# 16

# MAMMALIAN HIBERNATION: BIOCHEMICAL ADAPTATION AND GENE EXPRESSION

KENNETH B. STOREY AND JANET M. STOREY

## INTRODUCTION

The core body temperature ($T_b$) of most mammals is maintained remarkably constant (typically 36 to 38°C, depending on the species), regulated by a thermostat in the hypothalamus of the brain and with heating and cooling achieved, as needed, by a variety of physiological and biochemical mechanisms. All mammals, including humans, are endotherms (generating internal heat by metabolic reactions) and most are also good homeotherms (maintaining a highly constant core $T_b$), and this supports a variety of mammalian successes, including high-speed locomotion, range extension into cold environments, and advanced brain functions. However, endothermy is costly and the metabolic rate of mammals is typically 4 to 7 times higher than that of comparably sized reptiles. This must be supported by equally higher rates of fuel consumption, supplied by foraging or, if food supply is limiting, by food caches or body adipose reserves.

When winter approaches and environmental temperature falls, the metabolic rate, $T_b$, and food needs of an ectothermic (cold-blooded) organism decline along with it. However, a mammal in the same situation loses body heat faster at colder temperatures and, hence, needs a higher metabolic rate and a greater fuel consumption to support the increased thermogenesis needed to maintain a constant core $T_b$. Many mammals, even some very small ones such as shrews and some mice, can meet this challenge and remain active throughout the winter by spending as much time as possible in sheltered environments (e.g., under the snowpack and/or in insulated nests), increasing their body insulation (thicker fur, more body fat), and assembling adequate fuel supplies of forage, food caches,

and/or body fat. For others, the combination of cold temperatures and lack of food availability in winter makes survival as a homeotherm impossible. The problem is particularly acute for animals such as insectivorous bats or grazing herbivores (e.g., ground squirrels, marmots) that have little or no access to edible food in the winter.

The solution to this problem is hibernation. For 6 to 9 months of the year, many small mammals abandon one of the defining characteristics of mammalian life (homeothermy) and allow their $T_b$ to fall, tracking environmental temperature. By doing so, hibernators gain tremendous energy savings. For example, it has been calculated for ground squirrels that winter hibernation saves 88% of the energy that would otherwise be needed to maintain a euthermic $T_b$ of ~37°C over the winter.

The present chapter explores metabolic regulation as it applies to mammalian hibernation. The field of hibernation research is a huge one that examines the phenomenon at ecological, physiological, and biochemical levels and also includes a huge body of applied research that seeks to use the lessons learned from hibernation in hypothermic medicine, organ preservation (see Chapter 19), and understanding complex brain functions. These subjects fill books of their own so the treatment here is selective and focuses on recent advances in understanding the principles of metabolic regulation as they apply to hibernation.

## HYPOTHERMIA AND HIBERNATION

In Chapter 14 environmental stressors were said to affect metabolism in two ways: (a) by direct perturbation of the structure/function of biological molecules and biochemical

reactions or (b) by jeopardizing the energy currencies of the cell. Mammalian hibernation is an adaptation directed at maintaining cellular energetics over an extended period when the environment is inhospitable for normal life. However, by using this strategy, the animal is then subjected to the perturbing effects of temperature on its metabolism, a situation that is unusual for most mammals. Humans, for example, undergo severe metabolic injuries (often lethal) if our core $T_b$ drops below about 25°C, but small-mammal hibernators readily allow $T_b$ to fall to as low as 0 to 5°C without suffering injury. Hibernating Arctic ground squirrels (*Spermophilus parryii*) are even known to let $T_b$ fall below 0°C; a recorded minimum $T_b$ of −2.9°C is the lowest $T_b$ ever recorded for a living mammal (these squirrels are not frozen, but supercooled; see Chapter 17). Note that the animal that typically comes to mind first when hibernation is mentioned—the bear—actually shows only a very small drop in $T_b$ (to ∼35°C) and has a shallow torpor that is easily disturbed. Even though metabolic rate is lowered, the large bulk of the bear, especially its insulating fat and fur layers, prevents its body from cooling very much. Bears are fascinating in that they do not eat, drink, defecate, urinate, or lose muscle mass over the course of many months of winter hibernation. However, physiological data on bear hibernation are still pretty minimal and biochemical data are almost nonexistent, so our discussion here will center on the small mammals that make much better laboratory models.

The injurious effects of hypothermia on nonhibernating mammals arise from two main effects of cold on metabolic systems that have been optimized over millions of years of mammalian evolution for function within a narrow temperature window. The first is the differential effect of temperature change on the rates of thousands of cellular reactions that can culminate in a mismatch between the net rates of reactions that produce and utilize adenosine 5′-triphosphate (ATP). The result is that energy currencies are depleted and the major manifestation of this energy crisis is membrane depolarization, which sets off a range of catastrophic events that are much the same as those caused by anoxia-induced energy failure (see Chapter 15). This is the main biochemical reason for the often lethal effects of hypothermia in humans (see Chapter 19 for the problems that this causes during hypothermic organ storage in transplant medicine). Interestingly, summer-active individuals of hibernating species are just as susceptible to hypothermia-induced membrane depolarization as are nonhibernating species. Hence, the preparation for winter hibernation must include specific adaptations that correct hypothermia-sensitive metabolic processes.

The second main effect of hypothermia is a decrease in lipid fluidity in both membranes and adipose depots as temperature declines. Mammalian membranes and adipose depots are designed to function at about 37°C, and they solidify at about room temperature (think of the difference between olive oil and lard on the kitchen counter). Membrane lipid fluidity is crucial for allowing the protein movements within membranes and protein conformational changes associated with receptor and transporter functions. Impaired fluidity severely disrupts these functions. Hence, hibernation must include mechanisms that sustain or reestablish membrane-associated metabolic functions over a wide range of core $T_b$ values.

Hibernation occurs in multiple mammalian lineages and is believed to have arisen independently several times. For a typical small mammal, the winter hibernation season consists of multiple bouts of torpor interspersed with brief periods of arousal when euthermic $T_b$ is reestablished. The season begins with a series of "test drops" in which the core $T_b$ of the sleeping animal falls by 10 to 15°C for short periods before returning to normal. It is now believed that these test drops are important in triggering the induction of various metabolic adjustments and gene expression changes that support the longer and deeper torpor bouts that follow. Subsequently, the decrease in $T_b$ becomes greater (falling very close to ambient) and the length of each torpor bout increases so that in midwinter $T_b$ is often maintained between 0 and 5°C for 1 to 3 weeks (although an amazing 76 days was recorded in one species of bat). Intervening arousals between torpor bouts generally last 6 to 24 h, depending on the species. During torpor $T_b$ tracks ambient temperature, but with the exception of the Arctic ground squirrels, hibernators do not let $T_b$ fall below 0°C. If ambient temperature decreases to subzero values, the hibernating animal activates a low rate of nonshivering thermogenesis (NST) by its brown adipose tissue (BAT) to avoid any risk of freezing. Hence, despite the deep torpor and the very low $T_b$, sensory systems are still tracking $T_b$ and the animal is defending a set point temperature.

During torpor all aspects of the animal's physiology slow dramatically. For example, the heart beat of a ground squirrel can drop from 200 to 300 beats per minute to just 5 to 10 beats per minute. Breathing rate similarly declines and in some species changes from rhythmic to intermittent with extended periods of apnea (breath-hold) of an hour or more that are interspersed with brief bursts of breaths. Overall, metabolic rate in torpor at a $T_b$ of 0 to 5°C can be as low as 1 to 5% of the normal resting rate at 37°C. As spring approaches, torpor bouts become shorter and shallower until the animal ceases to hibernate any more. Seasonal hibernation is a longer, deeper, and more dramatic version of the daily torpor that occurs in many small mammals and birds. By allowing $T_b$ to decline by a few degrees during sleep, animals that employ daily torpor can accrue significant energy savings that are often the difference between "making it through the night" and death from starvation.

Metabolism during hibernation is fueled almost exclusively by lipid oxidation; even brain switches to a mixed dependency on glucose and ketone bodies, the latter being produced from fatty acids (see Chapter 9). The whole-body respiratory quotient (RQ; ratio of $CO_2$ output to $O_2$ consumption) during steady-state hibernation is typically $\sim 0.7$, which is indicative of lipid catabolism. However, studies with ground squirrels have shown that if stressed by ambient temperatures that fall below $0°C$

(and that necessitate NST to stabilize $T_b$), RQ values can rise to 0.85. This suggests that other fuels can also be called into play when needed. Fatty acids are particularly important as the fuel for the intense thermogenesis that is needed to rewarm the animal during each arousal from torpor. Heat production comes from two sources: NST due to high rates of uncoupled respiration by BAT mitochondria and shivering thermogenesis by skeletal muscles (see Text Box 16.1). The early minutes of arousal rely

---

**TEXT BOX 16.1   THERMOGENESIS**

Metabolism is inefficient and contains many exergonic reactions that release energy, generally as heat. For example, under physiological conditions of ATP, ADP, $P_i$ and $H^+$ and at cellular pH, the calculated efficiency of energy conservation from the aerobic catabolism of glucose to produce ATP is about 65% so about 35% is lost as heat. This significant heat release is further enhanced by energy release during the subsequent hydrolysis of ATP by ATP-utilizing metabolic reactions. These inefficiencies can be put to use to support either episodic heat production or sustained homeothermy. Thermogenesis can be accomplished by either (or both) of two mechanisms (1) increasing the rate of ATP turnover by speeding up selected ATP-utilizing reactions in a "futile" manner (i.e., no real net benefit except heat output) so that both ATP production and ATP utilization are increased, or (2) uncoupling ATP synthesis by oxidative phosphorylation from the electron transport system so that energy that would otherwise be trapped as ATP is released as heat. Both options are used by animals.

**Thermogenesis from Enhanced Rates of ATP Turnover**

The high basal metabolic rate of mammals and birds, four- to sevenfold higher than comparably sized reptiles, derives primarily from the first mechanism. Endotherms "waste" high amounts of ATP, supporting much higher rates of transmembrane ion pumping than occurs in ectotherms. All organisms maintain gradients of $Na^+$, $K^+$, $H^+$, $Ca^{2+}$, and other ions across their membranes, but the membranes of endotherms are much more "leaky" than those of ectotherms due to the presence of higher numbers of ion channels that facilitate ion movements down their concentration gradients. To counteract the dissipation of ion gradients that such leaky membranes cause, endotherms maintain many more ATP-driven ion pumps in their membranes to move ions against their concentration gradients. Although there are positive benefits of high sensitivity and rapid response to stimuli that are supported by this system, such futile cycling of ions across membranes requires high rates of ATP hydrolysis and high rates of substrate oxidation, generating large amounts of heat to support homeothermy. Indeed, as discussed elsewhere in this chapter, strong inhibition of the activities of ion-motive ATPases (and of corresponding ion channels) is one of the major mechanisms of metabolic rate depression that allows $T_b$ to fall during hibernation.

A similar form of futile ATP turnover is the basis of shivering, an episodic mechanism of heat generation used by both vertebrate and invertebrate animals. ATP hydrolysis by the myosin ATPase of skeletal muscles is unhooked from sarcomere shortening (muscle work) so that high rates of fuel and ATP consumption occur with only minor (as in humans) or sometimes no (e.g., honeybees) detectable physical movements of the muscle. For example, various silkmoths need to shiver to warm up their thoracic flight muscles to over $30°C$ before they can fly and honeybees hold the core temperature of

their colony (surrounding the queen) at 34 to 35°C throughout the winter by varying the rate and number of shivering bees and the density of packing of the cluster. The high rate of ATP production needed to support their shivering is supplied by aerobic sugar oxidation in muscle using the massive stores of honey that the colony laid down from summer foraging. Hence, honeybees use a social solution to winter warmth.

Another futile form of ATP turnover that supports thermogenesis occurs in bumblebees, which must also heat their flight muscles before take-off. Here, a futile hydrolysis of ATP occurs from the unregulated function of two enzymes of carbohydrate metabolism: the ATP-dependent 6-phosphofructo-1-kinase (PFK-1) reaction forms fructose-1,6-bisphosphate (F1,6P$_2$), whereas fructose-1,6-bisphosphatase (FBPase-1) immediately hydrolyses it back to fructose-6-phosphate (F6P). Normally, FBPase-1 activity is very low in muscle and the two enzymes are oppositely regulated by allosteric and reversible phosphorylation controls (see Chapter 14). Indeed, bumblebee FPBase-1 is inhibited during flight to allow high rates of unidirectional glycolytic flux (inhibition comes from the high Ca$^{2+}$ released into the cytosol to stimulate contraction). However, during preflight warmup both enzymes function at high rates, achieving a high net rate of ATP hydrolysis that warms the muscle.

### Uncoupling Oxidative Phosphorylation from Electron Transport

High rates of heat release from the oxidation of substrates can also be achieved by failing to utilize the proton-motive force to drive the synthesis of ATP; that is, by uncoupling oxidative phosphorylation from the electron transport system (ETS). As noted in Chapter 8, some plants do this by maintaining a second ETS that is never linked to ATP synthesis. In mammals, nonshivering thermogenesis (NST) by brown adipose tissue (BAT) uses an uncoupling protein (UCP) located in the inner mitochondrial membrane to bypass the F$_1$F$_0$–ATP synthase.

The mechanism of action of UCP isoform 1 (UCP1), the BAT-specific isoform, is as follows (see Fig. TB16.1). Proton pumping out of the matrix by complexes I, III, and IV of the ETS generates a proton gradient across the inner mitochondrial membrane. Normally this is used to drive ATP synthesis by the F$_1$F$_0$–ATP synthase, but, when UCP1 is active, proton reentry bypasses the F$_1$F$_0$–ATP synthase and energy that
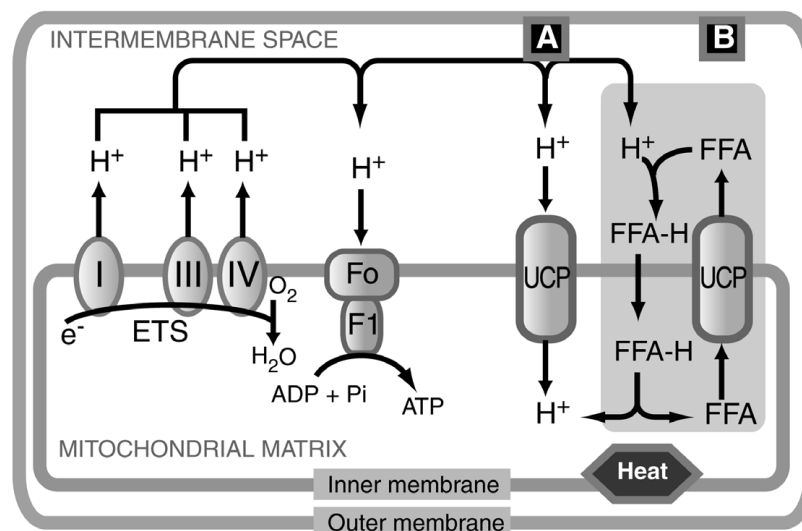


**Figure TB16.1** Mode of action of mitochondrial uncoupling protein 1 (UCP1) in heat production by brown adipose tissue. Both the (*a*) original theory and (*b*) current theory of UCP1 action are shown.

would normally be trapped as ATP is released as heat. Two theories of UCP1 action exist. The original theory was that UCP1 was a proton carrier that directly channeled protons back into the matrix. However, recent studies support a different mechanism but with the same net effect. These studies identify UCP1 as one member of a large gene family of mitochondrial inner membrane anion carriers, the physiological substrates of UCP1 being free fatty acid (FFA) anions. Fatty acid anions are transported out of the matrix by UCP1. They are protonated in the acidic intermembrane space, and then neutral FFA-H diffuse across the inner membrane and dissociate again in the more basic pH environment of the matrix. The net effect is that UCP1 functions as a protonophore to return $H^+$ to the matrix. Although both models can account for the well-known action of FFAs as uncouplers of mitochondrial oxidative phosphorylation, model B also accounts for two other observations: (1) long-chain alkylsulfonates are also transported by UCP1, supporting its role as an anion carrier, and (2) other related mitochondrial transporters (e.g., the dicarboxylate carrier, the ADP/ATP carrier) can also mediate FFA-dependent uncoupling activity.

exclusively on NST by BAT to warm the core thoracic organs, whereas shivering begins once the peripheral skeletal muscles are partly rewarmed. Arousal is a very intense process; for example, in the bat, *Rhinolopus ferrumequinum*, $T_b$ can rise from 7 to 35°C in just 30 min with an increase in oxygen consumption to a level that is 8.7-fold higher than the resting metabolic rate in euthermia. The purpose of the periodic arousals from torpor have frequently been questioned because these brief periods of arousal consume 60 to 80% of the total winter energy budget. This and other key mysteries of hibernation remain unanswered (see Text Box 16.2).

## PREPARATORY BIOCHEMICAL ADJUSTMENTS FOR HIBERNATION

### Fuel Reserves

The hibernation season can last as long as 9 months for Arctic and alpine mammals, and even with the low metabolic rate in torpor, this still requires a lot of metabolic fuel. Some species cache food in their burrows and eat during their periodic arousals, but many do not. For these latter species, all fuel reserves must be "on board" before hibernation begins. To do this, animals go through a period of intense eating (hyperphagia) in the late summer during which body mass increases by up to 50%, mainly due to the deposition of huge reserves of triglycerides in white adipose tissue. Biochemical adjustments supporting this preparatory phase include elevated activities of lipogenic enzymes that are sustained until autumn fattening is completed. For example, the activity of fatty acid synthase in liver of prairie dogs was 10-fold higher during the prehibernating versus hibernating phase of the year.

Normal hormonal controls on satiety and lipid storage by adipose tissue (see Chapter 9 for a review) are overridden during this period of fattening. In little brown bats (*Myotis lucifugus*) plasma leptin levels decrease during the period of prehibernating fattening, just the opposite of the normal response to increasing adiposity in humans and other nonhibernating species. However, administration of mouse recombinant leptin reduced food intake and body weight gain in Arctic ground squirrels (*S. parryii*) during the fattening period. This suggests that animals remain sensitive to leptin in the prehibernating phase, but the production of leptin has been inhibited or dissociated from the normal signals that indicate rising adiposity probably in response to overriding seasonal controls on body mass set point.

### Lipid Fluidity

Direct effects of temperature on the fluidity of triglyceride depots are a key concern for hibernators. To mobilize their fuel reserves when $T_b$ is close to 0°C, lipid depots must be in a fluid state and yet the body fats in most mammals have melting points (MPs) of ~25°C and, hence, are solid near 0°C. The MP of triglycerides depends on the degree of unsaturation of the fatty acids, and the greater the content of mono- and polyunsaturated fatty acids (PUFAs), the lower the MP. Studies have shown that a key element of hibernation success is the presence of sufficient amounts of two PUFAs, linoleic (18:2) and $\alpha$-linolenic (18:3) acids, which lower the MP of the depot fats of hibernators to between 5 and −6.5°C. Mammals can synthesize saturated and monounsaturated fatty acids but must get PUFAs from their diet. A natural diet for ground squirrels produces abdominal fat depots with a composition of 16:0, 18:0, 16:1, 18:1, 18:2, and 18:3 fatty acids that

---

**TEXT BOX 16.2   GREAT MYSTERIES IN HIBERNATION**

Key mysteries about hibernation remain. One is the nature of the signal that initiates the metabolic suppression to start a torpor bout. During entry into torpor, metabolic rate and $T_b$ drop in parallel, and early studies suggested that it was the drop in $T_b$ that caused the decrease in metabolic rate. However, is has now been proven that the opposite occurs. Careful monitoring of the time course of changes in metabolic rate (measured as oxygen consumption) and $T_b$ have shown that the metabolic rate suppression occurs first. So what triggers cells to reduce their metabolic rate? Part of the puzzle is a lowering of the hypothalamic set point for $T_b$ (the functional equivalent of turning down the thermostat in a home), but it is not yet known how or what the message is that is sent out to all cells/organs of the body to coordinate metabolic rate depression in each.

The existence of a blood-borne hibernation induction trigger (HIT) has been indicated. Some studies have reported that administration of the plasma from hibernating animals induced behavioral or physiological characteristics of hibernation in active animals. However, studies from other laboratories have not been able to confirm this. At present the HIT is believed to be an opiate, but it has never been isolated or purified. The effects of treatment with HIT-containing serum can be mimicked by administration of synthetic opioids, such as D-Ala(2),D-Leu(5)-enkephalin (DADLE), and are opposed by opiate antagonists, but much remains to be done to both identify the natural HIT and determine how it works at the cellular level.

The second great mystery is why hibernators go through periodic arousals during the winter season. These arousals are hugely expensive; it has been estimated that reheating the body and fueling the short periods of interbout euthermia consume, respectively, $\sim$19 and $\sim$52% of the total winter energy budget. Many species neither eat, urinate, or defecate during arousal and actually spend a large portion of their arousal time in REM (rapid eye movement) sleep. Indeed, based on EEG (electroencephalogram) recordings, it is believed that the deep torpor of hibernation is actually a state of sleep deprivation, and this is supported by the measured levels of oleamide, a marker of sleep debt, in ground squirrel brain; oleamide was 2.6-fold higher in brain from hibernating versus euthermic ground squirrels. Hence, periodic arousals may be needed to allow REM sleep in order to reset neuronal homeostasis and also, as winter draws to an end, to allow the animal to sense cues (both endogenous circannual stimuli and exogenous environmental cues) that indicate that it is time to terminate hibernation. Another theory supported by recent experiments is that periodic arousals are needed to reactivate a dormant immune system. Torpid ground squirrels did not activate the normal acute-phase response when injected with bacterial lipopolysaccharide but only developed fever and the immune response during their next arousal period, which they extended to six times longer than normal while they fought the infection.

---

is approximately 16, 4, 3, 41, 29, and 7% of the total, respectively. Animals fed artificial diets with minimum PUFA contents, resulting in <20% linoleic and 0% linolenic acids in their depot fats, would not enter torpor when placed in the cold. On the other hand, too many PUFAs in artificial diets also inhibited hibernation. When prairie dogs were compared after feeding high-versus-low PUFA diets, those receiving the higher PUFA contents entered torpor earlier, and had lower torpor $T_b$ values and longer bouts of torpor. Hence, a key element in hibernation success in nature is diet selection. However, the natural sensing mechanism involved in evaluating the PUFA content of depot fats remains unknown.

Although the fluidity of depot lipids is increased to allow them to be metabolized by the hibernator at low $T_b$, it is still not fully understood how the fluidity of phospholipids in cell and organelle membranes is adjusted to maintain membrane functions in hibernation. In ectothermic (cold-blooded) animals, acclimation to temperature change (either up or down) stimulates a rapid restructuring of membrane lipid composition that is termed homeoviscous adaptation (see Text Box 16.3). Interestingly, the quest for homeoviscous adaptation in the membrane lipids of hibernators has produced mixed results when either seasonal or hibernation-induced changes in membrane lipid composition were sought. There may be two reasons for the lack of obvious homeoviscous adaptation in hibernators. One is the metabolic cost. Restructuring all membranes to prevent cold rigidification at the low $T_b$ values in torpor (or alternatively to restore normal membrane fluidity during arousal) would be a huge energetic cost to the animal and a major task to accomplish within a short time frame of entry into or arousal from torpor. Instead of phospholipid restructuring, hibernators might use alternative mechanisms for adjusting membrane fluidity at low $T_b$. The production of specialized proteins that could affect membrane fluidity during torpor is one untested idea. Another reason for a lack of homeoviscous adaptation in hibernators could be that the rigidification of unadapted membranes that would develop at low $T_b$ values might be

**TEXT BOX 16.3   HOMEOVISCOUS ADAPTATION**

Lipids in biological membranes normally exist in a "liquid-crystal" state that is inter-mediate between a highly fluid material and a rather rigid structure. Membrane viscosity is sustained within a narrow window at any $T_b$ by establishing an optimal mix of saturated, monounsaturated, and polyunsaturated fatty acids in the membrane phospho-lipids. For example, in the phosphatidylcholine fraction of brain membranes the ratio of saturated to unsaturated fatty acids was 0.59 in Arctic fish (sculpin) at a $T_b$ of 0°C, 0.82 in goldfish at 25°C, and 1.22 in rats at 37°C. A temperature increase raises the fluid-ity of membrane lipids, whereas a temperature decrease causes a more ordered packing of lipids that increases rigidity. Ectotherms respond rapidly to temperature change to modify the composition of their membranes in order to maintain the optimal membrane viscosity, a process called homeoviscous adaptation. During cold acclimation, this involves a rapid increase in the proportion of unsaturated fatty acids that causes a "disordering" of the membrane hydrocarbon interior to offset the cold-induced ordering. This is accomplished by the rapid activation of desaturase enzyme activity. In particular, much attention has been focused on the $\Delta^9$-acyl-CoA desaturase that, in animals, incorporates the first double bond into saturated fatty acids at the C9–C10 position. A double bond at such as central position in a fatty acid chain (C16 and C18 are the most common chain lengths in animals) creates the maximum disordering effect on membrane properties. Figure TB16.2 shows the responses when carp were cooled from 30 to 10°C. The com-position of the phosphatidylinositol phospholipid class changed rapidly; monoene content was significantly elevated within 1 day, whereas saturates were reduced. Analysis of chil-ling effects on the $\Delta^9$-desaturase revealed two mechanisms at work: (a) an early acti-vation of latent enzyme activity (within 1 day) that may result from some sort of posttranslational modification of the enzyme, and (b) up-regulation of gene expression resulting in elevated mRNA transcripts within 2 days and increased $\Delta^9$-desaturase protein levels within 3 to 4 days. The central role of desaturases in homeoviscous adap-tation is demonstrated by the fact that the enzymes are induced by low temperature in a wide variety of organisms. For example, when the cyanobacterium *Synechocystis* is shifted from a growth temperature of 34 to 22°C, transcripts of three fatty acid desaturase genes, *desA* ($\Delta^{12}$), *desB* ($\omega^3$), and *desD* ($\Delta^6$), are induced within 30 min and rise rapidly by about 10-fold.
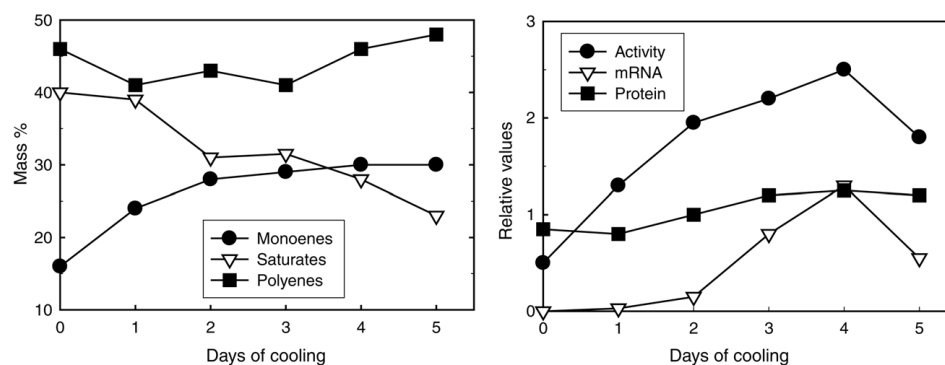


**Figure TB16.2**   Homeoviscous adaptation. Left panel shows the time course of changes in the composition of the phosphatidylinositol fraction in carp liver microsomes when warm-acclimated (30°C) fish were transferred to 10°C. Right panel shows responses of $\Delta^9$ desaturase. [Redrawn from R. J. Trueman et al. (2000). *J Exp Biol* **203**:641–650.]

exploited as one of the controlling elements in metabolic rate depression. The activities of multiple membrane transporters, channels, and pores are dependent on the fluidity of the membranes around them to permit the conformational changes that are key to protein function. These activities could be reversibly suppressed with ease as $T_b$ cools by simply "trapping" the proteins in increasingly rigid membranes (see section on metabolic rate depression below). Indeed, recent studies with nonhibernating species support this idea. $Na^+K^+$–ATPase activity correlated positively with the average membrane packing in both rat and toad microsomes. Similarly, $Ca^{2+}$ channel function responded oppositely to detergents that reduce stiffness versus cholesterol that increases the stiffness of the surrounding membrane.

## PUFAs, Oxidative Damage, and Antioxidant Defenses

To return to the discussion of depot lipids, there is a serious negative side to the presence of high PUFA contents in lipids and that is that PUFAs are highly susceptibility to autooxidation to produce lipid peroxides, as is explained in detail in Chapter 12. Hence, because of the high PUFA contents of their lipids, hibernators need well-developed antioxidant defenses to prevent oxidative damage (commonly known as rancidification) to their lipid depots over the long winter months. Indeed, ground squirrels fed on a high-PUFA diet showed a 50% increase in superoxide dismutase and a two-fold increase in catalase activities in BAT, compared with squirrels on a low-PUFA diet.

Furthermore, the preparation for natural hibernation in ground squirrels includes an elevation of antioxidant defenses in BAT, including enzymatic (superoxide dismutase, glutathione peroxidase), protein (metallothionein), and metabolite (ascorbate) antioxidants (see Chapter 12 for information on their functions) in order to deal with a surge of reactive oxygen species generation during arousal from torpor when rates of oxygen consumption rise very rapidly. Ascorbate levels are two- to fourfold higher than normal in plasma and cerebrospinal fluid of hibernating ground squirrels but drop quickly during arousal. Furthermore, the up-regulation of uncoupling protein (UCP) isoforms 2 and 3 during hibernation may serve an antioxidant function. Unlike UCP1, these isoforms are not involved in NST (see Text Box 16.1), but recall from Chapter 8 that new studies of UCP2 in mammalian mitochondria have shown that the protein functions to reduce damage by reactive oxygen species (ROS) such as the superoxide that is generated by the electron transport chain. UCP2 shifted the release of ROS from the mitochondrial matrix to the extramitochondrial space and inhibited ROS-triggered apoptosis.

Evidence of oxidative stress associated with hibernation is also found in other organs. During torpor the intestine of ground squirrels shows several signs of oxidative stress, including elevated levels of conjugated dienes (products of lipid peroxidation), a decrease in the ratio of reduced-to-oxidized glutathione, activation of redox-sensitive transcription factors such as the nuclear factor-$\kappa$B (NF-$\kappa$B), and expression of the stress protein HSP70.

## NONSHIVERING THERMOGENESIS

Metabolic heat production by NST in BAT is one of the hallmarks of hibernation. NST is used to reheat the body during arousal and can often return $T_b$ from near 0°C to 37 to 38°C within minutes. Low-level NST also maintains a minimum $T_b$ just above freezing when ambient temperature in the burrow or den of the hibernator drops below 0°C. BAT is found in large masses in the interscapular region and the perirenal area and surrounds the aorta and heart. BAT differs from white adipose tissue in the presence of many small lipid droplets and a very high number of mitochondria. It has a primary role as an energy-dissipating organ rather than the energy-storing function of white fat. BAT is richly supplied with capillaries that deliver substrates (oxygen, fatty acids) and carry away the product, heat. Brown adipose is not restricted to hibernators but is found in most neonatal mammals (including humans) and persists throughout life in rodents and all hibernating species (except bears). In neonates, NST is critical for maintaining body heat until insulating layers of subcutaneous fat and fur/hair can be grown.

Like white adipose, BAT proliferates during the prehibernation period, for example, increasing in mass by two- to threefold in ground squirrels during late summer and early autumn. Part of the proliferation response to cold exposure in rodents is an increase in vascular endothelial growth factor (VEGF), which stimulates capillary growth (angiogenesis). Recall that VEGF production in hypoxia was under the control of hypoxia-inducible factor (HIF-1) (see Chapter 15), whereas in proliferating BAT, VEGF responds to noradrenaline stimulation, which also triggers NST. Proliferation and differentiation of brown adipocytes is stimulated by a variety of extracellular signals, including noradrenaline, insulin, and insulin-dependent growth factor (IGF-I), each triggering both positive and negative signal transduction pathways. Thyroid hormones are also positive regulators of thermogenic differentiation. The unique protein in BAT that defines its thermogenic capacity is the 33-kD BAT-specific UCP1 that acts as a protonophore to dissipate the proton gradient across the inner mitochondrial membrane and uncouple ATP synthesis from electron transport (see Text Box 16.1 for an explanation of its action). Not surprisingly, then, huge numbers of studies have focused on the induction of UCP1 synthesis under

both proliferative and acute signals with multiple signal transduction pathways identified that impact on its synthesis. Major positive signal transduction pathways include protein kinase A (PKA) mediating noradrenaline signals (generally for acute situations) and protein kinase B (Akt) mediating insulin/IGF-I routes (generally for longer term proliferation).

Although most of the work on the molecular mechanisms of BAT thermogenesis has used cold acclimation in nonhibernating species (e.g., mice) as the model system, the mechanisms involved seem identical in hibernators. Acute activation of thermogenesis in brown adipocytes is triggered by noradrenaline released from sympathetic nerves that, in turn, are regulated from the hypothalamus. Noradrenaline activates $\beta_3$-adrenergic receptors on the BAT plasma membrane, causing an elevation of intracellular cyclic $3',5'$-adenosine monophosphate (cAMP) and stimulating the dissociation of the active catalytic subunits of PKA. PKA triggers various downstream events, two of the most important being activation of hormone-sensitive lipase (HSL) (see Fig. 9.14) and the up-regulation of UCP1 gene expression via the CREB (cAMP response element binding protein) transcription factor. HSL is activated by PKA-mediated phosphorylation and releases fatty acids from intracellular lipid droplets. These are loaded onto the adipose isoform of fatty acid binding protein (A-FABP) that can form a complex with HSL and are then transported through the cytoplasm to the mitochondria where fatty acids are oxidized by uncoupled respiration to produce heat. Studies in our laboratory showed that there are many additional protein targets of PKA-mediated phosphorylation in BAT. *In vitro* incubation of BAT extracts with cAMP + $^{32}$P-ATP showed strong labeling of multiple phosphoprotein peaks that were not labeled when a PKA inhibitor was included in the incubation. In addition, both the pattern and intensity of labeled protein peaks differed between extracts from euthermic-versus-hibernating animals and between incubations done at $37°C$ versus $5°C$, which suggests both hibernation-specific and temperature-specific differences in PKA targets. In particular, strong phosphorylation of proteins in the 40 to 50-kD range occurred in hibernator BAT extracts that were incubated at $5°C$.

Brown adipose tissue cells maintain a limited amount of lipid and when this is depleted; lipid fuel can also be derived from circulating triglycerides in chylomicrons or from fatty acids delivered from white adipose tissue. Use of circulating triglycerides depends on the action of lipoprotein lipase (LPL), which is also up-regulated by noradrenaline stimulation in brown adipocytes and exported to the capillary lumen where it attaches to heparan sulfate stalks and hydrolyzes passing triglycerides (see Chapter 9 for more details). Since triglycerides in this form come primarily from dietary intake, the involvement of LPL in NST

during arousal from hibernation may be minimal, but the mechanism is important in active rodents in the cold. Fatty acids released by HSL action in white adipose are transported to BAT on plasma albumin and when taken up across the BAT plasma membrane are carried by the heart isoform of FABP (H-FABP) to the mitochondria. Some hibernator organs (BAT, heart) are perhaps unique among mammals in expressing both A- and H-FABP isoforms, reflecting the use of both internal and external fatty acids to fuel NST in BAT and the need for uninterrupted fuel supply to support the rapid increase in heart power output needed to circulate heated blood during arousal. At the mitochondria fatty acids are converted to fatty acyl-CoA moieties, then transported across the inner mitochondrial membrane as carnitine derivatives and catabolized by the normal pathways of $\beta$ oxidation (to produce acetyl-CoA) and the tricarboxylic acid cycle (see Chapter 9). Electron transport through respiratory complexes I, III, and IV is coupled to the formation of an electrochemical proton gradient across the inner membrane. In conventional cells, this gradient is used to drive ATP synthesis by the $F_1F_0-ATPase$, but in BAT the presence of UCP1 uncouples ATP synthesis from electron transport (see Text Box 16.1) and energy is dissipated instead as heat.

Highly abundant in BAT, UCP1 can reach 5% of total mitochondrial protein in cold-adapted rats. Interestingly, UCP1 content was increased by 41% and total NST capacity was higher when mice were fed a diet high in PUFAs, suggesting that the elevated PUFA levels (required before animals can enter hibernation) may have a signaling role in optimizing BAT thermogenic capacity. By contrast, the amount of $F_1F_0-ATP$ synthase in BAT mitochondria is typically very low, the ratio of the synthase to the other respiratory chain components being about 10-fold lower than in other tissues. This clearly enhances the ability of UCP1 to "compete" with the $F_1F_0-ATP$ synthase for proton transport. The limiting factor in $F_1F_0-ATP$ synthase expression in BAT is the level of the $c$-$F_0$ subunit, one of the 10 subunits of the $F_0$ proton translocator.

Metabolic regulation of UCP1 comes from several sources. Purine nucleotides [ATP, adenosine $5'$-diphosphate (ADP), guanosine $5'$-diphosphate (GDP), and guanosine $5'$-triphosphate (GTP)] are negative modulators of UCP1 that can rapidly change its activity; GDP is particularly important in this regard. UCP1 shows temperature-independent properties with regard to both GDP maximal binding capacity and the dissociation constant ($K_d$) for GDP, but the $K_d$ is strongly pH-dependent, decreasing with decreasing pH. This would enhance inhibition of UCP1 by GDP under the state of respiratory acidosis that prevails during torpor, but as an initial event in arousal, animals hyperventilate and unload excess $CO_2$. Cytosolic pH rises and a higher $K_d$ for GDP at higher pH values would reduce the inhibitory effect of GDP on UCP1 and

contribute to the activation of NST during arousal. UCP1 is also sensitive to the level of oxidized ubiquinone, and elevated free fatty acids stimulate UCP1 by providing the vehicle for proton translocation (see Text Box 16.1). Once UCP1 is activated, lipid oxidation is no longer inhibited by high levels of ATP and reduced nicotinamide adenine dinucleotide (NADH) that normally integrate the rate of oxidation with organ demand for ATP.

## METABOLIC REGULATION IN HIBERNATION

Mammalian hibernation is a complex phenomenon. It includes strong metabolic rate depression with differential suppression of metabolic functions to virtually halt all non-essential functions while continuing to support essential functions with adequate levels of energy. Because hibernating animals can have body temperatures far below euthermic values, temperature effects on metabolism are also a key consideration; both compensation for and exploitation of temperature effects on metabolic reactions can contribute to the metabolic reorganization during hibernation. Hibernation is also a state of long-term starvation, and multiple metabolic adjustments are made to reorganize fuel metabolism for a primary dependence on the oxidation of stored lipids as the primary fuel source. Hibernators also have greatly reduced rates of breathing and heart beat; respiratory acidosis develops and blood flow is reduced to values that would cause severe ischemia at euthermic $T_b$. Low rates of blood flow increase the risk of spontaneous clot formation, so adjustments must also be made to anticoagulant systems. Long weeks of torpor also mean long weeks of muscle inactivity, so strategies must be in place to minimize disuse atrophy. All of these issues must be dealt with, often by exploiting, modifying, or exaggerating well-known mechanisms of metabolic regulation but also with selected new mechanisms. Regulation can take multiple forms, including exploitation of temperature effects on enzyme/protein functional properties, changes to the structure and/or properties of hibernator proteins/ enzymes, posttranslational modification of key proteins, and specific changes in gene and protein expression. The following sections will highlight some examples of these modes of metabolic control, but the reader will notice that there is also considerable overlap between these mechanisms. The mechanisms of metabolic regulation in hibernation are also of great interest to medical science (see Text Box 16.4).

## TEMPERATURE EFFECTS ON HIBERNATOR METABOLISM

Between euthermia and torpor, the core $T_b$ of a hibernator can change by over 30°C. Temperature change affects the

---

**TEXT BOX 16.4   HIBERNATION AND MEDICAL SCIENCE**

Medical researchers see multiple applications of hibernation research to humans. The molecular mechanisms that provide long-term stability to hibernator organs at temperatures close to 0°C are of great interest to researchers that want to improve the long-term viability of donor organs while they are transferred on ice to new recipients. Identification of key elements of low-temperature tolerance in hibernators could help to extend hypothermic preservation times from the current limits of about 4 h (heart, lung) to 48 h (kidney) (see Chapter 19). Hibernators are also viewed as possible models for ischemia tolerance because they experience no metabolic damage from very low rates of blood flow in torpor, rates that would normally cause extensive ischemic damage to tissues such as heart and brain. However, the rationale for this ischemia model is faulty. Although experiencing low rates of blood flow, hibernators are not oxygen-limited because their $T_b$ and metabolic rate are also very low; indeed, the fact that lipid oxidation fuels hibernation and lactate does not accumulate demonstrates that metabolism is not oxygen-limited during torpor. But hibernators do appear to be a good model for another ischemia-related problem, that of how to prevent blood clots from forming at extremely low perfusion rates. Hibernators also provide a model for studies of muscle disuse atrophy. Hibernating animals are inactive for many months but lack much of the muscle degeneration that occurs in medical situations of long-term immobility (limb casting, bed confinement) or other situations of disuse such as under the microgravity conditions of long-term space flight. Black bears, for example, lose <20% of their strength over 130 days of hibernation, whereas humans in a similar situation would lose more than 90%. Bears also show no loss of skeletal muscle cell number or size. The mechanism involved is still unknown but may involve periodic shivering or isometric exercise by the torpid animal, limitation of the normally high rates of muscle protein turnover (see Chapter 9), or active muscle protein resynthesis by drawing on labile protein reserves of other organs or recycling urea into new protein synthesis. The space agencies of several countries are keenly interested in hibernation research not only as it relates to muscle atrophy but also to uncover the secrets of metabolic arrest that could allow astronauts to be placed into torpor for long space flights to Mars and beyond.

rate of virtually all biological reactions and physiological functions, and in most cases reaction rates double with every 10°C increase in temperature (see Chapter 2). This is normally stated as a temperature quotient ($k_{(t+10°C)}/k_{t°C}$), where $k$ is the rate at two temperatures differing by 10°C, abbreviated as $Q_{10}$. Most metabolic reactions show $Q_{10}$ values of $\sim2$, which over a 30°C change in temperature would mean an 8-fold change in reaction rate. However, $Q_{10}$ values as high as 3 (producing a 27-fold increase in rate over a 30°C range) are quite common, and higher values occur in some cases; for example, the decrease in oxygen consumption of small mammals entering torpor can often have a $Q_{10}$ of about 4 (a 64-fold change in rate over a 30°C range). Indeed, such high $Q_{10}$ values during entrance into torpor have been taken as evidence of the use of active mechanisms of metabolic rate depression that cause the drop in $T_b$ (rather than vice versa). Temperature change also affects the strengths of weak bonds and, therefore, can alter multiple protein/enzyme properties, including conformation, affinity for ligands, substrate or allosteric effectors, subunit dissociation, and binding interactions with other proteins (see Chapter 14). Depending upon the numbers and types of bonds involved, protein/enzyme properties may range from temperature-insensitive to highly affected by temperature change. Hence, differential effects of temperature on reaction rates and protein/enzyme properties could cause havoc with both individual function and the integration of enzymatic pathways and other metabolic processes. This could necessitate the implementation of multiple mechanisms for temperature compensation in order to reestablish homeostasis at the low $T_b$ of the hibernating state. However, the potential havoc could be mitigated by various factors, for example: (a) unusual temperature effects on equilibrium enzymes with low-flux-control coefficients may have little or no effect on the overall activity of the pathway in which they participate, (2) targeted control over specific regulatory enzymes (with high-flux-control coefficients) could effectively override temperature effects and readjust the relative activities of metabolic pathways for function in the hibernating state, (3) temperature effects on membrane properties could alter the temperature sensitivity of membrane-associated enzymes, and (4) temperature effects could alter enzyme-binding interactions with structural proteins. Equally, however, natural effects of temperature change on selected enzymes and functional proteins could also be exploited to assist in the creation of the torpid state by allowing normal or even enhanced temperature sensitivities of various reactions to "effortlessly" shut off metabolic processes that are not needed in torpor (and equally effortlessly reactivate them during arousal). Several examples of temperature effects on enzyme/protein function in hibernating species illustrate the different principles involved.

## Maintenance of Low-Temperature Function

Multiple metabolic processes must continue in an integrated fashion during hibernation; for example, aerobic lipid oxidation fuels metabolism in all phases of hibernation (deep torpor, arousal, interbout euthermia), and BAT thermogenesis and the signal transduction pathways that activate it must be functional at all $T_b$ values. Because $T_b$ can range from 0 to 37°C within minutes to hours, a temperature-insensitive design for many proteins would seem to be logical. Indeed, several key enzymes/proteins are designed in this way. As noted above, GDP inhibition characteristics of UCP1 from BAT are temperature-independent. We found that the glycolytic enzyme aldolase from ground squirrel skeletal muscle also showed reduced temperature sensitivity as compared with a nonhibernator. When assay temperature was dropped from 37 to 5°C, the $K_m$ of ground squirrel aldolase for its substrate, fructose-1,6-bisphosphate (F1,6P$_2$), increased by only 50%, whereas the $K_m$ of rabbit aldolase increased threefold.

***Temperature-Insensitive Function of Fatty Acid Binding Proteins*** FABPs also show temperature-independent binding parameters. These intracellular carriers transport fatty acids through the cytoplasm, linking sites of fatty acid import/export (plasma membrane), internal storage (lipid droplets), and oxidation (mitochondria). They are crucial elements in hibernation success because fatty acids are the primary fuel utilized throughout the winter by all organs (even brain uses ketones that are synthesized from fatty acids). Perhaps not surprisingly, then, the genes for FABPs are among those that are strongly up-regulated during hibernation (see section on gene expression below), and key kinetic and structural changes to hibernator FABPs appear to provide the temperature insensitivity of function that is needed of a protein that must function "seamlessly" between low and high $T_b$ values. Indeed, we found that ground squirrel L-FABP shows temperature insensitive dissociation constants for both natural (oleate) and artificial (cis-parinarate) substrates at 5, 25, and 37°C (e.g., $K_d$ for oleate was $\sim1.5$ $\mu$M in all cases). By contrast, rat L-FABP showed its lowest $K_d$ (i.e., highest affinity) at 37°C, but $K_d$ values rose about twofold at the lower temperatures (i.e., ligand affinity was reduced by about half). The ability of L-FABP to bind fatty acids of different chain length and degree of unsaturation (16:0, 16:1, 18:1, 18:2, 18:3) was also analyzed by evaluating their ability to quench the fluorescence of bound *cis*-parinarate. In every case, hibernator L-FABP showed equal or better binding of fatty acids at 5°C compared with 37°C, whereas rat L-FABP showed much poorer fatty acid binding at low temperature (Fig. 16.1). Hence, the properties of ground squirrel L-FABP adapt it for function
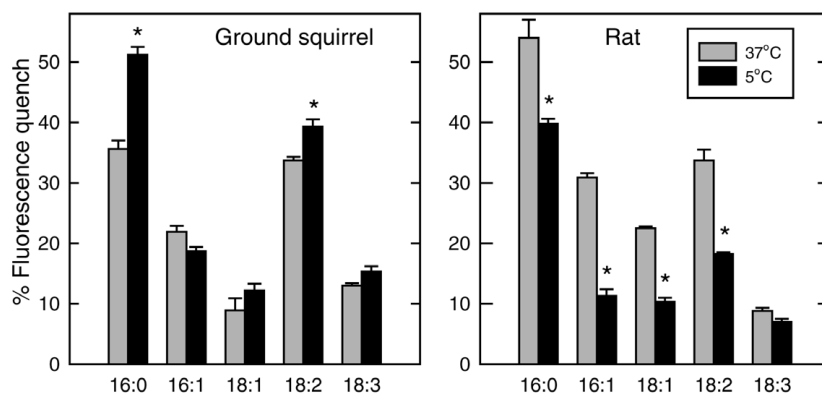
**Figure 16.1**   Binding of fatty acids to (a) ground squirrel and (b) rat fatty acid binding protein assessed by the ability of FABP to displace a fluorescent probe *cis*-parinarate at 37°C (shaded bars) or 5°C (black bars); the greater the fluorescence quench, the greater the fatty acid binding. Probe and fatty acids were both 1 μM. Palmitate 16:0, palmitoleate 16:1, oleate 18:1, linoleate 18:2, and linolenate 18:3. (*) Significantly different from the value at 37°C, $P < 0.05$. Data are means ± SEM, $n = 3–6$. [From J. M. Stewart, T. E. English, and K. B. Storey (1998). *Biochem Cell Biol* **76**:593–599.]

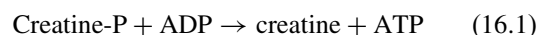over the entire $T_b$ change experienced by the hibernating animal.

The mechanism of this functional difference between ground squirrel and rat L-FABPs has not been directly determined, but parallel studies of the heart isoform of FABP (H-FABP) indicate that as little as three amino acid substitutions make all the difference. This 133-amino-acid protein is highly conserved with at least 90% sequence identity between hibernating and nonhibernating mammalian species. Of the few substitutions in hibernator H-FABP, three appear to be key. In bat H-FABP these are threonine 83, lysine 73, and leucine 71. Ground squirrel H-FABP shares the threonine 83 substitution but has two other polar amino acid substitutions (lysine 68, asparagine 109) that replace the nonpolar or hydrophobic amino acids present in H-FABP from nonhibernating mammals. The Lys 68 and Asn 109 substitutions in ground squirrel H-FABP occur at turns connecting $\beta$ sheets in the protein in a "gap" area of FABP that is believed to confer flexibility to the protein. Substitutions in this region could compensate for the effects of low temperature on both protein and ligand rigidity and provide for consistent protein function over the full range of $T_b$ values under which FABP must function.

**Improved Low-Temperature Function**

Selected enzymes from hibernating species also show temperature effects on substrate affinity that could help them to improve low-temperature function. One example is protein kinase A (PKA), which plays a critical role in the activation of NST. Table 16.1 shows the effects of temperature on the kinetic properties of PKA from ground squirrel BAT versus the rabbit enzyme. The $K_m$ values for both ATP and Kemptide (a peptide substrate) of the hibernator enzymes are sharply reduced at 5°C, compared with 37°C, showing significantly improved substrate affinity at low temperature. By contrast, the substrate affinity of rabbit PKA is unaltered by the temperature change.

A similar situation of improved substrate affinity at low temperature occurs for skeletal muscle creatine kinase (CK) and adenylate kinase (AK) [reactions (16.1) and (16.2)] from hibernators (see more information on these enzymes in Chapter 14). Both are centrally involved in muscle energy metabolism and would have particularly important roles in the supply of ATP for shivering thermogenesis during arousal from torpor:

$$\text{Creatine-P} + \text{ADP} \rightarrow \text{creatine} + \text{ATP} \qquad (16.1)$$
$$2\,\text{ADP} \rightarrow \text{ATP} + \text{AMP} \qquad (16.2)$$

As Table 16.1 shows, the $K_m$ values for all substrates of these enzymes (both forward and reverse reactions) decrease with decreasing assay temperature, an effect that would improve enzyme substrate affinity under the low $T_b$ values of torpor. What is different from the situation with PKA, however, is that CK and AK rabbit muscle also show the same effect, so it seems that the temperature effect on the properties of these enzymes is an inherent property of the mammalian enzymes, perhaps related to the fact that skeletal muscles naturally experience a much wider range of temperatures than do the core organs. The usefulness of enzyme affinities for adenylates that increase

**TABLE 16.1  Effect of Assay Temperature on Enzyme Substrate Affinity; Comparison with Hibernation-Associated Changes in Tissue Adenylate Levels**[a]

| | Hibernator | | Rabbit | |
|---|---|---|---|---|
| | 37°C | 5°C | 37°C | 5°C |
| *Protein Kinase A* | | | | |
| $K_m$ ATP.Mg (μM) | 49 | 23 | 27 | 29 |
| $K_m$ Kemptide (μM)[b] | 50 | 10 | 34 | 35 |
| *Adenylate Kinase* | | | | |
| $K_m$ ATP (mM) | 0.11 | 0.06 | 0.07 | 0.03 |
| $K_m$ ADP (mM) | 0.26 | 0.11 | 0.26 | 0.15 |
| $K_m$ AMP (mM) | 0.22 | 0.10 | 0.12 | 0.08 |
| *Creatine Kinase* | | | | |
| $K_m$ creatine-P (mM) | 2.12 | 1.76 | 2.98 | 2.11 |
| $K_m$ ADP (mM) | 0.20 | 0.01 | 0.25 | 0.01 |
| $K_m$ ATP (mM) | 0.48 | 0.20 | 0.51 | 0.20 |
| $K_m$ creatine (mM) | 3.79 | 2.29 | 11.0 | 2.79 |

| | Prairie Dog | | Jumping Mouse | |
|---|---|---|---|---|
| | 37°C | 5°C | 37°C | 5°C |
| *Adenylate Levels* | | | | |
| Creatine-P (mM) | — | — | 8.1 | 4.8 |
| ATP (mM) | 5.98 | 3.51 | 7.2 | 3.1 |
| ADP (mM) | 0.89 | 1.01 | 0.8 | 0.5 |
| AMP (mM) | 0.10 | 0.14 | 0.05 | 0.15 |
| Energy charge | 0.92 | 0.86 | 0.94 | 0.90 |

[a]Hibernator enzymes are ground squirrel (*Spermophilus richardsonii*) brown adipose PKA, prairie dog (*Cynomus leucurus*) skeletal muscle AK, and bat (*Myotis lucifugus*) CK. Adenylate levels are for skeletal muscle of prairie dogs and jumping mouse (*Zapus hudsonius*). All $K_m$ values at 5°C are significantly different than the corresponding values at 37°C.

[b]*Note*: Kemptide is a peptide substrate that is widely used for PKA assay; it mimics the phosphorylation site in pyruvate kinase.

*Sources*: Data are compiled from K. B. Storey (1997). *Comp Biochem Physiol A* **118**:1115–1124; T. E. English and K. B. Storey (2000). *Arch Biochem Biophys* **376**:91–100; J. A. MacDonald and K. B. Storey (1999). *J Comp Physiol* **168**:513–525.

as temperature decreases is apparent when the changes in muscle concentrations of the substrates of these enzymes during hibernation are considered.

As $T_b$ decreases, the creatine phosphate and total adenylate levels in hibernator organs also fall; Table 16.1 shows examples for the skeletal muscle of prairie dogs and jumping mice, but the same effect has been seen in other species and organs. However, despite an approximate 50% decrease in the total adenylate pool size, the energy charge of organs is undisturbed. Energy charge is defined as $[ATP + \frac{1}{2}ADP]/[ATP + ADP + AMP]$ and is effectively a measure of the ATP power available to metabolism

(the term $\frac{1}{2}[ADP]$ reflects the fact that the adenylate kinase reaction can produce 1 ATP from 2 ADP) (also see Chapter 1). Energy charge in most normal cells is around 0.9 but declines sharply under conditions that cause a rapid utilization of ATP (e.g., muscle exercise) or impair ATP synthesis. A high-energy charge promotes anabolic reactions whereas a drop in energy charge stimulates catabolic reactions to increase ATP synthesis. The decrease in the total adenylate pools of muscle and other organs during hibernation is not accompanied by a change in energy charge or a buildup of end products of AMP breakdown (IMP) such as would act to stimulate increased rates of ATP synthesis. Instead, depletion of the total adenylate pool (with energy charge remaining intact) appears to be one facet of metabolic rate depression. Parallel decreases in the ATP pool size and in the $K_m$ values for ATP of ATP-utilizing enzymes (e.g., PKA, CK, AK) between euthermic and hibernating states help to maintain a near-constant ratio of [ATP] to $K_m$ that may be key to sustaining enzyme function at low temperature. Note, however, that a very strong increase in the affinity of CK for ADP at low temperature ($K_m$ ADP changes by 20-fold) could be an important factor in promoting CK function when rapid ATP generation from creatine phosphate reserves is needed during shivering thermogenesis.

## Change or Suppression of Low-Temperature Function

Just as the interactions of temperature with enzyme/protein properties can facilitate the maintenance of consistent enzyme function across a wide temperature range, temperature effects can also be utilized to change or suppress enzyme function at low temperature. A striking illustration of the use of low temperature to change metabolic function is the disaggregation of polysomes in hibernator tissues when $T_b$ falls below 18°C (discussed in greater detail later).

***Protein Kinase C***  A good example of low-temperature-induced enzyme suppression in hibernators is the behavior of the $Ca^{2+}$-activated and phospholipid-dependent protein kinase, known as protein kinase C (PKC). PKC is a central enzyme of signal transduction in cells (see Chapter 4). The gamma isoform dominates in brain of the bat, *M. lucifugus*, and total activity decreases by about 40% when bats enter hibernation. Arrhenius plots of enzyme activity (log of velocity) versus 1/temperature (in Kelvin) show that activity of the bat brain enzyme declines slowly as temperature initially decreases below 37°C (indeed, $Q_{10}$ is only 1.25 for the 27 to 37°C interval) (Fig. 16.2). Below 10°C, however, activity drops very quickly, with $Q_{10}$ for the 0 to 10°C interval being 3.6 for PKCγ isolated from brain of euthermic bats and 8.1 for the enzyme from torpid bats. By contrast, rat brain PKCγ showed no "break" in the Arrhenius plot over the whole
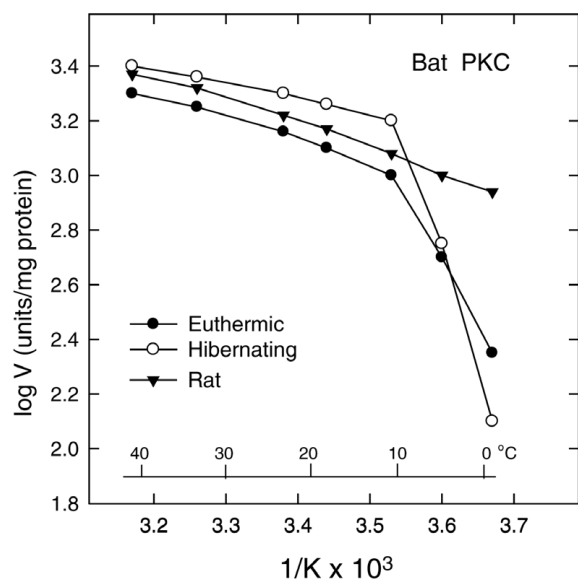
**Figure 16.2** Arrhenius plots showing the effect of temperature (in Kelvin) on the activity of PKC$\gamma$ isoform isolated from brain of euthermic and hibernating bats, *Myotis lucifugus*, and rat brain. Activities were assayed in 5°C increments between 0 and 42°C; note the sharp "break" in the lines for euthermic and hibernator PKC at ~10°C. Inset shows temperature in degrees Celsius. [Data are compiled from H. Mehrani and K. B. Storey (1997). *Neurochem Int* **31**:139–150.]

range. Hence, temperature effects on the activity of bat brain PKC$\gamma$ could be exploited to suppress signal transduction by this pathway at the low $T_b$ values of the torpid state. Temperature also affected the sensitivity of bat brain PKC$\gamma$ to phosphatidylserine (PS), a key activator of PKC; the fold activation of PKC$\gamma$ activity by PS was only 3.5-fold at 4°C compared with 14 to 18-fold at 33°C, a factor that could again aid suppression of PKC$\gamma$ activity during hibernation. However, bat brain PKC$\gamma$ showed a feature not found for the enzyme from other mammalian sources. Bat PKC$\gamma$ was activated by multiple phospholipid types, not just PS. In particular, phosphatidylinositol provided 80 to 90% of the activation seen with PS, whereas phosphatidylethanolamine and phosphatidylcholine were 35 to 45% as effective as PS. This suggests a major modification to the structure of bat PKC$\gamma$ that imparts to it the possibility of enzyme regulation by different phospholipid types, possibly changing at high versus low $T_b$ values.

***Glutamate Dehydrogenase*** Liver glutamate dehydrogenase (GDH) from the ground squirrel (*Spermophilus richardsonii*) presents another interesting case where the properties of the enzyme in a hibernating species are substantially different from those of the normal mammalian enzyme. Furthermore, a comparison of GDH purified

from euthermic (E-GDH) versus hibernating (H-GDH) animals provides strong evidence for a stable modification of the enzyme during hibernation. GDH is a central enzyme of amino acid metabolism; a reversible enzyme found in the mitochondria, it has both an anabolic role in amino acid synthesis and a catabolic role in amino acid degradation and the provision of $NH_4^+$ for the urea cycle [reaction (16.3)]:

$$\text{Glutamate} + \text{NAD}^+ \rightarrow \alpha\text{-ketoglutarate} + \text{NH}_4^+$$

$$+ \text{NADH} \qquad (16.3)$$
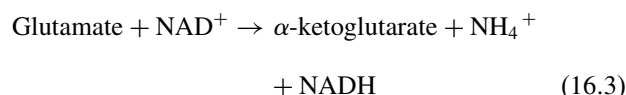
Purified E- and H-GDH from ground squirrel liver show distinct physical and kinetic differences, particularly when compared at assay temperatures that mimic the euthermic (37°C) and hibernating (5°C) states (Table 16.2). The two forms showed small differences in apparent molecular mass on high-pressure liquid chromatography (HPLC), which suggests that hibernation may induce a posttranslational modification of the enzyme. The mechanism of this modification is not yet known, but mammalian GDH has never been shown to be subject to reversible phosphorylation control. The forward (glutamate-utilizing) reaction may be the functional direction of GDH in liver during hibernation, helping to support gluconeogenesis from amino acids during torpor, and kinetic evidence tends to support this. Both E- and H-GDH showed strong reductions in $K_m$ for glutamate (i.e., increased affinity) at 5°C with H-GDH having the lowest $K_m$ glutamate overall. GDH is sensitive to multiple effectors, the two most important ones being activation by ADP and inhibition by GTP, that place amino acid synthesis versus degradation under control by the energy status of the cell. In the glutamate-utilizing direction, E- and H-GDH both showed strong decreases in $K_a$ ADP (6- and 50-fold, respectively) that greatly increase enzyme sensitivity to ADP activation at low temperature whereas inhibition by GTP was reduced at 5°C in both cases ($I_{50}$ values rose by 2 to 3-fold). Changes in both ADP and GTP effector properties at low temperature would promote the glutamate-utilizing reaction of GDH at low $T_b$ and aid a gluconeogenic function for the liver enzyme during torpor.

## METABOLIC RATE DEPRESSION

The decrease in $T_b$ during torpor contributes substantially to the overall reduction in metabolic rate. Indeed, because the $Q_{10}$ value for resting metabolic rate (oxygen consumption) between euthermic and hibernating states is often close to 2, early studies proposed that it was the drop in

**TABLE 16.2   Kinetic Properties of Glutamate Dehydrogenase Purified from Liver of Euthermic versus Hibernating Ground Squirrels, *S. richardsonii*, Measured at 37 and 5°C**[a]

| | Euthermic GDH | | Hibernating GDH | |
|---|---|---|---|---|
| | 37°C | 5°C | 37°C | 5°C |
| *Apparent $K_m$ (mM)* | | | | |
| Glutamate | $2.03 \pm 0.17$ | $0.5 \pm 0.05$[b] | $5.2 \pm 0.47$[c] | $0.25 \pm 0.02$[b] |
| $NH_4^+$ | $7.0 \pm 0.69$ | $24.7 \pm 0.01$[b] | $15.8 \pm 1.44$[c] | $12.1 \pm 0.95$[c] |
| $\alpha$-Ketoglutarate | $0.10 \pm 0.01$ | $3.66 \pm 0.34$[b] | $0.98 \pm 0.08$[c] | $0.43 \pm 0.02$[b,c] |
| *Effectors of Forward (Glutamate-Utilizing) Reaction* | | | | |
| $K_a$ ADP (mM) | $0.181 \pm 0.004$ | $0.032 \pm 0.002$[b] | $0.58 \pm 0.052$[c] | $0.011 \pm 0.001$[b] |
| $I_{50}$ GTP (mM) | $0.019 \pm 0.001$ | $0.061 \pm 0.0051$[b] | $0.076 \pm 0.007$[c] | $0.19 \pm 0.013$[c] |

[a]$K_m$ is the Michaelis constant (substrate concentration providing half-maximal enzyme velocity); $K_a$ is the activator constant (activator concentration providing half-maximal activation); $I_{50}$ is the inhibitor concentration that reduces enzyme velocity by 50% under defined substrate concentrations. Data are means $\pm$ SEM, $n = 4$. For $K_m$ glutamate, concentration of the co-substrate $NAD^+$ was 1.5 mM and for $K_m$ values in the reverse direction, NADH was 0.15 mM with either 100 mM $NH_4HCO_3$ or 7.5 mM $\alpha$-ketoglutarate.
[b]Significantly different from the value for the same enzyme at 37°C, $P < 0.05$.
[c]Significantly different from euthermic GDH at the same temperature, $P < 0.05$.
*Source*:  Data are compiled from B. J. Thatcher and K. B. Storey (2001). *Biochem Cell Biol* **79**:11–19.

$T_b$ that caused the metabolic depression of torpor. However, it is now known that the opposite is true—a regulated, coordinated, and differential suppression of the rates of multiple metabolic processes results in the drop in $T_b$. This was confirmed by careful measurements of the decrease in metabolic rate and $T_b$ during entry into a hibernation bout; the former always preceded the latter. Furthermore, $Q_{10}$ for metabolic rate during this entry period is often 3 to 4, indicative of active suppression of metabolic reactions over this period. The overall signal that triggers and coordinates the suppression is still not known (see Text Box 16.2), but one new idea suggests a hypoxia–hypothermia connection (see Text Box 16.5).

Recent studies support the conclusion that metabolic rate depression (MRD) is an active process because many instances of differential regulation of the rates of diverse metabolic processes have now been documented both within and between organs (examples will be discussed throughout this chapter). Protein synthesis provides an interesting case in point. Studies with ground squirrels showed that the rate of $^{14}$C-leucine incorporation into protein in brain of hibernating squirrels *in vivo* was only 0.04% of the value in active squirrels, indicating severe suppression of this metabolic function during hibernation (remember, if $Q_{10} = 2$, then a 30 or 40°C change in temperature should result in a rate decrease to 12 or 6% of the euthermic value). Part of this rate suppression was due to an extrinsic factor (the decrease in $T_b$) and part was intrinsic. Indeed, when measured *in vitro* at a constant 37°C intrinsic suppression was obvious, the rate of protein synthesis in brain extracts from hibernating animals was just 34% of the euthermic value. When comparable assessments using kidney extracts from hibernating squirrels were made, we found an *in vitro* rate at 37°C that was just 15% of the euthermic value, whereas *in vitro* protein synthesis by extracts from BAT were unchanged between torpor and euthermia. These data reinforce the idea that MRD is organ-specific and tailored to achieve the required level of function of each organ in the hibernating state. With its critical role in providing the thermogenesis needed for arousal, BAT clearly maintains its protein biosynthesis capacity even in deep torpor.

Coordinated MRD is one of the hallmarks of hibernation. Over the course of just a few hours, metabolic rate of the hibernator can sink to well below 5% of the corresponding resting rate of the euthermic animal. Although the central trigger that initiates and coordinates MRD is not yet known (see Text Box 16.2), many regulatory controls at the cellular level are now clear. Key among these is reversible protein phosphorylation, a mechanism of metabolic arrest that is widely conserved across phylogenetic lines and is broadly applicable to the control of almost every metabolic function (see Chapter 14). Metabolic functions that are known, to date, to be under reversible phosphorylation control during hibernation include those discussed below.

## Carbohydrate Oxidation

Fuel metabolism during hibernation is reorganized so that most organs depend on aerobic lipid oxidation for their energy needs. Carbohydrate catabolism in most organs is spared to save glucose as a fuel for the brain, and gluconeogenesis in liver directs glycerol (from triglyceride hydroly-

---

### TEXT BOX 16.5    THE HYPOXIA–HYPOTHERMIA CONNECTION

The organs of hibernating mammals are hypoperfused and, assessed by the standards of an active mammal at 37°C, would be considered to be severely ischemic; for example, cerebral blood flow is only ~10% of the euthermic value. This has led some researchers to argue that hibernators would make good models for studying ischemia, although this argument does not stand up to scrutiny (Text Box 16.4). Although apnoic breathing patterns may mean that blood oxygen content varies over a considerable range during torpor, their organs are never oxygen-limited; this is confirmed by their reliance on lipid oxidation for ATP production even when extremely high rates of heat production by BAT are required during arousal. However, having said this, there are now at least two lines of evidence that indicate that hypoxia has a role to play in hibernation. These are:

1. An ancient hypoxia–hypothermia interaction may contribute to the mechanism of metabolic rate depression in hibernation. When ectothermic animals are challenged with hypoxia, they will move to a cooler environment to reduce their metabolic rate. Endotherms can achieve the same result in a different way. Hypoxia exposure causes a drop in $T_b$ in many mammalian species. That is, if oxygen is limiting for ATP supply, then a reduction in ATP demand by lowering $T_b$ could keep the system in balance. Hibernating species show a more pronounced drop in $T_b$ in response to hypoxia than do non-hibernating mammals. Furthermore, studies with ground squirrels have shown that metabolic rate was not simply suppressed under hypoxia but was regulated to assist the initial fall in $T_b$ and then acted to stabilize $T_b$ at a new lower level. Indeed, a new set point was established for $T_b$ as long as hypoxia persisted. However, oxygen was not limiting in this situation since a drop in ambient temperature caused the animals to elevate their metabolic rate to maintain the new $T_b$ (this also occurs in hibernation if ambient temperature falls below 0°C). Hence, it is possible that hypoxia signals (perhaps generated from breath-hold episodes) may contribute to initiating and managing the drop in metabolic rate and $T_b$ that occurs during entry into torpor.

2. Hibernating animals show up-regulation of hypoxia-related genes. The hibernation-responsive genes that our laboratory has identified from cDNA array screening included several that code for hypoxia-related proteins in heart and skeletal muscle: HIF-1$\alpha$, HIF-1$\beta$ (or ARNT), ORP150 (oxygen-regulated protein) and proline hydroxylase. However, the role that these play is not yet known. HIF is a well-known inducer of glycolytic enzymes, but these do not change in hibernator organs, consistent with a lipid-based metabolism. Thus, it remains to be determined what the HIF signal is doing.

---

sis) and amino acids (some muscle wasting occurs) into glucose for export. Indeed, the measured rates of gluconeogenesis from lactate and glycerol in isolated hepatocytes were 60 to 100% higher (measured in 37°C incubations) in cells isolated from torpid versus summer-active ground squirrels, documenting one of only a handful of metabolic functions that are enhanced during hibernation. Ketogenesis in liver also augments the fuel supply for brain metabolism. The situation is virtually identical with the human metabolic responses to starvation that were described in Chapter 9.

The primary mechanism responsible for the suppression of carbohydrate catabolism is reversible protein phosphorylation. A key focus of control is the pyruvate dehydrogenase complex (PDC) that commits pyruvate, derived from glycolysis, into the oxidative reactions of the mitochondria. PDC is regulated in an on–off manner by reversible phosphorylation. The active enzyme is the dephosphorylated form, and enzyme activity is typically expressed as the percentage of total pyruvate dehydrogenase (PDH) that is present in the active $a$ form (Fig. 16.3). All hibernators examined to date exhibit strong suppression of PDH activity in multiple organs during hibernation, the %PDH$a$ typically falling from 60 to 80% active in euthermia to <5% in most tissues of hibernating individuals (Fig. 16.3). Similar strong control of the %PDH$a$ also characterizes the shallower daily torpor of hamsters (*Phodopus sungorus*). We found that PDH$a$ content in hamster heart and liver was generally only 20 to 40% in torpid animals with low metabolic rates (<40 mL $O_2$/h) but rose to 60 to 80% in aroused animals with metabolic rates of 75 to 100 mL $O_2$/h. Hence, a block on pyruvate
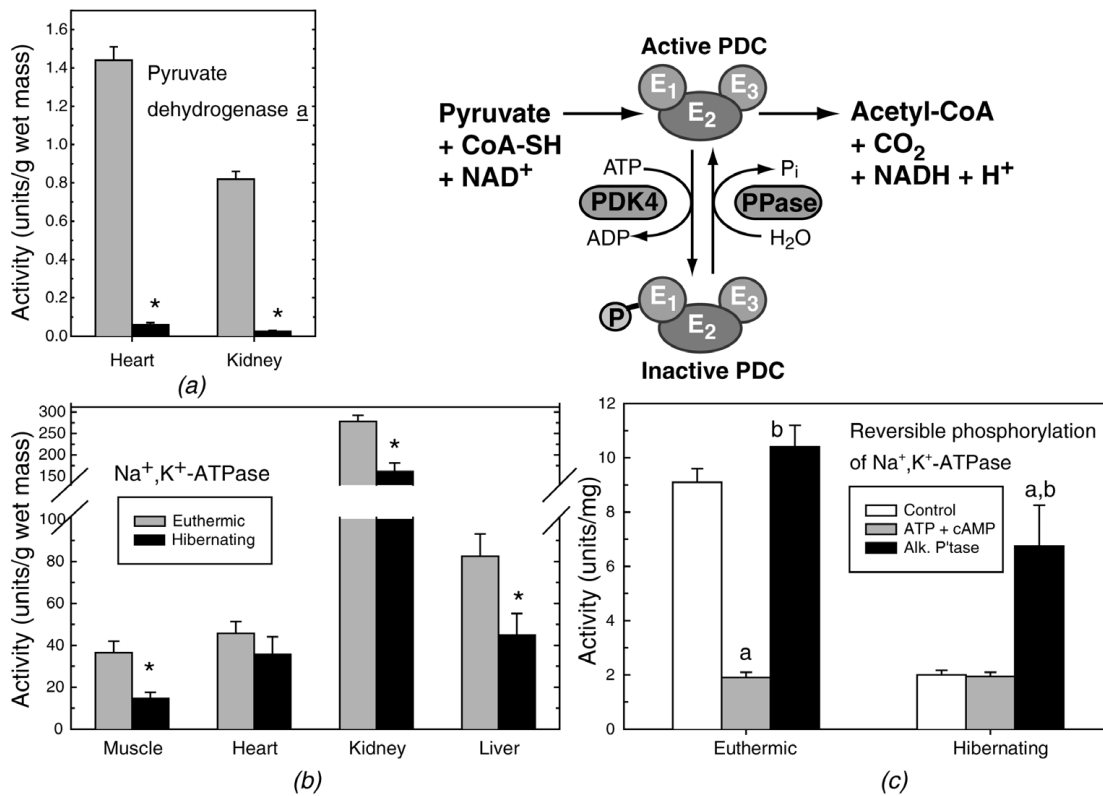
**Figure 16.3** Pyruvate dehydrogenase and sodium–potassium ATPase during hibernation in ground squirrel organs. The pyruvate dehydrogenase complex consists of three enzymes: $E_1$ is pyruvate dehydrogenase, $E_2$ is dihydrolipoyl transacetylase, and $E_3$ is dihydrolipoyl dehydrogenase. (*a*) The amount of active pyruvate dehydrogenase a is strongly suppressed during hibernation due to protein phosphorylation by pyruvate dehydrogenase kinase 4 (PDK4) that is strongly up-regulated in torpor. (*b*) Activity of the ion-motive pump, $Na^+,K^+$–ATPase, is reduced to 40 to 60% of the euthermic value in ground squirrel organs during hibernation. (*c*) The mechanism of $Na^+,K^+$–ATPase suppression is also protein phosphorylation as shown by the decrease in enzyme activity in extracts of euthermic skeletal muscle when incubated with $Mg/ATP + cAMP$ to stimulate endogenous protein kinase A. Subsequent treatment with alkaline phosphatase reverses the effect and also raises the activity of the enzyme in muscle extracts from hibernating animals. Significantly different from (*) the euthermic value, (a) the untreated control sample, (b) the protein kinase treated sample, $P < 0.05$. [Data are derived from S. P. J. Brooks and K. B. Storey (1992). *J Comp Physiol B* **162**:23–28; J. A. MacDonald and K. B. Storey (1999). *Biochem Biophys Res Comm* **254**:424–429.]

entry into mitochondrial metabolism is one of the central loci of metabolic inhibition in hibernation.

Phosphorylation of PDH is catalyzed by pyruvate dehydrogenase kinase (PDK), and gene screening techniques have repeatedly identified PDK isozyme 4 as one of the genes that is strongly up-regulated during hibernation in multiple organs and different species. For example, PDK4 messenger ribonucleic acid (mRNA) levels were about 10-fold higher in heart of hibernating animals sampled in November to January than in animals sampled in September to November before hibernating; comparable changes in PDK4 protein levels were about sixfold. PDK4 (and

PDK2) activities increase in several mammalian organs during starvation, which could indicate that the hibernation response is derived from a preexisting mammalian response that suppresses carbohydrate metabolism under conditions where lipid oxidation is optimized. A new study has shown that rat heart PDK also phosphorylates one of the subunits of NADH ubiquinone-oxidoreductase, also known as NADH dehydrogenase (ND) or complex I of the electron transport system. The result of phosphorylation was reduced ND activity and increased NADH-dependent production of superoxide. PDK phosphorylation of ND would provide a way of coordinating complex I activity

with the input of carbohydrate fuel into the tricarboxylic acid cycle and could obviously contribute to metabolic rate suppression during entry into hibernation by targeting the activity of the initial complex of the respiratory chain. Phosphorylation of the enzyme by PKA had exactly the opposite effect, and because PKA mediates signals (e.g., epinephrine) that can increase carbohydrate oxidation by heart, a coordinated activation of both the PDC and the respiratory chain could be achieved. Furthermore, as discussed in Chapters 8 and 12, production of superoxide by the respiratory chain is no longer viewed as a negative by-product of oxygen-based metabolism but is emerging as an important signaling metabolite. A role for superoxide signaling in transitions to and from the hypometabolic state of hibernation needs to be investigated.

Additional phosphorylation controls on liver carbohydrate metabolism closely regulate the disposition of the body's main carbohydrate (glycogen) reserve and the glucose derived from gluconeogenesis (for a review of liver metabolism during starvation see Chapter 9). Inhibitory control of glycolysis targets three loci: glycogen phosphorylase (GP), 6-phosphofructo-1-kinase (PFK-1), and pyruvate kinase (PK). Coordinated suppression of all three is well-illustrated by studies on liver of the jumping mouse, *Zapus hudsonius* (Table 16.3). GP activity is strongly suppressed by two mechanisms, a decrease in total enzyme activity (probably due to a reduced amount of GP protein) and a decrease in the percentage of enzyme present in the phosphorylated active *a* form. Overall, then, the amount of active GP*a* in liver of hibernat-

**TABLE 16.3   Kinetic Parameters of Liver Glycolytic Enzymes in Euthermic and Hibernating Meadow Jumping Mice, *Zapus hudsonius***

|  | Euthermic | Hibernating |
|---|---|---|
| *Glycogen Phosphorylase* | | |
| Total activity ($a + b$), U/g | 4.5 | 3.0 |
| Phosphorylase *a*, U/g | 3.0 | 0.42 |
| Percent active | 66 | 15 |
| *6-Phosphofructo-1-Kinase* | | |
| $K_m$ fructose-6-P, mM | 3.73 | 3.95 |
| $K_a$ AMP, mM | 0.48 | 0.05 |
| $K_a$ fructose-2,6-P$_2$, $\mu$M | 0.14 | 0.35 |
| $I_{50}$ Mg.ATP, mM | 11.4 | 2.84 |
| $I_{50}$ Mg.citrate, mM | 11.7 | 3.13 |
| *Pyruvate Kinase* | | |
| $S_{0.5}$ phosphoenolpyruvate, mM | 0.47 | 0.44 |
| $K_a$ fructose-1,6-P$_2$, $\mu$M | 0.27 | 1.18 |
| $I_{50}$ L-alanine, mM | 14.0 | 2.21 |

*Source*: Data are compiled from K. B. Storey (1987). *J Biol Chem* **262**:1670–1673.

ing mice (0.42 U/g) was only 14% of the value in euthermic animals (3 U/g).

Entry of hexose phosphates into the "lower" triose phosphate portion of glycolysis is controlled by PFK-1. The enzyme is closely controlled by both energetic signals (ATP inhibition, AMP activation) and fuel supply signals (activation by high fructose-2,6-P$_2$ signals plentiful carbohydrate that can be used for anabolism; inhibition by high citrate signals plentiful lipid oxidation and, hence, that carbohydrate can be spared). Stable changes to the kinetic constants for these activators and inhibitors occur during hibernation (Table 16.3) and can be interpreted as follows: (a) much greater sensitivity to AMP ($K_a$ decreases by 90%) puts the enzyme under strong control by the energy status of the cell, (b) increased sensitivity to ATP inhibition ($I_{50}$ decreases by 75%) has the same function but also recall that cellular ATP content typically falls by about 50% in torpor (Table 16.2) so part of the change in $I_{50}$ readjusts PFK-1 sensitivity to the prevailing ATP levels in hibernation, (c) reduced sensitivity to fructose-2,6-P$_2$ ($K_a$ increases by 2.5-fold) makes the enzyme less sensitive to anabolic signals, and (d) increased sensitivity to inhibition by citrate, a signal of high substrate availability in the tricarboxylic acid cycle, enhances the inhibition of glycolysis under conditions of high lipid oxidation.

Pyruvate kinase is the third control point in liver glycolysis. Strong inhibitory control of PK is needed under conditions when gluconeogenesis is needed, a situation that occurs in hibernation. Again, stable changes to PK kinetic parameters were found in liver of hibernating mice: (a) the $K_a$ for fructose-1,6-P$_2$ rises 4.4-fold, making the enzyme much less sensitive to feed-forward activation by the product of PFK-1, and (b) the $I_{50}$ for L-alanine drops to just 16% of the euthermic value, greatly increasing sensitivity to inhibition by this key gluconeogenic substrate.

In all cases, these kinetic changes to *Z. hudsonius* liver enzymes were stable during enzyme extraction, and studies in this species and others have identified the mechanism of hibernation-induced changes as protein phosphorylation. GP is dephosphorylated and largely returned to its inactive form, whereas PFK-1, PK, and PDH are converted to less active forms by phosphorylation. Not all species show such dramatic and coordinated changes to the properties of their glycolytic enzymes during hibernation (although all show PDH suppression). Such strong regulatory controls are particularly prominent is small species, such as mice and bats, with less consistent changes in larger species such as ground squirrels.

## Ion-Motive ATPases

As discussed in Chapter 15, membrane ion pumps consume a huge portion of the total ATP turnover of

any cell (5 to 40% depending on the tissue), but their job is crucial for the maintenance of membrane potential difference, which in turn is the basis of multiple activities, including nerve conductance, muscle contractility, the transmembrane movement of many organic molecules, and the sensitivity of multiple receptors. Among these, $Na^+K^+$–ATPase has a prominent role, especially in endotherms, because high rates of ATP turnover during $Na^+/K^+$ pumping are a major contributor to thermogenesis (see Text Box 16.1). Hence, $Na^+K^+$–ATPase activities in mammalian organs are two- to sixfold higher than in the same organs of comparably sized reptiles, and the opposing facilitated flux of $Na^+$ and $K^+$ ions through ion channels is similarly greater in mammals. Obviously, then, $Na^+K^+$–ATPase and other energy-driven pumps as well as the facilitative channels that oppose them represent critical targets for metabolic arrest during hibernation.

Analysis of $Na^+K^+$–ATPase activities in organs of hibernating ground squirrels confirmed this. We found that activities in extracts from hibernator organs were just 40 to 60% of the comparable values in organs from euthermic animals (all measured at 25°C) (Fig. 16.3). Activity of the sarcoplasmic reticulum (SR) Ca-ATPase that is responsible for $Ca^{2+}$ reuptake into the SR after muscle contraction is similarly suppressed during hibernation. Indeed, in ground squirrel skeletal muscle, the activities of $Na^+K^+$–ATPase and Ca–ATPase measured under optimal substrate conditions in extracts from hibernating animals were 40 and 41%, respectively, of the corresponding values in euthermic extracts, suggesting coordinate control over these two important ion pumps. Under *in vivo* conditions, suppression may be even stronger because muscle ATP concentration is 30% lower in torpor than in euthermia (reducing ATP substrate availability) at the same time as enzyme affinity for substrates is reduced (e.g., for Ca–ATPase, $K_m$ $Ca^{2+}$ is 75% higher and $K_m$ ATP is threefold higher in muscle extracts from hibernating versus euthermic animals). *In vitro* studies that incubated skeletal muscle extracts under conditions that stimulated protein kinase activity showed us that hibernation-induced suppression of $Na^+K^+$–ATPase was due to protein phosphorylation whereas $Na^+K^+$–ATPase activity was restored after alkaline phosphatase treatment (Fig. 16.3). Other proteins involved in SR calcium signaling are also suppressed during hibernation; SR calcium-release channels (ryanodine receptors) decreased by 50%, and most SR calcium binding proteins (e.g., sarcalumenin, calsequestrin) are three- to fourfold lower in hibernating, compared with summer-active, animals. Overall, then, targeted covalent modification of membrane ion pumps and associated proteins strongly suppresses ATP use by these proteins during torpor, but the primary mechanism used (reversible phosphorylation) allows for an immediate reversal and

reactivation of ion pumps via stimulation of protein phosphatases during arousal.

Similar controls may regulate other membrane-associated processes during hibernation. For example, rates of $K^+$ efflux are threefold lower in mitochondria isolated from torpid ground squirrels, compared with euthermic animals, and rates of state 3 respiration are 30 to 66% lower (measured at a constant temperature). By contrast, there are only minor changes in the contents of mitochondrial enzymes or cytochromes between euthermic and hibernating states, and this inhibition is rapidly reversed upon arousal. Again, this implicates reversible controls such as protein phosphorylation in the suppression of mitochondrial activity during hibernation.

## Protein Synthesis and Ribosomal Translation Machinery

Protein synthesis is another of the energy-expensive processes in cells and, as noted above, is strongly suppressed during hibernation. As discussed in the previous chapter, two factors could restrict protein synthesis in suppressed metabolic states: (1) mRNA substrate availability and (2) inhibition of the ribosomal translation machinery. Substrate availability does not appear to be a significant factor as there little evidence of reduced mRNA availability in hibernation (similar to the case in other hypometabolic systems; see Chapter 15). No major change in global mRNA transcript levels during hibernation were detected when the question was approached in several ways: total mRNA levels were unchanged, as were the levels of mRNA transcripts of selected constitutively active genes, and evidence from complementary deoxyribonucleic acid (cDNA) array screening showed only a very low percentage of genes whose transcript levels were up- or down-regulated during hibernation. Furthermore, mRNA appears to remain intact during hypometabolism with no reductions in transcript sizes or general shortening of poly(A) tail lengths as typically occurs when transcripts are degraded. Hence, global mRNA substrate availability does not appear to be a factor in protein synthesis inhibition in hibernation.

Instead, organ-specific suppression of the rates of protein synthesis during hibernation is regulated by control over ribosomal translation by two main mechanisms: (1) reversible phosphorylation of proteins in the translational machinery and (2) the state of ribosome assembly.

***Reversible Phosphorylation of Ribosomal Proteins*** Reversible protein phosphorylation regulates a variety of ribosomal proteins. The mechanisms and target proteins are the same as those involved in the suppression of protein synthesis under multiple other states in mammals (notably

starvation) (see Chapter 7 for a general discussion) and as also occur in anoxia-induced metabolic rate depression (Chapter 15). As in these other cases, the eukaryotic initiation factor 2 (eIF2), which introduces initiator methionyl–tRNA (transfer RNA) into the 40S ribosomal subunit, is a main target of inhibitory control during hibernation (Chapters 7 and 15). For example, in ground squirrel brain the percentage of total eIF2$\alpha$ that was phosphorylated rose from >2% in euthermic to 13% in hibernation. Hibernator kidney and skeletal muscle also showed severalfold higher levels of phospho-eIF2$\alpha$ than in euthermia.

Protein translation is also controlled at the level of polypeptide elongation. Studies with ground squirrels have shown that mean transit times for polypeptide elongation by ribosomes were twofold longer in extracts from hibernating animals than in control extracts incubated at the same temperature. The mechanism of this effect was traced to the eukaryotic elongation factor 2 (eEF2) (Fig. 16.4). Studies with other systems have established that eEF2 is one of the most prominently phosphorylated proteins in mammalian cells and is the major player in global protein synthesis control at the elongation stage. During hibernation the amount of phospho-eEF2 (the inactive form) increased substantially in brain and liver. Likewise, phospho-eEF2 content in skeletal muscle of hibernating bats was 15-fold higher than in euthermic animals. The mechanism of eEF2 control in ground squirrel brain included an ∼50% higher activity of eEF2 kinase during hibernation and a 20 to 30% decrease in protein phosphatase 2A (PP2A) activity (which opposes eEF2 kinase) as a result of a 50 to 60% increase in the levels of the specific inhibitor of PP2A, $I_2{}^{PP2A}$.

The eEF2 kinase is $Ca^{2+}$-dependent, and this initially suggested a way that changes in intracellular $Ca^{2+}$ concentrations could regulate protein synthesis. However, new work shows that this is just a minor part of the whole story for eEF2 kinase is subject to phosphorylation and activation by several of the major cellular protein kinases, including PKA, mitogen-activated protein kinases (MAPKs), p90$^{RSK1}$, and p70 S6 kinase. This puts global protein elongation under regulation by a host of other inputs. New research has also shown that eEF2 kinase is very sensitive to changes in pH. At normal physiological pH in mammals (pH 7.2 to 7.4) the phosphorylation state of the enzyme in liver homogenates was very low, but when the pH of extracts was lowered to 6.6 to 6.8, the amount of phosphoenzyme increased severalfold. This provides a mechanism for a global suppression of protein synthesis in response to cellular acidosis. Strikingly, every known system of natural hypometabolism includes cellular acidification as one of its traits—all facultative anaerobes develop acidosis as a result of a reliance on fermentative metabolism, hibernators and estivators develop respiratory acidosis due to apnoic (breath-hold) breathing patterns,

and many other dormant systems are also acidotic as compared with their active states. Hence, exploitation of pH effects on the activities of key enzymes can provide a way of making "effortless" transitions in the activity states of multiple cell functions to facilitate overall metabolic rate depression.

***Polysome Disaggregation*** Translational control in hibernation is also regulated by polysome aggregation state. Studies with brain, liver, and kidney of hibernating ground squirrels have all found a decrease in polysome content and an increase in monosomes in torpor along with a shift of the mRNA for constitutively active genes [e.g., actin, glyceraldehyde-3-phosphate dehydrogenase, cytochrome *c* oxidase subunit 4 (*Cox4*)] into the monosome fraction. This is the same principle that was described for anoxia-tolerant species in Chapter 15, and this confirms that polysome disaggregation is a general principle of metabolic rate depression across broad phylogenetic lines. Figure 16.5 illustrates the principle for ground squirrel tissues. *Cox4* transcripts are retained during hibernation but shifted into the translationally silent monosome fraction so that translation of this constitutively active gene is halted during torpor and its protein levels remain constant.

The trigger for polysome disaggregation during hibernation is not known with certainty, but new evidence indicates that temperature is a factor. When ground squirrels were sampled at multiple $T_b$ values during both entry into (cooling) and arousal from (warming) torpor, the distribution of ribosomal RNA (rRNA) (monitoring ribosomes) and actin mRNA (monitoring transcripts) in liver shifted distinctly when core $T_b$ reached 18°C. Actin mRNA was primarily in the polysome fraction of euthermic squirrels, but below 18°C a large portion of the transcripts, as well as rRNA, suddenly shifted to the monosome fraction, where they remained throughout torpor. Conversely, during arousal, polysome reassembly was first evident when $T_b$ rose to 18°C. Whether this temperature effect derives from a passive influence of temperature on polysome assembly or is due to temperature-stimulated regulation of one or more ribosomal proteins is not yet known.

The principle illustrated above is that an overall suppression of protein synthesis during hibernation is achieved by the dissociation of active polysomes and the storage of mRNA transcripts in the translationally silent monosome fraction. During arousal the reverse transition allows protein synthesis to be rapidly reinitiated without a need for *de novo* gene transcription. Note that by this mechanism an effective "life extension" of mRNA transcripts is achieved. However, this principle does not account for genes that are up-regulated during hibernation, and a growing number of these have been identified (see below). Generally, an increase in mRNA transcript levels goes hand in hand with increased protein synthesis, so
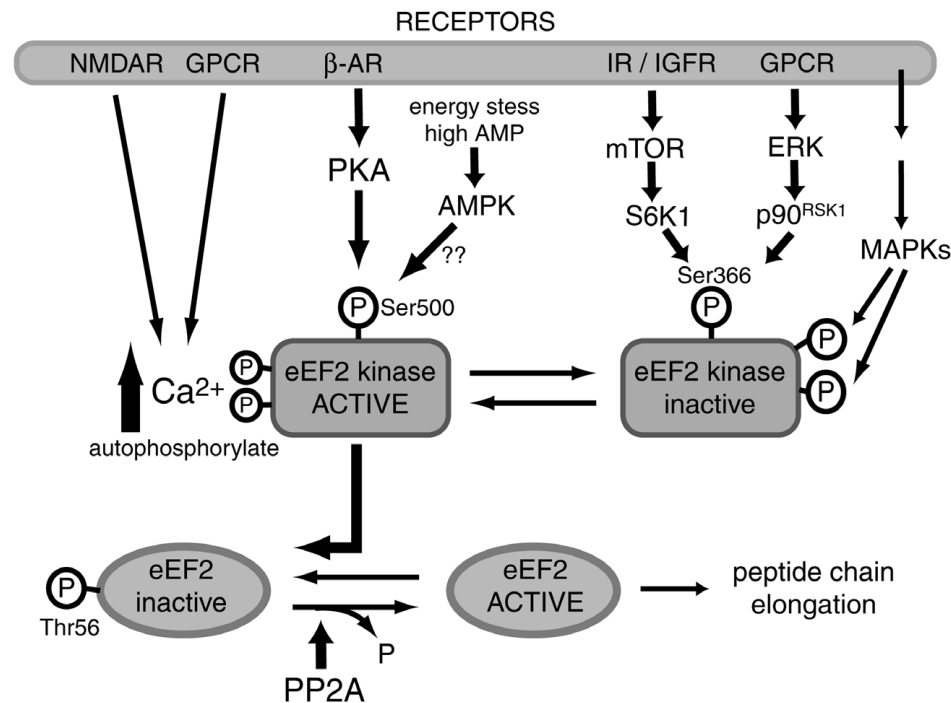
**Figure 16.4** Regulation of peptide elongation via controls on the eukaryotic elongation factor 2 (eEF2). eEF2 activity is controlled by reversible phosphorylation of Thr56 with inhibition mediated by eEF2 kinase and activation via dephosphorylation by protein phosphatase 2A (PP2A). eEF2 kinase is the only enzyme that directly phosphorylates eEF2, but the impact of many cellular signals is focused on eEF2 via reversible phosphorylation control over eEF2 kinase at multiple sites. Activation of eEF2 kinase (leading to phosphorylation and inhibition of eEF2) comes from several sources. Initial studies showed that high $Ca^{2+}$ activated the enzyme in a $Ca^{2+}$/calmodu-lin-dependent manner, stimulating autophosphorylation of eEF2 kinase at multiple sites. High $Ca^{2+}$ mediates signals from $N$-methyl-D-aspartate receptors (NMDARs) and various G-protein-coupled receptors (GPCRs). Signals such as epinephrine that act via $\beta$-adrenergic receptors ($\beta$-ARs) trigger protein kinase A (PKA) to phosphorylate eEF2 kinase on Ser500 and activate the enzyme. Stresses such as exercise or ischemia that deplete cellular ATP levels and elevate AMP also lead to increased phosphorylation of eEF2 by activating the AMP-dependent kinase (AMPK); however, whether AMPK acts via effects on eEF2 kinase or regulates eEF2 dephos-phorylation is not yet known. Inhibition of eEF2 kinase (leading to eEF2 activation and the promotion of protein synthesis) comes from phosphorylation of this kinase on different serine residues. Insulin and insulin-like growth factor 1 (IGF1) stimulate their respective receptors (IR, IGR1R) and activate the p70 S6 kinase (S6K1) in an mTOR (mammalian target of rapamy-cin)-dependent manner to phosphorylate Ser 366. MAPK pathways also lead to inhibitory phos-phorylation of eEF2 kinase including ERK-mediated signaling that acts via the p90[RSK] and SAPK/JNK signaling that phosphorylates other sites. [For a review of this topic see G. J. Browne and C. G. Proud (2002). *Eur J Biochem* **269**:5360–5368.]

how are transcripts that are induced by hibernation handled in a system where ATP availability for protein synthesis is low, polysomes are largely disaggregated, and ribosomal initiation and elongation factors are inhibited? Two cases highlight interesting variations on the general principle.

***Differential Distribution of Individual mRNA Species*** Gene transcripts (*fabp*) for FABP increase severalfold in

most organs during hibernation. Figure 16.6 shows the responses by *h-fabp* transcripts in BAT during hibernation; transcript levels are strongly increased and, despite the overall suppression of protein synthesis, H-FABP protein also increases during hibernation. This supports the need for high rates of fatty acid delivery to BAT mitochondria during thermogenesis. How is this accomplished? The answer is differential distribution of *fabp* transcripts in
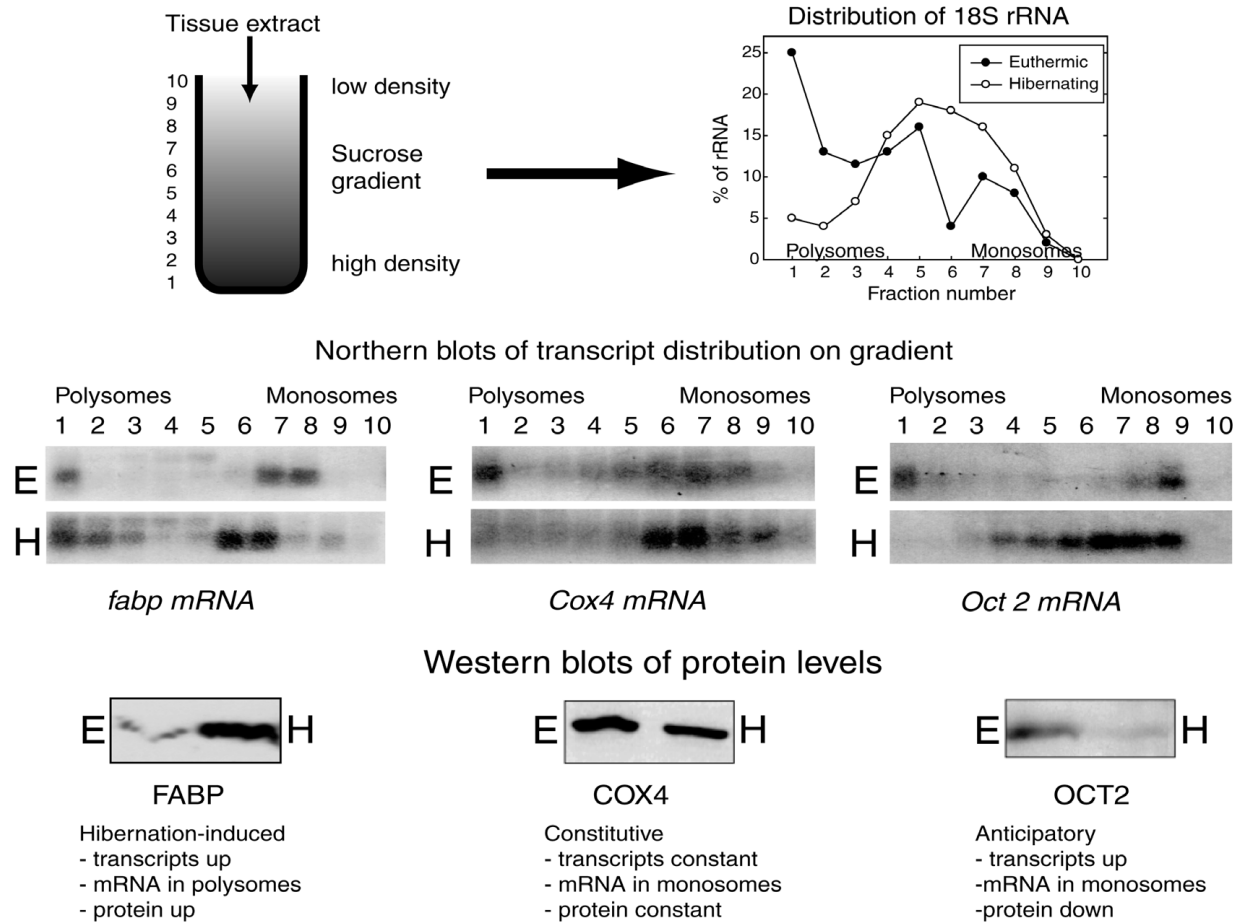
**Figure 16.5** Changes in the distribution of mRNA transcripts between translationally active polysomes and translationally silent monosomes during hibernation. Tissue extracts were fractionated on a sucrose gradient and assay of 18S ribosomal RNA content in each fraction shows the relative content of polysomes and monosomes in tissue from euthermic versus hibernating ground squirrels. Northern blots of RNA extracts from each fraction show the position on the gradient and relative abundance of mRNA for three genes: fatty acid binding protein (*fabp*) and cytochrome *c* oxidase subunit 4 (*Cox4*) from brown adipose tissue and the organic cation transporter type 2 (*Oct2*) from kidney. Western blots show the corresponding changes in protein content. The analysis shows (1) enrichment of *fabp* in the polysome fractions and an overall increase in mRNA abundance during hibernation that correlates with the strong increase in FABP protein in hibernator BAT, (2) a strong shift in the mRNA of *Cox4*, a constitutive gene, from the polysome to the monosome fraction during hibernation with unchanged protein levels, and (3) an increase in the abundance of *Oct2* mRNA during hibernation but transcripts are sequestered into the monosome fraction (probably for storage until arousal occurs) while protein content falls during hibernation. [Data compiled from D. S. Hittel and K. B. Storey (2002). *Arch Biochem Biophys* **410**:244–254.]

the monosome versus polysome pools. Whereas transcripts of the constitutively expressed gene *Cox4* are largely sequestered into the monosome fraction during hibernation, we found that *fabp* transcripts were associated with the remaining polysomes. This differential enrichment of *h-fabp* transcripts in the polysome fraction correlated with a threefold increase in H-FABP protein in BAT of hibernat-

ing animals. By contrast, COX4 protein levels were unchanged. This illustrates another principle of metabolic control—the rate of translation of individual mRNA species can be altered by differential distribution of transcripts between translationally active and inactive ribosomes. The polysomes in hibernator BAT contain disproportionately higher numbers of those mRNAs (such
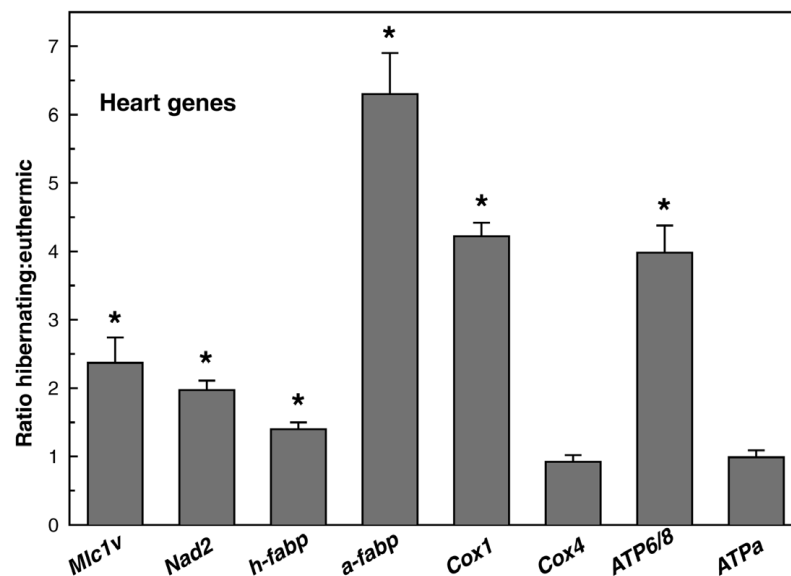
**Figure 16.6**   Gene up-regulation in ground squirrel heart during hibernation. Relative transcript levels (determined from Northern blots) in hibernating versus euthermic heart are shown for eight genes: $Mlc1_v$, myosin light-chain 1 ventricular isoform; $Nad2$, subunit 2 of NADH–ubiquinone oxidoreductase; $FABP$-$H$ and $FABP$-$A$, heart and adipose isoforms of fatty acid binding protein, respectively; $Cox1$ and $Cox4$, subunits 1 and 4 of cytochrome $c$ oxidase; $ATP6/$ $8$, ATPase 6/8 bicistronic mRNA; and $ATP\alpha$, $\alpha$ subunit of the mitochondrial ATP synthase. $Nad2$, $Cox1$, and $ATP6/8$ are mitochondria-encoded subunits and $Cox4$ and $ATP\alpha$ are nuclear-encoded subunits of mitochondrial proteins. Data are means $\pm$ SEM, $N = 3$. [Data compiled from D. S. Hittel and K. B. Storey (2002). *J Exp Biol* **205**:1625–1631; A. Falman, J. M. Storey, and K. B. Storey (2000). *Cryobiology* **40**:332–342.]

as *fabp*) that are crucial to the hibernation phenotype, whereas mRNA species that are not needed during hibernation are relegated into the translationally silent monosome fractions.

***Anticipatory Up-Regulation***   Another variation on translational control was identified in studies of the gene for the organic cation transporter type 2 (*Oct2*) in hibernator kidney. *Oct2* transcript levels rose by two- to threefold during hibernation, but OCT2 protein levels actually decreased during hibernation (Fig. 16.5). Analysis of *Oct2* distribution on polysome profiles showed that, although *Oct2* transcripts were much higher in hibernation, they were largely sequestered into the translationally silent monosome fraction. Why would this be? The probable reason for this is so that OCT2 protein can be produced very rapidly from existing *Oct2* transcripts as soon as torpor is broken. Kidney function is virtually shut down during hibernation, and it is possible that this includes an actual degradation of OCT2 protein (rather than a reversible inactivation) since immunoblotting showed much lower levels of OCT2 in hibernator kidney compared with euthermia. In such a situation, resumption of transpor-

ter action to support renewed kidney function during interbout arousals would require the rapid synthesis of OCT2. Hence, this suggests another possible principle of translational control—anticipatory up-regulation of selected transcripts during hibernation can support a rapid activation of protein synthesis during arousal that does not depend on enhanced gene expression. This mechanism has a similar functional outcome as the mechanism of ferritin transcript regulation described in the previous chapter. Ferritin transcripts are sequestered until an increase in intracellular iron stimulates their translation, whereas *oct2* transcripts are held in readiness until translation is reactivated during arousal from torpor, possibly as the consequence of polysome reaggregation when $T_b$ rises above 18°C.

***Stress Granules***   The mean half-life of mRNA transcripts in a vertebrate cell is about 3 h, but some transcripts may be stable for only a few minutes and others for a very long time. However, if untranslated mRNAs are to be maintained for days or weeks in hibernator cells, how are they protected from degradation so that translationally competent mRNA transcripts still remain when animals arouse? The poly(A) tail on vertebrate mRNAs is an important

protector of the transcript, and a major degradative pathway in eukaryotic cells involves first shortening the poly(A) tail, then decapping the $5'$ end and degrading the mRNA in a $5' \rightarrow 3'$ direction. As mentioned previously, analysis of mRNA poly(A) tail lengths has shown no reductions in transcript sizes or general shortening of poly(A) tail lengths during long periods of dormancy, so certainly transcripts are being protected from degradation.

Studies with mammalian systems have linked the suppression of protein synthesis in stress situations with the phosphorylation of eIF2$\alpha$; recall that increased amounts of phospho-eIF2$\alpha$ is a prominent feature of hibernation. This key initiation factor is phosphorylated by at least four different kinases that mediate different stress signals, and phosphorylation increases binding in an inactive complex with eIF2B, which stops protein synthesis by limiting the availability of the eIF2–GTP–Met–tRNA ternary complex (see Chapter 7). When translation is initiated in the absence of this complex, an eIF2/eIF5-deficient "stalled" 48S preinitiation complex is assembled. This preinitiation complex is joined by RNA-binding proteins, TIA-1 and TIAR, and routed to cytoplasmic foci known as stress granules. In stressed cells, mRNA is in a dynamic equilibrium between polysomes and stress granules. Storage of mRNAs in stress granules during hypometabolism would be an effective protective mechanism that could (a) preserve the valuable pool of untranslated mRNAs until normal conditions were reestablished and (b) provide for a very rapid reinitiation of the translation of key transcripts to provide protein products that are needed immediately during arousal from the hypometabolic state.

Prominent protein constituents of stress granules are poly(A)-binding protein 1 (PABP-1) and T-cell intracellular antigen-1 (TIA-1), a self-aggregating RNA-binding protein. In studies with ground squirrel kidney, Western blotting was used to assess the presence of these proteins in the ribosomal fractions separated on sucrose gradients (as per Fig. 16.5). TIA-1 was restricted to the monosome fractions in trials with kidney extracts from both euthermic and hibernating ground squirrels, but a significant redistribution of PABP-1 occurred in hibernation. In extracts from euthermic animals, PABP-1 was found only in monosome fractions (fractions 8 to 10), whereas in extracts from hibernating individuals PABP-1 was also detected in lower fractions (4, 5, and 7), fractions that also contained substantial amounts of *Cox4* and *Oct2* mRNA (Fig. 16.5). Although the evidence to date is only preliminary, it suggests that a significant amount of the mRNA in the cells of hibernating animals is sequestered into untranslatable stress granules where it is protected from degradation. Furthermore, the presence of ultrastructural changes in the nuclei of hibernating animals and indirect evidence for the binding of PABP to hibernator mRNAs strengthen the argument for the presence of stress granules during torpor.

## GENE AND PROTEIN EXPRESSION DURING HIBERNATION

Multiple protein adaptations support hibernation, although, to date, our knowledge of these is still fragmentary. Historically, hibernation research has focused on only a few selected topics, NST and UCP1 being "front and center," with episodic exploration of other events. However, major advances in gene screening technologies over the past few years (cDNA library screening, cDNA arrays, differential display polymerase chain reaction (PCR), reverse transcription (RT)-PCR) are now supporting a more comprehensive analysis of the changes in gene and protein expression that support hibernation. Several studies have examined gene expression differences between euthermic and hibernating states (or hibernating and aroused states) to seek acute changes that support entry into or arousal from hibernation. One consistent result of these studies is that entry to hibernation occurs with very few acute changes in gene expression. This is perhaps not unexpected because (a) entry into a state of cold torpid is not a time to undertake major projects in expensive protein synthesis, (b) cells and organs cannot undergo major modifications because they must remain fully competent to rapidly resume normal body functions during interbout arousals, and (c) many of the required protein changes are put in place well before hibernation begins as part of a strong circannual rhythm that is further cued by photoperiod and ambient temperature changes. Furthermore, it has also been suggested that the test drops in $T_b$ that occur during sleep in the early autumn are used to trigger various metabolic adaptations that support longer full-torpor bouts in the weeks to come.

Hibernation-responsive genes that are up-regulated in ground squirrels include UCP and FABP isoforms in multiple tissues, ($\alpha_2$-macroglobulin in liver, moesin in intestine, pancreatic lipase in heart, PDK4 in heart, skeletal muscle, and white adipose, the ventricular isoform of myosin light-chain 1 (MLC1$_v$) in heart and skeletal muscle, OCT2 in kidney, the melatonin receptor in brain, heart, and BAT, and four genes on the mitochondrial genome: NADH ubiquinone–oxidoreductase subunit 2 (ND2), cytochrome $c$ oxidase subunit 1 (COX1), and subunits 6 and 8 of the $F_1F_0$–ATP synthase. Although the genes identified to date represent a wide assortment of cellular proteins, some principles of adaptation are beginning to emerge and will provide interesting directions for new studies over the next few years.

A substantial number of hibernation-responsive genes have been identified in heart (Fig. 16.6). For the mitochondrially encoded genes (*Nad2*, *Cox1*, and *Atp6/8*) this compares with no changes in the transcript levels of nuclear-encoded subunits of these multimeric proteins. The substantial changes in gene responses in heart may

be related to the fact that heart must continue to work during torpor and adjustments in gene expression are undoubtedly needed to optimize cardiac function with respect to the changes in $T_b$, work load, and fuel availability that occur in torpor. Indeed, gene expression studies support substantial myosin restructuring in hibernators. Myosin is made up of two heavy chains (MHC) and four light chains (MLC) that are classified as alkali (MLC1, MLC3) or regulatory (MLC2) light chains; the latter are subject to reversible phosphorylation. Not only is $Mlc1_v$ expression up-regulated in ground squirrel heart and skeletal muscle (the ventricular isoform also occurs in skeletal muscle), but studies with hamster heart have shown that the phosphorylation state of MLC2 decreases from 45% in summer to 23% in torpor. In addition, the proportions of heart MHC isoforms changed from a high content of the $\beta$ isoform (79% of total) in summer hamsters and winter-active animals at 22°C to near-equal amounts of the $\alpha$ (53%) and $\beta$ (47%) isoforms in hibernators. Changes in the expression of myosin genes and in the mix of myosin isoforms represented in a muscle are a well-known response for optimizing the myosin motor for function under different conditions. Numerous stimuli (e.g., stretch, electrical stimulation, work load) trigger the response in mammals (see Chapter 11), and myosin restructuring in response to temperature change is well-known in ectotherms. It is not surprising, then, that myosin restructuring occurs in hibernator heart. During hibernation, the heart continues to beat but at a much lower rate and at a much lower $T_b$. However, peripheral resistance increases substantially, and to compensate for this, the force of contraction actually increases. Increased cardiac contractility in hibernation was originally postulated to result from changes in sarcoplasmic reticulum $Ca^{2+}$ storage and release, but an altered mix of myosin isoforms is another central factor in adjusting heart function for the new work load and thermal conditions of the torpid state.

Another metabolic function that is addressed by adaptive changes in gene expression during hibernation is the clotting capacity of blood. Thrombosis in the microvasculature is a serious risk under low-blood-flow conditions and is a major complication of ischemic conditions in humans (heart attack, stroke). Evidence that hibernators make adjustments to lower the risk of spontaneous blood clot formation during torpor came when studies of liver revealed the up-regulation and export of $\alpha_2$-macroglobulin during hibernation. $\alpha_2$-Macroglobulin is a protease inhibitor that binds and inhibits several of the proteases that catalyze steps in the clotting cascade. Other changes during torpor are now known to include reduced levels of several clotting factors as well as reduced platelet numbers (platelets are sequestered into the spleen until arousal), all of which would contribute to minimizing spontaneous clot formation during torpor.

Expression of UCP1 is well known to be induced by cold exposure in both hibernators and nonhibernating rodents. UCP1 levels in BAT are high before animals enter hibernation and remain constant as long as animals are hibernating at ambient temperatures above 0°C. However, when ambient temperature dips below 0°C, the torpid animal increases its metabolic rate and activates NST in order to maintain a constant core body temperature; for example, Arctic ground squirrels could defend a core $T_b$ of 0°C even when ambient temperature fell to $-16$°C although, to do so, metabolic rate increased about 10-fold. Under this high thermogenic demands, UCP1 transcript levels in BAT rapidly increased. This clearly shows that transcription of at least some genes can go forward at low $T_b$ values and correlates with the protein synthesis capacity of BAT, which appeared to be unaltered in hibernating animals, in contrast to the strong suppression of this function in brain and kidney. The same study also showed that UCP2 and UCP3 transcript levels rose in ground squirrel white adipose and skeletal muscle, respectively, and this suggested that tissues other than BAT could potentially contribute to NST during hibernation.

Supporting the change to a lipid-based economy during hibernation, the capacity for intracellular transport of fatty acids is elevated in most (probably all) tissues during hibernation by increasing the levels of FABPs. Our studies have found that expression of the heart and adipose isoforms of FABP increased in multiple tissues of both ground squirrels and bats during hibernation and up-regulation of other isoforms (at least seven isoforms are known) likely follows suit. Transcripts of A-FABP rose 2-fold in BAT and over 6-fold in heart (Fig. 16.6) of hibernating ground squirrels and were 4-fold higher in BAT of hibernating versus euthermic bats (Fig. 16.7). A-FABP protein levels similarly increased (Fig. 16.7). Transcripts of the heart isoform (that is also expressed in skeletal muscle) were 1.5 to 3-fold higher in BAT, heart, and muscle of hibernating versus euthermic ground squirrels (Fig. 16.6) and nearly 2-fold higher in BAT and skeletal muscle of bats. H-FABP protein also increased strongly, especially in skeletal muscle and BAT (Figs. 16.5 and 16.7).

New studies using cDNA array screening are offering up many more candidate genes that are potentially important to hibernation success. Arrays made with human or rat cDNAs have been used very successfully to analyze gene expression changes in hibernating ground squirrels and bats. Although interspecific sequence differences means that not all hibernator cDNAs will cross-hybridize with the arrays, our studies using human 19K microarrays achieved 85 to 90% hybridization after optimization of hybridization and washing conditions, which means that the responses to hibernation by over 16,000 genes were
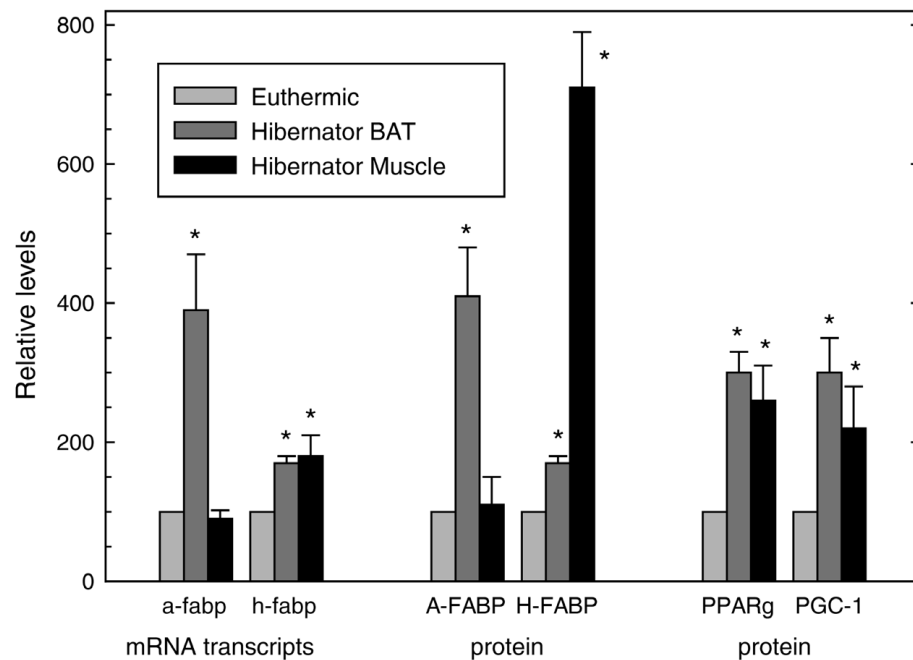
**Figure 16.7**    Effect of hibernation (36-h reentry into torpor) on transcript and protein levels of the adipose (A) and heart (H) isoforms of fatty acid binding protein and protein levels of the transcription factor, PPARγ and its coactivator, PGC-1, in brown adipose tissue and skeletal muscle of bats, *M. lucifugus*. Transcript levels were measured by Northern blotting; proteins by Western blots. [Data are means $\pm$ SEM, $n = 3$; (*) values are significantly higher in hibernator, compared with euthermic, tissues, $P < 0.05$. [Data on PPARγ and PGC-1 excerpted from S. F. Eddy and K. B. Storey (2003). *Biochem Cell Biol* **81**:269–274.]

assessed. Array screening has two key assets: (1) the opportunity to identify genes (and thereby implicate pathways or functions) that are key to hibernation but have never before been considered as participating in the phenomenon and (2) the opportunity to evaluate the overall responses to hibernation by functionally related groups of enzymes or proteins. For example, in our screening of skeletal muscle extracts from thirteen-lined ground squirrels, *S. tridecemlineatus*, we found that several genes encoding components of the small and large ribosomal subunits were coordinately down-regulated during hibernation, including L19, L21, L36a, S17, S12, and S29. Other insights from array screening are providing avenues for continuing research. For example, analysis of liver and kidney samples from both ground squirrels and bats consistently indicate up-regulation of genes involved in antioxidant defense during hibernation. Glutathione-S-transferase, glutathione peroxidase, and superoxide dismutase mRNA levels were all elevated in hibernator kidney, and these same enzymes as well as peroxiredoxin and metallothionein were up-regulated in liver. These data support our previous discussion of the importance of antioxidant defenses for hibernation success. Array screening of euthermic versus hibernator kidney also showed pronounced increases in transcript

levels of selected transport proteins in the hibernator: a twofold increase in aquaporin 3, a fivefold increase in sodium–proton exchanger isoform 2, and a sevenfold rise in the organic cation exchanger isoform 2 (*Oct2*) (discussed earlier).

Array screening revealed very few proteins that were actually down-regulated during hibernation. This correlates well with the results of studies on translational control that indicates that the majority of mRNA transcripts are not degraded during hibernation but are sequestered into translationally inactive monosomes and stress granules. One particularly interesting instance of hibernation-responsive down-regulation is the suppression of insulin-like growth factor (IGF) and its plasma-binding protein (IGFBP-3). The growth-regulatory IGF axis controls somatic growth in skeletomuscular and other tissues, and its suppression during hibernation indicates that energy-expensive growth is curtailed in the hypometabolic state.

### Signal Transduction and Transcriptional Control in Hibernation

Relatively little is known about the signaling mechanisms and transcriptional controls that are involved in mediating

metabolic and gene expression changes between euthermic and hibernating states. The effects of norepinephrine in the acute activation of BAT thermogenesis during arousal from hibernation have been discussed previously, but other signaling pathways operating to mediate metabolic rate depression and other aspects of cellular responses during hibernation are less well known. Multiple signaling pathways are undoubtedly involved and include well-known mammalian signaling networks that function in starvation to reduce energy expenditure on biosynthesis and shift metabolism to a high dependency on lipid catabolism for ATP generation. Metabolic responses during human starvation are outlined in Chapter 9, and key hormonal signals include a strong decrease in insulin and an increase in glucagon. During hibernation, liver metabolism switches from being an insulin-mediated consumer of glucose (for biosynthesis of glycogen, fatty acids, and many other molecules) to a glucagon-mediated supplier of glucose to other organs (via glycogenolysis or gluconeogenesis) as well as a producer of ketone bodies. Glucagon effects on vertebrate liver include phosphorylation-mediated inhibition of glycolytic enzymes (PFK-1, PK) as well as PDC, and it is likely that glycolytic suppression during hibernation is also mediated in this manner via cAMP as the second messenger and PKA as the kinase. Skeletal muscle $Na^+K^+$–ATPase activity was also strongly suppressed by PKA treatment, but the enzyme was also inhibited by treatments that stimulated protein kinases C and G activities. Clearly, the activity of this major energy-consuming reaction in cells is responsive to multiple inputs, and it remains to be determined which one mediates the coordinated suppression of this enzyme and other ion-motive ATPases during entry into the hypometabolic state.

Consistent with the starved state, serum insulin levels are very low during hibernation; serum insulin in midwinter hibernating ground squirrels was less than 25% of the values in the prehibernating period. However, insulin transcript levels in the pancreas were just the opposite (highest in hibernation, lowest in prehibernation). Low circulating insulin levels would mean inhibition of intracellular insulin-signaling pathways and that is just what is seen. A variety of insulin effects, especially on carbohydrate metabolism, are mediated by Akt (also called protein kinase B). For example, activation of Akt is linked with increased glucose uptake into muscle cells probably via stimulation of the glucose transporter (GLUT4) and with adipogenesis and lipogenesis in white adipose. Akt also promotes glycogen synthesis in liver by phosphorylating and inhibiting glycogen synthase kinase 3, which prevents the enzyme from inactivating glycogen synthase. During hibernation we found that phospho-Akt (the active form) content in *M. lucifugus* organs was reduced to 30 to 40% of the euthermic value in liver, kidney, brain, and white adipose and was unchanged in heart and skeletal muscle.

Furthermore, the total amount of Akt in white adipose also fell to 30% of its euthermic value. This indicates strong suppression of lipogenic pathways during hibernation, as is consistent with the opposite requirement for mobilizing lipid reserves to fuel metabolism during hibernation.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that play a major role in the regulation of cellular lipid metabolism (see Chapter 9). PPARγ has a primary role in the differentiation and metabolism of adipocytes, both white and brown. All genes involved in lipid catabolism are thought to contain a PPAR response element (PPRE) to which PPAR isoforms bind as a heterodimer complex with activated retinoic acid receptors; this is potentiated by a number of activating factors, including PPAR-gamma coactivator-1 (PGC-1) (Fig. 16.8). Notably, PGC-1 transcription is inhibited by insulin, and this is the mechanism by which the expression of lipolytic enzymes is suppressed when insulin is high. PGC-1 also stimulates the expression of gluconeogenic enzymes, which provides the link between increased expression of both lipolytic and gluconeogenic enzymes during fasting. During fasting, levels of insulin drop and glucagon and glucocorticoid levels increase. Glucagon stimulates an increase in cAMP levels and PKA activity. One of the targets that is phosphorylated by PKA is the CREB protein, a transcription factor. Levels of phosphorylated CREB (the active form) were elevated during hibernation by 2-fold in skeletal muscle of both bats and ground squirrels and by 3.4-fold in heart of squirrels (other organs were not tested). Phospho-CREB binds to the cAMP response element (CRE) that is upstream of the PGC-1 gene (among others) and induces its expression.

Figure 16.7 shows the strong elevation of PGC-1 content in BAT and skeletal muscles of hibernating bats (*M. lucifugus*) that correlates with comparable increases in PPARγ in these organs. Furthermore, coordinated increases in PGC-1 and PPARγ of 1.5 to 2.5-fold were also documented in heart, kidney, liver, and white adipose tissue of bats during hibernation, whereas both were suppressed to about 50% of aroused values in bat brain.

To date, about 50 genes are known to be up-regulated under PPARγ control in mammalian white and brown adipocytes, including large numbers of enzymes involved in both lipogenesis and fatty acid transport and oxidation and genes involved in glucose use for lipogenesis. Of particular relevance to hibernation is PPARγ-mediated up-regulation of the expression of A- and H-FABP, lipoprotein lipase, UCP1, PDK4, carnitine palmitoyl transferase, FATP-1 (the cell surface fatty acid transporter), and several of the enzymes of β oxidation, among others. The gene for leptin is down-regulated by PPARγ. Figure 16.7 shows that the strong up-regulation of PPARγ and
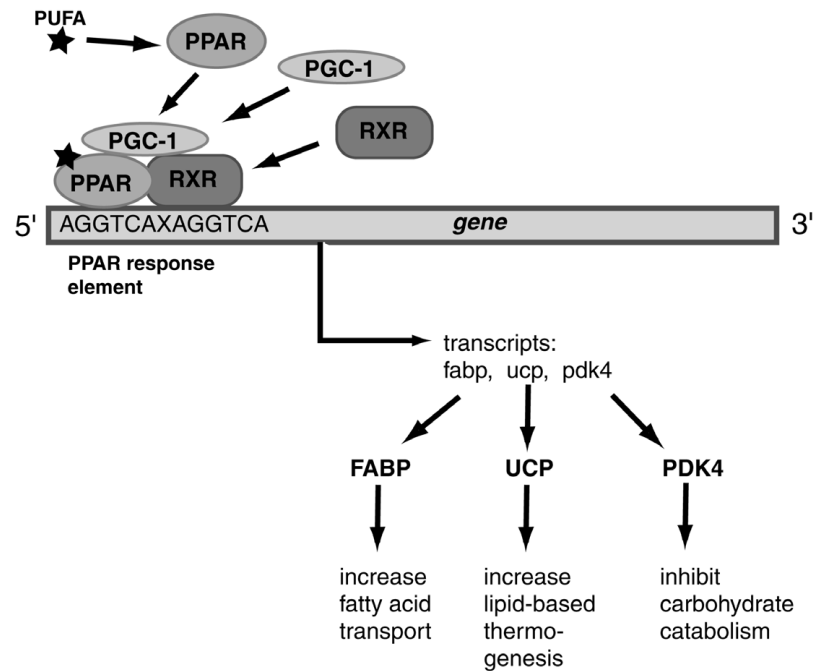
**Figure 16.8** When activated by the binding of a ligand such as a polyunsaturated fatty acid (PUFA), peroxisome-proliferator-activated receptors (PPAR) form heterodimers with retinoid X receptors (RXR) and bind to the PPAR response element (AGGTCAXAGGTCA; where X is a variable base) of selected genes. Binding is enhanced/stabilized by the PPAR coactivator, PGC-1. Binding activates transcription of selected genes. In hibernators, these probably include genes for fatty acid binding protein (FABP), mitochondrial uncoupling protein (UCP), and pyruvate dehydrogenase kinase 4 (PDK4).

PGC-1 levels in BAT and muscle of hibernating bats (*M. lucifugus*) correlates with the increase in H- and/or A-FABP mRNA transcripts and protein levels in those organs (skeletal muscle only has trace amounts of A-FABP that do not change in torpor). Thus, we have strong evidence that FABP and undoubtedly other proteins of lipid oxidation (e.g., UCP1, PDK4) are up-regulated in a coordinated fashion under the control of the PPAR transcription factor during hibernation (Fig. 16.8). Furthermore, it is interesting to note that the $\gamma$ isoform of PPAR is typically described as being abundant in adipose tissue and low in other tissues of nonhibernating mammals, although treatment with PPAR$\gamma$ agonists does up-regulate genes not only in brown and white adipose but also in other insulin-sensitive organs (liver, skeletal muscle). However, in bats the transcription factor was found in all seven tissues tested, the four already mentioned as well as kidney, heart, and brain. This may attest to an enhanced importance of PPAR$\gamma$ and PGC-1 in the regulation of fatty acid catabolism in hibernating species and may represent an adaptive modification of a signal transduction pathway to play a specific role in hibernation.

Mitogen-activated protein kinases also participate in hibernation. MAPKs have widespread roles in regulating gene expression, the p38 MAPKs being particularly involved in cellular responses to stresses. Organ-specific responses by all three MAPK modules [extracellular signal regulated kinases (ERKs), stress-activated kinases (SAPKs), p38 MAPKs] (see Chapter 5 for a review of these modules) were seen in hibernating ground squirrels. ERK1 and ERK2 activities were strongly increased in brain during hibernation, whereas SAPK activity was unchanged in brain but rose in all other organs tested. Western blotting with antibodies specific for the phosphorylated, active p38 showed that levels were twofold higher in heart and over sixfold higher in skeletal muscle of hibernating versus euthermic ground squirrels. Studies focused on brain during arousal from hibernation documented approximately sixfold increases in SAPK and ERK (but no change in p38) during the time that core $T_b$ rose from 7 to 35°C as well as three- and fivefold increases in Akt and PKC (recall both were suppressed in hibernator brain) and elevated levels of glucose-regulated proteins (GRP) and tumor necrosis factor alpha. Hence, multiple signaling pathways are involved in "reactivating" brain function during warming, and the induction of GRP proteins suggests a need to provide protection to multiple cellular proteins under the conditions of rapidly rising $T_b$ and

oxygen consumption (undoubtedly generating high levels of reactive oxygen species) over the arousal period.

## CONCLUSIONS

In the present chapter we have concentrated mainly on the molecular and biochemical aspects of hibernation, primarily related to fuel metabolism and the control of metabolic rate. The interested reader can explore many other aspects of hibernation; a huge literature is available on the ecological aspects of hibernation, the physiology of tissue and organ functions during torpor including extensive information on neurophysiology, neuroendocrine controls, the relationships between torpor and sleep, circannual rhythms in hibernators, and the regulation of development and proliferation of brown adipose tissue. Our understanding of the biochemical mechanisms that underlie metabolic rate depression in general and hibernation in particular is undergoing very rapid change at the moment due to amazing recent advances in biochemistry and gene expression. For example, gene screening studies (particularly with cDNA arrays) have identified more gene/protein targets of potential interest to hibernation researchers in the last couple of years than have probably been known in total over the past 20 to 30 years of hibernation research. The development of peptide antibodies and of phospho-specific antibodies is vastly improving our capacity to analyze changes in protein content and to trace signal transduction pathways from cell surface to gene or protein target. Overall, this is a hugely exciting time to be involved in hibernation research, and scientists are poised to elucidate many of the remaining mysteries of the phenomenon.

## SUGGESTED READING

Boyer, B. B., and Barnes, B. M. (1999). Molecular and metabolic aspects of mammalian hibernation. *BioScience* **49**:713–724.

*Easy to read outline of physiological and biochemical changes that support hibernation.*

Heldmaier, G., and Klingenspor, M. (2000). *Life in the Cold.* Springer, Berlin. *Proceedings of the 11th International Hibernation Symposium highlighting multiple advances in the ecology, physiology, and biochemistry of torpor and hibernation.*

Storey, K. B. (ed.) (2001). *Molecular Mechanisms of Metabolic Arrest.* BIOS Scientific, Oxford, UK. *Multiauthor book with 12 chapters assessing metabolic arrest in many forms including hibernation, estivation, anoxia tolerance, diapause, desiccation tolerance with discussion of multiple biochemical mechanisms supporting metabolic arrest.*

Storey, K. B. (2003). Mammalian hibernation: transcriptional and translational controls. In Roach, R. C., Wagner, P. D., and Hackett, P. H. (eds.). *Hypoxia: Through the Lifecycle.* Kluwer/Plenum Academic, New York. *Review of the controls on protein synthesis as they apply to hibernation and exploration of the hypoxia-hibernation connection.*

Storey, K. B., and Storey, J. M. (eds.) (2003). *Cell and Molecular Responses to Stress. Vol. 3: Sensing, Signaling and Cell Adaptation.* Elsevier, Amsterdam. *Includes reviews on uncoupling protein functions from thermogenesis to regulation of reactive oxygen species (Ch. 18), regulation of brown adipocyte proliferation (Ch. 19), hibernators as models for hypoxia-ischemia tolerance in mammalian brain (Ch. 1), and use of cDNA array screening for gene discovery in hibernators (Ch. 22).*

Storey, K. B., and Storey, J. M. (2004). Metabolic rate depression in animals: Transcriptional and translational controls. *Biol Rev Cambridge Philosophical Society* **79**:207–233. *Review of the principles of metabolic rate depression showing the conservation of mechanisms across phylogeny and between different stresses.*

Wang, L. C. H. (1989). Ecological, physiological and biochemical aspects of torpor in mammals and birds. In Wang, L. C. H. (ed.), *Advances in Comparative and Environmental Physiology. Vol. 4: Animal Adaptation to Cold*, Springer, Heidelberg, pp. 361–401. *Still an excellent resource of background information on many aspects of hibernation in a volume that includes many other articles about hibernation and mammalian responses to cold.*