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RESEARCH ARTICLE

Glycogen synthase kinase-3: cryoprotection and glycogen metabolism in the freeze-tolerant wood frog

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SUMMARY

The terrestrial anuran *Rana sylvatica* tolerates extended periods of whole-body freezing during the winter. Freezing survival is facilitated by extensive glycogen hydrolysis and distribution of high concentrations of the cryoprotectant glucose into blood and all tissues. As glycogenesis is both an energy-expensive process and counter-productive to maintaining sustained high cryoprotectant levels, we proposed that glycogen synthase kinase-3 (GSK-3) would be activated when wood frogs froze and would phosphorylate its downstream substrates to inactivate glycogen synthesis. Western blot analysis determined that the amount of phosphorylated (inactive) GSK-3 decreased in all five tissues tested in 24 h frozen frogs compared with unfrozen controls. Total GSK-3 protein levels did not change, with the exception of heart GSK-3, indicating that post-translational modification was the primary regulatory mechanism for this kinase. Kinetic properties of skeletal muscle GSK-3 from control and frozen frogs displayed differential responses to a temperature change (22 *versus* 4°C) and high glucose. For example, when assayed at 4°C, the K_m for the GSK-3 substrate peptide was ~44% lower for frozen frogs than the corresponding value in control frogs, indicating greater GSK-3 affinity for its substrates in the frozen state. This indicates that at temperatures similar to the environment encountered by frogs, GSK-3 in frozen frogs will phosphorylate its downstream targets more readily than in unfrozen controls. GSK-3 from skeletal muscle of control frogs was also allosterically regulated. AMP and phosphoenolpyruvate activated GSK-3 whereas inhibitors included glucose, glucose 6-phosphate, pyruvate, ATP, glutamate, glutamine, glycerol, NH_4Cl, NaCl and KCl. The combination of phosphorylation and allosteric control argues for a regulatory role of GSK-3 in inactivating glycogenesis to preserve high glucose cryoprotectant levels throughout each freezing bout.

Key words: glycogen synthase kinase-3 (GSK-3), freeze tolerance, *Rana sylvatica*, protein phosphorylation, glucose, cryoprotectant.

INTRODUCTION

Glycogen and glucose metabolism are of central importance to physiology. The monosaccharide glucose is a fundamental fuel for all cells. The polymeric form of glucose, glycogen, serves as the storage form of carbohydrate in all animals. The synthesis of glycogen from glucose and its eventual breakdown back to glucose monomers oscillates within fed/fasted and other physiological states (Shulman and Landau, 1992). The two pathways are reciprocally regulated by different signals appropriate to the proper metabolic response. Glycogenolysis (glycogen breakdown), catalysed by glycogen phosphorylase (GP), is stimulated by hormones such as adrenaline and glucagon that trigger production of cyclic adenosine monophosphate (cAMP) and activate the cAMP-dependent protein kinase (PKA). PKA phosphorylates glycogen phosphorylase kinase, which, in turn, phosphorylates GP and converts it from the inactive *b* form to the active *a* form (Johnson, 1992). Concurrently, PKA also phosphorylates and inactivates glycogen synthase (GS), shutting off glycogen synthesis (glycogenesis) (Roach et al., 1991). Thus, glycogenolysis and glycogenesis are prevented from directly competing with one another.

Glycogenesis, in turn, is mediated by insulin signalling. Phosphodiesterase is activated, hydrolysing cAMP to inactivate PKA. Protein phosphatase 1 (PP1) is activated and dephosphorylates both GP and GS, inactivating the former and activating the latter (Dent et al., 1990; Roach, 1990). Insulin signalling also activates GS *via* another mechanism: the phosphorylation and inactivation of glycogen synthase kinase-3 (GSK-3), a protein kinase that, when active, phosphorylates and inactivates GS (Sutherland et al., 1993; Summers et al., 1999). When GSK-3 is phosphorylated and inactivated, GS can be dephosphorylated and activated, allowing glycogenesis to occur. In addition to its role in glycogen metabolism, GSK-3 has been shown to be a master kinase and is recognized to have roles in: (i) the Wnt signalling pathway, known to regulate several physiological processes as well as development, embryogenesis and cancer (Ding and Dale, 2002); (ii) protein synthesis (Welsh and Proud, 1993); (iii) the response to DNA damage (Watcharasit et al., 2002); (iv) the immune response (Beals et al., 1997); and (v) regulation of Tau protein in neurodegenerative disease (Medina et al., 2011), among others.

The importance of glycogen metabolism in normal physiology is self-evident. However, conditions occur where atypical glycogen and glucose mobilization are necessary, requiring altered regulation of the above enzymes. The freeze-tolerant physiology of the wood frog, *Rana sylvatica* LeConte 1825, is one of these situations. During the winter, wood frogs can survive the freezing of 65–70% of total body water in extracellular and extra-organ spaces and, as one

consequence, heart beat and breathing are halted, leading rapidly to anoxia and ischaemia (Storey and Storey, 1984; Storey and Storey, 1988; Storey and Storey, 2004). One of the prominent adaptations that underlie wood frog freeze tolerance is the accumulation and distribution of high amounts of glucose as a cryoprotectant, derived from extensive glycogenolysis, mainly in liver (Storey and Storey, 1985). Plasma and tissue glucose can rise as high as 200–300 mmol l^{-1}, compared with normal levels of ~5 mmol l^{-1}. This uncharacteristically high breakdown of glycogen and production of glucose requires selective regulation of the enzymes controlling glycogen metabolism (Crerar et al., 1988; Mommsen and Storey, 1992; Russell and Storey, 1995). In particular an 'override' control on normal glucose homeostasis would need to be present to prevent the activation of glycogenesis that is normally triggered when glucose rises above about 10 mmol l^{-1}.

Post-translational modification of enzymes is a widespread mode of biochemical regulation in wood frog freeze tolerance, and recent studies have uncovered roles for reversible protein phosphorylation in controlling: (i) glycolysis (Dieni and Storey, 2011); (ii) antioxidant defence and reductive biosynthesis (Dieni and Storey, 2010); (iii) fuel storage and mobilization (Rider et al., 2006; Dieni and Storey, 2009); (iv) adenylate metabolism (Dieni and Storey, 2008); and (v) protein degradation (Woods and Storey, 2006). In the current study we investigated GSK-3 and its regulation by post-translational modification in response to freezing. Based on what is known about GSK-3 and metabolic controls in the frozen state, we propose that GSK-3 is activated during freezing and serves to shut off glycogenesis in order to facilitate both the synthesis and maintenance of high cryoprotectant glucose in wood frog organs throughout freezing episodes.

MATERIALS AND METHODS

Animals and chemicals

This research was approved by the Carleton University Animal Care Committee. Male wood frogs were captured from spring breeding ponds in the Ottawa area. Animals were washed in a tetracycline bath, and placed in plastic containers with damp sphagnum moss at 5°C for 2 weeks prior to experimentation. Control frogs were sampled from this condition. For freezing exposure, frogs were placed in closed plastic containers with damp paper towelling on the bottom, and put in an incubator set at –3°C. A 45 min cooling period was allowed during which body temperature of the frogs cooled to below –0.5°C and nucleation was triggered as a result of skin contact with ice crystals formed on the paper towelling (Storey and Storey, 1985; Layne et al., 1990). Subsequently, timing of a 24 h freeze exposure began. All frogs were killed by pithing, followed by rapid dissection and flash-freezing of tissue samples in liquid nitrogen. Tissues were then stored at –80°C until use.

Protein assay dye reagent, Kaleidoscope pre-stained protein markers and acrylamide were purchased from Bio-Rad (Hercules, CA, USA). Antibodies were purchased from Cell Signalling Technology (Danvers, MA, USA) or Santa Cruz Biotechnology (Santa Cruz, CA, USA) as indicated. The enhanced chemiluminescence (ECL) assay and GSK-3 substrate peptide were from Millipore (Bedford, MA, USA) and γ-^{32}P-adenosine triphosphate was from GE Healthcare (Piscataway, NJ, USA). All other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA).

Tissue extract preparation for SDS-PAGE and immunoblotting

Soluble protein extracts were prepared from tissues that had previously been stored at –80°C. Briefly, samples of frozen tissue were weighed and then quickly homogenized using a Polytron PT1000 homogenizer (Brinkmann Instruments, Rexdale, ON, Canada) at 50% of full power in a 1:5 w:v ratio with ice-cold buffer A containing 20 mmol l^{-1} Hepes, 200 mmol l^{-1} NaCl, 0.1 mmol l^{-1} EDTA, 10 mmol l^{-1} NaF, 1 mmol l^{-1} Na_3VO_4 and 10 mmol l^{-1} β-glycerophosphate. Protease and phosphatase inhibitors were added just prior to homogenization: 1:1000 v:v Sigma Protease Inhibitor Cocktail (P8340), 1:1000 v:v Sigma Phosphatase Inhibitor Cocktail 1 (P2850) and a few crystals of phenylmethylsulphonyl fluoride (PMSF). Samples were centrifuged at 10,000 ***g*** for 15 min at 4°C and then supernatants were removed and held on ice.

Soluble protein concentration was quantified by the Bradford assay (Bradford, 1976) using the Bio-Rad prepared reagent and a Dynatech MR5000 microplate reader (DYNEX Technologies Inc., Chantilly, VA, USA) set at 595 nm. Samples were then adjusted to equal soluble protein concentrations by the addition of small volumes of buffer A; this compensates for differences in the wet:dry ratio of tissues from control *versus* frozen frogs. Aliquots were mixed 1:1 v:v with SDS-PAGE sample buffer containing: 100 mmol l^{-1} Tris-HCl (pH 6.8), 4% w:v sodium dodecyl sulphate (SDS), 20% v:v glycerol, 5% v:v 2-mercaptoethanol and 0.2% w:v Bromophenol Blue. Following boiling for 5 min, samples were immediately cold-snapped on ice, and stored at –20°C until use.

SDS-PAGE and polyvinylidene difluoride membrane transfer

Aliquots of thawed samples containing 20 μg of protein were loaded into wells of SDS-polyacrylamide gels (8% resolving gel, 5% stacking gel). Samples were electrophoresed at 180 V in a Mini-PROTEAN III apparatus (Bio-Rad) using 1× running buffer (5× running buffer contained 15.1 g Tris-base, 94 g glycine and 5 g SDS per litre, pH 8.3). Proteins were then wet-transferred to polyvinylidene difluoride (PVDF) membrane (Millipore) using a current of 300 mA for 1.5 h at 4°C in a Bio-Rad Mini Trans-Blot Cell apparatus (Bio-Rad). Transfer buffer contained 25 mmol l^{-1} Tris-base pH 8.8, 192 mmol l^{-1} glycine and 20% v:v methanol, chilled to 4°C.

Immunoblotting of PVDF membranes

Primary antibodies, from Santa Cruz Biotechnology, were mouse anti-GSK3α/β (0011-A) raised against full-length GSK-3β of *Xenopus* origin (sc-7291), and goat anti-p-GSK3β (Ser9) with an epitope corresponding to phosphorylated Ser9 of GSK-3β of human origin (sc-11757). Stock primary antibodies were diluted 1:1000 in Tris-buffered saline supplemented with Tween-20 (TBST; 20 mmol l^{-1} Tris pH 7.5, 150 mmol l^{-1} NaCl, 0.05% v:v Tween-20). Secondary antibodies were the following: horseradish peroxidase (HRP)-conjugated horse anti-mouse (Cell Signalling Technology; catalogue no. 7076) or horseradish peroxidase (HRP)-conjugated mouse anti-goat (Santa Cruz Biotechnology; sc-2354). Stock secondary antibodies were diluted 1:2000 in TBST.

After transfer was complete, PVDF membranes were quickly equilibrated in TBST and then blocked with 5% w:v non-fat milk dissolved in TBST for 15 min at room temperature. The blot was rinsed with TBST and then incubated with primary antibody in TBST on a shaking platform overnight at 4°C. Blots were washed twice with TBST and incubated with secondary antibody for 1.5 h at room temperature. Immunoreactive bands were visualized using ECL following the manufacturer's protocol. The luminol and oxidizing reagents were mixed 1:1 v:v on the membrane for 1 min and the ECL signal was detected using a ChemiGenius (SynGene, Frederick, MD, USA). Band intensities were quantified using the associated Gene Tools program (v. 3.00.02). Kaleidoscope pre-

stained markers were run in selected lanes and used to estimate GSK-3 molecular mass.

Total protein was then visualized on the PVDF membrane by staining for 30 min with Coomassie Blue staining solution (0.25% w:v Coomassie Brilliant Blue R, 50% v:v methanol, 7.5% v:v acetic acid) followed by destaining with destain solution (25% v:v methanol, 10% v:v acetic acid). Three Coomassie-stained bands that did not differ in intensity between active and frozen conditions were used to normalize the corresponding intensity of the immunoreactive band in each lane to correct for any unequal protein loading, as described previously (Ramnanan and Storey, 2006).

Sample preparation for protein kinase assays

Samples of frozen skeletal muscle were homogenized 1:5 w:v using a Polytron PT1000 homogenizer in ice-cold buffer B containing 50 mmol l^{-1} Tris-HCl, pH 7.4, 50 mmol l^{-1} NaF, 4 mmol l^{-1} EDTA, 1 mmol^{-1} Na_3VO_4 and 0.1% v:v 2-mercaptoethanol. A few crystals of PMSF were added just prior to homogenization. Homogenates were centrifuged at 10,000 ***g*** for 25 min. Supernatants were then centrifuged through small columns of Sephadex G-50 to remove potential endogenous enzyme effectors. To do this, 5 ml syringe barrels were plugged with glass wool and packed with Sephadex G-50 equilibrated in buffer B. Packed syringes were centrifuged in a bench-top clinical centrifuge (Damon/IEC Division, Needham, MA, USA) at full power for 1 min and eluant was discarded. A 500 µl aliquot of enzyme supernatant was then layered onto the column followed by a second centrifugation as above. The eluant was collected and stored on ice. Soluble protein concentration was quantified by the Bradford assay as above. Samples were then adjusted to equal protein concentration (typically 5 mg ml^{-1} for protein kinase assays) using buffer B; because freezing causes cellular dehydration, this compensated for the use of wet mass in preparing homogenates because the wet:dry ratio of tissues is different in control *versus* frozen frogs.

GSK-3 enzyme assays

GSK-3 activity was determined using a radioactive assay that measured the incorporation of radiolabelled γ-^{32}P from γ-^{32}P-adenosine triphosphate (ATP; 3000 Ci mmol^{-1}, GE Healthcare) onto phospho-glycogen synthase peptide-2 (Millipore; 12-241). The peptide has a sequence similar to that around the phosphorylation site in human skeletal muscle GS: YRRAAVPPSPSLSRHSSPHQ(pS)EDEEE and contains one phosphoserine, corresponding to phosphorylated site 4, because GSK-3 preferentially acts on substrates that have already been phosphorylated by another kinase. The two underlined serines correspond to sites 3b and 3c, respectively, which can both be phosphorylated by GSK-3 (Rylatt et al., 1980). GSK-3 was assayed in a final reaction volume of 25 µl with 25 µg of total soluble protein from muscle extracts used per assay. Optimum assay conditions were 50 mmol l^{-1} Tris-HCl, pH 7.5, 2 mmol l^{-1} $MgCl_2$, 200 µmol l^{-1} ATP, 0.5 µCi γ-^{32}P-ATP, 1 mmol l^{-1} EGTA, 150 mmol l^{-1} NaCl and 0.1% v:v 2-mercaptoethanol. The assay also contained 0.5 mmol l^{-1} of specific peptide inhibitors of protein kinase A (PKA inhibitor fragment 5–24 amide trifluoroacetate salt; P7739) and protein kinase C (PKC fragment 19-31 amide; P2235). The optimal concentration of GSK-3 substrate peptide was 75 µmol l^{-1} but concentrations ranging from 0 to 75 µmol l^{-1} were used for studies of kinetic parameters; zero substrate blanks were always run to account for non-specific background signals. Assays were initiated by the addition of ATP + γ-^{32}P-ATP and incubations were 30 min at 22 or 4°C.

To determine the effects of metabolites on GSK-3 activity, reactions were run in the presence of varying concentrations of metabolites and a subsaturating (20 µmol l^{-1}) or saturating (70 µmol l^{-1}) concentration of substrate peptide. All metabolites were prepared in 50 mmol l^{-1} Tris buffer pH 7.5. The metabolites tested were: glucose, glucose 6-phosphate (G6P), fructose 1,6-bisphosphate ($F1,6P_2$), phosphoenolpyruvate (PEP), pyruvate, glycerol, glycerol 3-phosphate (G3P), lactate, Mg citrate ($MgCl_2$ and citrate in a 2:1 ratio), succinate, adenosine monophosphate (AMP), adenosine diphosphate (ADP), MgATP ($MgCl_2$ and ATP in a 1:1 ratio), aspartate (Asp), alanine (Ala), glutamate (Glu), glutamine (Gln), NH_4Cl, NaCl and KCl. Concentration ranges tested for each of the metabolites were as follows: 0–25 mmol l^{-1} for glucose; 0–10 mmol l^{-1} G6P, F1,6BP, PEP, pyruvate, glycerol, G3P, lactate, Mg citrate, succinate, AMP, ADP, MgATP, Asp, Ala, Glu and Gln; 0–250 mmol l^{-1} glycerol; 0–1 mol l^{-1} NH_4Cl, NaCl and KCl.

GSK-3 assay signal detection

GSK-3 activity was quantified based on previous methods (modified from Asensio and Garcia, 2003). Reactions were stopped by addition of one-half reaction volume of 0.2 mol l^{-1} EDTA containing 0.1% w:v Bromophenol Blue. Aliquots of 2 µl of the reaction mix were then spotted onto P81 paper (which binds the ^{32}P-labelled peptide; Whatman/GE Healthcare; 3698) that was sectioned with a pencilled grid. The P81 paper array was allowed to air dry under a fume hood followed by washing to remove unbound ^{32}P-ATP: four quick washes in 0.8% (v:v) phosphoric acid, two long washes (10 min) with 0.8% phosphoric acid, and one quick wash with 95% ethanol.

After washing, the paper array was allowed to air dry and was then placed under Saran wrap and exposed to a storage phosphor screen in autoradiography cassettes. Prior to use, storage phosphor screens were erased for at least 10 min using a white light box. The exposure time for visualizing ^{32}P-labelled GSK-3 peptide was 1 h. After exposure, phosphor screen signals were captured using a Personal Molecular Imager scanning system (Bio-Rad). Quantification, background correction and data analysis were performed using Quantity One software, and relative signal density was transformed into units of activity using a standard curve of γ-^{32}P-ATP that was prepared using the same conditions as those for GSK-3 activity (same spot volume, exposure time, etc.). One unit is defined as the amount of enzyme that phosphorylates 1 µmol of peptide per minute. Kinetic parameters were determined using a non-linear least squares regression computer program (Kinetics 3.51) (Brooks, 1992).

RESULTS

GSK-3 and phospho-GSK-3 (Ser9) protein levels

GSK-3 protein was detected in all wood frog tissues tested: brain, heart, kidney, liver and skeletal muscle (Fig. 1). The mouse anti-GSK3α/β antibody used, which was raised against GSK-3β of *Xenopus* (anuran) origin, detected a single, dense immunoreactive band at ~50 kDa, the expected molecular mass. It should be noted that while mammals have two GSK-3 genes with products of slightly different sizes (51 and 47 kDa for GSK-3α and β, respectively), evidence to date indicates that only GSK-3β is found in anurans (see Discussion). Whole-body freezing did not affect total GSK-3 protein levels in four tissues, but GSK-3 protein content decreased significantly in heart of frozen frogs by 42.5% of control values (P<0.05).

The anti-p-GSK3β (Ser9) antibody also detected a single immunoreactive band at ~50 kDa in all five tissues (Fig. 2). The

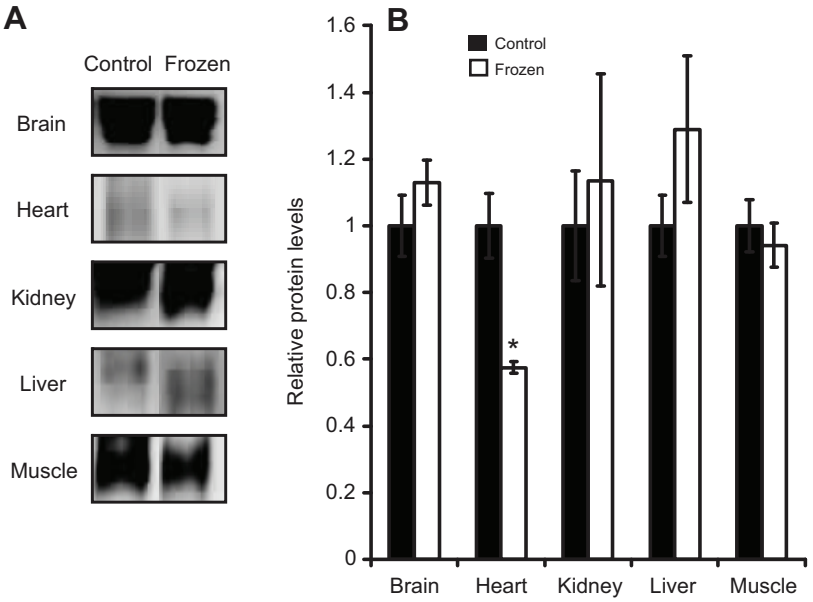

Fig. 1. Relative levels of total glycogen synthase kinase (GSK)-3α/β protein in control and frozen frogs as determined by SDS-PAGE and western blotting. (A) Representative images of enhanced chemiluminescence (ECL)-detected western blots. (B) Histogram showing relative protein levels in tissues from control and frozen frogs, as determined by quantifying band densities. Data are means ± s.e.m., N=4 independent preparations from control and frozen frogs. *Significantly different from the corresponding value in control frogs as assessed by Student's t-test, P<0.05.

relative amount of phospho-GSK-3 decreased significantly (P<0.05) in all organs during freezing. Mean values for phospho-GSK-3 content in the tissues of frozen frogs were just 50.7%, 27.3%, 50.0%, 43.1% and 15.0% of the corresponding control values for brain, heart, kidney, liver and skeletal muscle, respectively.

GSK-3 enzyme activity

GSK-3 activity and kinetic parameters were measured in skeletal muscle extracts from 5°C-acclimated control frogs *versus* frogs frozen for 24 h at –3°C (Table 1, Fig. 3). Enzyme maximal activity (V_{max}) at 22°C tended to increase in frozen frogs, but did not change significantly between control and frozen states, being 1.8–2.3 U mg^{-1}. The enzyme showed a hyperbolic (Hill coefficient n_H ~1), non-cooperative relationship between reaction rate and substrate concentration. GSK-3 affinity for its substrate peptide did not change significantly between control and frozen states at 22°C assay temperature, the K_m value being 23.5±4.2 μmol l^{-1} for skeletal muscle from control frogs and 33.1±4.4 μmol l^{-1} for frozen frogs.

Effects of temperature and glucose on GSK-3 enzyme activity

To determine whether glucose (the cryoprotectant accumulated in high intracellular levels during freezing) affected GSK-3 activity, assays at 22°C were repeated in the presence of 250 mmol l^{-1} glucose (Table 1, Fig. 3). This level of glucose was chosen as it represents a high concentration of cryoprotectant that can potentially be encountered within core organs and blood cells of frozen frogs, and has been in standard use for assessing the effect of high glucose on enzyme kinetic parameters in previous studies (Dieni and Storey, 2008; Dieni and Storey, 2010; Dieni and Storey, 2011). The addition of glucose significantly changed both V_{max} and K_m peptide values for skeletal muscle GSK-3 from both control and frozen frogs. For GSK-3 from control frogs, K_m peptide increased by ~73% and V_{max} increased by ~58% compared with the values obtained in the absence of glucose (both significant changes, P<0.05). Conversely, GSK-3 from muscle of frozen frogs showed a significant decrease in K_m peptide by ~34%, and V_{max} fell by 50% compared with assays without glucose. As a result, the comparison of kinetic parameters

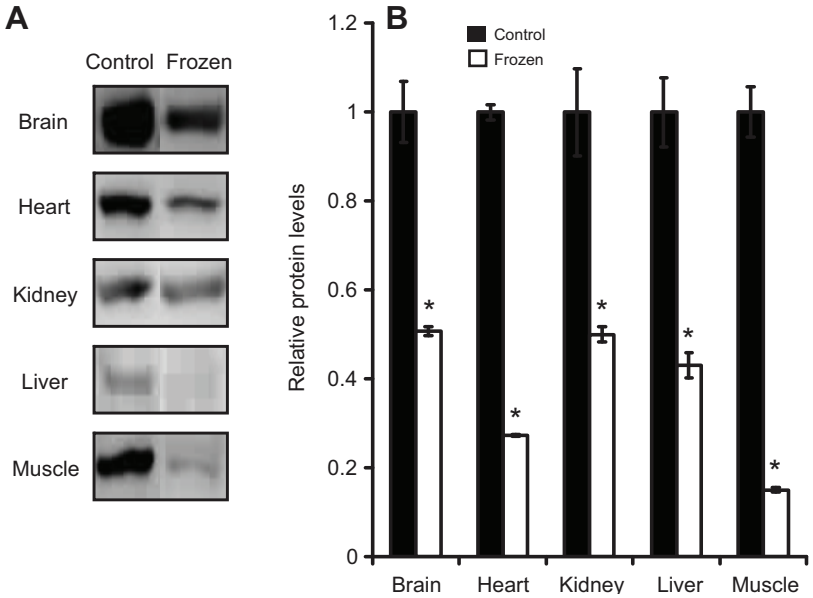

Fig. 2. Relative levels of phospho-GSK-3β (Ser9) protein in control and frozen frogs as determined by SDS-PAGE and western blotting. (A) Representative images of ECL-detected western blots. (B) Histogram showing relative protein levels in tissues from control and frozen frogs, as determined by quantifying band densities. Data are means ± s.e.m., N=4 independent preparations from control and frozen frogs. *Significantly different from the corresponding value in control frogs as assessed by Student's t-test, P<0.05.

Table 1. Kinetic parameters of GSK-3 in skeletal muscle extracts from control and frozen frogs assayed at 22°C, in the absence *versus* presence of 250 mmol l^{-1} glucose

	K_m peptide (μmol l^{-1})	V_{max} (U mg^{-1})	n_H
Control			
No glucose	23.5±4.2	1.83±0.30	1.34±0.13
250 mmol l^{-1} glucose	40.6±4.0*	2.89±0.64*	1.46±0.11
Frozen			
No glucose	33.1±4.4	2.27±0.18	1.53±0.20
250 mmol l^{-1} glucose	21.8±2.9*,†	1.14±0.35*,†	2.09±0.26*,†

Data are means ± s.e.m. for N=4 independent preparations of muscle extracts from different animals.
*Significantly different from the corresponding value in the same physiological state (control or frozen) but assayed in the absence of glucose, P<0.05.
†Significantly different from the corresponding value assayed at the same glucose condition, but in a different physiological state (control *versus* frozen frogs), P<0.05.

for GSK-3 in the presence of 250 mmol l^{-1} glucose showed substantial significant differences between control and frozen frogs. The K_m peptide of muscle GSK-3 from frozen frogs was ~46% lower than the control value, and V_{max} was reduced by ~61%. In addition, n_H increased by ~43% to a value of 2.09±0.26, indicating a more sigmoidal kinetic profile in contrast to the hyperbolic profile observed in GSK-3 assayed in the absence of glucose. This indicates that when assayed at 22°C in the presence of high glucose, GSK-3 from frozen frogs has a higher substrate affinity and greater cooperativity, but a lower maximal rate of reaction, than the control enzyme.

To examine the effect of temperature (and the compounded effects of temperature and glucose) on GSK-3 function, the enzyme from skeletal muscle of control and frozen frogs was also assayed at 4°C, in the absence and presence of glucose (Table 2, Fig. 3). In the absence of glucose, an expected temperature-dependent drop in V_{max} to values of 0.59–0.60 U $mg^{-1}$was observed for GSK-3 from both control and frozen frogs. While the K_m peptide from control muscle did not change significantly from the 22°C value (23.5±4.2 μmol l^{-1}, Table 1) when assayed at 4°C (29.7±2.6 μmol l^{-1}, Table 2), the corresponding value for the muscle enzyme from frozen frogs was significantly reduced (P<0.05) by ~50%, from 33.1±4.4 μmol l^{-1} (Table 1) to 16.6±0.07 μmol l^{-1} (Table 2), indicating greater GSK-3 affinity for its substrate peptide at lower temperatures close to the environmental temperatures encountered by frozen frogs (Fig. 3).

Further changes were observed in the presence of high glucose in 4°C assays (Table 2). V_{max} values for GSK-3 from both control and frozen frogs were further reduced in the presence of glucose, compared with assays at 4°C without glucose. Although high glucose had no effect on the K_m peptide for GSK-3 from control frogs at 4°C, the corresponding value for the enzyme from frozen frogs increased significantly (P<0.05) to a value approximately 2-fold higher than that in the absence of glucose at 4°C (Fig. 3).

GSK-3 effectors

The effects of different metabolites on the activity of GSK-3 from control frogs was assessed at 22°C and at 4°C, using concentrations of peptide that were either subsaturating (20 μmol l^{-1}) or saturating (70 μmol l^{-1}, i.e. a level that is potentially competitive to inhibitors). Metabolites that were predicted to change *in vivo* in muscle during freezing were tested over a range of concentrations to determine whether they had an effect on GSK-3 activity, and whether that effect was inhibitory or activating.

Several of the metabolites had no apparent effect on GSK-3 activity at either 22 or 4°C over the concentration ranges tested. These included: F1,6P_2, G3P, lactate, Mg citrate, succinate, ADP, Asp and Ala. Of the metabolites tested, AMP and PEP were the only two that had activating effects on GSK-3. The activation constants (K_a) for both these metabolites were also altered when assayed at 4°C compared with 22°C. An increase in K_a of AMP by ~100% was observed, yet the K_a value of PEP decreased by ~36% under the same conditions. Inhibition of GSK-3 activity was detected in the presence of glucose, G6P, pyruvate, MgATP, glycerol, Glu, Gln, NH_4Cl, NaCl and KCl (Fig. 4). For some of these metabolites, a lower assay temperature (4°C) led to an observed increase in the inhibitor concentration required for 50% inhibition of enzyme activity (IC_{50}). Glucose did not inhibit GSK-3 from skeletal muscle of control frogs when assayed at 22°C within the concentration range tested; however, when assayed at 4°C, glucose showed IC_{50} values of 14–15 μmol l^{-1} at both substrate peptide

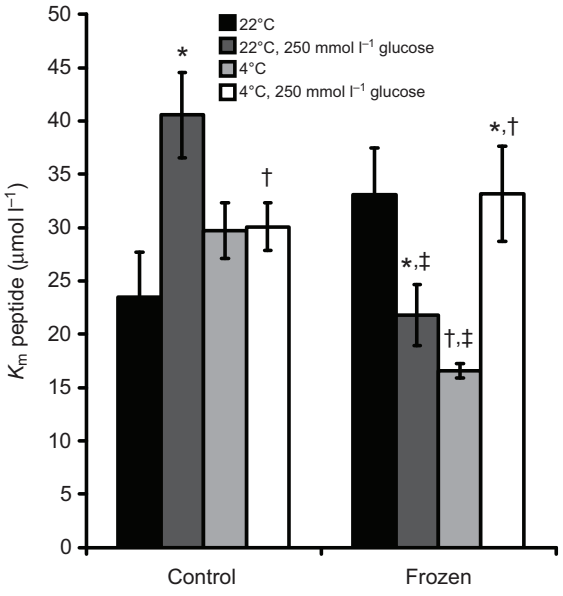

Fig. 3. Effect of temperature and glucose on substrate peptide affinity values (K_m peptide) of skeletal muscle GSK-3 from control and frozen frogs. Data are means ± s.e.m., N=4 independent preparations from control and frozen frogs. *Significantly different from the corresponding value in the same physiological state (control or frozen) and assayed at the same temperature (22 or 4°C) but in the absence of glucose, P<0.05. †Significantly different from the corresponding value in the same physiological state and assayed at the same glucose condition, but at different assay temperature (4 *versus* 22°C), P<0.05. ‡Significantly different from the corresponding value assayed in the same glucose condition and at the same temperature, but in a different physiological state (control *versus* frozen frogs), P<0.05.

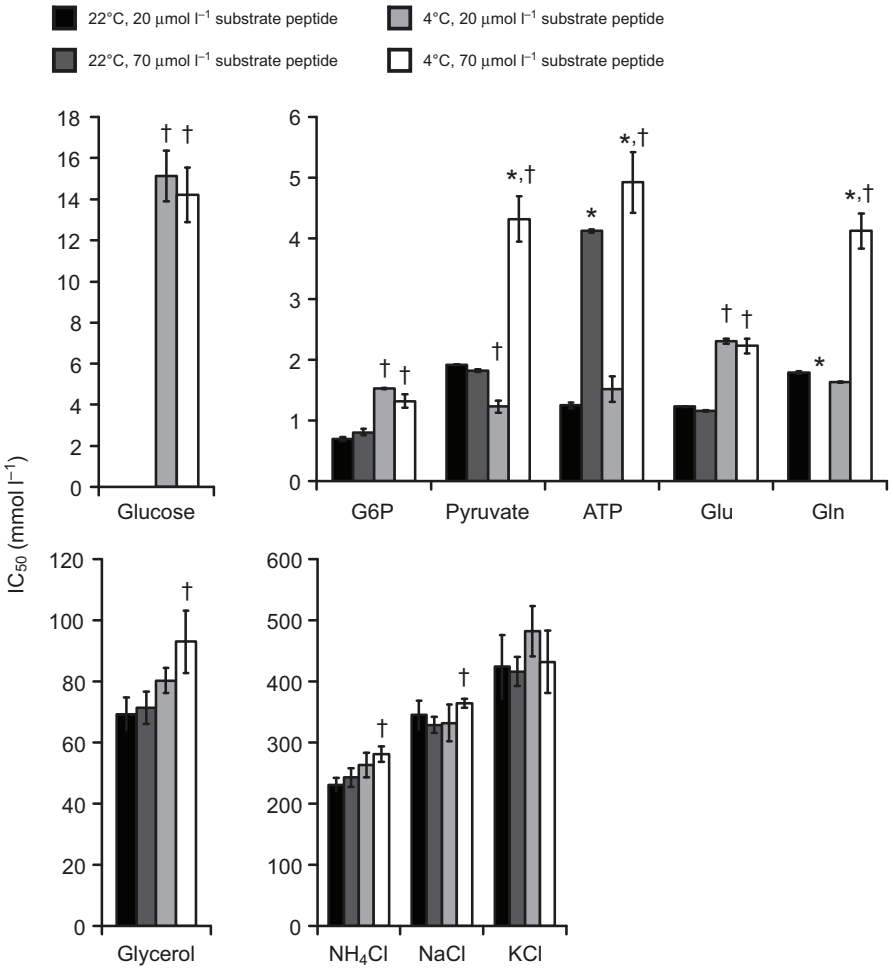

Fig. 4. Inhibitory effects of metabolites on GSK-3 activity from control frog skeletal muscle. Values plotted are the concentration needed to inhibit 50% of enzyme activity (IC_{50}). Data are means ± s.e.m., N=4 independent preparations. *Significantly different from the corresponding value assayed at the same temperature (22 or 4°C) but at the lower 20 μmol l^{-1} peptide concentration, P<0.05. †Significantly different from the corresponding assay with the same peptide concentration at 22°C, P<0.05. G6P, glucose 6-phosphate.

concentrations tested (20 and 70 μmol l^{-1}). IC_{50} values for G6P and Glu also increased at 4°C compared with 22°C when assayed at both higher and lower substrate peptide concentrations. Low temperature-dependent increases in IC_{50} were also observed for glycerol, NH_4Cl and NaCl, but only when assayed at the higher substrate peptide concentration.

Inhibition by other metabolites was dependent upon the concentration of substrate peptide (Fig. 4). IC_{50} values for MgATP increased in the presence of 70 μmol l^{-1} substrate peptide compared with 20 μmol l^{-1}, somewhat expectedly given that whatever allosteric effects ATP may have it is also a protein kinase substrate. At 70 μmol l^{-1} of substrate peptide, a further increase in IC_{50} of ATP was observed at 4°C compared with that at 22°C, indicating a possible combination of allosteric and substrate-mediated effects. A similar effect was observed for Gln. No inhibition of GSK-3 activity by Gln was detected over the Gln range tested at 22°C and 70 μmol l^{-1} of substrate peptide, whereas inhibition was observed at the same temperature and lower substrate peptide concentration. However, a higher IC_{50} for Gln was observed at 4°C and 70 μmol l^{-1} peptide, indicating that Gln inhibition of GSK-3 is also mediated by assay temperature and competition from the substrate peptide. An interesting phenomenon was observed for pyruvate inhibition. There was no change in IC_{50} of pyruvate between the higher and lower substrate peptide concentrations at 22°C. However, an assay temperature of 4°C caused a drop in IC_{50} of pyruvate at a lower substrate peptide concentration, whereas an increase of the same value was observed at 70 μmol l^{-1} peptide. This would seem to indicate that temperature decreases render GSK-3 more susceptible to inhibition by pyruvate, but that competition by the substrate peptide rescues GSK-3 from this inhibition.

DISCUSSION

GSK-3 was first identified as a protein kinase that phosphorylated GS (Embi et al., 1980) but is now known to act at the core of many signalling pathways and is a master regulator of cell growth and death with wide-ranging effects in all cells and organs, including a key role in *Xenopus* embryonic development (Green, 2004). In its role in the control of glycogen metabolism, phosphorylation of GS by GSK-3 is inhibitory, thereby inactivating GS and halting glycogenesis (Rylatt et al., 1980; Grekinis et al., 1995). Akt was later shown to be a prominent kinase responsible for the insulin-induced inhibition of GSK-3 by phosphorylating it at Ser9 (Cross et al., 1995). With GSK-3 inhibited, protein substrates including GS could be dephosphorylated and activated by protein phosphatases. This mechanism is integral to the insulin-induced activation of glycogen synthesis and other pathways, such as protein synthesis (Ingebritsen and Cohen, 1983; Parker et al., 1983; Ali et al., 2001). In the absence of insulin and other stimuli, GSK-3 is constitutively active (Cohen and Frame, 2001; Doble and Woodget, 2003) and free to phosphorylate its downstream targets, maintaining GS in an inactive state and glucose in a free, non-polymerized form.

Mammalian GSK-3 is encoded by two highly homologous genes; GSK-3α has a mass of 51 kDa and GSK-3β encodes a protein of

Table 2. Kinetic parameters of GSK-3 in skeletal muscle extracts from control and frozen frogs assayed at 4°C, in the absence *versus* presence of 250 mmol l^{-1} glucose

	K_m peptide (μmol l^{-1})	V_{max} (U mg^{-1})	n_H
Control			
No glucose	29.7±2.6	0.60±0.02	1.93±0.55
250 mmol l^{-1} glucose	30.1±2.2	0.30±0.04*	2.80±0.38
Frozen			
No glucose	16.6±0.7†	0.59±0.08	1.34±0.18
250 mmol l^{-1} glucose	33.2±4.5*	0.41±0.04*,†	2.56±0.80*

Data are means ± s.e.m. for N=4 independent preparations of muscle extracts from different animals.
*Significantly different from the corresponding value in the same physiological state (control or frozen) but assayed in the absence of glucose, P<0.05.
†Significantly different from the corresponding value assayed in the same glucose condition, but in a different physiological state (control *versus* frozen frogs), P<0.05.

47 kDa (Doble and Woodgett, 2003). The difference in size is due to a glycine-rich extension in the amino terminus of GSK-3α; this size difference is significant enough that the isozymes resolve separately under typical electrophoretic conditions. Our study used an anti-GSK3α/β antibody which was raised against full-length (420 amino acids) GSK-3β of *Xenopus* (anuran) origin, but has been shown to cross-react with both mammalian isozymes (α and β); indeed, GSK-3 isoforms from species as distant as flies and humans show over 90% homology within the kinase domain (Ali et al., 2001). However, this antibody detected only a single dense band of GSK-3 in all wood frog tissues tested, suggesting that only a single isoform is present. Similarly, in lysates of A6 cells (a cell line derived from epithelial cells of *Xenopus* kidney) this antibody, which was raised against an antigen from *Xenopus*, again cross-reacted with only a single band (Santa Cruz Biotechnology datasheet; http://www.scbt.com/datasheet-7291-gsk-3alpha-beta-0011-a-antibody.html). In support of this, a GenBank search for GSK-3 in *Xenopus laevis* (whose genome is fully sequenced) found only a β isozyme (NCBI accession NP_001083752); furthermore, embryology studies using *Xenopus* as a model only discuss a β isozyme (Dominguez et al., 1995; Green 2004). Hence, no literature is available to support the existence of more than one GSK-3 isozyme in any anuran species. Therefore, the accumulated evidence indicates that the anti-GSK3α/β antibody detected only GSK-3β in wood frog tissues, and strongly supports the contention that a GSK-3α is not present.

GSK-3 protein levels did not change significantly during freezing, with the exception of the heart, which showed significantly reduced GSK-3 protein. Maintenance of GSK-3 at fairly constant levels during freezing exposure has two implications: (i) the enzyme is always present to respond when needed, and (ii) any regulation of its kinetic parameters (activity, substrate affinity) is likely to be derived from post-translational controls. Indeed, the amount of phospho-GSK-3 (Ser9) was significantly reduced in all five tested tissues of frozen frogs. As the amount of phosphorylated enzyme decreased during freezing, this implies that GSK-3 was activated in liver, kidney, muscle and brain. The situation in heart is complicated by the concomitant decrease in both total and phospho-GSK-3. However, total GSK-3 was reduced by ~42% in heart, whereas the phospho-GSK-3 content was more strongly reduced by ~73%. Overall, this could suggest a higher relative amount of dephosphorylated, active enzyme. Thus, GSK-3 phosphorylation state appears to be globally reduced in frozen frogs, implying a more active GSK-3 in tissues during freezing. A more active GSK-3 in frozen frogs correlates well with a previous study of wood frog liver GS activity showing that GS was strongly suppressed during freezing (a decrease from 34% to 8% activity), following an inverse pattern to that of GP, which was strongly activated to support glucose synthesis (Russell and Storey, 1995).

Analysis of the enzymatic properties of wood frog skeletal muscle GSK-3 showed that alternative mechanisms for controlling the activity of the enzyme also exist, including effects of temperature and glucose. For example, when assayed at 22°C, high glucose decreased GSK-3 substrate affinity (K_m rose). Typically, a high glucose load must be catabolized or converted into glycogen for storage, and thus it is not surprising that high glucose or other glucose-derived metabolites decrease GSK-3 substrate affinity, an effect that would, in turn, help to keep GS active (Markuns et al., 1999; Halse et al., 2001). Notably, high G6P (the product of glucose phosphorylation by hexokinase) is also an allosteric activator of GS, so glucose and glucose derivatives affect GSK-3 and GS in a coordinated manner. However, at 4°C, the negative effect of high glucose on the affinity of control GSK-3 for its substrate peptide was no longer seen and the K_m value was the same in the absence or presence of high glucose. By contrast, high glucose effects on GSK-3 kinetics in muscle extracts from frozen frogs resulted in increased substrate affinity at 22°C, in contrast to the decreased affinity of the enzyme from control frogs. This could potentially increase phosphorylation of GSK-3 targets, including GS. However, a low temperature assay of the enzyme from frozen frogs reversed these effects; of all conditions tested, GSK-3 from frozen frogs assayed at 4°C without glucose exhibited the highest substrate affinity of all, which correlates with the increased amount of dephosphorylated active GSK-3 in this state (Fig. 3) and could lead to the greatest inactivation of GS. This is a particularly relevant finding as, traditionally, we would have expected that GSK-3 V_{max} would increase in frozen frogs as a result of dephosphorylation-dependent activation; surprisingly, we did not observe a significant V_{max} increase in frozen frogs when assaying GSK-3 at 22°C or at 4°C. Thus, in frog muscle, the 'activation' of GSK-3 by dephosphorylation may instead manifest itself as a stronger substrate affinity, and one that is only detected by assaying at lower temperatures close to environmental temperatures encountered by frozen frogs. Interestingly, the addition of high glucose at low temperature reduced GSK-3 substrate affinity to a level similar to that observed at 4°C in control frogs.

Several metabolites that change in wood frog tissues during freezing also affected GSK-3 activity as assessed with the muscle enzyme from control frogs. Both AMP and PEP were allosteric activators and increased GSK-3 activity. AMP, the end-product of ATP hydrolysis, is particularly interesting. AMP is well known to act as an intracellular allosteric activator of glycogen phosphorylase and high AMP under low energy conditions helps to trigger glycogen hydrolysis. AMP allosteric activation of GSK-3 would

further favour a net catabolic state of glycogen metabolism by promoting increased phosphorylation of GS and thereby inhibiting glycogen synthesis under metabolic situations where AMP is elevated. Notably, elevated AMP also activates the AMP-activated protein kinase (AMPK), which, in turn, triggers multiple other events that increase catabolism and decrease anabolism under energy stress conditions (Rider et al., 2006). Notably, K_a values for AMP of GSK-3 (0.21–0.42 mmol l^{-1}) were similar to *in vivo* AMP concentrations in wood frog muscle (0.11–0.15 μmol g^{-1} wet mass) (Storey, 1987a), indicating that changing AMP concentration could be a significant physiological regulatory mechanism.

Other metabolites inhibited GSK-3 activity. These included: G6P, a substrate for glycogen synthesis; the glucogenic metabolites glutamate, glutamine, glycerol and pyruvate; and several ionic salts including NaCl, KCl and NH_4Cl. Inhibition by each of these was found to be temperature sensitive and typically caused less inhibition at low temperature. G6P is particularly interesting because it is not only a substrate for glycogen synthesis but also an allosteric activator of GS, so it makes sense that it would inhibit GSK-3. Notably, the GSK-3 IC_{50} for G6P (0.8–1.5 mmol l^{-1}) was well within the *in vivo* concentration in wood frog muscle (1.6 and 3.3 μmol g^{-1} wet mass in control and frozen frogs, respectively) (Storey and Storey, 1984), indicating that G6P could have a major physiological role in GSK-3 regulation *in vivo*. Four inhibitors behaved differently. Pyruvate showed significantly higher inhibition at low temperature and low peptide concentration, and inhibition by ATP (both an allosteric effector and a GSK-3 substrate) and Gln was dependent on both temperature and peptide concentration. Interestingly, glucose inhibited GSK-3 from control frogs only at low temperature within the concentration range tested for allosteric effects.

The freezing-induced changes in GSK-3 found in this study can be interpreted with respect to what is already known about glycogen metabolism and the freezing process in wood frogs. Freezing triggers a rapid increase in GP activity, mainly in liver but also in muscle, initiating glycogen breakdown and a sharp increase in glucose production within minutes that ultimately leads to glucose levels of ~50 mmol l^{-1} in skeletal muscles and higher levels in core organs and blood (~75 mmol l^{-1} in kidneys, ~150 mmol l^{-1} in heart, ~200 mmol l^{-1} in liver, and as high as 250–300 mmol l^{-1} in blood) (Storey and Storey, 1988; Storey and Storey, 2004). With GP and glycogenolysis fully active, it is important to inactivate GS to avoid a wasteful ATP-dependent recycling of glucose into glycogen. Previous studies of wood frog GS showed that it was indeed reduced to very low activity levels during freezing, showing an inverse pattern to that of GP activity (Russell and Storey, 1995). A more active GSK-3 during freezing is therefore consistent with an inactivation of GS and would help preserve a high free glucose pool in tissues for cryoprotection. The highest substrate affinity (lowest K_m) of GSK-3 in this study was for the enzyme from frozen frogs assayed at low temperature in the absence of glucose. This is likely to be the consequence of the reduced level of GSK-3 phosphorylation in muscle of frozen frogs (Fig. 2) and would allow a more active GSK-3 to bind and phosphorylate GS to suppress its activity, particularly in the early stages of freezing when glucose cryoprotectant is being rapidly synthesized and accumulated. As energy stress increases over the long term under the ischaemic and anoxic conditions of the frozen state, ATP levels decrease and AMP accumulates in tissues (Storey, 1987b). This could have a further regulatory effect on GSK-3 by alleviating ATP inhibition and promoting AMP activation of GSK-3. At cold temperatures (4°C), high glucose was observed to inhibit GSK-3 activity from both control and frozen frogs and also decrease substrate affinity in frozen frogs (Table 2). Outwardly, this may seem counter-intuitive. However, glucose concentrations only reach high levels several hours after the onset of freezing (Storey and Storey, 2004) whereas the timescale of GS inhibition (and GP activation) is within minutes of the start of freezing (Russell and Storey, 1995; Storey and Storey, 2004). GSK-3 activation would presumably parallel GS inhibition and so during the initial stages of freezing GSK-3 would be unencumbered by inhibitory effects from high glucose. Furthermore, the inhibition of GSK-3 by high glucose may actually be advantageous when wood frogs thaw and glucose clearance is needed (Storey and Storey, 2004).

The activation of GSK-3 is probably an early event in freezing and potentially occurs in tandem with signals that activate GP. Previous studies have shown that glycogenoloysis is stimulated by peripheral freezing through hormone-based mechanisms, such as β-adrenergic signalling (Hemmings and Storey, 1994; Hemmings and Storey, 2001; Storey and Storey, 2004). It is possible that GSK-3 activation is mediated by similar hormonal action. Akt from the insulin signalling pathway is known to be a primary inhibitor of GSK-3 in vertebrates. Interestingly, wood frog insulin is structurally unique compared with that of other ranid frog species and could have impaired binding to the insulin receptor (especially at low temperature) that might contribute to allowing the anomalously high rise in glucose concentrations (Conlon et al., 1998). With reduced stimulation of the insulin receptor, intracellular signal transduction would be affected. Indeed, recent studies have shown reduced Akt phosphorylation in most tissues during freezing, with the exception of liver, where an increase was observed (J. Zhang and K.B.S., unpublished results). Another potential mediator of downstream Akt signalling is the AMPK pathway. AMPK signalling can antagonize Akt signalling in cancer cell proliferation (Rattan et al., 2005; Kim et al., 2009). In neuroblastoma cells, activators of AMPK caused the dephosphorylation of both Akt and GSK-3 (King et al., 2006). AMPK is activated by freezing in frog skeletal muscle and liver (Rider et al., 2006); in this study, the role of AMPK was postulated to be facilitation of cryoprotectant production by the inhibition of GS. The combined mechanisms of unique wood frog insulin structure and its downstream signalling, coupled with potential AMPK modulation of Akt targets, may constitute the proposed ‘override’ on normal glucose homeostasis that prevents the activation of glycogenesis that would normally be triggered under hyperglycaemia conditions.

In conclusion, GSK-3 is regulated in the transition to the frozen state by multiple mechanisms. The most prominent of these is the altered phosphorylation state; GSK-3 is phosphorylated in control frogs and dephosphorylated in frozen frogs. This post-translational modification causes differential responses to temperature and high glucose that facilitate the production of high concentrations of glucose for use as a cryoprotectant. Wood frog muscle GSK-3 also displayed reduced inhibition by allosteric effectors at low temperatures, and activation by end-products of energy stress (AMP) and gluconeogenic metabolites.

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