MOLECULAR EVOLUTION OF THE NEUROHYPOPHYSIAL HORMONES: THE ACTIVE PEPTIDES OF A PRIMITIVE BONY FISH *POLYPTERUS BICHIR*

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Neurohypophysial hormones have been so far identified in Neopterygii and Crossopterygii but not in species of the bird sub-class of bony fishes, the Palaeopterygii. Isolation and chemical characterization of the active principles of a primitive bony fish, *Polypterus bichir*, have been performed. Isotocin (Ser^4–Ile^8–oxytocin) and arginine vasotocin (Arg^8–oxytocin) have been identified. Because the same peptides were found in the recent Neopterygii, it can be deduced that neurohypophysial hormones have displayed a peculiar stability in the course of the evolution of bony fishes. However isotocin and vasotocin are replaced by oxytocin and vasopressins in mammals and therefore might be regarded as "old" molecules.

1. Introduction

According to Rothchild [1] the living bony fishes can be divided into three sub-classes the Palaeopterygii, the Neopterygii and Crossopterygii. Because isotocin (Ser^4–Ile^8–oxytocin) and arginine vasotocin (Arg^8–oxytocin) were chemically identified in seven species of the second group [2], and mesotocin (Ile^8–oxytocin) and arginine vasotocin in two species of the third group [3], it was of interest to isolate the neurohypophysial hormones of a few species of Palaeopterygii in order to characterize molecules possibly more primitive than those of teleost fishes.

The sub-class of Palaeopterygii has only two orders, Chondrostei and Cladistia [1]. In the second, two genera, Polypterus and Calamoichtys, have been studied for the anatomy of the pituitary gland [4], but there are pharmacological data about neurohypophysial hormones only for Polypterus. Mesotocin (Ile^8–oxytocin) was first thought to be present in *Protopterus senegalus* [5], but further work was suggested that the oxytocin-like hormone may be isotocin [6]. Our investigations were mainly performed on *Polypterus bichir* because this species is one of the biggest among the 8 *Polypterus* species [7].

375 *Polypterus bichir* were fished in the River Chari, near Lake Tchad, a first lot (70) in January 1969 and a second (305) in July 1969. The average weight of animals was about 700 g. Fishes were killed by decapitation and entire pituitary glands were immediately removed and placed in pure and cool acetone in order to prepare a dry powder. The weight of the dry whole hypophysis is about 1.1 mg. 7 *Polypterus senegalus* were caught in the same river and pituitary glands removed under the same conditions. The weight of animals was about 100–200 g and the weight of the dry gland about 0.14 mg.

Oxytocic activity was determined without magnesium according to Holton [8] and pressor activity according to Landgrebe et al. [9]. The biological activities of the acetone powders are shown in table 1.

2. Purification of hormones

Purification was attempted only for *P. bichir* neurohypophysial principles. Three preparations of hormone peptides were carried out respectively with 100, 100 and 200 mg of acetone powder. The powder was extracted for 10 min with 0.25% acetic acid (1 ml/mg) on a boiling water bath. The insoluble material was removed by centrifugation and the solution was concentrated in a rotary evaporator to one-tenth of its volume. Proteins were then precipitated twice with 5% trichloroacetic acid and removed by centrifugation, the active peptides remaining in the supernatant

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Table 1.
Biological activities of polypterus pituitary powders.

Species	Number of glands	Weight (mg)	Oxytocic activity (USP units/mg)	Pressor activity (USP units/mg)
Polypterus bichir	70	55	0.19 ± 0.03	0.24 ± 0.04
	305	350	0.26 ± 0.02	0.25 ± 0.02
Polypterus senegalus	7	1	0.08 ± 0.01	0.10 ± 0.01

solution. This solution was deacidified, freeze-dried and the hormones were purified by chromatoelectrophoresis on Whatman 3 MM paper under conditions previously described [3, 10]. Two active principles were disclosed by development with dilute ninhydrin. (0.01% in alcohol) as well as by pharmacological detection [11]. One of these occupied the common position of isotocin, mesotocin or oxytocin which are not clearly separated from each other under the conditions used (electrophoretic migration: 13 cm; chromatographic migration: 23 cm; migrations of tyrosine used as reference respectively 5 cm and 16 cm); the other took up on the paper the position of arginine vasotocin (electrophoretic migration: 24 cm, chromatographic migration: 7 cm).

3. Characterization of hormones

The amino acid composition of the peptides was determined by *in vacuo* hydrolysis with 6 N HCl in a sealed tube for 48 hr at 105° and analysis of the hydrolyzate with an analyzer Spinco 120 B [12]. About 20–30 nmole were used for each analysis. The results are given in table 2.

Isotocin: The hormone which has electrophoretic and chromatographic migrations of the oxytocin-like peptides has the amino acid composition of isotocin (table 2). Its biological properties are in agreement with this identification. The oxytocic activities without and with magnesium [8, 13], the pressor activity on the rat [9], and the depressor activity on the chicken

Table 2
Amino acid composition of *Polypterus bichir* neurohypophysial hormones.
Molar ratio using aspartic acid as reference

Amino acid	Isotocin		Vasotocin	
	Found (20 nmoles)	Expected (number of residues per mole)	Found (24 nmoles)	Expected (number of residues per mole)
Asp	1.00	1	1.00	1
Ser	0.94	1	0.29	–
Glu	0.23	–	1.04	1
Pro	1.23	1	0.95	1
Gly	1.11	1	1.04	1
Cys*	1.45	2	1.31	2
Ile	1.81	2	0.75	1
Tyr	0.76	1	0.72	1
Arg	–	–	0.83	1
Thr	0.10	–	–	–
Leu	0.05	–		–
Ala	0.17	–	0.08	–
Val	0.15	–	0.08	–

* Cystine undergoes partial destruction when the peptide is hydrolyzed after elution from paper; the determination was performed in a separate sample after oxidation into cysteic acid with performic acid.

Table 3
Biological properties of *Polypterus bichir* neurohypophysial hormones*.

Peptide	Ratios using oxytocic activity as reference		
	OMg^{2+}/O	D/O	P/O
Polypterus isotocin	2.67 ± 0.68	2.58 ± 0.36	
Hake isotocin	2.97 ± 0.81	3.01 ± 0.45	–
Synthetic isotocin	2.52 ± 0.39	2.50 ± 0.38	–
Ox oxytocin	0.73 ± 0.08	1.03 ± 0.16	–
Synthetic mesotocin	0.84 ± 0.09	2.06 ± 0.08	–
Polypterus bichir vasotocin	2.15 ± 0.34	2.30 ± 0.63	1.67 ± 0.25
Rana esculenta vasotocin	1.79 ± 0.28	2.54 ± 0.28	1.98 ± 0.42

* O: oxytocic activity without magnesium [8]; OMg^{2+}: oxytocic activity with magnesium [13].
D: depressor activity in the chicken [14]; P: pressor activity in the rat [9].

[14] were determined. The peptide has no pressor activity; the ratios of biological activities are similar to those found for hake isotocin or synthetic isotocin (table 3).

A partial determination of the amino acid sequence was performed by using the Edman degradation procedure as described by Fraenkel-Conrat [15]. Identification of phenylthiohydantoin (PTH) amino acids was carried out by thin layer chromatography according to Jeppsson and Sjöquist [16]. About 50 nmoles of peptide were used for the experiment. The first step of the recurrent degradation gave no PTH amino acid because the cystyl residue remained bound to the peptide by the disulfide bridge, but the next steps gave successively the PTH of Tyr, Ile, Ser and Asn so that the sequence Tyr–Ile–Ser–Asn could be deduced. After the sixth step, no PTH amino acid could be obtained, may be because of the hindrance of the disulfide bridge. The remaining peptide was hydrolysed. Proline, isoleucine and glycine were found in stoichiometric proportions. These results are compatible with the structure:

Cys–Tyr–Ile–Ser–Asn–Cys–Pro–Ile–Gly (NH_2)

assigned to isotocin [17].

Arginine vasotocin: The second peptide, which occupies the typical position of arginine vasotocin on chromatograms, has the amino acid composition of arginine vasotocin (table 2) and its biological properties are similar to those of this hormone (table 3).

Partial determination of the amino acid sequence was carried out as for isotocin and the results were compared with those obtained with hake arginine vasotocin. The first step of the recurrent degradation gave no PTH derivative but the next steps gave successively the PTH or Tyr, Ile, Gln and Asn so that the sequence Tyr–Ile–Gln–Asn can be established. The sixth step gave no PTH and the remaining peptide was hydrolysed. Proline, arginine and glycine were found in the hydrolyzate. Similar results were obtained with hake arginine vasotocin. There data are in agreement with the structure:

Cys–Tyr–Ile–Gln–Asn–Cys–Pro–Arg–Gly (NH_2)

assigned to arginine vasotocin [18].

Polypterus bichir therefore has the same hormones as the so-called recent bony fishes. Pharmacological data obtained with pituitary extracts of *Polypterus senegalus* suggest that isotocin and arginine vasotocin are present in this species [6]. If it is admitted that Polypterus is really an archaic bony fish, isotocin and arginine vasotocin could be regarded as primitive molecules which have remained in Neopterygii.

The structural stability of neurohypophysial hormones in the course of evolution is rather peculiar: isotocin was found in bony fishes except in Crossop-

terygians which are supposed to make a bridge between bony fishes and amphibians, mesotocin was found in lungfishes [10], amphibians [11] and reptiles [9] and oxytocin in mammals [19]. Arginine vasotocin is present in all the non-mammalian vertebrates and only two vasopressins are known in mammals. Although the physiological role of neurohypophysial hormones in lower vertebrates is not clear, this stability could be explained either by a strict adaptation of the structure to a precise function or by a peculiar immutability of the structural genes which control the amino acid sequences.

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