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TURNING DOWN THE FIRES OF LIFE: METABOLIC REGULATION OF HIBERNATION AND ESTIVATION

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1. Introduction

The ability to suppress basal metabolic rate and enter a hypometabolic or dormant state is a life-saver for many animals. When food is unavailable or environmental conditions too stressful to maintain normal life, many species reduce their energy consumption and, thereby, extend the time that fixed reserves of endogenous metabolic fuel stores can sustain life. Metabolic suppression can come in varying intensities, ranging from a relatively shallow 20-30 % reduction in metabolic rate for a few hours during nightly torpor in small birds and mammals to a >95% reduction in metabolic rate for many weeks during seasonal hibernation in mammals or diapause in insects through to a virtually ametabolic state that allows desiccated seeds, spores and cysts of many plant and animal species to remain viable for many years (Storey and Storey, 1990; Hand and Hardewig, 1996). The present review focuses on two forms of aerobic hypometabolism in vertebrates - mammalian hibernation and amphibian estivation (Geiser, 1988; Wang, 1989; Land and Bernier, 1995) - and describes some of our recent advances in understanding the metabolic regulation and changes in gene expression that underlie the phenomena.

Hibernation is a common winter survival strategy of many small mammals that allows them to deal with two serious problems: (1) limited food supply, and (2) the enormous energetic costs of maintaining a constant high body temperature in cold weather (Wang, 1989). To escape these problems, many bats, mice, ground squirrels and others hibernate. Metabolic rate is strongly reduced and the hypothalmic set point for core body temperature is lowered so that body temperature falls to the environmental ambient (although regulation is reinstated if there is a danger of core temperature falling below ~2°C). Animals fall into a deep torpor that can last for days or weeks. Hibernation bouts are longest and deepest in midwinter and are punctuated by periods of arousal that rewarm and awaken the animal. The overall energy savings achieved over the hibernating season (including the costs for periodic arousals) were reported to be 88% for ground squirrels as compared with the cost of remaining euthermic throughout the winter (Wang,

1989) and may be even greater for smaller mammals like bats and mice that show a higher fold-reduction in metabolic rate (Geiser, 1988).

Estivation is a torpid state that is probably best defined as a survival strategy for dealing with arid conditions but is also frequently associated with heat and lack of food (Pinder *et al*., 1992; Land and Bernier, 1995; Abe, 1995). The frequently cited examples among vertebrates are lungfish that retreat into underground burrows when river waters subside during the dry season and a variety of frog and toad species that live in arid regions and spend as much as 10 months of the year buried underground, emerging only briefly in the rainy season to feed and breed. Metabolic rate in estivating anurans typically falls to about 20-30% of the corresponding rate in resting but non-estivating individuals. Most estivating species also express adaptations that defend the body from water loss during dormancy. Frogs and toads all enter estivation with large reserves of water in the bladder that are used to maintain tissue hydration for as long as possible. Some species also use physical barriers to water loss by forming cocoons made of dried mucus or multiple layers of shed skin, whereas others retard water loss by chemical means by greatly elevating the osmolality of their body fluids through the production of high concentrations of solutes such as urea. Nonetheless, as the soil dries out over time, net water loss can rise as high as 60% of total body water (47-50 % of body mass) after several months (McClanahan 1967).

Both hibernation and estivation have in common a retreat into sheltered sites that provide both physical protection from predators and minimize variations in environmental conditions of temperature, humidity, oxygen, etc. Both are prefaced by the laying down of large reserves of endogenous fuels, primarily lipids, and in both situations lipids are used as the primary fuel source during dormancy. In the case of hibernating species, body mass can increase by up to 50% during late summer feeding as fat reserves are laid down and the composition of body fat depots changes to include a high percentage of polyunsaturated fatty acids that allow the lipid depots to remain fluid and metabolizable at body temperatures approaching 0°C (Kenagy and Barnes, 1988; Frank and Storey, 1995). Carbohydrate reserves make a fairly minor contribution to metabolism during hibernation or estivation but protein use (primarily due to skeletal muscle wasting) can be substantial with amino acids frequently channeled into gluconeogenesis to provide glucose for those tissues that need it. In spadefoot toads, the net contributions to the total energy budget over the estivating season are 72% from fatty acid oxidation, 23% from protein, and 5% from carbohydrate (Jones, 1980). However, protein use changes over time, being low during the early weeks of estivation but rising as the water potential of the soil declines and the demand for urea synthesis rises.

Estivation and hibernation also share the use of specific biochemical mechanisms that strongly reduce net ATP turnover, that change patterns of fuel use, and that initiate specific changes in gene expression. The transition to the hypometabolic state must be regulated in order to achieve a coordinated suppression of many metabolic functions and a new balance between ATP-producing and ATP-utilizing reactions in cells. The need for coordinated control is amply illustrated by the effects of hypothermia on non-hibernating mammals. Once core body temperature falls to only a few degrees below optimal in humans or rats, for example, energy balance is disrupted and ATP levels fall, membrane potential difference collapses due to

imbalances in the activities passive ion channels versus ATP-dependent ion pumps, and a variety of destructive events are unleashed (many of them Ca^{2+}-mediated) that quickly become irreversible. Such a collapse of homeostasis is the reason for the damaging or even lethal effects of cold on the core organs of most mammals, problems that manifest themselves medically in the injuries done by hypothermia and in the deterioration during cold storage of donor organs awaiting transplant. However, hibernating mammals with core body temperatures that may be <5°C show none of these injurious effects of cold due to the implementation of a suite of control mechanisms that coordinate both a steep metabolic suppression and rebalance cellular reaction rates. For example, protein synthesis, one of the energy-expensive functions of cells, is strongly suppressed in hypometabolic systems. In brain of hibernating ground squirrels the rate of protein synthesis was only 0.04 % of the mean rate in euthermic brain (Frerichs *et al*., 1998) whereas in liver slices from estivating frogs, *Neobatrachus centralis*, protein synthesis was reduced by 67% compared with the rate in slices from non-estivating frogs (Fuery *et al*., 1998). Other energy expensive cellular processes such as ion transport are also suppressed in hypometabolic systems. The biochemical mechanisms involved need to be both powerful and yet easily reversible to allow a quick return to normal metabolism during arousal.

One of these mechanisms is reversible protein phosphorylation. Major changes to the activity state of many enzymes and functional proteins can be made by the addition or removal of covalently bound phosphate due to the action of protein kinases or protein phosphatases and the role of this mechanism in suppressing the activities of multiple enzymes of carbohydrate metabolism has been well documented in hibernating mammals as well as in a variety of other hypometabolic systems (Storey and Storey, 1990; Brooks and Storey, 1997; Storey, 1996; 1997, 1998). For example, reversible phosphorylation coordinates the suppression of glycogen phosphorylase (GP), phosphofructokinase (PFK) and pyruvate kinase (PK) activities in liver of hibernating jumping mice (*Zapus hudsonius*) (Storey, 1987) and control over pyruvate dehydrogenase (PDH), the enzyme gating carbohydrate entry into the tricarboxylic acid cycle, suppresses the amount of active enzyme to <5% of the corresponding euthermic values in tissues of hibernating *Z. hudsonius* and ground squirrels (Storey, 1987, 1989; Brooks and Storey, 1992).

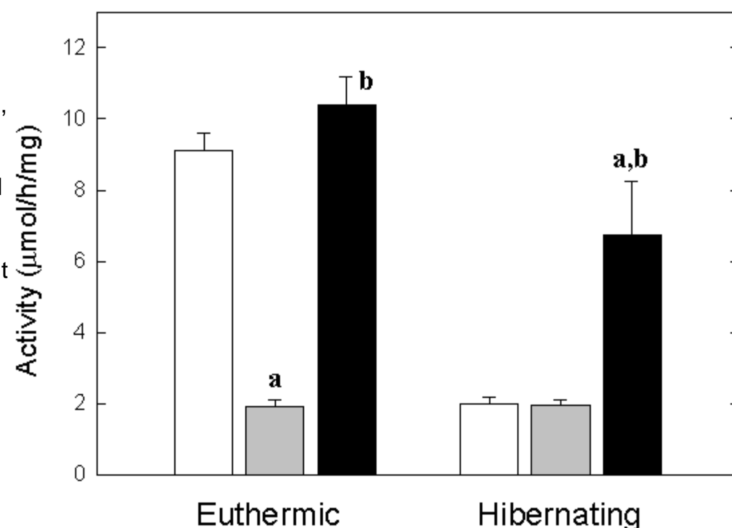
Recent studies in my lab continue to explore the biochemical mechanisms underlying metabolic rate depression in both hibernating and estivating systems including the role of reversible phosphorylation in enzyme control, the signal transduction mechanisms that mediate changes in metabolism, adaptive changes to the properties of selected proteins, and the role of gene expression in providing new protein variants for specific roles in the hypometabolic state. The primary model animals that we use for these studies are hibernating ground squirrels, *Spermophilus richardsonii* or *S. lateralis* (Richardson's and golden-mantled ground squirrels, respectively) and estivating spadefoot toads, *Scaphiopus couchii*. The current chapter highlights some of our recent findings.

2. Reversible phosphorylation and enzyme control

2.1 Hibernators

Our first studies of the role of reversible phosphorylation in the control of hibernation, summarized above, focused on enzymes involved in the ATP-producing pathways of catabolism. However, equally important to reducing net ATP turnover and re-balancing metabolism at a lower metabolic rate is control over the hundreds of diverse ATP-utilizing processes in cells. To determine whether reversible phosphorylation also played a role in the regulation of the energy-utilizing reactions in cells, we chose to look first at one of the largest consumers of cellular energy, the sodium/potassium ATPase (Na/K-ATPase), which is responsible for 5-40% of total ATP turnover depending on cell type (Clausen, 1986). Analysis of the Na/K-ATPase maximal activities in organs of hibernating versus euthermic ground squirrels, *S. lateralis*, found a strong reduction in activity during hibernation. Activities were only 40, 59 and 54% of the corresponding euthermic values in skeletal muscle, kidney and liver, respectively (MacDonald and Storey, 1999). However, Na/K-ATPase activity in heart was unaffected during hibernation. To determine whether reversible phosphorylation was the mechanism responsible for the hibernation effect, skeletal muscle extracts from euthermic and hibernating squirrels were incubated *in vitro* for 2 hours with added cAMP + ATP + Mg^{2+} to stimulate protein kinase A (PKA) action and were then treated with alkaline phosphatase for 2 more hours. Figure 1 shows that PKA treatment lowered Na/K-ATPase activity in euthermic muscle extracts to just 20% of the untreated value but had no effect on the enzyme in hibernator muscle. Subsequent treatment with alkaline phosphatase fully restored enzyme activity in extracts from euthermic muscle and also raised Na/K-ATPase activity in hibernator extracts by 3.5-fold. Treatments that stimulated protein kinases G or C also reduced Na/K-ATPase activity in muscle extracts of euthermic animals (MacDonald and Storey, 1999). These data indicate that suppression of Na/K-ATPase activity during torpor is brought about by phosphorylation of the enzyme whereas protein dephosphorylation reactivates the enzyme when the animal arouses and Na/K-ATPase is needed for its key role in shivering thermogenesis.

Figure 1. Effect of stimulating endogenous protein kinase A (incubation with 10 mM ATP, 10 mM $MgCl_2$, 0.3 mM cAMP) and subsequent alkaline phosphatase treatment (10 units) on Na/K-ATPase activity in skeletal muscle extracts of hibernating and euthermic ground squirrels. Bar fills are: untreated extracts (open), after protein kinase treatment (shaded), after phosphatase treatment (black). Data are means $\pm$ SEM, n=4. a - significantly different from untreated; b- from protein kinase treated, $P<0.05$. From MacDonald and Storey (1999).



2.2 Estivators

Reversible phosphorylation control over enzymes also plays a major role in the control of aerobic metabolic rate depression in estivating animals. For example, the suppression of carbohydrate catabolism in estivating snails includes reversible phosphorylation control over GP, PFK, PK and PDH (Brooks and Storey, 1997). Our recent studies with estivating toads show that the mechanism also extends to vertebrate estivation.

Cowan and Storey (1999) evaluated the effect of estivation on PK and PFK from skeletal muscle of the spadefoot toads, *S. couchii*. Both enzymes occur in two forms in toad muscle that are separable by isoelectrofocusing. Estivation for 2 months at 15°C resulted in a strong increase in the proportion of the total activity for both enzymes that was associated with peak II (pI = 6.2-6.4) as compared with peak I (pI = 5.3-5.4). Thus, for PK in muscle of control toads the distribution of activity was 23 % Peak I and 77 % Peak II whereas after estivation the proportions changed to 13% Peak I and 87% Peak II (Figure 2). For PFK, enzyme distribution in the two peaks changed from roughly 50-50 in controls to ~25 % of the recovered activity in Peak I and 75 % in Peak II in estivating animals. To determine whether the changes in peak ratios were due to covalent modification, crude muscle extracts were incubated *in vitro* with ^{32}P-ATP under conditions that promoted the activity of PKA. This led to strong radiolabeling of the enzymes in Peak I but not Peak II and reverse phase HPLC confirmed that the subunits of PK and PFK found in Peak I had ^{32}P associated with them. Confirmation that cAMP-dependent protein kinase A was responsible for the phosphorylation of peak I enzymes came from experiments where PK or PFK was incubated in the absence versus presence of the protein kinase A inhibitor, PKA-I (5-24). After incubation, specific antibodies were used to immunoprecipitate the individual enzymes. The amount of radiolabeled PK in immunoprecipitates was 36-fold higher when extracts had been incubated with ^{32}P-ATP and protein kinase A alone than when PKA-I was also included; the comparable value for PFK was 3.2 fold. Hence, toad skeletal muscle peaks I and II were identified as the phosphorylated and dephosphorylated forms, respectively, for both PK and PFK and the effect of estivation was to increase the proportion of dephosphorylated enzymes in muscle.

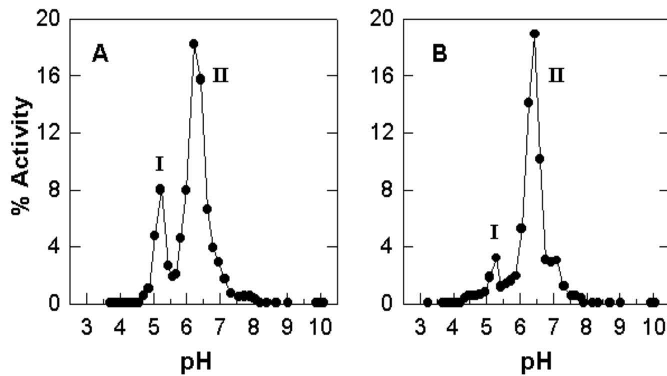


Figure 2. Pyruvate kinase elution in two peaks after isoelectric focusing of spadefoot toad leg muscle extracts. (A) extract from control toads, (B) extract from 2 month estivated toads. PK activity is expressed as a percentage of the total activity applied to the column. From Cowan and Storey (1999).

Kinetic analysis of the partially purified enzymes showed significant differences in properties between Peak I and II forms. Peak II (low phosphate) PK had a 1.6-fold higher Km for phosphoenolpyruvate and a 2.4-fold higher Ka for fructose-1,6-bisphosphate than did the Peak I (high phosphate) form (Cowan and Storey, 1999). These differences suggest that Peak II PK is a less active enzyme, and coupled with the shift to predominantly the Peak II form during estivation, the data are consistent with a suppression of PK activity in estivating muscle, as part of the overall metabolic rate depression of the estivating state. Phosphorylation of vertebrate skeletal muscle PFK typically occurs during exercise and the phospho-enzyme shows increased binding to myofibrils in active muscle (Foe and Kemp, 1982). The increased content of the Peak II, low phosphate, form of PFK in muscle of estivating toads would be consistent therefore with metabolic suppression. Peak II PFK also showed reduced sensitivity to inhibition by Mg:ATP (I_{50} 50 % higher) compared with Peak I PFK suggesting that the enzyme in estivating muscle is less tightly regulated by cellular adenylate status than in control toads.

3. Signal transduction

Given the widespread role of reversible phosphorylation in the control of enzymes in hibernating and estivating systems, we predicted that the activities of cellular protein kinases and protein phosphatases would change in the hypometabolic state. In recent work we looked at the effects of estivation on the activities of protein kinases and phosphatases in organs of spadefoot toads, *S. couchii* (Cowan *et al*., 2000). Given the evidence of PKA-mediated control over PFK and PK in organs of estivating toads (Cowan and Storey, 1999), we first assessed the organ-specific responses of PKA during estivation. Total PKA activity remained constant in all organs during estivation but the percentage that was present as the active free catalytic subunit (%PKAc) was strongly reduced in all seven organs of estivating toads that were tested. A common role for PKA is the activation of fuel catabolism, frequently as a response to hormones such as adrenaline or glucagon; for example, PKA mediates the activation of both glycogen phosphorylase and

hormone sensitive lipase to increase the breakdown of carbohydrate and lipid stores, respectively. Therefore, the reduced %PKAc in toad organs during estivation is consistent with lower rates of fuel catabolism during dormancy. However, total PKA activity was unaltered during estivation and this maintains organ sensitivity to PKA-mediated signals so that fuel catabolism and metabolic rate can be rapidly re-elevated during the arousal process.

Estivation was also associated with reduced activities of Ca^{2+}-activated, phospholipid-dependent protein kinase C (PKC) in toad organs. PKC mediates the intracellular responses that underlie a variety of events including cell proliferation, differentiation, exocytosis, and neural activity (Kikkawa *et al*. 1989). PKC is distributed between membrane-bound and cytosolic forms and in response to extracellular stimuli that elevate phosphatidylserine and Ca^{2+}, the enzyme is translocated from inactive cytosolic pools to the plasma membrane where it becomes active. Therefore, the key parameter in evaluating the state of PKC activity in tissues is the percentage that is membrane-bound. As Table 1 shows, the percentage of membrane-bound PKC was strongly reduced during estivation in all toad tissues that were tested. Furthermore, total PKC activity also dropped by more than half in liver during estivation (from 48.5 ± 6.05 to 20.8 ± 4.00 milliunits per gram wet weight) (Cowan *et al*., 2000). Overall, then, the amount of active membrane-bound PKC in estivating toads was reduced to 50 % of the control value in brain and kidney and to just 13 % of control in liver. This reduced content of active PKC in organs is consistent with a lower organ metabolic rate and implicates the suppression during estivation of various metabolic processes that are PKC-controlled. Furthermore, the similar responses of both PKA and PKC during estivation suggest that protein kinase-mediated signal transduction pathways are key regulators of the induction and maintenance of hypometabolism.

Table 1. Effect of estivation on protein kinases A and C in spadefoot toad tissues: the percentage of PKA present as the active catalytic subunit (PKAc) and the percentage of PKC present as the active, membrane-bound enzyme.

	% PKAc		**% PKC membrane bound**	
	Control	**Estivated**	**Control**	**Estivated**
Brain	57.0 ± 8.0	25.0 ± 3.0[a]	45.8 ± 1.6	21.5 ± 3.2[a]
Liver	97.0 ± 5.0	62.0 ± 8.0[a]	46.6 ± 5.7	16.4 ± 2.4[a]
Muscle	80.0 ± 6.0	47.7 ± 1.0[a]	29.6 ± 5.6	29.8 ± 3.9
Heart	38.0 ± 3.0	20.0 ± 5.0[a]	62.2 ± 1.4	65.6 ± 1.1
Lung	97.0 ± 2.0	63.0 ± 0.1[a]	-	-
Kidney	82.0 ± 2.0	62.0 ± 4.0[a]	70.8 ± 12.0	18.7 ± 4.7[a]
Gut	40.0 ± 6.0	21.0 ± 4.0[a]	-	-

Note that total PKA activity did not change in any tissue during estivation whereas total PKC activity changed

only in liver and kidney. Data are means ± SEM, n = 4; PKC was not analyzed in lung and gut. a- Significantly different from the corresponding control value, $P < 0.05$. From Cowan *et al.* (2000).

Protein phosphatases reverse the action protein kinases and typically changes in their activities behave oppositely to those of their protein kinase partners. When we analyzed the effects of estivation on the activities of protein phosphatase (PP) types 1, 2A and 2C in toad organs, we found only a few changes in the activities of these enzymes with the transition to the hypometabolic state (Cowan *et al*., 2000). PP-1 was quantified in dilute, trypsin-treated extracts to release any enzyme bound to inhibitor proteins and give a measure of total PP-1 activity and also in untreated extracts to measure the free, active enzyme only (Price *et al*., 1986). Changes in the total activity of PP-1 (in trypsin-treated extracts) and in the percentage of free, active enzyme (measured in untreated extracts) were seen in four organs of estivating toads but these changes were generally oppositely directed so that the net change in the amount of free, active enzyme in liver, heart and gut of estivating toads was small (Cowan *et al*., 2000). However, active PP-1 in kidney was strongly reduced during estivation, decreasing from 1.16 U/mg in control toads to 0.33 U/mg in estivated animals. What is striking about these results is that whereas PKA (and PKC) activities were uniformly suppressed in toad organs, PP-1 activities did not show an opposite rise but remained constant or were reduced. Similarly, PP-2A activity remained constant (brain, skeletal muscle, lung, kidney) or fell by 29-43% (liver, heart, gut) in estivating toads. PP-2C activity decreased by 50 % in heart, was unaffected in three organs, and rose in brain, muscle and gut of estivating animals. Overall, however, the data for both protein kinases and protein phosphatases suggests that a general suppression of all signal transduction mechanisms may occur as part of the metabolic depression of the estivating state.

Signal transduction during hypometabolism may also rely upon selected adaptations to the properties of protein kinases or phosphatases that adjust them for better function under the cellular conditions that prevail in the hibernating or estivating state. We wondered in particular about the influence of temperature on these enzymes in hibernating mammals and to analyze this we purified and characterized the catalytic subunit of protein kinase A from brown adipose tissue of ground squirrels (*S. richardsonii*) (MacDonald and Storey, 1998). PKAc has a particularly important role in brown adipose tissue of hibernators for it is integral to the activation of thermogenesis during arousal. ß-Adrenergic signals stimulate a rise in cAMP levels in brown fat cells which in turn cause the dissociation of the PKA holoenzyme to release the catalytic subunits which then phosphorylate a range of target proteins to activate lipolysis and the uncoupled respiration that generates heat. A number of factors were identified that could aid the activation of brown adipose PKA at low body temperature during the initiation of arousal from hibernation: (1) total PKA activity was 33% higher in brown adipose of hibernating squirrels compared with euthermic controls, (2) the percentage of free catalytic subunit (PKAc) was low (1-3%) in both resting euthermic and torpid animals so that the metabolic scope for increasing PKAc content upon stimulation was very high, (3) cAMP effects on PKA holoenzyme dissociation were hyperbolic at 37°C but sigmoidal at 5°C which would make the enzyme much more responsive to small changes in [cAMP] at low body temperatures, (4) PKAc substrate affinities increased at low temperature: Km ATP decreased by 50% when assayed at 5°C, compared with 37°C, and Km for the phosphate accepting model substrate, Kemptide, decreased

by 80%, and (5) enzyme inhibition by salts was reduced at low temperature (MacDonald and Storey, 1998). Furthermore, an analysis was made of the patterns of protein phosphorylation when crude extracts of brown adipose tissue from either euthermic or hibernating animals were incubated with ^{32}P-ATP and cAMP ($\pm$ PKA-I) at either 37 or 5°C. The resulting patterns of phosphoproteins differed both between hibernating and euthermic extracts and between the two incubation temperatures. This indicates that there are both different targets for PKAc action in the brown adipose of the hibernating animal and that temperature influences the capacity of PKAc to phosphorylate different target proteins (MacDonald and Storey, 1998). Overall, then, it is clear that a variety of different influences can regulate the function of PKAc in the hibernator and contribute to the action of the enzyme in the activation of thermogenesis.

4. Protein adaptation

Thousands of examples of protein adaptation are known that create variants with features tailored to multiple needs including cell-, organ- or species-specific jobs, as well as to deal the effects of environmental parameters including temperature, salinity, pH, and pressure to name a few (Hochachka and Somero, 1984). Hibernators face a challenge that is uncommon among mammals and that is that metabolism must function over a wide range of body temperatures, from euthermic values of 37-40°C down to near 0°C. Multiple adaptive strategies have been documented that can tailor the enzymes of hibernators for low temperature function including (1) reversible phosphorylation (discussed previously), (2) changes in the levels of substrates and effectors (e.g. organ ATP levels typically drop by 30-50% during hibernation; Storey, 1997; MacDonald and Storey, 1999; English and Storey, 2000), and (3) temperature-dependent changes in enzyme properties (Storey, 1997; English and Storey, 2000; Thatcher and Storey, 2000). Several recent studies of protein adaptation in hibernator and estivators provide new insights into the adaptive strategies used to modify proteins for function under stress conditions.

4.1 Glutamate dehydrogenase in hibernators

Glutamate dehydrogenase (GDH) is a key enzyme in intermediary metabolism for it represents one of the main links between carbohydrate and amino acid metabolism through its reversible reaction: glutamate + NAD $\longrightarrow$ NH_4^+ + α -ketoglutarate + NADH. During hibernation, which is a state of prolonged fasting, the enzyme likely functions primarily to catalyze the oxidation of glutamate that is derived from the breakdown of tissue protein as one of the fuel sources for torpor. All hibernators show a decrease in total muscle mass during torpor (Wang 1989) and gluconeogenic capacities rise in liver and kidney during hibernation to allow these organs to convert amino acids and glycerol (from triglyceride oxidation) into the glucose that is needed to fuel selected organs. The carbon skeletons from five amino acids are funneled into the oxidative reactions of the tricarboxylic acid cycle via GDH and GDH together with the transaminases process the amino groups from many amino acids to release NH_4^+ for use in urea biosynthesis.

GDH was purified from liver of both euthermic (37°C body temperature) and hibernating (torpid, 5°C body temperature) Richardson's ground squirrels (*S. richardsonii*) and kinetic and physical properties of the two enzymes were compared (Thatcher and Storey, 2000). Like GDH from

other sources, the enzyme was a hexamer with a subunit molecular weight of 59.5 ± 2 kDa. However, we found small differences in the native molecular weight of the euthermic (335 ± 5 kDa) versus hibernator (320 ± 5 kDa) enzymes on size exclusion HPLC and consistent small differences in subunit retention times on reverse phase HPLC that suggested that there is some physical difference between the euthermic and hibernator forms. The two enzymes showed substantial differences in kinetic properties in both the forward (glutamate-utilizing) and reverse (α -ketoglutarate-utilizing) reactions. Table 2 summarizes some of these differences, focusing on apparent Km values and selected effectors of the forward reaction. Kinetic properties of each enzyme were differentially affected by assay temperature (37 versus 5°C) but notably, euthermic versus hibernator GDH appeared to be optimized for function at warm versus cold temperature, respectively. Thus, the Km values for all three substrates (glutamate, α -ketoglutarate, NH_4^+) were lowest (ie. affinity highest) at 37°C for euthermic GDH and at 5°C for hibernator GDH. In particular, the very strong effects of low temperature on euthermic GDH (Km values for α -ketoglutarate and NH_4^+ rose by 37- and 4.5-fold, respectively, at 5° compared with 37°C) were not seen with hibernator GDH. A GDH that is less sensitive to temperature change would be more practical for the hibernating state where body temperature would be variable. Temperature effects on Ka ADP values of the enzymes followed a similar pattern. Although Ka values for both enzymes decreased at low temperature, the lower Ka at each temperature was displayed by euthermic GDH at 37°C and by hibernator GDH at 5°C (Table 2). By contrast, GTP inhibition was strongest with the euthermic enzyme at 37°C and weakest with hibernator GDH at 5°C. Other nucleotides (AMP as an activator, ATP as an inhibitor) lost their effects at 5°C, leaving ADP and GTP as the primary regulators of GDH. Because tissue ATP levels fall by 30-50% during hibernation, it is likely that levels of other nucleotide triphosphates are also reduced during torpor and that the role of GTP in controlling GDH would be lessened under these conditions. Interestingly, in comparing euthermic and hibernator GDH at 37 and 5°C, the lowest Ka ADP and the highest I_{50} GTP were found with hibernator GDH at 5°C which suggests that ADP assumes prominence in the regulatory control of GDH in the hibernating state, a situation that could link the rate of amino acid oxidation via GDH with cellular demand for energy production.

Table 2. Kinetic properties of glutamate dehydrogenase purified from liver of euthermic versus hibernating ground squirrels, *S. richardsonii*, measured at 37 and 5°C.

	Euthermic		**Hibernating**	
Apparent Km (mM)	**37°C**	**5°C**	**37°C**	**5°C**
Glutamate	2.03 ± 0.17	0.5 ± 0.05[a]	5.2 ± 0.47[b]	0.25 ± 0.02[a]
NH_4^+	7.0 ± 0.69	24.7 ± 0.01[a]	15.8 ± 1.44[b]	12.1 ± 0.95[b]
α -Ketoglutarate	0.10 ± 0.01	3.66 ± 0.34[a]	0.98 ± 0.08[b]	0.43 ± 0.02[ab]
Effectors of the	**forward (glutamate-**	**utilizing) reaction**		

Ka ADP	0.181 ± 0.004	0.032 ± 0.002[a]	0.58 ± 0.052[b]	0.011 ± 0.001[a]
I_{50} GTP (mM)	0.019 ± 0.001	0.061 ± 0.0051[a]	0.076 ± 0.007[b]	0.19 ± 0.013[b]

Data are means ± SEM, n=4. For Km glutamate, NAD^+ was 1.5 mM and for Km values in the reverse direction, NADH was 0.15 mM with either 100 mM NH_4HCO_3 or 7.5 mM α -ketoglutarate. a- Significantly different from the value for the same enzyme at 37°C, $P < 0.05$; b- significantly different from euthermic GDH at the same temperature, $P < 0.05$. Data from Thatcher and Storey (2000).

The differences in kinetic properties between the euthermic and hibernating forms of GDH from ground squirrel liver suggest that entry into hibernation leads to a stable change in GDH that allows the enzyme to function optimally at the lower body temperature. The mechanism of the change in enzyme properties has not yet been elucidated. Since both the euthermic and hibernating experimental groups were sampled at the same time after hibernators had gone through torpor cycles for 1 month, it seems likely that the mechanism must be one that can change the enzyme quickly during entry into torpor but be reversed during arousal. Synthesis of a new isozyme of GDH under the low metabolic rate conditions of hibernation would not be likely but a covalent modification of the enzyme could be made that would allow an easy interconversion of GDH forms between torpid and aroused states. GDH is not known to undergo reversible phosphorylation in other mammals but this mechanism or another form of covalent modification might have developed in hibernators to adjust the enzyme for optimal function at either high or low body temperatures.

4.2 FABP in hibernators

Given the high reliance on lipid oxidation as the primary metabolic fuel during hibernation as well as during the arousal process, it is obvious that proteins involved in lipid catabolism must be tailored to function over a wide range of temperatures in hibernating species. We recently became interested in a one such protein. Fatty acid binding proteins (FABPs) are the cytoplasmic carriers that transport long chain fatty acids through the aqueous cytosol. About 20 different gene products of these low molecular weight (14 kDa) proteins have been identified in mammals (Paulussen and Veerkamp, 1991; Stewart 2000), with homologues in other vertebrate and invertebrate groups (Londraville, 1996). Two broad categories exist: FABPs from muscle, heart and intestine mediate one-way, vectorial delivery of fatty acids to intracellular sites (e.g. the mitochondria) and use a collisional mechanism to load fatty acids from membranes or membrane-bound protein complexes. Liver FABP, by contrast, shows diffusion-limited loading and release of fatty acids in line with its role in two-way flux of fatty acids into or out of liver cells (Hirs *et al.*, 1995). Physical and functional properties of FABPs have been examined extensively in mammals, but few studies have explored the potential for adaptive modifications to FABPs that would allow them to respond to environmental stress or altered physiological demands. In hibernating species, FABPs must necessarily function at both euthermic (~37°C) and hibernating (often <5°C) body temperatures. However, the binding of fatty acids to FABPs involves both hydrophobic and hydrophilic bonds (Richieri *et al*. 1995) which are temperature-

sensitive and oppositely affected by decreasing temperature. Thus, the large change in body temperature between aroused and torpid states could have serious consequences for FABP function and cellular fatty acid transport.

To determine whether ground squirrel FABP showed any adaptive modifications that would aid its function over a wide temperature range and particularly at the low body temperatures of the torpid state, we purified the protein from *S. richardsonii* liver and compared it with rat liver FABP (Stewart *et al*. 1998). Like the rat protein, ground squirrel FABP is a monomer of ~14 kDa which binds oleate with 2:1 stoichiometry. However, rat and ground squirrel FABPs were quite different in the temperature sensitivity of substrate binding. Binding of a fluorescent model ligand, *cis*-parinarate, by hibernator FABP was temperature insensitive when tested at 5, 25, or 37°C with K_d values of 0.25-0.31 μM. By contrast, rat FABP showed its lowest K_d (0.18 μM) at 37°C and significantly higher values at 5 or 25°C (0.30-0.32 μM). Hence, rat FABP showed reduced affinity for its ligand when functioning below normal body temperature. Furthermore, when the temperature-dependence of fatty acid binding was tested in assays that measured the ability of different fatty acids to displace the fluorescent probe (Figure 3), rat FABP showed decreased fatty acid binding (ie. reduced displacement of the fluorophore) at 5°C compared with 37°C for all of the fatty acids tested except linolenate (18:3). By contrast, the fatty acid binding capacity of ground squirrel FABP at 5°C was equal to or better than that at 37°C. This was particularly true of palmitate, the most abundant fatty acid in mammalian tissues, and of two polyunsaturated fatty acids that are key components of hibernator fat depots, linoleate (18:2) and linolenate (18:3). The results suggest that ground squirrel FABP is unimpaired at low temperatures whereas the rat protein is considerably less functional at low temperature. The reason for the difference in temperature-dependent behaviour of these two liver FABPs is not yet known but will likely reside in just a few amino acid substitutions that allow the ground squirrel FABP to maintain optimal protein conformation and optimal fatty acid binding at both high and low temperatures.

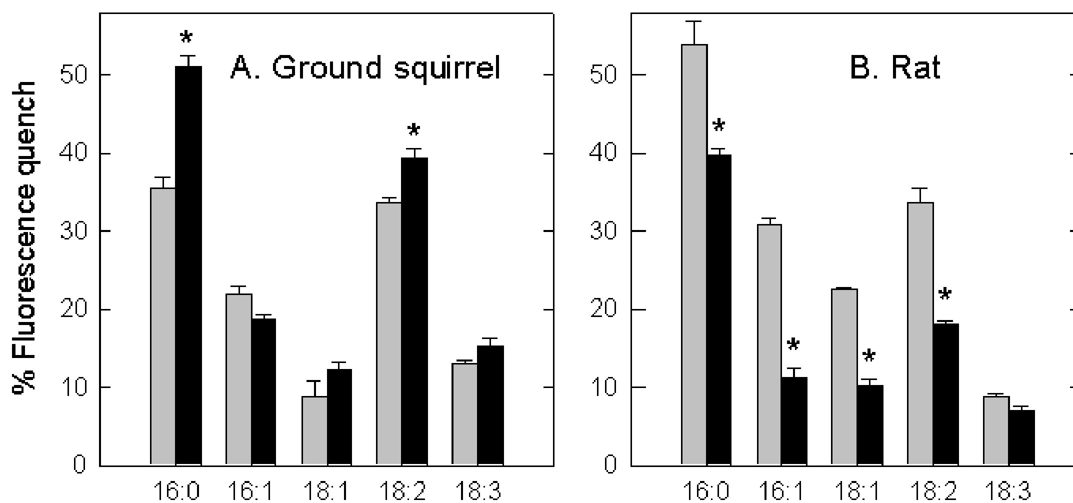


Figure 3. Binding of fatty acids to (A) ground squirrel and (B) rat fatty acid binding protein assessed by ability to displace a fluorescent probe *cis*-parinarate at 37°C (shaded bars) or 5°C (black bars). Probe and fatty acids are both 1μM. Palmitate 16:0, palmitoleate 16:1, oleate 18:1, linoleate 18:2, linolenate 18:3. * - significantly different from the value at 37°C, $P<0.05$. Data are means ± SEM, n=3-6. From Stewart *et al.* (1998).

4.2 FABP in estivators

Estivating toads also depend on lipids as their primary fuel source for long term survival and so we wondered about the nature of their FABPs and whether any adaptive changes to FABP properties might be necessary to support the functional role of the proteins during estivation. In a recent study we purified the FABP from skeletal muscle of spadefoot toads, *S. couchii* (Stewart *et al*., 2000). Little is known about amphibian FABPs, only two other reports having been published on amphibian liver FABPs (Schleicher and Santome, 1996; Baba *et al*., 1999). Spadefoot toad FABP had a molecular weight of ~14 kD, a blocked N-terminus (due to N-acetylation), bound fatty acids with a 1:1 stoichiometry, and showed K_d values for oleate and the fluorescent probe, *cis*-parinarate, of 1.04 ± 0.08 and 1.15 ± 0.16 μM, respectively, all properties consistent with FABPs from other sources (Paulussen and Veerkamp, 1991).

One of the interesting issues with respect to proteins in estivating toads is their sensitivity to high urea concentrations. To help retard the loss of body water as the soil dries out, toads accumulate urea (a product of protein catabolism) in concentrations that reach about 300 mM in body fluids after several months of torpor. Urea is well-known for its ability to denature proteins and a number of studies have examined the effects of urea on the properties of enzymes from elasmobranch fish, which have permanently high tissue urea levels (400-500 mM) in order to maintain isosmotic balance with seawater. These studies, summarized in Hochachka and Somero (1984), showed a number of disruptive effects of high urea on enzyme properties but these were counteracted by effects of other osmolytes, the methylamines, which are also present in elasmobranch body fluids. At the natural ratio of 2:1 urea:methylamines, net enzyme function was unperturbed.

Because of the situation in sharks, we wondered whether the properties of spadefoot toad proteins would also be altered by urea or whether urea insensitivity might have developed. Notably, no counteracting solutes accumulate along with urea in toad body fluids (McClanahan, 1967). In our analysis of toad FABP, we examined urea effects on the binding of *cis*-parinarate. Urea at 200 mM increased the binding of the fluorescent probe by ~60% which suggests that fatty acid binding and transport by FABP would not be impeded as cytoplasmic urea levels rose in estivating toads (Stewart *et al*., 2000). High levels of urea (1 M) also had no negative effect. By contrast, the addition of 200 mM KCl showed a trend (not significant) to reduce probe binding to FABP.

These results for FABP parallel our analysis of urea and salt effects on the activities of a variety of metabolic and antioxidant enzymes from spadefoot toad tissues (Grundy and Storey, 1994, 1998). Plasma and tissue urea levels can reach 200-300 mM in spadefoot toads (Jones, 1980; Grundy and Storey, 1998) to provide colligative resistance to body water loss. Typically, 300 mM urea had little or no effect on the maximal activities of enzymes. For example, out of three antioxidant enzymes assessed (glutathione-S-transferase, glutathione reductase, catalase) in six

organs of both awake (control) and estivating spadefoot toads, only three instances where 300 mM urea affected enzyme activity were found. Urea reduced GR activity in kidney and GST in lung of awake (but not estivating) toads by 19-20%, and urea lowered CAT activity in heart of estivated toads by 26% (Grundy and Storey, 1998). By contrast, the addition of 200 mM KCl reduced the maximal activities of GST and CAT in all tissues of both awake and estivating toads by 51-55% and also reduced GR activity in liver by 25-40%. The same general lack of effect of 300 mM urea but significant inhibition by 200 mM KCl was seen in an analysis of pyruvate kinase, phosphofructokinase, NAD-dependent isocitrate dehydrogenase, and glutamate dehydrogenase activities from spadefoot toad (Figure 4) and frog (*Rana pipiens*) skeletal muscle (Grundy and Storey, 1994). Our interpretation of these results is that as tissue water loss increases over time during estivation, the concentrations of all osmolytes will rise in toad tissues. Some of these, including ions such as K^+ or Cl^-, can have deleterious effects on enzyme activities and kinetic properties. The accumulation of urea during estivation not only helps to retard water loss by raising cellular osmolality but also minimizes the increase in cellular ionic strength that would otherwise occur. Therefore, the accumulation of an osmolyte (urea) that has little or no effect on protein/enzyme function effectively prevents the negative effect on many enzymes that would accrue if ion concentrations rose to high levels.

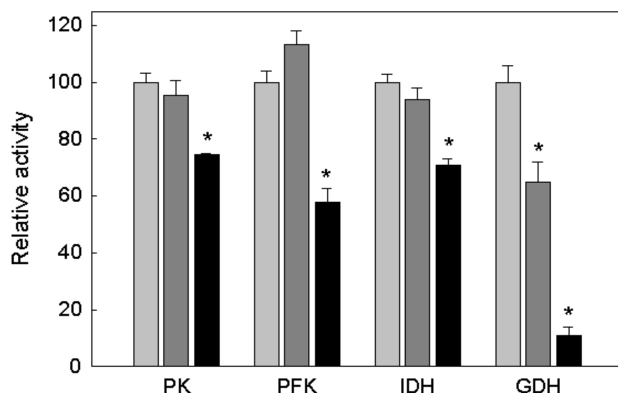


Figure 4. Effect of urea and KCl on activities of enzymes from spadefoot toad skeletal muscle. Bars are: control (light gray), + 300 mM urea (dark gray), + 200 mM KCl (black). Enzymes are: PK, pyruvate kinase; PFK, phosphofructokinase; IDH, NAD-dependent isocitrate dehydrogenase; GDH, glutamate dehydrogenase. Data are expressed relative to control activities and are means $\pm$ SEM, n=3-4. * - Significantly different from the corresponding control, P<0.01. From Grundy and Storey (1994).

5. Genes and hypometabolism

Changes in gene expression underlie virtually all cellular responses to external stress and readjust the protein complement of the cell to provide the appropriate response to any given

stress. Situations of long term hypometabolism or dormancy are by definition times of low metabolic activity and so it could be predicted that the transition from a normal to a hypometabolic state should not be a time of major metabolic reorganization with broad changes in gene expression. Indeed, rates of protein synthesis are strongly suppressed in tissues of hibernating or estivating individuals compared with controls (Frerichs *et al*., 1998; Fuery *et al*., 1998). Thus, it appears that entry into a hypometabolic state occurs with only a selected subset of metabolic changes that target processes that are key for survival under the new conditions of the dormant state. For example, the activity of liver carbamyl phosphate synthetase was twice as high in *S. couchii* emerging from estivation as in active, summer animals (Jones, 1980). Up-regulation of this key enzyme of the urea cycle would clearly be needed during estivation to enhance the capacity for urea synthesis under desiccating conditions. The maximal activities of selected enzymes of intermediary energy metabolism also rose during estivation, presumably due to biosynthesis (Cowan *et al.,* 2000).

New studies in our lab are examining the changes in gene expression that occur during hibernation and estivation to support the torpid state. Studies using cDNA library construction and screening, and more recently, cDNA microarray screening, are identifying selected genes that are up-regulated in the hypometabolic state to serve specific needs. It should be noted that our studies have shown that the levels of most of the mRNA transcripts that were evaluated by these techniques were unchanged or strongly down-regulated during hibernation or estivation, consistent with the quiescence of these hypometabolic states. Against this background, selected genes that were up-regulated during hypometabolism were of great interest.

5.1 Hibernators

Our initial studies with hibernating mammals focused on gene expression in heart of *S. lateralis*. Heart has a critical role that cannot be interrupted during hibernation despite the fact that the organ must function at a much lower T_b and higher peripheral resistance than in euthermia (Wang, 1989). Indeed, although heart rate in torpor may be less than 1/30 of euthermic values, the force of myocardial contraction is increased and cardiac muscle mass and oxidative capacity actually rise (Wickler *et al*., 1991). A cDNA library was constructed from hearts of squirrels that had been hibernating in the lab for 30 days at 3.5°C and that were 2-4 days into their current torpor bout with stable minimal body temperature. Differential screening using ^{32}P-labeled probes made from heart mRNA isolated from hibernating versus euthermic (sampled prior to the start of the 30 day hibernation period) animals revealed clones that were putatively up-regulated during hibernation and northern blots confirmed the up-regulation for two clones (Fahlman *et al*. 2000). Sequence comparisons with the Genbank data bases identified one clone as the nuclear gene *MLC1$_v$* which encodes the ventricular isoform of myosin light chain subunit 1 (MLC1$_v$) and other as the mitochondrial gene *Nad2* which encodes subunit 2 of NADH-ubiquinone oxidoreductase (ND2) (Genbank accession numbers AF170306 and AF169642, respectively). The amino acid sequence of ground squirrel MLC1$_v$ was nearly identical with that of the rat or human protein (97.5% identity) whereas squirrel ND2 showed 66% identity with its human counterpart.

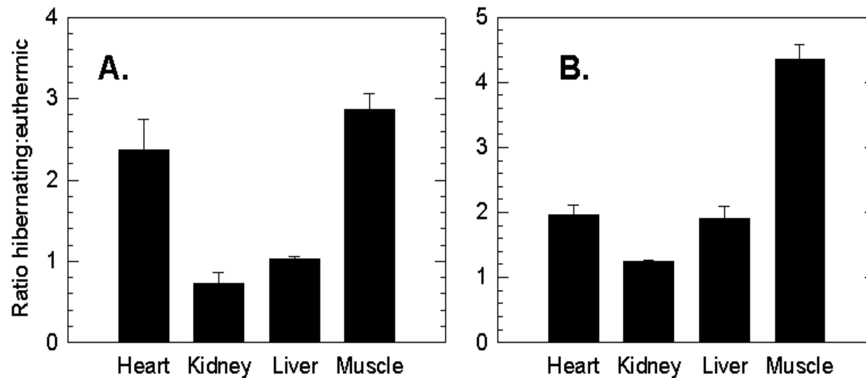


Figure 5. Histograms showing the ratio of relative amounts of mRNA transcripts for (A) ventricular myosin light chain 1 and (B) NADH-ubiquinone oxidoreductase subunit 2 in organs of hibernating versus euthermic ground squirrels, *S. lateralis*, as determined by northern blotting. Blots were scanned, band intensities were quantified using Imagequant, and then band intensities were normalized relative to their corresponding 28 S ribosomal bands. The ratios of normalized band intensities for hibernating versus control samples were then calculated. Data are means $\pm$ S.E.M., n=3 (except for n=2 for kidney and liver for *Nad2*) separately run northern blots; each trial contained an RNA sample prepared from a separate pool of tissue, each pool containing material from 5 different animals. From Fahlman *et al.* (2000).

Levels of $MLC1_v$ transcripts were assessed in several organs of hibernating versus euthermic squirrels. In heart, $MLC1_v$ transcript levels rose by 2.37 $\pm$ 0.64 fold (mean $\pm$ SEM, n=3) during hibernation compared with euthermic levels (Figure 5) (Fahlman *et al.*, 2000). A nearly 3-fold rise in $MLC1_v$ transcripts also occurred in hibernator skeletal muscle. The skeletal muscle results are explained by the fact that the ventricular MLC1 isoform and the slow skeletal muscle isoform are coded by the same gene (Barton *et al.* 1985). However, in other organs (kidney, liver) $MLC1_v$ transcripts remained low and unchanged during hibernation. Comparable northern blot analysis of *Nad*2 found increased transcript levels in three organs during hibernation; increases were ~2-fold in heart and liver and 4.4-fold in muscle (Figure 5).

The reasons for the up-regulation of these two genes in hibernation are not entirely clear but for myosin at least there are good arguments to be made. Myosin is composed of two heavy chains and four light chains (MLC), the MLC being either alkali (MLC1, MLC3) or regulatory (MLC2). Myosin restructuring in response to exercise or stress has been widely reported, especially for the heavy chains which do the actual work in contraction (Goldspink *et al.,* 1992). The function of the alkali light chains is still unclear (Barton *et al*., 1985). They are not essential for enzyme activity but their presence increases the velocity of contraction and they may also help to generate the force of contraction. Isomers of the alkali MLCs are differentially expressed in various muscles and changes in the proportions of heavy and light chains may determine

contractile velocities (Wada *et al*., 1993). The increased peripheral resistance to blood flow at the low body temperature of the hibernating state means that the force of heart contraction has to increase (Wang, 1989). Wang and Lee (1996) linked increased heart contractility in hibernating ground squirrels to changes in sarcoplasmic reticulum Ca^{+2} storage and release but our data as well as similar data for hamsters suggest that another mechanism is also involved. This is a change in the composition of muscle proteins. The increased abundance of $MLC1_v$ transcripts during hibernation implies increased synthesis of $MLC1_v$ subunits in heart at this time and this could alter the ratio of alkali light chain subunit types in the myosin molecule. As a result, the contractile properties of myosin could be altered to create a molecule that is better designed to supply the contractile strength needed during hibernation and operate at the low body temperatures of the torpid state. Hamster heart (*Cricetus cricetus*) also showed changes in myosin make-up during hibernation (Morano *et al*., 1992). The ratio of α to β isozymes the heavy chains was 21:79 in summer animals and winter-active hamsters at 22°C but changed to 53:47 in hibernating animals. Furthermore, the phosphorylation state of the regulatory myosin light chains deceased from 45% phosphorylated protein in summer animals to 23% during hibernation. All of these results suggest that myosin restructuring is an important feature of hibernation and one that deserves further detailed study.

The rationale for an up-regulation of *Nad2* transcripts is not yet known but stress-induced changes in transcript levels of various mitochondrially-encoded subunits of electron transport chain proteins have recently been found in several situations, suggesting that they may serve adaptive or functional needs. Transcripts of mitochondrial genes were up-regulated in rat liver by 4°C exposure and in turtle heart and brain by anoxia exposure, the latter including subunits 4 and 5 of ND (Immaculada *et al*., 1993; Cai and Storey, 1996; Willmore *et al.*, submitted). ND4 was also up-regulated in response to extracellular freezing (an ischemic stress) in liver of the freeze tolerant wood frog (S. Wu and K.B. Storey, unpublished). The common thread in all of these cases is probably oxygen limitation, the oxygen stress in the case of hypothermia in rats and hibernation in other species probably resulting from the hypoperfusion of organs at low body temperatures and also from apnoic breathing patterns in the hibernators.

Other examples of gene up-regulation during mammalian hibernation are also known and suggest a variety of gene products whose function benefits hibernation. Srere *et al*. (1992) reported up-regulation of α_2-macroglobulin in liver whereas other examples include pyruvate dehydrogenase kinase isozyme 4 and pancreatic lipase in heart (Andrews *et al*., 1998), moesin in intestine (Gorham *et al*., 1998), and isoforms of uncoupling protein in multiple tissues (Boyer *et al*., 1998). Each of these gene expression changes may have a key role in the induction or maintenance of torpor. For example, pyruvate dehydrogenase kinase is the enzyme that phosphorylates and turns off pyruvate dehydrogenase, the enzyme that gates carbohydrate entry into mitochondrial oxidative reactions. As mentioned earlier, pyruvate dehydrogenase is strongly suppressed in organs of hibernating animals to restrict carbohydrate oxidation (Storey, 1989; Brooks and Storey, 1992).

5.1 Estivators

Studies in our lab have provided the first analysis of the role of gene expression in estivation. A cDNA library was constructed from liver of 2 month estivating female spadefoot toads, *S. couchii*. Screening with probes made from liver of awake (control) toads versus toads that subsequently estivated for 2 months revealed one strong positive result (Storey *et al.*, 1999). The clone contained a complete open reading frame coding for a protein of 235 amino acids which was identified as riboflavin binding protein (RfBP). RfBP is a phosphoglycoprotein monomer that is synthesized by liver of female vertebrates and secreted into the plasma where it binds plasma riboflavin and loads the vitamin into the yolk of avian or reptilian eggs or the fetus of mammals (White and Merrill, 1988). In birds, synthesis also occurs in the oviduct to produce an isoform that is added to egg white. Our study was the first confirmation that this protein is also found in amphibians. Toad RfBP showed 50 % of residues identical with the chicken or turtle liver proteins including the conservation of essential structural features as identified in the chicken protein: 18 cysteine residues forming 9 disulfide bridges, two asparagine glycosylation sites and 6 tryptophan residues that appear to act in ligand binding (Monaco, '97; Blankenhorn, '78). However, one key element was missing from the toad protein, a highly phosphorylated region near the C-terminus that in the chicken protein extends from amino acids 186 to 197 and contains eight phosphoserines interspersed with glutamate residues. This region is the recognition site for the carrier that facilitates the transport of the vitamin-loaded protein into the yolk (Sooryanarayana *et al.*, '98). The toad protein has only three serine residues in this region, spaced by two glutamate residues. Hence, recognition and binding of RfBP to amphibian oocytes could be quite different than it is in birds and reptiles. Northern blots showed that toad RfBP was largely liver-specific with no mRNA transcripts detected in brain, gut, heart or kidney but low message levels in skeletal muscle of estivating, but not control, toads. The up-regulation of RfBP in toad liver during estivation could serve one of two purposes: (1) RfBP production may be associated with the maturation of eggs in preparation for the explosive breeding that occurs immediately upon emergence from estivation in this species, or (2) RfBP might function in the adult estivator to "cache" riboflavin and maintain a vitamin pool through the 9-10 months of annual dormancy. Significant wasting of the skeletal muscle mass occurs during extended dormancy (*S. couchii* loses 17 % of its body protein reserves) (Jones, 1980) and vitamin caching could be a conservation strategy for important nutrients in these desert animals. In this regard, the results of the northern blots that showed the presence of RfBP mRNA in leg muscle of estivated, but not control, toads are very interesting.

6. Conclusions

Not so long ago the prevailing opinion about mammalian hibernation was that the decrease in body temperature was the cause of the observed decrease in metabolic rate during torpor. Close inspection of the patterns of change in metabolic rate and body temperature in several species have now proven that just the opposite is true: it is the regulated decrease in metabolic rate that causes the fall in body temperature. The present review suggests some of the mechanisms that are involved in regulating and coordinating the decrease in metabolic rate in hypometabolic systems and re-establishing homeostasis for long term dormancy. These include changes in signal transduction, temperature-dependent controls on enzymes and functional proteins,

reversible phosphorylation of enzymes, and differential gene expression. Studies to date highlight both the complexity (the many different enzymes/proteins that must be targeted, the variety of gene expression) and the simplicity (common coordinating mechanisms such as reversible protein phosphorylation) of metabolic arrest but most importantly show us that much more remains to be learned about how nature turns down the fires of life.

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