# Chapter 2
## Metabolic Regulation and Gene Expression During Aestivation

**Kenneth B. Storey and Janet M. Storey**

<table>
<thead>
<tr>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Introduction .................................................................</td>
</tr>
<tr>
<td>2.2 Metabolic Control by Reversible Phosphorylation in Aestivation ................................</td>
</tr>
<tr>
<td>2.2.1 Glucose-6-Phosphate Dehydrogenase ..................................</td>
</tr>
<tr>
<td>2.2.2 Ion Motive ATPases .......................................................</td>
</tr>
<tr>
<td>2.2.3 Protein Synthesis .........................................................</td>
</tr>
<tr>
<td>2.2.4 Protein Degradation ......................................................</td>
</tr>
<tr>
<td>2.3 Signaling Cascades and Metabolic Control in Aestivation ............</td>
</tr>
<tr>
<td>2.3.1 AMP-Activated Protein Kinase ..........................................</td>
</tr>
<tr>
<td>2.3.2 Akt Mediated Signaling ...................................................</td>
</tr>
<tr>
<td>2.4 Gene Regulation ...............................................................</td>
</tr>
<tr>
<td>2.4.1 Global Suppression of Gene Expression ...............................</td>
</tr>
<tr>
<td>2.4.2 Gene Hunting and Stress Response .....................................</td>
</tr>
<tr>
<td>2.4.3 Aestivation-Responsive Gene Expression .............................</td>
</tr>
<tr>
<td>2.5 Conclusion ........................................................................</td>
</tr>
<tr>
<td>References .............................................................................</td>
</tr>
</tbody>
</table>

**Abstract** The biochemical regulation of aestivation, a state of aerobic hypometabolism, achieves actions including strong overall suppression of metabolic rate, re prioritization of energy use by diverse cell functions, and enhancement of defenses such as protein chaperones and antioxidants that aid long-term life extension. This is accomplished by mechanisms that include differential action of intracellular signaling cascades, reversible protein phosphorylation to alter the activity states of multiple enzymes and functional proteins, global suppression of transcription and translation, and selective gene upregulation. Recent advances in understanding the regulation of aestivation are discussed with a particular emphasis on land snail and anuran models.

K.B. Storey and J.M. Storey
Institute of Biochemistry, Carleton University, 1125 Colonel By Drive, Ottawa, Ontario, Canada, K1S 5B6
E-mail: kenneth_storey@carleton.ca

C.A. Navas and J.E. Carvalho (eds.), *Aestivation: Molecular and Physiological Aspects*, Progress in Molecular and Subcellular Biology 49, DOI 10.1007/978-3-642-02421-4_2, © Springer-Verlag Berlin Heidelberg 2010


2.1 Introduction

Many organisms experience highly variable environmental conditions ranging from those that are conducive to rapid growth and development to those that are incompatible with normal life. A critical option for promoting survival under inhospitable conditions is hypometabolism. By strongly suppressing metabolic rate to low values, organisms gain an extension of the time that they can resist stressful conditions and sustain viability using their only endogenous fuel reserves. Hypometabolism is the common element of numerous animal survival strategies including aestivation, torpor, hibernation, diapause, dormancy, anaerobiosis, dauer state, and anhydrobiosis. Principles of hypometabolism include (a) an overall strong suppression of metabolic rate, typically at least a 70–80% reduction as compared with normal resting rate but ranging up to virtually 100% in anhydrobiosis, (b) differential control over the rates of various metabolic processes so that energy use is reprioritized to favor core vital cell functions (e.g., membrane potential difference) while largely shutting off “optional” activities (e.g., protein synthesis, cell division), and (c) implementation of actions that protect cells and preserve viability over what could be many months of dormancy (Storey and Storey 2004, 2007). How is this accomplished? Suppression of overall metabolic rate during hypometabolism is partly due to reduced energy use by physiological functions because breathing and heart rate are lowered, little skeletal muscle work is done, digestion is halted and some tissues may atrophy (e.g., moderate skeletal muscle atrophy can occur and intestinal mass can decrease) (Guppy and Withers 1999; Hudson and Franklin 2002; Secor 2005; Hudson et al. 2006). In aestivating anurans, for example, intestinal masses were reduced by 44% and total intestinal uptake capacities were lowered by 60% in Ceratophrys ornata and Pyxicephalus adspersus after 1 month of aestivation (Secor 2005). Other extrinsic factors that influence metabolic rate depression include the partial pressures of O₂ and CO₂, pH, and reduced temperature (Barnhart and McMahon 1988; Guppy and Withers 1999); in aestivating snails, for example, CO₂ retention resulting from apnoic breathing patterns is a key factor in metabolic depression (Barnhart and McMahon 1988). However, intrinsic regulation of cellular metabolism is very important, particularly, for achieving a coordinated suppression of ATP-producing versus ATP-consuming pathways, for reprioritizing energy use in the hypometabolic state, for changing patterns of fuel consumption (e.g., states of aestivation or hibernation rely heavily on lipid reserves whereas anaerobiosis requires a switch to carbohydrate fuels), and for implementing cell preservation mechanisms. Furthermore, it is now clear from studies on a wide variety of animal systems of hypometabolism, that most of the molecular mechanisms of metabolic rate depression are highly conserved across phylogeny so that the principles of hypometabolism are becoming well-defined (Storey and Storey 2004, 2007).

This chapter focuses on recent advances in understanding the intrinsic mechanisms that control hypometabolism as they apply to aestivation. We will focus primarily on (a) the use of reversible protein phosphorylation (RPP) to change the activity
states of enzymes and functional proteins, (b) intracellular signal transduction cascades, and (c) differential gene expression. Studies on land snails and anurans will be featured since these are the major models that have been used for molecular studies of aestivation.

2.2 Metabolic Control by Reversible Phosphorylation in Aestivation

Isolated tissues from aestivating animals (snails, frogs) of several species show stable reductions in oxygen consumption compared with tissues from active animals indicating that stable intrinsic metabolic controls are involved in metabolic rate suppression (summarized in Guppy et al. 2000; Storey 2002). Studies with multiple forms of hypometabolism have shown that the major mechanism involved in intrinsic metabolic suppression is RPP (Storey 2002; Storey and Storey 1990, 2004, 2007). Entry into hypometabolic states including aestivation, hibernation, anaerobiosis, and others involves coordinated changes in the phosphorylation states of many cellular enzymes and functional proteins. The addition of phosphate groups via protein kinases or their removal via protein phosphatases can have multiple consequences for target proteins/enzymes including major changes in activity (ranging up to complete on/off control), changes in substrate affinity and susceptibility to allosteric activators or inhibitors, changes in binding to other proteins, subcellular structures or DNA, and changes in subcellular location, to name a few. In comparative biochemistry, the first indication of a role for RPP in adjusting metabolic response to stress came from studies of anoxia tolerance in marine mollusks, initially, with the demonstration that anoxia induced the phosphorylation of pyruvate kinase (PK), thereby inhibiting the enzyme and rerouting the catabolism of phosphoenolpyruvate into anaerobic pathways (Storey and Storey 1990; Brooks and Storey 1997). A broader role for RPP was appreciated when it was shown that other enzymes of carbohydrate catabolism were similarly controlled under anoxia. Finally, the global role of RPP in metabolic rate depression was demonstrated when studies showed that (a) glycolytic rate depression was mediated by RPP in multiple states of hypometabolism including aerobic systems of snail aestivation and mammalian hibernation, and (b) that RPP regulated and coordinated many other metabolic functions when animals entered hypometabolism (Storey 2002; Storey and Storey 1990, 2004, 2007). Both of these criteria have been amply demonstrated in research on aestivation.

RPP control over enzymes of carbohydrate catabolism has been clearly demonstrated for both vertebrate (toad) and invertebrate (land snails) aestivators (reviewed by Brooks and Storey 1997; Storey 2002). Initial studies showed phosphorylation-mediated control of two regulatory enzymes of glycolysis during aestivation: PK and phosphofructokinase (PFK) (Whitwam and Storey 1990, 1991; Cowan and Storey 1999). For example, in skeletal muscle of spadefoot toads, Scaphiopus couchii,
high and low phosphate forms of the enzymes were separated by isoelectric focusing and showed different kinetic properties (Cowan and Storey 1999). The proportions of low phosphate PK and PFK increased during aestivation and kinetic analysis showed that these were the less active forms. Both aestivation and anoxia also suppressed maximal activities and altered kinetic properties of PK and PFK in foot muscle and mantle of land snails, *Otaia lactea* (Whitwam and Storey 1990, 1991). Furthermore, *in vitro* incubations that stimulated cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG), or Ca^{2+}/phospholipid-dependent protein kinase (PKC) altered the properties of PK and PFK from control snails in a manner that mimicked the effects of aestivation/anoxia but did not affect the enzymes in extracts from aestivating snails. This indicated that phosphorylation was responsible for enzyme suppression during entry into hypometabolism. Aestivation-responsive changes in PK properties were similarly found in foot and mantle of another snail, *Helix aspersa* (Fields 1992) but in both species the properties of PK in hepatopancreas (digestive gland) were differently affected during aestivation, although changes were still phosphorylation mediated (Whitwam and Storey 1990; Fields 1992). Other enzymes of carbohydrate catabolism are also regulated by RPP during aestivation in *O. lactea*. Glycogen phosphorylase activity decreased as did the amount of pyruvate dehydrogenase in the active form; activity of both of these enzymes is well known to be controlled in an on–off manner by RPP (Brooks and Storey 1990a, 1992). In addition, the concentration of fructose-2,6-bisphosphate (F2,6P$_2$), a strong activator of PFK, fell to <10% of control in foot muscle and the enzyme that produces F2,6P$_2$ was inhibited during aestivation (Brooks and Storey 1990a; Storey 2002). These changes in kinetic properties are consistent with enzyme phosphorylation as also typically occurs during starvation and carbohydrate sparing in vertebrate species.

Subsequent work has indicated a global involvement of RPP in the control of many aspects of metabolism during aestivation in *O. lactea*. In general terms, a comparison of 32P-labelling patterns in active versus aestivating snails revealed aestivation-responsive changes in both the molecular masses and the subcellular distributions of phosphoproteins in *O. lactea* tissues (Brooks and Storey 1995a). Targeted studies of other enzyme systems have revealed what some of these RPP targets must be.

### 2.2.1 Glucose-6-Phosphate Dehydrogenase

The first demonstration of stress-responsive RPP control over glucose-6-phosphate dehydrogenase (G6PDH) in an animal system was supplied by a recent study of the *O. lactea* hepatopancreas enzyme (Ramnanan and Storey 2006a). G6PDH gates carbohydrate entry into the pentose phosphate pathway to produce sugar phosphates for anabolic purposes (e.g., nucleotide biosynthesis) and NADPH reducing power to drive many kinds of biosynthesis as well as antioxidant defense (to produce reduced forms of glutathione and thioredoxin).
Indeed, modulation of G6PDH activity (particularly in liver) has a known impact on cell growth, nutrient processing, antioxidant defense, and death (Tian et al. 1998). Therefore, regulation of G6PDH could be one way to exert general control over biosynthesis during hypometabolism. *O. lactea* hepatopancreas G6PDH showed significant stable changes in enzyme properties during aestivation: activity increased by 50%, substrate affinity improved (\(K_m\) of G6P decreased by 50%), and sensitivity to citrate activation increased (\(K_a\) of Mg.citrate decreased by 35%). Ion exchange chromatography revealed two peaks of activity that were identified as low and high phosphate forms; the low phosphate form dominated in active snails (57% of total activity) and the high phosphate form in aestivation (71%). PKG and protein phosphatase 1 (PP1) appeared to be responsible for the interconversion of the two forms. The high phosphate form of G6PDH was also less sensitive to urea inhibition and more resistant to thermolysin proteolysis; this suggested greater structural stability which could be advantageous during long-term dormancy. However, the kinetic properties of G6PDH from aestivating snails argue for a more active enzyme during dormancy. Why would this be? The prominent role of G6PDH in antioxidant defense may be the answer. Enhancement of antioxidant defenses is proving to be a universal feature of hypometabolism across phylogeny (Hermes-Lima et al. 1998; Storey and Storey 2007; Ferreira-Cravo et al. 2009). This helps to prevent/minimize oxidative damage to macromolecules during dormancy, a state in which organisms cannot afford the high ATP expenditures associated with repairing, degrading, or resynthesizing macromolecules damaged by reactive oxygen species (ROS). Since the backbone of antioxidant defense is NADPH reducing power, the key role of G6PDH in gating the pentose phosphate pathway can be appreciated. Indeed, G6PDH activity rises in response to oxidative stress in systems ranging from yeast to humans (Ursini et al. 1997) whereas inhibited and/or reduced G6PDH activity has been correlated with reduced antioxidant capacities, ROS-related cellular damage, and ROS-induced cell death (Tian et al. 1999).

### 2.2.2 Ion Motive ATPases

Successful entry into hypometabolism requires strong suppression of the rates of all energy-expensive metabolic processes. Ion-motive ATPases are certainly among the most expensive; indeed, the Na⁺K⁺ATPase ion pump alone can consume up to 40% of total ATP turnover in some cell types (Clausen 1986). Furthermore, the critical importance of ion pumps and channels to supporting a large number of cell functions that are linked to transmembrane potential difference (e.g., nerve conductance, muscle contraction, Ca²⁺ signaling, numerous transport mechanisms, etc.) means that control over their activity during entry into and arousal from hypometabolic states needs to be closely regulated and coordinated. Studies with *O. lactea* showed that RPP is critical to managing the activities of ion pumps during aestivation. Both the plasma membrane Na⁺K⁺ATPase and the sarco(endo)plasmic Ca²⁺ATPase (SERCA) are regulated in this manner in foot muscle and hepatopancreas during
aestivation in *O. lactea* (Ramnanan and Storey 2006b, 2008). The same mechanism is seen in other hypometabolic systems including hibernating mammals and dia-

pausing insects (MacDonald and Storey 1999; McMullen and Storey 2008). During aestivation in *O. lactea*, the maximum activities of both enzymes decreased by 33–50% in muscle and hepatopancreas, although Western blotting showed no change in the amounts of enzyme protein. Substrate affinities also decreased during aestivation; for example, $K_m$ Mg.ATP values were 40% and 30% higher for Na$^+$K$^+$ATPase and SERCA (Ramnanan and Storey 2006b, 2008). Foot muscle Na$^+$K$^+$-ATPase from aestivated snails also showed reduced affinity for Na$^+$ ($K_m$ for Na$^+$ rose by 80%) and for Mg$^{2+}$ as an activator ($K_a$ for Mg$^{2+}$ increased by 60%) and $K_m$ Ca$^{2+}$ of foot muscle SERCA doubled in aestivating animals. In vitro incubations that manipulated the activities of endogenous protein kinases and phosphatases confirmed that the aestivation-induced changes in activities were regulated by RPP, the enzymes in aestivating snails proving to be the high phosphate forms. Thus, incubation of foot muscle extracts under conditions that stimulated protein kinases A, C, or G mimicked the changes in Na$^+$K$^+$ATPase enzyme properties that were seen during aestivation, whereas treatments that stimulated PP1 or PP2A had the opposite effect (Fig. 2.1a,b) (Ramnanan and Storey 2006b). SERCA activity was sensitive to PKG or Ca$^{2+}$-calmodulin protein kinase (CaMK) in foot muscle (Fig. 2.1c,d) and PKA or PKG in hepatopancreas whereas reactivation of SERCA was facilitated by protein phosphatases PP2A and PP2C in foot muscle and hepatopancreas, respectively (Ramnanan and Storey 2008). The commonality of PKG effects on both ion-motive ATPases suggest that this may be the kinase that naturally mediates aestivation-induced suppression of these enzymes in vivo. PKG has also been implicated in regulating enzymatic responses during anaerobiosis (another hypometabolic state) in marine molluscs (Brooks and Storey 1990b; Michaelidis and Storey 1990; Larade and Storey 2002) and was singled out as the kinase mediating aestivation-induced phosphorylation of PK in *O. lactea* (Brooks and Storey 1994).

### 2.2.3 Protein Synthesis

Another major energy expense in cells is protein turnover, both synthesis and degradation being ATP-expensive processes. Indeed, suppression of protein synthesis is one of the first responses by most cells when put under stress or nutrient/energy limitation (DeGracia et al. 2002). It is no wonder then that suppression of global rates of protein synthesis is a consistent response in all systems of hypometabolism that have been studied to date (summarized in Storey and Storey 2004). With respect to aestivation, suppression of global protein synthesis has been documented in both vertebrate and invertebrate models. Using liver slices from the desert frog *Neobatrachus centralis*, Fuery et al. (1998) found that the rate of protein synthesis was reduced by 67% in aestivation as compared with slices taken from awake frogs and that this accounted for 52% of the metabolic depression in liver and 4.9% of the
Metabolic Regulation and Gene Expression During Aestivation

decrease in whole animal metabolic rate. In the snail, *H. aspersa*, the rate of protein synthesis was reduced by 77 and 47% during aestivation in hepatopancreas and foot muscle, respectively (Pakay et al. 2002). *O. lactea* showed similar results: ^3^H-leucine incorporation into protein in extracts of foot muscle and hepatopancreas was reduced by ~80% within 2 days when snails entered aestivation (Ramnanan et al. 2009).

One of the main mechanisms for regulating protein synthesis in all animal systems is RPP control over the activity of ribosomal initiation and elongation factors. Critical loci for regulation include the ribosomal initiation factor 2 (eIF2), that brings the initiating methionine into the assembling ribosome, eIF4 that brings in the mRNA, and elongation factor 2 (eEF2). Studies with *O. lactea* showed that all of these are regulated during aestivation. When unphosphorylated, the alpha subunit of eIF2 forms a complex with GTP and methionine-tRNA and carries both into the assembling ribosome where the GTP is used to drive the attachment of

---

**Fig. 2.1** Effect of in vitro incubations that stimulated the activities of endogenous protein kinases or protein phosphatases on the activities of Na^+^K^+^-ATPase or sarco(endo)plasmic Ca^{2+}-ATPase (SERCA) in foot muscle extracts from active or 10-day aestivated *O. lactea*. Incubation time was 4 h prior to assay of maximal activity at 22°C. Protein kinase incubations stimulated cAMP-dependent protein kinase (PKA), Ca^{2+}, and phospholipid-dependent protein kinase (PKC), cGMP-dependent protein kinase (PKG), AMP-activated protein kinase (AMPK), or Ca^{2+}-calmodulin protein kinase (CaMK). Appropriate combinations of stimulators and inhibitors allowed protein phosphatase incubations to promote total phosphatase activities (total PP), PP1, protein phosphatase 1 and 2A (PP1/2A), total phosphatases minus PP1/2A (T−PP1/2A), total phosphatases minus PP2A (T−PP2A). Incubations were also done with exogenous alkaline phosphatase added. Data are means ±S.E.M, n=4 determinations on separate tissue extracts. (a) Significantly different from the corresponding control incubation using the Student’s t-test, *P*<0.05. Compiled from Ramnanan and Storey (2006, 2008)
methionine as the first amino acid of the peptide chain. Subsequently, eIF2α-GDP dissociates and GDP must be recharged to GTP before the next methionine can be loaded. Phosphorylation of eIF2α blocks it from being recharged and thereby inhibits protein synthesis (Proud 2007). The relative amount of phosphorylated eIF2α is a sensitive indicator of protein synthesis activity in all eukaryotes. Analysis of the relative amount of P-eIF2α (Ser51) in O. lactea showed that levels soared when snails aestivated; amounts in hepatopancreas rose by 3.8-fold and 15-fold after 2 and 14 days of aestivation (Fig. 2.2) and foot muscle showed a 20-fold higher P-eIF2α (Ser51) content after 14 days (Rammnanan et al. 2009). Pakay et al. (2003) also found a approximately twofold increase in the relative amount of P-eIF2α in liver extracts from aestivating desert frogs (Neobatrachus sutor), but in H. aspersa the amount of P-eIF2α was below the detection limit in hepatopancreas of both aestivating and awake snails (Pakay et al. 2003).

The eIF4 initiation complex delivers the mRNA to the assembling ribosome and several subunits of the complex are subject to RPP regulation. Changes in the phosphorylation state of three of the eIF4 components were assessed in O. lactea (Rammnanan et al. 2009). Both eIF4GI and eIF4E can be phosphorylated and this is linked to enhanced translation (Proud 2007). Figure 2.2 shows that the amount of

![Graph](image)

**Fig. 2.2** Aestivation and the responses of ribosomal initiation and elongation factors in hepatopancreas of the O. lactea. Active snails are compared with those that aestivated 2 or 14 days. Immunoblotting was used to analyze both total protein and the amount of phosphorylated protein; phosphospecific peptide antibodies detected serine/threonine phosphorylation as follows: eIF2α (Ser51), eIF4E (Ser209), eIF4GI (Ser1108), 4E-BP1 (Ser65), and eEF2 (Thr56). Phosphoprotein band density was expressed as a ratio with total protein band density for the same sample; total protein did not change significantly during aestivation except for eIF4E where both total and P-eIF4E (Ser209) decreased by ~20% during aestivation. Data are shown relative to the active value; data are means ±S.E.M, n = 4–5 animals. (a) Significantly different from the value for active snails as determined by the Student’s t-test, P < 0.05. Compiled from Rammnanan et al. (2009)
phospho-eIF4GI (Ser1108) decreased strongly in hepatopancreas during aestivation. Similarly, the content of phospho-eIF4E (Ser209) decreased strongly in foot muscle after 14 days of aestivation (Ramnanan et al. 2009). Reduced phosphorylation of both proteins is consistent with translational inhibition during aestivation. Furthermore, eIF4E is closely regulated by reversible association with a specific binding protein, 4E binding protein (4E-BP1). Binding to 4E-BP1 prevents eIF4E from interacting with EIF4GI to help deliver mRNA to the ribosome. Binding capacity is regulated by phosphorylation of 4E-BP1 which prevents it from binding and inhibiting eIF4E. Indeed, 4E-BP1 (like eIF2α) is one of the most important targets for RPP control over eukaryotic translation and is regulated by a variety of positive and negative effectors (Proud 2007). A strong reduction in amount of phospho-4E-BP1 (Ser65) content occurred in both hepatopancreas (Fig. 2.2) and foot muscle of aestivating O. lactea (Ramnanan et al. 2009). This means an increase in the amount of eIF4E bound to 4E-BP1 during estivation and is again indicative of translational inhibition in the hypometabolic state.

Translation is also regulated at the level of peptide elongation and several protein kinases focus their attention on the eukaryotic elongation factor 2 (eEF2) with phosphorylation inhibiting the factor (Browne and Proud 2002). This protein is also a target for phosphorylation in O. lactea with levels of P-eEF2 (Thr56) increasing ~10-fold in hepatopancreas (Fig. 2.2) and fivefold in foot muscle during aestivation (Ramnanan et al. 2009). Suppression of peptide elongation by enhanced phosphorylation of eEF2 is also well known in mammalian hibernation (Chen et al. 2001).

### 2.2.4 Protein Degradation

Given that rates of protein synthesis are strongly suppressed during aestivation, rates of protein degradation must also be suppressed, presumably by a similar amount, so that the amounts of most cellular enzymes/proteins are stabilized over what could be many weeks or months of hypometabolism. Reduced rates of protein degradation have been reported in several hypometabolic systems (summarized in Storey and Storey 2004) and this is also true of aestivation. In O. lactea, activity of the 20S proteasome in hepatopancreas decreased by 60–80% after 2 days of aestivation, although Western blotting showed that the content of selected subunits of the proteasome did not change (Ramnanan et al. 2009). Some subunits of the 20S proteasome are known to be phosphorylated (Pereira and Wilk 1990) and so in vitro incubations were used to test kinase and phosphatase effects on proteasome activity. Suppression of activity in vitro was mediated by protein kinase G whereas PP2A activated the proteasome (Ramnanan et al. 2009). Furthermore, reduced proteolytic activity in hepatopancreas during aestivation was associated with approximately twofold increases in $K_m$ values (i.e., lower substrate affinity) for two out of three substrates tested.

However, although it is generally true that reductions in the rates of both protein synthesis and protein degradation would be needed to reduce ATP expenditure on protein turnover during aestivation, a situation where protein degradation is
specifically enhanced also occurs during long-term aestivation in anurans. Anurans enter aestivation with huge reserves of water in their bladders but after weeks or months they begin to experience water stress as the water potential of the soil declines. At this point, protein oxidation begins to supplement lipids as the fuel for metabolism (Jones 1980) and the nitrogen released is used to synthesize urea. Urea can build up as high as 300 mM and acts colligatively to defend against water loss from the body. It has been estimated that as much as 30% of total body protein may be catabolized for this purpose in *S. couchii* (McClanahan 1967; Jones 1980). The molecular regulatory mechanisms controlling this form of protein degradation have not yet been investigated for aestivating animals.

### 2.3 Signaling Cascades and Metabolic Control in Aestivation

The above discussion of reversible phosphorylation control suggests an important role for PKG in mediating various responses by metabolic enzymes during aestivation in *O. lactea*. This is also supported by a measured rise in cGMP levels in foot muscle and hepatopancreas over the first 24 h during entry into aestivation in these snails whereas, by contrast, tissue cAMP levels dropped (Brooks and Storey 1996). A study on spadefoot toads also indicated reduced reliance on cAMP signaling during aestivation with reductions (by one-third to one-half) in the percentage of PKA present as the active catalytic subunit in multiple tissues of aestivating animals (Cowan et al. 2000). Three organs also showed much lower percentages of active membrane-bound PKC during aestivation. Total tyrosine kinase activities were also reduced significantly during aestivation in most toad organs, including both membrane-bound receptor and cytoplasmic activities (Cowan and Storey 2001). In general, however, activities of protein phosphatases that oppose protein kinases were less affected during aestivation, including both serine/threonine and tyrosine phosphatases (Cowan et al. 2000; Cowan and Storey 2001). This subject is also reviewed in greater depth by Storey (2002). Recent studies have implicated additional signaling pathways in metabolic control during aestivation.

#### 2.3.1 AMP-Activated Protein Kinase

A need to conserve energy (ATP) to permit long-term life extension during aestivation could implicate the AMP-activated protein kinase (AMPK) as another signalling kinase to consider in the control of aestivation. AMPK plays a central role in sensing cellular energy status and appropriately regulating the relative rates of catabolic ATP-generating pathways versus anabolic ATP-consuming processes; indeed, it is often called the “fuel gauge” of the cell (Hardie 2007; Hue and Rider 2007). AMPK is activated under various situations; it responds to hypoxia, glucose
deprivation, and muscle exercise (all situations that deplete ATP and elevate AMP), elevated intracellular Ca\textsuperscript{2+}, various cytokines (e.g., adiponectin, leptin) and tumor suppressors (e.g., LKB1) (Horman et al. 2002; Alessi et al. 2006; Hardie 2007). A recent study suggests AMPK involvement in aestivation in *O. lactea*. Tissue AMP levels do not rise during aestivation (Churchill and Storey 1989) but AMPK activity increased at least twofold, resulting from a strong increase in the amount of active AMPK as assessed by immunoblotting with antibody recognizing the phospho-Thr172 site on AMPK (Ramnanan and Storey, unpublished data). Because AMPK activation was due to phosphorylation, this implicated upstream kinases in AMPK regulation during aestivation. Three known upstream kinases that phosphorylate AMPK were then tested and one of them, LKB1, showed a parallel, approximately twofold, increase in activity (as assessed by the amount of active phospho-Ser428 LKB1) in snail tissues during aestivation. LKB1 is a tumor suppressor and, as the name suggests, is known to inhibit cell proliferation in other systems (Alessi et al. 2006). The LKB1-AMPK pathway functions as a cellular energy-sensing checkpoint, enabling growth and proliferation of cells to be coupled to the availability of fuel supplies; indeed, medical interventions that manipulate this signaling pathway have promise in cancer treatment (Alessi et al. 2006). The data for *O. lactea* suggest that this signaling system also has a natural action in suppressing biosynthesis and proliferation when organisms enter hypometabolic states. Downstream functions under AMPK control include inhibitory regulation of energy-expensive activities such as lipid biosynthesis via phosphorylation of acetyl-CoA carboxylase (ACC) and protein synthesis by regulating protein factors that control mTOR (Horman et al. 2002; Alessi et al. 2006; Hardie 2007). Not surprisingly, ACC activity was strongly inhibited in aestivating snails, as a result of a threefold increase in the amount of phospho-Ser79 ACC (Ramnanan and Storey, unpublished).

### 2.3.2 Akt Mediated Signaling

The cell signaling cascade consisting of phosphoinositide-3-kinase, 3-phosphoinositide-dependent kinase, and Akt (also called protein kinase B) is a major pathway involved in mediating survival and proliferation responses, most importantly transducing the action of insulin in vertebrates (Brazil et al. 2004). The pathway remains intact in invertebrates, triggered by different growth factors, and has been well characterized in *Caenorhabditis elegans* (Baumeister et al. 2006). Akt generally mediates a variety of actions that go forward when fuel/energy supplies to cells are plentiful while also inhibiting cell death signals. For example, Akt mediates the anabolic and growth-promoting effects of insulin and insulin-like growth factor by actions that include stimulating glucose uptake, promoting glycogen synthesis by inactivating glycogen synthase kinase 3 (GSK-3), and stimulating lipogenesis and protein synthesis (via regulation of mTOR) (Whiteman et al. 2002; Brazil et al. 2004). Oppositely, Akt inhibits apoptosis by control of the pro-apoptotic protein BAD and suppresses expression of forkhead box, class O transcription factors
(FOXOs) that are involved in cell-cycle arrest, quiescence, stress resistance, life extension, and apoptosis in different systems (Greer and Brunet 2005). Given the importance of these latter functions for long-term aestivation, the patterns of expression of Akt, downstream proteins and kinases under Akt control, FOXO transcription factors, and the genes that they control are clearly important in aestivating species. Indeed, numerous studies with *C. elegans* have documented the key role of these signaling pathways in regulating the dauer state, a developmental arrest (diapause) that occurs in the third larval stage when environmental conditions are not conducive to adult reproduction of the nematode (Burnell et al. 2005; Mukhopadhyay et al. 2006).

A recent study has explored the responses of Akt and various downstream targets under its control during aestivation in *O. lactea* (Ramnanan et al. 2007). Multiple criteria were consistent in showing that Akt was activated in foot and hepatopancreas of aestivating snails: measured Akt activity was approximately twofold higher, substrate affinities were lower, immunoblotting showed higher levels of the active phosphorylated form (Ser473), and incubation studies showed that only control Akt was susceptible to phosphorylation in vitro whereas aestivated Akt could be modified by protein phosphatases. Proteins under Akt control were also analyzed. A key regulator of protein translation, mTOR, showed no change in phosphorylation state during aestivation, which suggests both that it is not under Akt control in aestivation and that mTOR is not the regulator of the changes in 4E-BPI phosphorylation, a known target of mTOR in other systems. Furthermore, the content of phospho-GSK-3β (Ser9) decreased by about half in both tissues during aestivation. Thus, it appears that growth/proliferation actions of Akt that are normally mediated via phosphorylation of mTOR and GSK-3 were uncoupled from Akt influence during aestivation. However, two other known targets of Akt action showed enhanced phosphorylation during aestivation: the amount of phospho-BAD (Ser136) was approximately twofold higher and phospho-FOXO3a (Ser253) was twofold and fourfold higher in hepatopancreas and foot muscle, respectively (Ramnanan et al. 2007). Phosphorylation of both of these proteins causes them to be sequestered in the cytoplasm. This prevents FOXO from moving to the nucleus to activate gene transcription and BAD from interacting with the apoptosis machinery in the mitochondria (Brazil et al. 2004). This suggests that proapoptotic signaling from either of these sources would be suppressed during aestivation. However, FOXO signaling also has key actions in cell-cycle arrest, enhancement of antioxidant defenses, stress survival, and life extension, and is key to dauer formation in *C. elegans* (Lam et al. 2006). These FOXO-mediated actions would predictably be beneficial during aestivation, so it remains to be determined if and how survival and life extension actions are accomplished in aestivating snails. It is possible that another protein kinase is involved such as the c-Jun activated kinase that has been linked to regulating FOXO with respect to its role in life extension in *C. elegans* and *Drosophila* (Lam et al. 2006). Thus, Akt and Akt-mediated downstream events are involved in snail aestivation, but there are clearly regulatory differences between aestivation and dauer formation that remain to be resolved.
2.4 Gene Regulation

2.4.1 Global Suppression of Gene Expression

Strong global suppression of gene expression is an integral part of metabolic rate depression in all systems of hypometabolism that have been studied to date (reviewed in Storey and Storey 2004, 2007). A variety of epigenetic controls are known that can mediate global gene silencing and have important roles to play in many aspects of life including development, differentiation, aging, and disease (Fraga et al. 2007). The best known epigenetic modifications are DNA methylation and posttranscriptional modification of histones by many methods including methylation, acetylation, phosphorylation, and SUMOylation, to name a few. Little is known to date about epigenetic controls in states of natural hypometabolism but the subject has begun to be explored in hibernating mammals (reviewed by Morin and Storey 2009) and comparable controls can be envisioned for aestivation. One method of global transcriptional repression is to reduce chromatin accessibility to the transcription apparatus. This is gated by histones which are subject to multiple forms of posttranslational modification that, in turn, affect chromatin packing. Both acetylation and phosphorylation of histones reduce chromatin packing to increase the accessibility. During mammalian hibernation, the level of histone acetylation and phosphorylation decreases consistent with translational silencing (Morin and Storey 2006). Furthermore, activities of histone deacetylases rose during torpor, providing the mechanism for reduced histone acetylation. Another factor is control over RNA polymerase II activity which is also reduced during hibernation, possibly via a RPP mechanism (Morin and Storey 2006). Global suppression of the action of transcription factors is another mechanism. Lee et al. (2007) showed that the levels of small ubiquitin-related modifier (SUMO) conjugated proteins rose dramatically in ground squirrel organs during hibernation. Transcription factors are primary targets of SUMOylation and this reversible posttranslational modification mainly has negative effects on gene expression (Girdwood et al. 2004). Hence, high levels of SUMOylation indicate strong global suppression of transcription factor action during torpor, and thus reduced transcription of the genes under their control. All of these methods of global transcriptional repression need to be explored in aestivation. A recent study took a specific look at epigenetic regulation in aestivation of green-striped burrowing frogs, Cyclorana alboguttata (Hudson et al. 2008). The authors quantified mRNA transcript abundance in cruralis muscle of control versus 6-month aestivated frogs to assess the expression of seven genes whose proteins have established roles in gene silencing: methyl CpG binding protein 2, chromodomain helicase DNA-binding protein 4, histone-binding protein rbbp4, histone deacetylase 1, nuclear receptor corepressor 2, transcriptional corepressor SIN3A, and DNA cytosine-5-methyltransferase 1. Transcript levels of the latter two were significantly elevated (by 1.7- and 3.5-fold, respectively) in aestivation, consistent with the idea that chromatin remodeling plays a significant role in long-term gene silencing in aestivators.
Gene expression is also regulated by mechanisms that are posttranscriptional but pretranslational – i.e., that affect mRNA processing and availability. One method is to sequester mRNA transcripts into protein–mRNA complexes (stress granules) or to store them in association with translationally silent monosomes (Bond 2006; Kedersha and Anderson 2007). This preserves existing mRNA transcripts so that they can be rapidly transcribed when organisms exit from stress conditions or a hypometabolic state. Polysome dissociation into monosomes is a documented feature of hypometabolism in hibernation and anaerobiosis (reviewed in Storey and Storey 2004). Anoxia-induced polysome dissociation occurs in marine snails (Littorina littorea) (Larde and Storey 2002) but only one study to date has examined aestivation. That study found that ribosomes were present almost exclusively as monosomes in O. lactea hepatopancreas from both active and aestivating snails (Hobbs et al. 1994).

Another principle of gene regulation that will probably prove to have a major influence in hypometabolic systems is the hot new topic in mRNA control. This is microRNA (miRNA). These small noncoding transcripts (19–25 nt long) regulate gene expression by binding to mRNA transcripts to block translation or target them for degradation (Bartel 2004). Dozens of studies are now linking miRNA to translational control in health and disease (O’Driscoll 2006) and new work on hibernators showed significant increases in the levels of selected miRNA types during torpor in ground squirrels, providing the first link between miRNA and hypometabolism (Morin et al. 2008). Comparable studies on aestivating species will be highly instructive.

### 2.4.2 Gene Hunting and Stress Response

Despite overall global suppression of transcription and translation in hypometabolic states, enhanced expression of selected genes and the proteins that they encode occurs in all hypometabolic systems that have been evaluated. Indeed, advances in molecular biology technologies in recent years have allowed huge leaps forward in our understanding not just of hypometabolism but of animal adaptation to environmental stress in general. A variety of approaches have been used for gene hunting in hypometabolism including screening of cDNA libraries, differential display PCR, and the now dominant technique, DNA array screening. Species-specific DNA arrays are now available for a variety of mammalian species as well as common “comparative” models including zebrafish (Danio rerio), African clawed frog (Xenopus laevis), fruit flies (Drosophila melanogaster), and the nematode (C. elegans). Furthermore, excellent results can be achieved by the gene hunter using heterologous screening (i.e., using an array developed for another species) (Eddy and Storey 2008). Although cross-reactivity is never 100% and additional validation is always needed to confirm gene/protein upregulation (via PCR or immunoblotting), heterologous probing of DNA arrays has been critical for identifying many genes whose protein products have never previously been linked
with hypometabolism and for finding conserved patterns of gene responses in multiple systems. For example, application of heterologous screening to several models of hypometabolism including hibernating ground squirrels, freeze-tolerant frogs, and anoxia-tolerant turtles has shown conserved gene expression responses that include enhanced expression of chaperones (heat shock proteins, glucose regulated proteins), antioxidant proteins, iron-binding protein, and serpins (serine protease inhibitors), among others (Storey 2004a,b; Storey 2006).

Several of the above gene expression responses are part of a conserved cellular stress response that is seen in all cells across phylogeny (Kültz 2003, 2005). The stress response includes (a) cell-cycle control including growth arrest through checkpoint control and translational controls, (b) proliferation of molecular chaperones to fold/refold and stabilize proteins, (c) DNA and chromatin stabilization and repair, and (d) degradation of damaged macromolecules. Although regulated entry into hypometabolic states would not be expected to generate cell stress, the coordinated implementation of the stress response could be of vital importance for two reasons. Firstly, the stress response could provide anticipatory preparation to deal with stresses that almost always accompany the hypometabolic state. For example, during aestivation this might include preparations to deal with anticipated stresses such as dehydration, wide variation in temperature, and oxidative stress. One of the earliest illustrations of this kind of anticipatory response was the upregulation of antioxidant enzymes in late gestation in mammalian lung preceding the oxidative stress that occurs when lung breathing begins at birth (Frank and Sosenko 1987). Secondly, activation of the stress response could aid life extension during dormancy by implementing or enhancing mechanisms that provide long-term stability of cellular macromolecules to deal with the realities of the hypometabolic state where energy/nutrient availability for macromolecular synthesis, repair, or degradation is limiting.

2.4.3 Aestivation-Responsive Gene Expression

Very little has been done to specifically explore aestivation-specific gene expression using molecular biology techniques. A recent study by Hudson et al. (2006) used PCR to evaluate mRNA transcript levels of selected genes in cruralis skeletal muscle of control versus 6-month aestivated C. alboguttata. Transcript levels of NADH ubiquinone oxidoreductase subunit 1, ATP synthase, and mitochondrial superoxide dismutase were suppressed by ~70%, consistent with mitochondrial quiescence during aestivation and reduced oxidant production, but transcripts of uncoupling protein type 2, catalase, and glutathione peroxidase were unchanged. PCR analysis of message levels for two extracellular matrix proteins in cruralis muscle also found no significant change during aestivation which supports the limited atrophy seen in this muscle (Hudson et al. 2007).

Enhanced expression of some specific genes during aestivation can be inferred in a number of cases. For instance, injection of $^{35}$S-methionine into O. lactea followed by analysis of radiolabeled proteins by polyacrylamide gel electrophoresis
or isoelectrofocusing showed strong differential radiolabeling of a few selected proteins in aestivating snails as compared with active animals. Proteins of 91, 70, 50, and 30 kDa were prominently elevated and these molecular masses suggest that they may be chaperone proteins (Brooks and Storey 1995b). Some of the prosurvival defenses associated with the stress response (discussed above) are likely upregulated during aestivation. Indeed, activities of a number of antioxidant enzymes were higher in foot muscle and hepatopancreas of aestivating O. lactea compared with active snails whereas selenium-dependent glutathione peroxidase rose substantially in tissues of aestivating H. aspera (Hermes-Lima and Storey 1995; Ramos-Vasconcelos and Hermes-Lima 2003). By contrast, antioxidant enzyme activities were generally lower in tissues of aestivating versus active spade-foot toads (S. couchii) (Grundy and Storey 1998). Chaperone proteins also have a role to play. Levels of several heat-shock proteins (HSP10, HSP60, HSP90, HSP110) were significantly elevated in hepatopancreas of 14-day aestivated O. lactea (Ramnanan et al. 2009) but HSP70 protein and mRNA levels were not altered in another Mediterranean snail, Cantareus apertus, during aestivation (other chaperones were not assessed) (Reuner et al. 2008). Gene expression that deals with specific stresses associated with aestivation can also occur. The prime example known is urea cycle enzymes; activities of these enzymes are typically elevated in liver when osmotic or desiccation stress becomes an issue in both aestivating and nonaestivating anurans (Jones 1980; Lee et al. 1982). Since the regulation of urea cycle enzymes is at the transcriptional level in virtually all cell types (Morris 2002), it is logical to assume that upregulation of the genes encoding these enzymes occurs when needed during aestivation.

To our knowledge, the only direct use of gene screening techniques to search for aestivation-responsive gene expression involved the construction and screening of a cDNA library from liver of 2-month aestivated female S. couchii. Differential hybridization using cDNA probes made from active versus aestivated toads revealed strong upregulation of the gene for riboflavin-binding protein (RfBP) in aestivating animals (Storey et al. 1999). Indeed, this study also provided the first identification of RfBP in an amphibian species. The protein was previously known to be synthesized only by liver of female birds, reptiles, and mammals; it is then secreted, binds plasma riboflavin and loads the vitamin into eggs or fetus (White and Merrill 1988). The amino acid sequence of toad RfBP was 50% identical to chicken or turtle RfBP including key functional residues but lacked some of the phosphoserine residues that are involved in RfBP binding to the oocyte membrane in other species. Possible reasons for RfBP upregulation of RfBP in aestivating toads can be suggested. RfBP gene expression might be linked with the maturation of eggs in female toads prior to the explosive breeding that occurs immediately upon emergence from aestivation. Alternatively, enhanced levels of RfBP in adult toads could serve to cache endogenous riboflavin over the 9–10 months that the animals are in aestivation each year. All hypometabolic states have in common a need to achieve long-term life extension without an input of nutrients. As such, conservation and recycling programs for important nutrients would be valuable and could potentially be part of a
conserved stress response. It would be interesting for future research to determine whether there are universal mechanisms for conserving valuable nutrients such as vitamins during hypometabolism, particularly, in species that have extended seasonal dormancies.

2.5 Conclusion

Much is already known about the molecular mechanisms that regulate metabolic suppression and support long-term viability in aestivation. Other mechanisms are implicated from common principles that have emerged in other systems of aerobic hypometabolism including hibernation, diapause, and dauer state. However, a number of discrepancies have been pointed out in this chapter between the metabolic responses/controls seen in snail versus anuran aestivation or in aestivation versus dauer state. Hence, it is important to continue studies of aestivation in order to gain a full understanding of both the common principles and the diversity of mechanisms that achieve hypometabolism in different systems. There are still many new avenues to explore in the biochemistry and molecular biology of aestivation, and, indeed, in hypometabolism in general. Of particular interest, currently, are global mechanisms of gene silencing, cell cycle suppression, and mechanisms that aid life extension by minimizing degradative programs of atrophy or apoptosis. The clear participation of multiple intracellular signaling pathways in coordinating subcellular responses during aestivation also leads us to question what the extracellular signal(s) are that trigger metabolic suppression and/or coordinate responses among cells, tissues, and organs. Extracellular (blood or haemolymph borne) regulators of hypometabolism have been highly elusive in most models except, perhaps, for diapause hormone in insects. Evidence from both hibernating mammals and anoxia-tolerant turtles suggests a role for delta-opioids, at least in the suppression of brain activity (Borlongan et al. 2004; Pamenter and Buck 2008), and it would clearly be a major advance to discover an extracellular trigger factor for aestivation. Finally, aestivation is, perhaps, the “purest” of the hypometabolic states in nature – an aerobic dormancy that can be induced and sustained without complicating factors such as the large decrease in body temperature during mammalian hibernation, the oxygen deprivation of facultative anaerobiosis, or the genetic/seasonal programming that can make diapause difficult to manipulate experimentally. So, aestivation may, in fact, provide the best model for identifying the basic principles, common regulatory mechanisms, and the core proteome that define hypometabolism in animal systems. The future is exciting!

Acknowledgments Thanks to the many past members of the Storey lab who have contributed to unraveling the molecular secrets of aestivation, especially, the novel advances made by C.J. Ramnanan, K.J. Cowan, R. Whitwam, M. Hermes-Lima, and S.P.J. Brooks. Research in the Storey lab is supported by a discovery grant from the NSERC Canada and the Canada Research Chair in Molecular Physiology
References


Ferreira-Cravo M, Welker AF, Hermes-Lima M (2009) The connection between oxidative stress and estivation in gastropods and anurans. This volume


MacDonald JA, Storey KB (1999) Regulation of ground squirrel Na\textsuperscript{+}\textsuperscript{-}K\textsuperscript{+}-ATPase activity by reversible phosphorylation during hibernation. Biochem Biophys Res Commun 254:424–429
McMullen DC, Storey KB (2008) Suppression of Na\textsuperscript{+}\textsuperscript{-}K\textsuperscript{+}-ATPase activity by reversible phosphorylation over the winter in a freeze-tolerant insect. J Insect Physiol 54:1023–1027
Aestivation
Molecular and Physiological Aspects
Arturo Navas, C.; Carvalho, J.E. (Eds.)
2010, XIV, 268 p. 38 illus., 3 illus. in color., Hardcover
ISBN: 978-3-642-02420-7