15

OXYGEN LIMITATION AND METABOLIC RATE DEPRESSION

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INTRODUCTION

Oxygen is crucial to the lives of most multicellular organisms on Earth. As humans, we are critically aware that an interruption of oxygen supply to our most oxygen-sensitive organ, the brain, for more than about 5 min can cause irreparable damage or death. Many other organisms also live highly oxygen-dependent lives. For example, the power requirements of high-speed hovering flight by hummingbirds and many insects are so great that without oxygen flight is impossible. Indeed, the flight muscles of bees and flies, which power flight with carbohydrate oxidation, have become so oxygen-dependent that lactate dehydrogenase has been deleted so that there is actually no way for these muscles to generate useful amounts of adenosine 5′-triphosphate (ATP) from anaerobic glycolysis. However, despite our anthrocentric view that oxygen is crucial to life, many other organisms actually survive equally well in the presence or absence of oxygen. These facultative anaerobes often live in environments where oxygen can become depleted either intermittently or at regular intervals. Many others are obligate anaerobes that never use oxygen; most of these are microbes but a variety of parasites (trematodes, nematodes, cestodes) that live in the gastrointestinal tract of other animals also lead anaerobic lives. In this chapter we will explore some principles of metabolic regulation as they apply to oxygen limitation. First, we will assess the reasons why inadequate oxygen supply causes metabolic injury to oxygen-sensitive species (such as ourselves) and examine the mechanisms that are used in attempts to regain adequate tissue oxygenation or to enhance glycolytic ATP production. Second, we will examine the biochemical mechanisms that allow facultative anaerobes to switch between oxygen-dependent and oxygen-independent life, with a particular emphasis on metabolic rate depression as the key survival strategy for enduring interruptions of oxygen supply.

The discussion in this chapter will be largely limited to what is termed *environmental* hypoxia (low oxygen) or anoxia (no oxygen), meaning limitation of oxygen availability to the organism. This can arise from a variety of circumstances, such as a move to a much higher altitude, breath-hold diving by lung-breathing animals, aerial exposure of gill-breathing animals, and oxygen depletion in ice-covered ponds or in polluted waters. Environmental anoxia is frequently differentiated from *functional* anoxia, which refers to situations where high-intensity work by muscles (e.g., sprint running) outstrips the capacity of muscle oxidative metabolism to produce ATP (see Text Box 15.1 and Chapter 11).

In the previous chapter environmental stresses were said to affect metabolism in two ways, by direct perturbation of the structure/function of biological molecules and biochemical reactions or by jeopardizing the energy currencies of the cell. Two main actions of oxygen were also listed—a "good" role in cellular energy metabolism and a "bad" role in the damage done by reactive oxygen species to macromolecules. The bad role of oxygen can be categorized as a direct perturbation by a stressor on biological molecules, and Chapters 12 and 13 discuss the damage to cellular macromolecules by reactive oxygen species (ROS) as well as the antioxidant defenses that mitigate this damage. Note, however, that the bad role of ROS is being seriously reevaluated at the present time, and

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TEXT BOX 15.1 FUNCTIONAL HYPOXIA OR ANOXIA: HIGH-INTENSITY MUSCLE WORK

Low-level exercise or even sustained medium- to high-intensity exercise (e.g., marathon running, hovering flight, cruiser swimming) can almost always be supported by ATP generation from mitochondrial oxidative phosphorylation using lipids or carbohydrates as the fuel (depending on the species). However, situations of high-intensity "burst" exercise (e.g., sprint running, the sudden leap of a frog) demand an instantaneous power output by contracting muscle fibers that outstrips the capacity of aerobic metabolism to supply ATP. Such high power demands are dealt with in two oxygen-independent ways: (1) a rapid mobilization of muscle phosphagen reserves provides an instant supply of ATP, and (2) glycolysis is rapidly activated to generate ATP from the catabolism of endogenous glycogen with the accumulation of a glycolytic end product (Fig. 15.1) (also see Chapter 11 on muscle metabolism). In vertebrate animals, the phosphagen is creatine phosphate (supplied by the creatine kinase reaction) and the glycolytic end product is lactate, produced by the reduction of pyruvate by lactate dehydrogenase. Both mechanisms supply high rates of ATP output for very short times (generally just a few seconds) but with a significant cost to oxygen-based metabolism that must replenish creatine phosphate and glycogen reserves during the recovery period after burst exercise (this is called an oxygen debt).

Some invertebrate animals use the same phosphagen and anaerobic end product to support burst muscle work, but a number of alternatives also exist. For example, in addition to creatine phosphate, there are seven other phosphagens (and corresponding phosphagen kinases) among invertebrates. The phosphagen kinase reactions show differences in thermodynamic poise, and the phosphagens differ in some physical properties including intrinsic diffusivity. Overall, the big "winners" in evolutionary terms are creatine phosphate and arginine phosphate, the latter being widespread in the "higher" invertebrate groups (insects, crustaceans, and other arthropods as well as mollusks). Alternatives to lactate also occur widely among invertebrates (outside of the Arthropoda); multiple other pyruvate oxidoreductases are used that couple the reductive condensation of pyruvate with an amino acid to form an end product called an imino acid or an "opine" (the alpha-amino nitrogen is linked with the C2 keto group of pyruvate) (Fig. 15.1). The most notable alternative system occurs in working muscles of mollusks. Here, arginine phosphate is the phosphagen and the glycolytic end product is octopine (named because it was first found in *Octopus*). The enzymes involved are arginine kinase (ArgK) and octopine dehydrogenase (ODH):

$$\text{Phosphoarginine} + \text{ADP} \rightarrow \text{arginine} + \text{ATP} \quad \text{(ArgK)}$$

$$\text{Pyruvate} + \text{arginine} + \text{NADH} + \text{H}^+ \rightarrow \text{octopine} + \text{NAD}^+ + \text{H}_2\text{O} \quad \text{(ODH)}$$

So, in a high-speed chase between a sperm whale and its prey, a giant squid, the whale will deplete creatine phosphate and accumulate lactate in its working muscle, whereas the jet propulsion swimming of the squid catabolizes arginine phosphate and accumulates octopine. Table TB15.1 shows an example of the effects of exhaustive exercise and hypoxia exposure on metabolite levels in the mantle muscle of a cephalopod mollusk. Both stresses deplete muscle glycogen, but exercise causes a greater mobilization of phosphagen pools (recall from Chapter 11 that phosphagen is mobilized first during burst muscle work) and ATP. Octopine accumulates as the glycolytic end product, and the total arginine pool (phosphagen + free arginine + octopine) is maintained constant.

The production of octopine actually appears to have some advantages over lactate as an end product including: (a) by consuming arginine, the product of phosphagen hydrolysis, octopine synthesis can help to "pull" the

TABLE TB15.1 Effects of Exhaustive Swimming Exercise or Severe Hypoxia on Muscle Metabolites in a Cephalopod Mollusk, the Cuttlefish *Sepia officinalis* [a]

	Control	Exercised	Hypoxic
Glycogen	23.7	4.5	2.2
Pyruvate	0.1	0.8	0.2
ATP	8.7	2.2	4.5
Arginine phosphate	33.6	3.8	18.8
Arginine	29.6	45.3	37.5
Octopine	0.2	8.6	3.6
Sum Arg-P + Arg + octopine	63.4	57.7	59.9

All values in μmol/g wet mass. [a]Both glycogen and phosphagen are substrates for exercise and hypoxic metabolism in cuttlefish, but, although glycogen depletion is similar in both exercise and hypoxia, exercise causes a greater depletion of ATP and arginine phosphate and a greater accumulation of octopine. Note that the sum of arginine phosphate, arginine, and octopine remains constant. For exercise animals were stimulated to jet repeatedly until exhausted. For hypoxia animals were given 30 min exposure to seawater that had been bubbled with nitrogen gas to lower P_{O_2} to 10 mm Hg from a normal value of 130 mm Hg.

Source: Data compiled from K. B. Storey and J. M. Storey (1979). *J Comp Physiol* **131**:311–319.

ArgK reaction toward ATP synthesis, (b) octopine accumulation causes less cellular acidification than does lactate, and (c) by combining the products of phosphagen hydrolysis and glycolysis into a single end product, cellular osmolality is not perturbed. This latter is quite important for the cells of marine invertebrates because these animals maintain isoosmotic balance with seawater; hence, a net accumulation of osmotically active particles (such as if lactate accumulated) would result in cell swelling due to water influx.

A number of other opines occur as glycolytic end products in different species including alanopine, strombine, lysopine, nopaline, and tauropine formed from the reductive condensation of pyruvate with alanine, glycine, lysine, proline, and taurine, respectively. These are almost exclusively found in marine organisms (although a few freshwater animals such as clams have low activities of one or more of these enzymes together with lactate dehydrogenase). However, a more universal replacement of lactate as a glycolytic end product has not occurred. This seems to be because marine invertebrates have large pools of the necessary amino acid substrates within their cells; these amino acids and other nitrogenous compounds provide about half of the osmotic balance needed to defend cell volume against the high ion concentrations in seawater. Freshwater and terrestrial animals lack these amino acid pools that would also provide the cosubstrates for opine synthesis.

whereas ROS clearly cause much damage to cellular macromolecules, many new studies are now showing that they also have key roles in intracellular signaling. In its good role, oxygen primarily affects energy currencies of the cell. When oxygen availability drops below a critical value, cellular energy supply can be compromised because the rate at which ATP is generated from oxidative phosphorylation in the mitochondria falls below the rate at which the many ATP-utilizing cellular reactions are consuming ATP. If this imbalance continues unabated, ATP availability for metabolism soon becomes limiting, metabolic damage accrues, and viability can be compromised.

There are two ways that metabolic regulation can be applied to deal with the effects of low oxygen availability on cellular energetics. The first is to increase the production of ATP by oxygen-independent mechanisms, primarily utilizing the glycolytic pathway, in order to meet the demands of ATP-consuming cellular reactions. The second is to reduce the cell's need for ATP production by suppressing the rate of ATP use by ATP-consuming cellular reactions. Virtually all organisms have some capacity, however short term, for increasing ATP output by oxygen-independent means (a capacity that is highly elevated in white muscles to deal with functional anoxia; see Text Box 15.1), but only facultative anaerobes have developed the aggressive metabolic controls necessary to strongly suppress metabolic rate to a level that can be sustained indefinitely by anaerobic ATP generation.

OXYGEN LIMITATION—THE PROBLEM

All cells strive to maintain homeostasis, a balance between the rate of ATP production by central catabolic pathways and the rate of ATP utilization by innumerable cellular processes. Oxygen-dependent ATP production by oxidative phosphorylation in the mitochondria makes the most efficient use of metabolic fuels, extracting their full energetic potential while fully "burning" fuels to CO_2 and H_2O. Not surprisingly, then, most organisms on Earth have taken up an aerobic lifestyle, but, in doing so, they can place themselves in jeopardy if oxygen availability is compromised. If oxygen is cut off, cells have two immediate energy supply problems: (1) lipid fuels become useless, as do most amino acids, because their catabolic pathways include no oxygen-independent substrate-level phosphorylation reactions by which ATP can be made and (2) phosphagen depletion plus greatly increased glycolytic flux rarely, if ever, meets the unmodified ATP demands of cellular metabolism for more than a few seconds or minutes.

The problem is particularly acute for organs such as brain that consume tremendous amounts of energy; for example, the human brain represents about 2% of our body weight but consumes about 25% of our energy budget. An interruption of blood supply (ischemia) to mouse brain causes an almost instantaneous increase in glycolytic rate of 4- to 7-fold, but this only partially compensates for the 18-fold decrease in ATP yield per glucose catabolized (a net of 2 ATP is produced per 1 glucose catabolized to 2 lactate versus 36 ATP if glucose is instead catabolized to CO_2 and H_2O). Brain is further compromised in its attempt at compensation because, with blood flow cut off, the brain is also deprived of its primary fuel source, blood glucose, and has only small reserves of endogenous carbohydrate (glycogen) to fuel glycolysis. Hence, within seconds, cellular ATP levels begin to fall, and within 5 min of oxygen deprivation as much as 90% of the ATP is depleted in mammalian brain. A high proportion of brain energy budget is committed to supporting ionmotive ATPases in their role in maintaining membrane potential difference; for example, the sodium–potassium ATPase alone is responsible for 5 to 40% of cellular ATP turnover, depending on cell type. When only 50 to 65% of brain ATP is lost, membrane depolarization occurs, and this sets off multiple negative consequences. Depolarization results in a rapid uptake of Na^+ (when ATP is limiting, Na^+ influx through ion channels is unopposed by

oppositely directed pumps) and water that is followed by an influx of Ca^{2+} through voltage-gated Ca^{2+} channels. The collapse of the sodium gradient causes the sodium–glutamate cotransporters to eject glutamate into the extracellular space where this neurotransmitter triggers a range of events, including activation of excitatory neurotransmission by stimulating *N*-methyl-D-aspartate (NMDA) receptors that are responsible for a significant part of the Ca^{2+} influx. Transient elevation of cytosolic Ca^{2+} is a critical signaling mechanism in the implementation of many normal cell functions, but sustained high Ca^{2+} triggers a range of pathological changes, including the activation of phospholipases and proteases that can lead to damage and death of neurons and all other cells (see also Chapter 8).

If the restoration of oxygen-rich blood flow occurs soon enough, these injuries caused by low cellular bioenergetics can be reversible, but the restoration of blood flow to previously ischemic tissues also stimulates a whole new set of injuries. Reperfusion injury, such as occurs after heart attack or stroke, was discussed in detail in Chapter 13 and is caused by a burst of ROS generation, chiefly superoxide radicals, from the highly reduced electron transport chain when oxygen is suddenly reintroduced. ROS-mediated damage arising from the bad role of oxygen can result in direct damage by free radicals to macromolecules, including deoxyribonucleic acid (DNA), proteins, and membrane lipids. Indirect damage can also arise such as from an inability of sarco- or endoplasmic reticulum membranes that are damaged by peroxidation to properly resequester Ca^{2+}, thereby exacerbating the Ca^{2+}-mediated damage caused by low energetics in the anoxic/ischemic phase. Hence, anoxia-tolerant animals need to have adaptations that deal with both the stresses on cellular energetics under anoxia and the potential for ROS-mediated damage during the return to aerobic life.

OXYGEN LIMITATION—RESPONSES IN OXYGEN-SENSITIVE SYSTEMS

Many organisms, including humans, are highly dependent upon oxygen and have little or no ability to survive if deprived of oxygen. If oxygen availability to tissues drops below a critical level, then a suite of compensatory responses, both physiological and biochemical, are activated with two goals: (1) to improve oxygen delivery to tissues and (2) to increase glycolytic ATP production to compensate for the reduced ATP output from oxidative phosphorylation. Both goals are addressed with immediate (seconds to minutes) and longer term adjustments (hours to days). Consider, for example, the case of an Acapulco resident taking a trip to Mexico City. Upon stepping off the airplane at an elevation of 2300 m, the traveler is deposited in an atmosphere that contains 30% less oxygen than at his sea-level home and immediate compensation for the reduced oxygen availability begins. Physiological responses include an immediate increase in ventilation rate and heart rate, an increase in hemoglobin unloading of oxygen, and a release of stored red blood cells from the spleen. These events attempt to increase oxygen uptake into the blood, oxygen-carrying capacity, and oxygen delivery to organs. The immediate biochemical event is an activation of oxygen-independent routes of ATP synthesis via an increase in glycolytic rate in all tissues as well as the consumption of creatine phosphate reserves in those tissues (e.g., muscles) that have significant reserves of phosphagen.

Control of Glycolysis

The activation of glycolysis in response to low oxygen stress has been extensively researched; in particular, this was initially driven by the desire to understand the molecular basis of the Pasteur effect (see Text Box 15.2). Initial work during the 1960s focused on the allosteric regulation

TEXT BOX 15.2 THE PASTEUR EFFECT

In 1861 Louis Pasteur published the results of experiments on yeast that showed that under anoxic conditions yeast consumed more sugar per unit mass than under aerobic conditions. His conclusion was that "oxygen inhibits fermentation," but in recent years the Pasteur effect is usually discussed as the corollary of the original "anoxia increases fermentation." In other words, in the presence of oxygen, yeast use mitochondrial oxidative phosphorylation to gain the maximum ATP yield from substrate catabolism; but, when oxygen is lacking, the rate of fermentation rises dramatically and ethanol is produced as the terminal product of glycolysis. The primary constraint on the Pasteur effect is substrate availability. Per mole of glucose consumed, aerobic metabolism produces 18 times more ATP than does fermentation. Hence, to sustain unaltered cellular ATP demands in anoxia, the rate of glucose utilization by glycolysis would have to increase by 18-fold. The virtually unlimited substrate availability that this requires is possible in some circumstances, for example, for yeast growing in a sugary mash of malted barley and hops! Excretion of end products (in this case, ethanol) also prevents a toxic buildup of intracellular waste (and yields a nice beer). However, for most multicellular organisms with fixed reserves of carbohydrate fuel in their tissues, the Pasteur effect is only a short-term solution to anoxic insult and fails as a long-term solution.

of enzymes and particularly on the control of 6-phosphofructo-1-kinase (PFK-1) as being central and rate-limiting to overall glycolytic rate [reaction (15.1)] (Fig. 15.1):

$$\text{Fructose-6-P} + \text{ATP} \rightarrow \text{fructose-1,6-P}_2 + \text{ADP} \quad (15.1)$$

The activity of PFK-1 is highly sensitive to substrate inhibition by high levels of ATP and to allosteric activation by

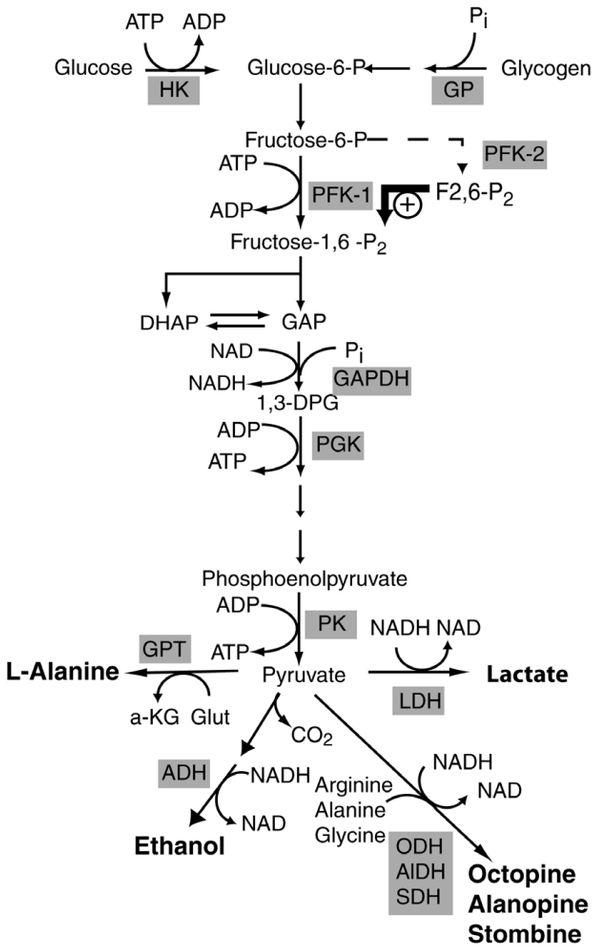

Figure 15.1 Anaerobic glycolysis showing inputs from glucose or glycogen and a range of possible end products derived from pyruvate. Also shown is the production of fructose-2,6-bisphosphate ($F2,6P_2$) and its activating effect on PFK-1. Enzymes are: HK, hexokinase; GP, glycogen phosphorylase; PFK-1, 6-phosphofructo-1-kinase; PFK-2, 6-phosphofructo-2-kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PK, pyruvate kinase; LDH, lactate dehydrogenase; GPT, glutamate-pyruvate transaminase; ADH, alcohol dehydrogenase; ODH, AlDH, and SDH, octopine, alanopine, and strombine dehydrogenases, respectively. Abbreviated metabolites are: GAP, glyceraldehyde-3-phosphate; 1,3-DPG, 1,3-diphosphoglycerate; α-KG, α-ketoglutarate; Glut, glutamate.

adenosine 5′-monophosphate (AMP). When ATP demand outstrips ATP availability in cells, ATP levels fall, and because of the near equilibrium of the adenylate kinase reaction ($2\ \text{ADP} \rightarrow \text{ATP} + \text{AMP}$) and the relative levels of ATP, adenosine 5′-diphosphate (ADP), and AMP in cells, a small percentage decrease in ATP concentration can cause an immediate severalfold rise in AMP levels. For example, recall from Chapter 1 that a 10% decrease in [ATP] in insect flight muscle during takeoff caused an immediate 2.5-fold increase in [AMP]. Allosteric control of PFK-1 was long regarded as the basis of glycolytic activation in the Pasteur effect. Control comes from strong allosteric activation of PFK-1 by AMP as well as ADP, inorganic phosphate (which rises when ATP and creatine phosphate hydrolysis is high), and NH_4^+ (produced from AMP deamination to inosine monophosphate (IMP), particularly in working muscles), all of which rise under hypoxic/anoxic conditions, as well as relief from inhibition by ATP and citrate, both of which decline in hypoxia/anoxia. When glucose is the substrate, increased glucose uptake (by GLUT transporters) and increased glucose phosphorylation by hexokinase are also regulatory factors in the Pasteur effect. Hexokinase control comes from activation by inorganic phosphate, inhibition by the hexokinase product, glucose-6-phosphate, and reversible binding of hexokinase to the mitochondrial membrane. In the 1980s the central role of PFK-1 in glycolytic rate control was further cemented with the discovery of fructose-2,6-bisphosphate ($F2,6P_2$), another powerful activator of PFK-1 (see Chapter 14 and Fig. 14.7). $F2,6P_2$, synthesized by 6-phosphofructo-2-kinase (PFK-2), rises during anoxia in yeast and by activating PFK-1 facilitates increased use of carbohydrate as a fermentative substrate. In vertebrates, $F2,6P_2$ involvement in activating glycolysis in hypoxia/anoxia is very much organ-specific; it occurs in heart but not in skeletal muscle or liver. In liver, $F2,6P_2$ actually falls under hypoxia/anoxia and contributes to the PFK-1 inhibition that is necessary to divert glycogenolysis into glucose export in order to feed the substrate needs of other organs.

In the late 1980s yet another factor in glycolytic rate control was discovered, the AMP-activated protein kinase (AMPK) (see Chapter 4). Ultimately, this enzyme now appears to be a major regulator of the low-energy signal in cells, and many effects of ATP depletion are now known to be mediated through this pathway rather than via the direct effects of adenine nucleotides on enzymes. Increased [AMP] in cells directly activates AMPK and also activates its upstream kinase (AMPK kinase) such that AMPK is strongly activated under cellular situations that either inhibit ATP production (hypoxia, glucose limitation, uncouplers, and other stresses) or stimulate ATP consumption (exercise) (see Fig. 14.13). Active AMPK switches on catabolic pathways and switches off anabolic

ones (e.g., 3-hydroxy-3-methylglutamyl coenzyme A (CoA) reductase and acetyl-CoA carboxylase, key enzymes in steroid and fatty acid biosynthesis, respectively). In ischemic heart, AMPK plays a key role in glycolytic activation with two main effects: (1) an activation of PFK-2 to elevate $F2,6P_2$ levels and stimulate PFK-1 and (2) stimulation of the translocation of GLUT4 to the plasma membrane. Hence, AMPK both improves glucose uptake into the ischemic heart and enhances PFK-1 activity by stimulating production of its potent allosteric activator, $F2,6P_2$. In skeletal muscle, AMPK does not affect $F2,6P_2$ production but increases the expression of GLUT4, hexokinase, and several mitochondrial enzymes, changes that are also features of endurance training.

Hypoxia-Induced Gene Expression

The response to hypoxia/ischemia stress by oxygen-sensitive systems includes not just acute activation of glycolytic rate but also gene expression responses that elevate the overall glycolytic capacity of cells and organs in preparation for possible prolonged hypoxia exposure. These gene expression responses are coordinated by the hypoxia-inducible transcription factor (HIF-1). The structure, regulation and action of HIF-1 is discussed in detail in Chapter 6 (see also Fig. 6.11). Recall that under oxygenated conditions the HIF-1α subunit is modified by an oxygen-dependent prolyl hydroxylase enzyme that targets the subunit for rapid degradation by the proteasome. When oxygen availability is low, this modification does not occur and HIF-1α is stabilized, forms a dimer with the HIF-1ß subunit, migrates to the nucleus, and activates the transcription of a suite of genes. Gene responses that improve glycolytic capacity include increased amounts of multiple enzymes in the glycolytic pathway (e.g., hexokinase, glyceraldehyde-3-P dehydrogenase, aldolase A and C, lactate dehydrogenase A, phosphoglycerate kinase-1, pyruvate kinase M) as well as increased levels of glucose transporter isoforms 1 and 3 to increase the capacity of cells to import glucose across the plasma membrane. Other genes that are up-regulated by HIF-1 include those whose products have angiogenic or vasodilatory functions such as erythropoietin that stimulates red blood cell synthesis, vascular endothelial growth factor that stimulates capillary growth, inducible nitric oxide synthase, and heme oxygenase.

Overall, then, the response to low oxygen by oxygen-sensitive organisms such as humans is a compensatory one. Mechanisms are activated that increase oxygen delivery to cells and that enhance ATP production by oxygen-independent routes. If the hypoxic stress is not too severe, acclimation will occur over time, and when the oxygen-carrying capacity of blood is sufficiently elevated, acute responses such as hyperventilation and high rates of glycolysis are abated. Many organisms are much more hypoxia tolerant than humans. They are prepared with better constitutive capacities, both physiological and biochemical, for dealing with hypoxic excursions and inducible responses are triggered at much lower pO_2 values (see Text Box 15.3). Our treatment of hypoxia tolerance here has been very brief, but this field is a huge one and the reader is referred to the suggested reading list for more information on the subject.

FACULTATIVE ANAEROBIOSIS

Life on Earth began in an anoxic environment, and glycolysis, the fundamental pathway of energy production from substrate fermentation, is still present in all organisms (see Chapter 20). However, although glycolysis remains integral to energy generation under low-oxygen conditions, the limitations of glycolysis as a general means of ATP supply are immediately obvious when compared with the advantages of oxygen-based fuel catabolism. These include the following:

1. *Substrate Options* Aerobic metabolism can make use of carbohydrate, lipid, and protein as oxidative fuels for mitochondrial pathways, whereas under anaerobic conditions organisms are restricted to the use of carbohydrates and a few amino acids as fermentative fuels. For many organisms, this means that their major stored fuel reserves (lipids) are useless if oxygen supply fails.

2. *Energy Yield* Glycolysis yields a net of only 2 mol ATP/mol glucose catabolized to lactate (or 3 mol ATP/mol glucose-1-phosphate cleaved from glycogen) compared with 36 (or 38) mol ATP produced if sugar is fully catabolized to CO_2 and H_2O by mitochondria. Hence, the normal rate of ATP use by aerobic cells can only be sustained under anoxia by a massive increase in the rate of fermentative glycolysis.

3. *Efficiency of Substrate Use* By rearranging the above statements, it is obvious that to generate equal amounts of ATP, anaerobic glycolysis (ending in lactate) would use 18 times more glucose than would be needed for aerobic ATP production from carbohydrate oxidation. Hence, anaerobic glycolysis can be viewed as a wasteful use of substrate, something that few organisms can normally afford.

4. *End Products* The incomplete catabolism of carbohydrates via anaerobic glycolysis results in the accumulation of waste products, generally lactate in animals and ethanol in plants, that if not excreted or "neutralized" in some manner can cause serious toxicity. By contrast, the volatile end product of mitochondrial respiration, CO_2, is readily excreted or exhaled.

TEXT BOX 15.3 HYPOXIA TOLERANCE

A distinction can be made between anoxia tolerance, the ability to survive without oxygen, and hypoxia tolerance, the ability to endure low-oxygen conditions. Many organisms can endure extended periods of low oxygen but cannot survive total oxygen lack. Hypoxia tolerance varies considerably between species and between organs and developmental stages within a given species. For example, no mammal is capable of sustained life without oxygen (anoxia), but many have a much greater tolerance for hypoxia than humans do. These include burrowing mammals (e.g., moles) whose tunnels are not always well-ventilated, alpine mammals that live at high altitudes, breath-hold divers such as whales and seals, and hibernating species such as ground squirrels that take only a few breaths per hour while in torpor (read more about hibernation in Chapter 15). Fetal and neonatal mammals are also much more hypoxia-tolerant than adults of the same species. In fetal mammals this is because oxygen must be derived from the maternal circulation and, indeed, oxygen tension in fetal brain is less than half the normal value for adults. The birth process also frequently imposes hypoxic and/or ischemic episodes.

However, although functioning under hypoxic conditions, such animals are generally not oxygen-limited. For example, the fact that hibernating mammals sustain torpor for many months by the slow oxidation of their huge adipose reserves shows that they cannot be oxygen-limited because lipids can only be catabolized aerobically. Similarly, the vast majority of dives by marine mammals are fully aerobic. These animals have made multiple physiological adjustments that enhance their "on-board" reserves of oxygen, including extremely high concentrations of the oxygen storage protein (myoglobin) in muscle, a high red blood cell count (hematocrit), and a huge reserve of red blood cells in the spleen. Selective vasoconstriction during the dive imposes hypoxic and ischemic conditions on those organs that can withstand the stress (e.g., kidney, intestine) and thereby enhances the oxygen available to sensitive organs (e.g., brain). Only in dives of unpredictably long durations is strong metabolic rate depression and a reliance on the high glycolytic capacity of diver organs needed to deal with low-oxygen stress. For more information on hypoxia tolerance, especially about mammalian diving and high altitude adaptations, consult P. W. Hochachka and G. N. Somero. *Biochemical Adaptation: Mechanism and Process in Physiological Evolution*, Oxford University Press, Oxford, 2002.

Despite the obvious limitations of a reliance on anaerobic glycolysis for ATP synthesis, there are numerous situations where this is necessary. As discussed in Text Box 15.1, intense muscle work (functional anoxia) is one of them. Another is the specialized needs of selected cell types. For example, to maximally pack each cell with hemoglobin, red blood cells have lost both mitochondria and nuclei. Their only option for ATP synthesis is the fermentation of glucose; fortunately, glucose is readily available to them from the surrounding plasma. Cells of the retina also function without oxygen because the infiltration of capillaries to bring oxygen to the retina would disrupt the visual field. Finally, many organisms experience environmental anoxia as part of their normal lives. They live in environments where oxygen can become depleted on either short-term or seasonal time scales or have lifestyles that cause them to be cut off from oxygen delivery intermittently. When oxygen supplies fail, these organisms simply switch to fermentative pathways and employ multiple intriguing biochemical adaptations to ensure their long-term survival without oxygen. Two of the most commonly studied animal models of facultative anaerobiosis are freshwater turtles that can live submerged underwater for many months over the winter (Text Box 15.4) and intertidal marine mollusks that are deprived of oxygen at each low tide (Text Box 15.5).

Mechanisms of Long-Term Anoxia Survival

The limitations of anaerobic glycolysis as an efficient method of ATP generation (listed above) also provide us with a basic list of factors that should be addressed to optimize anaerobic survival: (a) access to large supplies of fermentable fuels, (b) supplementing the basic glycolytic pathway with other reactions that increase ATP yield per glucose catabolized, (c) minimizing cytotoxicity of end products by enhancing buffering capacity or making products that can be excreted easily, (d) meeting the ATP demands of the anoxic cell by either greatly increasing glycolytic ATP output or greatly decreasing rates of ATP use in anoxia, and (e) maintaining or upgrading antioxidant defenses to deal with potential oxidative stress during the transition from anoxic to aerobic life. Each of these are discussed below.

Fermentable Fuels For most organisms, anaerobic metabolism basically means carbohydrate catabolism via glycolysis using polysaccharide reserves (glycogen in animals, starch in plants) or free sugars as substrates. A few selected amino acids can also be used in reactions that provide "substrate-level" phosphorylation of ADP, but lipid fuels, because they all feed into acetyl-CoA for catabolism in the tricarboxylic acid (TCA) cycle, can only be used to produce ATP by oxidative phosphorylation

TEXT BOX 15.4 CHAMPION OF VERTEBRATE ANAEROBIOSIS—THE FRESHWATER TURTLE

Many turtle species spend much of their lives underwater, diving for food or to evade predators. Winter survival for many freshwater turtles is also ensured by underwater hibernation that provides an escape from freezing temperatures. Aerobic metabolism can easily support short-term dives (although perhaps with some muscle lactate accumulation if swimming is vigorous), but for long-term hibernation, other strategies are needed to allow these lung-breathing animals to survive. Some species of turtles (mostly soft-shelled varieties) have solved the problem by using extrapulmonary mechanisms of oxygen uptake; oxygen is extracted from the water across the epithelium lining the throat or the cloaca. In cold water when metabolic rate is low, this strategy is sufficient to sustain aerobic metabolism. Other turtles have perfected facultative anaerobiosis. Turtles belonging to the *Trachemys* and *Chrysemys* genera, which include the common red-eared slider (*T. scripta elegans*) of the pet store trade and the painted turtles (*C. picta*) that are a familiar sight in ponds and rivers across North America, can live submerged in cold water for 3 to 4 months without oxygen. The secrets of their survival include strong metabolic rate depression to $\sim$10% of the aerobic rate at the same temperature, large stores of glycogen fuel loaded into all organs, and the ability to both buffer and store lactic acid in their bony shell. The extreme anoxia tolerance of turtle brain and heart has been appreciated by medical science, and these are widely used as models for understanding the molecular events that impart anoxia and ischemia resistance to organs.

TEXT BOX 15.5 INVERTEBRATE FACULTATIVE ANAEROBES—MOLLUSKS OF THE SEASHORE

Anoxia tolerance is critical for the survival of many kinds of marine invertebrates and has been particularly well-studied in mollusks, including bivalves (e.g., mussels, clams, oysters) and gastropods (e.g., periwinkles, whelks). For these animals, natural oxygen deprivation can result from a variety of factors: (1) gill-breathing species that live in the intertidal zone (especially sessile creatures such as mussels) are deprived of oxygen when the waters retreat with every low tide, (2) burrowing and benthic mollusks can encounter anoxic bottom sediments, (3) high silt or toxin levels in the water as well as predator harassment can force shell valve closure, leading to substantial periods of "self-imposed" anoxia, and (4) animals in small tide pools can be oxygen-limited when animal and plant respiration depletes the oxygen supply in the water. The anoxia tolerance of the intertidal species found at high latitudes (e.g., periwinkles, blue mussels) is also an important contributor to freezing survival by these animals for, when extracellular fluids freeze, cells must rely on anaerobic energy production to remain viable until they thaw again (see Chapter 17). The remarkable anoxia tolerance of these marine shellfish (that includes a profound anoxia-induced metabolic rate depression) accounts for the long shelf-life of fresh shellfish in seafood markets. Anaerobiosis in mollusks has several components including large tissue reserves of glycogen and fermentable amino acids, modified pathways of fuel catabolism that increase the ATP yield compared with glycolysis alone, production of volatile end products that are easily excreted, and strong metabolic rate depression.

in the mitochondria. For microorganisms, unlimited supplies of fermentable sugars are sometimes available from the extracellular medium, and indeed, work by Louis Pasteur studying yeast provided the first analysis of fuel use by organisms growing under aerobic versus anaerobic conditions (see Text Box 15.2). Among multicellular animals, however, carbohydrate catabolism under anoxia typically draws upon tissue glycogen reserves, which are generally much higher in organs of anoxia-tolerant species compared with intolerant species. Furthermore, in cases where anoxia exposure is seasonal (e.g., turtles that hibernate underwater; see Text Box 15.4), glycogen reserves are built up to high levels during prehibernation feeding. In vertebrates, glycogen reserves are particularly high in the liver, and this organ is the main supplier of carbohydrate, in the form of blood glucose, to other organs during anoxia. In many invertebrates, however, there is less centralization of glycogen stores and most organs retain their own supplies of fermentable fuels. Indeed, this sort of self-sufficiency has made the tissues of bivalve mollusks (e.g., gill, mantle, adductor muscle) excellent and widely used models for *in vitro* studies of anaerobic metabolism. Figure 15.2 shows a typical pattern of glycogen consumption and end-product accumulation in the gill of the marine clam, *Mercenaria mercenaria*, over the course of 4 days of anoxia exposure.

Some amino acids can also be used as anaerobic fuels. Aspartate is the most notable one, but asparagine, glutamate, and glutamine can also be used. Anoxia-tolerant marine invertebrates have been particularly successful in

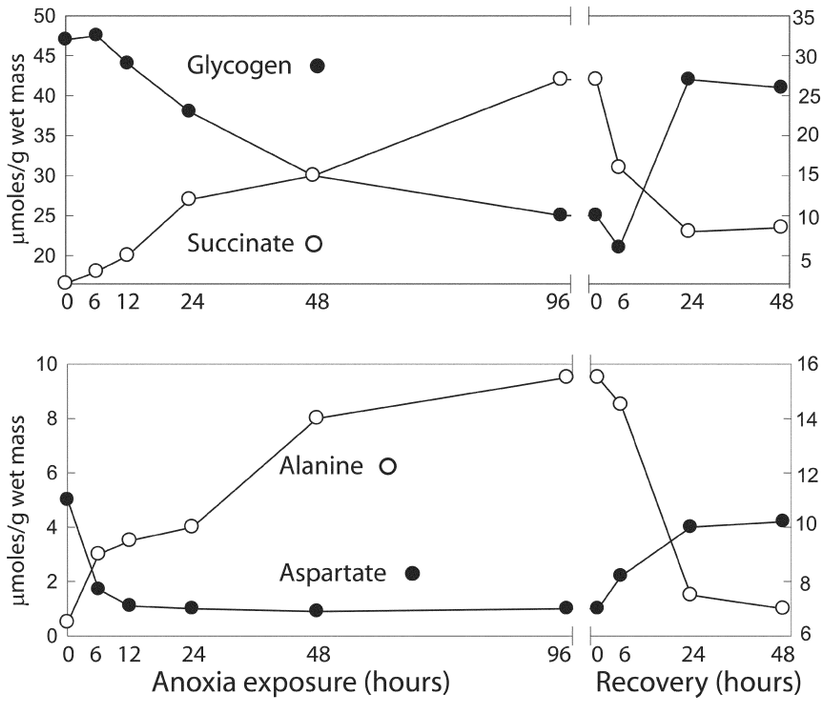

Figure 15.2 Metabolism in gill of the marine clam, *Mercenaria mercenaria*, over 4 days of anoxia exposure and 2 days of aerobic recovery. Glycogen and aspartate are utilized as substrates, whereas alanine and succinate accumulate as products. Note the rapid initial catabolism of aspartate, whereas glycogen fuels long-term anaerobiosis. All metabolites are largely restored to control levels within 24 h of aerobic recovery. [Data compiled from S. A. Korycan and K. B. Storey (1983). *Can J Zool* **61**:2674–2681.]

integrating the anaerobic catabolism of glycogen and aspartate (Fig. 15.3). Aspartate is rapidly mobilized early in anoxia exposure (Fig. 15.2 shows that aspartate pools are largely depleted within the first 6 h in *M. mercenaria*), whereas glycogen takes over as the sole fuel for longer term anaerobiosis. However, for intertidal invertebrates that are cut off from oxygen during twice-daily aerial exposures at low-tide, 6 h often represents the extent of a natural anaerobic excursion, and hence the pathways of coupled fermentation of glycogen and aspartate (Text Box 15.5, Fig. 15.3, and discussed below) may be the ones that support most normal aerial exposures at low tide. Aspartate is catabolized by reactions that reverse the segment of the TCA cycle running from oxaloacetate to succinate with ATP produced in a substrate-level phosphorylation by the fumarate reductase reaction (Fig. 15.3).

Improved Energy Yield and Alternative End Products

For most animals, the end product of anaerobic carbohydrate catabolism via glycolysis is lactate (in plants it is ethanol). The action of lactate dehydrogenase (LDH) [reaction (15.2)] in catalyzing the reduced nicotinamide adenine dinucleotide (NADH)-dependent reduction of pyruvate to form lactate regenerates the NAD that is needed by the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reaction and allows glycolysis to continue without a net depletion of NAD cosubstrate (Fig. 15.1). However, anaerobic glycolysis with lactate as the end product has two limitations: (a) a low yield of ATP per glucose catabolized and (b) significant cellular acidification. Several alternative end products to lactate have appeared in anoxia-tolerant species that provide enhanced ATP yield per glucose catabolized and/or a reduced acid buildup. These include several kinds of "opines," ethanol, alanine, succinate, propionate, and acetate (Fig. 15.1):

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \rightarrow \text{lactate} + \text{NAD}^+ + \text{H}_2\text{O} \qquad (15.2)$$

$$\text{Pyruvate} + \text{amino acid} + \text{NADH} + \text{H}^+ \rightarrow \text{opine} + \text{NAD}^+ + \text{H}_2\text{O} \qquad (15.3)$$

Alternative pyruvate oxidoreductases, the so-called opine dehydrogenases [reaction (15.3)], are quite widespread

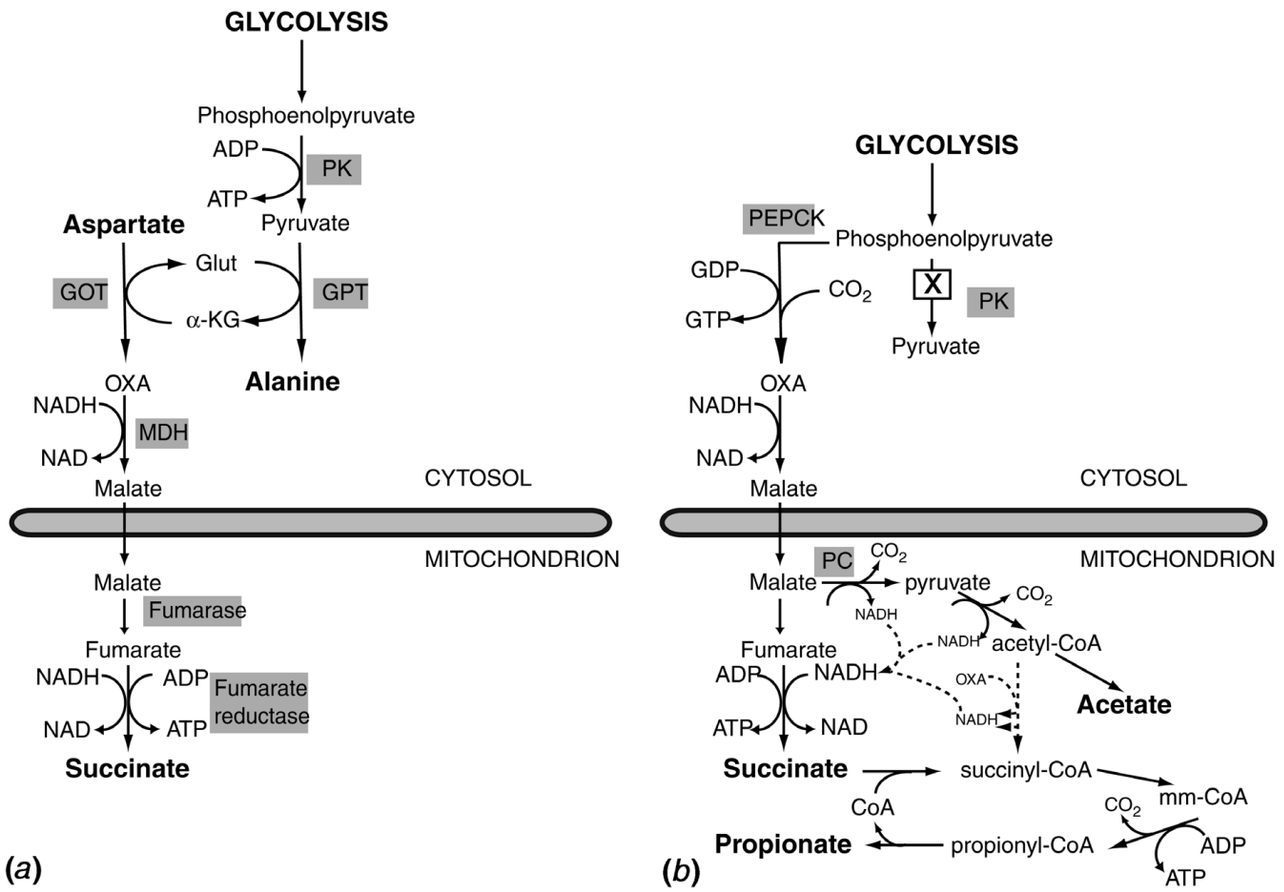

Figure 15.3 Patterns of anaerobic metabolism in marine molluscs. (*a*) Early in an anoxic excursion, the pyruvate produced by glycolysis is converted to alanine in a reaction that is coupled with aspartate conversion to oxaloacetate (OXA); the enzymes involved are glutamate–pyruvate transaminase (GPT) and glutamate–oxaloacetate transaminase (GOT). OXA reduction to malate by malate dehydrogenase (MDH) regenerates cytoplasmic NAD that would otherwise have been the function of lactate dehydrogenase. In the mitochondria, malate is converted to succinate with ATP generated at the fumarate reductase reaction. (*b*) Later in anoxia when aspartate pools are depleted, glycolytic carbon is shunted directly into the reactions of succinate synthesis. Inhibition of the pyruvate kinase (PK) reaction promotes the conversion of phosphoenolpyruvate (PEP) to OXA via the enzyme PEP carboxykinase. The intramitochondrial generation of NADH needed for succinate synthesis is still somewhat controversial but appears to involve malate dismutation with a portion of the malate used to generate NADH via malate conversion to acetyl-CoA using the enzymes pyruvate carboxylase and pyruvate dehydrogenase. The acetyl-CoA could then be processed via a low rate of forward functioning of the tricarboxylic acid cycle, generating more NADH, or acetyl-CoA could be converted to acetate. Production of the volatile end products, acetate and propionate, occurs only in prolonged anaerobiosis. The reactions linking succinate to propionate involve methylmalonyl-CoA (mm-CoA) as an intermediate and ATP generation at the propionyl-CoA carboxylase reaction.

among marine invertebrates where they may supplement or completely replace LDH. The structure of all opines is functionally equivalent to that of octopine (see Text Box 15.1), and the main amino acids used by these reactions are arginine, alanine, glycine, serine, lysine, proline, and taurine. There is no energetic advantage to producing these end products compared with lactate, but in the case of octopine or alanopine, their synthesis incorporates one of the other end products of anaerobic metabolism. For octopine this is the arginine released from phosphagen

hydrolysis (see Text Box 15.1), and for alanopine it is the alanine that is a major product of anaerobic glycolysis (see below). However, in general, the opine end products more commonly accumulate as products of muscle work (functional anoxia) than of environmental anoxia. For example, Figure 15.2 shows a net alanine accumulation by *M. mercenaria* gill of about 8 μmol/g wet mass over 4 days of anoxia exposure, but over the same time course we found that the net increase in alanopine was only about 0.5 μmol/g.

For many marine invertebrates (particularly bivalve or gastropod mollusks), a coupled fermentation of glycogen and aspartate occurs as the initial response to anoxia. Glycogen is catabolized to the level of pyruvate, and aspartate is converted to succinate (Fig. 15.3*a*). The pathways are linked in two ways: (a) amino group transfer—glutamate–oxaloacetate transaminase removes the amino group from aspartate to produce oxaloacetate and glutamate–pyruvate transaminase adds the amino group to pyruvate to produce alanine as the glycolytic product—and (b) cytoplasmic redox balance—the NADH generated by the GAPDH reaction of glycolysis is regenerated by the malate dehydrogenase reaction (replacing LDH). Aspartate levels are high in tissues of marine mollusks, and this paired catabolism of glycogen and aspartate can support several hours of anaerobiosis with alanine and succinate typically accumulating in a 1 : 1 ratio. Figure 15.2 illustrates this pattern for *M. mercenaria* gill and shows that aspartate reserves are depleted after about the first 6 h. Alanine production supports the same ATP output as does lactate synthesis, but the energetic advantage of the coupled system comes from succinate production because ATP is generated at the fumarate reductase reaction (Fig. 15.3). Hence, the coupled conversion of 1 mol glucose plus 2 mol aspartate into 2 mol alanine and 2 mol succinate produces a net of 4 mol ATP/mol glucose catabolized, compared with the 2 mol that would result if lactate was the sole product.

When anaerobiosis is prolonged and aspartate pools are depleted, the amino acids are "cut out" of the scheme and carbon from glycolysis is directed straight into the reactions of succinate synthesis. The energy yield of direct fermentation of glucose to succinate is also 4 mol ATP/mol glucose. In this scheme, glycolysis proceeds as normal to the level of phosphoenolpyruvate (PEP), but then, instead of continuing into the pyruvate kinase (PK) reaction, carbon is diverted into the PEP carboxykinase (PEPCK) reaction that yields oxaloacetate plus guanosine 5′-triphosphate (GTP) (or ATP) (Fig. 15.3*b*). This switch is regulated by the anoxia-induced phosphorylation of PK that strongly inhibits PK in all tissues (this is discussed more later). Note that in humans and most other animals PEPCK is typically a key player in gluconeogenesis, the coupled actions of pyruvate carboxylase and PEPCK circumventing the PK reaction to reconvert pyruvate to PEP. In many anoxia-tolerant invertebrates, however, the reverse reaction is emphasized under anoxia, and the enzyme converts the three-carbon PEP into the four-carbon oxaloacetate that can be fed into the pathway of succinate synthesis.

The energy yield per mole of glucose catabolized can be further raised to 6 mol ATP if succinate is further catabolized to produce propionate, a volatile fatty acid. A need to maintain redox balance in the anaerobically functioning mitochondria typically requires the dismutation of malate to produce both propionate and acetate. This scheme is found in many marine invertebrates (but typically only when anoxia is prolonged) and is also the basis for normal carbohydrate fermentation in parasitic helminths that live in the anoxic lumen of the intestines of other animals. Interestingly, the novel carbon metabolism of parasitic helminths was first suggested by the seemingly odd observation that CO_2 fixation was a required part of anaerobic glycogen catabolism in these animals; CO_2 incorporation was later found to occur at the PEPCK reaction.

End Product Excretion and Minimizing Cytotoxicity As is evident from the comparison of the net ATP yield from glucose fermentation to lactate (2 ATP) versus glucose oxidation to CO_2 and H_2O (36 ATP), the incomplete fermentation of glucose leaves a lot of potential energy trapped in the end product. Synthesis of selected alternative products from glucose such as succinate or propionate can extract considerably more energy and often have the bonus of creating less acidity and/or being easy to excrete (e.g., propionate and acetate are volatile). Anaerobic metabolism always results in tissue acidification, and low pH can have negative consequences for many cellular enzymes (recall from Chapter 1 that all enzymes are sensitive to pH change and normally show distinct pH optima). Hence, the synthesis of end products that enhance ATP yield, are less acidifying, or can be readily excreted are all bonuses for facultative anaerobes, and many species have adopted one or more of these options to minimize cytotoxicity during long-term anaerobiosis.

Before we proceed further, it is useful to digress briefly to note that, strictly speaking, it is not the production of "lactic acid" that causes cellular acidification in situations where ATP production is derived from glycolysis (e.g., muscle exercise, anoxia). Protons are actually produced as the products of ATPase action [ATP is hydrolyzed to ADP, inorganic phosphate (P_i), and H^+], and so it is the ATP-consuming reactions that actually generate H^+. Under aerobic conditions this proton output is balanced by proton consumption during ATP synthesis by oxidative phosphorylation, and net cellular pH is unchanged. However, when glycolysis is the only source of ATP synthesis, this balance breaks down because the glycolytic

pathway is not a net consumer of protons. Hence, ATP turnover in anoxia (glycolytic ATP production plus cellular ATP consumption by ATPases) results in the net production of H^+. For example, 1 mol glucose converted to 2 mol lactate and producing 2 mol ATP results in a net production of 2 mol H^+ when the ATP is hydrolyzed. Rearranged, this means that 1 mol ATP can be turned over for every mole of H^+ produced. When glucose is fermented to succinate, however, the net proton output is less because 2 mol ATP can be turned over per mole of H^+ produced, and for propionate this rises to 3 mol ATP turned over per mole of H^+ accumulated. Hence, there are clear advantages to succinate or propionate production for anaerobes: their synthesis yields more ATP per mole of glucose consumed and their production results in a lower net acid buildup as compared with lactate production. However, a slow acidification still occurs in all anaerobic systems, and so additional choices must be made to (a) elevate buffering capacity and/or (b) detoxify anaerobic end products by excreting them. Different organisms have made different choices.

Turtles have optimized buffering. Freshwater turtles of the *Trachemys* and *Chrysemys* genera are the champion facultative anaerobes of the vertebrate world (see Text Box 15.4). Anaerobiosis for them is simply glucose/glycogen catabolism to lactate, a high-acid buildup option. However, they turn a peculiarity of their morphology (their large shell) into a metabolic advantage in two ways. First, calcium and magnesium carbonates are released from the shell, the carbonate providing plasma buffering of H^+. Second, lactate is taken up and stored by shell and bone; for example, amounts of 136 and 164 mmol/kg wet mass were found in these compartments in 125-day anoxic turtles compared with plasma lactate levels of 155 mM (lactate is <2 mM in controls). Nearly half of the total body lactate that was accumulated during anoxia was sequestered and buffered within shell and bone, and combined with the buffering provided by carbonate released from shell and bone (determined from the rise in plasma Mg^{2+} and Ca^{2+}), nearly 75% of the total lactic acid buffering in anoxia could be attributed to this source. Hence, shell and bone are crucial to long-term anoxia survival for turtles. Furthermore, because this strategy requires no end-product excretion, turtles end their anaerobic excursions with a plentiful supply of lactate that can be consumed as an aerobic fuel or returned to glycogen stores when air breathing is again possible.

Species of anoxia-tolerant fish have optimized the strategy of end-product excretion. Goldfish and crucian carp show the highest anoxia tolerance among fish species and use this capacity to allow them to survive in small pools that can become oxygen-depleted, particularly when ice-locked in the winter. Lactate is still the product of anaerobic metabolism in most organs, but it is exported into the blood and delivered to skeletal muscles. There the LDH reaction is reversed and the pyruvate is converted in two steps to ethanol and CO_2. The enzymatic reactions involved are pyruvate decarboxylase [reaction (15.4)] and alcohol dehydrogenase [reaction (15.5)]:

$$\text{Pyruvate} \rightarrow \text{acetaldehyde} + CO_2 \tag{15.4}$$

$$\text{Acetaldehyde} + \text{NADH} + H^+ \rightarrow \text{ethanol} + NAD^+ \tag{15.5}$$

The pyruvate decarboxylase seems to be a novel function of the pyruvate dehydrogenase complex (and therefore the reaction may occur mostly in red muscle), and the conversion to ethanol is catalyzed by the unusual presence of very high activities of alcohol dehydrogenase in the skeletal musculature of these fish. Both ethanol and CO_2 are then excreted by the gills. Clearly, this scheme is wasteful in terms of the carbon reserves of the animal, but these fish still have the option of continuing to feed in anoxic waters, which is unlike the situation of most other facultative anaerobes that are either hibernating (e.g., turtles) or withdrawn into closed shells (e.g., intertidal mollusks). Notably, intestinal parasites that excrete propionate and acetate are also "wasteful" of the carbon reserve and energy potential of these products, but they can afford this because their hosts supply them with a virtually constant supply of sugars from digesting foodstuffs.

Most anoxia-tolerant marine invertebrates have discarded lactate as an anaerobic end product and, furthermore, they have combined multiple strategies in order to design highly efficient systems of fermentative metabolism. They use fermentative pathways that optimize energy output by coupling glycogen and aspartate catabolism and utilize alternative pathways of fermentation that produce extra ATP in substrate-level phosphorylations (Fig. 15.3). They have also optimized the synthesis of a range of low-acid products (e.g., alanine, succinate, propionate, acetate) that includes volatile products (propionate, acetate). Notably, the volatile products accumulate in large amounts only when anaerobiosis is prolonged to several days so that under normal tidal cycles of aerial exposure there would be very little net carbon loss from tissues because the main products of short-term anaerobiosis are alanine and succinate. Finally, mollusks mobilize calcium carbonate from their shell to enhance buffering capacity during anoxia.

Meeting ATP Demand The ATP output of fermentative pathways is always much lower than that of oxidative metabolism, and hence, there are only two options for reestablishing a balance between ATP consumption and ATP production in anoxia. One is to raise anaerobic ATP production up to a level that can meet the unaltered demands

of ATP-utilizing reactions. This can be accomplished over the short term by greatly accelerating the rate of glycolysis but often fails very quickly (human brain energetics are irreversibly compromised after <5 min of anoxia). Some organisms can sustain high rates of ATP production during long-term anaerobiosis in cases where substrate supply is virtually unlimited (e.g., microorganisms growing in a sugary broth; see Text Box 15.2). However, multicellular organisms would quickly and wastefully deplete their internal reserves of fermentable fuels by using this strategy, and therefore, long-term anoxia survival by most facultative anaerobes has come to depend on strong metabolic rate depression. By reducing net ATP use in anoxia to $<10\%$ of the corresponding aerobic rate, organisms can meet ATP demand using fermentative pathways of ATP production and gain a 10-fold or more extension of the time that their fixed internal carbohydrate reserves can fuel anaerobic survival. By reducing ATP turnover in anoxia, animals also reduce the extent of acidification during long-term anaerobiosis as well as the net buildup of end products (that need to be excreted or that must be restored to glycogen pools via ATP-expensive gluconeogenic reactions when oxygen returns). Anoxic excursions are often of unpredictable duration, and so the ability to ration carbohydrate reserves and sustain homeostasis for the longest possible time is critical. Metabolic rate depression is quantitatively the most important of the biochemical adaptations that support anaerobiosis and is discussed in detail below.

Antioxidant Defenses Although it may seem contradictory, all anoxia-tolerant animals that we have examined have very well developed antioxidant defenses, both enzymatic and metabolite, for dealing with the generation of ROS. This is true not only of anoxia-tolerant vertebrates and invertebrates but also of freeze-tolerant animals and hibernating mammals and, therefore, high antioxidant defenses may be generally associated with situations where organisms experience very wide variation in oxygen availability. Under ischemic or hypoxic situations, the electron carriers of the mitochondrial respiratory chain become reduced, and when oxygen is reintroduced, an immediate reoxidation of these carriers takes place and results in a transient overproduction (or burst) of ROS. This burst of ROS production is well known to be the basis of postischemic reperfusion injuries in mammalian organs recovering from an ischemic event. Anoxia-tolerant species appear to avoid ROS-triggered metabolic injury by sustaining high constitutive levels of antioxidant defenses and supplementing this, in some cases, with anoxia-induced increases in the activities of antioxidant enzymes or elevated glutathione pools. This subject is discussed in greater detail in Text Box 13.5.

METABOLIC RATE DEPRESSION

Metabolic rate depression (MRD) is a widespread response to many types of stress and is found throughout phylogeny (see Text Box 15.6). The biochemical mechanisms of MRD received the greatest initial study from researchers studying anoxia tolerance, and hence the subject is introduced here but the phenomenon will be explored further in the next chapter when we discuss mammalian hibernation. As mentioned earlier, there are two main solutions to situations that disrupt an organism's capacity to produce ATP at a rate that adequately supplies the ATP demands of metabolism. One is compensation, which works well in the short term, and the other is conservation. If ATP production is decreased due to low oxygen availability, then organisms turn down their rate of ATP consumption until a new rate of ATP turnover is established where ATP production again equals consumption. MRD is a key component of anoxia survival for all anoxia-tolerant organisms for it allows metabolic rate to be lowered to a level that can be supported by the ATP output from fermentative pathways alone. For example, turtles submerged under water suppress their anoxic metabolic rate to only 10 to 20% of their comparable resting metabolic rate when breathing air at the same temperature. In marine mollusks, anoxic metabolic rate is reduced to only 2 to 10% of the aerobic value.

How is metabolic rate depression achieved? Several extrinsic factors contribute to energy savings. For example, organisms in hypometabolic states typically show few voluntary movements by skeletal muscles, most do not eat so the costs of digestion and absorption are saved, and heart beat, breathing, and kidney filtration rates are all greatly reduced. Metabolic rate is also suppressed by accompanying factors that are typically present, including low pO_2, elevated pCO_2, low pH, and sometimes lower temperature. For instance, many ectothermic organisms voluntarily seek cooler temperatures when challenged by hypoxia and thereby use a decrease in body temperature to help reduce tissue demands for oxygen. Indeed, a hypoxia–hypothermia connection is also suspected as part of the mechanism by which hibernating mammals lower their metabolic rate and body temperature as they sink into torpor (see Text Box 16.5). However, intrinsic mechanisms within cells appear to account for at least half of the whole animal energy savings in the hypometabolic state. These induce stable suppression of the rates of multiple metabolic processes to provide both a net reduction in metabolic rate and selective targeting of specific cell functions that are unneeded in the hypometabolic state.

Metabolic suppression is not necessarily applied uniformly to all processes within a cell or to all organs within an organism. Priorities exist between organs; for example, the suppression of protein synthesis in different

TEXT BOX 15.6 METABOLIC RATE DEPRESSION—TORPOR, DORMANCY, HIBERNATION, DIAPAUSE, ESTIVATION, AND MORE

The ability to strongly suppress metabolic rate and sink into a hypometabolic state is a lifesaver for many organisms and is found in virtually all phylogenetic lineages. Metabolic rate depression (MRD) is, in fact, one of the most powerful defenses that animals have developed to deal with environmental stress of many kinds. The ability to sink into a dormant or torpid state allows the organism to effectively "wait out" the stress, extending by 10- or 20-fold or more the length of time that the animals can live off internal reserves of stored fuels (chiefly glycogen or triglycerides). MRD is widespread in nature as a way of ensuring long-term survival when environmental conditions are incompatible with normal life (e.g., too hot, too cold, too dry, no oxygen). It is an integral component of torpor, hibernation, estivation, diapause, dormancy, anaerobiosis, and cryptobiosis.

As discussed at length in this chapter, MRD is key to anaerobic survival, but it is also widely used in aerobic circumstances. Nightly torpor, during which metabolic rate drops by 20 to 30%, preserves just enough body fuel to allow many small birds and mammals to live to see the next day. Winter hibernation allows many mammals to "sleep" through the winter. Hibernation can last as long as 9 months in Arctic or alpine environments, and the profound MRD of the hibernator produces energy savings of as much as 90% compared with the costs of maintaining a high and constant body temperature throughout the winter (see Chapter 16). Life in arid regions of Earth is aided by estivation, an aerobic dormancy induced by dry and/or hot conditions; a 70 to 90% reduction in metabolic rate allows lungfish, frogs, toads, and snails, among others, to survive for many months until the next rainy season. Diapause arrests the developmental cycle of many insects and other invertebrates to allow them to "wait out" excessively hot, cold, or dry conditions or to synchronize the transformation of a whole population to the next developmental stage (e.g., adult emergence). Extremes of hypometabolism are found in cryptobiosis where a virtual ametabolic state in many seeds, spores, cysts, emrbyos, and eggs produces a life extension that can stretch to years, decades, or even centuries (for more information see Text Box 17.1).

organs of ground squirrels during hibernation ranged from 0 to 85%, the zero value belonging to brown adipose tissue that seems to need to maintain full biosynthetic potential to support its central role in thermogenesis (see Chapter 16). Priorities also exist within cells as to which metabolic activities are maintained in the hypometabolic state. Table 15.1 illustrates this with the fractional use of ATP by different metabolic processes in liver cells (hepatocytes) of turtles under aerobic versus anoxic conditions. In normoxic cells, five main ATP-consuming processes were identified with ion pumping by the Na^+K^+–ATPase and protein synthesis using the largest portions of cellular ATP. Under anoxic conditions, however, total ATP turnover in liver cells fell by 94%, and each of the ATP-consuming processes was differently affected. Na^+K^+–ATPase activity decreased by 75%, whereas protein synthesis was suppressed by 93% and gluconeogenesis was undetectable in anoxic cells. As a result of this reordering of metabolic priorities, the sodium/potassium pump became the dominant energy sink in anoxic hepatocytes, consuming 62% of total ATP turnover. These priorities appear to derive from the ATP sensitivity of different energy-consuming reactions; in isolated thymocytes the pathways of macromolecular biosynthesis were shown to be most sensitive to energy supply, followed by sodium cycling and then calcium cycling across the plasma membrane, and finally the mitochondrial proton leak was least sensitive to ATP.

The molecular mechanisms of MRD are a subject of much study at present, and while the full picture is not yet available, the critical importance of certain mechanisms, such as reversible protein phosphorylation, is known and is discussed below. In general, mechanisms of MRD need to be readily reversible so that the constitutive metabolic machinery of cells can be retained in readiness to respond rapidly when organisms arouse from the hypometabolic state. Hence, hypometabolism is not associated

TABLE 15.1 Fractional Use of Cellular ATP Turnover by Different Cellular Activities in Turtle Hepatocytes Incubated under Aerobic versus Anaerobic Conditions

	Normoxia	Anoxia	Suppression in Anoxia (%)
Na^+K^+–ATPase	28.5	62.3	75
Protein synthesis	36.1	20.8	93
Protein degradation	16.6	9.1	94
Urea synthesis	3.0	7.8	70
Gluconeogenesis	17.0	0	100

Source: Data reworked from P. W. Hochachka, et al. (1996). *Proc Natl Acad Sci USA* **93**:9493–9498.

with a major loss of metabolic capacity by cells, but instead reversible controls are applied to coordinately suppress the rates of all metabolic processes while leaving intact the potential to rapidly return to the normal state.

Reversible Protein Phosphorylation

The most powerful and widespread mechanism of MRD is control over the activities of enzymes and functional proteins via reversible protein phosphorylation. As discussed in Chapter 14, reversible phosphorylation can affect enzyme function in multiple ways, including on–off control over activity, marked changes in enzyme properties, or altering enzyme-binding interactions with partner proteins.

Control of Anaerobic Glycolysis in Marine Mollusks The first indication that reversible protein phosphorylation played a role in MRD came from studies of the control of fuel catabolism in anoxia-tolerant marine mollusks. Anoxia-induced phosphorylation of PK proved to be key to the control of the PEP branch point and the partitioning of glycolytic carbon between the aerobic (PEP is fed into the Krebs cycle) and anoxic (PEP is fed into the reactions of succinate synthesis) routes of carbohydrate catabolism. By strongly inhibiting PK, PEP can be rerouted into the PEPCK reaction to produce oxaloacetate that is then fed into succinate synthesis (Fig. 15.3*b*). Aerobic and anoxic variants of PK were found in anoxia-tolerant marine mollusks, and these were separable on ion exchange chromatography and showed stable differences in kinetic properties (see Figs. 14.5 and Table 14.4). As Table 14.4 showed, our studies of PK from both muscle and hepatopancreas of whelks (*Busycon canaliculatum*) showed that the anoxic form of PK has a much lower affinity for PEP as a substrate, is much less sensitive to fructose-1,6-bisphosphate ($F1,6P_2$) as an activator, and is much more susceptible to inhibition by the anaerobic end product alanine. We traced the differences between the aerobic and anoxic forms of PK to their contents of covalently bound phosphate. Aerobic PK is the low-phosphate form and anoxic PK is the high-phosphate form, and the two are interconvertible via treatments with protein kinases versus protein phosphatases. Anoxia-induced phosphorylation of PK coupled with anoxia-induced changes in the concentrations of substrates and effectors of PK *in vivo* (particularly the large accumulation of alanine that occurs in virtually all marine mollusks under anoxia; Fig. 15.2), produces a situation where PK activity is virtually shut off under anoxic conditions to allow effective rerouting of glycolytic carbon via PEPCK into the pathway of succinate synthesis. Anoxia-induced phosphorylation of PK has now been documented in many different kinds of marine invertebrates and appears to be a universal mechanism for controlling the PEP branch point in order to make the switch from aerobic to anoxic routes of carbohydrate catabolism. Interestingly, however, it is not always the phosphorylated enzyme that is the low-activity, anoxic form. In the periwinkle, *Littorina littorea* (a small intertidal gastropod mollusk), we found that the opposite situation occurred; anoxia exposure stimulated a dephosphorylation of PK and the low-phosphate enzyme form shows reduced substrate affinity and enhanced inhibition by alanine compared with the high-phosphate aerobic enzyme form.

Subsequently, we found that anoxia-induced phosphorylation regulated not just PK but also other loci in glycolysis in anoxia-tolerant marine mollusks. This changed our view of the importance of reversible phosphorylation control from that of a mechanism that controlled a single branch point to a mechanism that coordinated glycolytic rate. Ultimately, we broadened this view even more and know now that the mechanism is a widespread and general mechanism for the coordinated suppression of all aspects of metabolism in hypoxia/anoxia-tolerant organisms. Our studies of glycolytic rate control showed that, in addition to PK control, reversible phosphorylation regulated glycogen phosphorylase (GP), PFK-1, and PFK-2 under anoxia. Figure 15.4 shows the coordinated effects of anoxia-induced protein phosphorylation on these four enzymes in whelk gill. In this case, the activities of GP*a* (the active enzyme) and PFK-2 fell over 20 h of anoxia exposure to 48 and 41% of aerobic values, whereas PK maximal activity was suppressed to just 16%. Suppression of PFK-2 activity had a major impact on $F2,6P_2$ levels, which fell to just 2% of aerobic values, a change that would then have major consequences for PFK-1 activity. PFK-1 phosphorylation state was assessed by changes in one of its kinetic parameters, the I_{50} value for PEP, which was reduced during anoxia to 20% of its aerobic value. Similarly, the I_{50} value for alanine of PK fell to just 4% of the aerobic value in anoxia. It is important to notice that the time course of all these changes is very similar, showing the greatest change over the first 4 h of anoxia exposure. This emphasizes the coordinated nature of the glycolytic suppression response to anoxia.

Having confirmed the role of reversible enzyme phosphorylation as a key mechanism of anoxia-induced MRD in marine mollusks, the question became whether this was a universal mechanism of MRD. Multiple studies have now confirmed this in many ways with demonstrations that (1) glycolytic enzymes are also targets of anoxia-induced phosphorylation in anoxia-tolerant vertebrate animals (e.g., goldfish, turtles) (see below); (2) reversible phosphorylation control of glycolytic enzyme activities also occurs in situations of aerobic MRD, such as during estivation in terrestrial snails and toads (see Text Box 15.7) or during hibernation in mammals (see Chapter 17); and (3) reversible phosphorylation regulates

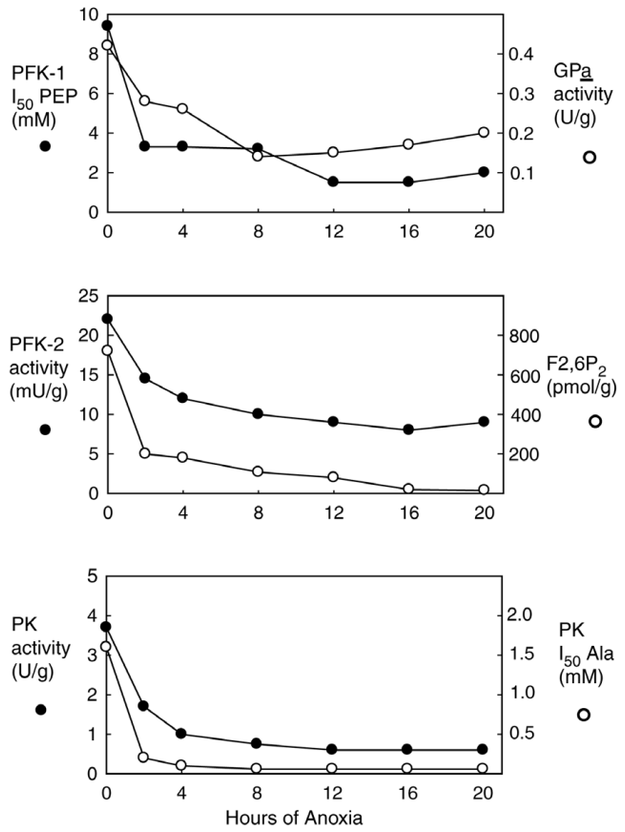

Figure 15.4 Coordinated changes in the activities and properties of glycolytic enzymes and fructose-2,6-bisphosphate ($F2,6P_2$) levels in gill of the whelk, *Busycon canaliculatum*, over the course of 20 h of anoxia exposure. Shown are (*a*) the activity of the active *a* form of glycogen phosphorylase (GP*a*) and the I_{50} value for phosphoenolpyruvate (PEP) of 6-phosphofructo-1-kinase (PFK-1), (*b*) the activity of 6-phosphofructo-2-kinase (PFK-2) and levels of its product, $F2,6P_2$, (*c*) the activity of pyruvate kinase (PK) and its I_{50} value for L-alanine. Activities are in units (or milliunits) per gram wet mass and concentrations are in millimolar or pmol/g wet mass. [Modified from K. B. Storey (1993). Molecular mechanisms of metabolic arrest in mollusks. In P. W. Hochachka, P. L. Lutz, T. J. Sick, M. Rosenthal, and G. van den Thillart, (eds.). *Surviving Hypoxia: Mechanisms of Control and Adaptation*. CRC, Boca Raton, FL, pp. 253–269.]

multiple other cell functions during hypometabolism, including suppression of pyruvate dehydrogenase activity (regulating pyruvate entry into the TCA cycle), the activities of ion-motive ATPases, and inhibition of the ribosomal translation machinery.

Control of Carbohydrate Metabolism in Anoxia-Tolerant Vertebrates Anoxia-induced reversible phosphorylation supplies coordinated control over the activities of

TEXT BOX 15.7 ESTIVATION

Estivation is a state of aerobic torpor. The underlying trigger for estivation is typically arid conditions, often accompanied by a lack of food availability and high environmental temperatures. Metabolic rate is typically reduced by 70 to 90%, and water conservation strategies are used including burrowing underground, apnoic breathing patterns, formation of cocoons or other physical barriers to water loss, and elevation of body fluid osmolality (see discussion of urea accumulation in Chapter 14). The physiology and biochemistry of estivation has been most extensively studied in two groups: pulmonate land snails and anuran amphibians (such as the spadefoot toad of the American Southwest). The first demonstration that the biochemical mechanisms of metabolic arrest that are used by anoxia-tolerant animals also underlie MRD in the aerobic state of estivation occurred during studies of land snail estivation. These studies confirmed that reversible protein phosphorylation that produced stable changes in the activity states of enzymes was a general principle of MRD in both aerobic and anoxic systems.

Land snails entering estivation in response to food and water deprivation showed stable changes in the properties of glycolytic enzymes (GP, PFK-1, PK) and strong suppression of $F2,6P_2$ levels in their tissues—responses that frequently parallel the patterns seen during anoxia in marine molluscs (see Fig. 15.4). For example, PK from foot muscle of estivating *Otala lactea* showed reduced affinity for PEP substrate (a 50% increase in K_m), increased sensitivity to inhibition by L-alanine and ATP (I_{50} values decreased by 60 and 40%, respectively), and a increase in isoelectric point from pH 5.85 to pH 6.2 as compared with control snails. Significantly, these same changes in PK properties were also achieved when land snails were given anoxia exposure under a nitrogen gas atmosphere. Treatments *in vitro* with protein kinases or phosphatases showed that, as in anoxia-tolerant mollusks, reversible phosphorylation of glycolytic enzymes was responsible for estivation-induced changes in enzyme function and a cGMP-dependent protein kinase was again implicated. However, in estivating snails an additional enzyme target of control over carbohydrate metabolism was found—pyruvate dehydrogenase (PDH). The percentage of PDH present in the active, dephosphorylated form decreased from ~98% in control snails to 60% over the first 1 to 1.5 days of estivation but rebounded within 1 h when snails were aroused by spraying with water. Control over PDH allows a coordinated suppression of both glycolytic and mitochondrial carbohydrate catabolism in the estivating states. The importance of

PDH control is discussed more extensively in Chapter 16 where it is a key element of MRD in mammalian hibernation.

Estivation in vertebrates also uses these same principles of metabolic control. Isoelectric focusing of skeletal muscle extracts from spadefoot toads, *Scaphiopus couchii*, revealed the presence of two forms of PK and PFK whose proportions changed during estivation and that were interconvertible by reversible phosphorylation. Interestingly, however, in spadefoot toad muscle the effect of estivation was to increase the proportion of the dephosphorylated enzymes, and kinetic analysis revealed that, contrary to the situation in mollusks, the dephosphorylated enzymes were, in this case, the less active enzyme forms. For muscle PFK this fits well with the known controls on vertebrate muscle PFK. Phosphorylation of vertebrate muscle PFK occurs during exercise and the phospho-enzyme shows increased binding to myofibrils in active muscle that helps to localize glycolytic ATP production near the sites of ATP use by the myofibrillar ATPase (see Chapter 14). Thus, the increased content of the low-phosphate form of PFK in muscle of estivating toads is consistent with a reduced glycolytic rate accompanying the overall MRD of the estivating state.

The changes in phosphorylation state of both PK and PFK in toad organs correlated well with the suppression of the activities of both protein kinases A and C during estivation. As in other situations of MRD, the metabolic potential of organs is largely retained in estivation with relatively few changes seen when the maximum activities of a wide variety of metabolic enzymes were surveyed. However, one general exception to this was the response of enzymes of antioxidant defense, which were generally lower in estivating animals than in aroused toads. This is consistent with the idea of reduced oxidative stress under the low metabolic rate of the estivating state and the need to restore defenses against ROS when metabolic rate rises by severalfold when animals arouse from torpor.

For more information on estivation consult K. B. Storey. Life in the slow lane: molecular mechanisms of estivation. *Comp Biochem Physiol A* **133**:733–754 (2002).

glycolytic enzymes in tissues of anoxia-tolerant vertebrates. Regulation of liver enzymes is particularly prominent, and this is related to the role of liver as the central store of carbohydrate fuel in the vertebrate body (see Chapter 9). Apart from liver, skeletal muscle is the only organ with major glycogen reserves, but muscle does not export glucose (its glucose transporter is for import only). The main way that muscle can export fuel is as end products, lactate or alanine, that are typically products of muscle work. These are significant aerobic fuels for organs such as heart, but they are useless under anoxic conditions. Hence, glucose supply by liver is key to the anoxia survival of the whole animal, particularly for the survival of brain, which has only low levels of endogenous glycogen. Not surprisingly, then, the percentage of GP present as the active *a* form in turtle liver increased from 14 to 26% after 5 h of anoxia exposure, but note that this increase is not large for an enzyme that is supplying glucose fuel to all other organs. Furthermore, GP activity was generally unaffected in other organs, which is consistent with the idea of strong MRD in anoxia that lowers ATP demand to a rate that can be fueled by low rates of carbohydrate fermentation. In recent years the application of metabolic control analysis to multiple systems has repeatedly highlighted the importance of substrate supply, rather than ATP consumption, in regulating metabolic rate, so this suggests that control by liver over substrate supply to the whole body may be a critical part of MRD.

Table 15.2 shows that anoxia exposure of goldfish triggers stable and coordinated changes to the properties of three liver glycolytic enzymes, very similar to the situation seen in marine mollusks. Glycogen phosphorylase activity is reduced by two mechanisms: decreases in the total amount of enzyme and in the percentage of enzyme in the active *a* form. In combination, this lowers the activity of GP*a* in anoxia to just 50% of the aerobic value (note

TABLE 15.2 Effect of 24 h in N_2 Bubbled Water on Properties of Glycolytic Enzymes in Goldfish Liver[a]

	Aerobic	Anoxic
Glycogen phosphorylase, U/g	3.6	2.5
% *a*,	80	55
Fructose-2,6-P_2, nmol/g	7.77	0.75
6-Phosphofructo-1-kinase		
$S_{0.5}$ fructose-6-P, mM	1.56	2.33
K_a fructose-2,6-P_2, μM	0.17	0.08
K_a AMP, μM	0.29	0.37
I_{50} ATP, mM	1.43	1.25
Isoelectric point	3.86	4.30
Pyruvate kinase		
$S_{0.5}$ PEP, mM	0.45	0.80
K_a fructose-1,6-P_2, μM	0.20	0.30
I_{50} L-alanine, mM	25.1	11.6
Isoelectric point	3.87	4.30

[a]All values are the means of 3–8 independent determinations and all anoxic values shown are significantly different from the corresponding aerobic values.

Source: Data are compiled from K. B. Storey (1987). *Physiol Zool* **60**:601–607 and M. S. Rahman and K. B. Storey (1988). *J Comp Physiol* **157**:813–820.

that, unlike the situation in turtle liver mentioned above, GP*a* activity was very high in liver of control fish). Anoxia exposure stimulated multiple changes in the properties of PFK-1, including reduced affinity for its substrate, fructose-6-P, and altered sensitivities to both activators and inhibitors. The K_a value for $F2,6P_2$ decreased by 50% in anoxia, which indicates that the enzyme is more sensitive to this activator, but liver $F2,6P_2$ content dropped 10-fold in anoxia so, overall, the influence of $F2,6P_2$ on PFK-1 is much reduced in anoxia. This makes sense because in liver $F2,6P_2$ acts as an anabolic signal to promote carbohydrate use for biosynthetic purposes, and such activity needs to be suppressed under anoxic conditions. Stable modification of PK properties was also seen with reduced substrate affinity, decreased sensitivity to activation by $F1,6P_2$, and increased sensitivity to alanine inhibition, all changes that would suppress PK activity in anoxia. Furthermore, both PFK-1 and PK show distinct changes in their isoelectric points under anoxia; this indicates a change in the net charge of the proteins and is frequently diagnostic of protein phosphorylation. Other organs of goldfish show selective changes in many of these parameters in anoxia, consistent with organ-specific metabolism in anoxia. We have also documented stable changes to the properties of GP, PFK-1, and PK as well as $F2,6P_2$ levels in response to anoxic submergence in the organs of freshwater turtles.

Signaling Mechanisms Inducing Events of Hypometabolism The clearly defined differences in kinetic parameters between phosphorylated and dephosphorylated enzyme forms (e.g., the 25-fold difference in the PK I_{50} for alanine seen in Fig. 15.4) provides a useful tool that can be exploited to explore the regulation of anoxia-induced enzyme phosphorylation. The effects of hormone second messengers and other treatments in mimicking anoxia-induced effects on mollusk enzymes have been explored *in vitro* with isolated tissues or tissue extracts. For both PK and PFK-1 such treatments were highly consistent in showing that anoxia-induced effects on the kinetic properties of the enzymes in marine mollusks were mimicked by treatments with cyclic 3′,5′-guanosine monophosphate (cGMP). For example, when extracts of radular retractor muscle from aerobic whelks were incubated with Mg–ATP plus the second messengers of protein kinases A [cyclic 3′,5′-adenosine monophosphate (cAMP)], C (Ca^{2+} + phorbol 12-myristate 13-acetate), or G (cGMP) and then PK kinetics were evaluated, the K_m value for PEP and the I_{50} value for alanine changed significantly only in response to cGMP. Treatment with cGMP raised the K_m from 0.05 mM (control) to 0.23 mM and lowered the I_{50} from 10 to 2.5 mM, replicating the effect of anoxia on the enzyme. This evidence and similar results for other species have clearly linked the events of anoxia-induced MRD in marine mollusks to control by the protein kinase G (PKG) mediated signal transduction pathway. However, despite this documented link, to date there has been little analysis of this protein kinase in facultative anaerobes. We have documented the presence of a PKG in marine whelks, *B. canaliculatum*, and shown that it stimulates the phosphorylation of PK. Furthermore, we also found a second-messenger-independent protein kinase that specifically phosphorylates PK but not PFK-1 or GP. This specific PK kinase is probably regulated by PKG, the cascade system of control allowing powerful regulation of the PEP branch point in anoxia. A serine/threonine PK phosphatase of the type 2C group reverses the effects of PK kinase. In other systems, the PKG signal transduction pathway is triggered by nitric oxide (NO). Although NO has not yet been explored as a possible regulator of aerobic–anaerobic transitions in anoxia-tolerant mollusks, this is clearly an area with exciting new research possibilities.

Another possible regulatory factor in MRD is acidosis. A reduction in cellular pH values accompanies all arrested states, in both anaerobic systems and situations of aerobic metabolic arrest such as hibernation and estivation. However, whether pH change triggers or controls MRD is questionable. Under anoxic conditions, the slow development of acidosis over the long term does not fit well with the rapid and early changes in enzyme phosphorylation patterns that trigger MRD or with the fact that anoxia-tolerant species clearly take steps to minimize the extent of tissue acidosis. Nonetheless, as we will see several times in the discussion of metabolic arrest in this and the next chapter, cellular acidosis clearly creates a metabolic context that facilitates various events of metabolic arrest. Associated with acidosis and likely the more important signaling influence on MRD is hypercapnia (high CO_2). Respiratory acidosis due to CO_2 retention as a result of breath-hold (apnoic) breathing patterns is a feature of most arrested states. The influence of high CO_2 has been strikingly illustrated with land snails, where the simple elevation of CO_2 in the air (pCO_2 raised to 65 mmHg) resulted in a decrease in the oxygen consumption of active snails by 50% within 1 h. However, as soon as CO_2 was removed, metabolic rate rebounded. Hypercapnia also stimulated a similar reduction in oxygen consumption by anoxia-tolerant marine worms. CO_2 retention is now a well known event during entry into many situations of MRD, and it is rapidly reversed when hypometabolism ends. For example, hyperventilation to clear accumulated CO_2 is one of the first events when hibernating mammals begin the arousal process. Signaling and regulatory events linking CO_2 and organ enzymatic responses during dormancy have received very little exploration to date, but some of our results with whelk muscle PK (Table 15.3) suggest that this could become a useful area of study.

TABLE 15.3 Effect of Anoxia, pH Change, and CO_2 on Pyruvate Kinase Kinetics During *in vitro* Incubations of Whelk Radular Retractor Muscle[a]

	Tissue pH	K_m PEP (mM)	I_{50} Alanine (mM)
Aerated, control	7.64	0.12	18.3
Aerated, pH 5.5	6.39	0.29	7.8
100% N_2	6.97	0.54	3.20
95 : 5% N_2 : CO_2	6.64	1.80	1.00
95 : 5% N_2 : CO_2, pH 9.5	7.35	1.62	0.70

[a]Incubations were in artificial seawater; in two cases, seawater pH was altered (pH 5.5, pH 9.5) in order to lower or raise tissue pH. Lowering tissue pH in aerated tissues had minimal effect on PK properties compared with the effects of anoxia (N_2 gas bubbling). The presence of CO_2 in anoxic incubations stimulated much greater changes in PK properties that were not altered when tissue pH was held artificially high.

Source: Data are reworked from S. P. J. Brooks and K. B. Storey (1989). *J Exp Biol* **145**:31–43.

These studies used *in vitro* tissue incubations and showed that the anoxia-induced increase in the K_m value for PEP and decrease in the I_{50} value for alanine of PK were much more pronounced when anoxia exposure was the result of bubbling seawater with a N_2–CO_2 (95 : 5) mixture rather than with 100% N_2 alone. Furthermore, these changes to enzyme properties still occurred even if tissue pH was kept artificially high in anoxia, indicating that it is not the acidifying effect of CO_2 that stimulated the enzyme modification.

Control of Membrane Transport and Related Functions

Membrane Ion-Motive ATPases and Ion Channels ATP consumption by ion-motive ATPases is one of the biggest energy expenditures in all cells as well as one of the most crucial. Not only does the failure of transmembrane ion gradients impair normal cell functions (e.g., signal transmission, neural conductivity, muscle contactility) but also membrane depolarization can trigger cascades of degenerative events, as explained earlier. It is not surprising, therefore, that anoxia-tolerant animals strongly reduce ATP expenditures on transmembrane ion movements in anoxia and coordinate a net suppression of both influx and efflux of ions by a process that has been called "channel arrest." This phenomenon has been particularly well studied in the brain of anoxia-tolerant turtles, and the summary here will pertain mostly to those animals.

Ion gradients across the plasma membrane (Na^+ and Ca^{2+} high outside, K^+ high inside cells) are maintained by ATP-dependent ions pumps that move ions against their concentration gradients versus facilitated movements of ions down their concentration gradients through ion channels. To reduce ATP expenditure without dissipating ion gradients, ion channel conductance is suppressed in anoxia. Studies have shown that K^+ leakage is significantly lower in brains of anoxic turtles compared with aerobic controls and that Ca^{2+} channel activity decreases progressively with length of anoxia. Closure of conductance channels is also accompanied by a decrease in sodium channel abundance in plasma membranes and silencing of NMDA receptors. NMDA receptors are a subfamily of glutamate receptors; they are high-flux, ligand-gated cation channels that are highly permeable to Ca^{2+} and a major source of Ca^{2+} entry into anoxic/ischemic brain. Strong inhibition of Ca^{2+} influx into turtle cells in anoxia is particularly crucial because Ca^{2+} levels in cerebrospinal fluid can rise six-fold within 10 days during anoxic submergence of turtles due to the mobilization of shell calcium carbonate to enhance plasma buffering of accumulating lactate. Activity of the Na^+K^+–ATPase ion pump in turtle brain was also reduced by 30 to 35% during anoxia but was rapidly reversible upon reoxygenation. The mechanism of Na^+K^+–ATPase suppression is likely to be reversible protein phosphorylation, which we have shown underlies the inhibition of this ion pump in hibernating mammals (see Chapter 16). Silencing of NMDA receptors has been linked with three mechanisms operating on different time scales: (a) short term—within 8 min of anoxia exposure NMDAR activity (Ca^{2+} influx and open probability) in turtle brain was reduced by 50 to 60% mediated by activation of protein phosphatases 1 or 2A; (b) intermediate term—by 2 h of anoxia a rise in intracellular Ca^{2+} of about 35% would trigger Ca^{2+}/calmodulin-mediated suppression; and (c) long term—reversible removal of NMDARs from the plasma membrane occurred when anoxia exposures exceeded 3 days.

Studies of anoxia-tolerant animals and of ischemia–reperfusion in hypoxia-sensitive mammals both agree on the importance of adenosine as a signaling molecule that triggers adaptations to protect tissues from injury. Adenosine is released by hypoxic or ischemic tissues and stimulates multiple protective effects. In the ischemic mammalian heart, for example, these include a reduction in myocardial oxygen demand through negative effects on the rate and force of contraction, promotion of ATP production from glycolysis, a reduction in oxygen free radical release, a stimulation of vasodilation, and an inhibition of neutrophil and platelet aggregation so that blood vessels do not become blocked during the interruption of blood flow. Adenosine is synthesized from AMP by the enzyme 5′-nucleotidase, and adenosine levels rise quickly under anoxia or ischemia stress and stimulate one of four subtypes of G-protein-coupled adenosine receptors. Signaling via the A1 adenosine receptor is particularly important and contributes to preconditioning, the phenomenon

whereby short initial periods of ischemia greatly reduce the metabolic damage that accrues from a subsequent extended ischemic challenge. The importance of adenosine to MRD was confirmed in studies with brain of anoxia-tolerant turtles. Adenosine in turtle brain peaked first after 2 to 3 h of anoxia exposure and then subsequent pulses of adenosine release occurred with longer anoxia exposures. Interestingly, adenosine (but not any of several other putative neurotransmitters) increased three- to four-fold under anoxia and hypercapnia in the anoxia-tolerant marine worm, *Sipunculus nudus*, and adenosine infusion into coelomic fluid of normocapnic worms suppressed oxygen consumption. These data suggest that adenosine plays a role in metabolic rate depression across phylogeny.

One of the known effects of adenosine action in turtle brain is a suppression of the activities of ATP-dependent ion channels, mediated by the A1 receptor. Suppression of K^+ leakage has been documented and, in particular, adenosine caused a strong suppression of excitatory neurotransmission in turtle brain by reducing Ca^{2+} entry into cells via NMDAR by more than 50%. The positive effects of adenosine during preconditioning are mediated intracellularly by at least two signal transduction pathways (in heart): protein kinase C (PKC) and the p38 mitogen-activated protein kinase (MAPK). In our studies of turtle liver and brain we found PKC activation during anoxia in both organs; the percentage of membrane-bound (active) PKC rose from 21 to 45% within 1 h of anoxia exposure in turtle liver, whereas in hindbrain the percent bound was 42% in controls and 69% in turtles after 5 h of anoxic submergence. Interestingly, PKC was suppressed in another part of the brain (the cerebrum) after 5 h of anoxia, a result that may indicate differential responses or differential timing of the development of anoxia-protective mechanisms in different parts of the brain. Hence, studies of the downstream effects of PKC and p38 signaling in anoxic organs are warranted. One of the intracellular end results of adenosine signaling (and of preconditioning) appears to be opening of the mitochondrial ATP-dependent K^+ (K_{ATP}) channel, which may have one of several protective consequences.

Adenosine also stimulates NO release in the vascular endothelium, and NO has multiple effects in ischemic/anoxic tissue, including stimulation of vasodilation, activation of PKC, and stimulation of K_{ATP} channel opening. Recall that NO can act via the cGMP signal transduction pathway and that cGMP has been shown to stimulate multiple adjustments by anaerobic energy metabolism in anoxia-tolerant mollusks. Hence, we now have good evidence of the types of signaling molecules, receptors, signal transduction pathways, and at least some of the subcellular targets that are involved in anoxia tolerance. What is needed now is a comprehensive characterization of all of these events within a single anoxia-tolerant animal model system in order to confirm or refute the importance of the many molecular mechanisms of receptor signaling and signal transduction that have been implicated from studies of both anoxia-sensitive and anoxia-tolerant systems.

Mitochondrial Functions The mitochondrion is known as the powerhouse of the cell because its primary function is the generation of ATP from the oxygen-dependent catabolism of fuels. But what happens to mitochondria under anoxic conditions? Clearly the electron transport system becomes reduced, TCA cycle activity is halted, and ATP synthesis stops, but during anaerobiosis the organelles must be maintained in a viable state in order that aerobic metabolism can be rapidly resumed and apoptosis avoided (see Chapter 8 for the role of mitochondria in apoptosis). Similar to the situation with the plasma membrane, key functions of the mitochondria are dependent on the maintenance of ion gradients across membranes, creating a membrane potential difference. In particular, maintenance of the proton-motive force is key to mitochondrial survival.

Under aerobic conditions, the proton-motive force is established by proton pumping out of the matrix into the intermembrane space via complexes I, III, and IV of the respiratory chain. Proton return is via the F_1F_0−ATPase and drives ATP synthesis. Under anoxic conditions, the respiratory chain becomes reduced and can no longer pump H^+. To prevent the collapse of the proton-motive force, mitochondrial "treason" occurs; the organelles switch roles from being major ATP producers to being potential major users of cellular ATP. The occurs because the F_1F_0−ATPase reverses its function and becomes an ATP-dependent proton pump that takes over the role of H^+ extrusion from the matrix. The ATP needed to fuel this function is imported from the cytosol by an accompanying reversal of the adenine nucleotide translocator. Clearly, then, it would be advantageous for organisms to limit this mitochondrial consumption of ATP in anoxia. Recent studies have considered two possibilities: (1) direct inhibition of F_1F_0−ATPase activity and (2) reduced proton conductance by the inner membrane. The data favors direct inhibition of the F_1F_0−ATPase in anoxia. In studies using intact mitochondria from skeletal muscle of anoxia-tolerant frogs, the calculated rate of ATP consumption by the F_1F_0−ATPase in anoxia was only ~4% of the enzyme's corresponding rate of ATP production under aerobic conditions. Even so, this amount of ATP consumption by the F_1F_0−ATPase could still consume ~9% of the total ATP turnover in anoxic frog muscle, and together with the ~75% of anoxic ATP turnover that is devoted to Na^+K^+−ATPase activity, it is obvious that anoxic tissues spend a huge proportion of their energy budget on maintaining ionic homeostasis. The

mechanism of F_1F_0−ATPase inhibition in anoxia-tolerant species is still being investigated but could involve an F_1−ATPase inhibitory subunit (IF_1) that has been identified in a number of species and is known to bind to the ATPase under low pH and nonenergizing conditions to inhibit ATP hydrolysis.

Another consideration for systems under oxygen limitation (e.g., severe hypoxia) is the mitochondrial proton leak, protons that leak back into the matrix without passing through the F_1F_0−ATPase and driving ATP synthesis. The futile cycling of protons that results partially uncouples respiration, reducing the efficiency of energy conservation. About 20% of mammalian standard metabolic rate has been attributed to such mitochondrial proton cycling. In situations of low oxygen availability (hypoxia) such a wasteful consumption of oxygen seems counterintuitive, especially in hypoxia-tolerant systems that use MRD to suppress ATP expenditure when oxygen is limiting. So, what happens to proton leak in hypoxia? Studies with muscle mitochondria from aerobic versus deeply hypoxic frogs indicated an ∼50% reduction in proton leak under hypoxic conditions (based on state 4 respiration rates). Furthermore, the reduction in proton leak seemed to be caused by a decrease in the activity of the electron transport chain and not by a change in inner membrane proton conductance. The data suggest that proton cycling is reduced in parallel with the reduction in metabolic rate and that this results from a decrease in the rate of substrate oxidation and a decrease in the size of the proton-motive force in hypoxia. Indeed, under low oxygen pressures such as actually occur *in vivo* in the intracellular microenvironment of the mitochondria in normoxia (estimated to be 0.3 to 0.4 kPa), recent studies have shown that proton leak and uncoupled respiration are actually very low and that phosphorylation efficiency is high. The implication is that the high-proton-leak rates measured in most *in vitro* studies of isolated mitochondria (that use air-saturated incubation medium at ∼20 kPa) may actually be a response of the organelles to hyperoxia that may contribute to minimizing oxidative stress under unusually high oxygen concentrations.

Control of Protein Synthesis

Protein synthesis consumes a substantial portion of cellular ATP turnover under aerobic conditions (Table 15.1), using about 5 ATP equivalents per peptide bond formed. Synthesis is well known to be sensitive to the availability of ATP and amino acids, and suppression of protein synthesis appears to be a proactive response to multiple stress situations, such as starvation and hypoxia in mammals. Inhibition of protein synthesis provides substantial energy savings to cells under stress. It is not surprising, therefore, that multiple studies have confirmed that protein synthesis is suppressed in an organ-specific manner as part of MRD in stress-tolerant animals, not only as part of anoxia tolerance but also in hibernation, estivation, and diapause. For example, in marine snails (*L. littorea*) ^{3}H-leucine incorporation into protein by hepatopancreas in anoxic snails was just 50% of the aerobic value. Furthermore, the inhibition of protein synthesis occurred very quickly, dropping within the first 30 min of anoxia exposure and remaining low for the remainder of a 48-h excursion. The proactive nature of this response is illustrated by the fact that ATP limitation for protein synthesis would not a factor within the first 30 min under the nitrogen gas atmosphere because ATP levels in tissues of anoxic snails only begin a slow decline after many hours of anoxia exposure. Protein synthesis inhibition also occurs under anoxic conditions in vertebrates; recall from Table 15.1 that the rate of protein synthesis was reduced by 93% in turtle hepatocytes under anoxia whereas a 95% inhibition was measured in liver of anoxic goldfish. Recall also that the fractional use of cellular ATP by protein synthesis is disproportionally reduced in anoxia; protein synthesis consumed 36.1% of cellular ATP turnover in aerobic turtle hepatocytes but only 21% in anoxic hepatocytes Similarly, when killifish embryos enter diapause, the fractional use of ATP by protein synthesis drops from 36% to negligible. Strong inhibition of protein synthesis in a hypometabolic state makes intuitive sense. The biosynthesis of new proteins is something of a "luxury item" that can be forfeited under stress in favor of the maintenance of more critical cellular functions such as sustaining central ATP-producing systems, the ability to sense and respond to stimuli, and osmotic and ionic balance.

Inhibition of protein synthesis during hypometabolism could be regulated in two ways: (1) by reduced messenger ribonucleic acid (mRNA) substrate availability and (2) by inhibition of the ribosomal translational machinery. Substrate availability is an influence on any metabolic process, but, in general, neither total mRNA content nor the specific mRNA transcript levels of most constitutively expressed genes are altered in hypometabolic states; this has been confirmed in studies with disparate systems, including anoxia survival in mollusks and brine shrimp and hibernation in mammals (Chapter 16). For example, when we used complementary DNA (cDNA) array screening to assess anoxia-responsive gene expression in *L. littorea*, 89% of genes assessed showed no change in transcript levels in anoxic snails, approximately 10% showed putative up-regulation, and only 0.6% showed suppressed mRNA levels in anoxia.

Instead, reversible control over the rate of protein synthesis in anoxia is vested primarily in the regulation of the ribosomal translation machinery. Two mechanisms are particularly important: (1) reversible phosphorylation of selected proteins of the translational machinery and (2)

the physical state of ribosome assembly. Ribosomes are large multimeric complexes of proteins; individual subunits have structural, enzymatic, and regulatory roles in protein synthesis. The regulation of translation is discussed in detail in Chapter 7, so the current discussion will focus on what is known about the targeted suppression of translation during anoxia-induced MRD. As noted above, the levels of most mRNA transcripts are maintained in arrested states, but some are up-regulated to provide new proteins with specific functions for survival under stress (see the later section on anoxia-induced gene expression). Therefore, control of translation in anoxia must accomplish three goals: (1) strong suppression of the overall rate of protein synthesis in cells, (2) stabilization and/or storage of most existing mRNA transcripts so that they are not degraded over long periods of hypometabolism and are available for translation when normal metabolic functions are reestablished, and (3) provide for the selected biosynthesis of a few stress-specific proteins. How is this accomplished?

Control of Translation by Reversible Protein Phosphorylation Both translation initiation and polypeptide elongation are inhibited as parts of MRD. Both have been traced to reversible phosphorylation control over specific ribosomal subunits (for a discussion of the regulation of elongation, see Chapter 16). A key element in translation initiation is the eukaryotic initiation factor 2 (eIF2), which introduces initiator methionyl–transfer RNA (tRNA) into the 40S ribosomal subunit (Fig. 15.5) (also see Chapter 7). Phosphorylation of the alpha subunit of eIF2 (eIF2α) blocks this function because phospho-eIF2α is an inhibitor of the guanine nucleotide exchange factor eIF2B and prevents the recycling of eIF2α between successive rounds of peptide synthesis. This mechanism of global protein synthesis inhibition is well known in all eukaryotic systems and is a response to stresses, including viral infection, heat shock, nutrient deprivation, induction of apoptosis, and hypoxia/ischemia. For example, when protein synthesis in rat brain was measured during recovery after a 10-min ischemic episode, net synthesis was reduced by 85%, and this corresponded with $\sim$23% of eIF2α in the phosphorylated state compared with $\sim$1% under normoxia. We found a similar response in marine snails under anoxia. Western blotting using antibodies that detected both total eIF2α and the phosphopeptide showed a 15-fold elevation of phospho-eIF2α in hepatopancreas of anoxic snails with no change in total eIF2α content (Fig. 15.5) (refer also to Text Box 14.1 for an explanation of the methodology used). This correlated with a 50% decrease in total protein biosynthesis in anoxia. However, this situation was rapidly reversed during aerobic recovery and, within 1 h after oxygen was reintroduced, phospho-eIF2α content was fully depleted.

To date, studies of the inhibition of translation initiation during metabolic arrest have focused mainly on the regulation of eIF2α, but protein synthesis inhibition during ischemia or starvation in mammals also involves controls on other initiation factors. These may also be targets of reversible suppression during MRD. For example, the eukaryotic initiation factor 5 (eIF5) is also regulated by reversible protein phosphorylation; it acts as a GTPase-activating protein to promote GTP hydrolysis within the 40S initiation complex [composed of 40S*eIF3*AUG*Met-tRNA(f)*eIF2*GTP] (see Fig. 6.4). Both ischemia and amino acid starvation also regulate eukaryotic initiation factor 4. Subunit eIF4E and its binding protein (4E-BP1) are both dephosphorylated under stress in rat brain (concurrent with eIF2α phosphorylation) with dephosphorylation of 4E-BP1 allowing it to bind to eIF4E and inactivate it. By contrast, subunit eIF4G shows proteolytic fragmentation after ischemia/reperfusion.

The involvement of protein phosphorylation in the control of translation inhibition during anoxia-induced MRD obviously implicates the actions of one or more protein kinases in carrying out this process and suggests the mechanism by which protein synthesis inhibition can be coordinated with the suppression of other metabolic functions in anoxia. However, it is yet known which protein kinases mediate the response to anoxia. Several protein kinases can phosphorylate eIF2α in mammals, representing multiple signals (e.g., ischemia, amino acid availability); these are discussed in Chapter 7.

Translation inhibition during anoxia may also involve suppression of the activities of the phosphatases that dephosphorylate the ribosomal phosphoproteins. Both protein phosphatase (PP) 1 and 2A can dephosphorylate phospho-eIF2α, and PP-1 seems to have this role *in vivo*. Studies of mammalian ischemia/reperfusion are inconclusive as to whether phosphatase control is an important factor in translation inhibition, but studies of phosphatase responses during natural MRD have shown a consistent reduction of PP-1 and PP-2A activities in tissues during anaerobiosis as well in estivation and freezing.

Ribosome Aggregation State The activity state of the protein-synthesizing machinery in a cell/tissue can generally be inferred from the state of ribosomal assembly. Active translation occurs on polysomes (aggregates of ribosomes moving along a strand of mRNA), whereas monosomes are translationally silent. Polysome dissociation has long been recognized as a cellular response to stress; for example, hypoxia, starvation, and diabetes all trigger polysome dissociation in rat tissues. Recently this mechanism has been confirmed as an integral feature of the suppression of protein biosynthesis during MRD in both anoxia-tolerant and hibernating systems (see Chapter 16).

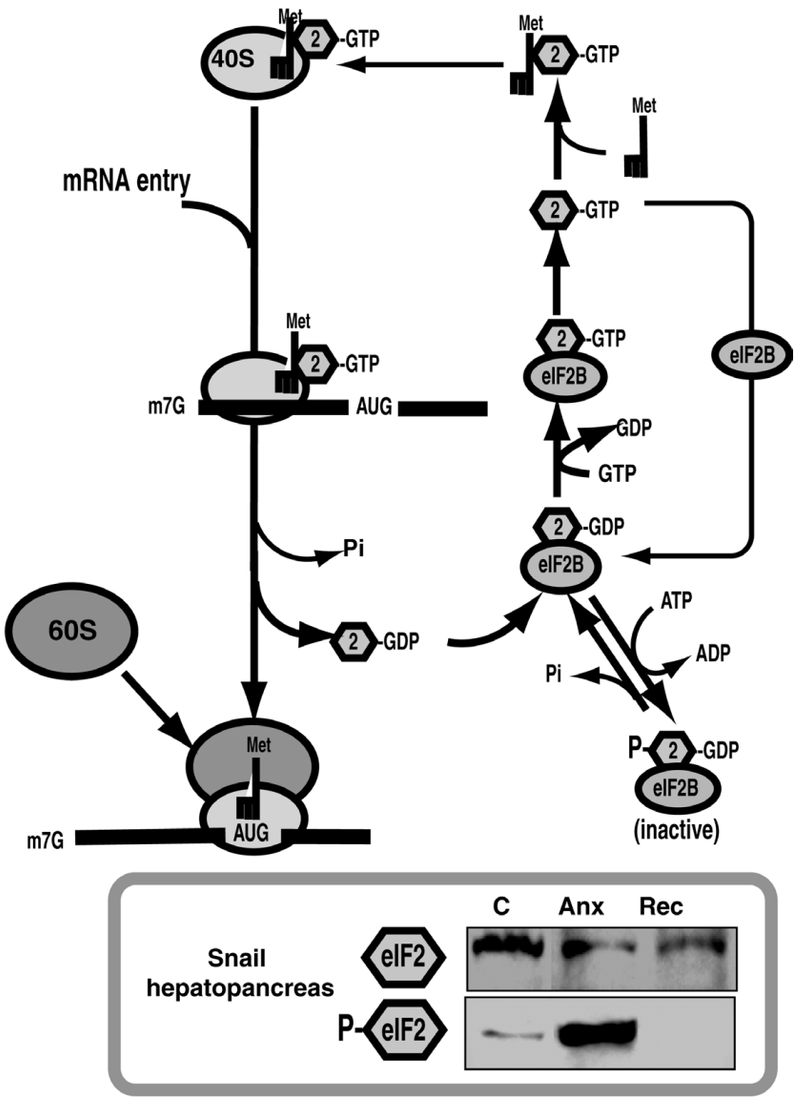

Figure 15.5 Reversible phosphorylation control of the eukaryotic initiation factor 2 (eIF2). eIF2 delivers the initiating methionine tRNA to the 40S ribosomal subunit. Phosphorylation of the α subunit of eIF2 inhibits translation because phospho-eIF2α acts as a dominant inhibitor of the guanine nucleotide exchange factor, eIF2B, and prevents the recycling of eIF2α between successive rounds of peptide synthesis. Inset shows the strong increase in the amount of phospho-eIF2α content, as determined by Western blots, in hepatopancreas from anoxic (Anx) marine snails, *Littorina littorea*, compared with aerobic controls (C). However, after 1 h of aerobic recovery (Rec) this is fully reversed and no phospho-eIF2α remains.

To assess the state of ribosomal assembly, tissue extracts are separated on a sucrose gradient. Polysomes migrate to the denser fractions, whereas monosomes are found in the less dense fractions. Figure 15.6 shows an example of the effects of anoxia exposure on the polysome profile in extracts of hepatopancreas from the marine snail, *L. littorea.* Under aerobic conditions, most ribosomes are present in the higher density polysome fractions (fractions 5 to 11 in the figure); RNA analysis via absorbance measurements at 254 nm (shown as bars) or ethidium bromide staining (not shown) both confirmed high RNA content in these fractions. Samples from each fraction were then separated on agarose gels and blotted to nylon membranes and Northern blotting was used to detect

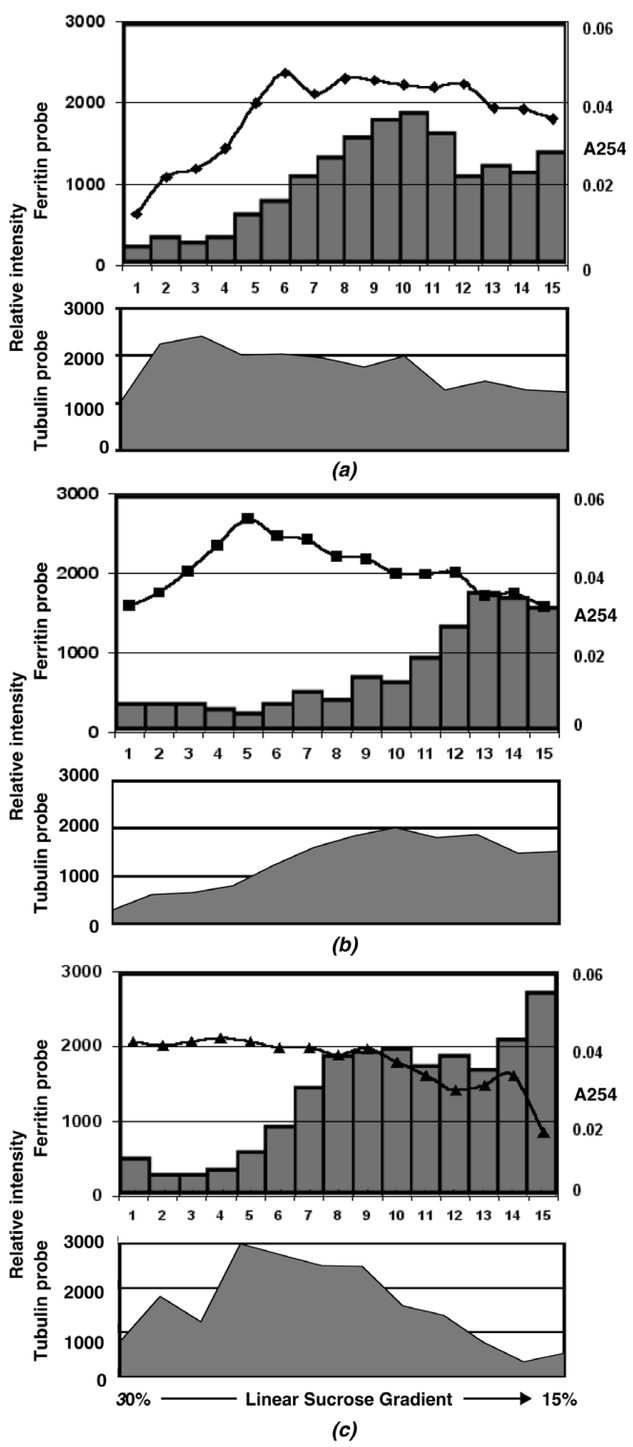

Figure 15.6 Polysome profiles of *L. littorea* hepatopancreas extracts prepared from snails under the following conditions: (*a*) aerobic, (*b*) 72-h anoxic, and (*c*) 6-h aerobic recovery after 72-h anoxia. Postmitochondrial supernatants were centrifuged on 15 to 30% continuous sucrose density gradients. Fractions were collected with high sucrose (30%) in fraction 1 and decreasing to 15% in fraction 15. In the upper panels bars show A_{254}, representing the relative amount of total RNA (mostly ribosomal RNA) in each fraction. Line graphs show the distribution of mRNA transcripts of ferritin heavy chain, a gene that is up-regulated in anoxia. Lower panels show the corresponding distribution of transcripts of a constitutively active gene, α-tubulin. To assess both ferritin and α-tubulin, mRNA was isolated from each fraction of the sucrose gradient, separated on agarose gels, blotted onto nylon membranes, and then hybridized with ^{32}P-labeled cDNA probe for each gene. Transcript levels were determined from the scanned intensity of bands on these Northern blots. [From K. Larade and K. B. Storey (2004) *J Exp Biol* **207**:1353–1360.]

mRNA transcripts of a constitutively expressed gene, α-tubulin, and an anoxia-induced gene, ferritin. Under aerobic conditions both α-tubulin (shown in the lower panels) and ferritin (line graph in upper panels) mRNA was found in the polysome fractions (Fig. 15.6*a*). This confirms a state of active translation under aerobic conditions. However, when anoxia was imposed by a nitrogen gas atmosphere, the peak of ribosomal RNA shifts to a lower density (fractions 12 and higher), indicating dissociation of polysomes. This began within 24 h of anoxia exposure and, as shown in Figure 15.6*b*, by 72 h there is little evidence of any polysomes remaining. Tubulin mRNA also shifted into the monosome fractions, which indicates that the mRNA pool of constitutively active genes is generally conserved in the hypometabolic state but not translated (a conclusion also reached from other evidence). However, transcripts of ferritin, a gene up-regulated by anoxia in *L. littorea* (see later section on gene expression), stayed in the high-density fractions in association with the few remaining polysomes. This suggests that ferritin transcripts are actively translated in the snail hepatopancreas under anoxia. Within 6 h of the shift back to aerobic conditions, Fig. 15.6*c* shows that the situation was normalized for renewed aerobic protein synthesis: Polysome reassembly had recurred, tubulin mRNA had returned to the polysome fraction, and ferritin mRNA remained with the polysomes.

Message Selection for Translation During Hypometabolism Although entry into a hypometabolic state is not the time for extensive ATP expenditure on the synthesis of new proteins, nonetheless all systems of natural MRD that have been examined (e.g., anoxia-tolerant organisms, hibernation, estivation, freezing) show up-regulation of selected gene transcripts and increased synthesis of selected proteins (see also Chapters 16 and 17). Transcript up-regulation does not always mean that levels of the protein also rise in the hypometabolic state; sometimes protein synthesis is delayed until the recovery period (see Chapter 16). However, various messages are translated during hypometabolism despite the general inhibition of ribosomal translation by the mechanisms discussed above. One way

that this is accomplished is via message selection. As mentioned earlier, subunit G of initiation factor 4 (eIF4G) undergoes proteolytic fragmentation in response to stress (e.g., ischemia/reperfusion, amino acid starvation) in mammalian systems and may be similarly affected in hypometabolic systems (see Fig. 7.4). Fragmentation changes the types of mRNAs that can be translated because intact eIF4G is needed to allow eIF4E-bound m^7G-capped mRNAs (the vast majority of cellular mRNAs) to bind to the small ribosomal subunit. When intact eIF4G is missing and eIF2α is phosphorylated and inactive, message selection changes dramatically. Only those messages that contain an internal ribosome entry site (IRES) can be translated because an IRES allows the message to bypass the normal mechanism of binding to the small ribosome subunit (see Chapter 7). For example, during amino acid starvation an up-regulation of selected gene transcripts containing an IRES occurs despite a general inhibition of the cap-dependent translational apparatus. In almost all cases, entry into a hypometabolic state is, in essence, a form of starvation since animals typically do not have access to food (consider shell valve closure in mollusks in response to aerial exposure or underground hibernation or estivation), and they typically switch to a dependence on internal fuel reserves. Therefore, it is likely that the protein synthesis inhibition response of MRD grew out of the preexisting mechanisms that regulate suppression of nonessential processes during starvation or other forms of stress. The IRES mechanism of mRNA message selection could then be employed to accomplish the specific up-regulation of selected genes that have protective functions during natural hypometabolism.

One gene that is well-known to use an IRES is the hypoxia-inducible factor-1 (HIF-1). As discussed earlier, HIF-1 mediates various gene responses to hypoxia in oxygen-sensitive organisms, and its activity is primarily regulated by the availability of the HIF-1α subunit. As such, HIF-1α must be translated under ATP-limited hypoxic conditions even if overall protein synthesis is inhibited. When the distribution of HIF-1α mRNA was assessed on a polysome profile, it was found that HIF-1α transcripts remained in the polysome fraction during hypoxia, unlike the majority of mRNA types. Sequence analysis showed that HIF-1α contains a long and guanosine-cytosine-rich 5′-untranslated region (5′UTR) that is typical of an IRES. Vascular endothelial growth factor (VEGF), one of the genes stimulated by HIF-1, also contains an IRES so this confirms the importance of an IRES for the selective up-regulation and translation of stress-responsive genes. These results suggest that a common characteristic of genes that are up-regulated during MRD and whose transcripts remain associated with polysomes for translation under stress conditions may be the presence of an IRES in the 5′UTR. To date, however, this has not been confirmed.

Protein Degradation

Given that protein synthesis is strongly suppressed in hypometabolic states but, upon arousal, animals show no significant deficit of cellular proteins, it is obvious that protein degradation must also be suppressed during hypometabolism. Indeed, Table 15.1 shows that this is the case for turtle hepatocytes when challenged with anoxia. Coordinated inhibition of the synthesis versus degradation of proteins results in a new lower net rate of protein turnover in hypometabolic states and effectively increases protein longevity. For example, the half-life of cytochrome oxidase increased 77-fold in anoxic encysted embryos of brine shrimp compared with normally developing embryos. Not only is suppressed protein turnover important for ATP savings as part of MRD but also the protein "life extension" that results minimizes the accumulation of nitrogenous end products of proteolysis during hypometabolism and, hence, the costs of processing, storing, and/or excreting wastes. Note, then, that Table 15.1 also shows that the rate of urea production drops by 70% in anoxic hepatocytes as compared with aerobic controls.

To date, very little is known about the mechanisms of proteolytic suppression during MRD. Ubiquitin-dependent proteolysis is a major mode of cellular proteolysis; conjugation with a polymer of ubiquitin tags proteins for degradation by the 26S proteasome (see Fig. 6.11), whereas monoubiquitinated proteins are targeted for endocytosis and degradation in lysosomes. Brine shrimp exposed to anoxic conditions showed ubiquitin conjugate levels that were just 7% of normoxic values, and this suggested a block on proteolysis under anoxia at the level of ubiquitin conjugation. Ubiquitination resumed in embryos when aerobic conditions were reestablished and cellular pH and ATP levels had normalized again. Our data from anoxia-tolerant turtles might also support control at the level of protein ubiquitination because we found that the activity of the multicatalytic proteinase complex (a key part of the 26S proteosome) did not change in liver of anoxic versus aerobic turtles, suggesting that inhibitory control over protein degradation was located elsewhere. However, in hibernating mammals, levels of ubiquitin-conjugated protein rose two- to threefold during torpor, suggesting that the inhibitory block in this case might be on the proteolysis machinery rather than the ubiquitin-tagging system. It is not yet known whether one or both of these mechanisms represent the common mode of proteolysis control during anaerobiosis or other modes of hypometabolism.

ANOXIA-RESPONSIVE GENE EXPRESSION

Control over the coordinated expression of subsets of genes is an excellent mechanism of biochemical adaptation and is

employed in many instances. As discussed earlier, the mammalian response to hypoxia is a coordinated up-regulation of the genes that improve oxygen delivery or glycolytic capacity of tissues, mediated by the HIF-1 transcription factor. The compensation response differs from the conservation strategies that are used by hypoxia/anoxia-tolerant species to lower the oxygen demands of tissues (e.g., MRD) and extend the time that organisms can live using endogenous reserves of fermentable fuels. The exploration of gene expression responses to anoxia by facultative anaerobes is still at an early stage but, not surprisingly, the anoxia-responsive genes identified to date are not the same as those that are up-regulated in hypoxia-sensitive organisms. For example, we found that anoxia exposure does not stimulate an increase in the activities of glycolytic enzymes in tissues of freshwater turtles or marine mollusks (oysters or littorines); the glycolytic capacities of tissues of anoxia-tolerant animals are apparently always optimized to allow smooth transitions between aerobic and anaerobic states. Indeed, until very recently, HIF-1 had not been found in anoxia-tolerant species, and it is still not known whether HIF-1 or an unknown transcription factor mediates anoxia-induced gene expression in these organisms. Logically, the involvement of a different transcription factor would make sense in order to allow anoxia-tolerant species to separate HIF-1-mediated angiogenic or erythropoietic responses that animals could need for a variety of purposes from anoxia-induced gene responses that stimulate and regulate long-term hypometabolism. To date, exploration of gene expression responses to anoxia in anoxia-tolerant species has focused on two main groups: freshwater turtles and marine mollusks.

Gene Expression Responses to Anoxia in Turtle Organs

Submergence in nitrogen-bubbled water is widely used to study both metabolic and gene expression responses to anoxia in turtles; in cold water, turtles can endure many weeks of this treatment, which mimics the conditions of underwater winter hibernation. Our first analysis of anoxia-responsive genes in turtles came from screening of a cDNA library prepared from heart of anoxia-exposed turtles, *Trachemys scripta elegans*. This revealed the anoxic up-regulation of four genes, and very surprisingly, three of these were encoded on the mitochondrial genome. One was homologous with the mitochondrial WANCY (tryptophan, alanine, asparagine, cysteine, and tyrosine) tRNA gene cluster, another was identified as the gene (*Cox1*) for cytochrome C oxidase subunit 1 (COI), and the third was *Nad5* encoding subunit 5 of NADH-ubiquinone oxidoreductase (ND5). When we performed comparable screening of a cDNA library made from brain of anoxic turtles, mitochondrially encoded genes were again up-regulated, this time represented by subunit 4 of ND as well as cytochrome *b* (CYTb). ND4/5, CTYb, and CO1 are components of complexes I, III, and IV, respectively, of the electron transport chain. All of these complexes are large polymers; for example, complex I can contain as many as 42 polypeptide subunits whereas complex IV has 13 dissimilar subunits. Six of the subunits of complex I and 3 of complex IV are encoded on the mitochondrial genome, whereas the rest are nuclear (see Chapter 8). Northern blots revealed a 3- to 5-fold increase in *Nad5* and *Cox1* transcript levels within 1 h of anoxic submergence in heart, whereas *cytb* and *Nad4* rose by about 6- and 12-fold in liver over the same time. All four transcripts showed differential up-regulation in other organs as well and transcript levels were reduced again within 1 to 5 h after a return to aerobic conditions. The significance of the up-regulation of these mitochondrially encoded proteins in response to anoxia remains unknown, but we are now finding that the phenomenon is widespread during entry into hypometabolism, occurring also during hibernation and freeze tolerance (see Chapters 16 and 17). Hence, there is growing evidence that mitochondrial gene expression as well as specific controls on mitochondrial enzymes/proteins is an integral part of anoxia tolerance. Recall the discussion earlier in the chapter about the regulation of the F_1F_0–ATP synthase in anoxia, and in Chapter 16 we outline reversible phosphorylation control of ND (complex I) by pyruvate dehydrogenase kinase (inhibiting) and mitochondrial protein kinase A (activating). ND is also now recognized as the primary site of superoxide production by the electron transport chain, and this superoxide produced by ND is known to have important signaling roles in cells. Thus, the combination of new information on the regulation of mitochondrial enzymes and new discoveries about mitochondrial gene up-regulation during transitions to hypometabolic states show that the mitochondria have important regulatory roles in the control of cellular metabolic rate via mechanisms that we are just only beginning to appreciate.

Gene Expression Responses to Anoxia in Marine Snails

Recent studies of anoxia-induced gene expression in our laboratory have focused on the marine gastropod, *L. littorina*, and have documented the anoxia responsiveness of genes including ribosomal protein L26, ferritin heavy chain, metallothionein, cytochrome *c* oxidase subunit 2 (*Cox2*), and several novel genes. *Cox2* joins the list of mitochondrially encoded genes that are up-regulated under stresses, including anoxia, that initiate MRD. One of the novel genes, named *kvn*, showed a six-fold up-regulation of transcript levels in *L. littorea* hepatopancreas during anoxia exposure that was reversed within 1 h of aerobic recovery. The gene codes for a protein of 99 amino acids with a predicted molecular weight of 12 kDa. A 15-residue

hydrophobic signal sequence at the N terminal, as well as a lack of any known retention signals or subcellular localization motifs, suggests that the protein is excreted into the hemolymph. Active translation during anoxia was inferred from the high proportion of *kvn* transcripts in the polysome fraction. Ongoing studies are seeking its function.

Transcript levels of the gene encoding the ribosomal protein L26 rose three- to fivefold in *L. littorea* tissues over the course of 2 to 4 days of anoxia exposure but fell again within 1 h of aerobic recovery. This pattern suggests a functional need for enhanced L26 protein in anoxia, and indeed, nuclear run-off assays (explained in Chapter 14) confirmed that the change in L26 transcript levels was the result of increased transcription of the L26 gene, not an alternative form of regulation such as transcript stabilization or inhibition of mRNA degradation. The L26 protein resides at the interface of the large and small ribosomal subunit, apparently at or near the ribosomal A site, where it is likely involved in subunit interactions. L26 can be crosslinked to elongation factor 2 (eEF-2), implicating it as a protein involved in forming the region that binds eEF-2 to the 60S ribosomal subunit preceding translocation of peptidyl-tRNA from the A to the P site during peptide bond formation. The link with eEF-2 is interesting because, as will be discussed in detail in Chapter 16, inhibitory control of eEF-2 is one of the important mechanisms involved in suppressing protein synthesis in hypometabolic states. The possibility exists that anoxia-induced up-regulation of L26 could contribute to eEF-2 control. Hence, once again, we have evidence that the control of transcription, via multiple mechanisms, is a critical component of anoxia survival.

Anoxia exposure of *L. littorea* also stimulated the up-regulation of two metal-binding proteins with roles in antioxidant defense, ferritin and metallothionein. Ferritin transcripts rose about twofold in anoxia (Western blots showed that protein also increased), whereas metallothionein transcripts increases three- to fivefold within 12 h of anoxia. As a resident of the intertidal zone at high latitudes, *L. littorea* is also freeze tolerant (see Chapter 17), and freezing is an anoxic/ischemic stress because ice formation halts the circulation of hemolymph to all tissues. Not surprisingly, then, metallothionein transcript levels were elevated just as strongly by freezing as by anoxia (ferritin response to freezing was not assessed). Ferritin sequesters iron and, by doing so, plays an important role in antioxidant defense because free iron is a major catalyst in the production of ROS via the Fenton reaction (see Chapter 12). Metallothionein is generally considered to contribute to the homeostasis of trace metals in the body and, in situations of heavy-metal challenge, to limit toxicity by sequestering metals such as cadmium, lead, and mercury. Much research has correlated metallothionein levels with heavy-metal pollution in aquatic environments, and changes in the levels of the protein are often used as a bioindicator of metal pollution. However, metallothionein is a low-molecular-weight cysteine-rich protein (27 out of 100 amino acids in the *L. littorea* protein are cysteines), and new research suggests that it has antioxidant properties either as a reducing agent (due to its many –SH groups) or due to binding of metals such as copper that, like iron, can catalyze ROS formation. Indeed, transgenic mice that overexpress metallothionein show much reduced damage during cerebral ischemia–reperfusion, whereas cultured embryonic cells that were deficient in metallothionein showed enhanced susceptibility to oxidative stress. Evidence of an antioxidant role in marine mollusks includes the fact that preexposure of mussels to cadmium to enhance their metallothionein levels also increased the subsequent survival of the animals when challenged by iron-triggered ROS production. In general, as discussed earlier, anoxia-tolerant animals maintain constitutively high levels of antioxidant defenses and/or improve these when stimulated by anoxic/hypoxic conditions; for example, we found that anoxia exposure stimulated a strong increase in the amount of reduced glutathione in *L. littorea* tissues. Measurements of conjugated diene contents in lipids suggested that snails are subject to some oxidative stress during the early minutes of recovery after anoxic excursions. Thus, the enhancement of thiol antioxidant defenses and elevated metal-binding capacities under anoxia may prepare the animals for dealing with enhanced levels of ROS when anoxic tissues are reoxygenated.

Some progress has been made in elucidating the regulatory controls on anoxia-induced gene expression in *L. littorea.* When we incubated hepatopancreas explants under aerated versus N_2-bubbled conditions *in vitro*, we found that L26, *kvn*, and ferritin transcripts were all up-regulated under anoxia *in vitro* just as they were *in vivo.* This validated the model for further use in testing the effects of stimulators of protein kinases on gene expression. The effects of dibutyryl cAMP, dibutyryl cGMP, calcium ionophore A23187, or phorbol 12-myristate 13-acetate on gene expression were tested in aerobic incubation. Transcript levels of all three genes were elevated when tissues were incubated with dibutyryl cGMP, whereas only ferritin message responded to any of the other treatments. This implicates a cGMP-mediated signaling cascade in the gene expression response to anoxia in *L. littorea* and agrees with the evidence presented earlier that cGMP-dependent protein kinase (perhaps responding to a nitric oxide signal) regulates a variety of metabolic responses to anoxia in marine mollusks, ranging from enzyme phosphorylation to gene expression.

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