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Review

Anoxia tolerance in turtles: Metabolic regulation and gene expression ☆

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Abstract

Freshwater turtles of the *Trachemys* and *Chrysemys* genera are champion facultative anaerobes able to survive for several months without oxygen during winter hibernation in cold water. They have been widely used as models to identify and understand the molecular mechanisms of natural anoxia tolerance and the molecular basis of the hypoxic/ischemic injuries that occur in oxygen-sensitive systems and underlie medical problems such as heart attack and stroke. Peter L. Lutz spent much of his career investigating turtle anaerobiosis with a particular focus on the mechanisms of brain ion homeostasis and neurotransmitter responses to anoxia exposure and the mechanisms that suppress brain ion channel function and neuronal excitability during anaerobiosis. Our interests intersected over the mechanisms of metabolic rate depression which is key to long term anoxia survival. Studies in my lab have shown that a key mechanism of metabolic arrest is reversible protein phosphorylation which provides coordinated suppression of the rates of multiple ATP-producing, ATP-utilizing and related cellular processes to allow organisms to enter a stable hypometabolic state. Anoxia tolerance is also supported by selective gene expression as revealed by recent studies using cDNA library and DNA array screening. New studies with both adult *T. scripta elegans* and hatchling *C. picta marginata* have identified prominent groups of genes that are up-regulated under anoxia in turtle organs, in several cases suggesting aspects of cell function and metabolic regulation that have not previously been associated with anaerobiosis. These groups of anoxia-responsive genes include mitochondrially-encoded subunits of electron transport chain proteins, iron storage proteins, antioxidant enzymes, serine protease inhibitors, transmembrane solute carriers, neurotransmitter receptors and transporters, and shock proteins.

Keywords: Anaerobiosis; Metabolic rate depression; Antioxidant defense; Calcium metabolism; Reversible protein phosphorylation; Gene expression

Contents

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1. Introduction

Oxygen is critical to the lives of most organisms on Earth and oxygen deprivation is often rapidly lethal. However, many animal species experience conditions of environment or lifestyle that have necessitated the development of long term anoxia tolerance. Among vertebrates, facultative anaerobiosis is most highly developed in freshwater turtles and supports survival during apnoic dives and winter hibernation underwater in ice-locked ponds and lakes. The mechanisms of anaerobiosis are not only fascinating in themselves but turtles of the *Trachemys* and *Chrysemys* genera are also widely used as models in medical science in the search for solutions to the injuries caused by anoxia/ischemia in heart attack and stroke (recent reviews: Bickler, 2004; Buck, 2004; Jackson, 2004; Lutz and Milton, 2004; Storey, 2004a). Lutz et al. (2003a) also proposed that the turtle could be an effective model for analyzing issues in aging versus longevity. They stated that "the processes that protect the turtle brain against anoxia and subsequent re-oxygenation might also contribute to turtle longevity since many of them are linked to age related neurodegeneration."

One of the major players responsible for unraveling the secrets of turtle life without oxygen was my colleague and friend, Dr. Peter L. Lutz, a transplanted Scot who built a strong career in southern Florida studying many aspects of the neurophysiology of freshwater and marine turtles. I first met Peter as a result of our common interest in turtle anaerobiosis. Peter and I came to similar places in science from different directions. Peter started out as a whole animal biologist with an interest in respiratory physiology who, over his career, moved increasingly towards molecular questions. I began as a protein chemist who was lucky enough to stumble into Peter Hochachka's office one day and be introduced to the idea that protein/enzyme adaptation could produce animals with some amazing abilities to endure environmental extremes. Peter Lutz's first foray into turtle studies was on sea turtle hemoglobin (Lutz et al., 1980; Lutz and Lapennas, 1982; Lapennas and Lutz, 1982) whereas mine looked at the properties of purified enzymes from turtle heart (Storey, 1975; Storey and Hochachka, 1974a, b). Peter moved through studies of respiratory physiology to a primary focus for most of his career on brain ion homeostasis and neurotransmitters (for review: Lutz, 1992; Hochachka and Lutz, 2001; Lutz and Nilsson, 2004). I focused on mechanistic biochemistry for the most part, but lately have morphed into studies of anoxia-induced gene expression (for reviews: Storey, 1996a,b, 2004a). In the intervening 20 plus years from our starts, we met at meetings, talked and wrote and he took pity on a Canuck from the frozen North by inviting me down to his tropical digs in the dead of winters. We both came to realize the central role that metabolic rate depression played in anoxia survival, Peter exploring ion channel arrest and the role of adenosine as the neurotransmitter mediating the suppression of neuronal activity (for review: Lutz and Nilsson, 1997, 2004) whereas my lab focused mainly on the protein kinases and phosphatases that mediate reversible suppression of enzyme/protein function during anoxia (for review: Storey, 1996a; Cowan and Storey, 2003; Storey and Storey, 2004). My lab also has a keen interest in the role that anoxia tolerance plays in freezing survival in terrestrially hibernating amphibians and reptiles (including *Chrysemys* hatchlings that overwinter in the natal nest) (Storey and Storey, 1992; Storey, 2004b, 2006a). Peter and I combined out interests in respiratory, neurological and biochemical adaptations to variations in oxygen tension for a 1997 review (Lutz and Storey, 1997) and, recently, we both began to explore the importance of selected changes in gene expression to turtle survival in anoxia (Cai and Storey, 1996; Willmore et al., 2001; Storey, 2004a, 2006b; Prentice et al., 2003, 2004).

In this review I will focus on some of the recent research on the biochemical and gene expression responses that underlie turtle anoxia tolerance. In particular, the review will highlight the turtle brain and the approaches taken by my lab and Peter Lutz's to understand the molecular mechanisms of anoxia tolerance in this most oxygen-sensitive vertebrate organ. Several common components of anoxia tolerance have been recognized and occur widely across phylogeny. These include (a) accumulation of high reserves of fermentable fuels, (b) strategies for buffering or excreting end products to minimize the acidosis that is a consequence of fermentative metabolism, (c) alternative routes of anaerobic carbohydrate catabolism with advantageous outcomes compared with glycolysis ending in lactate (e.g. higher ATP yields, easily excreted end products), (d) good antioxidant defenses to minimize oxidative injury when oxygen is reintroduced, (e) enhanced expression of selected genes, and (f) strong metabolic rate suppression (Storey, 1996a,b; Storey and Storey, 1990, 2004; Hermes-Lima et al., 2001). For turtles, huge organ reserves of glycogen provide the fermentable fuel for glycolytic ATP production and metabolic poisoning from lactate build-up is avoided by two measures — buffering by calcium and magnesium carbonates released by the shell, and storing a high percentage of the lactate produced in the shell (Jackson, 2004). Unlike many other facultative anaerobes, turtles have not turned to alternative routes of anaerobic carbohydrate catabolism, perhaps because of their unique solutions to storing and buffering lactate. Antioxidant defenses are extremely well-developed in anoxia tolerant turtles with organ enzyme activities and glutathione levels that are far higher than in other cold-blooded vertebrates and comparable to mammalian levels; hence, little or no oxidative damage is detected during anoxia exposure or in the aerobic recovery period (Willmore and Storey, 1997a,b; Hermes-Lima et al., 2001). Anoxia-responsive gene expression puts in place selected proteins with specific

protective functions; this rapidly expanding field will be discussed in detail later in this article.

Overall, however, the most important contributor to anoxia survival, not just in turtles but across phylogeny, is metabolic rate depression (Storey and Storey, 1990, 2004). The pioneering work of Dr. Don Jackson at Brown University first demonstrated the huge reduction in metabolic rate in turtles under anoxic conditions (Jackson, 1968). Calorimetry showed that metabolic rate of submerged turtles was only 10–20% of the corresponding aerobic rate at the same body temperature (Herbert and Jackson, 1985) and studies with Gordon Ultsch have demonstrated that very low metabolic rates in cold water allow painted turtles to survive submerged for as long as 3 months in anoxic water and 5 months in aerated water, sufficient time to ensure survival during winter hibernation under water in ice-locked ponds (Ultsch and Jackson, 1982; Ultsch, 1985, Ultsch et al., 1999).

The molecular basis of metabolic rate depression is a controlled and coordinated suppression of the rates of all ATP-generating and ATP-utilizing metabolic functions so that a new lower net rate of ATP turnover is achieved (Storey and Storey, 1990, 2004). In turtle brain, for example, the activity of Na^+K^+ATPase, the single greatest consumer of brain ATP, was reduced by 30–35% in different regions of the brain after 24 h anoxia (Hylland et al., 1997). Layered over the general suppression of metabolic functions are differential controls that reorganize the priorities for ATP use in the hypometabolic state (Hochachka et al., 1996). Studies with turtle hepatocytes effectively illustrated this. Incubation of cells under anoxia resulted in a net 94% decrease in ATP turnover and dramatically altered the proportion of ATP use by five main ATP-consuming processes: ion motive ATPases, protein synthesis, protein degradation, gluconeogenesis and urea synthesis. As a result the Na^+K^+ATPase became the dominant user of ATP in anoxic hepatocytes, consuming 62% of total ATP turnover compared with 28% in normoxia (Hochachka et al., 1996). By contrast, protein synthesis and protein degradation were largely shut down under anoxia (by >90%) and urea synthesis was halted. Lutz and collaborators reported similar findings in whole animal experiments with *Trachemys scripta elegans*; incorporation of radiolabeled amino acids into organ protein pools occurred during the first hour under a nitrogen gas atmosphere (the hypoxia transition period) but little, if any, further incorporation was seen with longer times up to 6 h of anoxia (Fraser et al., 2001).

In humans and most other mammals, a critical immediate effect of hypoxia/anoxia is the disruption of membrane potential difference. Falling ATP supply due to compromised ATP production causes a rapid imbalance in the opposing rates of ion transport across membranes by ATP-dependent ion pumps versus ATP-independent ion channels (Perez-Pinzon et al., 1992; Hochachka and Lutz, 2001). The result is the rapid dissipation of membrane potential difference which not only compromises physiological functions (e.g. nerve transmission, muscle contractility) but has multiple negative effects on intracellular metabolism. One of these is unregulated calcium release into the cytoplasm which triggers a variety of degenerative events including apoptosis and the activation of Ca^{2+}-dependent proteases (Hochachka, 1986; Wasser and Heisler, 1997; Bickler and Buck, 1998). The concept of "ion channel arrest" was put forward by Hochachka (1986) for anoxia tolerant species and states that flux through ion channels is reduced to a level that is balanced with the activity of ATP-limited ion pumps in anoxia so that potential difference is maintained across both plasma and organelle membranes. Indeed, considerable evidence for this mechanism has accumulated in studies with turtle brain as well as for the concept of "spike arrest" (a strong decrease in neuronal excitability under anoxia), much of it arising from research done by Lutz and collaborators (Perez-Pinzon et al., 1992; Sick et al., 1993; Bickler et al., 2001; Hochachka and Lutz, 2001; Lutz and Nilsson, 2004).

The biochemical mechanisms by which suppression of the activities of enzymes and functional proteins is achieved in hypometabolic systems has been the subject of much research by my laboratory. In general, we know that entry into a hypometabolic state does not involve major changes in the overall protein/enzyme make-up of cells, a logical occurrence since entry into an energy-limited state is not the time to undertake widespread energy-expensive protein synthesis or degradation. For example, anoxic submergence had very little effect on the maximal activities of 21 metabolic enzymes in six organs of adult turtles (*T. s. elegans*), with changes in only 0–4 enzymes per tissue, in most cases suppressing activities (Willmore et al., 2001). Rather, constitutive activities of enzymes in anoxia-tolerant species are well designed to meet the needs of anoxic excursions. This can be contrasted with the well-documented hypoxia-induced up-regulation of multiple glycolytic enzymes (coordinated by the hypoxia-inducible transcription factor, HIF-1) in hypoxia-sensitive species such as mammals (Wenger, 2002).

Instead of modifying the overall amounts of enzymes in response to hypoxia/anoxia, facultative anaerobes use post-translational mechanisms to alter the activity state and sub-cellular distribution of existing enzymes and functional proteins to facilitate the transitions to/from the hypometabolic state. For example, in *T. s. elegans* brain, the distribution of several glycolytic enzymes between soluble and particulate-bound states changes during anoxia to increase the amount of enzyme found associated with the particulate fraction (Duncan and Storey, 1992). This could provide better physical positioning of the ATP-generating enzymes of glycolysis with the major ATP-utilizing processes, such as membrane ion pumps. Post-translational modification by reversible protein phosphorylation is also key to the overall suppression of the activity states of many enzymes not just during anoxia-induced hypometabolism but also in aerobic states of hypometabolism including hibernation, estivation and torpor (Storey and Storey, 1990, 2004). In turtles, reversible phosphorylation controls have been linked with the suppression of multiple cell functions during anoxia including glycolytic enzymes, voltage-gated ion channels (Na^+, Ca^{2+}, K^+), membrane receptors (e.g. *N*-methyl-D-aspartate-type glutamate receptor), and protein synthesis (e.g. ribosomal initiation and elongation factors) (Hochachka and Lutz, 2001; Bickler et al., 2001; Storey, 1996a, Storey and Storey, 2004). The global rate of gene transcription is also

suppressed during hypometabolism, often with little net change in total mRNA but instead by sequestering mRNA in association with translationally-silent monosomes or ribonuclear proteins (Storey and Storey, 2004). However, levels of selected gene transcripts can also be reduced. For example, in anoxic turtle brain, mRNA transcripts of voltage-dependent potassium channels were reduced to just 18.5% of normoxic levels after 4 h of anoxia but rebounded after reoxygenation (Prentice et al., 2003).

Our initial studies of the role of post-translational control in turtle anoxia tolerance documented stable changes in the kinetic properties of selected enzymes of glycolysis during submergence anoxia in *T. s. elegans*. These included organ-specific changes in the kinetic properties of glycogen phosphorylase, PFK, or pyruvate kinase that were consistent with anoxia-induced posttranslational modification of the enzymes (Brooks and Storey, 1988, 1989; Mehrani and Storey, 1995a; Storey, 1996a). Furthermore, the activities of protein kinase A, protein kinase C and protein phosphatase 1 also showed coordinated organ-specific responses to anoxia exposure that could suppress the activities of enzymes under their control and reorganize metabolic functions in anoxia to contribute to overall metabolic rate depression (Brooks and Storey, 1993a; Mehrani and Storey, 1995b,c). Furthermore, studies with juvenile *T. s. elegans* injected with ^{32}P-orthophosphate to label the ATP pool *in vivo* revealed a substantial increase in ^{32}P labeling of the acid-precipitable protein fraction under anoxic conditions (e.g. a 60% increase overall in brain) suggesting that anoxia-induced protein phosphorylation is widespread in the cell (Brooks and Storey, 1993b). In brain, ^{32}P-labeling of proteins was 60 and 70% higher in the plasma membrane and cytosol fractions, respectively, under anoxia than in the same fractions from aerobic control turtles. These fractions also contained the greatest amounts of incorporated radioactivity, an average of 29 and 63%, respectively, of the total labeled protein in brain. Hence, the importance of protein phosphorylation to the regulation of both plasma membrane and cytosolic proteins in brain during anoxia can be appreciated. By contrast, radiolabeled proteins were concentrated in the cytosolic fraction of liver (~85%) and only this fraction showed a significant anoxia-responsive increase in ^{32}P incorporation into proteins (46%) (Brooks and Storey, 1993b). Thus, it appears that the primary site of action of anoxia-induced phosphorylation regulation of metabolism is directed to cytosolic proteins/enzymes in liver whereas in brain the phosphorylation of plasma membrane proteins is also of major importance.

2. Calcium metabolism in anoxia tolerant turtles

Our interest in the molecular mechanisms that coordinate the suppression of energy-expensive metabolic functions to facilitate hypometabolism has led us into some new studies of the regulation of proteins involved in ion regulation. We have used multiple models to show that protein phosphorylation is a key element in the suppression of Na^+K^+ATPase activity in systems as diverse as hibernating mammals and estivating land snails (MacDonald and Storey, 1999; Ramnanan and Storey, 2006). We have also shown that regulation of multiple proteins involved in Ca^{2+} metabolism is important in hibernation (Malysheva et al., 2001) and new studies, discussed below, suggest that similar mechanisms are used by anoxia tolerant turtles.

The level of free, ionized calcium in the cytoplasm is carefully regulated at very low levels, normally ~10^{-7} M, due to Ca^{2+} sequestering in the sarco(endo)plasmic reticulum and mitochondria and the action of various calcium binding proteins. In response to one of many different signals, calcium is released into the cytoplasm to trigger processes including gene expression, signal transduction, muscle contraction, cell proliferation, differentiation, and even death. As noted earlier, unregulated calcium release into the cytoplasm is a key factor in hypoxic/ischemic injury in mammalian tissues. Indeed, Ca^{2+} concentration in mature mammalian neurons rises by >10-fold within minutes in anoxia (Bickler et al., 2001). Turtle neurons show an ~2-fold increase in intracellular [Ca^{2+}] to about 240 nM over the first 2–3 h but then Ca^{2+} stabilizes at this level over weeks of anoxia exposure (Bickler, 1998). Hence, mechanisms that stabilize intracellular calcium levels must be part of turtle anoxia tolerance and such mechanisms must also be very powerful to deal with one of the peculiarities of turtle anaerobiosis — large increases in extracellular Ca^{2+} levels. For example, Reese et al. (2001) reported a 10–20 fold increase in plasma Ca^{2+} levels over weeks of anoxia at 3 °C, reaching levels as high as 60 mM whereas Cserr et al. (1988) found that Ca^{2+} in cerebrospinal fluid rose ~6 fold after 8–10 days of submergence anoxia at 10 °C. This occurs because calcium carbonate is mobilized from shell and bones to provide buffering — bicarbonate buffers the H^+ build-up associated with anaerobic glycolysis whereas calcium lactate formation sequesters the carbohydrate end product (Jackson, 2004). Hence, the regulation of intracellular calcium levels may be a crucial part of anoxia tolerance and may require adjustments of the activities or protein levels of various Ca^{2+} storage and Ca^{2+}-responsive proteins to help maintain the level of intracellular biologically active Ca^{2+} within appropriate limits.

To investigate this idea, we examined the responses to anoxia exposure by a range of proteins involved in calcium metabolism in brain of adult turtles (*T. s. elegans*). One of these was Ca^{2+}/calmodulin-dependent protein kinase (CaMK). Table 1 shows the activity of CaMK isozyme II in brain over the course of submergence anoxia at 7 °C and aerobic recovery after 20 h anoxia. CaMK II activity did not change during anoxia but during aerobic recovery activities decreased significantly by 37–40%, as compared with aerobic controls. Changes in enzyme activities can be due to post-translational modification of the existing protein or to changes in the amount of protein present. To evaluate the latter, we used Western blotting to quantify the levels of CaMK II and three other Ca^{2+}-responsive proteins (calsequestrin, CaMK IV, CaMKK) in turtle brain. Fig. 1 shows that CaMK II protein levels did not change over the course of anoxia and recovery which suggests that the activity changes were due to post-translational modification, possibly a result of protein phosphatase action since dephosphorylation is known to reduce CaMK II activity.

The responses of CaMK isozyme IV were quite different. Protein content decreased substantially during anoxia in brain,

Table 1
Total CaMK II activities in brain from control, anoxic, and recovered turtles

Condition	Time	CaMK II Activity
		U/mg protein
Control		29.4±3.5
Anoxic	1 h	31.1±4.1
	5 h	36.0±3.7
	20 h	31.6±2.2
Recovered	5 h	18.4±1.3[a]
	24 h	17.6±1.6[a]

Samples of frozen brain were homogenized (1:10 w/v) in 20 mM MOPS, pH 7.2, 25 mM β-glycerophosphate (β-GP), 1 mM sodium orthovanadate (Na_3VO_4), 1 mM dithiothreitol (DTT), 5 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin A, with 0.1 mM phenylmethylsulfonyl fluoride (PMSF) added immediately prior to homogenizing. After centrifugation at 18,000 ×*g* for 20 min at 4 °C, supernatants were removed and used immediately. Activity was measured using CaMK II kits from Upstate Biotechnology (Lake Placid, NY) that measure the incorporation of ^{32}P-ATP onto the peptide Auto Camtide II (KKALRRQETVDAL). One unit is defined as the amount of enzyme that transfers one nmol of phosphate onto peptide per minute at 30 °C. Data are U/mg soluble protein, means±SEM, $n=4$ animals. a-Significantly different from control, 1, 5 and 20 h anoxic values, $P<0.05$. (K.J. Cowan and K.B. Storey, unpublished data).

falling by 27% after 1 h and stabilizing at 36–40% below control values during long term anoxia; this could contribute to the general anoxia-induced metabolic rate depression in turtle brain. CaMK IV content remained at 31% below control values during recovery. CaMK IV is a monomer that translocates to the nucleus where it is then effectively activated (20–50 fold) by a specific CaMK kinase (CaMKK) (Anderson and Kane, 1998). The enzyme is also activated by Ca^{2+}/calmodulin and protein phosphatase 2A is responsible for CaMK IV inactivation. Enrichment and activation of CaMK IV in the nucleus leads to phosphorylation of various nuclear substrates such as the transcription factor CREB and hence, CaMK IV is effective in mediating gene expression responses to Ca^{2+} signals (Anderson and Kane, 1998). CaMKK content was also reduced in turtle brain by 25% when anoxia was prolonged to 20 h. This again supports the idea that suppression of calcium signaling processes, such as Ca^{2+} induced gene expression, is part of metabolic suppression. During recovery, levels of CaMKK rose significantly to 27% and 15% above controls after 5 and 24 h of recovery, respectively, indicating a return to normal functioning.

Calsequestrin is a major Ca^{2+}-binding protein located in the terminal cisternae of the sarcoplasmic reticulum. It can bind up to ~50 moles of Ca^{2+} per mole protein with moderate affinity, and acts to buffer free, ionized calcium in the cell to reduce the gradient against which the Ca^{2+}-ATPase pump must move Ca^{2+}, and to localize Ca^{2+} in the terminal cisternae for quick release into the cytosol. Calsequestrin also acts as a regulator of Ca^{2+}

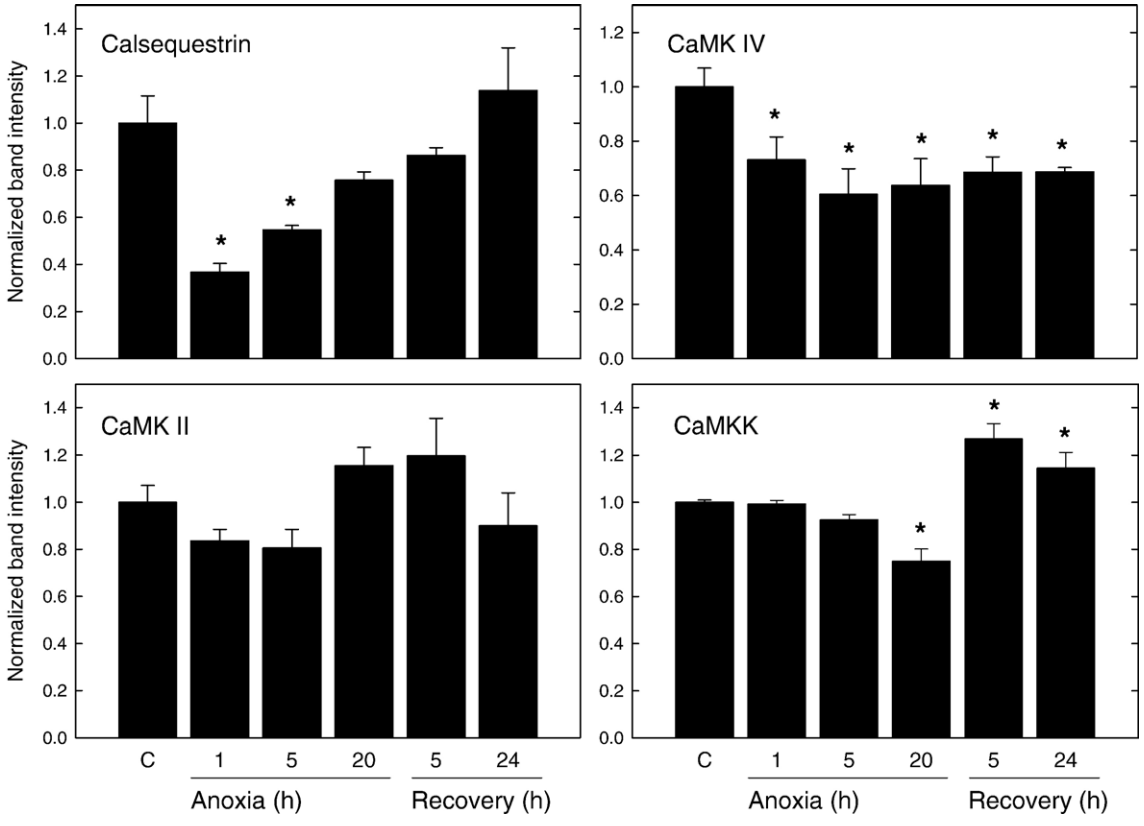

Fig. 1. Effects of anoxia exposure and aerobic recovery on the relative levels of calcium-binding proteins in turtle brain. Tissue extracts were prepared as in Table 1. Aliquots of 25 μg of soluble protein were separated on 12% SDS-PAGE gels and Western blots were probed with antibodies to rabbit cardiac calsequestrin, rabbit CaMK II or CaMK IV (Upstate Biotechnology), or mouse CaMKK (Transduction Laboratories). Band intensities were quantified using Imagequant and normalized against the intensity of the control band which was set at 1.0. Bars from left to right are: Control, 1 h anoxic, 5 h anoxic, 20 h anoxic, 5 h aerobic recovery after 20 h anoxia exposure, and 24 h recovery. Data are means±SEM, $n=3$. * — Significantly different from the corresponding control value after one-way analysis of variance and testing with the Student–Newman–Keuls test, $P<0.05$. (K.J. Cowan and K.B. Storey, unpublished data).

channels in the sarcoplasmic reticulum membrane (Kawasaki and Kasai, 1994). In turtle brain, calsequestrin levels decreased strongly and immediately in response to anoxic submergence, falling to just 37% of control values within 1 h and remaining low (55% of control) after 5 h. Levels began to rise again after 20 h anoxic submergence and were not significantly different from controls during the aerobic recovery period. At first glance, the early reduction of calsequestrin levels in anoxic turtle brain seems to be counter-intuitive at a time when greater calcium storage might be predicted. However, if the amount of calsequestrin determines the amount of calcium that is available in the terminal cisternae to respond to calcium release signals, then reduced calsequestrin could facilitate metabolic arrest. Thus, if less calcium is available for release through Ca^{2+} channels then less Ca^{2+} has to be returned to the sarco(endo)plasmic reticulum via ATP-dependent pumps. Other Ca^{2+} binding proteins also occur in sarco(endo)plasmic reticulum (e.g. sarcalumenin, histidine-rich Ca^{2+} binding proteins); whether these are elevated during anoxia to compensate for reduced calsequestrin levels remains to be determined.

3. Anoxia-responsive gene expression in turtle organs

Metabolic rate depression is key to anoxia survival and, as mentioned earlier, rates of transcription, translation and protein degradation are all strongly reduced during anaerobiosis. Hence, one would expect that anoxia-responsive gene expression would be focused on the production of only those gene products that are key to anaerobic survival. Early studies using ^{35}S-methionine labeling showed synthesis of some new proteins during anoxia in turtle organs but these were not identified (Brooks and Storey, 1993b; Douglas et al., 1994). More recently, we have used gene screening techniques to find anoxia-responsive genes with follow-ups including sequencing to determine identity, Northern blotting or PCR to quantify changes in mRNA transcript levels, and Western blotting to quantify protein changes. We have used two main approaches: cDNA library screening and DNA array screening. Interestingly, each has revealed that a surprisingly wide variety of genes are up-regulated in response to anoxia exposure, and notably, many of these represent cell functions that had never before been considered as involved in anaerobic survival. Hence, a key outcome of gene screening technology, not only in anoxia tolerance but also in our analyses of gene responses to other situations of adaptation to environmental stress (e.g. freeze tolerance, estivation, hibernation), has been the power of these technologies to provide an unbiased assessment of the full complement of genes that contribute to cellular adaptation to a given stress (Eddy and Storey, 2002; Storey, 2004c, 2006b).

Our initial study of anoxia-responsive gene expression screened a cDNA library made from heart of anoxia-exposed (20 h submergence in N_2-bubbled water at 7 °C) adult *T. s. elegans*. Differential screening produced a surprising result — up-regulation of two genes encoded on the mitochondrial genome. Transcripts of *Cox1* that encodes cytochrome *C* oxidase subunit 1 (COX1) and *Nad5* that encodes subunit 5 of NADH-ubiquinone oxidoreductase (ND5) rose within 1 h of anoxia exposure to 3–4.5 fold higher than aerobic control values, remained high after 20 h, and then declined during aerobic recovery (Cai and Storey, 1996). Both genes were also up-regulated during anoxia in muscle, brain and kidney. A follow-up study found other mitochondrially-encoded genes that were anoxia responsive in turtle liver: *Cytb*, encoding cytochrome *b*, and *Nad4*, encoding subunit 4 of ND (Willmore et al., 2001). The reason for mitochondrial gene up-regulation in anoxia is not yet known but the phenomenon also occurs in response to freezing (which imposes organ ischemia) in freeze tolerant turtle hatchlings (*Chrysemys picta*) and wood frogs (*Rana sylvatica*) (Cai and Storey, 1996; Storey, 2004b).

Our newest studies have used DNA array screening to analyze anoxia-responsive gene expression in adult *T. s. elegans* and hatchling painted turtles, *C .p. marginata* (Storey, 2004a, 2006b) as well as freeze-responsive gene expression in hatchling *C. p. marginata* (5 h freezing at −2.5 °C) (Storey, 2006a). We used heterologous screening with human 19 K gene chips to search for differential gene expression in adult *T. s. elegans* given 4 h anoxic submergence in nitrogen-bubbled water or hatchling *C. p. marginata* given 4 h exposure under a nitrogen gas atmosphere, each compared with aerobic controls and all animals held at 7 °C. Heterologous probing has some caveats. Cross-hybridization, especially across a considerable phylogenetic distance, will never give 100% cross-reaction but after optimizing binding and washing conditions, a high percentage hybridization can typically be achieved (Eddy and Storey, 2002; Storey, 2004c, 2006b). The potential for false positive and false negative results also exists although in our experience to date, genes showing substantial putative up-regulation from array screening (e.g. 2-fold or greater differences between control and experimental states) have always been validated either by prior results (genes previously found by cDNA library screening) or subsequent analyses (e.g. Northern blots, PCR, Western blotting).

Array screening of heart and liver of hatchling *C. p. marginata* for anoxia-responsive or freeze-responsive genes found three outstanding groups of genes that were consistently up-regulated: (1) iron storage proteins, (2) enzymes of antioxidant defense, and (3) selected serpins (Table 2) (Storey, 2006a,b). The functions of the proteins encoded by these genes provide some new ideas about anoxia tolerance.

3.1. Iron storage proteins

The heavy and light chains of ferritin, the transferrin receptor 2 (TfR2), and hemoglobin α and β subunits were up-regulated during anoxia or freezing exposures in heart and liver of hatchling turtles (Storey, 2006a,b). Iron is a vital component of many proteins but free ferrous iron (Fe^{2+}) participates in the Fenton reaction with H_2O_2 and lipid peroxides to generate reactive hydroxyl radicals and lipid radicals (Hentze et al., 2004). Hence, intracellular free iron is kept low by locking up excess iron in ferritin; the 24 heavy and light subunits of ferritin surround a core where as many as 4500 atoms of iron are stored in the low reactivity ferrihydrite state. Another protein, transferrin (Tf), binds iron in the plasma, and iron uptake into

Table 2
Anoxia-responsive gene expression in brain of adult turtles, *T. s. elegans*, as highlighted by DNA array screening

Gene	Protein function
Adenosine A1 receptor:	Cell surface adenosine detection and signal transduction
5′ Nucleotidase, cytosolic II isozyme (NT5C2):	IMP conversion to inosine
Gamma-aminobutyric acid A receptors: beta 3 (GABRB3), gamma 2 (GABRG2)	Ligand-gated ion channels
5-Hydroxytryptamine receptor 6	G-protein receptor; mediates intracellular signaling
Solute carrier family 6, member 12 (SLC6A12)	Betaine/GABA transporter
Solute carrier family 11, member 2	Fe^{2+} transporter
Shock proteins:	
HSP70-1A (inducible form, Hsp72)	Chaperone
HSP70-9B (mortalin-2 or Grp75	Mitochondrial, associated with lifespan extension
HSP40 (DnaJ or HSJ1)	Co-chaperone holdase
αB-Crystallin	chaperone

cells involves Tf docking with the transferrin receptor (TfR) followed by endocytosis. The TfR1 isoform is ubiquitous but in mammals TfR2 is found just in liver and erythroid cells. Both ferritin and TfR are induced by hypoxia in mammals and Tf and TfR genes are known to be regulated by HIF-1 (Wenger, 2002; Tacchini et al., 2002; Smith et al., 2003). Ferritin subunits are anoxia/ischemia responsive in other stress-tolerant species; for example, the H chain was up-regulated by anoxia in an anoxia tolerant marine snail (Larade and Storey, 2004) and the L chain was freeze responsive in wood frog heart (Storey, 2004b). The reason for ferritin and TfR2 up-regulation under anoxia in turtles remains to be confirmed but a role in iron sequestering as an aid to antioxidant defense is suspected. Various damaging effects of ischemia in mammals actually result from a burst of reactive oxygen species (ROS) generated when oxygen is reintroduced in the reperfusion phase because normal antioxidant defenses are overwhelmed (Hermes-Lima et al., 2001; Hermes-Lima and Zenteno-Savin, 2002). If anoxia tolerant species take steps to minimize free Fe^{2+} and maximize antioxidant defenses, then the potential for oxidative damage to cellular macromolecules during transitions to/from low/high oxygen conditions is reduced.

3.2. Antioxidant defense

Array screening also showed that several antioxidant genes were up-regulated by anoxia exposure in heart and liver of hatchling *C. p. marginata*: superoxide dismutase 1 (SOD-1), glutathione peroxidase (GPX) isozymes 1 and 4, glutathione-*S*-transferase (GST) isozymes M5 and A2, and peroxiredoxin 1 (Storey, 2006b). Except for SOD-1 and GPX4, the same genes were up-regulated in response to freezing in these hatchlings (Storey, 2006a) and a variety of antioxidant enzymes were also up-regulated by freezing in wood frogs (Storey, 2004b). Anoxia tolerant species typically show two strategies for dealing with oxidative stress during natural transitions from low to high oxygen availability: (a) high constitutive antioxidant defenses, both antioxidant enzymes (AOEs) and metabolites, and (b) anoxia induced increases in AOEs (Hermes-Lima et al., 2001; Hermes-Lima and Zenteno-Savin, 2002). Enzymatic studies have shown that the best facultative anaerobes that undergo routine anoxia excursions, such as adult turtles and freeze tolerant frogs, largely employ the first strategy, maintaining high constitutive AOE activities in their tissues at all time. Indeed, activities in *T.s. elegans* and *R. sylvatica* organs are several-fold higher than those in other cold-blooded vertebrates and comparable to mammalian levels (Joanisse and Storey, 1996; Willmore and Storey, 1997a,b; Hermes-Lima et al., 2001). By contrast, species that face anoxia less often show anoxia-induced enhancement of defenses; for example, this has been demonstrated for anoxia exposure of leopard frogs and freezing or anoxia exposures of garter snakes and (Hermes-Lima and Storey, 1993, 1996). This also appears to explain the gene expression responses of hatchling *C. p. marginata* to anoxia or freezing. Adults of this species would undoubtedly have high AOE activities, similar to *T. s. elegans*, due to their diving activities and underwater hibernation. However, hatchlings spend their first winter on land where they may or may not have to endure ischemia/reperfusion insults during freeze/thaw exposures. Hence, inducible antioxidant defenses seem to suit their needs and we see the up-regulation of several AOEs in response to experimental anoxia exposure.

Interestingly, our recent study of adult *T. s. elegans* liver GST activity adds another layer of complexity onto the story of antioxidant defense in anoxia. An earlier analysis of GST in liver of adult turtles showed reduced activity during anoxia that rose again during aerobic recovery (Willmore and Storey, 1997b). In a recent kinetic analysis we found two alpha class GSTs in adult *T. s. elegans* liver, a homodimer and a heterodimer. Both exhibited a major reduction (>50%) in specific activity (units/mg protein) in anoxic liver and the heterodimer showed a major change in substrate preference, these characteristics being indicative of an anoxia-responsive stable modification of the enzyme (Willmore and Storey, 2005). Hence, it appears likely that GST is modified, perhaps via reversible phosphorylation, in order to alter or optimize its function in anoxic tissues, possibly as part of the reorganization of metabolic priorities that is part of metabolic rate depression.

The anoxia- and freeze-responsive up-regulation of peroxiredoxin 1 is worthy of special mention since it is a novel finding that may suggest a new principle of metabolic adaptation. ROS are traditionally viewed as bad and, therefore, the purpose of antioxidant defenses is to prevent or repair the damage done by ROS to cellular components. This view began to change when reactive nitrogen species (nitric oxide, peroxynitrite) were shown to have second messenger functions and, more recently, when intracellular second messenger roles were assigned to both superoxide and hydrogen peroxide (Hermes-Lima, 2004; Rhee et al., 2005b). Support for H_2O_2 as an intracellular second messenger includes: (a) transient elevations of intracellular H_2O_2 occur in response to various cytokines and

peptide growth factors, (b) elevated H_2O_2 affects the function of various protein kinases and phosphatases, transcription factors, and G proteins, and (c) inhibition of H_2O_2 generation causes blocking of signals from growth factors (Rhee et al., 2005a). Therefore, it would be expected that one or more enzymes would be present in cells to modulate the levels of H_2O_2 with respect to its signalling function. Peroxiredoxins (Prx), relatively recent additions to the known antioxidant defenses of cells, may have this role (Rhee et al., 2005b). Prx has six isozymes in mammals, all containing a reactive Cys in a conserved region near the N-terminus that can reduce H_2O_2 or various alkyl hydroperoxides. Prx1, which is anoxia- and freeze-responsive in turtles, is a cytosolic dimer that uses thioredoxin as its electron donor. The enzyme is induced by oxidative and other stresses and cells transfected with Prx1 exhibit resistance to apoptosis caused by hydrogen peroxide (Fujii and Ikeda, 2002). As discussed earlier, a critical survival mechanism in anoxia (and freeze) tolerance is metabolic rate depression and biosynthetic processes (e.g. protein synthesis) are major targets of suppression when ATP is limiting (Storey and Storey, 2004). Predictably, then, metabolic suppression should include reduced cell responsiveness to growth signals. One way to do this could be to strongly suppress the levels of intracellular second messengers, such as H_2O_2, that mediate growth factor and cytokine effects. Up-regulation of Prx1 could have this action because Prx1 is known to catabolize H_2O_2 produced from cell surface signalling (Rhee et al., 2005b). Indeed, Prx1 is known to be regulated by cyclin dependent kinases. Interestingly, we have recently shown that Prx1 is also up-regulated in another situation of hypometabolism — mammalian hibernation (Eddy et al., 2005; Storey, 2006b).

3.3. Serpins

Another family of proteins that is consistently identified in cDNA array screening as up-regulated in response to environmental stress are the *ser*ine *p*rotease *in*hibitor*s* (serpins). These are irreversible covalent inhibitors of specific proteases that act as critical checkpoints in self-perpetuating proteolytic cascades; most function to control plasma proteases involved in blood coagulation, fibrinolysis, inflammation, and complement activation (Gettins, 2002). Most serpins are synthesized and secreted by liver. Anoxia exposure of adult *T. s. elegans* turtles triggered enhanced expression of selected serpins in liver and heart: SERPINC1 (antithrombin), D1 (heparin cofactor II; liver only), and F1 (pigment epithelium derived factor or PEDF) (Storey, 2004a). Hatchling *C. p. marginata* showed anoxia responsive up-regulation of SERPINC1 and D1 in liver and F1 and G1 (complement inhibitor) in heart (Storey, 2006b). All but F1 were also freeze responsive in the hatchlings (Storey, 2006a). Serpin C1 and D1 both inhibit thrombin to suppress the clotting cascade. Reduced clotting capacity during anaerobiosis would be adaptive to minimize the risk of thrombosis in the microvasculature under the low blood flow conditions caused by bradycardia during hypometabolism. Interestingly, multiple mechanisms that minimize the risk of thrombosis, including up-regulation of eight different serpins (including C1), is also a demonstrated part of hypometabolism in mammalian hibernation (Storey, 2004d).

With regard to anoxia-responsive gene expression, SERPINF1 (PEDF) is especially intriguing. PEDF is not a protease inhibitor but instead antagonizes the actions of vascular endothelial growth factor (VEGF); VEGF is controlled by HIF-1 and regulates an "umbrella" group of genes that are involved in vascular growth as a response to hypoxia. PEDF inhibits vascular growth responses to hypoxia when they are inappropriate by inducing apoptosis in actively dividing endothelial cells. It was first described as the factor that prevents aberrant blood vessel growth in models of ischemia-induced retinopathy (Stellmach et al., 2001) and is now known to inhibit vascular growth in several other tissues (Doll et al., 2003). Circulating levels of PEDF in humans are high enough to be physiologically relevant which suggests that systemic delivery of PEDF could affect angiogenesis throughout the body (Petersen et al., 2003). Anoxia tolerant animals clearly must retain HIF-1 mediated gene responses to allow for normal tissue growth and development and to respond to some changes in environmental oxygen conditions (e.g. changes in altitude, changes in water O_2 content), but most actions of HIF-1 are counter-intuitive to long term anaerobiosis. In turtles, for example, HIF-1 mediated responses such as enhanced capillary growth or the proliferation of red blood cells is unproductive and a waste of energy when low tissue oxygen levels are caused by long term apnea. Hence, a selective inhibition of certain HIF-1 mediated responses during breath-hold diving or submerged hibernation would be expected. The up-regulation of PEDF to inhibit VEGF would serve to counteract the angiogenic response to low oxygen in situations that cannot be aided by enhanced capillary growth. Other inhibitory effects that limit or modulate HIF-1 action in anoxia-tolerant species remain to be explored.

4. Anoxia responsive gene expression in turtle brain

To return to our earlier focus on turtle brain responses to anoxia, in recent work we have used DNA array screening to assess anoxia-responsive gene expression in brain of adult *T. s. elegans*, comparing aerobic controls with turtles given 20 h anoxic submergence (protocol as in Willmore et al., 2001). RNA extraction, quality assessment, and array screening were as in Storey (2006a) using the human 19,000 gene arrays produced by the Microarray Center of the University Health Network (Toronto, ON). Cross-hybridization between turtle cDNA and the array was about 33% and, of these, about 3% of genes (150–200) showed strong up-regulation (2-fold or more) although some of these "hits" represent duplicates of a single gene. Hence, expression of the majority of genes is unchanged or suppressed under anoxia. Discussed below are selected genes that showed a minimum 2-fold increase in expression in anoxic brain (also listed in Table 2) and that are linked with some of the known responses by turtle brain to anoxia.

4.1. Adenosine A1 receptor

Adenosine receptors are ubiquitous G-protein coupled receptors of four subtypes: A1 and A3 mediate inhibition of adenylyl cyclase whereas A2a and A2b stimulate the enzyme

(Yaar et al., 2005). Adenosine A1 receptor agonists are known to mediate neuroprotection by activation of K^+ channels, blockage of Ca^{2+} influx and suppression of excitatory neurotransmitter release, whereas antagonists of adenosine A2 receptors reduce ischemic damage in brain (Wardas, 2002). In turtle brain, adenosine causes a decrease in *N*-methyl-D-aspartate (NMDA) receptor activity and an associated decrease in Ca^{2+} permeability and appears to mediate anoxia-induced channel arrest (Pek and Lutz, 1997; Buck and Bickler, 1998). In mammalian brain, hypoxia/ischemia produces a rapid down-regulation of A1 receptor density but in turtle brain there was no significant change in the population of A1 receptors after 24 h anoxia exposure (Lutz and Manuel, 1999). Thus, turtles do not require enhanced numbers of plasma membrane adenosine receptors in anoxia but they may need to take steps to prevent the loss of receptors. If loss of A1 receptors from the membrane in mammalian systems is due to an imbalance between synthesis and degradation under low oxygen, then anoxia-tolerant species may compensate for this with a specific up-regulation of A1 receptor expression. Despite the obvious ATP cost for the synthesis of A1 receptor protein, it may be a necessary component of metabolic arrest. Indeed, array screening also highlighted adenosine A1 and A2A receptors as up-regulated during freezing in wood frog heart (Storey, 2004b).

4.2. 5′ Nucleotidase, cytosolic II isozyme (NT5C2)

The 5′ nucleotidases (NT5) are a family of enzymes that degrade 5′mononucleotides, removing the phosphate group to release the nucleoside. The best known are the enzymes that produces adenosine, a neuromodulator, which has a key role in natural metabolic rate depression in anoxia tolerant species and in ischemic preconditioning in mammals (Hunsucker et al., 2005). Adenosine may be formed intracellularly by cytosolic NT5C1 or by the membrane-bound ecto-NT5; this latter was up-regulated in heart of wood frogs during freezing (an ischemic stress) (Storey, 2004b). Both routes contribute to enhanced adenosine formation in ischemia/hypoxia and the stress is also known to induce ecto-NT5 in mammals (Hunsucker et al., 2005). The NT5C2 isozyme has a different role; it is the Mg^{2+}-dependent cytosolic form that prefers (d)IMP or (d)GMP as substrates (Bretonnet et al., 2005). Could IMP conversion to inosine have a role to play in turtle anoxic tolerance?

A common strategy for maintaining a high adenylate energy charge, AEC = [ATP + 0.5 ADP]) / [ATP + ADP + AMP], in cells/tissues under physiological stress, particularly in stress tolerant species, is to remove AMP from the adenylate pool by converting it to IMP via the enzyme AMP deaminase. This allows total adenylate concentration to drop but stabilizes AEC. Mommsen and Hochachka (1988) explored this system in fish muscle during exercise and showed the importance of the AMP deaminase products, IMP and NH_4^+, as regulators of glycolysis in working muscle. The same system – a decrease in total adenylates with a stabilization of AEC – occurs in turtle tissues during anoxia. Several studies have shown that ATP levels fall in turtle brain during anoxia (summarized by Buck et al., 1998) and Kelly and Storey (1988) showed a 32% decrease in total adenylates after 1 h of anoxic submergence but a stable AEC. Buck et al. (1998) found a 23% reduction in ATP over ~2 h of anoxic perfusion but estimated that only a 0.9% decrease in ATP was needed to account for the 10-fold rise in adenosine concentration (to 20 μM) that occurred in anoxic turtle brain. Hence, a large part of the decrease in brain ATP is unaccounted for and may be represented (at least partially) in IMP and inosine pools.

Given the important role of adenosine as a signaling molecule in anoxia, could inosine also have a signaling role? Furthermore, could anoxia-responsive NT5C2 up-regulation have a physiological function in anoxic turtle brain? Although information is fragmentary, new research suggests some intriguing possibilities that deserve study in both anoxia-tolerant and intolerant systems. Rats treated with inosine while under hyperoxia showed substantially less damage to their pulmonary epithelium, less DNA damage and increased antioxidant status; the data indicated that inosine provided protective signaling mediated via mitogen-activated protein kinase (MAPK) signaling (Buckley et al., 2005). Gelain et al. (2004) found that extracellular inosine levels rose strongly under H_2O_2 stress and inosine treatment reduced H_2O_2-induced lipid peroxidation. Tomaselli et al. (2005) found that inosine, adenosine and guanosine all significantly inhibited the loss of viability by neuronal pheochromocytoma cells caused by hypoxia insult and did so in a MAPK-mediated way. Hence, it is interesting to speculate that inosine has an independent role to play in cellular responses to variations in oxygen availability. Whereas adenosine mediates various metabolic arrest responses that help to conserve energy under hypoxia, inosine may mediate antioxidant defense responses which, as discussed earlier, have an important function in cellular protection during the recovery process after anoxia. An up-regulation of NT5C2 during anoxia could have functional significance in promoting inosine formation and thereby help to set the stage for inosine-mediated protective actions in the anoxic brain. It is interesting to note as well that selected MAPKs are known to change in an organ-specific manner in both adult and hatchling turtles under anoxia exposure (Greenway and Storey, 1999, 2000; Cowan and Storey, 2003).

4.3. Gamma-aminobutyric acid A receptors

Gamma-aminobutyric acid (GABA) is a major inhibitory neurotransmitter and plays an important role in turtle anaerobiosis (Lutz et al., 2003b). GABA(A) receptors (GABR) are ligand-gated ion channels that mediate the majority of fast synaptic inhibition; seven subclasses of GABR are known, each with multiple members, but alpha, beta and gamma subtypes dominate in brain (Brandon et al., 2002). Regulatory mechanisms include GABR phosphorylation as well as receptor internalization and recycling (Brandon et al., 2002). Turtle brain shows a sustained rise in extracellular GABA during anoxia, and an increase in the density of GABA(A) receptors in cerebral cortex (Lutz and Leone-Kabler, 1995). The elevated receptor levels probably act to increase the inhibitory effectiveness of the released GABA, resulting in a strengthening of inhibitory tone in the brain under anoxia. Our cDNA array

screening indicated up-regulation of two isoforms of the GABA (A) receptor in brain of anoxic turtles, the beta 3 (GABRB3) and gamma 2 (GABRG2) isoforms. This suggests that elevated receptor levels in turtle brain during anoxia are due, at least in part, to enhanced gene expression. By contrast, Gao et al. (2004) found reduced mRNA levels for four GABA(A) receptors, including GABRG2, under hypoxia in a human neuronal cell line. The GABRB3 and B1 isoforms in brain are phosphorylated by cAMP-dependent protein kinase (PKA) because the A-kinase anchoring protein 150 binds to these subunits but not to other family members (Brandon et al., 2002). Hence, this may make these GABR isoforms responsive to PKA-mediated signals in anoxia.

4.4. 5-Hydroxytryptamine receptor 6 (HTR6)

5-Hydroxytryptamine (serotonin) is an important neurotransmitter. Its intracellular actions are mediated by binding to membrane receptors, with 14 HTR members known to date (Pauwels, 2000). HTR6 has almost exclusive expression in the central nervous system (especially limbic and cortical regions) and appears to regulate glutamatergic and cholinergic neuronal activity (Woolley et al., 2004). Most HTR members couple to guanine nucleotide-binding proteins (G proteins) to produce second messengers that regulate cellular functions via phosphorylation/dephosphorylation of intracellular proteins. Five of the receptor types, including HTR6, stimulate two major intracellular second messenger pathways, adenylate cyclase and phospholipase C. Interestingly, two other receptor types from this group (HTR1A and HTR7) are implicated in hypoxia-induced hypothermia in mammals (Gargaglioni et al., 2005), a response that is also seen in ectotherms. To date, relatively little is known about the functional actions of HTR6 but some studies have implicated the receptor in cognition enhancement (Russell and Dias, 2002). Little is also known about the role of monoamines in turtle anoxia tolerance. Nilsson et al. (1990) found reduced dopamine levels but serotonin, norepinephrine, and epinephrine levels were maintained in brain over a thirteen-hour time course of anoxia exposure. Levels of monoamines in turtle brain were very high which suggested that they might be stockpiled; since monoamine synthesis requires molecular oxygen this could ensure that sufficient monoamine supplies remain available over long anaerobic excursions.

4.5. Solute carrier family 6, member 12 (SLC6A12)

The solute carrier family 6 transports a range of solutes while co-transporting sodium or chloride down their concentration gradients (Chen et al., 2004). Many of the SLC6 subtypes transport neurotransmitters. The A1, A11, A12 and A13 subtypes are betaine/GABA transporters and, in mammals, the A12 subtype which is particularly prominent in brain and kidney, is believed to function mainly in betaine transport (Chen et al., 2004). This suggests a physiological role for SLC6A12 in osmoregulation. SLC6A12 was up-regulated in turtle brain during anoxia. The functional significance of this remains to be determined because the relative role of SLC6A12 in GABA versus betaine transport in lower vertebrates is not known. In a GABA transporter role, SLC6A12 up-regulation could contribute to the important role of GABA in neural inhibition as part of anoxia-induced metabolic rate depression. However, an osmoregulatory role could also be envisioned because during long term anaerobiosis turtles show substantial increases in extracellular ion concentrations, particularly calcium (Reese et al., 2001). Import of betaine to increase intracellular osmolality could provide another way of resisting ion influx into brain cells when plasma osmolality rises due to the release of calcium and magnesium carbonates from the shell.

4.6. Solute carrier family 11, member 2

This transporter, also known as the divalent metal ion transporter 1 (DMT1), has an important role in iron metabolism. In brain, iron is taken up after docking of transferrin with transferrin receptors which are then internalized in endosomes. Iron is then detached and transported into the cytosol via the DMT1. In mammals, the amount of DMT1 in four brain regions did not change in response to high versus low iron diets although iron content and transferrin receptor levels did (Ke et al., 2005). However, another study found a general correlation between the distribution of DMT1 and ferrous iron in multiple regions of monkey brain (Huang et al., 2004). DMT1 and TfR expression appear to be linked in neuronal cells (Wang et al., 2005) which suggests that DMT1 may be under HIF-1 regulation as TfR is. DMT1 up-regulation in turtle brain during anoxia may be another element in the general enhancement of proteins involved in iron transport and storage under anoxia as was discussed earlier for heart and liver.

4.7. Shock proteins

Array screening of turtle brain for anoxia-responsive proteins also highlighted the putative up-regulation of several shock proteins. These were HSP70-1A (the inducible form, also known as Hsp72), HSP70-9B (also known as mortalin-2 or Grp75), HSP40 (also known as DnaJ or HSJ1), and αB-crystallin. Elevated levels of Hsp72 protein have been reported previously as a response to anoxia in turtle brain and other organs (Prentice et al., 2004; Ramaglia and Buck, 2004) and the up-regulation of the HSP70-1A gene indicates that the mechanism of Hsp72 enhancement is due in whole or part to transcriptional activation. αB-Crystallin acts as a molecular chaperone to suppress inappropriate protein–protein interactions, particularly among cytoskeleton proteins, that would otherwise lead to protein aggregation and precipitation and has been strongly linked with a variety of physiological stresses and neurodegenerative diseases in brain (Head and Goldman, 2000). Both HSP70-1A and αB-crystallin levels rose in rat brain under heat stress and αB-crystallin also responded to oxidative stress (Goldbaum and Richter-Landsberg, 2001). Hence, it is probable that αB-crystallin up-regulation in turtle brain during anoxia indicates a protective function for this protein during hypometabolism.

HSP40, also known as DnaJ or HSJ1, is a co-chaperone or "holdase" that works together with the ATP-dependent HSP70

chaperone or "foldase" (Winter and Jakob, 2004). Conversion between the ADP-bound high substrate affinity state of HSP70 and the ATP-bound low affinity state is mediated by co-chaperones that stimulate ATP-hydrolysis (DnaJ) and nucleotide exchange (GrpE). Co-chaperones appear to bind to unfolded substrate proteins or folding intermediates via hydrophobic interactions but are usually unable to directly support refolding to the native state, transferring their protein substrates instead to the foldase. Under non-permissive folding conditions (e.g. heat stress), the unfolded substrate proteins remain bound to chaperone holdases and after the return to non-stress conditions they are relayed to the foldase system for refolding (Winter and Jakob, 2004). Recent studies have also revealed another function for the HSP70/HSP40 system. Isoforms of HSP40 act as neuronal shuttling factors; their J domain stimulates substrate loading onto HSP70 whereas their ubiquitin interaction motifs allow them to bind to ubiquitinylated proteins to prevent their aggregation and facilitate their correct delivery to the proteasome system (Westhoff et al., 2005). Anoxia-induced metabolic rate depression in turtles includes suppression of multiple ATP-utilizing metabolic functions including both protein synthesis and protein degradation (Hochachka et al., 1996); logically, ATP-dependent protein refolding would be another function that is restricted in the hypometabolic state. Hence, a proliferation of holdase proteins may provide a way to scavenge and retain valuable proteins in anoxic cells until they can be refolded and reused when aerobic ATP generation is again possible. Alternately, HSP40 could be involved in managing a pool of ubiquitinylated proteins that may continuously accumulate under anoxic conditions when protein degradation mechanisms are suppressed.

HSP70-9B or mortalin-2 is a chaperone that is induced by a variety of stresses in mammalian cells. Many transformed and tumor-derived cells have a high level of mortalin expression and, indeed, overexpression of mot-2 cDNA resulted in malignant transformation of cells (summarized by Wadhwa et al., 2002). Mortalin is found predominantly in mitochondria but depending on its particular subcellular niche and binding partner, it appears to perform multiple functions relevant to cell survival, control of proliferation and stress response. It plays roles in both senescence and immortalization and its induction by heat stress or transgenic over-expression increased nematode lifespan by >40% (Yokoyama et al., 2002). The role in lifespan extension seems to be due to its ability to inactivate the tumor suppressor protein p53 (Wadhwa et al., 2002). It will be interesting to determine how mortalin contributes to long term anoxia survival by turtles and whether its up-regulation also occurs in other organisms that live for extended times in hypometabolic states.

5. Concluding remarks

The molecular mechanisms that provide anoxia tolerance to turtles are a fascinating study in biochemical adaptation. Much is already known, particularly about the vertebrate brain without oxygen, due in no small part to the extensive research contributions of Peter Lutz. Much more remains to be learned, as our recent results from gene screening attest. We are moving from an era where research advances were made step by step by researchers following individual trails to a new era where genomic and proteomic screening technologies provide the means to broadly assess the full range of gene expression and protein synthesis adaptations that underlie stress tolerance. The gene screening results discussed here show that anoxia tolerance includes modifications of a wide variety of metabolic proteins implicating the adaptive regulation of various cell functions that have never before been considered to contribute to anoxia tolerance. Coordinated controls, triggered by critical low oxygen concentrations, must be initiated to readjust all cell functions to a new lower rate of ATP turnover during hypometabolism and to prioritize ATP use by the most vital functions. Other new screening technologies such as transcription factor arrays will allow us to identify the signal transduction pathways that transmit low oxygen messages and the transcription factors that regulate the coordinated expression of groups of genes whose protein products enhance anoxia survival. Peter Lutz found himself in 2005 still at the forefront of metabolic research on turtle anoxia tolerance, fully 25 years after his first paper on the subject (Lutz et al., 1980). He always looked at his work in the larger context of whole animal physiology, even while pursuing molecular level details. The new multi-functional approaches must keep this focus in mind — we want the next 25 years of research in this area to make Peter proud.

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