Signal transduction and gene expression in the regulation of natural freezing survival

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1. Strategies of winter survival in animals

Winter poses severe challenges to the survival of ectothermic organisms for exposure to subzero temperatures is frequently lethal. Most living things are killed when their tissues freeze and some are even chill intolerant and killed by simple exposure to temperatures below 0°C. For many organisms, the optimal way to survive the winter is to find ways to avoid having to deal with subzero temperatures. Behavioral options can be used including migration to a warmer climate or a retreat into thermally buffered sites such as under water or deep underground. Life cycles may also be modified so that many organisms overwinter in a relatively undifferentiated embryonic form (e.g. egg, cyst, spore, seed) that can often be engineered to contain little or no freezable water. However, many other cold-blooded species still spend the winter in terrestrial habitats that offer little or no protection from ambient temperatures. There they may need to endure exposures to temperatures that are far below the freezing point of their body fluids. In general, two strategies of cold hardiness have developed: freeze avoidance and freeze tolerance (Storey and Storey, 1988, 1989; Duman et al. 1991; Zachariassen, 1985).

Freeze avoiding animals use adaptations that maintain their body fluids in a liquid state down to temperatures that are well below the expected environmental minima for their habitat. They do so by exploiting the phenomenon of supercooling - the ability of watery solutions to chill well below their equilibrium freezing point (FP, the temperature at which an introduced ice crystal begins to grow). For example, many invertebrates living in the leaf litter and upper soil layers can stay liquid down to -10 or -15°C, temperatures that are substantially below the minima that are typically experienced in the insulated environment under the snow. Others that winter in sites that are exposed above the snowline (e.g. under the bark of trees) or in extremely cold polar or alpine environments have even more impressive abilities and can often stay liquid down to -40°C or lower. Freeze avoiding organisms use a variety of adaptations to lower both the FP and the crystallization temperature (Tc, temperature at which spontaneous freezing occurs) as well as to widen the gap between these two parameters (Zachariassen, 1985; Storey and Storey, 1989; Duman et al. 1991; Lee and Denlinger, 1991). Firstly, animals minimize their contact with potential nucleators - any molecules, particles or surfaces that could seed ice formation at temperatures at or below the FP. Gut contents are emptied to expel foreign bacteria and food

particles, selected blood proteins may be deleted during the winter months, and animals may wrap themselves in waterproofing (e.g. cocoons) to avoid contact with the most potent nucleator of all, ice crystals growing in their external microhabitat. Secondly, specific antifreeze proteins are synthesized and loaded into blood or hemolymph (see Duncker et al., 2001 this volume). These adhere to any microscopic ice crystals that may form and prevent them from growing to a size that could do physical damage. By doing so, they effectively lower the FP without changing melting point (MP), resulting in a thermal hysteresis between FP and MP that is the key to the detection and analysis of these proteins by researchers. Thirdly, many organisms also accumulate extremely high levels of sugar alcohols in their body fluids. In some insects, for example, glycerol levels can rise to over 2 M and may represent ~20% of the total body mass over the winter months (Storey and Storey, 1989, 1991). Like the ethylene glycol used in the radiator of a car, polyols strongly depress both the FP and the Tc of body fluids by colligative means. Overall, these strategies form an effective way to prevent body fluids from freezing. The freeze avoidance strategy has one major downfall, however, and that is that if environmental temperature drops below the Tc or if supercooled body fluids come in contact with a powerful nucleator, freezing will occur instantaneously and freezing is lethal for these animals. Hence, the freeze avoidance strategy is a bit of a gamble but one which serves many species well, ensuring the winter survival of the overall population, although not always of all individuals.

Freeze tolerant animals have an even more amazing strategy of winter survival. They can survive for days or weeks with as much as 65% of total body water converted to ice (Storey and Storey, 1988, 1992, 1996a). For example, in wood frogs (*Rana sylvatica*) ice propagates through the lumen of blood vessels, fills the ventricles of the brain, freezes the lens and bladder water, grows in sheets between skin and skeletal muscle, and fills the abdominal cavity. Only intracellular water remains unfrozen, its liquid state defended by high concentrations of sugars or sugar alcohols. Freeze tolerance is less common than freeze avoidance, probably because it is a more complex strategy to implement but, nonetheless, it has developed in diverse groups of organisms. Hundreds of insect species and other terrestrial invertebrates are freeze tolerant as are a variety of marine invertebrates that inhabit the intertidal zone on northern seashores (Storey and Storey, 1988). Various terrestrially-hibernating amphibians and reptiles also endure freezing during hibernation (Storey and Storey, 1992, 1996a). In some cases, freeze tolerance appears to be the only suitable winter hardiness strategy for species that can neither escape a subzero thermal environment nor protect themselves from contact with environmental ice. For example, terrestrially-hibernating frogs, such as wood frogs, have a highly water permeable skin and must hibernate in the moist microhabit of the forest leaf litter to prevent their bodies from desiccating. When the leaf litter freezes, however, so must the frogs for it is impossible for them to resist nucleation when they come in contact with environmental ice. Various species of intertidal molluscs and barnacles living at high latitudes also tolerate freezing which can occur at every low tide when the animals are exposed to winter air temperatures. Even when their shells are closed, their tissues are still bathed in seawater which freezes at about -2°C so seeding cannot be avoided. Frozen animals show no vital signs (no movement, no breathing, no heart beat) yet within minutes after thawing, all these processes resume. Studies of the adaptations that support freezing survival have been a focus of our research for many years including extensive work with freeze tolerant frogs and insects as well as exploration of freezing survival by turtles, snakes and lizards and by marine bivalve and gastropod molluscs (Storey and Storey, 1988, 1992, 1996a, 1999).

At least three serious problems threaten freezing survival: (1) ice crystals can cause physical damage, especially because water expands on freezing so that ice can rupture delicate tissues such as capillaries, (2) freezing halts delivery of oxygen and nutrients to organs via the blood, and (3) the conversion of up to two-thirds of body water into ice has major osmotic effects on cells including extreme cell volume reduction and a large increase in cellular osmolality and ionic strength. Specific adaptations of freeze tolerant animals help to deal with each of these problems (Storey and Storey, 1988, 1996a, 1999; Lee and Costanzo, 1998; Lee et al., 1998). These include: (1) methods to trigger nucleation just below 0°C (via ice nucleating proteins or heterologous nucleators) so that ice growth can be slow and controlled as it propagates through body fluid spaces, and to minimize recrystallization, the tendency of small crystals to regroup into larger crystals over time, (2) good ischemia resistance to aid tissue viability while frozen including ATP production via fermentative reactions, metabolic rate depression, and antioxidant defenses to deal with oxyradical stress when oxygen is reintroduced upon thawing (see Hermes-Lima et al., 2001 in this volume), (3) accumulation of high levels of sugars or polyhydric alcohols that act as cryoprotectants to minimize intracellular volume reduction during extracellular freezing, and (4) synthesis of other low molecular weight cryoprotectants (e.g. trehalose, proline) that stabilize membrane bilayer structure against the compression stress of cell shrinkage.

The current review focuses on new advances in understanding the cell and molecular responses to freezing stress in animals. Particular emphasis will be placed on new studies of the role of gene expression in supporting freeze tolerance and the mechanisms of signal transduction that mediate freeze-induced responses.

2. Freeze-induced Gene Expression

Changes in gene expression underlie the seasonal acquisition of cold or freeze tolerance in both animal and plant systems (Storey and Storey 1999; Thomashow 1998; Warren 2000). Evidence for this has been available for many years because the levels of selected proteins with functions in cold hardiness typically rise during the autumn months. For example, the activity of glycogen phosphorylase (GP) and various other enzymes involved in polyol synthesis in insects increase in the early autumn prior to the induction of cryoprotectant synthesis by cold exposure (Joanisse and Storey, 1994a,b). Antifreeze proteins or ice nucleating proteins also appear in the blood or hemolymph of various species during the autumn (Duman et al., 1991; Davies et al., 1999). Other proteins disappear over the winter; for example, no antimicrobial peptides could be detected in skin of wood frogs upon emergence from winter hibernation but a peptide of the brevinin-1 family was induced rapidly when the animals were warmed to higher temperatures and began to eat again (Mattute et al., 2000). The above cited examples represent changes of a preparative nature that are designed to alter the metabolic make-up of the organism prior to the arrival of cold weather. Often the trigger for these preparations is decreasing daylength; for example, induction of antifreeze protein synthesis in insects is triggered by a critical photoperiod (Duman et al., 1991). In some species, however, the induction of cold hardiness adaptations is obligately linked with a particular developmental stage; such is often the case in univoltine insects. In addition, whereas preparative measures (e.g. enzyme levels, glycogen accumulation) occur prior to cold exposure, the actual synthesis of carbohydrate cryoprotectants is typically triggered either by a critical temperature (5°C exposure triggers glycerol synthesis in many insects) or by freezing itself (glucose synthesis by wood frog liver is triggered within 2 minutes after the skin begins to freeze) (Storey and Storey, 1988).

Until recently, the identification of cold- or freeze-induced genes relied on identifying metabolic adaptations that support cold hardiness (e.g. cryoprotectant synthesis) and then working backwards to determine which proteins/enzymes were induced or up-regulated to support this function. For example, the use of glucose as a cryoprotectant by frogs suggested that increased numbers of plasma membrane glucose transporters would be needed during the winter months in freeze tolerant species and, indeed, this was found to be the case (King et al., 1995). It became increasingly obvious, however, that this approach is limited because of its dependence on a fore-knowledge of which adaptations are important for freezing survival. Techniques were needed that allow an unbiased evaluation of cold- or freeze-induced changes in gene expression. We began with an evaluation of freeze-induced protein/gene expression. Because freezing is an ischemic stress where energy is limited (cellular ATP levels fall to about 50 % of normal; Storey and Storey, 1985; 1986), it seems reasonable to assume that the frozen state should be one where energy-expensive biosynthetic reactions, such as protein synthesis, are generally minimized. Hence, examples of freeze-stimulated gene expression and protein synthesis should represent protein products that have critical functions for freezing survival and the identification of these proteins should lead to critical advances in understanding the mechanisms of freeze tolerance.

Initial studies analyzed patterns of freeze- or thaw-induced protein synthesis in wood frog organs using ^{35}S-methionine labeling techniques. Intraperitoneal injection of ^{35}S-methionine was used to evaluate protein synthesis *in vivo* under two forms of water stress, thawing after 12 h frozen at -1.4°C and dehydration/rehydration (27 or 40% of total body water lost and rehydration after 40 % dehydration) (Storey et al., 1997). Wood frogs can readily withstand the evaporative loss of 40-50% of total body water which mimics one aspect of freezing (the steep reduction in cell volume that occurs when up to 65% of total body water freezes as extracellular ice) and we have previously shown that dehydration of wood frogs at 5°C stimulates the same massive liver glycogenolysis and hyperglycemia as occurs during freezing (Churchill and Storey, 1993). Changes in protein patterns during freezing or thawing were also evaluated by isolating the mRNA transcripts present in the tissues of control (5°C acclimated), frozen (24 h at -2.5°C) and thawed (24 h at 5°C after 24 h frozen) frogs and subjecting these to translation *in vitro* in the presence of ^{35}S methionine/cysteine (White and Storey, 1999). For both experimental approaches, analysis of radiolabeled protein products using isoelectrofocusing and SDS-gel electrophoresis showed both freeze- and thaw-stimulated changes in the synthesis of selected proteins. Of special interest in both studies was the strong labeling of proteins of 15-20 kDa (Storey et al., 1997; White and Storey, 1999). For example, *in vitro* translation of mRNA isolated from liver of freeze-exposed frogs showed the presence of several new translation products (proteins of 45, 33.9, 21.5, 16.4, 15.8 and 14.8 kDa) as compared with controls (Fig. 1) (White and Storey, 1999). However, *in vitro* translation of mRNA from liver of thawed frogs showed no new protein peaks in comparison with either control or frozen profiles and the loss of several proteins of 16-22 kDa that were present in frozen and/or control frogs. However, neither of these methods was conducive to easy identification of the newly synthesized proteins and so we turned to techniques of cDNA library construction, differential screening, northern blotting, and DNA sequencing to isolate and identify genes, and their protein products, that are up-regulated during freezing.

2.1. Freeze-induced gene expression in wood frogs

In our first studies, a cDNA library was prepared from liver of wood frogs that were frozen for 24 h at -2.5°C. After differential screening with ^{32}P-labeled single-stranded total cDNA probes from liver of control (5°C-acclimated) vs frozen frogs, several unique freeze-responsive cDNA clones were found. DNA sequencing and Genbank searches identified two of these as the genes for the α and γ subunits of fibrinogen, a plasma protein involved in clotting that is synthesized by liver (Cai and Storey, 1997a). Both showed >70% identity of amino acid residues in the translated protein sequence with the corresponding sequences of the mammalian proteins. The gene for ADP/ATP translocase (AAT) was also freeze up-regulated (Cai et al. 1997); this protein of the inner mitochondrial membrane mediates the exchange transport of ADP and ATP. Another clone could not be identified from Genbank searches but its cDNA sequence of 457 bp had a single open reading frame that could encode a small protein of 90 amino acids with a molecular weight of ~10 kD (Cai and Storey 1997b). The deduced amino acid sequence of this novel protein, which we named FR10, showed an N-terminal region of 21 residues that contained ~80 % hydrophobic residues and had a potential nuclear exporting signal (LALVVLVIAISGL). The predicted secondary structure contained long sections of α helix as well as coiling structures distributed in four narrow regions and ß sheet structures in the N-terminus.

Changes in the levels of the mRNA transcripts of these four genes in wood frog liver were monitored by northern blotting over the course of a 24 h freezing exposure at -2.5°C followed by 24 h thawing at 5°C. As Fig. 2 shows, the genes for the two fibrinogen subunits were coordinately expressed with mRNA transcript levels of both rising by more than 3-fold after 8 h freezing and remaining at ~70 % of this maximum after 24 h frozen (Cai and Storey, 1997a). When frogs were thawed, however, fibrinogen transcript levels fell and were again near control values within 24 h. The time-dependent expression of *Fr10* transcripts followed a similar pattern (Cai and Storey, 1997b) but AAT expression was different. AAT transcripts rose 4.5-fold after 8 hours freezing but declined sharply with longer freezing and fell to less than control values after the 24 h thaw. AAT protein levels in liver were also monitored using immunoblotting and these followed an offset pattern with the maximum increase in protein content being ~2-fold after 24 h freezing (Cai et al., 1997).

Organ-specific patterns of gene up-regulation were also revealed. *Fr10* transcripts were found in all 8 organs tested and strong up-regulation by freezing was seen in all organs except kidney and muscle (Fig. 3a). This suggests a near universal expression of FR10 protein in frog organs and hints at a role in freezing protection in all organs. For example, a possible role as a freeze-specific transcription factor might be proposed, accounting for the wide organ distribution of FR10, its small size and its nuclear exporting signal. This idea is currently being pursued. On the other hand, mRNA transcripts for fibrinogen α and γ subunits showed a much narrower organ distribution. Fibrinogen is viewed as liver-specific in mammals and not surprisingly, frog liver showed the highest transcript levels of all organs tested. However, low levels of fibrinogen transcripts were also found in lung, bladder and gut (Fig. 3 b,c) and, as in liver, transcript levels in these three organs rose significantly in 24 h frozen frogs. AAT transcripts showed another pattern. They were elevated in liver, lung and bladder during freezing, fell in kidney, heart and gut and did not change in brain and muscle; western blots revealed that AAT protein levels followed much the same pattern (Cai et al., 1997).

Freezing involves multiple stresses on cells and organs (ischemia and dehydration being two major ones) and freeze-stimulated metabolic adaptations may, in fact, be targeted to address only one of the consequences of freezing. One way to help determine the role of genes/proteins that are up-regulated during freezing is to look at their responsiveness to the various component stresses of freezing. We took this approach first when examining the control of cryoprotectant synthesis and found that the extreme hyperglycemia that is triggered by freezing is stimulated just as strongly when autumn frogs were dehydrated at 5°C (at a rate of ~1% body water lost per hour) (Churchill and Storey, 1993). Like freezing, dehydration also stimulated the rapid increase in cAMP and an activation of protein kinase A in liver that stimulated glycogenolysis whereas, by contrast, anoxia exposure (N_2 atmosphere at 5°C), had no effect on this signal transduction cascade (Holden and Storey 1997). This suggested that the activation of cryoprotectant biosynthesis responded to cell volume signals and supported the demonstrated role of glucose is minimizing cell volume reduction during freezing (Storey et al., 1992).

When the same comparison of dehydration versus anoxia effects was applied to characterize freeze-induced gene expression in wood frog liver, two types of responses were found. Fibrinogen and *Fr10* transcripts were up-regulated just as strongly by dehydration as by freezing but under anoxic conditions, their transcript levels were down-regulated and virtually undetectable after as little as 30 min (Cai and Storey, 1997a,b). However, AAT transcripts in liver responded oppositely with levels increasing strongly under anoxic conditions (1-24 h) but showing no response to dehydration or rehydration (Cai et al., 1997). Combining these data with the responses to freezing, we can suggest that both fibrinogen and FR10 may have roles in dealing with some aspect of water balance during freezing which could include functions in cell volume regulation or in the accommodation of extracellular ice. By contrast, AAT probably has a role in ischemia resistance.

A probable reason for fibrinogen up-regulation during freezing could be its known role in repairing tissue injury. Fibrinogen is an acute-phase plasma protein. It is synthesized mainly by liver and secreted into the plasma with production stimulated by stresses including infection, inflammation, and tissue injury (Huber et al., 1990). The protein has two halves, each made of three subunits (Aα, Bβ and γ) and as the final step in the coagulation cascade, thrombin cleaves near the N-termini of the Aα and Bβ chains to release the A and B fibrinopeptides and expose sites for polymerization into the fibrin mesh of a growing blood clot. Notably, although our first study retrieved clones for just the α and γ fibrinogen subunits (Cai and Storey, 1997a), in new work we have isolated clones encoding the β and γ subunits of fibrinogen when a liver cDNA library made from glucose-loaded frogs was screened (K.B. Storey, unpublished). Hence, coordinate expression of all three subunits appears likely as a response to both freezing and high glucose. A stimulation of fibrinogen biosynthesis when frogs freeze would ready the animal to deal with any internal bleeding injuries that occur upon thawing. Ice crystals can do serious physical damage to tissues, particularly as a result of ice expansion within the lumen of microcapillaries. Ice can easily rupture vessel walls so that, upon thawing, vascular integrity is destroyed. Indeed, vascular injury is one of the most widespread and devastating problems faced in cryomedicine by researchers that are trying to develop mammalian organ freezing technology (Rubinsky et al., 1987).

Freeze tolerant animals need to address this problem to minimize ice damage to their tissues. One way to do this is to limit ice growth within organ capillaries. This is done by substantially dehydrating organs during freezing and moving water out of organs to freeze in extra-organ ice masses. This is readily evident upon examination of frozen frogs. The animals have a huge mass of ice in the abdominal cavity that surrounds very shrunken organs; large sheets of ice are also sandwiched between skin and skeletal muscle layers. Quantitatively, up to 25 % of organ water can be lost during freezing (Costanzo et al., 1992) and this significantly reduces the amount of extracellular ice that can form within organs. However, some physical damage by ice is still likely to occur and, indeed, hematomas are quite commonly seen in leg muscles after thawing. Thus, a freeze-induced elevation of plasma clotting capacity (involving fibrinogen synthesis and possibly other proteins of the clotting cascade) would enhance the frog's ability to deal with any internal bleeding during thawing.

The role of ADP/ATP translocase in wood frog liver during freezing is not yet clear. The enzyme catalyzes the transmembrane exchange of cytosolic ADP with mitochondrial ATP generated via oxidative phosphorylation. AAT probably has a role in dealing with freeze-induced ischemia, possibly in regulating the intramitochondrial adenine nucleotide pool size. However, what is interesting is the fact that enzymes of mitochondrial energy metabolism are appearing frequently as we search for stress up-regulated genes among freeze-tolerant and anoxia-tolerant animals.

2.2. Freeze-induced gene expression in hatchling turtles and mitochondrial gene expression

Other examples of freeze-induced gene expression come from our studies of anoxia tolerance and freeze tolerance in freshwater turtles. Adult turtles of the *Trachemys* and *Chrysemys* genera have the best developed anoxia tolerance among vertebrates and use this ability to hibernate for 3-4 months each winter on the bottom of ponds without breathing air and with only a minor ability to take up oxygen by extrapulmonary means (Ultsch, 1989). Anoxia tolerance is also put to use when diving at other seasons of the year and, intriguingly, by newly hatched juvenile turtles to aid in their winter freezing survival (Storey et al., 1992). Young turtles hatch in September but instead of exiting their terrestrial nests, they stay hidden underground for their first winter. This strategy lowers their risk of being eaten by aquatic predators but it means that they must have a way to survive when temperatures in their shallow nests drop below 0°C. For Canadian populations of the painted turtle, *C. picta*, this has meant the development of freeze tolerance (Storey et al., 1988; Churchill and Storey, 1992a), a capacity that is also rudimentary in the southern U.S. species, the red-eared slider *T. scripta elegans* (Churchill and Storey, 1992b).

Screening of a cDNA library made from heart of adult *T. scripta elegans* searched for genes that were induced or up-regulated when animals were given anoxia exposure (20 h submergence in N_2-bubbled water at 7°C) (Cai and Storey, 1996). Up-regulation was confirmed for two genes of the mitochondrial genome that encode subunits of electron transport chain proteins: subunit 5 of NADH-ubiquinone oxidoreductase and subunit 1 of cytochrome c oxidase. Transcripts of both increased by 3-4.5 fold in heart within 1 h of anoxic submergence, remained high over 20 h of anoxia and fell to control values again during aerobic recovery. Anoxic up-regulation of *Cox1* also occurred in brain, kidney and red skeletal muscle and *Nad5* transcripts were high in anoxic kidney and skeletal muscle. What was interesting is that ND5 and CO1, which are up-regulated by anoxia in adult turtles, were also up-regulated by freezing in juvenile *C. picta* (Cai and Storey, 1996). In heart, *Cox1* transcripts were 3.5-fold higher in frozen (24 h at -2°C) hatchling turtles than in 5°C-acclimated controls whereas *Nad5* transcripts were increased by about 70%. Freeze up-regulation of both transcripts also occurred in gut and kidney.

New studies are expanding the links between freeze tolerance and mitochondrially-encoded genes even further. Further analysis of the wood frog liver cDNA library highlighted freeze up-regulation of subunit 4 of NADH-ubiquinone oxidoreductase whereas differential screening of a cDNA library made from wood frog brain showed freeze up-regulation of the mitochondrially-encoded genes for ATPase subunits 6 and 8 of the F_0F_1ATPase complex (S. Wu and K.B. Storey, unpublished). This new data is providing us with many new avenues to explore to determine why and how the up-regulation of mitochondrially-encoded genes contributes to anoxia and freezing survival.

3. Freeze tolerance, glucose metabolism and signal transduction

3.1 Unique glucose metabolism of freeze tolerant frogs

Freeze tolerant frogs have harnessed liver glycogen metabolism for a unique purpose, the synthesis of massive amounts of glucose for use as a cryoprotectant. Within 2-5 min after freezing begins on the skin surface of the frog, GP in liver has been activated, glycogenolysis is stimulated and glucose levels in liver and blood are rapidly rising. Blood and liver glucose rises from control values of ~5 mM to levels of ~40 mM within an hour and continues upwards to reach 150-300 mM in core organs fully frozen animals (Fig. 4) (Storey and Storey, 1986, 1988). Glucose export from liver is facilitated by seasonally high numbers of glucose transporters in liver plasma membranes that are about 8.5-fold higher in autumn- than in summer-collected frogs (King et al., 1995). Blood glucose is readily taken up by all other organs but a gradient is seen between core organs and brain where glucose is highest and the peripheral skeletal muscle and skin where glucose is lower (30-60 mM) (Fig. 4) (Storey and Storey, 1988). Differential organ glucose contents result because the freezing front propagates inward from the periphery and progressively cuts off blood flow (and hence glucose delivery) to tissues as ice moves inward. ^{1}H-NMR images show that heart and liver are the last organs to freeze (Rubinsky et al., 1994) and, hence, these have the highest final glucose concentrations. Interestingly, because the higher the glucose concentration, the lower the MP of cellular fluids, frogs thaw from the inside out when temperature is raised (Rubinsky et al., 1994), an effect that has the advantage of allowing the heart to recover and resume beating as soon as possible.

Although glucose is readily taken up by all organs during freezing, its catabolism must be strictly limited. This is necessary in order to sustain the cryoprotectant pool and so, despite the fact that glucose is normally a very good anaerobic fuel for cells, its catabolism must be inhibited under the ischemic conditions of the frozen state. A study with wood frog erythrocytes confirms this. Figure 5a shows that D-[U-^{14}C] glucose was readily taken up by isolated wood frog erythrocytes at all incubation temperatures between 23 and 4°C (Brooks et al., 1999). Once taken up, ^{13}C-NMR revealed that glucose in erythrocytes was also readily catabolized at higher incubation temperatures with a linear rates of 0.91 ± 0.02 and 1.27 ± 0.02 mol/h/10^{16} cells at 12 and 17°C, respectively (Fig. 5b). However, when incubated at 4°C, glucose was not catabolized by wood frog red cells (Fig. 5b), suggesting that some form of metabolic inhibition occurs to block the catabolism of glucose in situations where a high pool size must be retained to provide cryoprotection. The site of this blockage is likely the hexokinase reaction (glucose + ATP → glucose-6-P +ADP) the necessary phosphorylation step that converts glucose into a hexose phosphate that can enter the glycolysis (or the reactions of glycogen synthesis). This must be so because glucose uptake by transporters is not blocked at low temperature nor is glycolysis itself since products of anaerobic glycolysis (lactate, alanine) accumulate over time in frozen tissues (Storey, 1987; Storey and Storey, 1986). Although it has never been proven experimentally, endogenous glycogen in organs is undoubtedly the fermentative substrate that supports anaerobic glycolysis during freezing whereas glucose use must be restricted to a cryoprotectant role by inhibiting hexokinase. Brooks et al. (1999) also noted a probable restriction on overall glycolytic flux at low temperatures. ^{13}C-NMR was used to monitor the incorporation of label from D-[2-^{13}]glucose into glycolytic intermediates in wood frog erythrocytes incubated at 4°C. The results showed that label mixed into the hexose and hexose phosphate pools but ^{13}C-labeled fructose-1,6-bisphosphate was not found. This suggests a metabolic block at the phosphofructokinase (PFK) locus at low temperature in red blood cells, an inhibition that may help to block glucose catabolism but would also contribute to an overall metabolic rate suppression at low or freezing temperatures. The same metabolic block at the PFK locus is prominent in liver during freezing where its purpose is to inhibit glycolysis so that glycogenolysis is directed into glucose synthesis and export (Storey, 1987). A key component of PFK inhibition in liver is the freeze-induced suppression of fructose-2,6-bisphosphate levels (a strong activator of PFK) and inhibition of the enzyme (6-phosphofructo-2-kinase) that synthesizes it (Vazquez-Illanes and Storey, 1993).

Glucose levels in vertebrates are normally strictly controlled within narrow limits (typically ~5 mM in blood) for a good reason. Sustained high glucose (10-50 mM), such as in diabetes, causes severe metabolic injuries, several of which are due to chemical effects of high glucose. Two of these are the nonspecific glycation of long-lived proteins and the pro-oxidant actions of glucose in generating reactive oxygen species (Ruderman et al., 1992; Kristal and Yup, 1992). Indeed, the pro-oxidant nature of high glucose may be the reason that wood frogs show stronger antioxidant defenses (higher activities of antioxidant enzymes and higher glutathione concentrations) than do comparably cold-acclimated

leopard frogs (Joanisse and Storey, 1996; Hermes-Lima and Storey, 1996; see also Hermes-Lima et al., 2001 this volume). The potential damage that can be done by high glucose is also probably the reason that frogs do not maintain high cryoprotectant levels over the entire winter as cold-hardy insects do with the polyhydric alcohols (e.g. glycerol, sorbitol) that are their cryoprotectants. Instead, glucose production is triggered immediately when freezing begins, is sustained throughout the freeze, and then reconverted to liver glycogen reserves when the animals thaw (Fig. 4) (Storey and Storey, 1986).

The unique glucose metabolism of wood frogs during freezing appears to arise from adaptations of both a quantitative (e.g. higher enzyme activities) and a qualitative (e.g. changes in regulatory mechanisms) nature. Some adaptations are clearly quantitative. For example, the activity of GP in freshly isolated hepatocytes of autumn wood frogs is ~13-fold higher than in hepatocytes of leopard frogs (*R. pipiens*), a species that shares a similar range with wood frogs but is freeze intolerant and hibernates underwater (Mommsen and Storey, 1992). Similarly, the number of glucose transporters in liver plasma membranes of wood frogs was 5-fold higher than in identically acclimated (5°C) leopard frogs (King et al., 1993). Responses to hyperglycemic stimuli are also more pronounced in wood frogs than in leopard frogs. For example, in wood frogs both freezing and dehydration stimulate a rapid increase in the percentage of liver protein kinase A that is present as the active catalytic subunit (PKAc); this rose from 7-10 % in controls (5°C acclimated) to 62 % within 5 min after freezing began or to 31 % when frogs were dehydrated by 20 % (Holden and Storey, 1996, 1997). Freezing effects cannot be assessed in leopard frogs but 20 % dehydration had no significant effect on the % PKAc, values being 22% in controls versus 28% in dehydrated animals (Holden and Storey, 1997). Such quantitative differences as well as 6-7 fold higher glycogen reserves in wood frog liver (Mommsen and Storey, 1992) help to make the difference between a 4-fold rise in liver glucose stimulated by the loss of 25% of body water in autumn *R. pipiens* and a 300-fold increase in liver glucose in *R. sylvatica* under the same conditions (Churchill and Storey, 1993, 1996). It should be noted, however, that the low but pronounced hyperglycemic effect of dehydration in leopard frogs led us to suggest that the cryoprotectant synthesis response to freezing by freeze tolerant frogs probably grew out of a pre-existing hyperglycemic response to dehydration that may be common to all anuran species (Churchill and Storey, 1993).

Qualitative changes in the regulation of glucose metabolism in freeze tolerant frogs are also needed to control glucose levels within a narrow range under normal (unfrozen) circumstances, just like in other vertebrates, versus to allow the development of extreme hyperglycemia during freezing. Glycogen metabolism in vertebrate liver is controlled externally by hormones (insulin, glucagon, adrenaline) and internally by a cascade of protein kinases and phosphatases that allow responses to hormonal and other stimuli. At least one part of the regulatory system must be altered to override the normal homeostatic control of glucose levels during freezing and permit the massive glycogen breakdown that allows glucose to soar during freezing. Several new studies shed light on the controls involved.

3.2. Structural modification of insulin in wood frogs

The regulation of glucose in vertebrates is normally strictly regulated by the opposing actions of two pancreatic hormones, insulin and glucagon. Insulin is quickly secreted as blood sugar levels rise so that in healthy humans, glucose rarely rises above 8 mM. Insulin stimulates the uptake and storage of glucose by organs either as glycogen or as fat via stimulation of fatty acid biosynthesis. Wood frogs, however, allow blood glucose to soar to concentrations as high 150-300 mM in core organs and blood during freezing. How this extreme hyperglycemia is permitted and how frogs avoid the negative metabolic consequences of prolonged exposure to high glucose remains to be determined but some clues have been found.

The loss of homeostatic control over glucose levels during freezing might be linked to a change in the hormonal regulation of glucose at this time. Insulin secretion from the pancreas might be inhibited during freezing or, alternatively, frog liver may become refractory to insulin stimulation at this time via a mechanism that interferes with insulin receptors on liver cell membranes. Another possibility could be a change in the structure of wood frog insulin that alters its function, particularly under subzero or freezing conditions. A recent study provides evidence for this latter possibility (Conlon et al. 1998). Table 1 shows the N-terminal sequences of insulin from four frog species compared with human insulin. Wood frog insulin shows some unusual features. Firstly, the wood frog hormone has a two amino acid extension (lysine-proline) on the N-terminus of the A chain. Although shared by other ranid frogs, this extension does not occur in other vertebrates. Its role remains unknown. Secondly, wood frog insulin shows some unique amino acid substitutions. The serine residue at position A23 in wood frog insulin (A21 of human) is an asparagine in all other species and the aspartic acid at B13 in wood frog insulin is glutamic acid in nearly all tetrapods. Both residues are known to play key roles in insulin function. The A21 (bonding to B22/23) helps to maintain the biologically active protein conformation and B13 is involved in binding to the insulin receptor (Markussen et al. 1988; Kristensen et al. 1997). One or both of these substitutions in wood frog insulin may impair its function. Notably, in the only other known instance of a Glu to

Asp substitution at B13 (in the coypu; Bajaj et al. 1986), the change creates a low potency insulin. It is also conceivable that these substitutions may be particularly effective in disrupting insulin conformation or receptor-binding ability at subzero temperatures but might be of lesser consequence to hormone action at warmer temperatures where a functional insulin would definitely be needed to regulate the disposition of glucose arising from dietary intake. The probable importance of these novel features are also underlined by the fact that whereas wood frog insulin shows unique features, wood frog glucagon does not; it is identical to glucagon from bullfrogs and has only one amino acid substitution as compared with the human hormone (Conlon et al. 1998).

3.3. Adrenergic control of freeze-induced glucose production

The signals involved in regulating the freeze-induced production of glucose as a cryoprotectant in wood frogs have been extensively studied by our lab. Synthesis is triggered by ice nucleation on the skin and signals are immediately transduced to the liver to activate glycogenolysis. The signal appears to be mediated through ß-adrenergic receptors on hepatocyte plasma membranes because intraperitoneal injection of propranolol (a ß-adrenergic blocker) just before freezing exposure reduced the hyperglycemic effect of freezing by ~50% whereas administration of the α-adrenergic blocker, phentolamine, had no effect on glucose accumulation (Storey and Storey, 1996b). Propranolol also reduced the strong activation of liver GP that supports freeze-induced hyperglycemia so that the activity of phosphorylase a rose by only 8-fold in propranolol-injected frogs during freezing as compared with the 30-fold increase seen in control animals (Storey and Storey, 1996b). The participation of ß-adrenergic receptors in mediating freezing-induced cryoprotectant synthesis by wood frog liver was also confirmed by a study that quantified the numbers of α_1, α_2 and $ß_2$ adrenergic receptors in liver plasma membranes by monitoring the binding of radiolabeled inhibitors of the three receptor types (prazosin, yohimbine, and iodopindolol, respectively) (Hemmings and Storey, 1994). This analysis demonstrated that $ß_2$ adrenergic receptors dominated in the plasma membranes of control frogs, with levels several-fold higher than those of α_1 and α_2 receptors. $ß_2$ receptor levels remained high (not different from controls) over the early hours of freezing (1, 12 h at -2.5°C) when rates of glucose production are highest but when frogs were fully frozen after 24 h $ß_2$ receptor binding had decreased by 73%. After 24 h thawing at 4°C, $ß_2$ receptor levels were even further suppressed (by 83% as compared with controls) (Hemmings and Storey, 1994). This suggests that the capacity to respond to hyperglycemic stimuli is reduced in thawed animals, a situation that would favor the clearance and restorage of cryoprotectant as hepatic glycogen. This agrees well with the rapid suppression of GP activity after thawing (Storey and Storey, 1988) and the strong resurgence of glycogen synthase, the amount of active, glucose-6-phosphate independent glycogen synthase activity rising from 0.38 U/g wet mass in liver of 24 h frozen frogs to 3.72 U/gwm after 24 h thawing (Russell and Storey, 1995).

3.4. Protein kinase A

Cyclic AMP binding to the two regulatory subunits of protein kinase A (PKA) causes the dissociation of the inactive tetramer to release the two catalytic subunits (PKAc) of the enzyme. PKAc then phosphorylates and activates glycogen phosphorylase kinase which in turn phosphorylates and activates GP. This normal pattern of activation of glycogenolysis in vertebrate liver is also stimulated by freezing in wood frogs. Within 5 min after freezing begins, the % PKAc rose from 7% in controls to over 60% (Holden and Storey 1996) and was closely followed by a rise in the percentage of GP present as the active a form and by elevated glucose output (Fig. 4). Although tetramer dissociation to release the catalytic subunits is the primary mode of PKA control *in vivo*, analysis of the properties of wood frog liver PKAc suggest that these could also influence enzyme function under freezing conditions (Holden and Storey 2000). Thus, the kinetic properties of purified wood frog liver PKAc were significantly affected by assay temperature with low temperature having positive effects on the enzyme (Table 2). Affinity for both Mg-ATP and the phosphate-accepting substrate, kemptide, increased at low temperature; Km Mg-ATP was 50% lower and Km kemptide 33% lower at 5°C, compared with 22°C. PKAc also showed reduced sensitivity to KCl and NaCl inhibition at low temperature with I_{50} values 45 and 25% higher at 5°C than at 22°C (Table 2). This lower sensitivity to salt would allow better enzyme function under the conditions of rising cytoplasmic ionic strength that occur as more and more body water freezes out in extracellular ice masses.

3.5. Protein phosphatase-1

Opposing PKA in the homeostatic control of glycogen metabolism is protein phosphatase-1 (PP-1). Under normal conditions in vertebrate liver if glucose rises above about 7-8 mM, PP-1 intervenes to halt further glycogen breakdown by dephosphorylating GPa and phosphorylase kinase. However, in the liver of freezing frogs, glucose production continues unabated to levels of 200-300 mM. Obviously, the off-switch must be missing or inactivated during freezing. To determine whether differential regulation of PP-1 was responsible for the apparent loss of regulatory control over glycogenolysis during freezing, we analyzed the properties of this phosphatase in wood frog liver. Opposite to our expectations but in line with the normal behaviour of the enzyme when glucose concentrations rise, we found that the amount of active PP-1 actually rose progressively over the first hour of freezing exposure, reaching 2.4-fold higher than control values (MacDonald and Storey, 1999). But, despite this, the normal effects of PP-1 action (GP inactivation, glycogen synthetase activation) do not occur during freezing although they are re-instated rapidly when animals thaw (Storey and Storey 1988; Russell and Storey 1995). To determine why this is so, we looked at other aspects of PP-1 regulation.

Further studies revealed that the key factor in liver PP-1 control is probably the physical location of the enzyme. Under normal conditions in vertebrate liver, PP-1 is distributed between cytosolic and glycogen-bound pools. To permit glycogen binding, the PP-1 catalytic subunit must first bind to a G subunit (glycogen binding protein) and this dimer then binds to glycogen. All three isoforms of the PP-1 catalytic subunit (α, δ, $\gamma 1$) can bind to the G subunit, but the α and δ subunits are the main ones associated with glycogen *in vivo* (Alessi et al. 1993). To analyze the distribution of PP-1 between free and glycogen-bound forms in liver of control (5°C -acclimated) and frozen (12 h at -2.5°C) wood frogs we used differential centrifugation to separate cytosolic and glycogen particle fractions, followed by PP-1 isolation from each fraction using microcystin-agarose affinity chromatography, and then SDS-PAGE and western blotting using antibodies to the three PP-1 isoforms of rat liver (MacDonald and Storey, 1999). The effects of freezing on PP-1 distribution were dramatic. In control frog liver the α and δ isozymes of PP-1 were predominantly localized in the glycogen fraction with little or no crossreacting material detected in the cytosolic fraction (Fig. 6). However, the distribution of PP-1 isoforms changed radically with freezing. In frozen animals, virtually all of the α and δ isozyme protein was translocated into the cytosolic fraction where it could no longer regulate GP. By contrast, the distribution of the $\gamma 1$ isoform did not change and was primarily cytosolic in both control and frozen frogs.

With the translocation of PP-1 α and δ to the cytosol during freezing, GPa can then function unrestrained in frog liver during freezing and provide a continuous output of glucose for use as a cryoprotectant until glycogen is depleted. The mechanism that regulates PP-1 translocation is not yet known but two possibilities exist. One is a down-regulation of the G subunit during freezing. Studies have shown that G subunit levels can affect glycogen metabolism; for example, in insulin-dependent diabetes, low levels of the G subunit impair liver glycogen synthesis (Doherty et al.; 1998). Hence, freezing might stimulate a rapid decrease in the amount of the G subunit in wood frog liver which would reduce the ability of PP-1 to bind to glycogen particles and lead to the appearance of PP-1 in the cytosolic fraction of liver in frozen frogs. A second possibility is reversible control over the G subunit during freezing, perhaps via protein phosphorylation and affecting its ability to either bind PP-1 or bind glycogen. A reversible system, rather than a change in G subunit amount, provides the capacity for a rapid reversal during thawing when GP activity drops by as much as 100-fold and glycogen synthase activity soars (Storey and Storey, 1988; Russell and Storey, 1995). PKAc is known to phosphorylate the G subunit in mammalian liver which increases the rate of PP-1 inactivation by inhibitor-1 and promotes PP-1 release from glycogen (Hubbard and Cohen 1989). Thus, the sustained high PKAc activity in liver of freezing frogs (Holden and Storey, 1996) could be responsible for both GP activation and PP-1 inhibition. However, normally this system is very sensitive to rising glucose levels so a missing piece of the puzzle still remains - specifically, how is the normal sensitivity of this system to high glucose overridden during freezing yet reinstated immediately upon thawing.

3.6. PKG, PKC and MAPKs

Recent studies in our lab have also evaluated the possible role of other signal transduction pathways in mediating events of freeze tolerance. An analysis of second messenger responses to freeze/thaw showed differential responses by both cGMP and inositol 1,4,5-trisphosphate (IP_3), the second messengers of protein kinases G and C, respectively, to freezing and thawing in five wood frog organs (Holden and Storey 1996). IP_3 levels in liver were especially intriguing, rising by 70% within 2 min after freezing began but then continuing to reach a peak of 11-fold higher than controls after 24 hours of frozen. This contrasts with the pattern of cAMP changes which jump 2-fold within 2 min, are sustained over the first hour of freezing and then begin to fall. IP_3 also rose in brain to a maximum of 75% higher than controls after 8 hours frozen. The longer response time for the rise in IP_3 and the sustained high levels of this second messenger during prolonged freezing suggests a possible role for PKC in events that occur over the longer term during freezing such as

ischemia resistance or cell volume regulation. Notably, IP_3 levels also rose in wood frog liver when animals were under dehydration stress; IP_3 had increased by 70% in liver of frogs that had lost 5 % of total body water and peaked at 4-fold above control values in 40 % dehydrated animals (Holden and Storey, 1997). Liver IP_3 levels fell again in both 24 h thawed frogs and fully rehydrated frogs. These very similar responses to freezing versus dehydration suggest that PKC may be involved in regulating cell responses to volume changes.

Mitogen-activated protein kinases (MAPKs) mediate a vast number of cellular responses including gene transcription, cytoskeletal organization, metabolite homeostasis, cell growth and apoptosis in response to many different extracellular signals (Kyriakis 1999; Hoeflich and Woodgett, 2001 this volume). Subfamilies include the extracellular signal regulated kinases (ERKs), Jun N-terminal kinases (JNKs) (also called stress-activated protein kinases) and p38. The latter is the vertebrate counterpart of the yeast Hog1 which was named for its role in the high osmolarity glycerol response. To gain an initial assessment of the roles of MAPKs in freeze tolerance, organ-specific responses of the enzymes were analyzed in wood frogs and hatchling turtles (*T. s. elegans*) (Greenway and Storey 1999, 2000). ERK activities did not change in frog organs over freeze/thaw and in turtles changed only in brain where the amount of active, phosphorylated ERK2 doubled after 30 min freezing and remaining high through 4 h frozen (Greenway and Storey 1999, 2000). This limited responses suggests that ERKS are not involved in transducing signals from freezing stress, which is perhaps not surprising since ERKs appear to primarily transduce signals from growth factors and mitogens.

However, both JNK and p38 responded to freeze/thaw. JNK activities did not change in wood frog organs over a 12 h freeze but fell by 40-50% in turtle liver and heart over a 4 h freeze (Greenway and Storey 1999, 2000). JNK activity showed a strong increase after 90 min thawing in both liver and kidney of wood frogs (rising ~5- and 4-fold, respectively) suggesting a role in responses activated by thawing. JNK activity was also elevated in frog heart during thawing, doubling after 4 h thawing. The p38 MAPK was the only one to show a widespread response to freezing in frogs (Greenway and Storey 2000). The amount of active, phosphorylated p38 rose by 5-7 fold in liver and kidney within 20 min post-nucleation (as measured by densitometry of immunoblots) but this was reversed by 60 min (Fig. 7). A role for p38 in one of the rapid, initial responses to freezing in these organs is therefore implicated. However, in heart, phospho-p38 content rose on a slower time course by about 4-fold after 1 h of freezing and 7-fold after 12 h. Heart experiences a progressive increase in work load as freezing progresses because blood viscosity increases as does peripheral resistance and hence changes in signal transduction in heart may be linked to either adjustments to heart function or implementation of freeze tolerance adaptations. Changes to the phosphorylation state of p38 also occurred during thawing and in brain, p38 was the only MAPK that was activated by thawing. A comparable analysis the effects of anoxia stress (0.5-12 h under N_2 at 5°C) or dehydration (10-40% of total body water lost) on phospho-p38 content in wood frog liver and kidney showed no changed under these stresses. This suggests that p38 might mediate metabolic responses that are unique to freezing survival.

4. Conclusions and future directions

Much remains to be learned about the cell and molecular responses to freezing stress and the multi-faceted adaptations that allow freezing survival. Identification of genes that are up-regulated under freezing stress are leading to whole new areas of research. For example, we are finding that a common response to many forms of stress (freezing, anoxia, hibernation) is the up-regulation of genes that are encoded on the mitochondrial genome and much work remains to be done to determine why this is so. Studies with freeze tolerant insects and marine gastropods are also opening up new venues. For example, in other recent work, we have identified a metallothionein as up-regulated in response to freezing and anoxia stresses in marine snails (*Littorina littorea*) (T.E. English and K.B. Storey, unpublished results). Injury caused by reactive oxygen species (ROS) is a serious problem in mammalian systems of ischemia/reperfusion but freeze tolerant animals that undergo ischemia/reperfusion with every cycle of freeze/thaw should have adaptations that address this problem. In previous studies we have shown that freeze tolerant species have high constitutive activities of antioxidant enzymes that could minimize damage by ROS when oxygen is reintroduced during thawing (see Hermes-Lima et al., 2001 this volume). The new finding of freeze-induced up-regulation of metallothionein could also contribute to antioxidant defenses by increasing the capacity of tissues to sequester iron, which as a catalyst for Fenton reactions, plays a major role in the generation of ROS in cells. Furthermore, a general elevation of metal binding capacity in cells during freezing could be of importance to the maintenance of homeostasis in the frozen state. If two-thirds of total body water is converted to ice, then the concentrations of all ionic species in the cytoplasm will rise by about 3-fold and this could cause problems for reactions that are influenced by metal ion concentrations. Increased metal binding capacity could lower cytoplasmic metal ion concentrations back into the normal range in shrunken, freeze-concentrated cells. Hence, these recent results suggest a new potential type of freezing adaptation for exploration.

Much more also remains to be learned about how cells perceive and transmit freezing signals and coordinate both general and organ-specific responses to freezing stress. The regulation of the synthesis and distribution of

cryoprotectants is now quite well understood but very little is yet known about how cells manage and regulate the very large changes in water flux, cell volume, ionic strength and osmolality that occur as a consequence of freezing. The molecular mechanisms that underlie the recovery of physiological functions (breathing, heart beat, nerve activity) after thawing are also a mystery awaiting to be explored.

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Thaw time

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Table 1. N-terminal sequences of insulin A and B chains in wood frog, bullfrog, green frog, clawed toad and humans. From Conlon et al. (1998).

	Insulin A-chain
Rana sylvatica	KP GIVEQ CCHNM CSLYD LENYC S…
Rana catesbeiana	-- ----- ----T ----- ----- N…
Rana ridibunda	-- ----- ----T ----- ----- N…
Xenopus laevis	----- ---ST --F-- --S-- N…
Human	----- --TSI ----Q ----- N…

	Insulin B-Chain
Rana sylvatica	FPNQH LCGSH LVDAL YMVCG DRGFF YSPRS…
Rana catesbeiana	----Y ----- --E-- ----- ----- -----…
Rana ridibunda	----Y ----- --E-- ----- E---- -----…
Xenopus laevis	LV--- ----- --E-- -L--- ----- -Y-KV…
Human	-V--- ----- --E-- -L--- E---- -T-KT…

Table 2. Kinetic parameters of the purified free catalytic subunit of PKA from *Rana sylvatica* liver.

	22°C	5°C
K_m kemptide (μM)	9.0 ±0.1	6.4 ±0.3[a]
K_m Mg-ATP (μM)	51.8 ± 1.0	24.8 ± 1.4[a]
I_{50} KCl (mM)	495 ± 10	720 ± 10[a]
I_{50} NaCl (mM)	562 ± 16	700 ± 12[a]

Data are means ± SEM, n = 3 separate preparations of purified frog liver PKAc enzyme. Kemptide (LRRASLG) is a synthetic peptide with the sequence of the phosphorylation site in pyruvate kinase. [a]-Significantly different from the corresponding value at 22°C, P<0.05. From Holden and Storey (2000).

Figure legends

Figure 1. Effect of freezing and thawing on the pattern of *in vitro* translation products produced from mRNA in wood frog liver. Total RNA was isolated from liver samples of control (5°C), frozen (24 h at -2.5°C) and thawed (24 h at 5°C after 24 h frozen) wood frogs and translated in a cell free system (wheat germ extract) followed by separation of ^{35}S-labeled proteins by SDS-PAGE. After autoradiography, densitometry scans showed the distribution of ^{35}S-labeled proteins. Peaks representing proteins that were new or enhanced in the frozen state are indicated by arrows along with their approximate molecular weights. Lines are: (thin), control; (thick), frozen; (dashed), thawed recovery. The positions of molecular weight standards are shown on the inner side of the x-axis. From White and Storey (1999).

Figure 2. Effect of freezing and thawing on mRNA transcript levels of four genes in wood frog liver as determined by relative band intensities on northern blots. Symbols are: (circles) fibrinogen α; (squares) fibrinogen γ; (triangles up), ATP-ADP translocase; (triangles down), FR10. Control frogs (0 h) were held at 5°C; freezing was at -2.5°C for up to 24 h, and thawed frogs were frozen for 24 h followed by 24 h back at 5°C. Compiled from Cai and Storey (1997a,b) and Cai et al. (1997).

Figure 3. *Fr10* (A) and fibrinogen α and γ (B) mRNA transcripts levels in organs of control (open bars) and 24 h frozen (solid bars) wood frogs. Controls were held at 5°C; frozen frogs were at -2.5°C. Total RNA was isolated from each tissue and transcript levels were analyzed via northern blots followed by autoradiography and densitometry; the 18S rRNA band was used for standardizing. Freezing had no effect on the negligible levels of fibrinogen transcripts in heart, brain, kidney or skeletal muscle. Data compiled from Cai and Storey (1997a) and Cai et al. (1997).

Figure 4. Glucose levels in frog organs over a course of freezing at -2.5°C and thawing at 5°C. Data are means $\pm$ SEM, n=3. Symbols are: (circles), blood; (squares), liver; (triangles up), skeletal muscle; (triangles down), heart; (diamonds), kidney. Data compiled from Storey and Storey (1986).

Figure 5. (A) Glucose depletion from the incubation medium due to uptake by *R. sylvatica* erythrocytes. Aliquots of 130 µl erythrocytes (66% hematocrit) were incubated with 10 mM glucose containing 0.6 µCi [U-^{14}C]D-glucose at four temperatures. Samples were removed at timed intervals, centrifuged to pellet cells, and the percentage of radioactivity remaining in the supernatant (SNT) was measured. Lines show theoretical fits. (B) ^{13}C-NMR determination of glucose utilization by wood frog erythrocytes at three temperatures. Samples were matched for equal numbers of cells and then given 10 mM [2-^{13}C]D-glucose. Glucose concentration remaining at any given time was determined by comparison of the glucose peak height at 76.7 ppm with the *p*-DP standard peak height at 127.5 ppm and confirmed by comparison with known concentrations of [2-^{13}C]D-glucose measured under identical conditions. From Brooks et al. (1999).

Figure 6. Protein phosphatase-1 (PP-1) isozymes in liver of control and 12 h frozen wood frogs. PP-1 was partially purified from cytosolic (C) and glycogen (G) fractions of frog liver by microcystin-agarose affinity chromatography followed by SDS-PAGE and blotting onto PVDF membranes. Immunoblotting used antibodies to rat liver α, δ and γ1 PP-1. Recombinant PP-1-γ1 was present in the fifth lane as a positive control. Gels show PP-1 bands at 37-39 kDa. From MacDonald and Storey (1999).

Figure 7. Changes in the amount of the phosphorylated (tyr 182) form of p38 in spring wood frog tissues sampled from control frogs (5°C-acclimated) and frogs frozen for 20 min, 1 h (or 4 h frozen for brain only), or 12 h at -2.5°C. Phospho-p38 content was determined on western blots (equal amounts of protein loaded in each lane) and then blots were scanned and subjected to densitometry. Data were standardized relative to control values levels and are shown as means ± SEM, n=3 except for n=4 for control and 12 h frozen brain, and n=2 for 1 h and 12 h frozen heart. * - Significantly different from the corresponding control value, $P < 0.05$. From Greenway and Storey (2000).

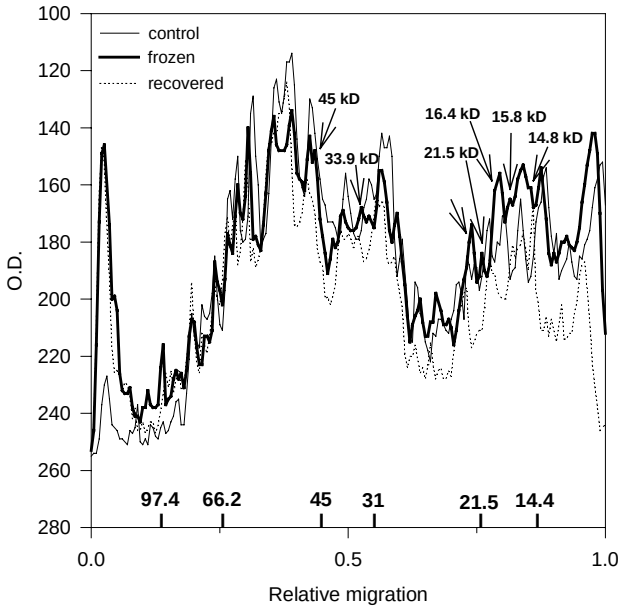
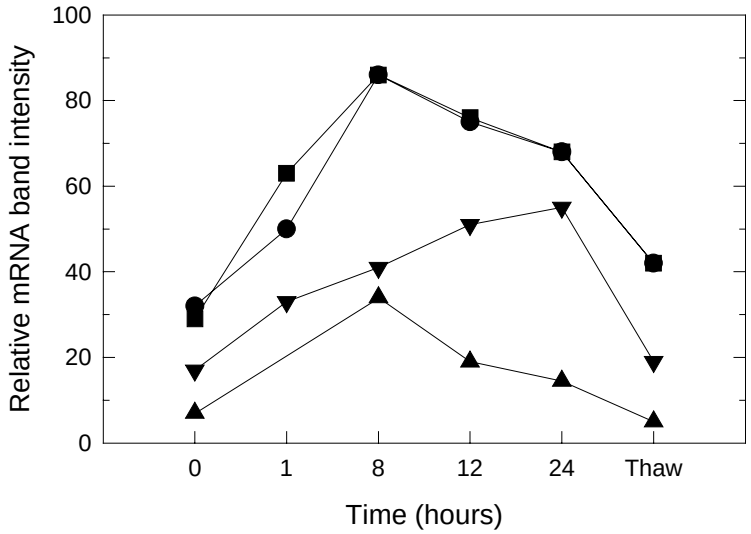


Figure 1

Figure 2



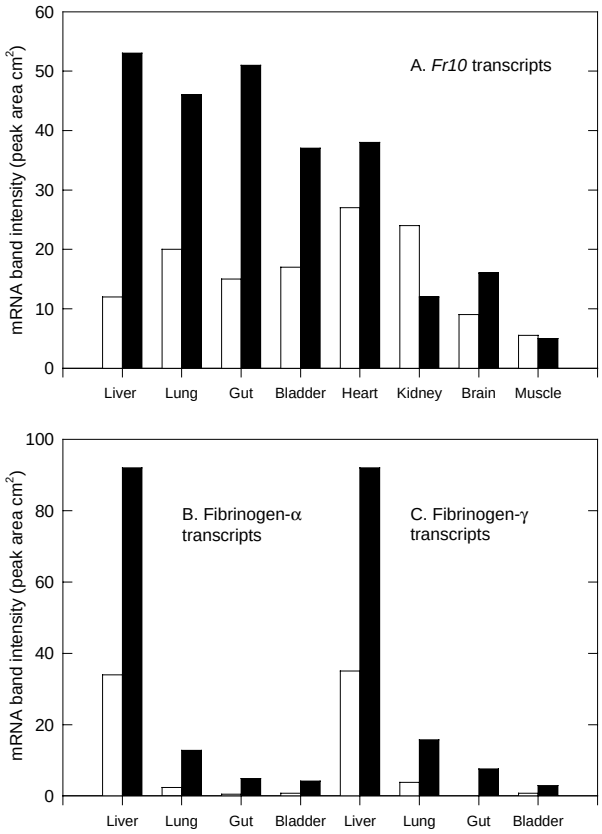


Figure 3

Figure 4

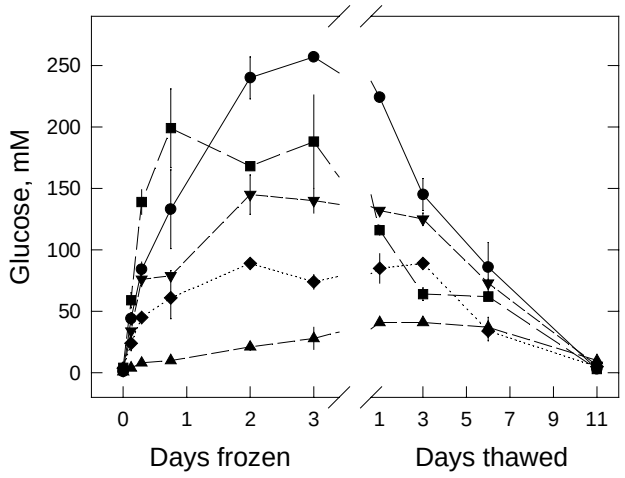


Figure 5

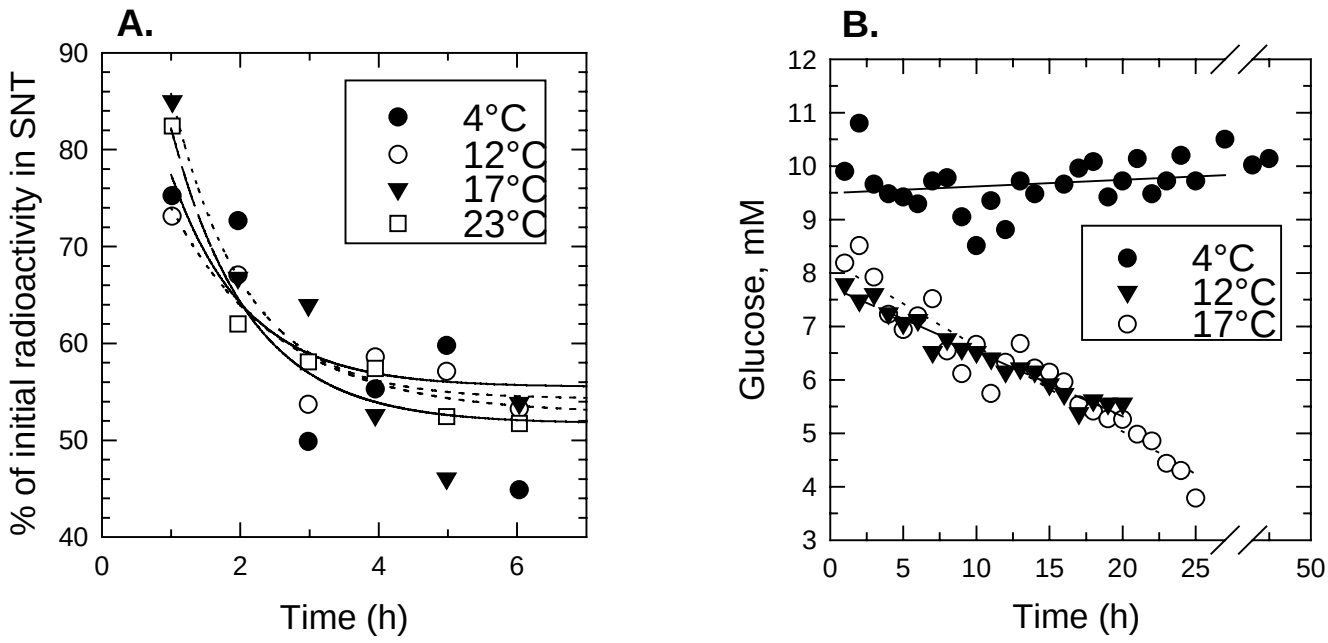


Figure 6

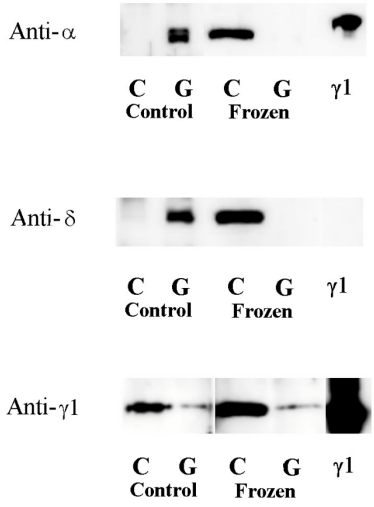
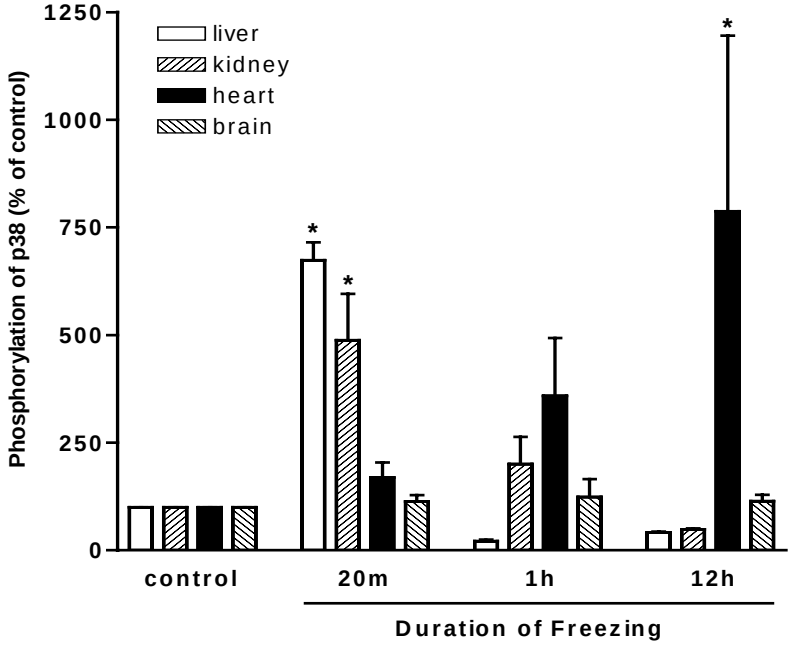


Figure 7


1250
1000
750
500
250
0
Phosphorylation of p38 (% of control)
liver
kidney
heart
brain
*
*
*
control
20m
1h
12h
Duration of Freezing