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Structure and receptor-binding activity of insulin from a holostean fish, the bowfin (*Amia calva*)

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The holostean fishes are the extant representatives of the primitive ray-finned fishes from which the present-day teleosts may have evolved. The primary structure of insulin from a holostean fish, the bowfin (*Amia calva*), was established as:
A-chain: Gly-Ile-Val-Glu-Gln-Cys-Cys-Leu-Lys-Pro-Cys-Thr-Ile-Tyr-Glu-Met-Glu-Lys-Tyr-Cys-Asn
B-chain: Ala-Ala-Ser-Gln-His-Leu-Cys-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Phe-Leu-Val-Cys-Gly-Glu-Ser-Gly-Phe-Phe-Tyr-Asn-Pro-Asn-Lys-Ser
This amino acid sequence contains several substitutions (methionine at A16, phenylalanine at B16 and serine at B22) at sites that have been strongly conserved in other vertebrate species and that may be expected to influence biological activity. Consistent with this prediction, bowfin insulin was approx. 14-fold less potent than pig insulin in inhibiting the binding of [^{125}I-Tyr-A14](human insulin) to transfected mouse NIH 3T3 cells expressing the human insulin receptor.

INTRODUCTION

The holostean fish occupy an important position in phylogeny as the surviving representatives of a group of primitive ray-finned fishes from which present-day teleosts may have evolved. Although abundant in the Mezozoic era, the superorder Holostei now comprises only two common genera, *Lepisosteus*, the gar, and *Amia*, the bowfin. The morphology of the pancreas in the bowfin (*Amia calva*) has been described in detail (Epple & Brinn, 1975). The pancreatic tissue is widely disseminated, accompanying the abdominal blood vessels and bile ducts and covering regions of the liver. Ultrastructural analysis indicates that the bowfin pancreas contains the four cell types (A, B, D and PP) found in the endocrine pancreata of teleost fish and the higher vertebrates (Epple & Brinn, 1975; Falkmer, 1985). A peptide of the pancreatic polypeptide family has been isolated from the bowfin pancreas and structurally characterized (Conlon *et al.*, 1991), but the primary structures of the other islet hormones are not known.

In a comparison of the abilities of partially purified insulins from a wide range of vertebrate species to increase glucose utilization by the mouse hemidiaphragm (Falkmer & Wilson, 1967), bowfin insulin displayed a remarkably low biological potency [0.02 i.u./mg of protein compared with 6.7 i.u./mg for a teleost insulin (cod) and 29.4 i.u./mg for ox insulin]. The activity of bowfin insulin, however, was completely abolished by an excess of an antiserum raised against ox insulin. Although data obtained with impure preparations of hormones must be treated with caution, these results suggest that bowfin insulin may contain unusual structural features in its receptor-binding region that reduce its affinity for a mammalian insulin receptor while retaining a common antigenic determinant with mammalian insulin. The present paper describes the purification and characterization of bowfin insulin and demonstrates that the peptide indeed shows reduced affinity for a mammalian insulin receptor.

EXPERIMENTAL

Preparation of bowfin pancreas extract

Bowfin (20 adult specimens of both sexes; 35–65 cm long) were collected at Hay Bay, Lake Ontario, Canada, during September and October 1989. Pancreatic tissue (145 g) was extracted with ethanol/0.7 M-HCl (3:1, v/v; 900 ml) as previously described (Conlon *et al.*, 1987). After centrifugation (10000 ***g*** for 1 h at 4 °C), ethanol was removed from the supernatant under reduced pressure. Peptide material was isolated from the extract by using Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA, U.S.A.) as previously described (Conlon *et al.*, 1987). Bound material was eluted with acetonitrile/water/trifluoroacetic acid (70:29:1, by vol.) and freeze-dried.

Radioimmunoassay

Insulin-like immunoreactivity was measured by use of an antiserum raised against pig insulin as previously described (Bailey & Ahmed-Sorour, 1980).

Purification of bowfin insulin

The pancreatic extract (20% of the total), after partial purification on Sep-Pak cartridges, was redissolved in 0.1% (v/v) trifluoroacetic acid (5 ml) and injected on to a Vydac 218 TP reversed-phase C_{18} h.p.l.c. 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. The peak denoted by the arrow (Fig. 1*a*) was rechromatographed on an Ultrapore RPSC reversed-phase C_3 column (1 cm × 25 cm) (Beckman/Altex, Berkeley, CA, U.S.A.) equilibrated with acetonitrile/water/trifluoroacetic acid (210:789:1, by vol.) at a flow rate of 2 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 42% (v/v) over 50 min. The peak denoted by the asterisk (Fig. 1*b*) was rechromatographed on a Vydac 214 TP reversed-phase C_3 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 35% (v/v) over 40 min.

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Structural characterization of bowfin insulin

Bowfin insulin (approx. 6 nmol) was reduced (with dithiothreitol) and pyridylethylated (with 4-vinylpyridine) as previously described (Conlon & Hicks, 1990). The modified A-chain and B-chain were separated by reversed-phase h.p.l.c. on a Vydac C_{18} column under the conditions used for the purification of insulin (Fig. 1*a*). Amino acid compositions were determined by pre-column modification with phenyl isothiocyanate with an Applied Biosystems model 420A apparatus followed by reversed-phase h.p.l.c. with an Applied Biosystems model 130A separation system. Hydrolysis (24 h at 110 °C in 5.7 M-HCl) of approx. 1 nmol of peptides was carried out. Cysteine and tryptophan residues were not determined. The primary structures of the pyridylethylated A- and B-chains were determined by automated Edman degradation in a Applied Biosystems model 471A Sequenator modified for detection of amino acid phenylthiohydantoin derivatives under gradient elution conditions. Approx. 2 nmol portions of the peptides were used.

Insulin-binding studies

Competitive binding studies were carried out with the NIH 3T3 HIR3.5 cell line, which was obtained by transfecting mouse NIH 3T3 cells with a full-length human kidney insulin-receptor cDNA inserted into a bovine papilloma virus vector (Whittaker *et al.*, 1987). The cells had approx. 6×10^6 insulin receptors per cell. The ability of bowfin insulin (purity > 98 %) and pig insulin to inhibit the binding of [3-[^{125}I]iodotyrosine-A14](human insulin) (specific radioactivity 74 TBq/mmol) (Amersham Corporation, Arlington Heights, IL, U.S.A.) to the cells was determined by using a procedure previously described (Whittaker *et al.*, 1987). Briefly, incubations in the presence of increasing concentrations of either bowfin or pig insulin were carried out for 16 h at 4 °C in 24-well culture dishes containing 2×10^4–3×10^4 cells per well and ^{125}I-labelled insulin (30 pmol/l) in a total volume of 200 μl. All determinations were performed in triplicate.

RESULTS

Purification of bowfin insulin

The elution profile on reversed-phase h.p.l.c. of the extract of bowfin pancreas, after partial purification on Sep-Pak cartridges, is shown in Fig. 1(*a*). The retention time and ratio of absorbances at 214 nm and 280 nm of the prominent peak denoted by the arrow were similar to the corresponding parameters of human insulin, and this was the only peak in the chromatogram to show weak insulin-like immunoreactivity in a radioimmunoassay system with an antiserum raised against pig insulin. Partial purification of the peptide was accomplished by reversed-phase h.p.l.c. on a semi-preparative C_3 column (Fig. 1*b*). Purification to apparent homogeneity of bowfin insulin was achieved by rechromatography of the major peak denoted by the asterisk on an analytical reversed-phase C_4 column (Fig. 1*c*). The final yield of pure peptide was approx. 11 nmol.

Characterization of bowfin insulin

The results of amino acid analysis demonstrated that the A-chain of bowfin insulin had the following composition (mol of residue/mol of peptide): Asx, 1.0; Glx, 3.9; Gly, 1.0; Thr, 0.8; Pro, 1.0; Tyr, 1.8; Val, 0.5; Met, 0.9; Ile, 1.5; Leu, 1.0; Lys, 1.6. The composition of the B-chain (mol of residue/mol of peptide) was as follows: Asx, 2.1; Glx, 2.7; Ser, 4.1; Gly, 3.0; His, 2.0; Ala, 2.7; Pro, 1.2; Tyr, 1.1; Val, 1.1; Leu, 3.1; Phe, 2.8; Lys, 1.1. The results of automated Edman degradation are shown in Table 1. It was possible to assign without ambiguity phenylthiohydantoin derivatives of amino acids for 21 cycles of operation during sequence analysis of the A-chain and for 31 cycles in the case of the B-chain. The results of Edman degradation indicated that the bowfin insulin was > 98 % pure. With the exception of low values for the amount of valine, agreement between the sequence analysis and amino acid composition data was good, demonstrating that the full sequence of the peptides had been obtained. When a sample of bowfin insulin was hydrolysed for 24 h, 48 h and 72 h and the amino acid composition data were extrapolated to zero time, the observed content of valine (2.8 mol/mol of insulin) was consistent with the proposed structure.

Competitive binding studies

The abilities of bowfin insulin and pig insulin to inhibit binding of ^{125}I-labelled human insulin to the human insulin receptor are compared in Fig. 2. The mean concentration of bowfin insulin producing a 50 % inhibition of binding was 48 nM (range 40–60 nM). The corresponding value for pig insulin, in

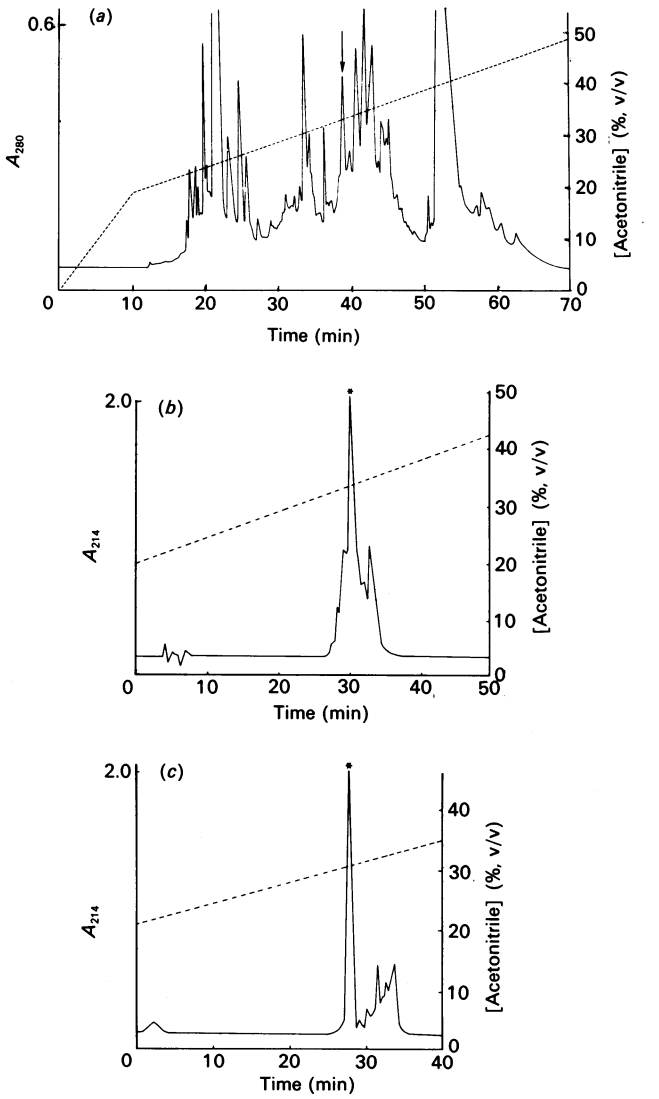

Fig. 1. Purification of bowfin insulin by reversed-phase h.p.l.c.

The extract of bowfin pancreas, after partial purification on Sep-Pak C_{18} cartridges, was chromatographed on (*a*) a Vydac 218 TP column, (*b*) an Ultrapore RPSC column and (*c*) a Vydac 214 TP column. Details of the elution conditions are given in the text. The broken line shows the concentration of acetonitrile in the eluting solvent. The peaks containing insulin are denoted by the arrow (panel *a*) and by the asterisks (panels *b* and *c*).

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

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

	A-chain		B-chain	
Cycle no.	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)
1	Gly	1450	Ala	1241
2	Ile	1360	Ala	1233
3	Val	1410	Ser	255
4	Glu	953	Gln	786
5	Gln	1137	His	519
6	PE-Cys	977	Leu	876
7	PE-Cys	1053	PE-Cys	717
8	Leu	1167	Gly	665
9	Lys	1312	Ser	167
10	Pro	911	His	398
11	PE-Cys	727	Leu	553
12	Thr	115	Val	520
13	Ile	714	Glu	292
14	Tyr	680	Ala	512
15	Glu	356	Leu	486
16	Met	629	Phe	436
17	Glu	317	Leu	469
18	Lys	617	Val	453
19	Tyr	533	PE-Cys	341
20	PE-Cys	345	Gly	303
21	Asn	213	Glu	228
22			Ser	67
23			Gly	275
24			Phe	348
25			Phe	378
26			Tyr	229
27			Asn	298
28			Pro	157
29			Asn	244
30			Lys	180
31			Ser	31

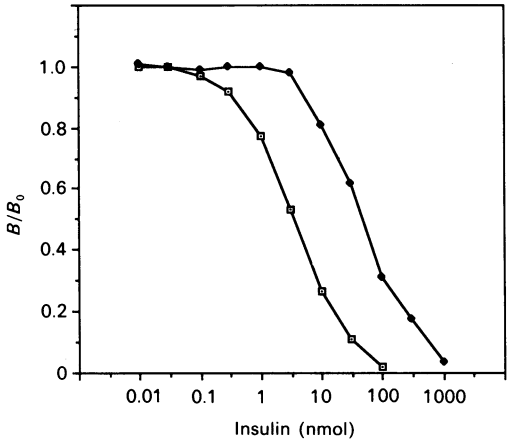

Fig. 2. Comparison of the abilities of bowfin insulin and pig insulin to inhibit the binding of [^{125}I-Tyr-A14](human insulin) to the human insulin receptor

Mouse NIH 3T3 HIR3.5 cells expressing the human insulin receptor were incubated at 4 °C for 16 h with ^{125}I-labelled human insulin (30 pM) and either bowfin insulin (◆) or pig insulin (⊡) in 200 μl of Hanks balanced salts solution supplemented with 50 mM-Hepes (pH 7.6) and 10 mg of BSA/ml. 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. The data are from one experiment out of three.

incubations carried out at the same time and under identical conditions, was 3.3 nM (range 3.0–3.5 nM).

DISCUSSION

The primary structure of bowfin insulin is compared with the structures of insulin from a second holostean fish, the alligator gar (*Lepistoseus spatula*) (Pollock *et al.*, 1987), from a teleost, the cod (*Gadus callarias*) (Reid *et al.*, 1968), and from a mammal, the domestic pig (*Sus scrofa*) (Chance *et al.*, 1968), in Fig. 3. In bowfin insulin, most of the amino acid residues that are important in maintaining the tertiary structure of insulin have been maintained (the six half-cysteine residues, glycine residues at B8 and B23 and the residues at B6, B11, B12, B15, B18, B26 and A2 that constitute the hydrophobic core) (Baker *et al.*, 1988). Bowfin insulin, however, contains amino acid substitutions at several sites that have been strongly conserved in other vertebrate species studied. In particular, the leucine residue at A16, which is important in maintaining the three-dimensional structure of insulin, is replaced by methionine. This substitution has previously been observed only in insulin from the lamprey (*Petromyzon marinus*) (Plisetskaya *et al.*, 1988). The tyrosine residue at position B16, which is involved in dimer formation, is replaced by phenylalanine. However, the other residues that are important in dimerization (B12, B20, B24, B26 and B28) have been conserved in bowfin insulin, together with residues at B4, B6, B10, B14, B17, B20, A14 and A17, which are believed to be involved in the formation of zinc-containing hexamers. Residues at A1, A19, A21 and B23, which are implicated in the formation of the receptor-binding region of insulin, are conserved, but the substitution of arginine at B22 by serine would be expected to result in a reduced binding affinity to a mammalian insulin receptor. This prediction is confirmed by the fact that bowfin insulin is approx. 13-fold less potent than pig insulin in inhibiting the binding of ^{125}I-labelled human insulin to the human insulin receptor. Guinea-pig insulin contains an aspartic acid residue at B22, and the reduction in receptor binding affinity of this insulin is comparable with that of bowfin insulin in assays with membranes prepared from mammalian tissues (Zimmerman & Yip, 1974). In contrast, insulin from the elasmobranch spiny dogfish (*Sqalus acanthias*) is about one-third as potent as pig insulin in a radio-receptor assay with rat liver plasma membranes (Bajaj *et al.*, 1983), and insulin from the teleost daddy sculpin (*Cottus scorpius*) is about 40% as potent as pig insulin in stimulating lipogenesis in rat fat-cells (Cutfield *et al.*, 1986). Bowfin insulin

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                    A-chain
Bowfin    GIVEQ CCLKP CTIYE MEKYC N
Gar       ----- --H-- ----- L-N-- -
Cod       ---D- --HR- -D-FD LQN-- -
Pig       ----- --TSI -SL-Q L-N-- -

                    B-chain
Bowfin    AASQH LCGSH LVEAL FLVCG ESGFF YNPNK S
Gar       --N-- ----- ----- Y---- -K--- ----- V
Cod       M-PP-- ----- --D-- Y---- DR--- ---K
Pig       FVN-- ----- ----- Y---- ER--- -T-KA
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Fig. 3. Comparison of the primary structures of insulins from the bowfin, alligator gar, cod and pig

- denotes residue identity.

contains other unusual substitutions, such as leucine at A8 in the hyper-variable A8–A10 region and lysine for asparagine at A18. Although the residue at position A18 is not believed to be involved directly in the interaction of insulin with its receptor, the substitution may lead to an altered conformation of the ligand and hence have an indirect effect on biological activity.

As shown in Fig. 3, the unusual amino acid substitutions in bowfin insulin are not found in the insulin from the other extant holostean fish, the gar (Pollock *et al.*, 1987). Gar insulin contains histidine at A8, leucine at A16, asparagine at A18, tyrosine at B16 and lysine at B22. In this respect, gar insulin may be described as possessing structural features that are typical of a teleost insulin (Blundell & Wood, 1975). Thus the data in the present paper suggest the hypothesis that the bowfin and the gar are probably not descended from a common Mesozoic ancestor but represent the results of parallel evolutionary development.

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