Freeze tolerance in the wood frog *Rana sylvatica* is associated with unusual structural features in insulin but not in glucagon

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**ABSTRACT**

The wood frog *Rana sylvatica* utilises glucose, derived from hepatic glycogen, as a cryoprotectant in order to survive freezing during winter hibernation, and glycogenolysis is initiated by hormonal and/or neural stimuli. The primary structure of insulin was determined from *R. sylvatica* and from two species of freeze-intolerant Ranid frogs *R. catesbeiana* (American bullfrog) and *R. ridibunda* (European green frog). All three insulins contain a dipeptide (Lys-Pro) extension to the N-terminus of the A-chain. The amino acid sequences of insulins from *R. catesbeiana* and *R. ridibunda* differ by only one residue (Asp for Glu at B21) but *R. sylvatica* insulin differs from *R. catesbeiana* insulin at A12 (Thr → Met), A23 (Asn → Ser), B5 (Tyr → His) and B13 (Glu → Asp). The residue at A23 (corresponding to A21 in human insulin) has been otherwise fully conserved during evolution and the residue at B13 has been strongly conserved in tetrapods. Insulin isolated from specimens of *R. sylvatica* that had been frozen for 24 h and from control animals that had not been frozen had the same structure, showing that freezing did not alter the pathway of post-translational processing of proinsulin. *R. sylvatica* glucagon was isolated in two molecular forms. Glucagon-29 was identical to *R. catesbeiana* glucagon-29 and contains only one amino acid substitution (Thr → Ser) compared with human glucagon. Glucagon-36 represents glucagon-29 extended from its C-terminus by Lys-Arg-Ser-Gly-Gly-Ile-Ser and is identical to *R. catesbeiana* glucagon-36. We speculate that selective changes in the structure of the insulin molecule may contribute to the anomalous regulation of glycogen phosphorylase in the wood frog.


**INTRODUCTION**

The wood frog *Rana sylvatica* is the only North American frog found north of the Arctic Circle and it has developed a survival strategy that enables it to withstand the freezing of extracellular body fluids during winter hibernation (Storey 1990). The animal accumulates glucose (plasma concentrations between 200 and 500 mM) as a cryoprotectant that is derived primarily from massive hepatic glycogen stores (up to 180 mg/g wet tissue weight) built up before hibernation. Glycogenolysis is triggered immediately when ice begins to form on the skin of the frog and glucose is distributed to all organs by the action of the cardiovascular system before heartbeat and blood flow finally stop (Storey 1987a, Layne et al. 1989). Freezing is associated with a rapid and sustained activation of hepatic glycogen phosphorylase, the enzyme responsible for glycogen mobilization (Storey & Storey 1985), an increase in the number and/or activity of glucose transporters in the animal’s vital organs (King et al. 1993), and a decrease in the intracellular concentration of glucose 2,6-bisphosphate, the major regulator of metabolic flux through the glycolytic pathway (Storey 1987b).

The extreme and sustained hyperglycemia in the freezing-exposed wood frog suggests that the hormonal regulation of the enzymes involved with
glucose metabolism in this species must be modified from the usual mechanisms operating in vertebrates that efficiently control the rates of glucose production and disposal. The precise hormonal and/or neural stimuli that trigger hepatic glycogenolysis are not known. Studies with dispersed hepatocytes have shown that neither low temperature exposure nor extracellular freezing directly stimulates the activation of phosphorylase (Storey & Mommsen 1994). *R. sylvatica* hepatocytes respond to porcine glucagon and epinephrine with an increase in glucose output (Mommsen & Storey 1992) but attempts to measure changes in circulating levels of these hormones during freezing have not been successful (Storey *et al.* 1992). The aim of the present study was to purify insulin and glucagon from an extract of the pancreas of the wood frog in order to determine whether anomalous structural features in the endogenous hormones may contribute to the anomalous regulation of glucose homeostasis in this species. The primary structures of these hormones are compared with the corresponding amino acid sequences of insulin and glucagon isolated from two species of freeze-intolerant Ranid frogs, the European green frog *R. ridibunda* and the American bullfrog *R. catesbeiana*.

**MATERIALS AND METHODS**

**Tissue extraction**

One-year-old male specimens of *Rana sylvatica* (5-7 g) were collected from the Ottawa region in April 1997. Pancreatic tissue (0·27 g wet weight) was removed from animals (*n*=200) that had been acclimated at 5 °C in a moist environment without food. Animals were killed by a single pithing and the tissues were immediately frozen in liquid nitrogen. Animals were killed while still frozen. Adult male specimens (*n*=200) of *Rana ridibunda* (40–50 g) and adult specimens (*n*=40) of *Rana catesbeiana* (400–500 g) of both sexes were obtained from a commercial source. The pancreas (7·3 g from *R. ridibunda* and 13·0 g from *R. catesbeiana*) was removed from pithed animals and the tissues were immediately frozen on dry ice. Tissues were homogenized with ethanol/0·7 M HCl (3/1, v/v; 20 ml/g) using a Waring blender. After centrifugation (1600 g for 30 min at 4 °C), ethanol was removed from the supernatants under reduced pressure. Peptide material was isolated from the extracts using Sep-Pak C-18 cartridges (Waters Associates, Milford, MA, USA) as previously described (Conlon *et al.* 1996). Bound material was eluted with acetonitrile/water/trifluoroacetic acid (70:29:9·0:1) and lyophilized.

**Radioimmunoassay**

Insulin-like immunoreactivity was measured using an antiserum raised against pig insulin as previously described (Flatt & Bailey 1981). Glucagon-like immunoreactivity was measured with an antiserum directed against a site in the central region of porcine glucagon as previously described (Conlon & Thim 1985).

**Purification of *R. sylvatica* and *R. ridibunda* insulins**

The same procedure was used for the purification of insulin from both species and so only the method used for the isolation of the peptide from the extract of pancreas from specimens of *R. sylvatica* that had not been frozen is described in detail. The extract, after partial purification on Sep-Pak cartridges, was redissolved in 0·1% (v/v) trifluoroacetic acid/water (5 ml) and injected onto a 1 × 25 cm Vydac 218TP510 C-18 reversed-phase HPLC column (Separations Group, Hesperia, CA, USA) equilibrated with 0·1% trifluoroacetic acid/water at a flow rate of 2 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 21% over 10 min and to 49% over 60 min using linear gradients. Absorbance was measured at 214 and 280 nm and fractions (1 min) were collected. Insulin-like immunoreactivity was measured at a dilution of 1:30. *R. sylvatica* insulin was purified to near homogeneity, as assessed by a symmetrical peak shape, by successive chromatographies on a 0·46 × 25 cm Vydac 214TP54 C-4 column and a 0·46 × 25 cm Vydac 219TP54 phenyl column. The columns were eluted at a flow rate of 1·5 ml/min and the concentration of acetonitrile in the eluting solvent was raised from 21% to 42% over 40 min using a linear gradient.

**Purification of *R. catesbeiana* insulin**

The pancreatic extract, after partial purification on Sep-Pak cartridges, was redissolved in 1 M acetic acid (2 ml) and chromatographed on a 1·6 × 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 24 ml/h and fractions (2·0 ml) were collected. Absorbance was measured at 280 nm. The concentration of insulin-like immunoreactivity in the fractions was determined at a dilution of 1:30. Fractions containing insulin were pooled and injected onto a 1 × 25 cm Vydac 218TP510 C-18 reversed-phase HPLC
Purification of insulin

The elution profiles on a semipreparative Vydac 214TP54 C-4 column and on a 0·46 × 25 cm Vydac 219TP54 phenyl column under the same conditions used for the purification of insulin.

Structural analysis

Purified insulin samples (approximately 1 nmol) isolated from frozen and control specimens of *R. sylvatica* and from *R. catesbeiana* and *R. ridibunda* were separately incubated for 6 h at room temperature with dithiothreitol (2 mg) in 0·1 M Tris–HCl–6 M guanidine hydrochloride buffer pH 7·5 (0·4 ml) under an atmosphere of argon. Cysteine residues were derivatized by addition of 4-vinylpyridine (3 μl) and the pyridylethylated A- and B-chains of insulin were separated on a 0·46 × 25 cm Vydac C-4 column under the conditions used for the purification of intact insulin (Fig. 2A). Amino acid compositions were determined by precolumn derivatization with phenylisothiocyanate using an Applied Biosystems model 420A derivatizer (Foster City, CA, USA), followed by separation of the phenylthiohydantoin amino acids by reversed phase HPLC (Bidlingmeyer et al. 1984). Hydrolysis in 5·7 M hydrochloric acid (24 h at 110 °C) of approximately 500 pmol peptide was carried out. The primary structures of the peptides were determined by automated Edman degradation using an Applied Biosystems model 471A sequenator (Foster City, CA, USA), followed by separation of the phenylthiohydantoin amino acids under gradient elution conditions. Standard operating procedures were used (Applied Biosystem model 471A Protein Sequencer User’s Manual) and the detection limit was 1 pmol.

RESULTS

Purification of insulin

The elution profiles on a semipreparative Vydac C-18 column of the extracts of the pancreata of *R. sylvatica* (non-frozen animals) and *R. ridibunda*, after partial purification on Sep-Pak cartridges, and of the extract of *R. catesbeiana* pancreas after partial purification by gel permeation chromatography, are shown in Fig. 1 (panels A-C). In each case, relatively prominent peaks were identified in the chromatograms that were associated with insulin-like immunoreactivity. The elution profile of the extract of the pancreata of viable specimens of *R. sylvatica* that had been frozen for 24 h was almost superimposable on that shown in Fig. 1A and the retention time of the insulin-containing peak was the same. Consequently, the peaks of *R. sylvatica* insulin from the frozen and control specimens were combined for further purification. *R. sylvatica* insulin was purified to near homogeneity, as assessed by a symmetrical peak shape, by successive chromatographies on an analytical Vydac C-4 column (Fig. 2A) and an analytical Vydac phenyl column (Fig. 2B). The final yield of pure peptide was 2·5 nmol. The final yield of pure insulin from *R. ridibunda* was 5 nmol and from *R. catesbeiana* was 4 nmol.

Purification of glucagons

The glucagon-like immunoreactivity in the extract of *R. sylvatica* pancreas was eluted from the semipreparative Vydac C-18 column as two peaks designated in Fig. 1A as G1 (subsequently shown to contain glucagon-36) and G2 (subsequently shown to contain glucagon-29). Glucagon-29 was purified to near homogeneity by successive chromatographies on an analytical Vydac C-4 column (Fig. 3A) and an analytical Vydac phenyl column (Fig. 3B). The final yield of pure peptide was 2·5 nmol. Glucagon-36 was purified under the same conditions (chromatograms not shown) and the final yield of pure peptide was 4 nmol.

Structural characterization

The primary structures of the pyridylethylated A-chains and B-chains of insulins from *R. sylvatica*, *R. ridibunda* and *R. catesbeiana* were determined by automated Edman degradation and the results are shown in Fig. 4. In each case, it was possible to identify without ambiguity phenylthiohydantoin-coupled amino acid for 21 cycles of operation of the sequenator during sequence analysis of the A-chain and for 30 cycles during analysis of the B-chain. The results of amino acid analysis were consistent with the proposed structures and demonstrated that full sequences of the peptides had been obtained. The primary structures of *R. sylvatica* glucagon-29 and glucagon-36 were determined without ambiguity by automated Edman degradation and the results are shown in Fig. 5. The amino acid compositions were determined by precolumn derivatization with phenylthiocarbamyl amino acids by reversed phase HPLC (Bidlingmeyer et al. 1984). Hydrolysis was performed as described above. The primary structures of the pyridylethylated A-chains and B-chains of glucagons from *R. catesbeiana* and others was shown in Fig. 4. In each case, it was possible to identify without ambiguity phenylthiohydantoin-coupled amino acid for 21 cycles of operation of the sequenator during sequence analysis of the A-chain and for 30 cycles during analysis of the B-chain.
compositions of the peptides were consistent with the proposed structures. The data indicated that glucagon-36 represents glucagon-29 extended from its C-terminus by the sequence -Lys-Arg-Ser-Gly-Gly-Ile-Ser.

DISCUSSION

The primary structures of insulins isolated from the three species of Ranid frog are compared with human insulin and with the structures of the other known amphibian insulins (the clawed toad *Xenopus laevis* (Shulinder et al. 1989), the urodeles the three-toed amphiuma *Amphiuma tridactylum* (Conlon et al. 1996) and the lesser siren *Siren intermedia* (Conlon et al. 1997), and the caecilian *Typhlonectes natans* (Conlon et al. 1995) (Fig. 4)). In view of the small amount of pancreatic tissue
available (<0.6 g), it was not possible to purify sufficient insulin from the wood frog to study its biological activity but we speculate that structural features in the molecule may influence conformation and biological potency relative to insulins from the freeze-intolerant Ranids.

Traditionally, the receptor-binding region of human insulin is considered to involve contributions from amino acid residues at positions A1-A3, A5, A19, A21 and B22-B24 (Baker et al. 1988). These residues have been fully conserved in the insulins from the freeze-intolerant Ranids but *R. sylvatica* insulin contains the substitution (Asn→Ser) at the position corresponding to A21. In the crystal structure of pig insulin, the carboxylate group of A21 forms an ionic bond with B22 arginine and a hydrogen bond bridges the α-nitrogen of A21 asparagine with the backbone carbonyl group of B23 glycine (Baker et al. 1988). It has been shown that these bonds are essential to the formation and maintenance of the biologically active conformation (Markussen et al. 1988). An asparagine residue is present at position A21 in all other vertebrate insulins yet characterized. More recently, however, an alanine scanning mutagenesis study has shown that the substitution (Asn→Ala) at A21 in human insulin had only a small effect on binding to the human insulin receptor suggesting that the importance of this residue may have been exaggerated (Kristensen et al. 1997). The same study showed that alanine substitutions at residues A19, B8, B11 and B13 are disruptive for binding to the insulin receptor.
receptor and so the substitution Glu→Asp) at B13 of \textit{R. sylvatica} insulin may affect biological activity. This residue has been strongly conserved in amphibia (Fig. 4) and in mammals, with the exception of the low-potency insulins from hystricormorphs which also contain the Glu→Asp mutation (Bajaj \textit{et al.} 1986).

The residues in human insulin involved in dimer formation (B12, B16, B20, B24, B26 and B28) and hexamer formation (B6, B10, B14, B17, B18, A13, A14) (Baker \textit{et al.} 1988) have been well conserved in all three Ranid insulins with the exception of the substitution (Leu→Met) at B17. The Ranid insulins share with insulins from \textit{Xenopus} (Shulinder \textit{et al.} 1989) and the amphiuma (Conlon \textit{et al.} 1996) the presence of a histidine residue at position A8. This residue, by forming stabilizing structural motifs in the insulin molecule that are of critical importance for receptor recognition, is responsible for the observed increase in binding affinity of these insulins for mammalian insulin receptors. The effect, if any, of the substitution (Thr→Met) at position A12 on the stabilizing motif is not known.

Each Ranid insulin contains a dipeptide extension (Lys-Pro) to N-terminus of the A-chains indicative of an anomalous pathway of post-translational processing of proinsulin. A similar dipeptide extension (Ala-Arg) to the N-terminus of the A-chain was identified in the insulin of the urodele \textit{Amphiuma tridactylum} (Conlon \textit{et al.} 1996) and it was speculated that a mutation (Lys→Ala) had occurred in the dibasic residue (Lys-Arg) linking the C-peptide region of proinsulin to the A-chain of insulin so that the PC-2 prohormone convertase is obliged to cleave at an alternative site. It is probable, therefore, that a mutation (Arg→Pro) has occurred at the same site in the proinsulins from the Ranid frogs. Amphiuma insulin was fivefold more potent than human insulin in inhibiting the binding of radiolabeled human insulin to the human insulin receptor (Conlon \textit{et al.} 1996) suggesting that the presence of a dipeptide extension does not result in a diminution of biological activity. Insulin isolated from viable specimens of \textit{R. sylvatica} that had been frozen for 24 h was identical to insulin from control animals, demonstrating that freezing does not affect the pathway of proinsulin processing. In contrast to the anomalous structure of \textit{R. sylvatica} insulin, the amino acid sequences of both glucagon-29 and glucagon-36 are identical to those of the corresponding peptides from \textit{R. catesbeiana} (Pollock \textit{et al.} 1986). \textit{R. sylvatica} glucagon differs from human glucagon by a single conservative substitution (Thr→Ser) at position 29 (Fig. 5). In general, it appears that glucagon and insulin play similar roles in the regulation of glycogenolysis in amphibia and in mammals. For example, glucagon induces an increase in the concentration of cAMP, conversion of glycogen phosphorylase to the active phosphorylated form (a form) and increases the rate of glycogenolysis in hepatic tissue from the axolotl \textit{Ambystoma mexicanum} and these effects are counteracted by insulin (Janssens & Maher 1986). The rise in intracellular glucose concentrations results in allosteric inhibition of phosphorylase a, and insulin by activating protein phosphatase-1 is responsible for the conversion of phosphorylase to the inactive b form. The mechanism of phosphorylase regulation in the liver of \textit{R. sylvatica} is anomalous (Storey 1990). Exposure to freezing results in an immediate rise in the proportion of phosphorylase in the a form which is followed by a slower rise over several hours in the total activity of the enzyme expressed. This results in a 7- to 13-fold increase in active phosphorylase a that supports the massive glucose output of the early hours of freezing (Storey & Storey 1988). Our present data, demonstrating changes in the amino acid sequence of \textit{R. sylvatica} insulin that are not found in insulins from freeze-intolerant Ranid frogs, lead us to speculate that this insulin has an impaired ability to activate protein phosphatase-1 and thereby convert phosphorylase a to the inactive form in the liver. In contrast, the primary structure of \textit{R. sylvatica} glucagon is similar to that of other amphibian and mammalian glucagons so that the ability of the hormone to stimulate glycogenolysis is not impaired.

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