Characterization of an insulin from the three-toed amphiuma (Amphibia: Urodela) with an N-terminally extended A-chain and high receptor-binding affinity

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Insulin was isolated from an extract of the pancreas of a urodele, the three-toed amphiuma (*Amphiuma tridactylum*), and its primary structure established as Ala-Arg-Gly-Ile-Val-Glu-Gln-Cys-Cys-His¹⁰-Asn-Thr-Cys-Ser-Leu-Asn-Gln-Leu-Glu-Asn²⁰-Tyr-Cys-Asn for the A-chain and Ile-Thr-Asn-Gln-Tyr-Leu-Cys-Gly-Ser-His¹⁰-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys-Gly²⁰-Asp-Arg-Gly-Phe-Phe-Tyr-Ser-Pro-Lys for the B-chain. The N-terminus of the A-chain is extended by two amino acids (Ala-Arg) relative to all other known insulins suggesting an anomalous pathway of post-translational processing in the region

of the C-peptide/A-chain junction of proinsulin. In common with chicken and *Xenopus* insulins, which contain a His^{A8}, amphiuma insulin was more potent (approx. 5-fold) than porcine insulin in inhibiting the binding of [¹²⁵I-Tyr^{A14}]insulin to the soluble human insulin receptor from transfected 293EBNA cells (an adenovirus-transformed human kidney cell line). This result is consistent with previous data showing that insulin analogues extended at Gly^{A1} by uncharged groups have reduced binding affinity whereas high affinity is preserved in analogues extended by basic amino acid residues.

INTRODUCTION

The amphibians, organized into three orders Anura (frogs and toads), Urodela (salamanders) and Gymnophiona (caecilians), occupy a pivotal position in vertebrate phylogeny as representatives of the first terrestrial vertebrates. The evolutionary history of present-day amphibians is unclear, largely because of the incomplete fossil record, so that the question of whether each of the modern orders arose from a common ancestor or from a distinct group of Paleozoic amphibians remains unresolved [1]. Although numerous regulatory peptides have been purified from skin and neuroendocrine tissues of frogs (reviewed in ref. [2]), our knowledge of the structure and biological activity of neurohormonal peptides from salamanders and caecilians is very limited.

The amino acid sequence of insulin has been established for at least 70 species (reviewed in ref. [3]) but structural studies have focused largely on mammals, birds and teleost fish. The primary structures of amphibian insulins are known only for two closely related peptides isolated from the South African clawed toad *Xenopus laevis* [4] and, more recently, for one peptide from the caecilian, *Typhlonectes natans* [5]. The present paper describes the purification and receptor-binding properties of insulin from a salamander of the family Amphiumidae, the three-toed amphiuma *Amphiuma tridactylum*. This aquatic species is found in the swamps of the southeastern U.S.A. and is popularly known as the Congo eel because of its long cylindrical body and slippery skin.

EXPERIMENTAL

Tissue extraction

Amphiuma (ten adult specimens of both sexes; 40–60 cm long; weight 1.5–2.5 kg) were supplied in April by Charles D. Sullivan

Company (Nashville, TN, U.S.A.). Pancreatic tissue (11.4 g) was homogenized with ethanol/0.7 M HCl (3:1, v/v; 200 ml) using a Waring blender and stirred for 2 h at 0 °C as previously described [6]. After centrifugation (4000 g for 30 min), ethanol was removed from the supernatant under reduced pressure. After a further centrifugation (4000 g for 30 min), the extract was pumped at a flow rate of 2 ml/min through eight Sep-Pak C₁₈ cartridges (Waters Associates, Milford, MA, U.S.A.) connected in series. Bound material was eluted with acetonitrile/water/trifluoroacetic acid (70:29:1, by vol.) and freeze-dried.

Radioimmunoassay

Insulin-like immunoreactivity was measured by using an anti-serum raised against pig insulin as described previously [7].

Purification of amphiuma insulin

The pancreatic extract, after partial purification on Sep-Pak cartridges, was redissolved in 0.1 % (v/v) trifluoroacetic acid/water (5 ml) and chromatographed on a Sephadex G-25 gel-permeation column (1.6 cm × 90 cm) (Pharmacia, Uppsala, Sweden) equilibrated with 1 M acetic acid at a flow rate of 24 ml/h. Fractions (2 ml) were collected and assayed for insulin-like immunoreactivity at a dilution of 1:30. Fractions containing insulin were pooled and injected on to a Vydac 218 TP510 C₁₈ reverse-phase HPLC column (1 cm × 25 cm) (Separations Group, Hesperia, CA, U.S.A.) equilibrated with 0.1 % (v/v) trifluoroacetic acid 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 and to 49 % (v/v) over 60 min with linear gradients. Absorbance was measured at 214 nm and 280 nm and individual peaks were collected by hand. The peak designated I (containing

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insulin-like immunoreactivity) (see Figure 1a) was rechromatographed on a Vydac 214 TP54 C₄ reverse-phase column (0.46 cm × 25 cm) equilibrated with acetonitrile/water/trifluoroacetic acid (210:789:1, by vol.) at a flow rate of 1.5 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 42% (v/v) over 40 min using a linear gradient. Amphiuma insulin was purified to near-homogeneity, as assessed by peak symmetry, by chromatography on a Vydac 219 TP54 phenyl column (0.46 cm × 25 cm) under the same conditions as those used for the C₄ column.

Structural characterization

Amphiuma insulin (approx. 4 nmol) was reduced (dithiothreitol) and pyridylethylated (4-vinylpyridine) as previously described [6]. The derivatized A-chain and B-chain were separated by reverse-phase HPLC on a Vydac C₄ column under the conditions used for the purification of insulin (see Figure 1b).

Amino acid compositions were determined in duplicate by precolumn derivatization with phenylisothiocyanate using an Applied Biosystems model 420A derivatizer followed by reverse-phase HPLC with an Applied Biosystems model 130A separation system. Hydrolysis (24 h at 110 °C in 5.7 M HCl) of approx. 1 nmol of peptide was carried out. Cysteine and tryptophan residues were not determined. The primary structures of the peptides were determined by automated Edman degradation in an Applied Biosystems model 471A sequencer modified for detection of phenylthiohydantoin amino acid derivatives under gradient elution conditions.

Insulin-binding studies

Competitive binding studies were carried out using both the secreted recombinant extracellular domain and the soluble full-length recombinant human insulin receptor, expressed in 293EBNA cells (an adenovirus-transformed human kidney cell line expressing EBV nuclear antigen) [8]. It has been shown that porcine insulin binds to a single population of binding sites with a dissociation constant K_d of 0.46 nM in the secreted extracellular domain and to two populations of binding sites in the full-length receptor with K_d values of 2.8 pM and 0.51 nM (D. C. Mynarcik and J. Whittaker, unpublished work). The abilities of amphiuma insulin (purity greater than 98%) and porcine insulin to inhibit the binding of human [3-¹²⁵I]iodotyrosine^{A14}insulin (specific radioactivity 74 TBq/mmol; Amersham Corporation, Arlington Heights, IL, U.S.A.) to the soluble forms of the insulin receptor were determined using a procedure previously described in detail [8] except that Tween 20 was omitted from the incubation buffer and the concentration of Triton X-100 was reduced to 0.025% (w/v). All determinations were performed in triplicate.

RESULTS

Purification of amphiuma insulin

The crude extract of amphiuma pancreas contained only a very low concentration of insulin-like immunoreactivity (approx. 1 pmol/g tissue weight), measured with an antiserum raised against pig insulin, and the immunoreactivity in serial dilutions of the extract did not diminish in parallel with the pig insulin standard curve in radioimmunoassay.

The insulin-like immunoreactivity in the extract, after partial purification on Sep-Pak cartridges, was eluted from a Sephadex G-25 gel-permeation column as a broad trailing peak immediately after the void volume of the column. Fractions with K_{av} between 0.1 and 0.4 were pooled and injected on to a semipreparative

Figure 1 Purification of amphiuma insulin by reverse-phase HPLC

The extract of amphiuma pancreas, after partial purification on Sep-Pak C₁₈ cartridges and on a Sephadex G-25 gel-permeation column, was chromatographed on (a) semi-preparative Vydac C₁₈, (b) analytical Vydac C₄ and (c) analytical Vydac phenyl HPLC columns under the conditions described in the text. The fractions denoted by I contained insulin. The broken line shows the concentration of acetonitrile in the eluting solvent and the arrows show where peak collection began and ended.

Vydac C₁₈ reverse-phase HPLC column (Figure 1a). The major UV-absorbing peak in the chromatogram, designated I, was the only peak containing detectable insulin-like immunoreactivity. This material was rechromatographed on an analytical Vydac C₄ column (Figure 1b) and the peptide was eluted as a distinct well-resolved peak. Amphiuma insulin was purified to near-homo-

Table 1 Determination of the primary structures of the A-chain and B-chain of amphiuma insulin by automated Edman degradation

PE-Cys refers to the vinylpyridine derivative of cysteine. The detection limit for phenylthiohydantoin derivatives was 0.5 pmol.

Cycle no.	A-chain		B-chain	
	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)
1	Ala	1310	Ile	2241
2	Arg	365	Thr	1147
3	Gly	963	Asn	1523
4	Ile	786	Gln	2290
5	Val	1003	Tyr	2145
6	Glu	657	Leu	1864
7	Gln	560	PE-Cys	1815
8	PE-Cys	861	Gly	1693
9	PE-Cys	880	Ser	239
10	His	531	His	730
11	Asn	495	Leu	705
12	Thr	182	Val	701
13	PE-Cys	567	Glu	459
14	Ser	78	Ala	629
15	Leu	440	Leu	587
16	Asn	465	Phe	527
17	Gln	298	Leu	612
18	Leu	324	Val	508
19	Glu	162	PE-Cys	341
20	Asn	317	Gly	383
21	Tyr	297	Asp	297
22	PE-Cys	146	Arg	318
23	Asn	51	Gly	284
24			Phe	148
25			Phe	179
26			Tyr	113
27			Ser	19
28			Pro	42
29			Lys	69

geneity, as assessed by symmetrical peak shape, by a final chromatography on an analytical Vydac phenyl column (Figure 1c). The final yield of pure peptide (determined by amino acid analysis) was 31 nmol.

Characterization of amphiuma insulin

The primary structures of the pyridylethylated A-chains and B-chains of amphiuma insulin were determined by automated Edman degradation and the results are shown in Table 1. It was possible to identify without ambiguity phenylthiohydantoin derivatives of amino acids for 23 cycles of operation during sequence analysis of the A-chain and for 29 cycles in the case of the B-chain. The results of amino acid analysis demonstrated that the A-chain and B-chain of amphiuma insulin had the following composition (mol of residue/mol of peptide): A-chain: Asx 3.7 (4), Glx 4.1 (4), Ser 0.9 (1), Gly 1.0 (1), His 1.0 (1), Arg 1.2 (1), Thr 0.9 (1), Ala 1.0 (1), Tyr 1.0 (1), Val 0.6 (1), Ile 0.6 (1), Leu 1.9 (2); B-chain: Asx 2.1 (2), Glx 2.2 (2), Ser 1.8 (2), Gly 2.7 (3), His 1.1 (1), Arg 1.2 (1), Thr 1.0 (1), Ala 1.0 (1), Pro 1.0 (1), Tyr 2.8 (3), Val 1.9 (2), Ile 1.1 (1), Leu 4.4 (4), Phe 1.8 (2), Lys 1.0 (1). The values in parentheses show the number of residues predicted from the proposed structure. Agreement between the results of Edman degradation and amino acid composition analysis was good, demonstrating that the full sequences of the peptides had been obtained. The data indicated that the amphiuma insulin was more than 98% pure.

Figure 2 Comparison of the abilities of amphiuma and porcine insulins to inhibit the binding of [125 I-Tyr A14]insulin to (a) the full-length and (b) the secreted extracellular domain of the recombinant human insulin receptor

Data are presented as the B/B_0 ratio where B is 125 I-labelled insulin specifically bound at the indicated insulin concentration and B_0 is the binding in the absence of added insulin. Each point represents the mean $\pm$ S.D. of three independent experiments with amphiuma (■) and porcine (●) insulins.

Receptor-binding properties

The abilities of amphiuma insulin and porcine insulin to inhibit binding of 125 I-labelled human insulin to the soluble full-length human insulin receptor are compared in Figure 2(a). The mean concentration of amphiuma insulin producing a 50% inhibition of binding was 0.026 nM (range 0.022–0.030 nM). The corresponding value for porcine insulin, in incubations carried out at the same time and under identical conditions, was 0.13 nM (range 0.12–0.14 nM). The mean concentrations producing 50% inhibition of binding of labelled insulin to the secreted extracellular domain were: amphiuma insulin 0.26 nM (range 0.25–0.27 nM) and porcine insulin 1.66 nM (range 1.54–1.86 nM) (Figure 2b).

DISCUSSION

The primary structure of amphiuma insulin is compared with the structures of other known amphibian insulins and with human insulin in Figure 3. Despite the fact that the line of evolution leading to salamanders diverged from that leading to mammals

	A-chain							
Amphiuma	AR	GIVEQ	CCHNT	CSLNQ	LENYC	N		
Caecilian	----	K	--LS-	----	YE	--S--	-	
<i>Xenopus</i> I	-----		S-	----	F-	--S--	-	
<i>Xenopus</i> II	-----		S-	----	F-	-----	-	
Human	-----		TSI	-----		-----	-	

	B-chain							
Amphiuma	ITNQY	LCGSH	LVEAL	YLVCG	DRGFF	YSPK		
Caecilian	-A--H	-----	-----	----	A	-----	-T--S	
<i>Xenopus</i> I	LV--H	-----	-----	-----		-----	-Y--V	
<i>Xenopus</i> II	LA--H	-----	-----	-----		-----	-Y--I	
Human	FV--H	-----	-----	-----	E----	-----	-T--T	

Figure 3 Comparison of the primary structures of amphiuma insulin with insulins from other orders of amphibia and with human insulin

-, Residue identity.

at least 300 million years ago, the amino acid sequence of amphiuma insulin is remarkably similar to that of human insulin. Residues considered to comprise the receptor-binding region (A1–A3, A5, A19, A21, B22–B26) as well as those involved in dimer formation (B12, B16, B21) and hexamer formation (B6, B10, B14, B17, B20, A13, A14) [9] have been conserved between mammalian and amphiuma insulins. A recent phylogenetic study [10] comparing mitochondrial DNA sequences of the 12 S and 16 S rRNA genes has supported the conclusion of an earlier morphological investigation [11] that the gymnophonians and the urodeles are sister taxa. Amphiuma insulin, however, contains ten substitutions compared with the corresponding region of a caecilian insulin but only six substitutions compared with *Xenopus* insulin II (Figure 3). It would appear therefore that the molecular clock based on the amino acid sequences of insulin has once again 'told the wrong time' [12].

The most unusual feature of the amphiuma insulin structure is the presence of a dipeptide (Ala-Arg) extension to the N-terminus of the A-chain which suggests an anomalous pathway of post-translational processing of proinsulin in the region of the C-peptide/A-chain junction. Insulins from the Agnatha, the sea lamprey, *Petromyzon marinus* [13] and river lamprey *Lampetra fluviatilis* [14] contain polypeptide extensions to the N-terminus

	P ₄	P ₃	P ₂	P ₁
Amphiuma	Xaa-Xaa-Xaa-Xaa-Ala-Arg-Gly-Ile-Val-Glu.....			
Rat	Val-Ala-Arg-Gln-Lys-Arg-Gly-Ile-Val-Glu.....			
Chicken	Tyr-Glu-Lys-Val-Lys-Arg-Gly-Ile-Val-Glu.....			
<i>Xenopus</i>	Tyr-Gln-Lys-Met-Lys-Arg-Gly-Ile-Val-Glu.....			
Hagfish	Val-Leu-Lys-Ser-Lys-Arg-Gly-Ile-Val-Glu.....			

Figure 4 The amino acid sequence in the neighbourhood of the dibasic-residue (Lys-Arg)-processing site linking the C-peptide region of proinsulin to the A-chain of insulin in species of different vertebrate taxa

It is proposed that the residues in amphiuma proinsulin that are underlined represent the processing site.

of the B-chain but such a modification to the A-chain has not been observed previously. The family of cellular endoproteases involved in the processing of prohormone precursors has now been well characterized (reviewed in ref. [15]). Transfection studies using the constitutively secreting COS-7 cell line have shown that the prohormone convertase SPC3 (PC1) preferentially cleaves rat proinsulin I at the Arg-Arg site located at the B-chain/C-peptide junction whereas the convertase SPC2 (PC2) specifically cleaves at the Lys-Arg site at the C-Peptide/A-chain junction [16]. We propose that a mutation (Lys → Ala) has taken place in the Lys-Arg-processing site in amphiuma proinsulin so that the site is no longer recognized by the SPC2-type convertase. However, as shown in Figure 4, in several, although by no means all, proinsulins from different vertebrate classes (e.g. rat [17], chicken [18], *Xenopus* [19] and hagfish [20]) the residue at the P₄ site relative to the Lys-Arg-scissible bond is either lysine or arginine. The residue at the P₄ site is believed to play an important role in substrate selectivity in the prohormone convertase family [21]. We speculate therefore that a second substitution of an amino acid at the P₃ site may have occurred, generating an alternative dibasic-residue-processing site (-Lys-Xaa-Ala-Arg- → -Lys-Arg-Ala-Arg-) that is recognized by the SPC2 enzyme. Determination of the nucleotide sequence of cloned amphiuma proinsulin cDNA is required to verify this hypothesis. This proposed pathway of post-translational processing is reminiscent of the anomalous pathway of processing of proinsulin in the holocephalan fish, Pacific ratfish *Hydrolagus coliei* [22]. In this species a mutation in the site linking the B-chain and C-peptide regions (Arg-Arg → Ile-Arg) results in the generation of multiple forms of insulin each arising from cleavages at different sites within the C-peptide region by a putative enzyme with chymotrypsin-like specificity.

Chicken [23] and *Xenopus* [4] insulins bind to insulin receptors on mammalian cells, e.g. rat liver membranes [4,23] and human IM-9 lymphocyte [4], with 2–3-fold increased binding affinity compared with pig insulin. This increase in affinity has been ascribed to the presence of the His^{As} residue which is also present in the equivalent position (A10) in amphiuma insulin. This residue may form stabilizing interactions with residues in the receptor and/or may induce a small conformation change in the receptor-binding region of the ligand [24]. The binding affinity of amphiuma insulin is approximately twice that of chicken/frog insulin and we speculate that this further increase in affinity may result from the substitution Ser → Asn at A11 (corresponding to A9 in other insulins). A 2-fold increase in binding affinity corresponds to a small (4–8 kJ/mol) change in free energy, consistent with the different abilities of serine and asparagine to form hydrogen bonds. Amphiuma insulin, in common with insulins from teleost fish, e.g. salmon [25], contains a residue deletion at the C-terminus of the B-chain and also the unusual substitution (His → Tyr) at B6. It has been shown, however, that modifications in these regions of the insulin molecule have little or no effect on biological potency [26–28].

Schaffer [29] has recently proposed a novel model of insulin-receptor interaction that explains the complex kinetics of insulin binding. In this model, insulin has two receptor-binding sites and the receptor heterodimer has two ligand-binding sites. High-affinity binding is generated by the asymmetrical binding of one insulin molecule to the two receptor heterodimers constituting the holoreceptor. In Schaffer's nomenclature, Site 1 of insulin binds to Site 1 on the first heterodimer and Site 2 of insulin binds to Site 2 on the second heterodimer. In the secreted receptor extracellular domain, only Site 1 on the insulin molecule binds to the corresponding site on the receptor producing a low-affinity interaction. The increase in binding affinity of amphiuma insulin

relative to porcine insulin is comparable for both forms of the receptor (full-length and extracellular domain) and so we propose that this increase is mediated by changes in the binding affinity of Site 1 of the insulin molecule.

A study of the pH-dependence of the binding of insulin to its receptor on IM-9 lymphocytes has concluded that a positive charge on the α -amino group of Gly^{A1} is important for high-affinity binding [30]. Consistent with this hypothesis, it was found that bovine insulin analogues modified at Gly^{A1} by uncharged residues, e.g. [acetyl-Gly^{A1}]insulin, [Boc-Gly^{A1}]insulin and [thiazolidine-Gly^{A1}]insulin, show 5–20-fold decreased binding to bovine liver plasma membranes whereas high-affinity binding is preserved in analogues extended from the A1 residue by one or more basic residues [31]. [Arg-Gly^{A1}]Insulin, for example, has $107 \pm 3.5\%$ of the receptor-binding activity of insulin in this system. The data suggest therefore that the presence of the arginine residue at position A2 in amphiuma insulin is important in maintaining a strong interaction between ligand and receptor.

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