EVOLUTION OF NEUROPHYSIN PROTEINS: PARTIAL AMINO ACID SEQUENCES OF RAT NEUROPHYSINS

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1. Introduction

Neurophysins (NPs) are proteins elaborated by cells of the hypothalamo–neurohypophysial system, and within this system there is a close association between the biosynthesis of each neurophysin and the biosynthesis of each neurohypophysial hormone [1,2]. This lead to the proposal of a classification based on this association [3–5]. In the rat, there are 3 major forms of these proteins which were formerly named rat neurophysins I, II and III, according to their electrophoretic mobility at pH 9.5 [6,7]. Rat neurophysin I has been renamed vasopressin-associated neurophysin (VP-RNP), and rat neurophysin II renamed oxytocin-associated neurophysin (OT-RNP) [1,3]. Rat neurophysin III was found [8,9] to be a metabolic derivative of OT-RNP, and is now referred to as OT-RNP′ [2]. The close relationship between one neurophysin and one hormone is perhaps best exemplified by Brattleboro homozygotes, rats with hereditary diabetes insipidus [10]. These rats have an apparent inability to synthesize vasopressin and VP-RNP (RNP I), while their synthesis of oxytocin, OT-RNP and OT-RNP′ is unimpaired [1,2,6]. The view now generally accepted is that each neurophysin and its hormone initially arise as parts of a common precursor molecule [1,2,11]. Amino acid sequences have been described for neurophysins of the ox [12,13], the pig [14,15], and the sheep [16] (mammals of the order Artiodactyla); for one neurophysin of the horse [17] (order Perissodactyla); and for one neurophysin of the whale [18] (order Cetacea). Also, the amino acid sequence of the N-terminal 54-residues of oxytocin-associated human neurophysin has also been determined [4,19] (order Primates). The N-terminal 30-residues of rat neurophysin I and rat neurophysin II (order Rodentia) have been identified in [20].

This report concerns the determination of the N-terminal sequence of VP-RNP through 44 residues of OT-RNP through 40 residues, and of OT-RNP′ through 69 residues. Results are discussed in relationship to evolution of this group of proteins.

2. Materials and methods

Rat neurophysins were purified by described methods and their amino acid compositions reported [7]. Vasopressin-associated rat neurophysin was characterized by its identity with the protein absent from the neural lobes of Brattleboro homozygotes by electrophoresis at pH 9.5, and by use of specific radioimmunoassays. Oxytocin-associated neurophysins were also characterized by electrophoresis and radioimmunoassay [2,8].

Samples of VP-RNP, OT-RNP and OT-RNP′ (200 nmol, 2mg each) were reduced with 2-mercaptoethanol (0.7 mmol) for 4 h and *S*-alkylated with ^{14}C-labelled iodoacetamide (1.44 mmol; 0.17 mCi/mmol) in the presence of 8 M urea in 0.6 M Tris–HCl buffer at pH 8.6. The *S*-alkylated proteins were separated from other reaction products in the absence of light on 1.0 × 30 cm columns of Sephadex G-25 equilibrated with 0.2 M acetic acid, and the acid was removed by lyophilization. The extent of *S*-alkylation was examined by amino acid analysis on a Beckman 121MB amino acid analyser.

Automated amino acid sequence analysis of each *S*-alkylated neurophysin was performed on a Beckman model 890C Sequencer utilizing a Quadrol buffer system [21]. All PTH-amino acids were analyzed by high-pressure chromatography (HPLC) and ^{14}C was counted in a Packard scintillation spectrometer, model 3255. HPLC analysis of PTH-amino acids involved the use

of 6 gradient systems at ambient temperature on columns of octadecasilyl-silica resins:

System 1: A system modified from that in [22] which employed a μBondapak C18 column (3.9 mm × 30 cm) from Waters Assoc. It comprised two solvents and a linear gradient over 23 min from 5–45% solvent B and 10 min at final conditions. Solvent A was 100 ml methanol, 900 ml water, 2.5 ml acetic acid, 50 μl acetone, adjusted to pH 4.1 with 10 M NaOH; solvent B was 900 ml methanol, 100 ml water, 0.25 ml acetic acid. Flow rate was 1.7 ml/min. This system resolved all PTH-amino acids except glycine–threonine, valine–methionine and phenylalanine–isoleucine. Absorbance was monitored at 254 nm and 269 nm. Using this system and absorbance at 323 nm, oxidation products of PTH-threonine and PTH-serine are clearly identified.

System 2: This system was performed on μBondapack C18 columns. Conditions were identical to those described for system 1, except that solvent B comprised 900 ml methanol, 100 ml water and 2.5 ml acetic acid, the flow rate was 2.0 ml/min, and the gradient was over 20 min. This system resolved all PTH-amino acids except alanine–glutamic acid, isoleucine–phenylalanine and methonine–valine.

System 3: This system was performed on μBondapack C18 columns. It consisted of a two solvent linear gradient from 20–55% solvent B over 30 min. Solvent A was water and solvent B was 90% acetonitrile–10% water (v/v). The flow rate was 2.0 ml/min and runs were performed at ambient temperature. Absorbance was monitored at 254 nm and 269 nm. This system clearly resolved PTH valine (27.63 ± 0.11 min) from PTH-methionine (29.46 ± 0.10 min); and PTH-isoleucine (30.19 ± 0.08 min) from PTH-phenylalanine (30.56 ± 0.10 min).

System 4: A system was developed for Altex Ultrasphere (5 μm particle size) ODS columns (4.6 mm × 25 cm) which was based on gradient 2, above. This comprised two solvent linear gradient from 20–70% solvent B over 23 min. Solvent A was identical to that in system 1. Solvent B consisted of 450 ml methanol, 375 ml acetonitrile, 175 ml water, and 0.25 ml acetic acid. Flow rate, 1.7 ml/min. This system resolved all PTH-amino acids except glycine–glutamine and valine–methionine. Absorbance was monitored at 254 nm and 269 nm.

System 5: This system, performed on Ultrasphere ODS columns comprised solvents A and B of system 4 and had 3 linear gradients from 15–25% solvent B over 15 min, from 25–32% solvent B over 7 min, then from 32–70% B over 3 min. The flow rate was 1.7 ml/min and absorbance was measured at 254 nm and 269 nm. This system clearly resolved glycine (15.78 ± 0.10 min), glutamine (17.15 ± 0.12 min), and glutamic acid (21.45 ± 0.12 min).

System 6: This system, of solvents A and B from system 4, was performed on Ultrasphere ODS columns. It consisted of 3 linear gradients from 35–37% solvent B over 10 min, 37–45% solvent B over 10 min and 45–80% B over 10 min. The flow rate was 2.0 ml/min and absorbance was measured at 254 nm and 269 nm. This system resolved valine (26.62 ± 0.09 min) and methionine (26.91 ± 0.08 min).

All residues were identified by using at least two of the above systems. When doubt remained about the identity of a PTH product, that product was co-chromatographed with each of the pertinent PTH-amino acid standards. PTH amino acids used for reference were obtained from the Sigma Chemical Co. HPLC was performed on a Beckman Unit, model 332 plus model 100A pump, an Hitachi model 155-40 variable wavelength spectrophotometer with flow cell, a model BD 41 dual pen recorder, and a model C-RIA Shimadzu Integrator recorder. Automation in sample handling for HPLC was possible through use of a WISP 710A automatic sample injector from Waters Assoc.

3. Results

The combination of gradients on HPLC and counts for ^{14}C utilized in this study unambiguously identified all PTH-amino acids, and afforded high sensitivity so that as little as 30 pmol PTH-amino acid could be clearly recognized at 254 nm. This sensitivity is increased by two when absorbance at 269 nm is used for detection.

The identification of the N-terminal residues from 1–44, with only ambiguity for residues 41 and 42 was achieved from a single run on the sequencer of 1 mg (100 nmol) VP-RNP. Similarly, an unambiguous identification of the N-terminal residues 1–40 was made for 1 mg (100 nmol) OT-RNP. Using 2 mg (200 nmol) OT-RNP, we achieved identifications

through 1–69 residues, with failure to clearly identify residues at positions 52, 53, 58, 59, 66 and 67 from the N-terminus. The partial sequences obtained for these proteins of the rat [order Rodentia] are compared in table 1 with the partial sequences of: bovine neurophysins I and II (residues 1–69) representing order Artiodactyla [12,13], and proposed respective oxytocin-associated neurophysin (OT-VNP) and vasopressin-associated neurophysin (VP-BNP) of the ox; and human neurophysin I (residues 1–54) representing the order Primata, and the oxytocin-associated neurophysin (OT-BNP) of man [4,19].

4. Discussion

The amino acid sequence of VP-RNP (RNP I) obtained here is identical to that given through 33 residues (including their uncertain assignment of Thr_2, Ser_3) for rat NP-II [20]. Likewise, there is sequence identity through 33 steps with our OT-RNP (RNP II) and OT-RNP′ (RNP III), and the sequence found in [20] for rat NP-I. Rat neurophysin-I, the predominant neurophysin found in rat neural lobe, is VP-RNP [1,2,6], and the mistaken assignment of sequence data in [20] has led to erroneous comparisons with the oxytocin-associated and vasopressin-associated neurophysins of other species [18,23].

Neurophysins characteristically have variable and constant regions in their sequence. The N-terminal 9 residues is a variable region, while the region from 10–70 is highly conserved. This would appear to hold true for the rat neurophysins, because VP-RNP and OT-RNP have 6 differences in their sequences through the N-terminal nine residues, and only 3 differences in the next 30 residues. The N-terminal sequence identity of OT-RNP and OT-RNP′ through the sequenced 43 residues confirms the established metabolic relationship of these proteins [2,8,9]. The NPs of the order Rodentia show many similarities to NPs

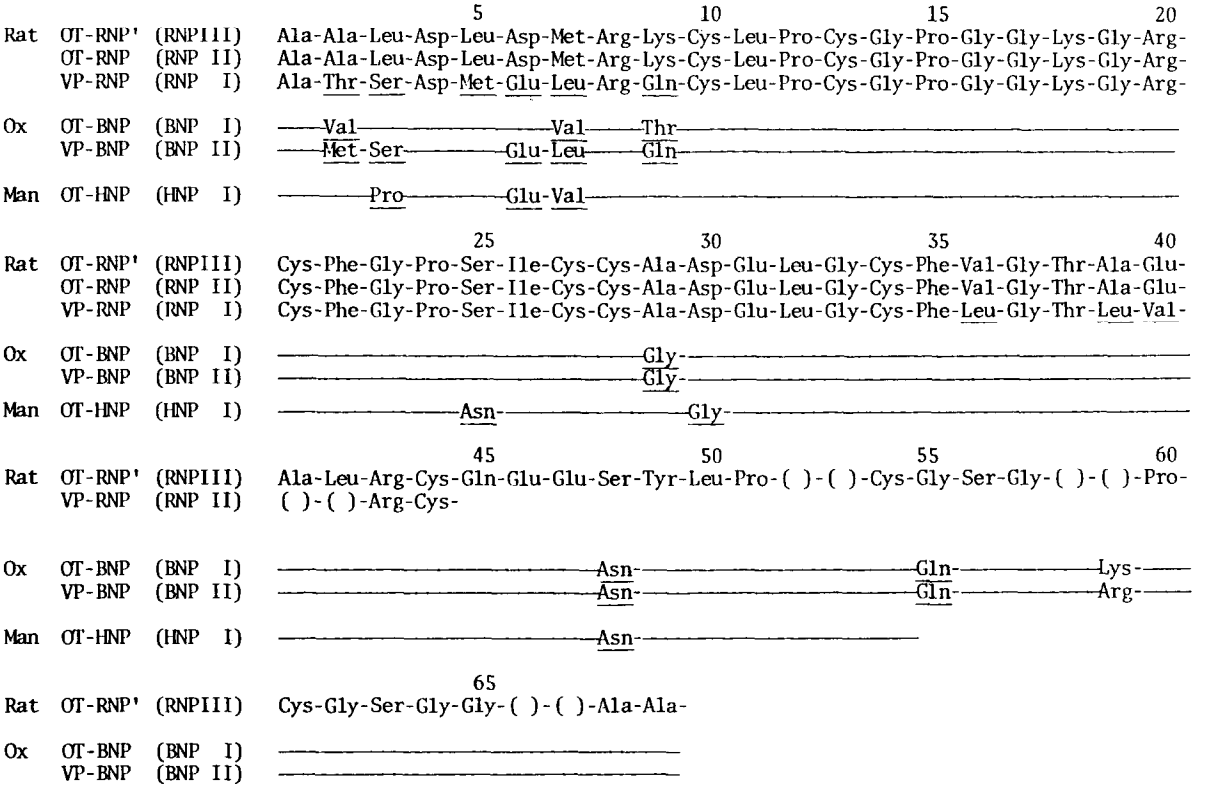

Fig.1. Comparison of rat, ox, and human oxytocin-associated and vasopressin-associated neurophysins. The partial sequence data for 3 rat proteins is shown, while sequence identity with OT-RNP′ is shown by solid lines for the ox and human proteins. Sequence differences with OT-RNP′ are shown and underlined. Unidentified residues in rat proteins are indicated by parentheses.

of the orders Artiodactyla, Perissidactyla, Cetacea and Primata. Of even more significance are the similarities between OT-NPs and between VP-NPs which, for the NPs of rat, ox and man, are greater than within-species similarities. Considering the N-terminal variable region, VP-RNP and OT-RNP show 6 differences while there are only two differences (positions 2,5) between rat and ox VP-NPs. The OT-NPs of rat and man have 3 differences in this region (positions 3,6,7), and the OT-NPs of rat and ox also have 3 differences (positions 2,7,9): The ox and human proteins have 4 differences among their N-terminal 9 residues. This similarity between VP-NPs and between OT-NPs suggests that neurophysins arose by gene duplication prior to speciation in the eutherian mammals. The data, however, do not support the MSEL- and VLDV-neurophysin concept advanced in [24]. Methionine is placed in position 5 of VP-RNP, and this would reduce their scheme for naming, at best, to SEL-neurophysins. Moreover, the OT-NP structures of fig.1 reduce the VLDV-scheme completely, as none of the residues 2, 3, 6 and 7 are the same for all 3 OT-NPs. For the conserved region 10–70, the rat neurophysins show variation at those 'permissible' positions such as position 36, shown for whale neurophysin [18]; and position 48, shown for sheep neurophysin [16]. They also introduce residues 39, 40 and 55 as other possible sites of variation among the NPs of eutherians.

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