Structure and biological activity of glucagon and glucagon-like peptide from a primitive bony fish, the bowfin (*Amia calva*)

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The bowfin, *Amia calva* (order Amiiformes) occupies an important position in phylogeny as a surviving representative of a group of primitive ray-finned fishes from which the present-day teleosts may have evolved. Glucagon and glucagon-like peptide (GLP) were isolated from an extract of bowfin pancreas and their primary structures determined. Bowfin glucagon shows only four amino acid substitutions compared with human glucagon, and bowfin glucagon was equipotent and equally effective as human glucagon in stimulation of glycogenolysis in dispersed hepatocytes from a teleost fish, the copper rockfish, *Sebastes caurinus*. In contrast, bowfin GLP shows 15 amino acid substitutions and three amino acid deletions compared with the corresponding region of human GLP-1-(7–37)-peptide. In particular, the bowfin peptide contains an N-terminal tyrosine residue rather than the N-terminal histidine residue found in all other glucagon-related peptides so far characterized. Bowfin GLP stimulated glycogenolysis in rockfish hepatocytes, but was 3-fold less effective and 23-fold less potent than human GLP-1-(7–37)-peptide. We speculate that selective mutations in the GLP domain of bowfin preproglucagon may be an adaptive response to the previously demonstrated low biological potency of bowfin insulin.

INTRODUCTION

Nucleotide sequence analysis of cDNAs and/or genomic fragments encoding preproglucagons from several mammalian species has shown that glucagon is co-synthesized with two structurally related peptides, termed glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2) (Bell, 1986). GLP-1 is further processed in the mammalian intestine to the truncated form GLP-1-(7–37)-peptide [and/or C-terminal α-amidated GLP-1-(7–36)-peptide] (Holst et al., 1987) and that this form circulates in blood (Kreymann et al., 1987). In contrast with mammals, the nucleotide sequence of the cDNA encoding preproglucagon from the anglerfish (*Lophius americanus*) has revealed that this species is associated with two non-allelic genes encoding distinct preproglucagons, neither of which encode the region corresponding to mammalian GLP-2 (Lund et al., 1983). Subsequently, GLP has been isolated from the pancreas of several species of teleost fish [catfish (*Ictalurus punctata*), salmon (*Oncorhynchus mykiss*), sculpin (*Cottus scorpius*) and eel (*Anguilla anguilla*) (reviewed in Conlon et al., 1991a)] and characterized structurally.

Data has accumulated to show that GLP-related peptides exhibit very different biological activities in mammals and fish (reviewed in Mommsen and Moon, 1989; Fehmann et al., 1992). GLP-1-(7–37)-peptide is a potent stimulant of insulin release from the rat pancreas (Mojsov et al., 1987) and potently elevates the concentration of cyclic AMP (Goke and Conlon, 1988) and stimulates proinsulin gene expression (Fehmann and Habener, 1992) in rat insulinoma-derived cells. However, receptors for GLP-1 or GLP-2 are not present in rat liver (Ghiglione et al., 1985), and the peptides exert no effect on intracellular cyclic AMP formation, intracellular Ca^{2+} concentrations, glycogen phosphorylase activity or glucose release from dispersed rat hepatocytes (Blackmore et al., 1991). In contrast, salmon GLP and human GLP-1-(7–37)-peptide are more potent stimulators of gluconeogenesis than salmon glucogen in dispersed salmon hepatocytes (Mommsen et al., 1987) and exposure of hepatocytes from several teleost species to the peptides results in an increase in glucose production from endogenous glycogen (Mommsen and Moon, 1989). Effects *in vivo* of salmon GLP on glycogen and triacylglycerol mobilization and on the activities of glycogen phosphorylase and pyruvate kinase were demonstrated in juvenile coho and chinook salmon but the peptide failed to stimulate insulin release from the pancreas (Plisetskaya et al., 1989).

The Amiiformes 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 Mesozoic era, the order Amiiformes now comprises only one genus, *Amia*, the bowfin. A previous study described the structure and receptor-binding properties of insulin from *Amia calva* (Conlon et al., 1991c). We now extend this work to describe the isolation and structural characterization of glucagon and GLP from an extract of the bowfin pancreas and compare their abilities to stimulate glycogenolysis in teleost hepatocytes.

EXPERIMENTAL

Materials

Synthetic peptides were supplied by Peninsula Laboratories (Belmont, CA, U.S.A.), except human GLP-1-(7–37)-peptide which was kindly provided by Dr. S. Mojsov, Rockefeller University, New York, NY, U.S.A. [3-[^{125}I]iodotyrosine-10] glucagon (sp. radioactivity 74 TBq/mmol) was supplied by Amersham Corp., Arlington Heights, IL, U.S.A.).

Tissue extraction

Bowfin (20 adult specimens of both sexes; 35–65 cm long) were collected at Hay Bay, Lake Ontario, Canada, during September

Abbreviation used: GLP, glucagon-like peptide.

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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., 1991c). 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 using Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA, U.S.A.). Bound material was eluted with acetonitrile/water/trifluoroacetic acid (70:29:1, by vol.) and freeze-dried.

Radioimmunoassay

Glucagon-like immunoreactivity was measured by radioimmunoassay using an antiserum directed against a site in the central region of pig glucagon (probably residues 10–18) and ^{125}I-labelled pig glucagon as tracer in a procedure that has been described previously (Conlon and Thim, 1985).

Purification of bowfin glucagon and GLP

The pancreatic extract (50% of total), after partial purification on Sep-Pak cartridges, was redissolved in 0.1% (v/v) trifluoroacetic acid (5 ml) and chromatographed on a Bio-Gel P-10 gel-permeation column (1.6 cm × 90 cm) (Bio-Rad, Richmond, CA, U.S.A.) equilibrated with 1 M acetic acid at a flow rate of 24 ml/h. Fractions (2 ml) were collected and assayed for glucagon-like immunoreactivity at a dilution of 1:100. Fractions denoted by the bar (Figure 1 below) were pooled and injected on to a Vydac 218 TP510 C_{18} reversed-phase 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 peaks designated G (containing glucagon) and GLP (containing glucagon-like peptide) (Figure 2a below) were separately rechromatographed on a Vydac 214TP54 C_4 reversed-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 30 min using a linear gradient. Bowfin glucagon and GLP were purified to apparent homogeneity, as assessed by peak symmetry, on a Vydac C_{18} reversed-phase column (0.46 cm × 25 cm) under the same conditions used for the C_4 column.

Structural characterization of bowfin glucagon and GLP

Amino acid compositions were determined in duplicate by precolumn derivatization with phenyl isothiocyanate using an Applied Biosystems model 420A derivatizer 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. 500 pmol of peptide was carried out. Cysteine and tryptophan residues were not determined. The primary structures of the bowfin peptides were determined by automated Edman degradation in an Applied Biosystems model 471A sequenator modified for detection of phenylthiohydantoin amino acid derivatives under gradient elution conditions. Approx. 3 nmol portions of the peptides were used.

Glycogenolytic activity of bowfin glucagon and GLP

Adult copper rockfish (*Sebastes caurinus*) of both sexes (200–350 g body weight) were caught by hook and line around Galiano Island, British Columbia, Canada, and maintained in aerated seawater at the University of Victoria for up to 1 year. Hepatocytes were prepared from fed animals by *in vitro* perfusion of the liver with collagenase as previously described in detail (Danulat & Mommsen, 1990). Dispersed cells were suspended in modified Hanks medium containing 1.5 mM $CaCl_2$ and 4% (w/v) defatted BSA and were not provided with exogenous substrates (Mommsen and Moon, 1990). Cell suspensions (200 μl containing 10–20 mg of packed cells) were divided into portions in 1.5 ml Microfuge tubes. At the start of the incubations, bowfin glucagon, mammalian glucagon, bowfin GLP or human GLP-1-(7–37)-peptide, at appropriate concentrations, or buffer only (50 μl) were added. Peptide concentrations were verified by amino acid analysis. The suspensions were lightly agitated and the incubation mixtures were kept at 20 °C for 30 min. Reactions were terminated by addition of 35% (v/v) perchloric acid (20 μl). After 30 min in an ice bath, the precipitated protein was removed by centrifugation (16000 *g* for 4 min) and glucose concentrations in the supernatant were measured enzymically (Danulat and Mommsen, 1990). All determinations were performed in triplicate. Concentration–response curves were generated using the P.fit Scientific Parameter Fitter For Non-linear Equations (Fig.P Software Corporation, Durham, NC, U.S.A.). Potency is defined as the concentration of peptide producing half-maximum activation of glucose production, and effectiveness is the maximum response produced by the peptide. Data are expressed as means ± S.E.M. for three independent experiments.

RESULTS

Purification of bowfin glucagon and GLP

The elution profile on a Bio-Gel P-10 gel-permeation column of the extract of bowfin pancreas, after partial purification on Sep-Pak cartridges, is shown in Figure 1. Glucagon-like immunoreactivity was eluted as a single peak with maximum immunoreactivity at the same elution volume as porcine glucagon. Fractions denoted by the bar were pooled and injected on to a Vydac semi-preparative reversed-phase C_{18} h.p.l.c. column (Figure 2a). The peak designated G showed glucagon-like immunoreactivity and was purified to apparent homogeneity by

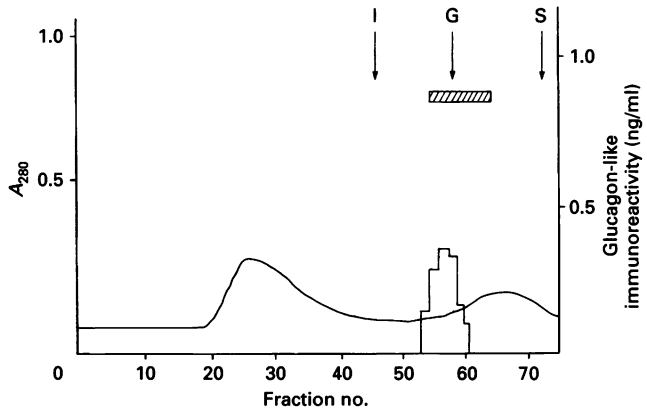

Figure 1 Fractionation of an extract of bowfin pancreas by gel permeation chromatography

The extract, after partial purification on Sep-Pak C_{18} cartridges, was chromatographed on a Biogel P-10 gel permeation column under the conditions described in the text. The fractions denoted by the bar were pooled and subjected to further purification. The arrows show the retention times of porcine insulin (I), glucagon (G) and somatostatin-14 (S).

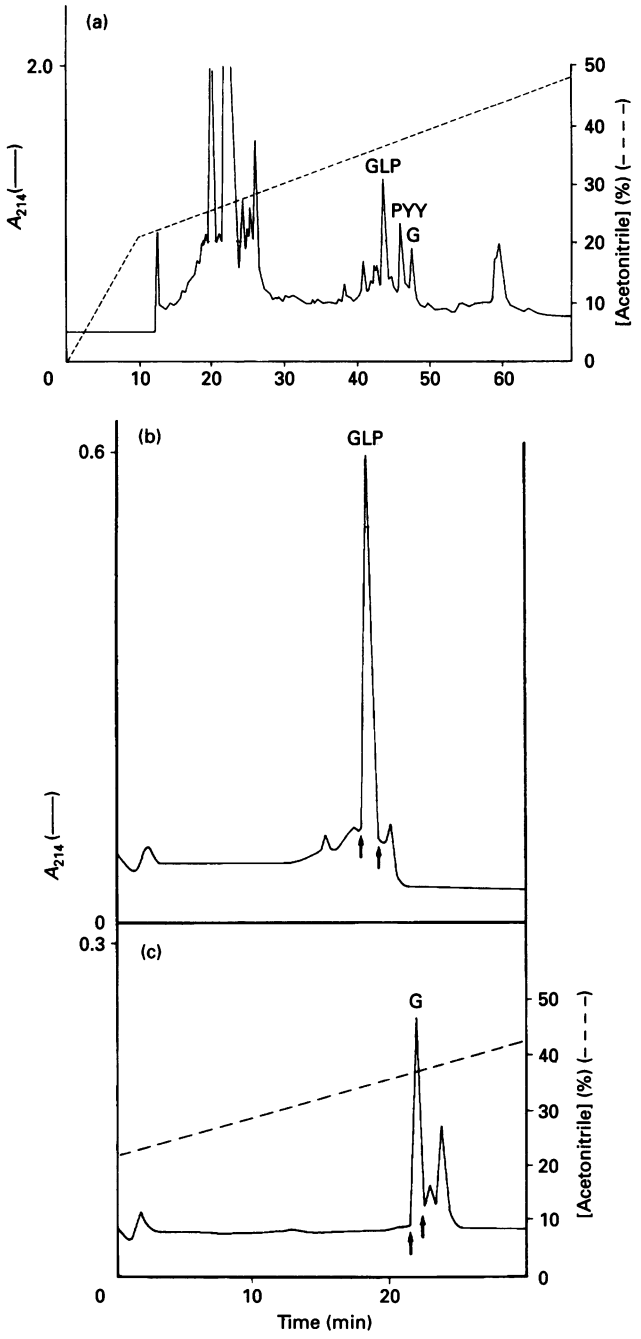

Figure 2 Purification of bowfin glucagon and GLP by reversed-phase h.p.l.c.

(**a**) Chromatography of the extract, after partial purification by gel permeation chromatography, on a Vydac 218TP column, (**b**) chromatography of bowfin glucagon-like peptide (GLP) on a Vydac 214TP column and (**c**) chromatography of bowfin glucagon (G) on a Vydac 214TP column. The broken line shows the concentration of acetonitrile in the eluting solvent and the arrows show where peak collection began and ended. The peak designated PYY contains peptide tyrosine-tyrosine.

chromatography on analytical Vydac C_4 (Figure 2c) and C_{18} (results not shown) columns. The final yield of pure bowfin glucagon was approx. 5 nmol. The prominent peak designated GLP in Figure 2(a) (subsequently shown to contain GLP) was also purified to apparent homogeneity by successive chromatographies on analytical Vydac C_4 (Figure 2b) and C_{18} columns. The final yield of pure bowfin GLP was approx. 16 nmol. The peak designated PYY was shown to contain peptide-tyrosyltyrosine, previously isolated from bowfin pancreas and structurally characterized (Conlon et al., 1991b).

Table 1 Determination of the primary structures of bowfin glucagon and GLP by automated Edman degradation

The detection limit for phenythiohydantoin derivatives was 0.5 pmol.

	Glucagon		GLP	
Cycle no.	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)
1	His	868	Tyr	1480
2	Ser	226	Ala	1794
3	Gln	1223	Asp	634
4	Gly	1445	Ala	1486
5	Thr	277	Pro	922
6	Phe	1474	Tyr	1149
7	Thr	274	Ile	922
8	Asn	865	Ser	156
9	Asp	706	Asp	587
10	Tyr	1177	Val	712
11	Ser	142	Tyr	734
12	Lys	966	Ser	113
13	Tyr	977	Tyr	674
14	Met	923	Leu	401
15	Asp	603	Gln	340
16	Thr	133	Asp	301
17	Arg	464	Gln	326
18	Arg	512	Val	303
19	Ala	928	Ala	331
20	Gln	588	Lys	210
21	Asp	461	Lys	235
22	Phe	550	Trp	31
23	Val	538	Leu	219
24	Gln	510	Lys	165
25	Trp	82	Ser	32
26	Leu	380	Gly	140
27	Met	358	Gln	100
28	Ser	28	Asp	93
29	Thr	43	Arg	78
30	–	–	Arg	95
31	–	–	Glu	17

Characterization of bowfin glucagon and GLP

It was possible to assign without ambiguity phenylthiohydantoin derivatives of amino acid for 29 cycles of operation during sequence analysis of bowfin glucagon and for 31 cycles during sequence analysis of bowfin GLP (Table 1). The data indicated that both peptides were > 95% pure. The results of amino analysis were consistent with the proposed structures and demonstrated that the full sequences of the peptides had been obtained (Table 2). The slightly low value for the methionine content of bowfin glucagon is probably a consequence of the partial oxidation of the residue to the sulphoxide derivative.

Glycogenolytic activity of bowfin GLP and glucagon

Under the conditions of incubation, the rate of glucose release from endogenous glycogen in the rockfish hepatocytes treated with vehicle only was linear over 30 min. This rate (6.4 ± 1.1 μmol of glucose produced/h per g of cells) was used as the baseline value. In the presence of hormone, the rate of production of

Table 2 Amino acid compositions of bowfin glucagon and GLP

Values in parentheses are those predicted from the proposed amino acid sequences determined by Edman degradation.

	Relative amount (residues/molecule)	
Amino acid	Glucagon	GLP
Asx	3.9 (4)	4.0 (4)
Glx	3.1 (3)	4.2 (4)
Ser	3.4 (3)	3.0 (3)
Gly	1.3 (1)	1.2 (1)
His	1.0 (1)	–
Arg	2.2 (2)	2.3 (2)
Thr	3.7 (4)	–
Ala	1.3 (1)	3.0 (3)
Pro	–	1.1 (1)
Tyr	2.0 (2)	3.8 (4)
Val	1.0 (1)	2.0 (2)
Met	1.5 (2)	–
Ile	–	1.0 (1)
Leu	1.3 (1)	2.0 (2)
Phe	1.7 (2)	–
Lys	1.3 (1)	2.9 (3)

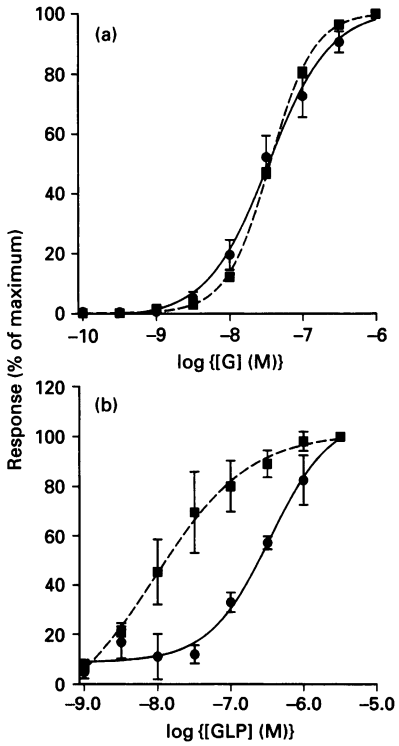

Figure 3 Comparison of the abilities of glucagon and GLP from the bowfin and human to stimulate glycogenolysis in rockfish hepatocytes

Dispersed hepatocytes from fed copper rockfish (*Sebastes caurinus*) were incubated with (**a**) bowfin glucagon (●–●) and human glucagon (■–■) and (**b**) bowfin GLP (●–●) and human GLP-1-(7–37)-peptide (■–■) under conditions described in the text. Data are presented as a percentage of the maximum rate of release of glucose into the medium. Each point represents the mean ± SEM of three independent experiments. G denotes glucagon; GLP denotes glucagon-like peptide.

glucose showed a sigmoidal pattern of increase up to 30 min and declined thereafter. Previous studies have shown that this decrease probably arises from down-regulation of hormone receptors rather than exhaustion of endogenous glycogen (Mommsen and Plisetskaya, 1993).

Maximum stimulation of glucose release from rockfish hepatocytes (6.3 ± 0.7-fold greater than basal release) was produced by 10^{-6} M concentrations of both bowfin and human glucagons. The concentration-dependence of the hormone-induced release is compared in Figure 3(a). The concentration of bowfin glucagon and human glucagon producing half-maximum stimulation of glucose release was the same (2.8×10^{-8} M).

The maximum amount of glucose released from the cells in 30 min was produced by 3.2×10^{-6} M concentrations of human GLP-1-(7–37)-peptide (7.1 ± 1.6-fold greater than basal release). This value was not significantly ($P < 0.05$) different from the maximum release produced by glucagon. The maximum amount of glucose released in 30 min was also produced by 3.2×10^{-6} M bowfin GLP, but this amount (2.1 ± 0.8-fold greater than basal release) was only 30% of the maximum produced by human GLP-1-(7–37)-peptide. These responses represented 99 ± 3% and 94 ± 8% respectively of the theoretical maximum response calculated from the curve-fitting program. There was insufficient peptide to compare the kinetics of the glycogenolytic responses produced by bowfin and human GLP, and so the effectiveness of the bowfin peptide may be underestimated if it acts more slowly than human GLP. As shown in Figure 3(b), the potency of human GLP-1-(7–37)-peptide was 1.3×10^{-8} M compared with a potency of 3.0×10^{-7} M for bowfin GLP.

DISCUSSION

The primary structures of bowfin glucagon and GLP are compared with the structures of corresponding peptides from other classes of vertebrates in Figure 4. Although glucagon and GLP are products of post-translational processing of the same biosynthetic precursor (preproglucagon), evolutionary pressure to conserve the amino acid sequence of the glucagon domain has been much stronger than that acting on the GLP domain. Bowfin glucagon contains only one substitution (Met^{14}→Leu) compared with glucagon from the alligator gar, *Lepisosteus spatula* (Pollock et al., 1988), another primitive bony fish that belongs to a different division and is perhaps more ancient than the bowfin (Lauder and Liem, 1983). In contrast, bowfin GLP contains a deletion and seven substitutions compared with GLP from the

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                          Glucagon
Bowfin          HSQGT FTNDY SKYMD TRRAQ DFVQW LMST
Gar             ----- ----- ---L- ----- ----- ----
Salmon          --E-- -S--- ---QE E-M-- ----- --NS
Human           ----- --S-- ---L- S---- ----- --N-

                      Glucagon-like peptide
Bowfin            YADAP YISDV YSYLQ DQVAK K***W LKSGQ DRRE
Gar               H--GT -T--- S---- --A-- -FVT- --Q-- ----
Salmon            H--GT -T--- STV-- --A-- DFVS- ----R A
Human GLP-1       H-EGT FT--- S---E G-A-- EFIA- -VK-R G
Human GLP-2       H--GS FSDEM DTI-D NLA-R DFIN- -IQTK ITDR
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Figure 4 Comparison of the primary structures of glucagon and glucagon-like peptide from the bowfin, gar, salmon and human

–denotes residue identity, * denotes residue deletion.

gar. Of particular note is the presence of a tyrosine residue at position 1 in bowfin GLP instead of the histidine residue that is found in all other glucagon-related peptides so far characterized. However, an N-terminal tyrosine residue is present in gastric inhibitory polypeptide and in growth-hormone-releasing factor, and it has been suggested that these peptides are homologous with glucagon (Bell, 1986).

Bowfin glucagon is sufficiently similar to human glucagon that both peptides are equally potent and equally effective in activating glycogenolysis in dispersed hepatocytes from a teleost fish. In view of the considerable difference in structures between bowfin and human GLP (a three-amino-acid deletion and 15 substitutions), it was surprising that bowfin GLP was able to elicit approximately one-third of the maximum glycogenolytic response of human GLP and was only 23-fold less potent. Previous studies have shown that, in this system, the potency and effectiveness of human GLP-1(7–37)-peptide is not significantly different from that of salmon GLP (Mommsen and Plisetskaya, 1993). The concentration of GLP in the bowfin circulation is not known, and so the physiological significance of this reduction in potency remains to be assessed. The mechanism by which GLP stimulates glycogenolysis (and gluconeogenesis) in fish liver is unknown, as are the structural features in the peptide that are responsible for biological activity. Only eleven residues (Ala^2, Ser^8, Asp^9, Val^{10}, Leu^{14}, Gln^{17}, Ala^{19}, Lys^{20}, Trp^{25}, Leu^{26}, Gly^{29}) are common to human, salmon and bowfin GLP, suggesting that the structural requirements of the putative GLP receptor in fish liver are not stringent. In contrast, des-His^7-GLP-1-(7–37)-peptide and GLP-1-(1–37)-peptide are > 100-fold less potent in inhibiting the binding of radiolabelled GLP-(7–37)-peptide to its receptor on rat insulinoma-derived cells (Goke and Conlon, 1988; Mojsov, 1992). The fact that the Asp^9 residue has been conserved in bowfin GLP is consistent with the observation that synthetic salmon [Asn^9]GLP is metabolically inactive in fish (T. P. Mommsen, unpublished work).

The biological activity of guinea-pig insulin is appreciably less than that of other mammalian insulins, even when tested in the guinea pig (Zimmerman et al., 1974). Determination of the nucleotide sequence of a cDNA encoding guinea-pig preproglucagon has shown that the molecule has undergone selective mutations in the C-terminal region of the glucagon sequence that result in production of a hormone with reduced biological potency (Seino et al., 1986). In this species, the primary structures of GLP-1 and GLP-2 have been well conserved. A previous study demonstrated that uncommon amino acid substitutions in bowfin insulin resulted in a 14-fold reduction in potency compared with pig insulin in inhibiting the binding of radiolabelled human insulin to the human insulin receptor (Conlon et al., 1991c). Similarly, bowfin insulin had the lowest potency of any insulin tested in stimulating glucose utilization by the mouse hemidiaphragm (Falkmer and Wilson, 1967). It is tempting to speculate, therefore, that a similar adaptive response to the reduced biological activity of insulin has taken place in the bowfin, except that an accelerated rate of mutation in the GLP rather than the glucagon domain has occurred.

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