Molecular evolution of peptide tyrosine–tyrosine: primary structure of PYY from the lampreys *Geotria australis* and *Lampetra fluviatilis*, bichir, python and desert tortoise

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Received 30 July 1998; accepted 20 October 1998

Abstract

Peptide tyrosine–tyrosine (PYY) has been isolated from the intestines of two species of reptile, the desert tortoise *Gopherus agassizii* (Testudines) and the Burmese python *Python molurus* (Squamata), from the primitive Actinopterygian fish, the bichir *Polypterus senegalensis* (Polypteriformes) and from two agnathans, the Southern-hemisphere lamprey *Geotria australis* (Geotriidae) and the holarctic lamprey *Lampetra fluviatilis* (Petromyzontidae). The primary structure of bichir PYY is identical to the proposed ancestral sequence of gnathostome PYY (YPPKPENPGE¹⁰ | DAPPEELAKY²⁰ | YSALR HYINL³⁰ | ITRQRY). Tortoise and python PYY differ by six and seven residues, respectively, from the ancestral sequence consistent with the traditional view that the Testudines represent an earlier divergence from the primitive reptilian stock than the Squamates. The current views of agnathan phylogeny favor the hypothesis that the Southern-hemisphere lampreys and the holarctic lampreys arose from a common ancestral stock but their divergence is of a relatively ancient (pre-Tertiary) origin. The *Geotria* PYY-related peptide shows only two amino acid substitutions (Pro¹⁰ → Gln and Leu²² → Ser) compared with PYY from the holarctic lamprey *Petromyzon marinus*. This result was unexpected as *Petromyzon* PYY differs from *Lampetra* PYY deduced from the nucleotide sequence of a cDNA (Söderberg et al. J. Neurosci. Res. 1994;37:633–640) by 10 residues. However, a re-examination of an extract of *Lampetra* intestine revealed the presence of a PYY that differed in primary structure from *Petromyzon* PYY by only one amino acid residue (Pro¹⁰ → Ser). This result suggests that the structure of PYY has been strongly conserved during the evolution of Agnatha and that at least two genes encoding PYY-related peptides are expressed in *Lampetra* tissues. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Agnatha; Peptide tyrosine–tyrosine; *Polypterus*; Squamata; Testudines

1. Introduction

Peptide tyrosine–tyrosine (PYY) is a hormone that was first identified in an extract of pig intestine using a chemical assay that permitted identification of peptides containing a C-terminal α -amidated amino acid residue

[1]. In mammals, PYY is produced primarily by endocrine-like cells in the ileum and colon and exercises an inhibitory action on pancreatic and gastric exocrine secretion, pancreatic and intestinal blood flow, and insulin release (reviewed in Ref. [2]). The primary structure of PYY is known for several species of mammals (reviewed in Refs. [3–5]) and sequence similarity of the peptides and a similar structural organization of the corresponding genes have suggested that PYY is homologous to pancreatic

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polypeptide (PP), produced in the pancreatic islets, and neuropeptide Y (NPY), distributed throughout the central and peripheral nervous systems [4–6]. The peptides are frequently classified together as members of the PP family and are believed to have arisen from successive duplications of an ancestral gene [3–6].

Our knowledge of the amino acid sequences of PYY-related peptides in non-mammalian species is fragmentary. PYY has been purified from the intestines of the chicken [7], a frog, *Rana ridibunda* [8], rainbow trout *Oncorhynchus mykiss* [9], and the holarctic sea lamprey *Petromyzon marinus* [10] and the peptides have been characterized structurally. The nucleotide sequence of a cDNA encoding a PYY-related peptide from a second holarctic lamprey, the river lamprey *Lampetra fluviatilis*, has also been determined [11]. It has been proposed that the PP family peptide identified in the pancreas of teleost, e. asmbbranch and holostean fish is the piscine equivalent of PYY rather than mammalian PP [5,8], although it is possible that the Brockmann bodies of certain Acanthomorph teleosts (e.g. anglerfish, sculpin, tilapia) synthesize a PP-related peptide [5]. With the exception of chicken PYY, which contains an additional residue, these lower vertebrate peptides comprise 36 amino acid residues and terminate in a C-terminally α -amidated residue. In this study, we extend our understanding of the molecular evolution of PYY by isolating and characterizing the peptide from extracts of the intestines of two species of reptile, the desert tortoise *Gopherus agassizii* and the Burmese python *Python molurus*, from the primitive Actinopterygian (ray-finned) fish, the bichir *Polypterus senegalensis*, and from two agnathans, the Southern-hemisphere lamprey *Geotria australis* and the holarctic lamprey *Lampetra fluviatilis*.

2. Materials and methods

2.1. Tissue extraction

Adult upstream-migrant *Geotria australis* (113 individuals of both sexes) were collected from the Donnelly River in southwestern Australia. The entire intestine (92 g) was removed immediately after capture, washed and the tissues were stored at -70°C . Desert tortoise *Gopherus agassizii* were collected in the Las Vegas area of Nevada and were euthanized due to the presence of upper respiratory disease. Small intestines (1060 g) were taken from 10 adult animals and immediately frozen on dry ice. The tissues were separately extracted with 10 volumes of ethanol/0.7 M HCl (3:1 v/v) using a Waring blender. After centrifugation ($1600 \times g$ for 1 h at 4°C), ethanol was removed from the supernatant under reduced pressure. Peptide material was isolated from the extract using Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA) as

previously described [10]. Bound material was eluted with 70% (v/v) acetonitrile/water and freeze-dried.

Lampetra PYY was isolated from side-fractions from the purification of *Lampetra* glucagon using entire small intestine (183 g) from 603 individuals collected during their spawning run in tributaries of the River Neva, Russia, during November 1993 [12]. Python PYY was isolated from side-fractions from the purification of tachykinins and neurotensin using whole small intestine (125 g) from three juvenile animals [13]. Bichir PYY was purified from side-fractions from the purification of insulin and glucagon using disseminated pancreatic tissue and anterior intestine combined (10.0 g) from 29 juvenile animals [14]. Full details of tissue collection and extraction from these three species have been provided previously.

2.2. Radioimmunoassay

PYY-like immunoreactivity was detected using antiserum 8999 which was raised against the cysteine-extended COOH-terminal hexapeptide of human NPY (Cys-Ile-Thr-Arg-Gln-Arg-Tyr-NH₂) in a radioimmunoassay procedure that has been described previously [15]. The antiserum reacts strongly with both human PYY and NPY but shows <1% reactivity with human pancreatic polypeptide. To maintain consistency of terminology, the lamprey peptides are referred to as PYY despite the fact that the N-terminal residue is methionine.

2.3. Purification of *Geotria* PYY

The same procedure was used for the isolation of PYY from all species and so only the purification of *Geotria* PYY will be described in detail. The intestinal extract, after partial purification on Sep-Pak cartridges, was redissolved in 1 M acetic acid (4 ml) and chromatographed on a 2.5×90 -cm column of Sephadex G-25 (Pharmacia Biotech, Uppsala, Sweden) equilibrated with 1 M acetic acid. The column was eluted at a flow rate of 48 ml/h and fractions (8 ml) were collected. Absorbance was measured at 280 nm. The concentration of PYY-like immunoreactivity in the fractions was determined at a dilution of 1:30. Fractions containing immunoreactivity were pooled and injected onto a 1×25 -cm Vydac 218TP510 C_{18} reversed-phase HPLC column (Separations Group, Hesperia, CA) equilibrated with 0.1% (v/v) trifluoroacetic acid/water at a flow rate of 2 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 21% (v/v) over 10 min, held at this concentration for 30 min and then raised to 49% (v/v) over 60 min using linear gradients. Absorbance was measured at 214 and 280 nm. Fractions (2 ml) were collected and assayed for PYY-like immunoreactivity at a dilution of 1:30.

The fraction designated PYY (containing PYY-like immunoreactivity) (Fig. 1A) was rechromatographed on a 0.46×25 -cm Vydac 214TP54 C_4 reversed-phase column

Fig. 1. Purification by reversed-phase HPLC on (A) semipreparative Vydac C₁₈, (B) analytical Vydac C₄, (C) analytical Vydac phenyl and (D) analytical Vydac C₁₈ columns of peptide YY in an extract of *Geotria* intestine after partial purification by gel permeation chromatography. The concentration of acetonitrile in the eluting solvent is shown by a broken line and the arrows show where peak collection began and ended. In panel D, peak 1 contains the 36 amino-acid-residue PYY and peak 2 contains the 38-amino-acid-residue PYY.

equilibrated with acetonitrile/water/trifluoroacetic acid (210:789:1) at a flow rate of 1.5 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 49% over 60 min using a linear gradient. *Geotria* PYY was purified to near homogeneity by successive chromatographies on (A) 0.46 × 25-cm Vydac 219TP54 phenyl, (B) 0.46 × 25-cm Vydac 218TP54 C₁₈ and (C) 0.46 × 25-cm Supelcosil LC-18-DB C₁₈ (Supelco Inc., Bellefonte, PA) columns under the same conditions used for the C₄ column.

2.4. Structural characterization

The primary structures of the peptides were determined by automated Edman degradation using an Applied Bio-

systems model 471A sequencer modified for on-line detection of phenylthiohydantoin amino acids under gradient elution conditions as previously described [10]. Mass spectrometry was performed on a Voyager RP MALDI-TOF instrument (Perspective Biosystems Inc., Framingham, MA) equipped with a nitrogen laser (337 nm). The instrument was operated in linear mode with delayed extraction and the accelerating voltage in the ion source was 25 kV. The accuracy of the mass determinations was within 0.05%.

3. Results

3.1. Purification of the PYY-related peptides

The PYY-LI in the extract of *Geotria* intestine tract, after partial purification on Sep-Pak cartridges, was eluted from a Sephadex G-25 column as a single peak with K_{AV} between 0.29 and 0.37. These fractions were pooled and injected onto a semi-preparative Vydac C₁₈ column (Fig. 1A). PYY-LI was associated with the single fraction denoted by the bar. After rechromatography on an analytical Vydac C₄ column (Fig. 1B), the PYY-LI was associated with the major peak in the chromatogram. Chromatography on analytical Vydac phenyl (Fig. 1C) demonstrated that the material was heterogeneous and PYY-LI was associated with the descending limb of the major peak in the chromatogram. After rechromatography on an analytical Vydac C-18 column (Fig. 1D), the material was eluted as an asymmetric peak and PYY-LI was associated with the ascending (peak 1) and descending shoulders (peak 2). Peak 1 and 2 components were separately rechromatographed on a Supelcosil C₁₈ column and both peptides were eluted as symmetrical peaks. The final yield of pure peak 1 material (subsequently shown to comprise 36 amino acid residues) was approximately 2 nmol and the final yield of peak 2 material (subsequently shown to comprise 38 amino acid residues) was approximately 500 pmol.

The PYY-related peptides from the other species were purified to near homogeneity, as assessed by a symmetrical peak shape, and the approximate final yields of purified peptides were: tortoise 4 nmol, python 2 nmol, bichir 1.5 nmol and *Lampetra* 600 pmol.

3.2. Structural characterization of the peptides

The amino acid sequences of the peptides were determined without ambiguity by automated Edman degradation. The results of the sequence analyses indicated the following primary structures:

3.2.1. Python

Tyr-Pro-Pro-Lys-Pro-Glu-Ser-Pro-Gly-Glu¹⁰-Asn-Ala-Thr-Pro-Glu-Glu-Leu-Ala-Lys-Tyr²⁰-Ile-

Ala–Asp–Leu–Arg–His–Tyr–Ile–Asn–Leu³⁰–Val–Thr–Arg–Gln–Arg–Tyr.

3.2.2. Tortoise

Tyr–Pro–Pro–Lys–Pro–Glu–Asn–Pro–Gly–Asp¹⁰–Asn–Ala–Ser–Pro–Glu–Glu–Met–Ala–Lys–Tyr²⁰–Phe–Ser–Ala–Leu–Arg–His–Tyr–Ile–Asn–Leu³⁰–Val–Thr–Arg–Gln–Arg–Tyr.

3.2.3. Bichir

Tyr–Pro–Pro–Lys–Pro–Glu–Asn–Pro–Gly–Glu¹⁰–Asp–Ala–Pro–Pro–Glu–Glu–Leu–Ala–Lys–Tyr²⁰–Tyr–Ser–Ala–Leu–Arg–His–Tyr–Ile–Asn–Leu³⁰–Ile–Thr–Arg–Gln–Arg–Tyr.

3.2.4. *Geotria* peak 1

Met–Pro–Pro–Lys–Pro–Asp–Asn–Pro–Ser–Gln¹⁰–Asp–Ala–Ser–Pro–Glu–Glu–Leu–Ser–Lys–Tyr²⁰–Met–Ser–Ala–Val–Arg–Asn–Tyr–Ile–Asn–Leu³⁰–Ile–Thr–Arg–Gln–Arg–Tyr.

3.2.5. *Geotria* peak 2

Thr–Ala–Met–Pro–Pro–Lys–Pro–Asp–Asn–Pro¹⁰–Ser–Gln–Asp–Ala–Ser–Pro–Glu–Glu–Leu–Ser²⁰–Lys–Tyr–Met–Ser–Ala–Val–Arg–Asn–Tyr–Ile³⁰–Asn–Leu–Ile–Thr–Arg–Gln–Arg–Tyr.

3.2.6. *Lampetra*

Met–Pro–Pro–Lys–Pro–Asp–Asn–Pro–Ser–Ser¹⁰–Asp–Ala–Ser–Pro–Glu–Glu–Leu–Ser–Lys–Tyr²⁰–Met–Ser–Ala–Val–Arg–Asn–Tyr–Ile–Asn–Leu³⁰–Ile–Thr–Arg–Gln–Arg–Tyr.

The proposed sequences were confirmed by MALDI-TOF mass spectrometry: python observed molecular mass (M_r) = 4229.6, calculated M_r = 4231; tortoise observed M_r = 4271.0, calculated M_r = 4269; bichir observed M_r = 4290.0, calculated M_r = 4292; *Geotria* peak 1 observed M_r = 4209.6, calculated M_r = 4210; *Geotria* peak 2 observed M_r = 4381.6, calculated M_r = 4382; *Lampetra* observed M_r = 4169.7, calculated M_r = 4169. The theoretical molecular masses are calculated on the assumption that the peptides terminate in a C-terminally α -amidated residue.

4. Discussion

The amino acid sequences of the PYY-related peptides determined in this study are compared with known sequences of PYY from species from a range of vertebrate taxa in Fig. 2. Cladistic analysis of such amino acid sequences from species of jawed vertebrates has led Larhammar to propose the 'ancestral' PYY sequence shown, from which all PYY molecules in gnathostomes have evolved [5]. Evidence in support of this sequence is strong as it constitutes the primary structure of PYY in the European spotted dogfish *Scyliorhinus canicula* (Elasmob-

	Ancestral	YPPKP	ENPGE	DAPPE	ELAKY	YSALR	HYINL	ITRQR	Y	Ref.
Pig	--A--	--A--	--S--	--SR--	--AS--	--L--	V----	-		[1]
Chicken	A	----	--S--	A--S--	--I-Q--	F----	V----	-		[7]
Python	----	--S--	N--T--	----	IAD--	----	V----	-		This study
Tortoise	----	--D	N--S--	--M--	F----	----	V----	-		This study
Frog	----	----	--S--	--MT--	LT----	----	V----	-		[8]
Trout	----	----	----	----	--T--	----	----	-		[9]
Bowfin	----	----	----	----	--R--	----	----	-		[18]
Gar	----	----	----	----	----	----	----	-		[17]
Bichir	----	----	----	----	----	----	----	-		This study
Dogfish	----	----	----	----	----	----	----	-		[16]
Skate	----	----	--A--	----	----	----	----	-		[18]
<i>Geotria</i>	M----	D--SQ	--S--	--S--	MS-V-	N----	----	-		This study
<i>Petromyzon</i>	M----	D--SP	--S--	--S--	ML-V-	N----	----	-		[10]
<i>Lampetra</i> I	M----	D--SS	--S--	--S--	ML-V-	N----	----	-		This study
<i>Lampetra</i> II	F----	D--D	N--S--	QM-R-	KA-V-	----	----	-		[11]

Fig. 2. Comparison of the primary structures of the putative ancestral sequence of PYY in gnathostomes with the corresponding peptides from representatives of various vertebrate taxa. (–) denotes residue identity. The amino acid sequence of *Lampetra* II PYY is deduced from the nucleotide sequence of a cloned cDNA.

ranchii) [16] and in the Alligator gar *Lepisosteus spatula* (Semiontiformes) [17]. Similarly, the primary structures of PYY from the skate *Raja rhina* (Elasmobranchii) [18], bowfin, *Amia calva* (Amiiformes) [18] and trout *Oncorhynchus mykiss* (Teleostei) [9] differ from the proposed ancestral sequence by only one amino acid residue. We now show that PYY from the bichir *Polypterus senegalensis* (Polypteriformes) is identical in structure to the ancestral peptide. The Polypteriformes are generally considered to represent a highly specialized survivor of the early stages in the evolution of the Actinopterygii (ray-finned fish). This group has evolutionary connections to the Acipenseriformes (sturgeons and paddlefish) which, like the Neopterygii (gars, bowfin and teleosts), also belong to the Actinopterygii [19]. The data in Fig. 2 show that the structure of PYY has been remarkably well conserved amongst non-tetrapod gnathostomes, indicating that the amino acid sequence of the peptide is not useful in cladistic analyses to infer phylogenetic relationships between species at this level of evolution.

Current ideas concerning the origins and phylogenetic relationships between extant reptiles are in a state of flux. Traditionally, turtles and tortoises (Testudines) have been separated from other orders of living reptiles on the basis of temporal fenestration. Testudines lack temporal fenestrae (anapsid condition) while crocodilians, lizards and snakes possess two openings on each side of the skull (diapsid condition). Thus, the Testudines are considered to represent survivors from the earliest anapsid reptiles which date to the late Pennsylvanian about 280 million years B.P. [20]. More recently, however, a cladistic analysis by Rieppel and deBraga [21] relying on 168 osteological characters found support for inclusion of turtles within the

diapsida. Although this conclusion has been challenged by other morphologically based analyses [22], a phylogenetic assessment comparing the amino acid sequences of PP resulted in the generation of most parsimonious trees in which the desert tortoise nested within the diapsida. In all cases, the branch order determining the placement of the tortoise was after the squamate representative (python) [23]. As shown in Fig. 2, the primary structure of desert tortoise PYY shows six amino acid changes from the ancestral sequence compared with seven changes for python PYY, eight changes for chicken PYY and nine changes for pig PYY. This result, therefore, provides support for the traditional view that Testudines represent an earlier divergence from the primitive reptilian stock than the Squamates. It is generally accepted that snakes evolved from lizards and the identification of the mid-Cretaceous fossil *Pachyrhachis problematicus* as a snake with legs suggests that mosasauroids (a group of extinct marine lizards) and snakes are sister taxa [24].

The lampreys (Petromyzontiformes), along with the hagfishes, are the only surviving groups from the agnathan phase of early vertebrate evolution [25]. Present-day lampreys are organized into three families [26]. The holarctic lampreys (34 species) are placed in the single family Petromyzontidae, whereas those of the southern hemisphere (four species) are placed in either the Moraciidae or Geotriidae. As the fossil record is incomplete, the phylogenetic relationships between the different families remain uncertain but it has been proposed that the southern-hemisphere lampreys evolved at different times in the pre-Tertiary period from stocks similar to those represented today by the holarctic genus *Ichthyomyzon* [27]. In this light, the observation that the *Geotria* PYY-related peptide shows only two amino acid substitutions (Pro¹⁰ → Gln and Leu²² → Ser) compared with PYY isolated from the intestine [10] and brain [28] of the holarctic lamprey *Petromyzon marinus* was unexpected as it had previously been shown that *Petromyzon* PYY differs from *Lampetra* PYY, deduced from the nucleotide sequence of a cDNA, by 10 residues [11] (Fig. 2). The isolation of a variant form of *Geotria* PYY extended from its N-terminus by Thr–Ala suggests either two alternative sites of cleavage of prepro PYY by the signal peptidase or the rapid conversion of the 38 amino acid residue primary product to the 36 residue form by the action of a dipeptidylaminopeptidase. Molecular clocks based upon the structures of other lamprey hormones have ‘told the right time’. *Geotria* pancreatic somatostatin contains 11 substitutions compared with *P. marinus* and 12 substitutions compared with *L. fluviatilis*, whereas the somatostatins from the Petromyzontidae differ by only eight residues [29]. Likewise, the amino acid sequence of *Geotria* insulin differs by 17 residues from the identical sequence of insulin from *Petromyzon* and *Lampetra* [29].

This discrepancy prompted us to re-examine an extract of the intestine of *Lampetra* for the presence of additional

PYY-related peptides with the result that a component was identified that differed from *Petromyzon* PYY by only one amino acid substitution (Pro¹⁰ → Ser) and from *Geotria* PYY by two substitutions (Gln¹⁰ → Ser and Ser²² → Leu) (Fig. 2). The data suggest, therefore, that at least two genes encoding PYY-related peptides are expressed in *Lampetra* tissues with strong evolutionary pressure acting to conserve the structure of one of the gene products during the radiation of the Petromyzontiformes. Although a component corresponding to *Lampetra* II PYY (Fig. 2) was not isolated from the intestinal extract in this study, its presence is not precluded, especially as the mRNA directing its synthesis was detected in gut cells [11]. The PYY-related peptides isolated from the intestines of three species of lamprey, despite their similarity to each other, show 10 amino acid changes compared with the ancestral gnathostome sequence. At this time, therefore, the evolutionary relationship between the PYY-related peptides of Agnatha and the PYYs of gnathostomes is unclear.

Acknowledgements

This work was supported by an award from the National Science Foundation (IBN-9806997). We thank Stephen M. Secor, U.C.L.A. School of Medicine, for a gift of python tissues and Drs Vera Bondereva and Yuri Rusakov, Sechenov Institute St. Petersburg, Russia, and Dr Stacia Sower, University of New Hampshire, USA, for help in collection of lamprey tissues.

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