cDNA was synthesized with the oligo(dT)-anchor primer supplemented by the manufacturer’s protocol.

**Northern Blot Hybridization**—A full-length digoxigenin (DIG)-labeled annetocin precursor cDNA was synthesized using a DNA labeling kit (Boehringer Mannheim) and was used as a probe for Northern blot analysis. Total RNA was separated on a denaturing formaldehyde-containing 1% agarose gel and fixed onto Hybond N+ membrane (Amersham Pharmacia Biotech) by UV irradiation. Hybridization and detection were carried out according to the manufacturer’s standard procedure (Boehringer Mannheim). RNA size was estimated using DIG-labeled RNA molecular markers (Boehringer Mannheim).

*In Situ Hybridization*—An earthworm head was dissected and incubated in 4% paraformaldehyde/PBS (10 mM sodium phosphate buffer (pH 7.5) and 0.9% NaCl) at 4°C overnight. After washing five times with 10 mM sodium phosphate buffer (pH 7.5) and 0.1% Tween 20 at 4°C for 30 min, the fixed head was dehydrated in ethanol and benzene and embedded in 96% polyester wax (BDH). Transverse sections 6-μm thick were cut, arranged on 3-aminopropyltriethoxysilane-coated slides (Mathunani), and dried for 5 h at 50°C and further overnight at room temperature. The sections were deparaffinized in xylene (2×5 min), rehydrated in ethanol and PBS, followed by sequential treatment with a 10 μg/ml proteinase K solution (Nakalai Tesque; 10 min, room temperature), PBS (3×5 min, room temperature), 4% paraformaldehyde/PBS (10 min, room temperature), PBS (3×5 min, room temperature), 0.2 N HCl (10 min, room temperature), PBS (3×5 min, room temperature), 0.25% acetic anhydride in 0.1 M triethanolamine HCl buffer (pH 8.0) (10 min, room temperature), and PBS (3×5 min, room temperature). The sections were then incubated for 2 h at 50°C in prehybridization medium containing 50% formamide, 10% dextran sulfate (Sigma), 1% blockimg reagent (Boehringer Mannheim), 5× SSC (1× SSC = 0.15 M NaCl and 0.15 M sodium citrate (pH 4.5)), and 50 μg/ml denatured herring sperm DNA. To prepare a sense or antisense probe, 53-mer oligonucleotides complementary or identical to a presynaptic cDNA located between nucleotides 234 and 286 were synthesized using the oligo(dT)-anchor primer and the gene-specific primer 1 (TGGAGCAGATGTGGTCTGGAG, complementary to nucleotides 349–368), followed by re amplification of the first-round PCR products using the anchor primer and the gene-specific primer 2 (GTTGTTGACAGGAGCAAGAG, complementary to nucleotides 309–328). Both first- and second-round PCR products were performed for 30 cycles consisting of 30 s at 94°C, 30 s at 55°C, and 1.5 min at 72°C. The second-round PCR products were cloned, and the inserts were amplified as described in 3’-RACE.

**DNA Sequencing**—All nucleotide sequences were determined using Big-Dye sequencing kits (Perkin-Elmer) and an automated DNA sequencer (Perkin-Elmer Model 373A) and analyzed on GENETYX-MAC software (Software Development). Universal M13 primers or gene-specific primers were used to sequence both strands.

| Peptide | Sequence | Animal (species) |
|---------|----------|------------------|
| Annetocin | C F V R N C P T G-amide | Earthworm (*E. foetida*) |
| Lys-conopressin | C F I R N C P K G-amide | Pond snail (*Lymnaea stagnalis*), geography cone (*Conus geographus*), sea hare (*Aplysia kurodai*), leech (*Erpobelia octoculata*) |
| Arg-conopressin | C I R N C P R G-amide | Striped cone (*Conus striatus*) |
| Cephalotocin | C F P I R N C P I G-amide | Octopus (*Octopus vulgaris*) |
| Lom-DH | C L I T N C P I G-amide | Locust (*Locusta migratoria*) |

**Characterization of annetocin cDNA Encoding an Annetocin Precursor Polypeptide**—In an attempt to obtain annetocin precursor polypeptide cDNA fragments, we performed a 3’-RACE experiment using degenerate primers corresponding to the N-terminal part of annetocin (Val-Ar-Gly-Ser-Cys-Pro) and the anchor primer (see “Experimental Procedures”). To increase abundance and specificity, the first-round PCR products were further amplified with degenerate primers corresponding to the C-terminal part of annetocin (Val-Ar-Gly-Ser-Cys-Pro-Thr-Gly-Gly) and the same anchor primer. Here, the C-terminal
amide group was thought to be derived from a C-terminal Gly residue that is well known as the typical amidation signal. Electrophoresis of the second-round PCR mixture revealed a single product of ~0.5 kilobases (data not shown). Sequencing of the subcloned second-round PCR products showed that all clones had essentially identical nucleotide sequences, except for minor differences in the 3'-terminal sequence, probably attributable to utilization of the alternate polyadenylation signal AATAAA and various lengths of the poly(A) tract. The predicted amino acid sequence comprised a cysteine-rich domain preceded by the endoproteolytic dibasic sequence Lys-Arg after a partial annetocin sequence derived from the second-round PCR primers, indicating that an annetocin precursor might be organized similarly to precursors of the VP/OT superfamily. To determine the 5'-end sequence, we performed 5'-RACE using specific primers for the clone (see “Experimental Procedures”). A single product of ~0.3 kilobases (data not shown) was obtained and sequenced after subcloning and amplification as described for the 3'-RACE products and contained two putative ATG initiation codons in addition to a TGA stop codon upstream of the first ATG codon. PCR products amplified using different polymerases had identical nucleotide sequences, confirming that these cDNA clones were not generated by artifacts. By combining nucleotide sequences determined by 3'- and 5'-RACE, the entire cDNA sequence encoding a preproannetocin was identified. Fig. 1 shows the complete sequence of the longest cDNA. The annetocin precursor cDNA is composed of 668 nucleotides containing a 417-base pair single open reading frame flanked by a short 5'-untranslated sequence of 58 base pairs and a 3'-untranslated sequence of 193 base pairs followed by various lengths of poly(A) tail. The open reading frame region begins with two putative start codons present at positions 59 and 86 and terminates with a stop codon at position 473. Both putative initiation codons conform to the Kozak rule (AAAATGG and AACATGA) (22). Two polyadenylation signals (AATAAA) were found in the 3'-untranslated region at positions 637 and 647. Nucleotide sequence analysis of all clones indicated that the second polyadenylation signal was used relatively more frequently than the first one; however, the biological significance of this remains to be elucidated. Northern blot analysis of total RNA using a DIG-labeled preproannetocin cDNA as a probe detected a single band of ~0.8 kilobases (Fig. 2A) even after longer exposure (data not shown), suggesting that the annetocin gene produces a single transcript. The apparent migration of the 0.8-kilobase sequence was well in accordance with the estimated length of the cDNA, confirming that the longest cDNA sequence identified by combination of 3'- and 5'-RACE includes a full-length nucleotide sequence encoding an annetocin proprecursor.

Amino Acid Sequence of the Deduced Annetocin Preprohormone—The open reading frame region encodes a 139-residue...
polypeptide with a predicted molecular mass of −14.6 kDa. Amino acid sequence analysis revealed that the structural organization of the annetocin precursor polypeptide was quite homologous to that of the VP/OT-related preprohormones: the precursor was composed of a signal peptide, a nonapeptide, and a neurophysin domain, as shown in Fig. 2B. The annetocin transcript was predicted to be translated with the Met present at position 1 or 10 since the nucleotide sequences surrounding the two start codons are entirely consistent with the Kozak rule (Fig. 2). Furthermore, seven amino acids are present between the ninth and tenth cysteines of the invertebrate prohormones, whereas the vertebrate counterparts contain five or six amino acids in the corresponding region. Generally, lower animal prohormones seem to include longer sequences in these regions. Comparative sequence analyses showed that several amino acids such as Arg23, Gly34, Glu67, Pro73, and Gly83 in the annetocin prohormone are completely conserved in the neurophysin domains of any species, in addition to the 14 cysteine residues. Other than the nonapeptide sequences flanked by the Gly C-terminal amidation signal and the endoproteolytic sites are shown as black lines. In C, the plot was generated according to the method of Kyte and Doolittle (21) using GENETYX-MAC software.

**FIG. 2.** Northern blot analysis of total RNA (A), schematic representation of the annetocin precursor polypeptide (B), and hydrophathy plot analysis of the predicted precursor (C). In A, total RNA was extracted from the anterior part of the earthworm, and −25 μg of RNA was subject to Northern blot hybridization using a DIG-labeled annetocin cDNA probe. RNA molecular markers are shown on the left in kilobases (kb). In B, the hydrophobic leader sequence is labeled S. The annetocin sequence and neurophysin-like domain are represented by the hatched and black bars, respectively. The endoproteolytic sites are shown as black lines. In C, the plot was generated according to the method of Kyte and Doolittle (21) using GENETYX-MAC software.

processing sequence Lys-Arg, was found to follow a signal sequence. The following moiety showed the properties of a neurophysin domain. The striking feature of this neurophysin domain is that 14 cysteine residues are positioned almost identically to those in the neurophysin domains of other VP/OT-related preprohormones (Fig. 3). This result suggests that an essential tertiary structure of the Eisenia neurophysin domain is also highly conserved because disulfide pairings by 14 cysteine residues in the neurophysin domain are believed to play a significant role in conformation essential for interaction with a hormone peptide (27–30). A copeptin-like region (another typical domain in the prohormones of the VP family, but not in those of the OT family) is entirely absent in preproannetocin since a TGA stop codon is found immediately after the fourteenth cysteine. These findings indicate that the principal architecture of VP/OT superfamily preprohormones is also highly conserved in Annelida and that a precursor polypeptide of annetocin is structurally closer to precursors of OT-related peptides since a C-terminal extension is not seen in the preprohormones. In addition, no consensus sequence for any other post-translational modification such as glycosylation or phosphorylation was found in the precursor polypeptide.

**Amino Acid Sequence Comparison of a Proannetocin with Other Prohormones of the VP/OT Superfamily**—The amino acid sequence of the annetocin prohormone is aligned with the sequences of other species’ prohormones of the VP/OT superfamily in Fig. 3. The neurophysin domain of the annetocin prohormone includes the longest sequence between the cleavage site Lys-Arg and the third conserved cysteine residue (position 41). Furthermore, seven amino acids are present between the ninth and tenth cysteines of the invertebrate prohormones, whereas the vertebrate counterparts contain five or six amino acids in the corresponding region. Generally, lower animal prohormones seem to include longer sequences in these regions. Comparative sequence analyses showed that several amino acids such as Arg23, Gly34, Glu67, Pro73, and Gly83 in the annetocin prohormone are completely conserved in the neurophysin domains of any species, in addition to the 14 cysteine residues. Other than the nonapeptide sequences flanked by the Gly C-terminal amidation signal and the endoproteolytic sites Lys-Arg site, only the Glu67-Asn68-His69-Leu70-Ser71-Thr72-Pro73 region is relatively homologous to the corresponding regions in vertebrates and Lymnaea. The total amino acid sequence of the annetocin prohormone is 37.4–45.8% homologous to the sequences of other prohormones (Table II). Interestingly, the similarity of the annetocin prohormone to the Lymnaea Lys-conopressin prohormone is not significantly distinct from the similarities to the prohormones of vertebrates. This result is somewhat surprising because we expected the amino acid homology of the invertebrate prohormone to another relatively close invertebrate or lower vertebrate (for example, cyclostomes) prohormone to be much higher than to advanced vertebrate prohormones. However, the highest and lowest similarities were found with the bovine vasopressin prohormone (45.8%) and the white sucker vasotocin I prohormone (37.4%), respectively. These findings suggest that the amino acid sequence of the invertebrate VP/OT superfamily prohormone may show a remarkable interphyletic difference that is not correlated with phyletic distance.

**Expression of the Preproannetocin Gene in the Central Nervous System**—Localization of annetocin precursor mRNA in the central nervous system of E. foetida was directly observed by in situ hybridization to 6 μm serial sections of the earthworm anterior end using an antisense DIG-labeled 53-mer oligonucleotide probe. Positive staining in the cytoplasm of neurons was observed only when using the antisense probe, but was not
seen either when using the sense probe or no probe or in RNase-treated sections (data not shown). Furthermore, specificity of the antisense oligomer for hybridization was confirmed by Northern blot analysis (data not shown). Taken together, the positive signals observed in the cytoplasm of neurons represented specific hybridization with the annetocin mRNA. As shown in Fig. 4, several positively stained neurons were detected exclusively in the subesophageal ganglia, whereas no neurons present in the cerebral ganglia or ventral ganglia were stained (data not shown). Moreover, the annetocin gene was shown to be expressed almost symmetrically in two separate regions of the subesophageal ganglia, although the physiological significance of this phenomenon has yet to be examined. A total of at least 10 positively stained neurons were observed in these regions. These results demonstrate specific expression of the annetocin gene in the subesophageal ganglia. A recent immunohistochemical study has also demonstrated that annetocin-like immunoreactive neurons are localized in the same regions (31), which is in agreement with our data. In addition, it has been well established that the subesophageal ganglion plays an important role in regulation of egg-laying behavior and that the anterior part of the earthworm (rather than the posterior part) is involved in reproductive movement (32). Thus, localization of the annetocin transcript in the subesophageal ganglia and restricted distribution of annetocin-like immunoreactivity in the anterior part are compatible with the involvement of annetocin in reproductive behavior.

DISCUSSION

Although VP/OT-related peptides have been isolated from several invertebrates, the organizational structure of the precursor polypeptide has never been characterized, except for that of the Lymnaea preconopressin (20). Consequently, the molecular evolution or diversity of the invertebrate VP/OT superfamily peptides and the phylogenetic relationships between vertebrates and invertebrates remain to be clarified. This is the first report of the characterization of cDNA encoding an invertebrate OT-related peptide, annetocin isolated from the earthworm E. foetida. The preproannetocin was found to consist of a signal peptide, annetocin (flanked by a Gly C-terminal amidation signal and a Lys-Arg dibasic endoproteolytic sequence), and a neurophysin domain. Of particular significance is that 14 cysteine residues that play a crucial role in constructing the correct tertiary structure of a neurophysin are completely conserved in the Eisenia neurophysin domain. All cysteines are positioned almost identically to those of the known neurophysin domains. These are all typical characteristics of the VP/OT superfamily precursors, leading to the conclusion that annetocin is a member of the VP/OT superfamily. This is the first evidence for the presence of the VP/OT superfamily in Annelida, supporting the presumption that the ancestral gene encoding the VP/OT superfamily precursors with the principal structure was present in the stem group Archaemetazoa, from which invertebrates diverged ~600 mil-

### Table II

| Species (peptide)          | Homology |
|----------------------------|----------|
| Lymnaea (Lys-conopressin)  | 42.1     |
| Bovine (vasopressin)       | 45.8     |
| Bovine (oxytocin)          | 44.9     |
| Lungfish (vasotocin)       | 40.2     |
| Lungfish (Phe-lmesotocin)  | 44.9     |
| White sucker (vasotocin)   | 37.4     |
| White sucker (isotocin)    | 39.3     |
| Lamprey (vasotocin)        | 40.7     |
analysis. Nevertheless, this classification is unlikely to be true of the invertebrate VP/OT superfamily because invertebrates are believed to contain only one VP/OT-related peptide. Van Kesteren et al. (15) showed that Lymnaea Lys-conopressin, structurally related to VP, exhibits an oxytocin-like activity and that only a single VP/OT superfamily gene is present. Similar data have been obtained in our study of the physiological effects of VP/OT-related peptides on the leech,2 demonstrating that annetocin induces egg-laying behavior to almost the same extent as Lys-conopressin that was isolated from the leech (17). These data support the notion that the amino acid at position 8 is not essential for physiological functions of the invertebrate VP/OT superfamily peptides (15).

Thus, classification of the invertebrate VP/OT-related precursors into the VP or OT family on the basis of the physiological function of the peptide and the residue at position 8 is impossible. Taken together, it appears most likely that the apparent OT-related structure of the annetocin precursor was generated probably due to a point mutation in the ancestral gene, causing incidental interruption of the precursor sequence immediately after the fourteenth cysteine, which was not a selective pressure.

However, the possibility of unknown structural generality for precursors of the invertebrate VP/OT-related peptide cannot be excluded. Identification of structures of precursor polypeptides from other invertebrates including Aplysia, octopus, and leech would contribute to the establishment of universal characteristics of VP/OT-related precursors of invertebrates or at least in the same phylum.

Proannetocin shows 37.4–45.8% amino acid homology to the VP/OT superfamily prohormones. Unexpectedly, the amino acid sequence of proannetocin is not so highly homologous to that of another invertebrate prohormone, Lymnaea conopressin. These data indicate the interphylectic high molecular diversity of the invertebrate VP/OT superfamily peptide precursors. Thus, to understand the invertebrate molecular diversity of the VP/OT superfamily and the phylogenetic relationship between vertebrates and invertebrates, investigation of intraphyletic molecular evolution is necessary. Comparative studies of the VP/OT superfamily prohormones also revealed that Arg122, Gly124, Glu127, Pro132, and Gly132 plus the 14 cysteines are conserved in all neurophysin domains, despite amino acid diversity among species, as shown in Fig. 3. These findings suggest that the 14 cysteines and the amino acids described above are strictly involved in the function(s) of the Eisenia neurophysin common to vertebrates or that substitutions of

2 Y. Fujino, T. Nagahama, T. Oumi, K. Ukena, F. Morishita, Y. Furukawa, O. Matsushima, M. Ando, H. Takahama, H. Satake, H. Minakata, and K. Nomoto, manuscript in preparation.
these amino acids can be a critical selective pressure. It is well known that specific disulfide bridges formed by highly conserved cysteine residues are responsible for functional conformation of the vertebrate neurophysin requisite for interaction with the hormone peptide (27–30). This suggests that the neurophysin of the annetocin precursor can construct a conformation principally similar to those of vertebrates. Recently, the crystal structure of the complex of bovine neurophysin with oxytocin has been determined, revealing amino acid residues in neurophysin and oxytocin essential for electrostatic and multiple hydrogen bonding interactions with each other (28–30). In particular, the y-carboxyl group of Glu59 in the bovine prohormone (corresponding to Glu67 in proannetocin) has been shown to participate in an electrostatic interaction with the N-terminal amino group of oxytocin. Moreover, involvement of the side chain of Arg20 (corresponding to Arg23) in the optimal location of the y-carboxyl group of Glu59 has been proposed (28, 30). Conservation of these amino acid residues in all prohormones suggests that the Eisenia neurophysin can bind to annetocin in a manner similar to the oxytocin-bovine neurophysin complex. In addition, a homology modeling study generated the putative tertiary structure of the annetocin–Eisenia neurophysin complex, quite similar to that of the oxytocin-bovine neurophysin complex. In this putative complex, most hydrogen bonds corresponding to those of the oxytocin-bovine neurophysin complex and the electrostatic interaction of Glu57 with the y-amino group of annetocin were observed. Previously, Tyr at position 60 or 61 in the vertebrate prohormones was thought to be requisite for binding to the nonapeptide (44), and the Lymnaea neurophysin was proposed to fail to interact with Lys-conopres because the sequence Asn-Tyr-Leu in most vertebrate neurophysin was proposed to fail to interact with Lys-conopressin (45). The results described in this study of annetocin have shown that Tyr61 is not involved in the interaction between a neurophysin and a nonapeptide hormone (27–30). This suggests that the invertebrate VP/OT superfamily precursors can interact with the nonapeptide owing to the presence of amino acid residues essential for oxytocin-neurophysin complex formation.