S. Afr. J. Zool. 1998, 33(2) 55–64

Survival under stress: molecular mechanisms of metabolic rate depression in animals

Kenneth B. Storey
Institute of Biochemistry, Departments of Biology and Chemistry, Carleton University, Ottawa, Ontario, Canada K1S 5B6
E-mail: kbstorey@ccs.carleton.ca

Received 1 September 1997; accepted 4 March 1998

For many species, survival under harsh environmental conditions includes metabolic rate depression, an escape into a hypometabolic or dormant state. Studies in my laboratory are analysing the molecular mechanisms and regulatory events that underlie transitions to and from hypometabolic states in systems including anoxia-tolerant turtles and molluscs, estivating snails and toads, hibernating small mammals, and freeze tolerant frogs and insects. Our newest research targets two areas: the role of protein kinases in regulating metabolic adjustments and the role of stress-induced gene expression in producing specific adaptive proteins. Protein kinases A, C and G are all linked to stress-induced signal transduction in various systems, and new studies also show tissue-specific activation of mitogen-activated protein kinases (ERK, JNK, see list of abbreviations p38). Protein adaptations supporting stress tolerance are being sought using cDNA library screening, differential display PCR and Northern blotting to analyse gene expression. These techniques offer new insights into the types of cellular targets that must be coordinated to achieve metabolic suppression and facilitate easy analysis of organ-, time-, and stress-specific gene expression. For example, freeze-induced gene expression in frog liver includes upregulation of genes for subunits of fibrinogen and ADP/ATP translocase, whereas mitochondrial genes coding for subunits of NADH-ubiquinone oxidoreductase subunit 5 and cytochrome C oxidase subunit 1 were upregulated during anoxia in turtle heart.

For many animal species the ability to enter a hypometabolic or dormant state is a lifesaver. When environmental conditions become stressful and threaten normal life, many organisms simply retreat into a state of suppressed metabolism and remain in this state until favourable conditions return. Metabolic rate depression is a common mechanism for dealing with environmental stress and can range from the nightly torpor of small birds and mammals that lowers metabolic rate by 30–40% for a few hours to the completely ametabolic state of encysted brine shrimp that allows them to survive in a desiccated state for many years. Metabolic rate depression is an integral part of many animal survival strategies including hibernation, torpor, estivation, diapause, anaerobiosis and anhydrobiosis and can be induced by various environmental stresses such as heat or cold, desiccation and oxygen deprivation (Hochachka & Guppy 1987; Wang 1989; Storey & Storey 1990; Crowe, Hoekstra & Crowe 1992; Pinder, Storey & Ultsch 1992). The molecular mechanisms regulating transitions to and from the hypometabolic state have been the subject of research in my laboratory for a number of years and have been reviewed both in general (Storey & Storey 1990) and with respect to particular forms of metabolic suppression (Storey 1993, 1996, 1997; Brooks & Storey 1993a, 1997; Lutz & Storey 1997). The goal of the present article is to discuss some of our recent advances in understanding the biochemistry and metabolic regulation of the phenomenon. I will focus on two areas: protein kinases and their role in controlling transitions to and from the hypometabolic state, and the role of gene expression in creating adaptive changes in specific proteins to enhance stress endurance.

Protein kinases and metabolic regulation

Our studies of the role of protein kinases in the regulation of reversible metabolic suppression arose originally from an exploration of the regulation of pyruvate kinase (PK) in anoxia-tolerant marine molluscs. Control over the activity of PK regulates the catabolism of phosphoenolpyruvate (PEP) through aerobic (via PK) versus anaerobic (via PEP carboxykinase) pathways and gates the entry of carbohydrate into the tricarboxylic acid cycle for aerobic oxidation versus into reactions of succinate and propionate synthesis for anaerobic fermentation (De Zwaan 1977). Allosteric and pH controls on the enzyme had been explored initially as the mechanisms responsible for PK suppression during anoxia (De Zwaan 1977; Hochachka & Mustafa 1972). However, it was then realized that PK existed in two stable forms in tissues of marine molluscs and that the proportions of the two forms changed in response to environmental factors including oxygen availability, temperature and season (Siebenaller 1979; Holwerda, Veenhof, van Heugten & Zandee 1983). The two forms differed in their amounts of covalently bound phosphate and high and low phosphate forms had very different kinetic properties. For PK from whelk radular retractor muscle, for example, the high phosphate form that predominates under anoxic conditions has a much lower affinity for PEP substrate (Km 12-fold higher than the aerobic form of PK), is less sensitive to the activator fructose-1,6-bisphosphate (Ka 24-fold higher than aerobic PK), and is much more sensitive to inhibition by L-alanine (I_{50} 490-fold lower than aerobic PK) (Plaxton & Storey 1984). Coupled with the large accu-mulation of alanine as a metabolic end product during anoxia, these changes in the properties of PK in anoxic muscle render the enzyme virtually inactive in the anoxic cell and, hence, facilitate carbon flow into pathways of anaerobic fermentation.

It is now known that reversible phosphorylation control of PK as the means of regulating the aerobic versus anaerobic dismutation of glycolytic carbon applies widely to many species of marine molluscs as well as to many other invertebrates that ferment carbohydrate to succinate and volatile fatty acids under anoxic conditions (Storey 1993). It then became appar-

ent that reversible protein phosphorylation had wider functions than just the control of the PK locus. Studies showed that the phosphorylation state of other enzymes of glycolysis (e.g. glycogen phosphorylase, 6-phosphofructo-1-kinase, 6-phosphofructo-2-kinase) changed in response to aerobic versus anoxic signals, and furthermore, that PK conversion to a less active, high phosphate form also occurred with the transition into a state of aerobic dormancy (e.g. during estivation) (Storey & Storey 1990; Storey 1993). Hence, it was clear that reversible protein phosphorylation had a broader role in metabolic control and was central to the control of enzymes of energy metabolism, and by implication, the net rate of ATP production, in various arrested states. Changes in the phosphorylation state of enzymes, causing changes in enzyme properties and activity state, have now been linked with glycolytic control in numerous invertebrate systems as well as in anoxia-tolerant vertebrates including freshwater turtles and goldfish (for review Storey & Storey 1990; Storey 1993, 1996). Furthermore, comparable regulation of glycolytic enzymes has been documented in several systems of aerobic dormancy including estivation in land snails and spadefoot toads and hibernation in small mammals (Storey & Storey 1990; Storey 1997; Brooks & Storey 1997; Cowan & Storey 1998). In aerobic systems, reversible phosphorylation also suppresses the activity of pyruvate dehydrogenase in the dormant state thereby providing the means of coordinating carbohydrate flux through glycolysis and the tricarboxylic acid cycle.

Control over the rate of energy production by cells is only one side of the story, however, and to achieve a stable metabolic suppression that is sustainable over long periods of time also requires control over the rate of energy utilisation by cells, so that net rates of ATP production and utilisation are reduced in concert. Hence, various of the major energy-consuming functions of cells (e.g. ion pumps, ion channels, protein synthesis, protein degradation) have also been analysed in recent years in anoxia-tolerant, hibernating, and estivating animal systems. In general, all appear to be suppressed in the hypometabolic state and in many cases reversible protein phosphorylation is implicated as one of the key regulatory mechanisms involved (Storey 1996; Lutz, Nilsson & Perez-Pinzon 1996; Hand & Hardewig 1996; Hochachka, Buck, Doll & Land 1996). In particular, reversible phosphorylation control over the relative activities of membrane ion channel proteins versus ATP-driven membrane ion pumps is central to the maintenance of membrane potential difference in the face of sharply reduced ATP production in hypometabolic systems.

Given the major importance of reversible protein phosphorylation in regulating transitions to and from the hypometabolic state, we turned our attention in various recent studies to analyses of the protein kinases and protein phosphatase enzymes involved. Some of our findings for anoxia-tolerant molluscs and turtles are summarised below.

Protein kinases and phosphatases in anoxia-tolerant marine molluscs

Anoxia-induced phosphorylation of PK in the marine whelk *Busycon canaliculatum* (as well as in various other molluscan species) is mediated through the action of the second messenger, cyclic guanosine 3',5' monophosphate (cGMP), and cGMP-dependent protein kinase. This was confirmed through experiments that monitored PK kinetics after incubation of crude muscle extracts with Mg^{2+} + ATP in the presence of cAMP, cGMP or Ca^{2+} + phorbol 12-myristate 13-acetate, the second messengers of protein kinase A (PKA), protein kinase G (PKG) and protein kinase C (PKC), respectively (Brooks & Storey 1990). Isolated tissue incubations further confirmed a lack of PKA or PKC involvement (Brooks & Storey 1989a). However, PKG itself does not appear to directly phosphorylate PK but, rather, appears to phosphorylate a PK-kinase enzyme which in turn phosphorylates PK. This PK-kinase has been purified and characterised from whelk foot muscle. It shows some properties that are similar to casein kinase type I in mammalian systems but lacks the activation by spermine or salt that characterises the mammalian kinase (Brooks & Storey 1991). PK-kinase activity separated from the PKG activity in whelk muscle during purification and did not phosphorylate either phosphofructokinase or glycogen phosphorylase, which are other targets of anoxia-induced phosphorylation. This suggests that PK-kinase is part of a regulatory cascade and that its role is quite specific to the control of PK. Hence, the mechanism of anoxia-induced PK inactivation in molluscs is this. Anoxia exposure stimulates cGMP formation, which activates PKG. PKG catalyses phosphorylation of several protein targets, among them PK-kinase which in turn phosphorylates and inactivates PK.

Anoxia-induced phosphorylation of PK is reversed by the action of a specific phosphatase and we have recently examined this enzyme from whelk foot muscle (Brooks & Storey 1996). Detection of the PK-phosphatase was via its ability to relieve L-alanine inhibition of the enzyme, for the dephosphorylation of foot PK elevates the inhibitor constant (I_{50}) for L-alanine about 200-fold. PK-phosphatase is a serine-threonine neutral phosphatase with a pH optimum of 6.5 and a molecular weight of 41 kdaltons. The enzyme was very sensitive to Mg^{2+} ions with a very low K_m (7.9 µM) and was not affected by spermine or fluoride. Hence, its properties fit those of a Type 2C protein phosphatase. PK-phosphatase was also unaffected by cAMP or cGMP or by conditions that would promote its phosphorylation *in vitro*. This is again typical of a Type 2C protein phosphatase and shows that the enzyme differs from the Type 1 phosphatase that is responsible for PK dephosphorylation in mammalian organs. Type 1 phosphatases are integrated into complex regulatory cascades via their susceptibility to phosphorylation by protein kinases which alters their activities (Cohen 1989). The net result is that activities of kinases and phosphatases are often inversely regulated. In whelk tissues, however, it appears that PK-phosphatase may not be regulated and, hence, that control over PK activity during aerobic-anoxic transitions may be primarily due to changes in kinase activities (PKG and PK-kinase). This means that anoxia-induced PK phosphorylation would occur owing to enhanced kinase activities against a 'background' of constant PK-phosphatase activity.

Protein kinases and phosphatases in anoxia-tolerant turtles

The response of turtles to anoxic submergence and/or nitrogen gas inspiration has two distinct phases. The first is a compen-

satory phase that occurs during the hypoxia transition period, where glycolytic rate is elevated to make up for a shortfall in ATP availability from oxidative phosphorylation. However, when low oxygen stress becomes severe, compensation is abandoned in favour of conservation and a coordinated suppression of the rates of all metabolic processes occurs, including glycolytic rate. Although not as prominent and consistent a regulatory mechanism as in molluscs, anoxia-induced reversible phosphorylation also alters the activities of various enzymes in freshwater turtles, *Trachemys scripta elegans*, in an organ-specific manner. Anoxia-induced changes are particularly evident in liver where they promote a 2-phase response, an initial activation of glycogen phosphorylase and inhibition of PFK that promotes glucose output as a fuel for other organs, followed by a later suppression of metabolic activity as part of the overall metabolic rate depression (Brooks & Storey 1989b; Mehrani & Storey 1995a). In another series of studies we have investigated the components of intracellular signal transduction in turtles, analysing the roles of protein kinases and protein phosphatases in mediating anoxia-induced metabolic responses.

Table 1 shows that turtle liver responds to anoxia exposure with coordinated changes in the activities of two protein kinases (PKA, PKC) and protein phosphatase type-1 (PP-1) (Mehrani & Storey 1995b,c, 1996). The short-term response, apparent after 1 h anoxic submergence at 7°C, is an activation of PKA and PKC and an inhibition of PP-1. PKA changes are the result of elevated cAMP levels (by 57%) which stimulate the dissociation of the inactive tetramer of PKA (containing two regulatory and two catalytic subunits) to release the free catalytic subunits. Thus, the percentage of PKA present as the free catalytic subunit rose from 6.4% in control liver to 14.7% after 1 h anoxia exposure.

PKC is regulated *in vivo* by its distribution between an inactive cytosolic pool and a membrane-bound active enzyme (Kikkawa, Kishimoto & Nishizuka 1989). Total PKC in turtle liver remained constant at 38–49 U/mg during anoxia but the amount of membrane-bound activity rose 2.4-fold after 1 h of anoxia (and the percentage bound doubled) (Table 1). Oppositely, the activity of liver PP-1 was suppressed by one-third within the first hour of submergence. PP-1 is the major phosphatase involved in glycogen metabolism, and when active, it catalyses the dephosphorylation-mediated inactivation of glycogen phosphorylase and phosphorylase kinase (and the opposite activation of glycogen synthetase) (Cohen 1989).

With longer submergence, however, the amounts of active protein kinases fell again; the percentage of membrane-bound PKC reverted to control levels whereas the percentage of PKA present as the active catalytic subunit (PKAc) fell to about half of the control value (Table 1). However, PP-1 activity did not respond oppositely by increasing but remained suppressed during prolonged anoxia. Activities of PKAc and PP-1 are typically regulated oppositely in mammalian cells since they generally mediate opposing catabolic versus anabolic signals. The fact that activities of both enzymes were suppressed in a coordinated manner with longer term anoxic submergence supports the idea that both catabolic and anabolic pathways are targeted as part of the overall 5–10-fold suppression of metabolic rate that constitutes the energy conservation response to long term anoxic submergence in turtles (Herbert & Jackson 1985).

In general, turtle liver PKA, PKC and PP-1 share many properties in common with the enzymes that have been characterised from mammals indicating that properties of these signal transducing enzymes are highly conserved among vertebrates (Mehrani & Storey 1995b,c; 1996). The same is true of turtle brain PKC which is present in three major isoforms, as in mammalian brain, and which also changes its distribution between cytosolic and membrane-associated forms in response to anoxia (Brooks & Storey 1993b). Thus, the elements of a signal transduction system can remain highly conserved even while very different net effects of the signals can be achieved in different tissues and organisms by changing the target proteins of kinase or phosphatase action and/or

Table 1 Effect of anoxic submergence at 7°C on the concentration of cAMP and the activities of cAMP-dependent protein kinase (PKA), protein kinase C (PKC) and protein phosphatase type 1 (PP-1) in turtle liver

	Total PKA U/gww	% PKAc	[cAMP] pmol/gww	PKC-membrane U/mg	% membrane bound	PP-1 U/gww
Aerobic	15.8 ± 1.42	6.4 ± 1.0	872 ± 76	9.25 ± 1.97	21	31.1 ± 0.7
Anoxic						
1 h	17.4 ± 2.43	14.7 ± 2.8*	1371 ± 71*	22.3 ± 1.45*	45	19.8 ± 3.9*
5 h	22.9 ± 2.50	2.1 ± 0.8**	892 ± 50**	12.5 ± 1.27	28	22.8 ± 0.8*
20 h	20.3 ± 4.35	3.7 ± 1.1**	907 ± 66**	7.61 ± 2.19	20	19.4 ± 2.0*

One unit of PKA activity is defined as the amount of enzyme that catalyses the incorporation of 1 nmol of phosphate onto kemptide per minute at 21°C; percentage of the enzyme present as the free catalytic subunit (PKAc) is determined from assays in the absence versus presence of 10 μM cAMP. One unit of PKC activity is defined as the amount of enzyme that catalyses the incorporation of 1 pmol of phosphate onto histone per minute at 30°C. Membrane versus cytosolic fractions of PKC activity were separated via centrifugation at 100,000 × *g*. Total PKC (membrane + cytosolic) did not vary over the course of anoxia, ranging from 38–49 U/mg protein. One unit of PP-1 activity is defined as the amount of enzyme that catalyses the release of 1 nmol of phosphate from ^{32}P-labeled phosphorylase per minute at 30°C. Data are from Mehrani & Storey (1995a,b; 1996).

* — Significantly different from the corresponding control value, $p < 0.01$; ** significantly different from the value after 1 h anoxia exposure, $p < 0.01$.

modifying the consequences of phosphorylation on the functions of the target proteins.

However, turtle PKAc also showed some interesting low temperature effects on its activity which could be significant with respect to the winter hibernation of the animals underwater. When temperature effects on enzyme maximal velocity were plotted as an Arrhenius plot (log V versus 1/°K), two distinct linear sections were seen with a break in the slope at about 15°C. These gave calculated activation energies for the enzyme of 27.9 ± 1.85 kJ/mol at temperatures greater than 15°C and 115 ± 2.5 kJ/mol at temperatures less than 15°C. The 4-fold increase in activation energy at low temperatures means that PKAc activity drops sharply within the temperature range (generally 0–10°C) that is commonly experienced by turtles during submerged winter hibernation. Such strong low-temperature effects on the enzyme could contribute to metabolic arrest by minimising various PKA-mediated cellular responses during hibernation. Indeed, it is well known that the extent of anoxia-induced metabolic rate depression (measured by whole body calorimetry) is much greater at temperatures below 10°C (reduced to about 10% of aerobic rate at the same temperature) than at warmer temperatures (about 20% of aerobic rate) (Herbert & Jackson 1985). Strong negative effects of low temperature on PKAc, modifying the enzyme's normal role in signal transduction, could be one factor that underlies this greater suppression of metabolic rate at low temperatures.

Calcium and phospholipid dependent protein kinase C in turtle liver was present in two isoforms, α and β, which closely resembled their mammalian equivalents. The β form of PKC predominated and in liver of control turtles the membrane-associated, active enzyme was virtually all of the β type. As described above, 1 h anoxic submergence greatly increased membrane-associated PKC activity and this represented a translocation of 40% of PKC β and more than 80% of PKC α from the cytosol to the membrane (Mehrani & Storey 1997). However, with longer anoxia exposures, the PKC distribution reverted to one that was similar to the control. Turtle PKC was responsive to the typical inhibitors of the mammalian enzyme and both isoforms were activated by micromolar levels of Ca^{2+} and by 12-O-tetradecanoylphorbol-13-acetate and phospholipids. Unlike mammalian PKC, however, the turtle liver enzymes were not activated by trypsin treatment and they were substantially activated by alternative phospholipids in addition to phosphatidylserine (PS). Phosphatidylinositol (PI) could substitute for PS in giving maximal activation of PKC α, and PKC β reached 82% of maximal activity in the presence of PI. Lysophosphatidylinositol was also an effective activator. This indicates that there is scope for regulating turtle PKC *in vivo* by phospholipids other than just PS and that the role of alternative phospholipids in PKC control may possibly be in regulating the enzyme at different body temperatures. Although we have not pursued this idea farther with turtle PKC, a recent study of PKC from another source supports this.

Protein kinase C in hibernating bats

Hibernating small mammals exhibit a strong metabolic rate depression; while torpid, oxygen consumption is typically lowered to only 1–5% of the euthermic, resting rate and body temperature can fall to near 0°C (Geiser 1988). Although early studies ascribed the drop in metabolic rate during hibernation to the decrease in body temperature, it is now known that the opposite is true. Entry into torpor is due to the coordinated regulation and suppression of multiple cellular functions whose net result is the decrease in metabolic heat production that lowers body temperature. One of the mechanisms involved in metabolic suppression is the reversible phosphorylation of key target proteins (Storey & Storey 1990; Storey 1997), mediated by the controlled actions of protein kinases and protein phosphatases.

PKC may be one of the kinases involved and recently we analysed the enzyme from brain of the hibernating bat, *Myotis lucifugus* (Mehrani & Storey 1997). Maximal activity of bat brain PKC γ was reduced in the torpid state to 63% of the value in awake, euthermic bats, consistent with metabolic rate depression, but enzyme distribution between cytosol and membrane was unaltered. Mammalian brain typically exhibits three isoforms of PKC (α, β and γ) (Kikkawa *et al.* 1989) but, intriguingly, brain tissue sampled from bats during the hibernating season (January) contained only the γ isoform of PKC. Furthermore, rat and bat brain PKC differed strongly in their responses to phospholipid activators. Rat brain PKC γ was strongly activated by PS but largely insensitive to other phospholipids. However, bat brain PKC γ was activated by PS, PI, phosphatidylcholine (PC) and phosphatidylethanolamine (PE). The ratio of V_{max} activities (in the presence of 1,2-dioleoyglycerol) was 100:81:33:42 for PS:PI:PC:PE for PKC γ from brain of euthermic bats and 100:91:45:35 for the enzyme from hibernating bats (phospholipids present at 50 μM each) (Mehrani & Storey 1997). In addition, activation of bat brain PKC γ by PS was temperature sensitive. PS was a much less effective activator at 4°C (hibernating body temperature) where it produced only a 3.5-fold maximal activation, compared with a 14–18-fold activation by PS when assayed at 33°C (near euthermic temperature). Furthermore, Arrhenius plots of bat brain PKC γ maximal activity showed a sharp break in slope at about 10°C. Again, as for turtle PKC, activation energies increased greatly at low temperature; brain PKC from hibernating bats showed a mean activation energy of 12.6 ± 1.6 kJ/mol over the 10–42°C range compared with an 11.5-fold higher value, 145 ± 8.6 kJ/mol over the 0–10°C range (Mehrani & Storey 1997). This latter gave a Q_{10} value for the reaction of 8.13 over the 1–11°C range. By contrast rat brain PKC γ showed a linear Arrhenius plot over the full temperature range tested from 0 to 42°C with a calculated Q_{10} of about 1.2.

Clearly, then, PKC γ from brain of a hibernating mammal is substantially different from the enzyme in brain of a nonhibernating mammalian species and exhibits high sensitivity to cold temperatures and strong suppression at temperatures < 10°C. These differences may lead to significant changes in the functional capacity of the PKC signal transduction pathway at the low body temperatures of the hibernating state and this could be important to the development and maintenance of metabolic rate depression. Furthermore, one of the 'layers' of regulatory control on PKC that may be important in determining enzyme responsiveness in the hibernating state may come from modulation of phospholipid make-up. Given that bat PKC is responsive to multiple phospholipid types and that

activation by at least one of these (PS) is highly temperature-sensitive (temperature effects on enzyme activation by other phospholipids have not been tested), changes in the types of phospholipids present in the environment of PKC or changes in enzyme sensitivity to different phospholipids at different body temperatures could provide differential control over enzyme activity. Interestingly, a recent study of ground squirrel sarcoplasmic reticulum showed changes in the composition of PE, PC and PI fractions between the hibernating and aroused states (Pehowich 1994) which suggests that PKC may be exposed to different phospholipid types in these two states.

Other types of stress-induced protein kinases

Overall, then, we have strong evidence of the importance of protein kinases and protein phosphatases in mediating and regulating transitions to and from hypometabolic states in a variety of animal types and in response to a variety of different environmental stresses. Whereas roles for the protein kinases and phosphatases commonly associated with intermediary energy metabolism (e.g. PKA, PKG, PKC, PP-1) are well-established in several systems, whole new 'worlds' of protein kinases remain to be explored to seek out their roles in mediating metabolic suppression. In recent years, research on protein kinases has grown explosively and multiple new families of protein kinases have been identified whose members increase continuously. Protein kinases and protein phosphatases participate in the regulation of virtually every aspect of metabolism and for the comparative biochemist offer rich opportunities to explore the adaptation of both the enzymes themselves and the regulatory roles that they assume in facilitating organismal survival in stressful environments. We are now exploring the possible roles of tyrosine kinases and of members of the mitogen-activated protein kinase (MAPK) family in mediating adaptive responses to environmental stress. These are producing fascinating results. For example, we are finding both organ-specific and stress-specific (anoxia versus freezing) responses in turtles by three members of the MAPK family, extracellular signal-regulated protein kinases (ERKs), c-Jun NH_2-terminal kinases (JNKs), and p38 (S. Greenway and K. Storey, manuscripts submitted for publication). In mammalian systems, ERKs are primarily responsive to growth factors and mitogens but can also be activated by hypoxia or osmotic stresses (Seko, Tobe, Ueki, Kadowaki and Yazaki, 1996; Matsuda, Kawasaki, Moriguchi, Gotoh, & Nishida 1995). JNKs are weakly activated by growth factors but respond to UV irradiation, heat shock, and protein synthesis inhibitors (Kyriakis, Banerjee, Nikolakaki, Dai, Rubie, Ahmad, Avruch & Woodgett 1994) and are activated by reperfusion following ischemia, mechanical stretch, or osmotic shock (Pombo, Bonventre, Avruch, Woodgett, Kyriakis & Force 1994; Galcheva-Gargova, Dérijard, Wu, Davis 1994; Knight & Buxton 1996). The third MAPK, p38, is typically activated by various environmental stresses, including hyperosmolarity (Han, Lee, Bibbs & Ulevitch 1994; Raingeaud, Gupta, Rogers, Dickens, Han, Ulevitch & Davis 1995). When initial assessments of these three MAPKs were made in multiple organs of adult red-eared slider turtles (*T.s. elegans*), we found that activities of ERKs were elevated only in spleen during anoxia exposure with activities 2.8-fold higher in 20 h anoxic animals compared with controls. This suggests a very specific role for ERKs in this organ. However, JNK was activated by anoxia in liver, kidney and heart with peak activity (4-fold higher than aerobic controls) measured after 5 h of anoxic submergence. The same responses to anoxia exposure by ERKs and JNK were documented with newly-hatched juvenile turtles but when the stress was freezing, the results were quite different. Hatchling *T.s. elegans* have some rudimentary freeze tolerance although it is not as well-developed as in their northern cousins, the painted turtles (Storey & Storey 1992). Analysis of responses to freezing exposure (up to 4 h post-nucleation at –2.5°C) by juvenile *T.s. elegans* showed that ERKs were stress-activated in brain of frozen turtles whereas JNK activities were generally suppressed during freezing. The third MAPK type, p38, was activated in both liver and heart during freezing, perhaps in response to the osmotic stress associated with extracellular freezing. It is obvious, then, that many more facets of protein kinase regulation in the control of stress-induced metabolic rate depression remain to be explored. Significantly, most of the known major functions of MAPKs are in the regulation of gene expression (Karin 1995) which suggests that we may ultimately find links between the actions of these protein kinases and the stress-induced expression of selected genes which is currently another major focus of ongoing research in my lab.

Stress-induced protein synthesis and gene expression

Until very recently, one of the constraints on the search for biochemical adaptations supporting animal endurance of environmental stress was that, to some degree, prior knowledge of the adaptations that you were seeking was necessary. Thus, the major or most 'visible' adaptations supporting stress tolerance have received the most attention and studies have progressed from these 'entry' points to follow different leads. For example, studies of cold tolerance in insects have put major emphasis on cryoprotectant metabolism, studying the regulation, synthesis and function of the polyhydric alcohols that accumulate in molar levels to provide antifreeze protection. Studies of anoxia tolerance focused for a long time on mechanisms of fermentative ATP production that enhanced glycolytic output and studies of mammalian hibernation have focused strongly on the regulation of heat production by brown fat and on the neuroendocrine control of body temperature. What has been missing is a way to know whether these 'highly visible' adaptations are, in fact, the only important adaptations that are required to confer stress tolerance on an organism. Conceivably, various key adaptations could be missed if these leave no readily detectable 'footprint'. Specific synthesis of selected key proteins is clearly a part of animal response to environmental stress both for stress-intolerant species (e.g. heat-shock proteins; Schlesinger, Ashburner & Tissieres 1982) and stress-tolerant organisms. For example, several recent studies have assessed anoxia-induced protein synthesis in turtles (Brooks & Storey 1993c; Land & Hochachka 1995; Douglas, Giband, Altosaar & Storey 1994) and freeze-induced protein synthesis in freeze-tolerant wood frogs *Rana sylvatica* (Lobsinger 1996; Storey, Storey & Churchill 1997). These studies took two approaches to com-

pare control versus experimental animals: *in vivo* labeling of newly synthesized proteins with radiolabeled amino acids and *in vitro* translation of tissue mRNA in the presence of ^{35}S-methionine. Following polyacrylamide gel electrophoresis to separate labeled protein products, autoradiography and scanning, the different patterns of labeled proteins in control and experimental situations were analysed. Studies with wood frogs compared ^{35}S-labeled protein patterns in organs 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 both techniques revealed freeze-induced and thaw-induced proteins with prominent labeling of low molecular weight proteins, 15–20 kD, in both cases (Storey, Storey & Churchill 1997; Lobsinger 1996). Studies of anoxia effects on protein synthesis *in vivo* in hatchling *T. s. elegans* failed to detect differences in ^{35}S-methionine protein labeling patterns under anoxia, but a new protein of 72 kdaltons was present in both liver and skeletal muscle of animals after 20 h aerobic recovery (following 20 h anoxia) (Brooks & Storey 1993c). However, in a different approach, *in vitro* incubation of isolated turtle hepatocytes with ^{14}C-leucine showed enhanced expression of proteins of 83, 70, 43, 35 and 16 kdaltons during anoxic incubations as well as suppression of five other protein types (Land & Hochachka 1995). *In vitro* translation of mRNA isolated from tissues of aerobic versus anoxic adult turtles showed an organ-specific appearance of new protein types in anoxic organs including, in liver, a novel 19.5 kdalton protein and increased amounts of 28.6 and 79.9 kdalton proteins (Douglas *et al.* 1994). New proteins of 28.6 kdaltons in heart and 37.5 kdaltons in red muscle also appeared during anoxia whereas kidney showed a new 32.8 kdalton protein during the aerobic recovery period. Overall, although results are conflicting, these studies clearly showed that multiple new proteins were induced in organ-specific patterns by anoxia or freezing exposure in stress-tolerant animals. However, the studies did little to characterise the proteins (beyond providing isoelectric points and subunit molecular weights) and could not identify them.

New techniques of molecular biology now offer the key to identifying the full range of genes and proteins that are expressed as adaptive responses to stress as well as excellent techniques for studying the regulation of expression and for producing recombinant protein in large amounts for analysis of protein properties. A cDNA library is first constructed from tissue sampled under an experimental stress condition (such as anoxia or freezing). The library is then screened with ^{32}P-labeled cDNA probes made from mRNA isolated from control versus experimental tissues, and clones that are differentially induced or upregulated under the stress condition are identified. These are isolated and cross-hybridised to eliminate duplicates. Unique clones are then confirmed as stress-positive by Northern blotting and finally sequenced. Gene sequences and/or deduced protein sequences can be compared with thousands of gene and protein sequences now stored in sequence banks and, in most cases, this allows identification. Studies can then be designed to assess the regulation of gene (e.g. via Northern blots) or protein (via Western blots) expression and to determine the adaptive functions that the proteins play in cellular response to stress. Recent studies in my lab have been using molecular biology techniques for just such purposes with novel and unexpected results that are contributing new advances to their fields.

Freeze-induced gene expression in frogs

An excellent case in point was afforded by our first foray into this new technology, which we used to search for genes that were induced by freezing exposure in liver of the freeze tolerant wood frog *Rana sylvatica*. The hibernation strategy of the wood frog, which winters on the forest floor, includes the ability to endure the freezing of up to 65% of its total body water as ice in extracellular and extraorgan fluid spaces (Storey & Storey 1992). The biochemical adaptations that underlie this capacity have been the subject of numerous studies in my laboratory and others, with a major emphasis to date on the regulation and functions of the natural cryoprotectant, glucose, which accumulates in levels up to 200–300 mM in plasma and cells. Among the known protein adaptations that we had identified over the years as aiding freeze tolerance were seasonal and/or freeze induced increases in the amounts of several enzymes in liver that are involved in glucose synthesis, elevated amounts of glucose transporter proteins in the plasma membranes of organs to facilitate glucose distribution, and the appearance of ice nucleating proteins in blood that help to guide ice formation (Storey, Mosser, Douglas, Grundy & Storey 1996). In addition, both *in vivo* labeling of newly synthesized proteins with ^{35}S-methionine and *in vitro* translation of message RNA revealed the existence of freeze-specific proteins without identifying them (discussed above).

We began our studies of freeze-induced gene expression in wood frogs by constructing and screening a cDNA library made with mRNA isolated from liver of freezing-exposed frogs (24 h at –2.5°C) (Cai & Storey 1997a). The library was screened with ^{32}P-labeled single-stranded total cDNA probes made from poly(A$^+$)RNA isolated from control (5°C-acclimated) versus freezing-exposed frogs. From this, five unique cDNA clones that were upregulated by freezing were isolated when about 80,000 plaques were screened. Two clones showed sequence homology with the genes for the α and β subunits of fibrinogen (Cai & Storey 1997a), a plasma clotting protein that is produced by liver (Weissbach & Grieninger 1990). The gene for ADP/ATP translocase was also upregulated (Cai, Greenway & Storey 1997) as was a gene encoding a novel 10 kD protein with a nuclear exporting sequence (Cai & Storey 1997b). None of these proteins had any obvious relationship to any of the known adaptations associated with freeze tolerance, but each now suggests new avenues of research to be explored. Hence, these results show the clear advantages of a molecular biology approach for identifying both new proteins and new cellular functions involved in adaptation to environmental stresses.

The upregulation of fibrinogen biosynthesis by freezing we interpret as indicating that enhanced mechanisms of tissue damage repair are an important part of freeze/thaw survival. This represents a totally new function that is part of the overall package of adaptations that support freeze tolerance. Most studies in the field of freeze tolerance to date have focused on the cryoprotective mechanisms that are used to regulate ice formation and minimise the impact of extracellular freezing on cells. These new results, which suggest that plasma clotting capacity is elevated during freezing, provide the first

indication that freeze tolerant animals probably experience some amount of tissue damage by ice; for example, we sometimes observe hematomas in leg muscles after thawing. Hence, natural freeze tolerance probably includes damage repair mechanisms that deal with any physical injuries to organ microvasculature as the result of ice expansion within delicate capillaries. Indeed, physical damage by ice to organ microcapillary beds, and the resulting loss of vascular integrity after thawing, is a primary cause of freezing damage in cryopreserved tissues and organs (Rubinsky *et al.* 1990). It is intriguing, therefore, that a naturally freeze tolerant species shows an adaptation that expressly deals with this issue.

The technique of Northern blotting hybridises radiolabeled probes from the clones of interest against total RNA isolated from tissues and separated on agarose gels. When RNA loading is standardised, the relative intensities of bands on autoradiograms provide a good estimate of changes in the message RNA content of the genes under study as the result of experimental manipulation (e.g. a time course of stress/recovery) or in different tissues of the animal. Thus, it is easy to screen through multiple tissues or experimental conditions to find where and when a gene is most strongly expressed. The results of this analysis provide added information about the function and the regulation of the induced genes and their associated proteins. Again, our studies with freeze tolerant frogs illustrate the power of these techniques. Northern blotting was used to assess the expression of the genes for α and γ subunits of fibrinogen in three situations: (1) in liver sampled over a time course of freeze-thaw (expression reached maximum after 8 h freezing), (2) in different organs of control, frozen and thawed frogs (substantial freeze-induction was found in lung and gut, trace amounts in heart and bladder), and (3) in response to other stresses imposed on frogs: anoxia and dehydration (Cai & Storey 1997a). The results of the latter experiments are the most interesting with respect to our topic of metabolic depression. Extracellular freezing places two major stresses on cells and organs: dehydration owing to the outflow of water into extracellular ice crystals, and ischemia imposed by the interruption of blood flow. The different biochemical adaptations that are expressed as part of natural freeze tolerance may be triggered by, and have their actions affect, one of these component stresses. Wood frogs can endure both anoxia exposure and dehydration when administered as individual stresses (Holden & Storey 1997) and, hence, we analysed the expression of fibrinogen genes in liver samples taken from frogs that were either (a) exposed to a nitrogen gas atmosphere for up to 48 h at 5°C or (b) dehydrated to 20 or 40% of total body water lost. In liver from anoxic frogs, mRNA transcripts for fibrinogen genes were virtually completely absent. However, in dehydrated frogs, mRNA transcript levels were greatly enhanced with induced levels similar to the maximum found in liver of frozen frogs. Hence, it was clear that dehydration, like freezing, strongly upregulated fibrinogen gene expression, suggesting that the fibrinogen response is part of a suite of adaptations that appear to be cell-volume regulated in freeze-tolerant frogs (Storey *et al.* 1996). Anoxia exposure, however, downregulated the genes, and this may be part of a general suppression of protein biosynthesis in liver in the energy-starved state.

Exactly the opposite response was found when mRNA levels for mitochondrial ADP/ATP translocase, another gene that was upregulated by freezing exposure, were assessed (Cai *et al.* 1997b). Northern blots revealed that mRNA levels were strongly elevated within 1 h of anoxia exposure in wood frog liver and remained elevated after 24 h anoxia. By contrast, the loss of 40% of total body water had no effect on the expression of the gene. These results indicate that induction of the ADP/ATP translocase gene by freezing (maximal after 8 h freezing exposure) is associated with the progressive oxygen limitation that builds as ice penetrates towards the body core and not with changes in cellular hydration or volume.

Anoxia-induced gene expression in turtles

Other studies have begun to explore the role of gene expression in anoxia tolerance of the freshwater turtle *T. s. elegans* (Cai & Storey 1996, 1997c). We constructed a cDNA library from heart of anoxia-exposed adult turtles and differentially screened with ^{32}P-labeled single stranded cDNA probes from heart of control versus anoxic animals to clone genes induced by anoxia stress. Four unique cDNA clones were obtained and confirmed to be upregulated in response to anoxic submergence (20 h in N_2 bubbled water at 7°C). Interestingly, three of these were derived from the mitochondrial genome. The clone, pBTaR914, was homologous to the mitochondrial WANCY (tryptophan, alanine, asparagine, cysteine and tyrosine)tRNA gene cluster (Cai & Storey 1997c). The clone pBTaR20 had a 1597-bp cDNA sequence containing a single open reading frame that was very close to full length and could potentially encode a polypeptide with 508 amino acids (Cai *et al.* 1996). Its deduced polypeptide sequence showed approximately 83% of the residues identical with the sequence of cytochrome C oxidase subunit I (COI) that is encoded by the mitochondrial gene *Cox1*. The clone pBTaR63 contained a 1837-bp sequence with a single, potentially full length, open reading frame that could encode a polypeptide of 591 residues (Cai *et al.* 1996). This was highly homologous to another mitochondrial protein, NADH-ubiquinone oxidoreductase subunit 5 (ND5), encoded by the mtDNA gene *Nad5*. NADH-ubiquinone oxidoreductase (complex I) and cytochrome c oxidase (complex IV) are two key enzymes of the electron transport chain that catalyse the transfer of electrons from NADH to ubiquinone and from cytochrome c to dioxygen, respectively. They are complex polymeric enzymes, the largest known complex I in bovine heart containing 41 polypeptide subunits (Ohnishi 1993) whereas typical mammalian complex IV has 13 dissimilar subunits as well as two heme and two copper moieties (Azzi, Muller & Labonia 1989). Six of the subunits of complex I and three of complex IV are encoded on the mitochondrial genome and the rest are nuclear.

Analysis of the time course of expression of *Cox1* and *Nad5* by Northern hybridisation analysis showed that mRNA transcripts for both accumulated rapidly (within 1 h) in response to anoxic submergence at 7°C (Figure 1). Transcript levels were 4.5- and 3-fold higher after 1 h anoxia exposure for *Cox1* and *Nad5*, respectively. After longer anoxia exposures (5 or 20 h) *Nad5* mRNA levels remained high whereas *Cox1* mRNA content declined somewhat; mRNA transcripts of both genes had returned to near control levels when turtles

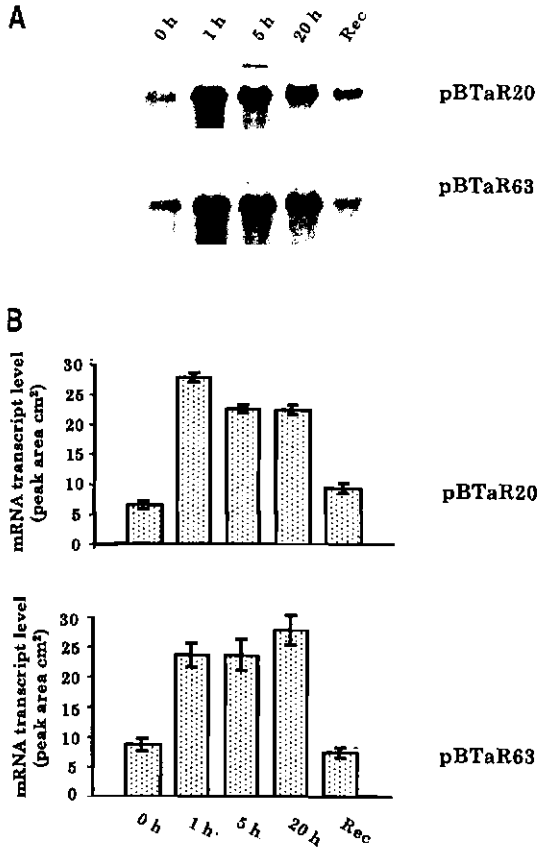

Figure 1 Effect of anoxia exposure and aerobic recovery on the expression of *Cox1* and *Nad5* genes in turtle (*Trachemys scripta elegans*) heart. Hearts were sampled from turtles over a course of up to 20 h submergence in N_2 bubbled water or 20 h aerobic recovery (all at 7°C) and total RNA was extracted and separated on formaldehyde agarose gels (16 µg per lane). Northern blot hybridization was conducted using ^{32}P-labeled clones pBTaR20 and pBTaR63 to probe *Cox1* and *Nad5* mRNA transcripts, respectively. After X-ray autoradiography, films were scanned (shown in A), quantified and converted to a histogram format (shown in B); histogram of band intensities shows the mean results from three separate trials. From Cai & Storey (1996).

were returned to aerobic conditions (20 h aerobic recovery after 20 h anoxia). Northern-blot hybridisation also revealed differential expression of the two genes in five other organs of *T. s. elegans* after 20 h anoxia exposure. A particularly large, 4-fold increase in mRNA transcripts of both genes occurred in anoxic red muscle and induction was also observed in brain and kidney but not in liver or white muscle. Interestingly, Northern blotting also showed that both genes were upregulated after freezing exposure (24 h at –2°C) in heart, kidney and gut of freeze tolerant hatchling turtles, *Chrysemys picta marginata*, indicating that the genes are also responsive to the ischemia that develops when blood plasma freezes (Cai & Storey 1996). In most cases, the proportional increases in *Cox1* and *Nad5* transcripts were very similar but this is probably a consequence of the way in which the mitochondrial genome is regulated. Vertebrate mitochondrial DNA contains only one promoter on each of the L and H strands and, in theory, all genes are transcribed as one RNA precursor from the same initiation site (except for rRNA genes) (Gilham 1994). Individual mRNA transcripts are then rescued through RNA processing. Turtles presumably have this same mechanism and, hence, it appears that the entire mitochondrial genome may be upregulated as an early response to anoxic submergence, probably triggered by a critical level of hypoxia.

The reasons for the induction of *Cox1* and *Nad5* during anoxic submergence remain to be fully explored but may be related to optimising the use of remaining oxygen supplies during the hypoxia transition period after submergence. Indeed, the rapid activation of gene expression within 1 h of submergence suggests that these are responses to declining oxygen availability, not complete oxygen lack. It remains to be determined, however, whether coordinated upregulation of all of the subunits of these multi-subunit respiratory chain enzymes occurs or whether CO1 and ND5 are specifically affected. The exact biological functions of these subunits are unknown but it has been suggested that CO1 can bind not only cytochrome but also O_2 (Azzi *et al.* 1989). Thus, an enhanced level of CO1 induced in the hypoxia transition period might increase the efficiency of O_2 binding to help optimise the use of remaining oxygen supplies in order to support oxidative phosphorylation for as long as possible.

Hypoxia-induced gene expression in mammals is a hot topic at the moment; upregulated genes identified to date include erythropoietin, glucose transporters, selected glycolytic enzymes, tyrosine hydroxylase and vascular growth factors (Guillemin & Krasnow 1997). Interestingly, a recent study found that ischemia suppressed the expression of mitochondrial genes encoding subunits of cytochrome b but did not affect expression of the subunits encoded by nuclear genes; mRNA levels were restored, however, after 2 h reperfusion (Itallie, Why, Thulin, Kashgarian & Siegel 1993). Mammalian renal mitochondria suffer pathological damage during ischemia and the results of Itallie *et al.* (1993) suggest that one of the mechanisms of postischemic recovery is the new synthesis of respiratory chain proteins. Along with our study of turtles, then, this suggests a key role for regulation of mitochondrially encoded genes of respiratory chain proteins in response to changing oxygen availability. The opposite direction of these changes, upregulation in turtles and downregulation in mammals, may characterise hypoxia-resistant versus hypoxia-sensitive systems.

Overall, then, these initial examples illustrate the power of molecular biology techniques for comparative biochemistry, for they offer an excellent mechanism for surveying and pinpointing critical changes in gene expression and protein synthesis that support animal adaptation to diverse environments. Continuing studies in my lab are following up on these initial systems as well as examining changes in gene expression induced by estivation in desert toads, hibernation in small mammals, and freezing in insects. A molecular biology approach will clearly be in the forefront of comparative biochemistry as we move into the new millenium.

List of Abbreviations

PKA	cyclic AMP dependent protein kinase
PKG	cyclic GMP dependent protein kinase
PKC	Ca^{2+} and phospholipid dependent protein kinase
PCR	polymerase chain reaction
PK	pyruvate kinase
PEP	phosphoenolpyruvate
PFK	phosphofructokinase
PP-1	protein phosphatase 1
Ka	activator constant
Km	substrate affinity constant
PS	phosphatidylserine
PI	phosphatidylinositol
PC	phosphatidylcholine
PE	phosphatidylethanolamine
ERK	extracellular signal-regulated protein kinase
JNK	c-Jun NH_2-terminal kinases
SAPK	stress-activated protein kinase
MAPK	mitogen-activated protein kinase
cDNA	complimentary DNA
COI	cytochrome C oxidase subunit I
ND5	NADH-ubiquinone oxidoreductase subunit 5

Acknowledgements

Thanks to several recent members of my laboratory for their work which is summarised here, including Dr. Q. Cai, Dr. S.P.J. Brooks, S. Greenway, H. Mehrani, T. Churchill, D. Douglas and D. Lobsinger, and to J.M. Storey for editorial comment on the manuscript. Research in my lab was supported by a grant from the N.S.E.R.C. Canada.

References

AZZI, A., MULLER, M. & LABONIA, N. 1989. The mitochondrial respiratory chain. In: Organelles in eukaryotic cells, (eds.) J.M. Tager, A. Azzi, S. Papa & F. Guerrieri, pp. 2–8, Plenum Press, New York.

BROOKS, S.P.J. & STOREY, K.B. 1989a. Influence of hormones, second messengers and pH on the expression of metabolic responses to anoxia in a marine whelk. *J. Exp. Biol.* 145: 31–43.

BROOKS, S.P.J. & STOREY, K.B. 1989b. Regulation of glycolytic enzymes during anoxia in the turtle *Pseudemys scripta*. *Am. J. Physiol.* 257: R278–R283.

BROOKS, S.P.J. & STOREY, K.B. 1990. cGMP-Stimulated protein kinase phosphorylates pyruvate kinase in an anoxia-tolerant marine mollusc. *J. Comp. Physiol. B* 160: 309–316.

BROOKS, S.P.J. & STOREY, K.B. 1991. The role of protein kinases in anoxia tolerance in facultative anaerobes: purification and characterization of a protein kinase that phosphorylates pyruvate kinase. *Biochim. Biophys. Acta* 1073: 253–259.

BROOKS, S.P.J. & STOREY, K.B. 1993a. Control of metabolic rate by multienzyme complexes: is glycolysis in hypoxia and anoxia regulated by complex formation? In: Surviving hypoxia: mechanisms of control and adaptation, (eds.) P.W. Hochachka, P.L. Lutz, T.J. Sick, M. Rosenthal & G. van den Thillart, pp. 281–293, CRC Press, Boca Raton.

BROOKS, S.P.J. & STOREY, K.B. 1993b. Protein kinase C in turtle brain: changes in enzyme activity during anoxia. *J. Comp. Physiol. B* 163: 84-88.

BROOKS, S.P.J. & STOREY, K.B. 1993c. *De novo* protein synthesis and protein phosphorylation during anoxia and recovery in the red-eared turtle. *Am. J. Physiol.* 265: R1380–R1386.

BROOKS, S.P.J. & STOREY, K.B. 1996. Purification and characterization of a protein phosphatase that dephosphorylates pyruvate kinase in an anoxia tolerant animal. Biochem. Mol. Biol. Intl. 38: 1223-1234.

BROOKS, S.P.J. & STOREY, K.B. 1997. Glycolytic controls in estivation and anoxia: a comparison of metabolic arrest in land and marine molluscs. *Comp. Biochem. Physiol. A* 118: 1103–1114.

CAI, Q. & STOREY, K.B. 1996. Anoxia-induced gene expression in turtle heart: up-regulation of mitochondrial genes for NADH-ubiquinone oxidoreductase subunit 5 and cytochrome C oxidase subunit 1. *Eur. J. Biochem.* 241: 83-92.

CAI, Q. & STOREY, K.B. 1997a. Freezing-induced genes in wood frog (*Rana sylvatica*): fibrinogen upregulation by freezing and dehydration. *Am. J. Physiol.* 272: R1480–R1492.

CAI, Q. & STOREY, K.B. 1997b. Upregulation of a novel gene by freezing exposure in the freeze-tolerant wood frog (*Rana sylvatica*). *Gene* 198: 305–312.

CAI, Q. & STOREY, K.B. 1997c. A novel RNA species from the turtle mitochondrial genome: induction and regulation of transcription and processing under anoxic and freezing stresses. *Genome* 40: 534–543.

CAI, Q., GREENWAY, S.C. & STOREY, K.B. 1997. Differential regulation of the mitochondrial ADP/ATP translocase gene in wood frogs under freezing stress. *Biochim. Biophys. Acta* 1353: 69–78.

COHEN, P. 1989. The structure and regulation of protein phosphatase. *Annu. Rev. Biochem.* 58: 453–508.

COWAN, K.J. & STOREY, K.B. 1998. Reversible phosphorylation control of skeletal muscle pyruvate kinase and phosphofructokinase during estivation in the spadefoot toad. *Scaphiopus couchii*. *Mol. Cell. Biochem.* (in press).

CROWE, J.H., HOEKSTRA, F.A. & CROWE, L.M. 1992. Anhydrobiosis. *Annu. Rev. Physiol.* 54: 579–599.

DE ZWAAN, A. 1977. Anaerobic energy metabolism in bivalve molluscs. *Oceanogr. Mar. Biol. Annu. Rev.* 15: 103–187.

DOUGLAS, D.N., GIBAND, M., ALTOSAAR, I. & STOREY, K.B. 1994. Anoxia induces changes in translatable mRNA populations in turtle organs: a possible adaptive strategy for anaerobiosis. *J. comp. Physiol. B* 164: 405–414.

GALCHEVA-GARGOVA, Z., DERIJARD, B., WU, I. & DAVIS, R.J. 1994. An osmosensing signal transduction pathway in mammalian cells. *Science* 265: 806–808.

GEISER, F. 1988. Reduction of metabolism during hibernation and daily torpor in mammals and birds: temperature effect or physiological inhibition? *J. Comp. Physiol. B* 158: 25–37.

GILHAM, N.W. 1994. Organelle genes and genomes. Oxford University Press, Oxford.

GUILLEMIN, K. & KRASNOW, M.A. 1997. The hypoxic response: huffing and HIFing. *Cell* 89: 9–12.

HAN, J., LEE, J-D., BIBBS, L. & ULEVITCH, R.J. 1994. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* 265: 808–811.

HAND, S.C. & HARDEWIG, I. 1996. Downregulation of cellular metabolism during environmental stress: mechanisms and implications. *Annu. Rev. Physiol.* 58: 539–563.

HERBERT, C.V. & JACKSON, D.C. 1985. Temperature effects on the responses to prolonged submergence in the turtle *Chrysemys picta belli*. II. Metabolic rate, blood acid-base and ionic changes and cardiovascular function in aerated and anoxic water. *Physiol. Zool.* 58: 670–681.

HOCHACHKA, P.W., BUCK, L.T., DOLL, C.J. & LAND, S.C. 1996. Unifying theory of hypoxia tolerance: molecular/metabolic

defense and rescue mechanisms for surviving oxygen lack. *Proc. Natl. Acad. Sci. USA* 93: 9493–9498.
HOCHACHKA, P.W. & GUPPY, M. 1987. Metabolic arrest and the control of biological time. Harvard University Press, Cambridge.
HOCHACHKA, P.W. & MUSTAFA, T. 1972. Invertebrate facultative anaerobiosis. *Science* 178: 1056–1060.
HOLDEN, C.P. & STOREY, K.B. 1997. Second messenger and cAMP-dependent protein kinase responses to dehydration and anoxia stresses in frogs. *J. Comp. Physiol. B* 167: 305–312.
HOLWERDA, D.A., VEENHOF, P.R., VAN HEUGTEN, H.A.A. & ZANDEE, D.I. 1983. Modification of mussel pyruvate kinase during anaerobiosis and after temperature acclimation. *Mol. Physiol.* 3: 225–234.
ITALLIE, C.M. VAN, WHY, S. VAN, THULIN, G., KASHGARIAN, M. & SIEGEL, N.J. 1993. Alterations in mitochondrial RNA expression after renal ischemia. *Am. J. Physiol.* 265: C712–C719.
KARIN, M. 1995. The regulation of AP-1 activity by mitogen-activated protein kinases. *J. Biol. Chem.* 270: 16483–16486.
KIKKAWA, U., KISHIMOTO, A. & NISHIZUKA, Y. 1989. The protein kinase C family: heterogeneity and its implications. *Annu. Rev. Biochem.* 58: 31–44.
KNIGHT, R.J. & BUXTON, D.B. 1996. Stimulation of c-Jun kinase and mitogen-activated protein kinase by ischemia and reperfusion in the perfused rat heart. *Biochem. Biophys. Res. Comm.* 218: 83–88.
KYRIAKIS, J.M., BANERJEE, P., NIKOLAKAKI, E., DAI, T., RUBIE, E.A., AHMAD, M.F., AVRUCH, J. & WOODGETT, J.R. 1994. The stress-activated protein kinase subfamily of c-jun kinases. *Nature* 369: 156–160.
LAND, S.C. & HOCHACHKA, P.W. 1995. A heme-protein-based oxygen-sensing mechanism controls the expression and suppression of multiple proteins in anoxia-tolerant turtle hepatocytes. *Proc. Natl. Acad. Sci. USA* 92: 7505–7509.
LOBSINGER, D.H. 1996. Differential gene expression in response to subzero temperatures in the freeze tolerant wood frog *Rana sylvatica*. M.Sc. thesis, Carleton University.
LUTZ, P.L. & STOREY, K.B. 1997. Adaptations to variations in oxygen tension by vertebrates and invertebrates. In: Handbook of Physiology, Section 13: Comparative physiology, (ed.) W.H. Dantzler, Vol. 2, pp. 1479–1522, Oxford University Press, Oxford.
LUTZ, P.L., NILSSON, G.E. & PEREZ-PINZON, M.A. 1996. Anoxia tolerant animals from a neurobiological perspective. *Comp. Biochem. Physiol. B* 113: 3–13.
MATSUDA, S., KAWASAKI, H., MORIGUCHI, T., GOTOH, Y. & NISHIDA, E. 1995. Activation of protein kinase cascades by osmotic shock. *J. Biol. Chem.* 270: 12781–12786.
MEHRANI, H. & STOREY, K.B. 1995a. Enzymatic control of glycogenolysis during anoxic submergence in the freshwater turtle *Trachemys scripta*. *Int. J. Biochem. Cell. Biol.* 27: 821–830.
MEHRANI, H. & STOREY, K.B. 1995b. cAMP-Dependent protein kinase and anoxia survival in turtles: purification and properties of liver PKA. *Mol. Cell. Biochem.* 145: 81–88.
MEHRANI, H. & STOREY, K.B. 1995c. Effects of anoxia on protein phosphatase in turtle organs: purification and properties of protein phosphatase type-1 from turtle liver. *Arch. Biochem. Biophys.* 316: 836–843.
MEHRANI, H. & STOREY, K.B. 1996. Liver protein kinase C isozymes: properties and enzyme role in a vertebrate facultative anaerobe. *Int. J. Biochem. Cell. Biol.* 28: 1257–1269.
MEHRANI, H. & STOREY, K.B. 1997. Protein kinase C from bat brain: the enzyme from a hibernating mammal. *Neurochem. Int.* 31: 139–150.
OHNISHI, T. 1993. NADH-quinone oxidoreductase, the most complex complex. *J. Bioenerg. Biomemb.* 25: 325–329.
PEHOWICH, D.J. 1994. Modification of skeletal muscle sarcoplasmic reticulum fatty acyl composition during arousal from hibernation. *Comp. Biochem. Physiol. B* 109: 571–578.
PINDER, A.W., STOREY, K.B. & ULTSCH, G.R. 1992. Estivation and hibernation. In: Environmental biology of the Amphibia, (eds.) M.E. Feder & W.W. Burggren, pp. 250–274, University of Chicago Press, Chicago.
PLAXTON, W.C. & STOREY, K.B. 1984. Purification and properties of aerobic and anoxic forms of pyruvate kinase from red muscle tissue of the channeled whelk, *Busycotypus canaliculatum*. *Eur. J. Biochem.* 143: 257–265.
POMBO, C.M., BONVENTRE, J.V., AVRUCH, J., WOODGETT, J.R., KYRIAKIS, J.M. & FORCE, T. 1994. The stress-activated protein kinases are major c-jun amino-terminal kinases activated by ischemia and reperfusion. *J. Biol. Chem.* 269: 26546–26551.
RAINGEAUD, J., GUPTA, S., ROGERS, J.S., DICKENS, M., HAN, J., ULEVITCH, R.J. & DAVIS, R.J. 1995. Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J. Biol. Chem.* 270: 7420–7426.
RUBINSKY, B., LEE, C.Y., BASTACKY, J. & HAYES, T.L. 1990. The process of freezing and the mechanism of damage during hepatic cryosurgery. *Cryobiology* 27: 85–97.
SCHLESINGER, M.J., ASHBURNER, M. & TISSIERES, A. 1982. Heat shock from bacteria to man. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
SEKO, Y., TOBE, K., UEKI, K., KADOWAKI, T. & YAZAKI, Y. 1996. Hypoxia and hypoxia/ reoxygenation activate Raf-1, mitogen-activated protein kinase kinase, mitogen-activated protein kinases, and S6 kinase in cultured rat cardiac myocytes. *Circ. Res.* 78: 82–90.
SIEBENALLER, J.F. 1979. Regulation of pyruvate kinase in *Mytilus edulis* by phosphorylation-dephosphorylation. *Mar. Biol. Lett.* 1: 105–110.
STOREY, K.B. 1993. Molecular mechanisms of metabolic arrest in mollusks. In: Surviving hypoxia: mechanisms of control and adaptation, (eds.) P.W. Hochachka, P.L. Lutz, T.J. Sick, M. Rosenthal & G. van den Thillart, pp. 253–269, CRC Press, Boca Raton.
STOREY, K.B. 1996. Metabolic adaptations supporting anoxia tolerance in reptiles: recent advances. *Comp. Biochem. Physiol. B* 113: 23–35.
STOREY, K.B. 1997. Metabolic regulation in mammalian hibernation: enzyme and protein adaptations. *Comp. Biochem. Physiol. A* 118: 1115–1124.
STOREY, K.B. & STOREY, J.M. 1990. Facultative metabolic rate depression: molecular regulation and biochemical adaptation in anaerobiosis, hibernation, and estivation. *Quart. Rev. Biol.* 65: 145–174.
STOREY, K.B. & STOREY, J.M. 1992. Natural freeze tolerance in ectothermic vertebrates. *Annu. Rev. Physiol.* 54: 619–637.
STOREY, K.B., MOSSER, D.D., DOUGLAS, D.N., GRUNDY, J.E. & STOREY, J.M. 1996. Biochemistry below 0°C: nature's frozen vertebrates. *Brazilian J. Med. Biol. Res.* 29: 283–307.
STOREY, K.B., STOREY, J.M. & CHURCHILL, T.A. 1997. De novo protein biosynthesis responses to water stresses in wood frogs: freeze-thaw and dehydration-rehydration. *Cryobiology* 34: 200–213.
WANG, L.C.H. 1989. Ecological, physiological and biochemical aspects of torpor in mammals and birds. In: Advances in Comparative and Environmental Physiology, Vol. 4 Animal Adaptation to Cold, (ed.) L.C.H. Wang, pp. 361–401. Springer-Verlag, Berlin.
WEISSBACH, L. & GRIENINGER, G. 1990. Bipartite mRNA for chicken α-fibrinogen potentially encodes an amino acid sequence homologous to β- and γ-fibrinogens. *Proc. Natl. Acad. Sci. USA* 87: 5198–5202.