Biochemical Adaptation

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BIOCHEMICAL ADAPTATION

KENNETH B. STOREY

> Living creatures press up against all barriers; they fill every possible niche all the world over. . . . We see life persistent and intrusive—spreading everywhere, insinuating itself, adapting itself, resisting everything, defying everything, surviving everything!
>
> —John Arthur Thomson, 1920

All organisms strive to remain alive, to maintain homeostasis, and to optimize growth and reproductive potential in the face of continual challenges from both their internal and external environments. The concept of *biochemical unity* (Chapter 1) tells us that all organisms have the same basic biochemical components—the same building blocks (macromolecules, fuels, substrates, cofactors), and the same basic metabolic pathways, membrane structure, and transcription and translation machinery. Yet metabolism is called upon to adjust to innumerable stresses, major and minor, on timescales that range from instantaneous to evolutionary. That organisms can do this is obvious from the fact that life on Earth has radiated into every conceivable environment. Living organisms are found in the frigid Antarctic and boiling hot springs, in the ocean depths and at the tops of mountains. Life abounds in anoxic sulfurous mud flats, in hypersaline lakes, and in the driest deserts. All this is possible because of biochemical adaptation—changes to the structure, function, regulation, and integration of biological molecules and metabolic processes. By means of biochemical adaptation, organisms can maintain both *metabolic control* (adjusting the output of a metabolic pathway in response to an external signal) and *metabolic regulation* (maintaining metabolic parameters relatively constant over time, despite fluctuations in external conditions). To review these concepts see Chapter 1.

Biochemical adaptation is necessary for two main reasons: (1) all biological molecules and all biochemical reactions are directly susceptible to perturbation by multiple environmental parameters—for example, temperature, pressure, pH, ionic strength, solute concentrations, water availability, radiation, and attack by free radicals, and (2) all cells and organisms, in order to remain viable, must maintain an adequate level of energy turnover through an adequate supply of the energy currencies of the cell, primarily adenosine triphosphate (ATP), which is used to drive thermodynamically unfavorable reactions, and reduced nicotinamide adenine dinucleotide phosphate (NADPH), which is used for reductive biosynthetic reactions. Environmental stresses can disrupt the physical properties of biological molecules, the activities and regulatory properties of functional proteins (e.g., enzymes, transporter proteins), and the integrated functioning of the biochemical reactions in individual pathways, individuals cells, and even intact organisms. These concepts and many other aspects of biochemical adaptation and physiological evolution are treated more fully by Hochachka and Somero (2002), and this excellent resource is highly recommended to the reader.

The vast majority of organisms on Earth are highly susceptible to multiple changes in their physical environment. For example, most organisms are ectotherms (cold-blooded) and, hence, a change in environmental temperature means a change in body temperature. A change in temperature dictates an automatic change in the rates of virtually all metabolic reactions, as well as conformational changes to the structure of proteins and changes in the fluidity of membranes. Living within our mammalian bodies, humans do not often fully appreciate the environmental stresses that rule the lives of most other organisms on Earth. This is because a large evolutionary effort has been

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put into the creation of a near-constant internal environment for our cells and organs. The temperature of our core organs rarely deviates from ~37°C, oxygen supply is rarely interrupted, our skin has good resistance to water loss, and our bodies rigorously regulate cellular conditions of pH, osmolality, and ionic strength. This relative freedom from environmental constraints comes at a high cost—a high rate of food consumption is needed to support the metabolic rates of mammals that are about four- to sevenfold higher than those of equivalently sized reptiles. However, a constant internal environment and high body temperature provide key advantages for mammals and birds, including an ability to remain active under adverse environmental or seasonal conditions as well as support for advanced brain functions. This latter has brought humans even greater freedom from environmental constraints for it has given us the capacity to substitute man-made technology for metabolic adaptation. Other organisms are not so lucky.

ENVIRONMENTAL STRESSES

We will begin with a brief outline of some of the effects of environmental parameters on biological molecules and metabolic functions. This is a selective list, focusing only on those stresses that are utilized in the examples of biochemical adaptation discussed in this chapter and the next three.

Oxygen

Oxygen availability has two main actions in metabolism—essentially, one good and one bad!

Oxygen the Good Oxygen is a substrate of many biological reactions. Most famously, it is the terminal electron acceptor of the cytochrome *c* oxidase reaction of the mitochondrial respiratory chain, but oxygen is also a substrate in about 200 other cellular reactions. Because of the huge difference in ATP output from oxidative phosphorylation compared with fermentative reactions, oxygen-dependent ATP synthesis is utilized by most multicellular organisms. Many organisms, including humans, are highly sensitive to reduced oxygen levels with variable abilities to endure low oxygen (hypoxia) and little capacity to survive full oxygen depletion (anoxia). Other organisms are good facultative anaerobes that can switch easily between aerobic and anoxic lifestyles, whereas others function as obligate anaerobes (mainly various microbes and some animals such as intestinal parasites). Biochemical adaptations for dealing with oxygen availability will be dealt with in greater detail in Chapter 15.

Oxygen the Bad Reactive oxygen species (ROS) (e.g., superoxide, hydrogen peroxide, hydroxyl radical) are by-products of the electron transport chain and products of selected enzymatic and nonenzymatic reactions in cells. It has been estimated, for example, that 1 to 4% of all O_2 consumed by vertebrates is "lost" in the production of superoxide radicals, chiefly from a "leaky" electron transport chain. ROS cause damage to many kinds of cellular macromolecules, and all cells must have effective antioxidant defenses to quench their production and repair or catabolize damage products. Changes in oxygen availability as well as other stresses (e.g., iron overload, radiation, xenobiotics) increase ROS production and necessitate adaptive responses by antioxidant defenses. Metabolic adaptations for dealing with ROS are dealt with in Chapter 12.

Temperature

The vast majority of organisms on Earth are ectotherms whose body temperature always closely matches ambient temperature. Temperature change affects biological molecules in multiple ways.

1. Temperature affects the rates of all chemical and biochemical reactions. In most cases, reaction rates double for every 10°C increase in temperature; this is designated as a temperature quotient or $Q_{10} = 2$. However, some metabolic reactions have Q_{10} values <2, whereas others range up to 3 or 4, and so differential effects of temperature change on the rates of cellular reactions can disrupt metabolic homeostasis.

2. Temperature alters the strength of the weak bonds that are critical to the conformation of macromolecules and to the conformational changes that occur during ligand binding and catalysis in enzymes. A decrease in temperature increases the stability of hydrogen bonds, van der Waals interactions, and electrostatic bonds but decreases the stability of hydrophobic bonds, and vice versa for a temperature increase. Because each protein/enzyme differs in the number and type of weak bonds that are important to its conformation, ligand binding, or other properties, a change in temperature affects each protein/enzyme slightly differently with the potential to disrupt the integrated functioning of multicomponent pathways. For example, a temperature increase may increase the K_m for the substrate of one enzyme while decreasing the K_m of another.

3. Both high and low temperatures can denature proteins. Cold denaturation is often reversible, but heat can irreversibly denature proteins at temperatures not too far about the normal biological range. For example, exposure to 55 to 60°C denatures many types of proteins from mesothermic organisms, including humans, but many thermophilic microorganisms live happily near 100°C.

4. Temperature affects the flexibility of fatty acids and phospholipids and changes the fluidity of biological membranes.

5. Below 0°C biological water can freeze, and organisms must take steps to either avoid freezing (e.g., with antifreezes) or endure freezing (e.g., cryopreservation). Biochemical adaptations for dealing with life below 0°C will be dealt with in greater detail in Chapters 16 and 17.

Water and Ions

Water is the universal solvent of all biological reactions and a substrate or product of many, its bulk is a key structural element in the bodies of all organisms, and the physical properties of water (e.g., melting point, surface tension, weak bonding) impact on many biochemical and physiological parameters. The structures and functions of most biological macromolecules are sensitive to changes in the type and concentration of inorganic ions as are selected vital functions, such as the maintenance of membrane potential difference. Stresses caused by variation in water and ion contents can have multiple effects including:

1. Variation in organismal water content necessarily changes ion concentrations and thereby affects biochemical reactions and metabolic functions, all of which have both high and low limits on the tolerable concentrations of Na^+, K^+, Ca^{2+}, and other ions.
2. Cell volume changes due to hyper- or hypo-osmotic stresses can cause physical damage to cells and destroy the integrity of the cell membrane. Shrinkage beyond a critical minimum can cause a breakdown of membrane bilayer structure due to excessive compression whereas excessive volume increase can burst cells.
3. All biological macromolecules are coated by a "shell" of vicinal water that contributes to their conformation and, in cases of extreme desiccation or freezing, the loss of vicinal water may cause denaturation of macromolecules.

MECHANISMS OF METABOLIC REGULATION

Previous chapters have discussed multiple mechanisms of metabolic regulation, and all of these are employed in biochemical adaptation. Table 14.1 lists many of these mechanisms, and the remainder of this chapter illustrates the principles of how these mechanisms can be used in biochemical adaptation with selected examples from studies of animal adaptation to environmental stress. Central to biochemical adaptation is the regulation of enzymes, and most of the discussion below relates to enzymatic adaptation, although the mechanisms are typically applicable to most proteins. In the field of biochemical adaptation, the regulation of central enzymes of intermediary energy metabolism (particularly those of glycolysis) has received by far the most attention because of the critical role that the maintenance of energy supply has in the adaptation to any environmental stress. It is these enzymes that we will also use as our primary examples.

TABLE 14.1 Some Mechanisms of Biochemical Adaptation

Enzyme/Protein Level
Changes in protein or enzyme amount
Changes in isoform/isozyme type and properties
Changes in the concentrations of substrates and effectors of enzymes
Changes in the kinetic and regulatory properties of enzymes
Covalent modification of enzymes and proteins
Protein–protein binding and changes in subcellular location
Influence of low-molecular-weight stabilizers
Transcriptional and Translational Level
Evolution of novel protein or enzyme types
Changes in response elements and transcription factors
Control over protein translation

ENZYME ADAPTATION

Enzymes are the catalysts of cells and most of the "business" in cells is conducted by enzymes. Central to biochemical adaptation is the control of enzyme function, and multiple mechanisms have been designed to tailor enzymes for optimal function in the cells/tissues/organisms in which they reside and to provide an appropriate range of responses to deal with metabolic demands and environmental stresses. Adjustments to enzymes in response to environmental stress can make use of any and all of the mechanisms of metabolic regulation that have been discussed in previous chapters. The discussion here will review many of these mechanisms, summarized in Table 14.1, and highlight instructive examples of biochemical adaptation.

Changes in Enzyme or Protein Amount

The amount of each enzyme or protein in a cell is a primary determinant of the capabilities of different cells, tissues, and species, and changes in enzyme/protein amount provides the coarse control of metabolism that is a key part of cellular response to many external signals (hormones, environmental stress, etc.). On an evolutionary timescale, differences in

TABLE 14.2 Activities of Muscle Enzymes[a]

	Rat Red Fibers	Rat White Fibers	Hummingbird Red Muscle	Tuna White Muscle
Glycogen phosphorylase	21	29	31	106
Hexokinase	1.3	0.7	9.2	0.8
Pyruvate kinase	246	405	672	1295
Lactate dehydrogenase	555	849	230	5492
Citrate synthase	19.5	5.5	343	2

[a]Selected glycolytic enzymes as well as citrate synthase from the tricarboxylic acid cycle are compared in the red and white muscle fibers of the vastus lateralis muscle of rats, the red pectoral muscle of hummingbirds, and the white muscle of tuna. All data are units per gram wet weight.

the amounts of selected enzymes (typically quantified as enzyme maximal activity per gram wet weight) helps to define the metabolic capacities of different cell/tissue types and the differences in these capacities between homologous cells/organs of different species.

Skeletal Muscle: A Study in Evolutionary Design The above concepts can be illustrated using the constitutive activities of selected enzymes of carbohydrate catabolism in skeletal muscles of different species. Recall from Chapter 11 that red muscle (slow twitch, type I) is designed for endurance work and primarily generates ATP from the aerobic catabolism of substrates and mitochondrial oxidative phosphorylation, whereas white muscle (fast twitch, type II) is designed for high-intensity burst work and relies mainly on ATP generated from creatine phosphate hydrolysis and anaerobic glycolysis (ending in lactate accumulation).

Data for the vastus lateralis muscle in rats show typical differences between the red and white fibers of mammalian skeletal muscle (Table 14.2). White fibers clearly have the higher glycolytic capacity with a greater capacity for glycogen degradation via glycogen phosphorylase (GP) and higher activities of the terminal enzymes of glycolysis, pyruvate kinase (PK), and lactate dehydrogenase (LDH) (Fig. 14.1). By contrast, red fibers have a higher activity of hexokinase (HK) and a greater HK : GP ratio (0.062 in red vs. 0.024 in white), which indicates a greater reliance on blood-borne substrates (glucose) as fuel. Red muscle also has a very much higher citrate synthase (CS) activity, which correlates with its greater capacity for ATP generation via mitochondrial oxygen-dependent metabolism.

The comparison between species in Table 14.2 shows the extremes that can be achieved by evolution when optimizing muscle enzyme activities for the very high power outputs needed for hovering flight by hummingbirds or for high-speed burst swimming by predatory tuna. Hummingbird red muscle still shows a well-developed glycolytic capacity, but the extremely high CS activity (which is matched by other mitochondrial enzymes) shows the tremendous capacity of the muscle for oxidative metabolism

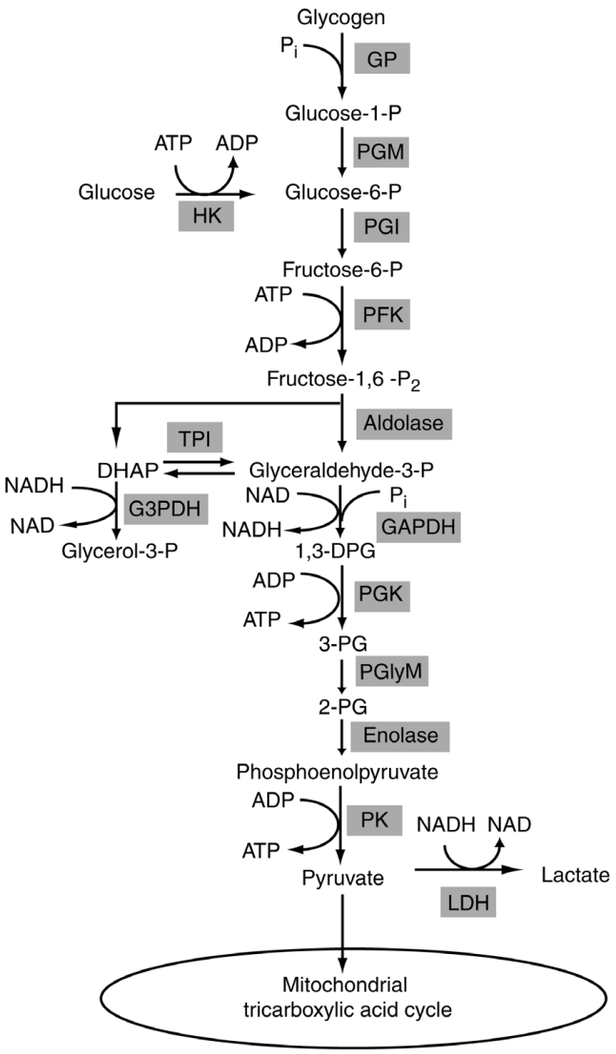

Figure 14.1 The pathway of glycolysis. Enzyme abbreviations are: GP, glycogen phosphorylase; HK, hexokinase; PGM, phosphoglucomutase; PGI, phosphoglucoisomerase; PFK, 6-phosphofructo-1-kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TPI, triosephosphate isomerase; PGK, phosphoglycerate kinase; PGlyM, phosphoglyceromutase; PK, pyruvate kinase; LDH, lactate dehydrogenase.

and correlates with a mitochondrial density in hummingbird muscle that is the highest found in any vertebrate muscle. By contrast, the swimming muscles of tuna contain the highest LDH activities ever measured and provide the muscle with the ability to generate enormous bursts of power output fueled by anaerobic glycolysis. The extremely low HK : GP ratio (0.0075) also indicates a reliance on huge reserves of glycogen in tuna muscle for substrate support of anaerobic glycolysis.

Stress-Induced Changes in Enzyme Amount Changes in the amount of an enzyme in a cell redefines the maximum capacity of its catalyzed reaction and, when the enzyme involved has a high flux control coefficient (see Chapter 1 for metabolic control theory), also exerts a major influence on overall metabolic flux through the pathway in which it resides. Numerous signals or stresses can stimulate changes in the amount of an enzyme or protein in a cell. This coarse control typically occurs over a relatively long time frame for it involves adjustments to protein synthesis (transcription and/or translation) or protein degradation and differs from the immediate or short-term responses to the same stress that can be achieved via fine controls on the activity of individual enzyme molecules (discussed in later sections). Examples of coarse control over enzyme amount include the effects of exercise training or electrical stimulation in elevating the activities of glycolytic and/or tricarboxylic acid cycle enzymes in muscle (see Chapter 11) and the actions of hormones in stimulating changes in the enzymatic profiles of tissues (see Chapter 10). Environmental stresses can similarly induce changes in enzyme activities in order to overcome the perturbing effect of the stress or to restore homeostasis when the stress is prolonged. For example, in hypoxia-sensitive species such as humans a low oxygen signal, acting via the hypoxia-inducible transcription factor (HIF), stimulates increased synthesis of multiple glycolytic enzymes as well as plasma membrane glucose transporters. This raises the capacity for anaerobic ATP synthesis when ATP production by oxidative phosphorylation is limited by low O_2 availability (see Chapters 6 and 15 for more on HIF function and hypoxia adaptation, respectively).

The process of cold acclimation in fish provides another good example of stress-induced changes in enzyme amount. When fish are transferred from warm to cold water, multiple changes are made to readjust metabolic functions for the new temperature. Several involve coarse controls on enzyme amount with two of the prominent changes being: (1) a rapid increase in the synthesis of desaturase enzymes that functions to restore fluidity to cold-rigidified membranes by increasing the number of unsaturated phospholipids in membranes, and (2) an increase in the activities of multiple mitochondrial enzymes as part of a general proliferation of mitochondria in the cold. For example, trout acclimated to 4 versus 18°C showed 40% higher activities of citrate synthase and cytochrome *c* oxidase in red muscle of cold-acclimated fish and 70% higher activities in white muscle. This adjustment occurs because the combination of low animal metabolic rate and higher dissolved oxygen content in cold water permits aquatic ectotherms to rely more heavily on aerobic ATP production to support muscle work at colder temperatures and, hence, mitochondrial enzymatic capacity responds accordingly.

Many organisms also show seasonal adjustments to enzyme activities that track predictable changes in environmental conditions. Such adjustments are frequently triggered via photoperiod cues that modulate production of one or more hormones that, in turn, alter gene expression and protein synthesis. In cold-hardy insects, for example, the activities of glycogen phosphorylase and polyol dehydrogenase rise sharply during the early autumn to support a massive biosynthetic effort that converts as much as 20% of the insect's body weight into the antifreeze agent, glycerol, to provide winter cryoprotection (see Chapter 17).

Changes in Enzyme and Protein Properties

Changes to the properties of proteins, particularly enzymes, is a major instrument of biochemical adaptation. Alterations to enzymes can include changes in substrate/ligand affinity, in the types of effectors to which they are sensitive, in their affinity for activators and inhibitors, and in the influence of physical parameters (temperature, pH, ion concentrations) on enzyme/protein properties. As a result, homologous enzymes or proteins in different species may show considerably different kinetic and regulatory properties that tailor the enzyme/protein to the particular metabolic needs of the cell/tissue/organism or allow it to function optimally under the range of environmental conditions that the organism normally experiences. Evolutionary time has also produced isoforms of proteins (encoded on different genes yet catalyzing the same reaction) and alloforms (protein variants of a single gene that can have slightly different properties). Isoforms may differ in their tissue distribution (e.g., liver versus muscle isoforms), subcellular location (e.g., cytoplasmic versus mitochondrial isoforms), expression patterns at different developmental stages, functional roles, and regulatory mechanisms and can play critical roles in defining the metabolic capabilities of different tissues.

Enzyme Properties Chapters 1 and 2 outlined enzyme kinetics and multiple mechanisms of enzyme regulation including modulation of substrate availability, the actions of activators and inhibitors, and the effects of changes in parameters such as pH, temperature, and ionic strength. One of the most powerful and plastic mechanisms of biochemical adaptation is the tailoring of enzyme kinetic and

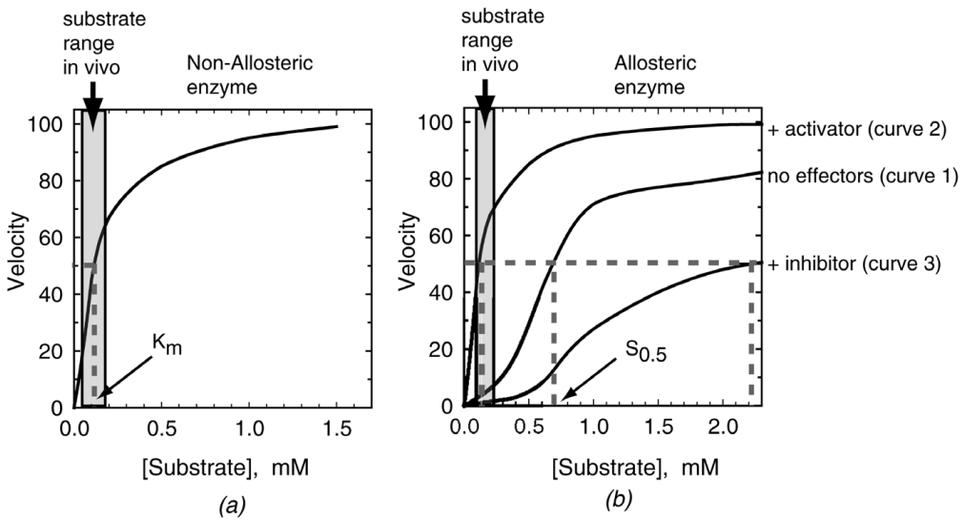

Figure 14.2 Relationship between substrate concentration and enzyme velocity for (a) nonallosteric and (b) allosteric enzymes. The nonallosteric enzyme shows hyperbolic substrate saturation kinetics, in this case with a K_m value (substrate concentration that produces half-maximal velocity) of 0.12 mM. The allosteric enzyme shows a strongly sigmoidal relationship between [S] and velocity in the absence of effectors (curve 1), in this case with a $S_{0.5}$ value (equivalent to K_m for a sigmoidal relationship) of 0.7 mM. Addition of an activator shifts the V vs. [S] curve to the left, creates a hyperbolic relationship and lowers $S_{0.5}$ to 0.12 mM. Addition of an inhibitor causes a right shift in the V vs. [S] curve and raises $S_{0.5}$ to 2.2 mM. The shaded bar shows the range of cellular substrate concentration *in vivo.*

regulatory properties to adjust enzyme function to meet the metabolic demands placed on cells, tissues, and organisms. Changes in the kinetic and regulatory properties of enzymes, integrated with the cellular environment in which the enzymes function, can produce a range of adaptive responses that can create both compensatory adjustments to allow metabolic functions to continue under stress conditions or opportunistic adjustments that alter metabolism under new environmental conditions.

Figure 14.2 shows a schematic of some of the common patterns of enzyme function. Many enzymes show hyperbolic substrate saturation curves and function under near-equilibrium conditions with very low control coefficients (Fig. 14.2*a*). They are often present in high amounts in tissues (relative to the pacemaker enzymes of their pathway) and act primarily as throughput conduits in pathway flux. In glycolysis, enzymes such as phosphoglucoisomerase, phosphoglyceromutase, aldolase, enolase, and lactate dehydrogenase function in this manner (Fig. 14.1). Studies of such enzymes have frequently demonstrated that their K_m values (substrate concentration producing half-maximal velocity) are well-matched with the concentration range of their substrates *in vivo.*

Compensatory adjustments to the K_m values of such enzymes have been demonstrated in a number of instances, and these presumably help to fine-tune enzyme function, particularly in the face of environmental stress. Temperature change is one such stress that affects the activities, conformations, and ligand binding properties of all enzymes, and adjustments to enzyme properties are often seen in the responses of ectothermic animals to temperature change. For instance, on an evolutionary timescale enzymes can be modified to optimize their function for the mean thermal environment in which they must operate. For example, extensive studies of LDH [reaction (14.1)] from multiple species of fish ranging from the Antarctic to the tropics have shown that an optimal K_m value for pyruvate of about 0.2 mM is invariably found when the enzyme is assayed at temperatures within the normal environmental range for the species, be it 0 to 2°C for Antarctic fish or 30 to 35°C for tropical fish. However, within as little as 5 to 10°C above the normal range, the K_m value rises rapidly (i.e., enzyme affinity for pyruvate declines); for example, the K_m pyruvate of Antarctic fish LDH was $\sim$0.4 mM when assayed at 15°C. The molecular basis of this phenomenon is small changes to the amino acid sequences of LDH in different species that readjust the ratios of hydrophobic and hydrophilic bonds within the protein for optimal function within a given thermal window. For example, LDH from Antarctic fish, has several more glycine residues in

key areas of the protein than does the enzyme from temperate fish, and these increase the flexibility of the cold-adapted orthologs [refer to Hochachka and Somero (2002) for an expanded treatment].

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \longleftrightarrow \text{Lactate} + \text{NAD}^+ + \text{H}_2\text{O} \quad (14.1)$$

Allosteric enzymes offer many more opportunities for adaptive control (Fig. 14.2*b*). Substrate binding is modulated in one of several ways. First, most such enzymes are multimeric and show homotropic allosterism, meaning that substrate binding to one subunit alters the conformation of other subunits to positively enhance substrate binding to them. This results in a sigmoidal relationship between enzyme activity and substrate concentration that makes the enzyme highly sensitive to small changes in substrate (see Chapter 1). These enzymes are also typically sensitive to regulation by multiple nonsubstrate effector molecules. Effectors bind at allosteric sites away from the active site and, by doing so, change enzyme conformation and alter enzyme–substrate binding properties. Activators alter enzyme conformation to increase substrate affinity, creating a left shift in the velocity (V) versus substrate concentration [S] curve whereas inhibitors shift the V versus [S] curve to the right to greatly reduce substrate affinity (Fig. 14.2*b*). Binding by allosteric activators can also modify enzyme sensitivity to inhibitors and vice versa. Such heterotropic allosterism offers multiple opportunities for influencing enzyme rate by metabolites other than substrates, products, and cofactors. Feedforward activation can come via allosteric effects by products of earlier reactions in the pathway whereas feedback inhibition can result from sensitivity to the ultimate end product of a pathway. Sensitivity to the energy status of the cell can be imparted via the presence of allosteric sites for ATP, adenosine diphosphate (ADP), or adenosine monophosphate (AMP) and metabolites from competing pathways can exert effects. With the multiple opportunities for allosteric regulation of such enzymes (often combined with additional control of the enzyme by reversible phosphorylation; see below), it is not surprising that allosteric enzymes are often the pacemaker enzymes of pathways. The ability to modify allosteric regulatory properties of enzymes also provides a huge range of opportunities for biochemical adaptation. Enzyme control in different species/tissues can be modified by deleting or adding regulation by different allosteric effectors, by changing enzyme sensitivity to one or more effectors, and by modifying enzyme/effector interactions with respect to parameters such as pH and temperature. For example, later in this chapter we will discuss the effects of widely differing sensitivities to L-alanine inhibition by pyruvate kinases in the regulation of glycolysis in different species. In the next section, the interactions of the enzyme 6-phosphofructo-1-kinase [PFK; reaction (14.2)] with its effectors and pH is emphasized, whereas in Chapters 16 and 17 several examples are given of the interactions between temperature and enzyme properties in the initiation and regulation of various biochemical adaptations that support cold-hardiness.

$$\text{Fructose-6-P} + \text{ATP} \rightarrow \text{fructose-1,6-P}_2 + \text{ADP} \quad (14.2)$$

The PFK of animal tissues is an excellent example of an allosteric enzyme and is frequently considered to be a pacemaker of glycolysis. PFK is the first committed step of glycolysis, its ATP-utilizing reaction gating the entry of hexose phosphates into the triose phosphate portion of glycolysis (Fig. 14.1). The reaction is functionally irreversible *in vivo* and during gluconeogenesis is bypassed by the fructose-1,6-bisphosphatase reaction. PFK is regulated in a multitude of ways: by allosteric effectors, by reversible phosphorylation, via reversible polymerization of the active tetramer, and in muscles via binding interactions with myofibrillar components. We will return to PFK many times in this chapter to provide examples of different principles of metabolic regulation.

The regulation of PFK by substrates, allosteric effectors, and parameters such as pH and temperature is complex and provides multiple opportunities for adaptive control of enzyme activity. In general, PFK shows a sigmoidal relationship between fructose-6-phosphate (F6P) substrate concentration and enzyme velocity and, in the absence of effectors, and the $S_{0.5}$ value for F6P is far above the physiological concentrations of F6P. ATP is the second substrate of PFK ($K_m \sim 0.05$ mM) but is also a powerful inhibitor of the enzyme at higher concentrations that are closer to physiological ATP levels (typically 2 to 8 mM). Hence, under normal cellular levels of F6P and ATP, net PFK activity would be extremely low, and the enzyme is highly dependent on the influence of powerful activators to produce the major changes in PFK activity that are needed when the demand for glycolytic flux is high. These activators include AMP, which is a key signal of low energy status in cells. Powerful AMP effects on PFK are a major factor in the activation of glycolytic ATP output under situations where cellular energy supply is stressed (e.g., muscle work, hypoxia). PFK is also activated by inorganic phosphate (the net product of creatine phosphate conversion to ATP followed by ATP hydrolysis) and by NH_4^+ and inosine monophosphate (IMP) (the products of AMP deaminase action on AMP), all of which also signal high energy demand, particularly in working muscle. Glycolysis also has another role in the supply of carbohydrate for biosynthetic reactions and a second powerful activator, fructose-2,6-bisphosphate ($F2,6P_2$), mediates

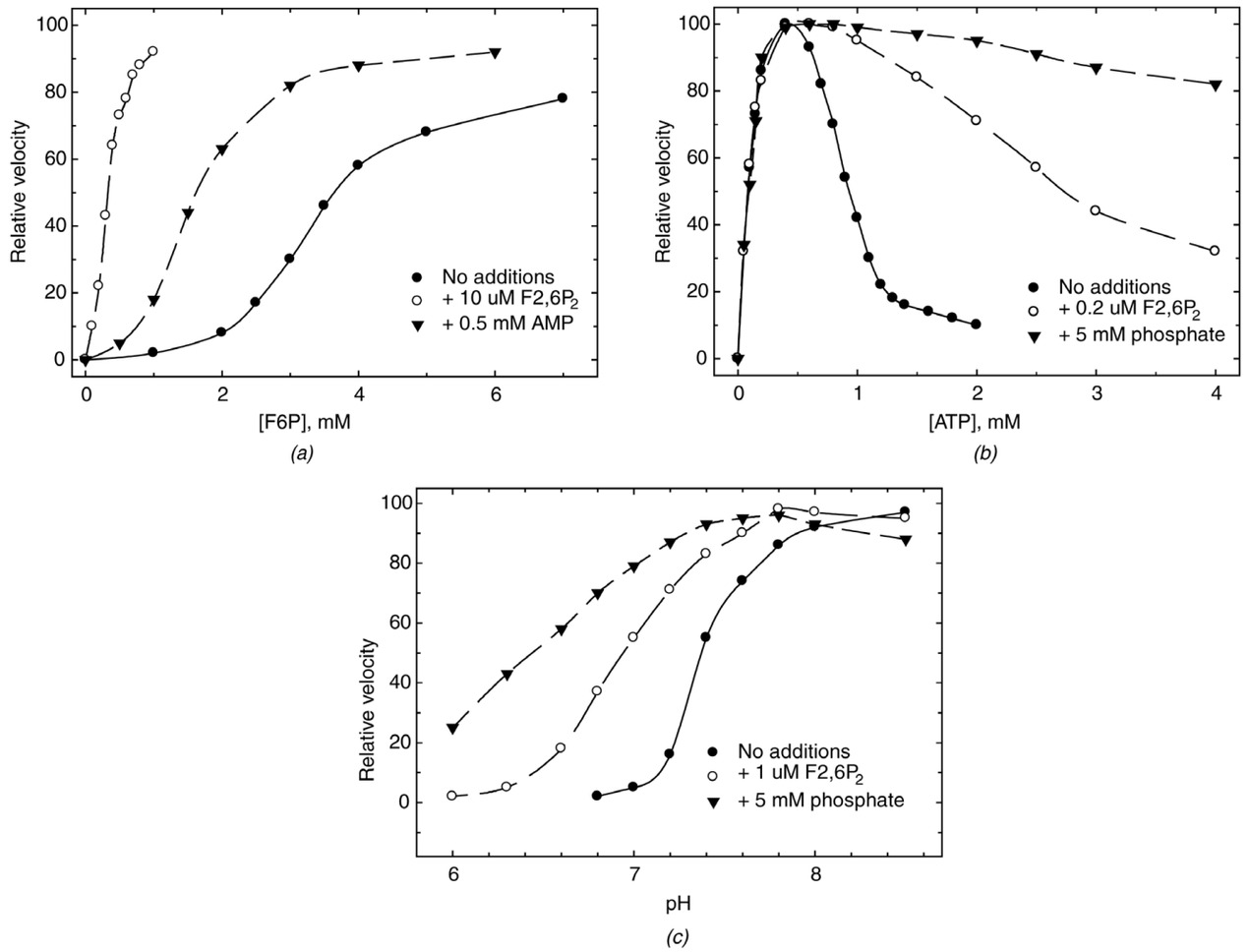

Figure 14.3 Effect of allosteric activators on properties of trout white muscle phosphofructokinase under simulated metabolic conditions in resting muscle: (*a*) fructose-6-phosphate substrate saturation curves at pH 7.2 with 7.5 mM ATP as cosubstrate, (*b*) ATP substrate saturation curves at pH 7.2 with 0.2 mM F6P as cosubstrate, and (*c*) pH profile. [Data compiled from J. Y. Su and K. B. Storey (1994). *Int. J. Biochem* **26**:519–528.]

PFK activity in this role. The enzyme is also affected by inhibitors that slow glycolytic rate when energy levels are high (ATP inhibition) or when biosynthetic demands decrease and cause a buildup of glycolytic (phosphoenolpyruvate) or tricarboxylic acid cycle (citrate) intermediates. Furthermore, PFK is highly sensitive to changes in pH within the physiological range with acidification reducing affinity for F6P, increasing ATP substrate inhibition, and reducing the effects of activators.

The PFK from the white (type II) muscle of trout provides an excellent example of the complexities of PFK regulation. In the absence of effectors, with a physiological level of ATP cosubstrate (7.5 mM), and an assay pH of 7.2 (the pH typical of resting muscle), PFK shows very low affinity for F6P; the $S_{0.5}$ value was 3.9 mM with a Hill coefficient, n_H, of 4 indicating high sigmoidicity (remember that $n_H = 1$ for a hyperbolic relationship; Chapter 1) (Fig. 14.3*a*, Table 14.3). When compared with the *in vivo* concentration of F6P in fish muscle, which is <0.20 mM, it is obvious that enzyme activity under these conditions would be very low. Furthermore, ATP substrate inhibition of PFK is high under resting conditions in muscle with an ATP I_{50} value (concentration producing 50% inhibition) of 0.9 mM when measured at physiological F6P levels (Fig. 14.3b, Table 14.3). However, in the muscle at rest low levels of activators such as $F2,6P_2$ can profoundly change PFK kinetics to regulate low rates of carbohydrate use for biosynthesis. As little as 0.2 μM $F2,6P_2$ strongly activates the enzyme and in the presence of 10 μM $F2,6P_2$ the $S_{0.5}$ for F6P was lowered from 3.9 to 0.2 mM

TABLE 14.3 Effects of Metabolite Modulators on the $S_{0.5}$ and n_H for Fructose-6-Phosphate of Trout Muscle PFK under ATP Concentrations and pH Values Representing "Resting" (ATP = 7.5 mM, pH 7.2) and "Exercised" (ATP = 3.5 mM, pH 6.6) Cellular Conditions[a]

	Resting Conditions (Modulator)	$S_{0.5}$ (mM)	n_H	Exercised conditions (Modulator)	$S_{0.5}$ (mM)	n_H
Control (no additions)	—	3.4	4.1	—	18.5	4.8
+ AMP	0.05 mM	1.3	3.5	0.50 mM	2.2	1.8
+ Inorganic phosphate	25 mM	0.2	1.0	50 mM	0.5	1.8
+ NH_4Cl	1 mM	0.9	4.0	5 mM	4.3	2.5
+ $F2,6P_2$	10 μM	0.2	1.8	10 μM	1.0	2.0
+ All 4 modulators	As above	0.07	1.4	As above	0.15	1.0

[a]The modulator concentrations chosen represent those found *in vivo* in resting versus exercised trout muscle.
Source: Data modified from J. Y. Su and K. B. Storey (1994). *Int J Biochem* **26**:519–528.

(Table 14.3) whereas the I_{50} for ATP was increased from 0.9 to 19 mM.

High-speed swimming demands a huge increase in power output from working muscle (10- to 100-fold), and in white muscle this is supplied by the rapid depletion of creatine phosphate reserves and by an immediate activation of glycolysis to produce ATP from the catabolism of glycogen to lactate. During muscle work, intracellular pH falls by about 0.6 units. Obviously, PFK must be activated during exercise, but how is this achieved? PFK activity falls rapidly at pH values below 7 (Fig. 14.3*c*) and the enzyme's kinetic parameters are negatively affected by reduced pH. For example, when measured at pH 6.6, the values for $S_{0.5}$ F6P and I_{50} ATP were 26.3 and 0.2 mM (compare these with the values for pH 7.2 assays above). Here is where activators that signal low energy levels play a huge role. Activators including inorganic phosphate and AMP lessen enzyme sensitivity to low pH and reduce the inhibitory effects of ATP (Figs. 14.3*b* and 14.3*c*). For example, the I_{50} value for ATP rose by 10-fold in the presence of 5 mM inorganic phosphate; notably, inorganic phosphate levels can increase *in vivo* by at least 20 mM during intense muscle exercise as the result of creatine phosphate hydrolysis. Coupled with a fall in cellular ATP levels of about 50% during exercise, net PFK inhibition by ATP is greatly reduced in working muscle. Enzyme affinity for F6P is also vastly improved by the actions of allosteric activators. Table 14.3 shows the influence of four activators on the $S_{0.5}$ for F6P and the n_H of F6P binding comparing both individual effects and the additive effects of multiple activators and utilizing conditions of effector concentrations and pH that mimic either "resting" or "exercised" muscle conditions. All activators individually lower both $S_{0.5}$ and n_H but, more importantly, strong additive effects of activators occur so that $S_{0.5}$ for F6P can be lowered from 18.5 mM in the absence of effectors to 0.15 mM in the presence of the four activators. This 123-fold change lowers the $S_{0.5}$ of the enzyme to a value that closely matches in vivo F6P concentrations (0.16 mM) in working muscle.

ISOFORM/ISOZYME PROPERTIES

A wide range of functional demands on an enzyme or protein cannot always be met by a single enzyme/protein form, and in many cases, isozymes/isoforms have evolved that may differ in kinetic properties, sensitivity to effectors, susceptibility to covalent modification, binding interactions with other macromolecules, subcellular location, tissue distribution, and so forth. The five isoforms of the vertebrate plasma membrane glucose transporter (GLUT) provide an instructive example of differential isoform distribution and function. The primary mode of glucose movement across cell membranes is facilitated diffusion mediated by GLUT proteins that move the sugar down its concentration gradient. Multiple GLUT isoforms are known, each with properties geared for different functions. GLUT1 is the passive glucose transporter of erythrocytes and many other tissues and is probably the "ancestral" form. It facilitates one-way uptake of glucose from the plasma into cells. GLUT2 is found chiefly in liver, and unlike the other GLUT2 isoforms functions effectively in two-way glucose transport into or out of hepatocytes. This capacity is crucial for its central role in liver, the organ that maintains plasma glucose homeostasis under both fed and starved conditions. GLUT3 is expressed in brain and nervous tissue and appears to act in concert with GLUT1 to ensure that glucose, the primary substrate for oxidative metabolism in the brain, is always in adequate supply. Muscles and adipose tissue have another isoform, GLUT4. This isoform mediates the insulin-dependent uptake of glucose into cells under fed conditions to provide sugar for energy metabolism and the synthesis of fuel reserves (glycogen in muscle, triglycerides in adipose). The action of insulin is to stimulate a transloca-

tion of inactive transporters from intracellular vesicles to the cell surface and thereby increase transport capacity. Finally, GLUT5 has a unique role in the small intestine, functioning in the uptake of a variety of dietary hexoses, particularly fructose. By diversifying into many different isoforms, a wide variety of specialized needs and organ-specific functions can be met.

Among enzymes, differences in the kinetic and regulatory properties of isozymes are critical to their function and to the control of the pathway in the tissue in which they reside. A good example of differential isozyme properties is the muscle and liver isozymes of pyruvate kinase (PK):

$$\text{Phosphoenolpyruvate} + \text{ADP} \rightarrow \text{pyruvate} + \text{ATP} \quad (14.3)$$

In skeletal muscle PK is typically a high activity enzyme (Table 14.2) and, as the second ATP-producing reaction of glycolysis (Fig. 14.1), PK has a critical role to play in the high rates of ATP output demanded by working muscle, particularly in white muscle. In liver, by contrast, PK is present in lower activities and is closely regulated. Glycolysis in liver plays a substantial role in providing carbohydrates for biosynthesis when glucose is plentiful, but the pathway must be shut down under either of two conditions: (a) when glycogenolysis must be directed into the synthesis and export of glucose, such as under starved conditions, and (b) when high levels of gluconeogenic substrates need to be converted to glucose. Differences in PK properties between skeletal muscle and liver isozymes emphasize these organ-specific roles. Table 14.4 shows how this is done.

Muscle PK is generally a nonallosteric enzyme that shows a velocity (V) versus substrate concentration (S) relationship that is hyperbolic (i.e., Hill coefficient=1). The enzyme shows a high maximal activity (Table 14.2) and a low control coefficient that supports its role in "high-throughput" glycolysis in working muscle. The enzyme is designed with a high affinity (low K_m) for its carbohydrate substrate, phosphoenolpyruvate (PEP), the K_m value typically falling well within the range of *in vitro* PEP concentrations. These properties of skeletal muscle PK are much the same in many different phylogenetic groups, as can be seen by comparing mammalian and squid muscle PK in Table 14.4.

By contrast, the liver isozyme of PK in mammals is an allosteric enzyme that shows a sigmoidal relationship between PEP concentration and enzyme velocity (Hill coefficient is ~1.5). Substrate affinity for PEP by liver PK is lower than that of the muscle enzyme [in Table 14.4 compare muscle PK with the values for dephosphorylated (DeP) liver PK] with K_m values that are typically higher than the normal range of PEP concentrations *in vivo*. The high K_m keeps enzyme activity low except when the activator, fructose-1,6-bisphosphate (FBP), is present. The enzyme is also inhibited by L-alanine. The two effectors shift the V versus [S] curve to the left or right, respectively, and alter the shape of the curve. A rise in FBP, the product of the PFK reaction (an earlier step in glycolysis) (Fig. 14.4), signals an activation of glycolysis, and feedforward activation of PK coordinates the rates of these two loci. On the other hand, a rise in alanine signals the presence of amino acids that can fuel gluconeogenesis and, under those circumstances, PK needs to be inhibited in order to allow pyruvate reconversion to glucose. Table 14.4 also shows how phosphorylation of the PK protein alters the properties of liver PK, and this is dealt with in the next section.

A dramatic example of the adaptation of the properties of PK isozymes is illustrated by the data for whelk muscle PK in Table 14.4. Whelks are large marine snails that have a high tolerance for oxygen deprivation. Adaptation of PK properties plays a key role in anoxia tolerance, as will be discussed in Chapter 15, but here it is instructive to see how very much the properties of PK can be altered when there is a specific need. In contrast to muscle PK from mammals or squid (another mollusk), whelk muscle PK is both an allosteric enzyme and susceptible to reversible phos-

TABLE 14.4 Kinetic and Regulatory Properties of Pyruvate Kinases from Mammalian and Mollusk Sources[a]

	Mammalian Muscle	Squid Muscle	Mammalian Liver		Whelk Muscle		Whelk "Liver"	
			DeP	P	DeP	P	DeP	P
K_m PEP (mM)	0.02	0.15	0.3	0.8	0.07	0.85	0.38	1.10
K_m ADP (mM)	0.30	0.40	0.25	0.25	0.27	0.25	0.21	0.22
K_a Fructose 1,6-P_2 (μM)	N.E.	N.E.	0.06	0.13	0.05	1.3	0.16	0.48
K_i L-alanine (mM)	N.E.	N.E.	0.70	0.35	24.5	0.05	3.9	0.48

[a]The liver and whelk enzymes are interconverted by reversible phosphorylation between dephosphorylated (DeP) and phosphorylated (P) forms. The "liver" of whelks is an organ called the hepatopancreas. The DeP form of whelk PK is the form present in aerobic tissues whereas the enzyme is converted to the P form under anoxic conditions (see Chapter 15). N.E. = no effect.

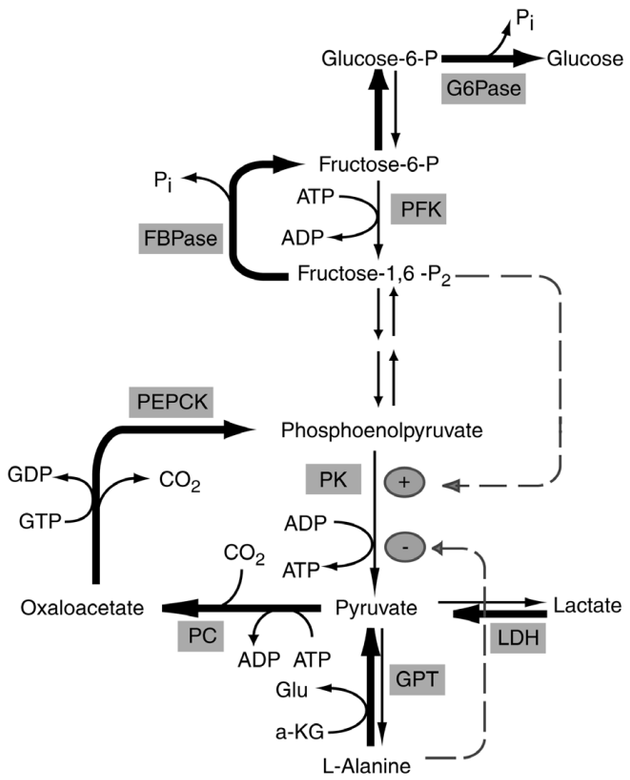

Figure 14.4 Gluconeogenesis using lactate or alanine as substrates, showing the reactions that bypass the phosphofructokinase (PFK) and pyruvate kinase (PK) loci of glycolysis. Other abbreviations are: G6Pase, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; PC, pyruvate carboxylase, GPT, glutamate-pyruvate transaminase; LDH, lactate dehydrogenase; Glu, glutamate; α-KG, alpha-ketoglutarate. Two modes of allosteric control of PK are also shown (gray dashed lines): feedforward activation by fructose-1,6-P_2 and inhibition by L-alanine.

phorylation, much like the mammalian liver enzyme. In its dephosphorylated form it probably does not function very differently from the mammalian or squid muscle PK; its low K_m PEP would facilitate its participation in muscle glycolysis during exercise and, although inhibited by alanine, the very high K_i value means that alanine effects would be of limited consequence *in vivo.* However, once the enzyme is phosphorylated, it becomes highly sensitive to alanine inhibition, and this is a critical factor in PK control in anoxia-tolerant species. PK from the "liver" (in mollusks, its an organ called the hepatopancreas) of whelks also shows this enhanced sensitivity to L-alanine in the phosphorylated form, but in other properties it is not unlike the mammalian liver enzyme.

Thus, we can see several principles of enzyme adaptation from this examination of PK properties: (1) isozymes can be designed with very different kinetic properties that suit their organ-specific functions, (2) enzymes that perform similar "jobs" display similar kinetic properties across wide phylogenetic groups, (3) selected kinetic properties (such as the K_m PEP) are highly malleable, whereas others (such as the K_m ADP) are not, and (4) enzyme sensitivity to one or more allosteric effectors can be enhanced or reduced to place enzymes under different regulatory controls in different circumstances.

Enzyme Control via Reversible Protein Phosphorylation

Cellular proteins can undergo posttranslation modification via multiple kinds of covalent modification (see Chapter 1). Of these, the mechanism of greatest importance to metabolic regulation in animal cells is reversible protein phosphorylation. Protein kinases transfer the γ-phosphate group from ATP onto an amino acid residue in a covalent attachment that is stable unless it is cleaved by a protein phosphatase (for review see Chapter 4). The first protein kinase to be appreciated was cyclic 3′,5′-adenosine monophosphate (cAMP)-dependent protein kinase (known as PKA), that phosphorylates serine or threonine (Ser/Thr) residues on a wide variety of enzymes, notably enzymes

of glycogen metabolism that were the first targets of PKA research. In recent years, the number of known protein kinases has grown explosively. Indeed, in 2002, an electronic analysis of sequences in the human genome revealed 448 sequences that contained the critical residues essential for kinase function. The major groups of human protein kinases are listed in Table 14.5, and the roles and regulation of protein kinases in signal transduction pathways are discussed in much greater detail in Chapter 4. Here, we are primarily concerned with the effects of phosphorylation and dephosphorylation on the control of metabolic enzymes and other functional proteins.

The covalent attachment of one or more negatively charged phosphate groups onto a protein changes its conformation and its charge. The difference in charge between phosphorylated and dephosphorylated protein forms is often exploited for the separation or purification of the two forms via techniques such as ion exchange chromatography or isoelectric focusing (Fig. 14.5). These separation techniques, as well as selected key differences in kinetic behavior, are also used for judging the effects of signals or stresses on the interconversion of the enzyme/protein between high and low phosphate forms. A change in phosphorylation state can affect a protein in one of several ways, including (a) on–off control over activity, (b) changes to kinetic properties, (c) changes in enzyme binding to other proteins, and (d) other effects.

TABLE 14.5 Major Subfamilies of Protein Kinases Encoded on the Human Genome, Classified by Kinase Catalytic Domain

Subfamily Name	Number of Members	Representatives in the Group
AGC	59	Protein kinases A, G, C
CAMK	81	Calcium/calmodulin-dependent kinases
CMGC	72	Cyclin-dependent kinases, mitogen-activated protein kinases, glycogen synthase kinase, casein kinase 2
TK	94	Receptor and nonreceptor tyrosine kinases
CK1	12	Casein kinase 1

Source: From Krupa and Srinivasan (2002).

On–Off Control over Activity Many enzymes are converted between active and inactive states by reversible phosphorylation. The classic example of this is glycogen phosphorylase (GP) [reaction (4.4)], which cleaves glu-

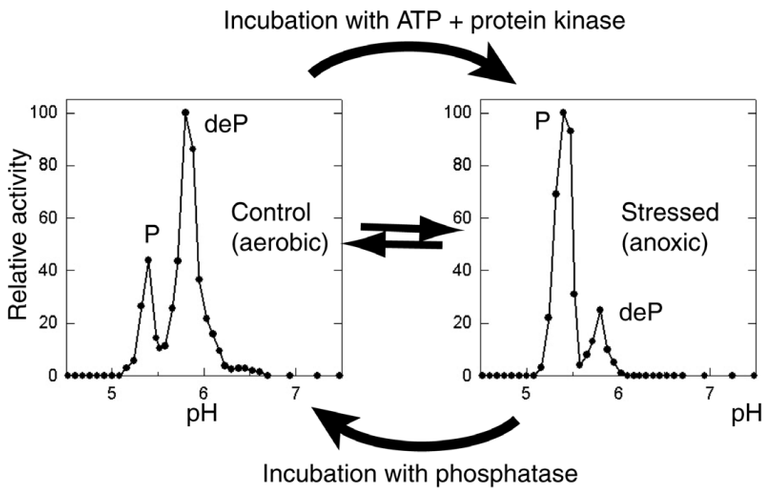

Figure 14.5 Assessment of stress effects on enzyme phosphorylation state. Tissue extracts from control and stressed animals are subjected to isoelectrofocusing, in this case producing two peaks of enzyme activity eluting with isoelectric points of 5.4 and 5.8. Environmental stress (anoxia exposure in this case) changes the relative amount of activity in each peak. To determine which is the phosphorylated form, tissue extracts are pretreated *in vitro* with either (1) protein kinase + ATP, which shifts activity into the peak at pI 5.4 and identifies it as containing the phosphoenzyme (P), or (2) phosphatase incubation, which shifts activity into the pI 5.8 peak and identifies it as the dephosphorylated (deP) enzyme. The figure is derived from the effects of anoxia exposure on pyruvate kinase in muscle of a marine mollusk; the deP form predominates under aerobic conditions and is converted to the P form under anoxic conditions (see Chapter 15).

cose-1-P units off the glycogen polymer:

$$\text{Glycogen}_{(n)} + P_i \rightarrow \text{ glucose-1-P + glycogen}_{(n-1)} \quad (14.4)$$

Phosphorylation of GP converts the inactive *b* form to the active *a* form. Simultaneously, phosphorylation has the opposite effect on glycogen synthase, inactivating that enzyme and thereby providing reciprocal control over glycogen synthesis versus breakdown. Although GP*b* can have activity *in vitro* when presented with high concentrations of AMP as an allosteric activator, it is unlikely that the *b* form contributes in any significant way to glycogen breakdown *in vivo.* Hence, phosphorylation of GP by phosphorylase kinase and its reversal by protein phosphatase 1 (PP-1) provides effective on–off control of the enzyme.

On–off control via reversible phosphorylation allows cells to maintain a high enzymatic potential that can be unleashed virtually instantaneously whenever it is needed. It provides the speed of response that longer-acting coarse controls (protein synthesis or degradation) cannot and a range of response (fold-activation) that is virtually impossible to achieve with other means of enzyme regulation such as changes in the concentrations of enzyme substrates or effectors. For example, when a fly or bee takes off, the percentage of GP in the active *a* form in flight muscle can rise from <20% to 80 to 100% within 15 s (triggered via Ca^{2+}/calmodulin activation of phosphorylase kinase) (see Fig. 1.6 and 4.11) in order to support the 20- to 100-fold increase in metabolic rate that is needed to power flight (dipteran and hymenopteran insects power flight with carbohydrate fuels).

Reversible phosphorylation providing on–off control is also found at many other key loci in cellular metabolism. For example, another critical locus in carbohydrate catabolism is regulated in this way. The pyruvate dehydrogenase complex (PDC) [reaction (14.5)] gates carbohydrate entry into the oxidative reactions of the tricarboxylic acid cycle:

$$\text{Pyruvate} + \text{CoA} + \text{NAD}^+ \rightarrow \text{acetyl-CoA} + \text{CO}_2 + \text{NADH} + \text{H}^+ \quad (14.5)$$

In this case, phosphorylation turns *off* the PDC, and control at this locus is important in regulating the relative use of carbohydrate versus lipid as aerobic fuels for the mitochondria. Indeed, phosphorylation-mediated inactivation of the PDC is a key element in the "carbohydrate sparing" that occurs during starvation or torpor (see discussion of hibernation in Chapter 16). It promotes the switch to lipid fuels by most organs in order to conserve carbohydrates for organs such as brain that have few other alternatives. It is not surprising, then, that the initiating enzyme in the mobilization of lipid reserves, triglyceride lipase (also called hormone-sensitive lipase), is oppositely affected by phosphorylation and turned *on*.

On–off control via reversible phosphorylation is also extremely important in the regulation of signal transduction cascades. Numerous protein kinases are themselves controlled in this manner by upstream kinases. For example, the activities of mitogen-activated protein kinases (MAPKs) increase about 1000-fold when they are phosphorylated. Hence, the dephosphorylated forms are effectively inactive *in vivo.* This sort of on–off control when applied to multiple enzymes in a signal transduction cascade is also highly effective in producing a huge amplification of a signal (see Chapter 4). The exploration of kinase cascades and many other proteins that are regulated via reversible phosphorylation (e.g., ribosomal initiation factors and elongation factors) has been greatly aided by a recent technological advance, the development of phospho-specific peptide antibodies (see Text Box 14.1).

Phosphorylation-Induced Changes to Kinetic Properties

Reversible phosphorylation provides on–off control for some enzymes, but for others it provides the means of making major adjustments to enzyme kinetic and regulatory properties, often changing the sensitivity of the enzyme to selected allosteric effectors and frequently facilitating large changes in pathway flux.

Table 14.4 illustrates this for the PK reaction. Compare the properties of the dephosphorylated and phosphorylated forms of mammalian liver PK. It can be seen that phosphorylation of the PK protein reduces enzyme affinity for substrate ($S_{0.5}$ PEP increases by 2.7-fold), reduces enzyme sensitivity to the activator fructose-1,6-P_2 (K_a increases by 2.2-fold), and increases enzyme sensitivity to inhibition by L-alanine (K_i decreases by 50%). Phosphorylation also changes the relationship between velocity and PEP substrate concentration, increasing the sigmoidicity of the relationship (the Hill coefficient increases). These changes are illustrated graphically in Figure 14.6. The relationship between [S] and *V* for the dephoshorylated and phosphorylated enzyme forms is reminiscent of the relationships seen in Figure 14.2 for an allosteric enzyme in the absence versus presence of an activator, but the key difference is that phosphorylation is a stable modification that is independent of changes in metabolite effector concentrations and permanent until reversed by the action of a phosphatase. All of the effects of phosphorylation on PK properties produce an enzyme that is less active *in vivo* because the phosphoenzyme has reduced substrate affinity, is less affected by activators, and is more strongly affected by inhibitors.

In addition, the reduced activity of the phosphoenzyme is frequently compounded *in vivo* by changes in the cellular levels of effectors. For example, in mammalian liver, PK is phosphorylated when it is necessary to turn off glycolysis and activate gluconeogenesis instead. Under these

TEXT BOX 14.1 PHOSPHO-SPECIFIC PEPTIDE ANTIBODIES

The development of phospho-specific peptide antibodies has created a minirevolution in the study of biochemical regulation, particularly for evaluating signal transduction pathways. The activity states of many proteins/enzymes are controlled in a virtual on–off manner by reversible phosphorylation so changes in the relative amount of phosphoprotein provides an excellent indicator of relative protein activity and of protein response to a stimulus or stress. Protein activity state is assessed using two kinds of antibodies, one that is raised against the whole protein and one that is raised against a short peptide that contains the phosphorylated amino acid residue (e.g., serine, threonine, tyrosine). The latter reacts only with the phosphoprotein and not with the dephosphorylated form. As Figure TB14.1 shows, a stress typically elicits a change in the relative amount of the phosphoprotein with little or no change in total protein content. An excellent source for further information on this topic is the website of Cell Signaling Technology, Inc., at www.cellsignal.com.

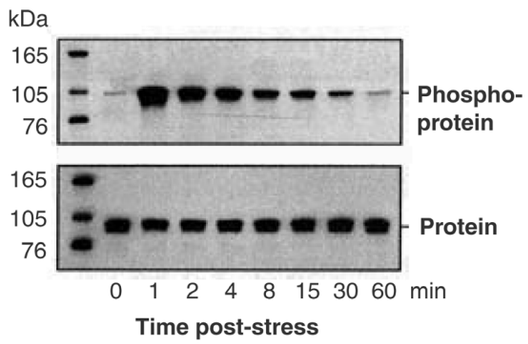

Figure TB14.1 Western blots showing the time course of activation of a 105-kDa protein by protein phosphorylation. Upper panel shows stress-induced changes in the amount of phosphoprotein determined with a phospho-specific polyclonal antibody (activation is maximal within 1 min and the declines to return to control levels by 60 min). Lower panel shows that total protein remains constant.

conditions, the concentration of L-alanine in liver cells is elevated because lactate and alanine, produced by working muscle, are two major substrates for gluconeogenesis in the liver. A severalfold increase in alanine concentrations, combined with the conversion of PK to its phosphorylated form that is strongly inhibited by alanine, create a powerful net suppression of PK activity *in vivo* so that carbon flow can be directed through the gluconeogenic reactions

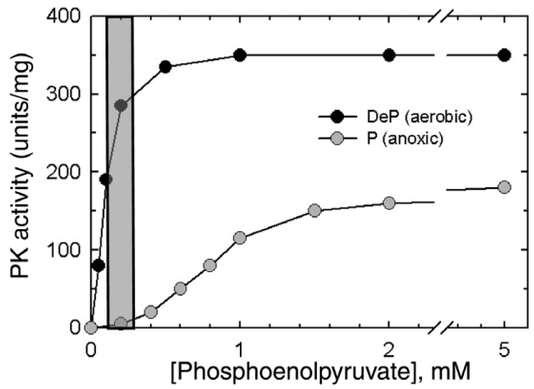

Figure 14.6 Relationship between pyruvate kinase (PK) activity and phosphoenolpyruvate (PEP) substrate concentration for the dephosphorylated (DeP) and phosphorylated (P) forms of the enzyme isolated from muscle of a marine mollusk exposed to aerobic versus anoxic conditions. The vertical bar shows the *in vivo* range of PEP substrate concentrations, 0.15 to 0.30 mM, in the muscle and indicates that the anoxic enzyme form would be virtually inactive *in vivo*. The calculated $S_{0.5}$ values (substrate concentration giving half-maximal activity) are 0.07 mM for the aerobic form and 0.85 mM for the anoxic form.

that bypass PK, namely pyruvate carboxylase and PEP carboxykinase (Fig. 14.4).

Phosphorylation-Dependent Changes in Binding to Other Macromolecules The conformational changes induced by protein phosphorylation or dephosphorylation often alter the ability of a protein to bind to another subcellular component. An excellent early example of this effect was provided during studies of mammalian skeletal muscle PFK. Muscle exercise stimulated PFK phosphorylation, but the kinetics of the purified phospho-PFK differed very little from those of the dephosphorylated form. This was puzzling until it was determined that the primary consequence of the phosphorylation of muscle PFK was to increase PFK binding to F-actin. This positioned this regulatory enzyme of glycolysis (the ATP-producing pathway) into proximity with the myofibrils (the ATP-utilizing motor) to potentially increase the efficiency of ATP turnover during exercise. A number of other muscle enzymes also associate with myofibrils, as will be discussed in a later section.

Reversible protein phosphorylation is also involved in hundreds of other binding interactions. For example, many transcription factors are phosphorylated via protein kinases (often by the MAPKs) to produce active factors that are then capable of binding to deoxyribonucleic acid (DNA) (see Chapter 6). Protein kinases and protein phosphatases are frequently compartmentalized to selected subcellular locations via binding interactions with targeting proteins. Phosphorylation of targeting proteins can either

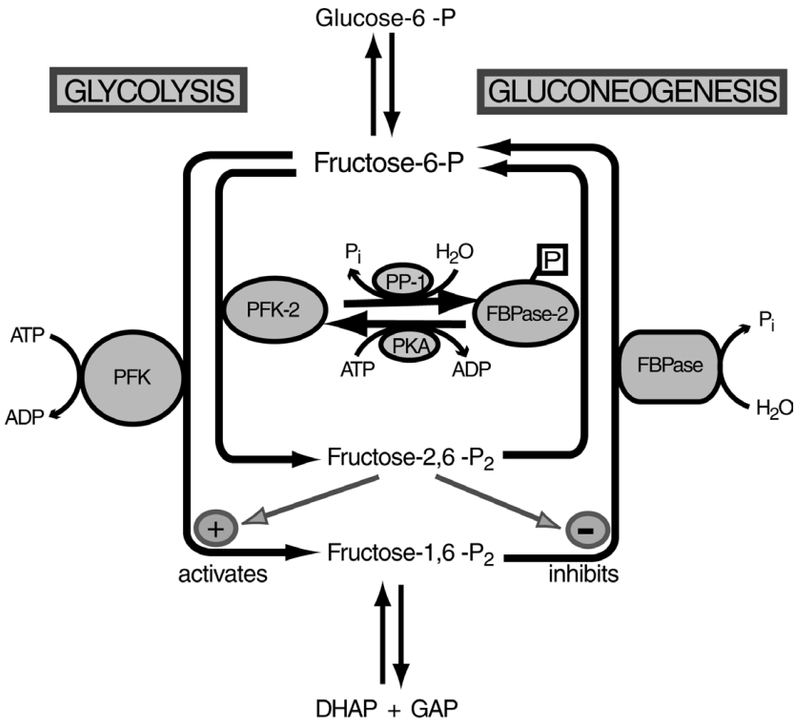

Figure 14.7 Reciprocal control of glycolysis versus gluconeogenesis via the allosteric effects of fructose-2,6-bisphosphate on phosphofructokinase (PFK) and fructose-1,6-bisphosphatase (FBPase). Phospofructokinase-2 (PFK-2), the enzyme that produces fructose-2,6-P_2, is bifunctional and synthesizes fructose-2,6-P_2 in its dephosphorylated form; but after phosphorylation by protein kinase A (PKA) it acts as a fructose-2,6-bisphosphatase to hydrolyze fructose-6-P.

disrupt or promote such binding. For example, PP-1 is targeted to the glycogen particle by its interaction with a glycogen-binding (G) targeting subunit. When glycogenolysis is activated under adrenergic stimulation (PKA mediated), phosphorylation of the G subunit by PKA causes PP-1 to dissociate from the glycogen particle and prevents the phosphatase from dephosphorylating GP at the same time that PKA is stimulating its phosphorylation (see Figure 4.14).

Other Enzyme-Specific Effects An excellent example of this is the case of the enzyme 6-phosphofructo-2-kinase (PFK-2). Only discovered in the early 1980s, PFK-2 and its product, fructose-2,6-bisphosphate (F2,6P_2), caused a minirevolution in our understanding of the control of glycolysis by identifying F2,6P_2 as an extremely potent effector of glycolytic versus gluconeogenic flux due to its reciprocal control over PFK and fructose-1,6-bisphosphatase (FBPase) [reaction (14.6)] (Fig. 14.7). This unique monomeric enzyme is bifunctional and capable of catalyzing either the synthesis or breakdown of F2,6P_2 depending on the phosphorylation state of the protein.

$$\text{Fructose-1, 6-P}_2 + \text{H}_2\text{O} \rightarrow \text{fructose-6-P} + \text{P}_\text{i} \qquad (14.6)$$

When dephosphorylated, the enzyme produces F2,6P_2, which is a powerful activator of PFK and a strong inhibitor of FBPase, the gluconeogenic enzyme that bypasses the PFK reaction. Phosphorylation of the enzyme by PKA causes a reversal of the reaction, causing the enzyme to function as a fructose-2,6-bisphosphatase (FBPase-2) and catabolize F2,6P_2. In mammalian liver it has been concluded that high F2,6P_2 mediates a "fed" signal that allosterically activates PFK to allow carbohydrate to be processed via glycolysis into various biosynthetic outputs. Under "starved" or stressed conditions, however, liver biosynthesis is inhibited and glycogenolysis (activated by glucagon or epinephrine stimulation of PKA) is directed into the synthesis of glucose for export. Inhibition of PFK-2 by phosphorylation stops F2,6P_2 synthesis so levels of the activator drop and, combined with the direct effects of phosphorylation of the kinetic properties of liver PFK (discussed above), this leads to a strong inhibition of the PFK locus that promotes glucose export. Under the same conditions, gluconeogenesis is typically activated to provide glucose output from this second source, and the phosphorylation-mediated promotion of the FBPase-2 reaction drains remaining

$F2,6P_2$ and releases FBPase from inhibition so that it can participate in gluconeogenesis (Fig. 14.7).

Biochemical Adaptation via Reversible Phosphorylation Given the wide array of actions and consequences that protein phosphorylation can cause, it is obvious that there are innumerable opportunities for using reversible phosphorylation as a mechanism of biochemical adaptation. Many specific examples are discussed in the next three chapters, but some of the principles for the adaptive use of reversible phosphorylation include:

1. *Susceptibility to Phosphorylation* An enzyme/protein may be susceptible to phosphorylation in some species but not in others, and the development of reversible phosphorylation control can provide a mechanism for exerting strong control on an enzyme under unusual or novel stress conditions. For example, skeletal muscle pyruvate kinase (PK) in mammals is typically not a phosphoenzyme. However, whelk muscle PK is a phosphoenzyme, and anoxia-induced phosphorylation produces major changes in enzyme properties that strongly suppress enzyme activity (Table 14.4). Another example provides a twist on this story. PK in the skeletal muscle of most amphibians follows the typical vertebrate pattern, but in species that estivate (estivation is a seasonal torpor induced by arid conditions), such as spadefoot toads, skeletal muscle PK has become a phosphoprotein. In this case, when toads enter estivation, the amount of dephosphorylated PK rises substantially and dephospho-PK shows kinetic differences (higher K_m for PEP, higher K_a for $F1,6P_2$) that would suggest that it is the less active form *in vivo.* Conversion of skeletal muscle PK to a less active form during estivation is one component of a general suppression of metabolic rate during estivation, many other aspects of which are also regulated by reversible phosphorylation of enzymes. It is interesting, however, that it is the dephosphorylated form of toad muscle PK that is the less active form, whereas in whelk muscle and vertebrate liver it is the phosphorylated form of PK that is less active. Hence, there are no fixed rules about whether phosphorylation of a given protein must activate or inactivate an enzyme.

2. *Consequences of Phosphorylation for Enzyme Kinetic Properties* Adaptive change in the quantitative effect of phosphorylation on enzyme properties can be critical for altering enzyme and/or pathway function in response to an imposed stress. Table 14.4 shows that the effects of phosphorylation on mammalian liver PK and molluskan muscle PK are qualitatively the same: In both cases, the phosphoenzyme shows a higher K_m for PEP, a higher K_a for $F1,6P_2$, and a lower K_i for L-alanine. However, the quantitative effects of phosphorylation on enzyme properties are hugely different for each of these parameters. Most notably, a two-fold difference in the K_i for alanine between phosphorylated and dephosphorylated forms of mammalian liver PK has been magnified to a 490-fold difference between the two enzyme forms in the mollusk. As will be discussed more fully in Chapter 15, the kinetic differences between the two PK forms in mollusk muscle provide a virtual on–off control over PK *in vivo* that is critical to redirecting glycolytic carbon flow into an alternative pathway of fermentative ATP production in anoxia-tolerant marine mollusks.

3. *Susceptibility to Different Protein Kinases* Enzymes may be phosphorylated by more than one kind of protein kinase, each with different consequences for enzyme function. Each protein kinase mediates enzyme response to a different signal so alone or in combination, enzyme phosphorylation by multiple protein kinases can create a wide range of enzyme responses to different stresses. Continuing with the example of PK, mammalian liver PK is well-known to be phosphorylated by PKA, which contributes to glycolytic inhibition when gluconeogenesis is needed. In marine mollusks, however, PK is susceptible to phosphorylation by protein kinases A, G, and C, but the major changes in PK kinetic properties that are induced by anoxia exposure (such as seen for whelk muscle in Table 14.4) are mimicked only by the actions of cyclic-3′,5′guanosine monophosphate (cGMP)-dependent protein kinase (PKG) on the protein.

Enzyme Control via Reversible Binding to Subcellular Macromolecules

The early history of enzymology explored the kinetic and regulatory properties of purified enzymes to characterize each individual enzyme catalyst and lay the framework for our understanding of how enzymes catalyze reactions and how they are influenced by allosteric and other effectors. The subsequent identification of posttranslational modification of enzymes provided the key to understanding how changes to the activity states of enzymes could be made in a manner that was independent of changes in cellular substrate and effector concentrations and provided the mechanism by which external stimuli could make large and stable changes to the activities and properties of selected enzymes. Coincident with the explosion of research on the role of posttranslational modification in enzyme control has been interest in a third mode of regulation, that of the spatial or three-dimensional control of metabolism in cells. This mode of control also provides multiple opportunities for biochemical adaptation, various examples of which will be discussed in this and the next chapter. Below, we will first briefly explore three issues:

1. Protein–protein binding interactions between enzymes and their regulatory proteins

2. Targeting of enzymes to specific subcellular sites via enzyme binding interactions with structural macromolecules
3. Formation of multienzyme complexes

Protein–Protein Regulatory Interactions Binding interactions between two (or more) proteins are now well-known as mechanisms of metabolic regulation. Binding interactions frequently regulate enzyme activity. The classic example is that of PKA. In its inactive form, PKA is a tetramer made up of two regulatory and two catalytic subunits. However, the binding of cAMP to the regulatory subunits causes their dissociation from the catalytic subunits, which are then free to phosphorylate target proteins. Numerous other signal transduction enzymes also have binding interactions that affect their activity (Chapter 4). For example, PP-1 activity is regulated by one of two inhibitor proteins that bind to it. The transcription factor nuclear factor kappa B (NFκB) shows another principle in its interactions with its inhibitor protein, IκB. Not only does the interaction with IκB hold NFκB in an inactive form, it also keeps the protein in the cytoplasm. Stimuli that activate NFκB do so by causing the phosphorylation and ubiquitination of IκB, causing it to dissociate from NFκB and allowing NFκB to migrate into the nucleus where it activates the transcription of various target genes.

Targeting of Enzymes to Subcellular Locations Protein–protein binding interactions are also important in targeting enzymes to very specific subcellular locations. Again, this is an extremely important mechanism for controlling enzymes involved in signal transduction (Chapter 4) and obviously presents many opportunities for adaptive change. Interaction with a targeting protein is well-known in the regulation of both PKA and PP-1 as well as the MAPKs. The regulatory subunits of PKA bind with A-kinase anchoring proteins (AKAPs) that feature a conserved "anchoring motif" to bind the regulatory subunit of PKA and a "targeting domain" that interacts with a particular subcellular component. Multiple AKAP proteins are known that interact with different structural proteins, membranes, or cellular organelles. As a result, PKA can be positioned at multiple subcellular sites where it is ready to respond to changes in local cAMP concentrations.

A similar system of targeting proteins is also key to PP-1 regulation. Indeed, this mechanism provides the answer to a paradox that has puzzled researchers for some time [the reader is referred to Cohen (2002) for an expanded treatment]. The human genome codes for $\sim$300–350 serine/threonine kinases but only $\sim$40 serine/threonine phosphatases. So how is the specificity and independent regulation of target enzymes maintained if this low number of phosphatases must serve all these kinases? The answer is with regulatory subunits. More than 50 regulatory subunits have been identified that can interact with the catalytic subunit of PP-1, and the formation of each of these complexes confers distinct phosphoprotein substrate specificities, restricted subcellular locations, and diverse regulation to PP-1. Regulatory subunits have been found that target PP-1 to glycogen particles, myosin, the nucleus, ribosomes, mitochondria, the cytoskeleton, and centrosomes, to name a few. In each location the regulatory subunit positions the enzyme near to its potential phosphoprotein targets. For example, association of PP-1 with the glycogen binding (G) subunit targets the enzyme to the glycogen particle where it can dephosphorylate active GP and halt glycogenolysis. Phosphorylation of the mammalian muscle G subunit by PKA (in response to epinephrine) triggers dissociation of the PP-1 catalytic subunit from G subunit, releasing it from the glycogen particle and interrupting its ability to dephosphorylate its three glycogen-bound substrates: GP, phosphorylase kinase, and glycogen synthase (see Figure 4.11).

Many metabolic enzymes and functional proteins are also localized to specific sites in the cell via protein–protein binding interactions, typically with structural macromolecules. For example, as mentioned previously, the GLUT4 glucose transporter is distributed between the plasma membrane and intracellular vesicular compartments. One of the actions of insulin stimulation on muscle cells is to translocate GLUT4-containing vesicles to the plasma membrane by stimulating a release of these vesicles from intracellular tethering sites and allowing them to move to the plasma membrane where docking and fusion of the vesicles is mediated via interaction with specific proteins.

Several other enzyme binding interactions are also well-known. Hexokinase isozymes I and II bind to mitochondria, whereas type III has a perinuclear localization. Type I hexokinase binds to mitochondria through an interaction with porin, the protein that forms channels through which metabolites traverse the outer mitochondrial membrane. Experiments have suggested that in this location, hexokinase selectively uses intramitochondrially generated ATP and that this provides a way of coordinating glucose entry into glycolysis with cellular ATP demands. Kinetic properties of the type I form (inhibition by G6P that is relieved by inorganic phosphate) also fit the enzyme for a catabolic function that is sensitive to the energy needs of the cell. By contrast, types II and III appear to serve anabolic roles such as providing G6P for glycogen synthesis or for the pentose phosphate pathway.

In skeletal muscle there is strong evidence of the association of enzymes involved in ATP production with elements of the ATP-utilizing myofibrillar contractile apparatus and that this association is key to muscle function. For example, the enzyme creatine kinase [CK; reaction (14.7)], which delivers ATP from creatine phosphate reserves, is localized in muscle cells at key subcellular sites where ATP demand

is high during muscle work: near the contractile machinery (the site of actomyosin ATPase), at the sarcoplasmic reticulum (where ATP is used for Ca^{2+} reuptake), and near the sarcolemma (where ATP is used to fuel the Na^{+}-K^{+} pump). A further pool of the enzyme is located on the inner mitochondrial membrane where it serves to regenerate PCr from ATP produced by oxidative phosphorylation. The MM-dimer of CK specifically interacts with the sarcomeric M-line, and a recent study showed that binding was localized to two pairs of lysine residues that are present in M-CK but are not found in the B-CK isozyme that is widely distributed in other tissues. The principle here is that addition or removal of protein–protein binding capabilities can fulfill organ-specific needs for the placement of selected enzymes or isozymes at specific subcellular locations.

Another enzyme involved in muscle adenylate metabolism, AMP deaminase [reaction (14.8)], is also regulated in this way. AMP deaminase binds to myosin, where it is positioned to help optimize the supply of ATP to the myosin ATPase. AMP deaminase drains the pool of AMP that forms as a result of the adenylate kinase reaction [AK; reaction (14.9)]:

$$\text{Phosphocreatine} + \text{ADP} \rightarrow \text{creatine} + \text{ATP} \qquad (14.7)$$

$$\text{AMP} \rightarrow \text{IMP} + \text{NH}_4^+ \qquad (14.8)$$

$$\text{ADP} \longleftrightarrow \text{ATP} + \text{AMP} \qquad (14.9)$$

The AK reaction helps to increase ATP supply to myosin ATPase if the rate of ATP production from muscle phosphagen pools, glycolysis, or mitochondrial oxidative phosphorylation is less than optimal and ADP begins to accumulate. The AK reaction scavenges ADP and converts it to ATP and AMP. Removal of AMP from the adenylate pool via the AMP deaminase reaction helps to keep the AK reaction running in the direction of ATP synthesis, and, in addition, the production of ammonium ion by this reaction has two effects: (a) It uses up protons that would otherwise further increase the acidification of the working muscle cell, and (b) NH_4^+ is a strong activator of PFK and provides an additional signal to stimulate PFK activity and glycolytic rate when ATP supply is limiting. Note that PFK also responds to two other signals of energy limitation: AMP is a strong activator and declining ATP reduces ATP substrate inhibition of the enzyme. The importance of AMP deaminase binding to myosin has been documented in multiple studies that have shown an increase in the amount of enzyme bound to myosin under physiological conditions where muscle ATP demand is increased (exercise) or where ATP supply is compromised (hypoxia). For example, a 90-min exposure of fish to hypoxia (low oxygen) increased the percentage of bound AMP deaminase in muscle from $\sim$20 to 50%.

There is also good evidence that some of the glycolytic enzymes have binding interactions with myofilaments that are important to the control of glycolysis in working muscle. Recall that the most prominent effect of protein phosphorylation on skeletal muscle PFK was to increase its affinity for F-actin, an action that would bring this regulatory enzyme of glycolysis into a close association with the myofibrils. The physiological relevance of PFK binding is supported by data that show that (a) PFK retains significant binding to subcellular particulate matter under the ionic strength and metabolite concentrations found *in vivo* (various other glycolytic enzymes bind at low ionic strength but are solubilized at physiological ion concentrations), (b) PFK binding increased at lower pH values, which would enhance binding under the increasingly acidic conditions of working muscle, and (c) PFK binding to F-actin has a net activating effect due to positive effects on enzyme properties (higher affinity for F6P substrate, greater sensitivity to AMP and inorganic phosphate as activators, less sensitivity to ATP and citrate as inhibitors). By contrast, the kinetic effects of enzyme-F-actin binding interactions were all inhibitory when aldolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), PK, and LDH were assessed. New studies also show that PFK, CK, and AK are targeted to the I-band and M-band regions of the sarcomere through association with a protein called DRAL/FHL-2, an adapter protein that, in turn, interacts with the elastic filament protein, titin. Another study has also shown that the presence of PFK, and to a lesser extent aldolase (but not PK or phosphoglycerate kinase), is necessary to achieve MM-CK binding to the myofibrillar M-line, which further demonstrates the importance of ordered enzyme binding to muscle structural components for the supply of ATP to the contractile apparatus.

Other advances in understanding the role of enzyme binding interactions in muscle function have come from studies with *Drosophila* flight muscle. In fruit fly muscle the enzyme glycerol-3-phosphate dehydrogenase (G3PDH) isozyme 1 (the muscle-specific isozyme) was found localized at Z-disk and M-lines and co-localized with it (and dependent on G3PDH-1 presence) were GAPDH, aldolase, triosephosphate isomerase, phosphoglycerate kinase, and phosphoglyceromutase. In mutant flies that were null for G3PDH-1, none of the other enzymes localized to either location. When G3PDH-3 (the isozyme of nonmuscle tissues) was substituted in transgenic flies, there was similarly no enzyme localization at either the Z-disks and M-lines; this isozyme lacks the C-terminal tripeptide (Glu-Asn-Leu) that is characteristic of G3PDH-1. Furthermore, these transgenic flies could not fly. The conclusion from this research is that the presence of a full complement of glycolytic enzyme activities is not enough to support muscle work. These enzymes must also be co-localized appropriately to create the correct structural

organization of the glycolytic enzymes in relationship to the ATP-utilizing muscle fibers.

Support for the physiological importance of glycolytic enzyme binding to subcellular structural elements has also come from multiple studies that documented changes in the percentages of bound versus free enzyme in response to experimental states (e.g., muscle exercise, hypoxia) that change the demand for ATP production. These studies used several different methods of cell disruption to preserve (theoretically) glycolytic enzyme binding interactions with particulate matter. A commonly used method, for example, is extraction in an isomotic sucrose solution that should not disrupt ionic binding interactions between proteins and allow the separation of enzymes into soluble (in the supernatant) versus bound (in the pellet) fractions. (Note: After centrifugation, enzyme in the pellet is subsequently released for assay by a second extraction in a dilute buffer.) Other methods used high pressure or ultracentrifugation of minced tissue pieces to retrieve free enzymes in extruded cellular "juice" while leaving behind bound enzymes. In quantitative terms the results from these techniques were often very different but, qualitatively, there was general agreement across multiple species and tissues that changes in glycolytic rate were positively correlated with changes in the percentages of bound glycolytic enzymes. Figure 14.8 shows two such studies. Exhaustive swimming exercise by trout (strongly increasing glycolytic rate) increased the amounts of several glycolytic enzymes that were associated with the particulate fraction of the cell whereas anoxia exposure of whelk heart (reducing glycolytic rate due to metabolic rate depression) reduced the content of bound enzymes. Note that in both cases changes in the amount of bound PFK occurred. Increased enzyme binding to the particulate fraction under conditions of increased cellular ATP demand would theoretically place glycolytic enzymes into close association with the ATP-utilizing myofibrillar apparatus. Thus, these sorts of studies produced strong evidence for the physiological importance of glycolytic enzyme distribution between bound and free states as a means of metabolic regulation and biochemical adaptation.

However, difficulties arose when attempts were made to reconcile these data with the kinetic and binding behavior of glycolytic enzymes *in vitro.* Several studies evaluated glycolytic enzyme binding with F-actin and, except for PFK (described above), the results were generally unimpressive. F-actin interactions with several glycolytic enzymes were shown to be ionic in nature with good binding demonstrated under the low ionic strength and low protein conditions of an *in vitro* assay but with binding virtually eliminated when ionic strength, metabolite levels, or protein concentrations were raised to mimic cytoplasmic conditions. Furthermore, several enzymes were kinetically inhibited when bound; for example, aldolase binding to F-actin was competitively inhibited by the aldolase substrate, $F1,6P_2$, so that there is no way that aldolase could both catalyze its reaction and remain bound to F-actin. PK and LDH binding to particulate matter were similarly disrupted by substrates (PEP, NADH).

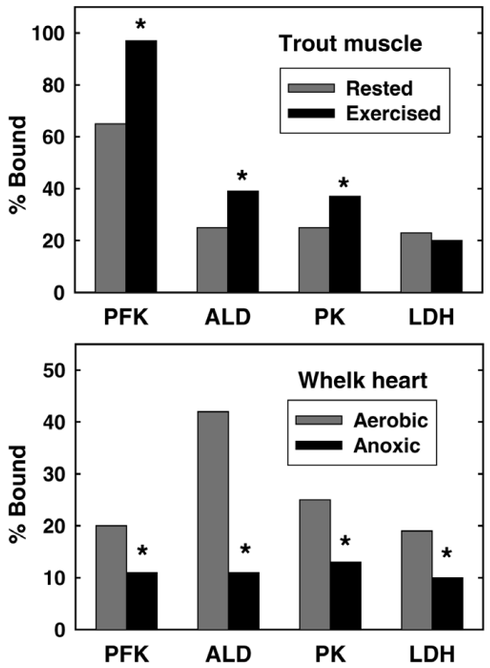

Figure 14.8 Effect of stresses on the binding of glycolytic enzymes to the subcellular particulate fraction. Fresh tissues were gently homogenized in a sucrose solution and centrifuged to separate free (supernatant) from bound (pellet) enzyme fractions; the pellet was then reextracted in dilute buffer to release bound enzymes for assay. Metabolic situations that increase glycolytic rate (e.g., muscle exercise in trout) typically increase the percentage of bound enzyme, whereas situations that decrease metabolic rate (e.g., anoxia-induced metabolic rate depression in whelks) are accompanied by reduced enzyme binding. *, Significantly different from the corresponding control state, $P < 0.05$.

Multienzyme Complexes The classic view of metabolism within a cell was that the vast majority of enzymes were freely soluble entities within the cytoplasm or within the lumen of various organelles. Compartmentation of selected enzymes/pathways into organelles was accepted as was the presence of a number of enzymes as integral membrane proteins, but the vast majority of metabolic enzymes, as well as their substrates, were considered to be randomly distributed in a cytoplasmic "soup." Pathway flux depended upon diffusion of substrates from one enzyme to the next. This view arose because a vast majority of enzymes, no matter how gentle the cell disruption or homogenization technique, always appeared in the soluble cytoplasmic fraction. This view began to change from the 1960s onward with two

types of advances: (1) the development of cell disruption techniques that apparently preserved delicate binding interactions and (2) histochemical techniques that showed uneven distribution of enzymes in the cytoplasm and preferential association of certain enzymes near structural elements in the cell. It is now known that the cytoplasm is very heterogeneous and that multiple mechanisms exist for creating subcellular associations of enzymes with other macromolecules in order to localize selected reactions to specific subcellular locations (see above). The reader is also referred to Chapter 3 for detailed treatments of the consequences of enzyme complex formation for enzyme activity and pathway flux.

Some types of tightly bound multienzyme complexes are unquestionably integral to selected cell functions. For example, the ribosome is clearly a complex of multiple enzymes and structural proteins that synthesizes new proteins just as the proteasome is a multicomponent digestive complex that degrades proteins; multienzyme complexes are also involved in DNA synthesis (see Chapters 6 and 7). As we will also see in Chapters 15 and 16, the association versus dissociation of multienzyme complexes such as the ribosome is a major mechanism of cellular response to stress; for example, the regulated disaggregation of polysomes is a key element in the strong suppression of protein synthesis under stress conditions that limit cellular ATP availability. Fatty acid synthase, pyruvate dehydrogenase, and cytochrome *c* oxidase are also good examples of metabolic "enzymes" that are, in fact, multienzyme complexes. For example, the pyruvate dehydrogenase complex is an association of three enzymes that carry out the reduction, decarboxylation, and thioester formation steps of the net reaction.

But what about enzymes in pathways that are traditionally thought of as being soluble, such as glycolysis. The idea of a *glycolytic complex* involving an association of all of the enzymes of glycolysis together has resurfaced in the scientific literature a number of times. Such a complex could serve to channel substrate from one enzyme to the next through the entire pathway to facilitate efficient carbohydrate catabolism and ATP output. If also anchored close to cellular sites with high ATP demand (e.g., myofilaments), a highly efficient coupling of ATP production and ATP use could be created that could, for example, power high-intensity white muscle work. During the 1980s and early 1990s several lines of evidence were developed to support the existence of a glycolytic complex, including (a) positive correlations between glycolytic rate and the percentage of bound glycolytic enzymes under multiple metabolic states that either increased or decreased ATP demand (Fig. 14.8) and (b) glycolytic binding interactions *in vitro* with structural proteins such as F-actin. However, selected glycolytic enzymes could be bound to structural elements without necessarily creating a full glycolytic complex, and as discussed above, several enzymes were actually inhibited when bound to F-actin. Indeed, mathematical modeling using the properties of free and bound enzymes and considering the percentages of each glycolytic enzyme that could be bound to F-actin under physiological ionic strength showed that a functional glycolytic complex that could channel substrate from glucose input to lactate output was unlikely. Hence, the idea of a glycolytic complex has been out of favor for the last several years. What is interesting, however, is that new studies are reopening interest in this idea. The data from *Drosophila* that show that flies cannot fly if G3PDH and its associated enzymes are not localized to specific areas of the contractile apparatus and the studies showing that PFK, CK, and AK bind to the DRAL/FHL-2 protein that associates with titin both support the idea that (a) glycolytic enzymes form binding associations with myofibrillar proteins that benefit ATP supply during contraction, and (b) a glycolytic complex is probably not built around enzyme associations with F-actin. Instead, the model seems to be that selected enzymes (e.g., G3PDH, PFK) interact with specific anchoring proteins and that these enzymes in turn can associate with other enzymes, potentially ultimately building an entire glycolytic complex. Hence, an older idea (glycolytic complex formation) seems poised for a come-back but one in which there will likely be many more players and much more complexity, including multiple myofibrillar proteins that can act as binding sites, multiple anchoring or targeting proteins, various modulating proteins, and also highly specific sequences in which different enzymes can bind to the complex. Studies over the next few years should be intriguing.

BIOCHEMICAL ADAPTATION AND MACROMOLECULAR PROTECTANTS

In most of the cases of enzymatic regulation discussed above, the purpose of biochemical adaptation is to make selective changes to the regulation of one or more individual enzymes or proteins in order to make very specific changes to metabolism or compensate for very specific effects of stress on individual enzymes/proteins. However, environmental stress can also have generalized consequences for many cell functions and/or macromolecules. In these situations, it is inefficient to consider modifying each protein to optimize it for stress resistance and more efficient to utilize one of several types of protectants or stabilizers for "global" stress relief. Metabolite protectants are reviewed briefly below and those involved in cold adaptation are discussed in greater detail in Chapter 17.

A variety of low-molecular-weight metabolites are used as protectants in the face of environmental stresses such as dehydration, freezing, or salinity change. These have two

main functions: (1) cell volume regulation and (2) stabilization of protein conformation or membrane bilayer structure. Metabolite protectants generally fall into two groups, carbohydrates and nitrogenous compounds. Carbohydrates include monosaccharides (e.g., glucose), disaccharides (e.g., trehalose), and a variety of polyhydric alcohols (e.g., glycerol, sorbitol, and others). Nitrogenous small molecules include urea, trimethylamines, betaine, and multiple amino acids including alanine, glycine, and proline. The same metabolite can be used in different contexts across phylogeny. For example, high glycerol levels provide antifreeze protection to cold-hardy insects (see Chapter 17), minimize cell water loss for algae placed in hyperosmotic media, and stabilize macromolecular structures in organisms that endure extreme desiccation (e.g., brine shrimp cysts). Glycerol is also well known to experimental biochemists as an excellent stabilizer of purified proteins and is widely used in medical science for the cryopreservation of cells and tissues.

Cells strive to maintain homeostasis, which includes maintaining an optimal cell volume and holding the ionic strength of body fluids within strict limits. Beyond these limits irreparable harm can occur; for example, mammals show nervous system dysfunction when plasma sodium rises by 30 to 60 mM above normal. Stresses that cause excessive water loss from cells (e.g., desiccation, freezing, hyperosmotic stress) create multiple problems because: (a) water is a key structural element in all cells, particularly for organisms without bones or exoskeletons, (b) water loss (or water gain) puts structural strain on membranes that can lead to the breakdown of the bilayer structure, (c) multiple cell functions (e.g., nerve signal conductance, cell sensitivity to external stimuli), and enzymatic activities require both overall ionic strength and the concentrations of individual ions to be maintained within narrow limits, and (d) maintenance of a surrounding vicinal shell of water is critical for native protein conformation and situations of extreme dehydration cause protein denaturation.

The majority of metabolite protectants have a primary physiological role in cell volume regulation and a secondary role, when cell water loss is unavoidably extreme, in the physical protection of macromolecules. The function in cell volume regulation is due to the colligative effects of high concentrations of low-molecular-weight metabolites in retarding water loss from cells. Physical stabilization of proteins typically arises because of the preferential exclusion of protectants from contact with the surface of proteins in aqueous solutions; this stabilizes the native conformation of proteins surrounded by their "shells" of water (see Chapter 17 for more about the action of cryoprotectants on both proteins and membranes).

Certain metabolites such as polyhydric alcohols and neutral amino acids are favored as protectants for several reasons. High solubility in the aqueous cytoplasm is a must in order to achieve the high concentrations (0.5 to 2 M) that are typically needed to have a substantial colligative effect in retarding cell water loss. But such high concentrations of many solutes (e.g., inorganic ions) would have hugely negative effects on protein conformation and enzymatic activities. Hence, useful protectants must be *compatible solutes*, which means that their presence in high concentrations must have little or no effect on the functions of cellular enzymes and pathways. That this is true has been shown for many different enzymes but is illustrated in Table 14.6 for the enzyme 6-phosphogluconate dehydrogenase, the second enzyme in the pentose phosphate pathway. In present case, the Michaelis constant (K_m) for substrate of the enzyme was assessed at two temperatures and in the presence/absence of glycerol, sorbitol, and KCl. At 22°C, the addition of high levels of glycerol (500 mM), sorbitol (250 mM), or both polyols had little effect on the K_m, but high KCl greatly reduced enzyme affinity for substrate by increasing K_m by sevenfold. However, when glycerol and sorbitol were added to assays containing high KCl, the negative effects of KCl on K_m were largely overridden. The same relationship was seen when the enzyme was assayed at 5°C, but, additionally, the low-temperature-stimulated rise in the K_m value under control conditions (no additions) was also partially reversed by the presence of polyols.

Nitrogenous metabolites are also frequently used as protectants. All animals maintain an intracellular ionic strength (made up mainly of K^+ but also Na^+, Cl^-, Ca^{2+}, and other ions) of 200 to 300 mM with other metabolites and proteins adding about another 200 mM. However, marine animals

TABLE 14.6 Effects of Glycerol and Sorbitol on the Michaelis Constant (K_m) for Substrate of 6-Phosphogluconate Dehydrogenase from a Cold-Hardy Insect: Reversal of Negative Effects of Low Temperature and High Salt[a]

	K_m 6-Phosphogluconate (μM)	
	Assay at 22°C	Assay at 5°C
Control (no additions)	64	119
+ Glycerol	53	60
+ Sorbitol	52	52
+ Glycerol and sorbitol	38	64
+ KCl	451	471
+ Glycerol, sorbitol and KCl	119	232

[a]When present, the concentrations of additions were 500 mM glycerol, 250 mM sorbitol (both similar to natural winter levels in the insect), and 500 mM KCl. Compared with the control K_m at 22°C, a temperature decrease to 5°C doubles the K_m whereas addition of KCl raises K_m by ~sevenfold. In the presence of polyols, the temperature effect is reversed and the KCl effect is greatly reduced.

have to reconcile this with a seawater ionic strength of ~1000 mM (mostly Na^+ and Cl^-). Marine invertebrates make up the difference in osmolality with high intracellular pools of amino acids, betaines, and other nitrogenous metabolites. These pools are raised or lowered as needed to allow animals to compensate for increases or decreases in seawater salinity. Again, the nitrogenous metabolites used are typically compatible solutes that do not disrupt enzyme kinetic or regulatory properties. However, one interesting situation deserves comment. Elasmobranch fishes (sharks, rays) use high concentrations of urea and trimethylamines/betaines in their body fluids to balance their internal osmolality with that of seawater. Urea is well-known to biochemists as a protein denaturant, and so researchers wondered how shark enzymes would function in the presence of constant high urea, about 400 mM in animals adapted to full-strength seawater. Indeed, the kinetic properties of a number of shark enzymes were negatively affected by high urea when assayed *in vitro.* However, these same enzymes responded positively to the addition of trimethylamines/betaines and, when added together in the physiological ratio (approximately 2 : 1), the negative effects of urea on enzyme properties were fully counteracted by the positive effects of trimethylamines/betaines. Hence, sharks have evolved a balanced system of multiple osmolytes that, as a whole, is compatible with unperturbed enzyme function although individual components can perturb some enzymes.

Urea is also commonly accumulated by terrestrial organisms that undergo desiccation stress. For example, urea accumulation is key to water retention during estivation (torpor) in toads and frogs that live in arid environments. These animals are only active briefly during a short summer rainy season, but then they retreat underground to estivate for 9 to 10 months of the year. As the desert soil dries out, the animals respond to increasing desiccation stress by breaking down muscle protein and accumulating urea, the nitrogenous end product of protein catabolism in their body fluids (see Chapters 8 and 9 for urea cycle function). Urea levels can reach 300 mM in plasma and tissues of spadefoot toads, but unlike the elasmobranch situation no counteracting solutes accumulate. However, in this case, no counteracting solute system is needed because natural levels of urea in estivating toads actually have little or no effect on estivator enzymes. Figure 14.9 shows the effect of 200 mM KCl on four toad enzymes. In all cases, enzyme activity was inhibited including a 90% reduction in the activity of glutamate dehydrogenase, a key enzyme of amino acid catabolism. However, urea inhibited only one enzyme. The comparable situation was documented for many other metabolic enzymes from liver and muscle of spadefoot toads. Because toads can lose about half of their total body water during estivation, intracellular K^+ and Cl^- concentrations could rise during estivation to a level that can have serious inhibitory effects on cellular enzymes. The accumulation of urea in estivator tissues helps to prevent this. Acting as a colligative osmolyte, the accumulation of high amounts of urea retards water loss from cells and from the organism as a whole. This, in turn, minimizes the increase in intracellular inorganic ion

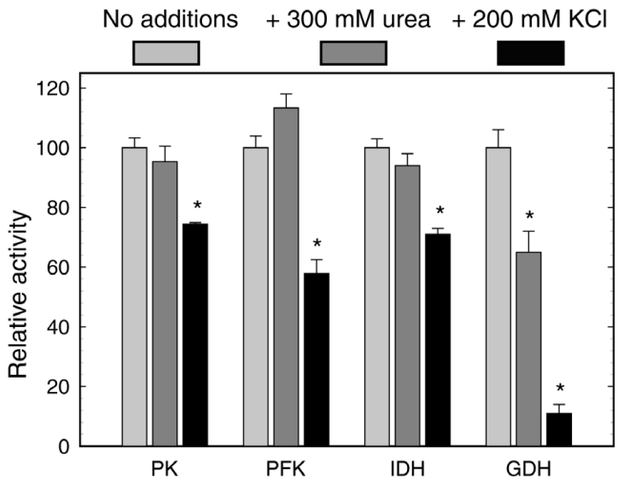

Figure 14.9 Effect of the addition of 300 mM urea or 200 mM KCl on the maximal activities of enzymes from spadefoot toad skeletal muscle. Enzymes are: PK, pyruvate kinase; PFK, phosphofructokinase; IDH, NAD-dependent isocitrate dehydrogenase; GDH, glutamate dehydrogenase. Data are expressed relative to control activities (no additions) and are means ± SEM, $n = 3$-4. *, Significantly different from the corresponding control, $P < 0.01$. See Storey (2002) in the suggested reading list.

concentrations and prevents ion concentrations from rising into a range that would inhibit enzymatic activity.

ADAPTIVE CONTROL OF TRANSCRIPTION

Organisms maintain an astonishing array of genes; many are constitutively expressed either in all or selected cell types, but many others are differentially expressed only in certain situations such as during growth, differentiation, and sexual maturation or in response to external stimuli or stressors including pathogens, changes in food availability, and changes in environmental parameters. The ability to detect and analyze stress-induced changes in gene expression has improved immensely in recent years and has led a revolution in the way that researchers can approach problems in biochemical adaptation. Previously, the main approach to a problem in biochemical adaptation was to identify some outstanding metabolic feature that was present in stress-tolerant organisms but not stress-intolerant forms and then work "from the bottom-up" to determine how that feature functioned and how it was regulated at both a protein and gene level. Examples from the field of cold-hardiness illustrate this point (see Chapter 17 for greater expansion). One of the first discoveries in the field of insect cold-hardiness was that animals produced huge quantities of glycerol (reaching $\sim$2 M in concentration or as much as 20% of the animal's body weight) that protected cells for survival at -40°C or lower. This initial discovery has led to thousands of research studies that have explored the distribution of glycerol and other cryoprotectants in hundreds of species, the regulation of cryoprotectant synthesis/degradation, the actions of cryoprotectants in protecting cells and proteins, and the applied use of these metabolites in medical organ cryopreservation. Similarly, a measured difference (hysteresis) between the freezing and melting points of the blood plasma of cold-water marine fish led to the discovery of antifreeze proteins (AFPs) and spawned another huge field that has explored AFPs in multiple ways: physiological function, molecular binding interactions with ice, and, most recently, the control of gene expression and the identification of novel mechanisms of gene evolution (see below).

However, the recent explosive growth in gene screening technologies, including new complementary DNA (cDNA) arrays (Text Box 14.2) has now made it possible to explore biochemical adaptation "from the top-down" by providing the means to identify those genes that are up-regulated in response to a stress and then analyze the corresponding response of protein levels and determine the action of the protein in contributing to stress tolerance. This approach has been particularly useful in two situations: (a) identifying gene/protein changes that are important but do not have a major metabolic "footprint" that would allow them to be

TEXT BOX 14.2 NEW FRONTIERS IN BIOCHEMICAL ADAPTATION

A revolution is underway in biology and medicine supported by two major advances: (1) the sequencing of the genomes of multiple species and (2) the development of cDNA microarrays. These have been combined to provide the ability to simultaneously assess the responses to an experimental parameter by thousands of genes and provide a broad-based and comprehensive analysis of cell/organ responses to stimuli (e.g., hormones, nutrition, development, aging), environmental stresses, or disease. For decades, the experimental sequence for studying organismal response to stress has been (a) identify a perturbed or novel physiological function, (b) seek an underlying protein/enzyme adjustment, (c) characterize the protein/enzyme adaptation, and (d) where appropriate, analyze gene structure and/or transcriptional control. Gene screening technology inverts this sequence and provides researchers with a second way of tracking down biochemical adaptations based on the identification of genes that are strongly up- or down-regulated by an imposed stress. Very importantly, array screening is "opening the eyes" of researchers to the coordinated involvement of many aspects of cellular metabolism in the response to an external stimulus. Major advances provided through gene screening can include: (1) discovery of genes/proteins and their cell functions/pathways that were never previously suspected as being part of an adaptive response, (2) analysis of the responses to a stress by functionally related groups of genes/proteins (e.g., mitochondrially encoded genes, genes for transmembrane transporters), and (3) identification of responses by multiple genes that are under control by a specific signal transduction pathway or that share a common response element.

MICROARRAY TECHNOLOGY

State-of-the-art glass slide microarrays can have up to 31,500 nonredundant cDNAs bound to them, most of which are identified genes, whereas others are expressed sequence tags representing genes of, as yet, unknown function. Figure TB14.2 outlines the basic procedure of array screening showing the following steps: (1) mRNA is isolated from control versus experimental tissues, (2) reverse transcriptase is used to create first-strand cDNA from the mRNA template in the presence of one of two fluorescent dyes (either Cy3 or Cy5) conjugated to dCTP, (3) equal amounts of control and experimental cDNA samples are mixed and applied to the microarray for hybridization, (4) slides are washed to remove nonspecific binding and centrifuged to dry, (5) slides are placed in a microarray reader and scanned twice to provide fluorescent intensity profiles for each dye (excitation and emission wavelengths are, respectively, 550 and 570 nm for Cy3 and 649 and 670 nm for Cy5), and (6) false coloring is applied to each intensity profile, and then images are superimposed and typically show differences in signal intensity as follows: red (experimental signal $>$ control), yellow (experimental $=$ control), or green (experimental $<$ control). A ratio experimental : control (E : C) transcript levels of about 2 : 1 or greater is generally considered to be significantly up-regulated. A color image of a hybridized array and a more detailed technical explanation and animated tutorial are available from the Microarray Centre (www.microarrays.ca).

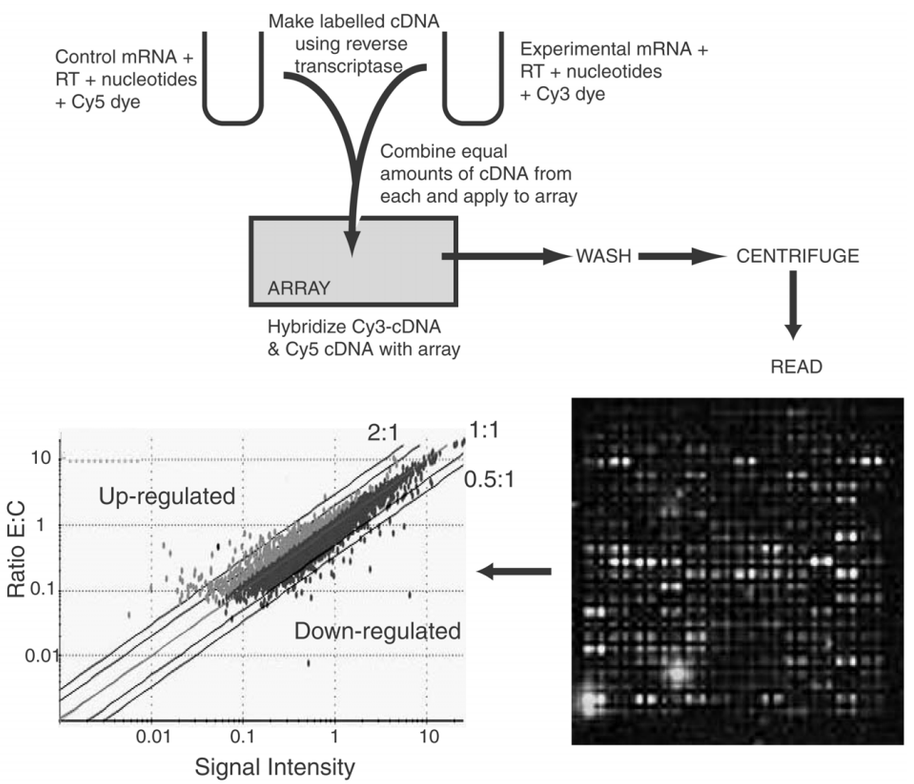

Figure TB14.2

HOMOLOGOUS VERSUS HETEROLOGOUS SCREENING

Whereas homologous screening is obviously best (e.g., human tissue samples assessed with human cDNA arrays), comparative biochemists can make good use of heterologous screening to assess differential gene expression in unusual animal systems. For example, we have used human arrays to assess gene expression changes in hibernating ground squirrels and bats (see Chapter 16). The gene sequence differences between species do limit the degree of cross-hybridization but still allow analysis of thousands of genes. For example, we found 85 to 90% cross-reaction when cDNA prepared from ground squirrel tissues was screened on a human 19,000-gene cDNA array but only 18.4% cross-reaction when cDNA from a marine snail was used. The low cross-reaction of snail cDNA is disappointing, but the analysis still revealed over 300 genes that were putatively up-regulated in response to anoxia stress (including protein phosphatases and kinases, mitogen-activated protein kinase-interacting factors, translation factors, antioxidant enzymes, and nuclear receptors), providing a plethora of new "leads" in the search for the regulatory mech-anisms underlying anoxia tolerance (see Chapter 15).

found through a bottom-up approach, and (b) finding novel proteins that were never before implicated in stress tolerance.

Figure 14.10 shows the application of this top-down approach in the discovery and assessment of a novel gene called *liver 16* or *li16*, which contributes to freezing survival by the wood frog, *Rana sylvatica.* The wood frog is one of only a handful of amphibians and reptiles that naturally endures whole-body freezing during winter hibernation (see Chapter 17). The search for freeze-responsive genes began with the construction of a cDNA library made from messenger ribonucleic acid (mRNA) isolated from liver of frozen frogs. The library was screened using cDNA probes prepared from mRNA of control frogs (held at 5°C) and frozen frogs (24 h at −2.5°C) and a freeze-responsive clone was isolated. Nucleotide sequencing provided a partial sequence, and then this was extended using the technique of 5′RACE (rapid amplification of cDNA ends) to provide the full open reading frame that encoded a 12.8-kDa protein. Both nucleotide and putative protein sequences were compared with all others stored in GenBank but had no matches. This suggested that the *li16* gene encodes a protein with a unique function in vertebrate freeze tolerance. The technique of Northern blotting was used to confirm that the *li16* gene was up-regulated during freezing. Samples of mRNA from liver of control frogs and frogs frozen for 4, 8, or 24 h were separated on formaldehyde gels, transferred to nylon membranes, and then hybridized with *li16* probe, the results showing a mean 3.6-fold increase in *li16* transcript levels after 24 h. A stress-induced elevation of transcripts is most likely due to increased gene transcription but can also arise from post-transcriptional controls (e.g., decreased rate of transcript degradation). Hence, the nuclear runoff assay was used to directly test the rate of transcript synthesis. Freshly isolated nuclei from both control and frozen frogs were incubated with ^{32}P-uridine triphosphate to label elongating transcripts and after 30 min the levels of radiolabeled *li16* transcripts were assessed by dot blots using *li16* probe. As Figure 14.10 shows, nuclei from frozen frogs produced substantially more *li16* transcripts, levels being 2.4-fold higher than in control frogs; this confirmed increased gene transcription during freezing (however, transcript levels of a constitutively expressed gene were unchanged). Finally, since stress-induced up-regulation of a gene is of little or no functional consequence if it is not translated into protein, Li16 protein levels were examined via Western blotting. Li16 protein was quantified using antibodies raised against the C-terminal decapeptide of the protein. Western blots revealed the presence of an appropriately sized 13- to 14-kDa protein in wood frog liver, and the content of Li16 protein increased progressively during freezing to a final 2.4-fold higher than control levels. The function of the Li16 protein awaits further experimentation but, to date, some facts are helping to narrow its possible functions: (a) The protein has no signal sequence and is therefore not exported from hepatocytes, (b) expression is limited to liver, heart, and gut, (c) the protein is also induced by anoxia exposure, which suggests a possible role in aiding ischemia resistance during freezing, and (d) transcript levels in tissue slices respond to stimulation by cGMP but do not respond to cAMP.

Changes in the amounts of selected proteins in cells is a major mechanism of biochemical adaptation, and regulation of gene transcription and protein translation are integral to biochemical adaptation. Multiple opportunities for transcriptional control exist (summarized in Chapter 6), and these can all be utilized to achieve biochemical adaptation.

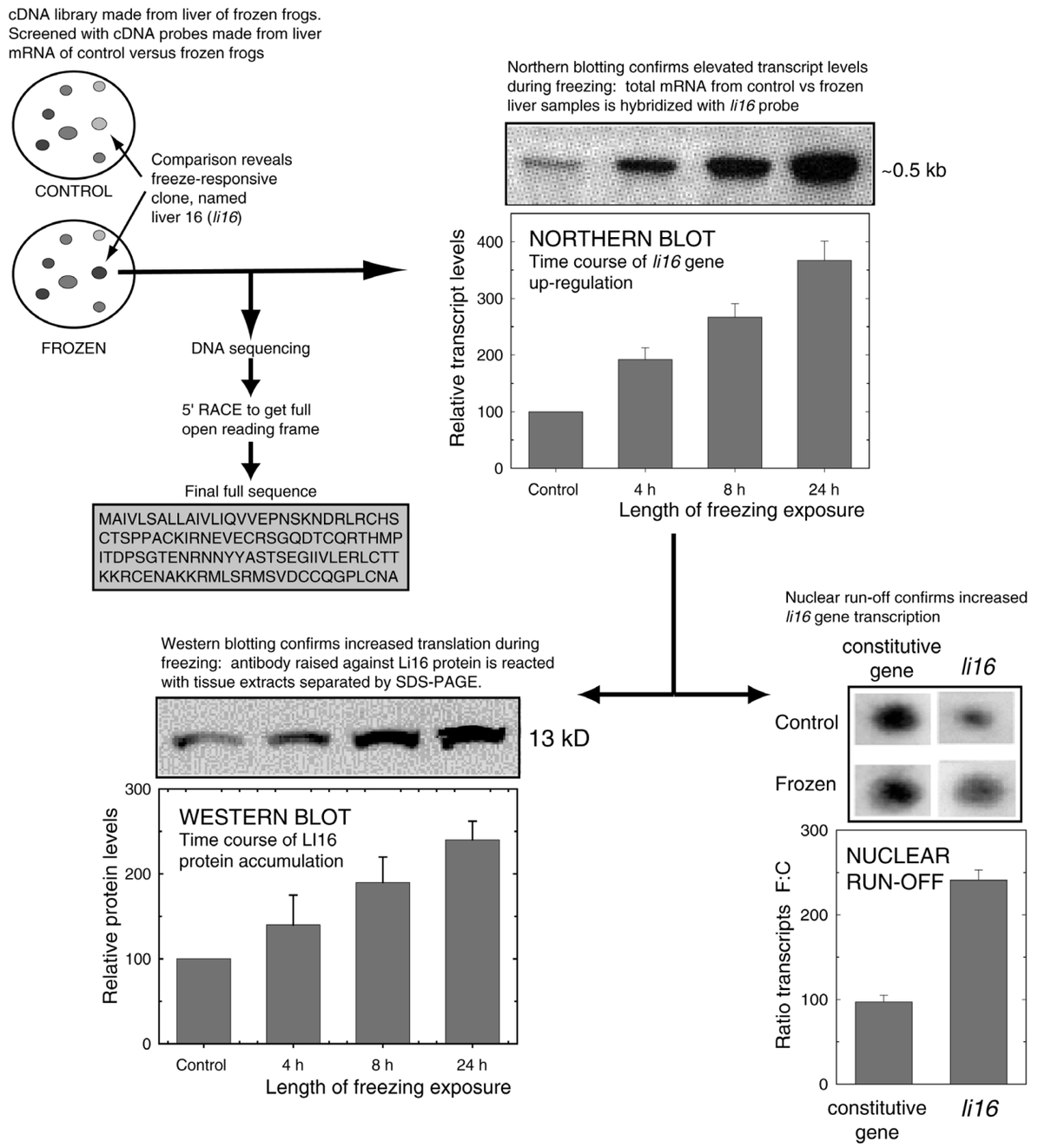

Figure 14.10 Methods for discovery and analysis of stress-induced genes highlighting the analysis of a freeze-induced novel gene, *li16*, from liver of the wood frog, *Rana sylvatica.* Techniques are explained in the text. [Example data are excerpted from J. D. McNally, S. Wu, C. M. Sturgeon, and K. B. Storey (2002). *FASEB J* **16**:902–904.]

These include:

1. Evolution of new genes encoding new protein types that address key adaptive needs.

2. Duplication of genes to insert multiple copies in the genome to greatly increase the number of mRNA transcripts that are produced in response to a signal.

3. Modification of existing or elaboration of new response elements in the 5′ untranslated region of a gene so that transcription of an individual gene can respond to a new transcription factor or to new combinations of factors.

4. Elaboration of new transcription factors or modification of the stimuli that activate transcription factor synthesis to provide a new or modified transcription factor response to a signal. Modification of transcription factor response (in either type or amount) would typically alter the responses of all target genes under its control to provide a modified yet coordinated response by multiple genes to a signal.

5. Modification of one or more elements of the signal transduction pathway leading from cell surface to gene expression that may include: the cell surface receptor, a second messenger metabolite, and one or more protein kinases often linked in a cascade that ultimatly regulate transcription factor synthesis. Multiple components in the pathway allow for multiple regulatory inputs that could permit:

   a. One signal to be spread out to initiate multiple effects on different aspects of cell metabolism such as up-regulation of selected genes, stimulation of fuel metabolism, and modulation of membrane ion conductance
   b. Diverse signals to activate the same coordinated set of responses or subsets of the responses
   c. Signal response to be modulated by inputs from other extracellular or intracellular sources

Two examples drawn from the field of animal cold-hardiness provide excellent illustrations of the principles of biochemical adaptation at the translational level. The first shows how the elaboration of new transcription factors (mechanism 4 above) can be utilized to bring preexisting gene responses to dehydration under the control of a cold trigger, whereas the second shows how new genes can be constructed and proliferated (mechanisms 1 and 2 above) to fulfill a requirement for an antifreeze protein.

Modification of Transcription Factors and Response Elements

Many plants and animals that live in temperate, polar, and alpine regions of Earth have developed freeze tolerance, the ability to endure the accumulation of ice in extracellular fluid spaces while defending the liquid state of the cytoplasm (see Chapter 17). Several of the adaptations that aid freeze tolerance are concerned with cell volume regulation, including mechanisms that defend a critical minimum cell volume against cell water loss into growing extracellular ice crystals, and the proliferation of stabilizers that help to preserve the structural integrity of membranes when cells shrink. In plants, these same mechanisms are found worldwide in drought-resistant plants (to help cells resist evaporative water loss), and this gave rise to the idea that adaptations for freeze tolerance grew out of preexisting strategies of desiccation resistance. How was this accomplished? The answer lies in gene regulation.

Genes involved in desiccation resistance in plants all contain a drought-responsive element (DRE) in their 5′ promoter region that binds a DRE-binding (DREB) transcription factor that is produced in response to a dehydration signal. When researchers looked at cold-responsive genes, they identified a cold-repeat element (CRT) in the 5′ promoter of cold-responsive genes that bound a CRT binding factor (CBF). Interestingly, when the DRE and the CRT sequences were finally compared in the model freeze-tolerant plant, *Arabidopsis thaliana*, they were found to be identical. Hence, the same set of cell volume regulatory genes could be turned on by either the DREB or CBF transcription factors (Fig. 14.11). However, although the DREB transcription factor is widely distributed, only cold-hardy plant species possess the CBF transcription factor. Transcription and synthesis of CBF is stimulated by cold or freezing exposure through a signal transduction pathway that has not yet been worked out. Thus, through the development a single new transcription factor (CBF), plants were able to exert coordinated control over a diverse group of genes whose products aid drought resistance and place the expression of these genes instead under the control of a cold signal. For more about this topic, see Chapter 17.

Elaboration of New Genes and Proteins

As much as possible, metabolism is conservative, making use of existing genes and proteins and modifying them as little as possible to adapt to new situations. For example, as little as one amino acid substitution in the sequence of the enzyme LDH can differentiate the pressure-sensitive enzyme of shallow-water fish from the pressure-insensitive form displayed by deep sea fish.

In some cases, however, a truly novel protein is needed for a unique new function and then some very creative solutions are found. Two instructive examples of how new genes and proteins are created comes from recent research on the origin of antifreeze proteins in coldwater marine fishes. The freezing point of vertebrate plasma is about -0.5°C, whereas that of seawater is -1.86°C. Nonetheless, polar fish live year-round in water that is colder than the freezing point of their blood; in temperate regions, surface-dwelling and inshore fish also show the same anomaly during the winter months. In these situations, body fluids are prevented from freezing by the presence of antifreeze proteins (AFPs) that bind to microscopic ice crystals and prevent them from growing (AFP action is discussed in greater detail in Chapter 17). In evolutionary terms, the

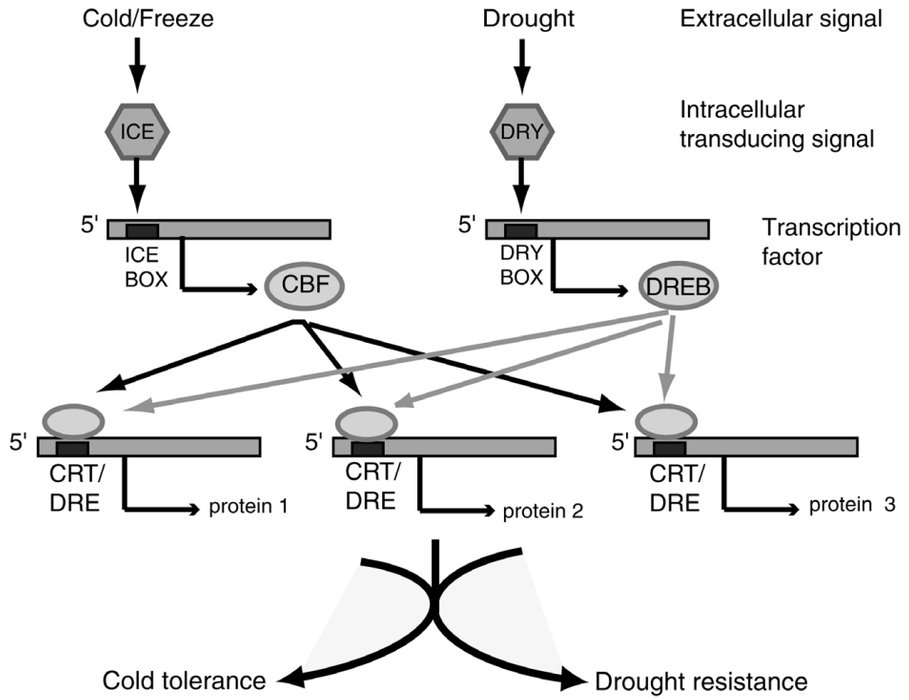

Figure 14.11 Schematic illustrating how a group of plant genes that aid drought resistance were put to a new use in freeze tolerance through the elaboration of a new transcription factor that allowed the genes to respond to a second environmental signal, cold. Genes involved in drought resistance contain a drought-responsive element (DRE) with a sequence TACCGACAT in their 5′ promoter region. This responds to the DREB transcription factor that is produced in response to a "DRY" signal. The same response element occurs in genes that respond to cold stimuli where it is known as the cold-repeat (CRT) element. Through the elaboration of a single new transcription factor, CBF (CRT binding factor), these multiple drought-responsive genes were made responsive to cold. CBF responds to an "ICE" (meaning "inducer of CBP expression") signal that interacts with a cold-regulatory element, the "ICE-box," present in the promoter region of the CBF. [Redrawn from M. F. Thomashow (1999). *Ann Rev Plant Physiol Plant Mol Biol* **50**:571–599. Used with permission.]

pressure to develop antifreezes was rapid and recent. The need for them arose only 10 to 14 million years ago when sea level glaciation began to recur after an interruption of >200 million years. How are new genes produced?

Five main types of AFPs are known, each in different families of fish, and two of these provide intriguing stories of gene adaptation. The cysteine-rich type II AFPs illustrate the evolution of an existing gene/protein into a new function. Type II AFPs are found in sea raven, smelt, and herring, species that diverged long before the glaciation that stimulated the need for AFPs. Nonetheless, both structural and sequence analysis has shown that the AFPs of all three species were derived from the Ca^{2+}-dependent (C-type) lectins, proteins that play a role in immunity by recognizing surface carbohydrates on pathogens. AFPs from all three fish show up to 30% sequence identity with the carbohydrate recognition domains (CRDs) of C-type lectins. Apparently, the ability of the CRDs to recognize and bind to galactose or other sugars was modified to create an AFP that recognizes and binds to ice. Interestingly, in two of the three species, the AFP retained one of the two calcium-binding domains of the C-type lectin and is Ca^{2+}-dependent, whereas the sea raven AFP lost both domains and is Ca^{2+}-independent.

Interestingly, plant AFPs also appear to be derived from older protein types. Carrot AFP is leucine rich and shows high sequence similarity with polygalacturonase inhibitor proteins that are secreted by plants as antifungal agents. AFPs in winter rye also show derivation from pathogenesis-related secretory proteins, the progenitor proteins apparently being ß(1-3)endoglucanase and chitinase. As in fish, therefore, plant AFPs appear to have arisen quite recently on the evolutionary timescale through modification of preexisting protein types.

The antifreeze glycoproteins (AFGPs) of Antarctic notothenioids and Arctic cod illustrate a different strategy

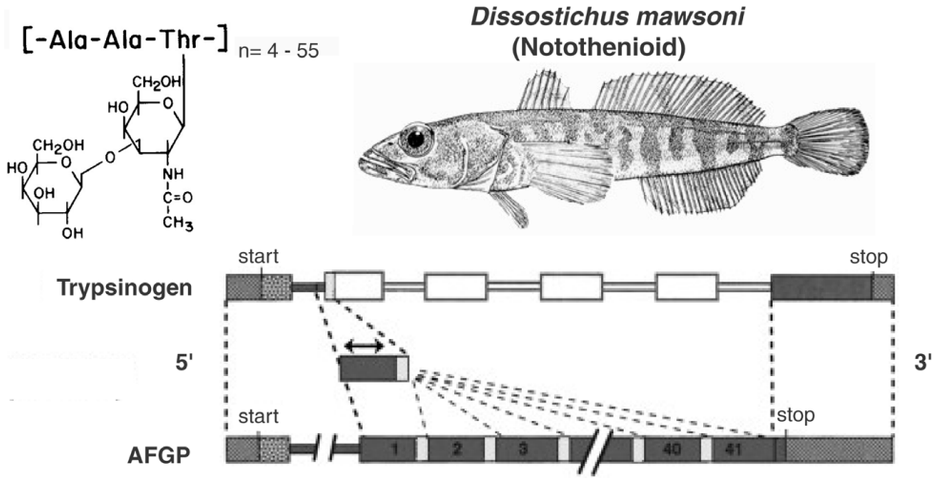

Figure 14.12 Evolution of the antifreeze glycoprotein (AFGP) gene from the pancreatic trypsinogen gene in the Antarctic notothenioid fish *Dissostichus mawsoni.* Upper left panel shows the structure of the glycotripeptide repeat with the disaccharide galactose-*N*-acetylgalactosamine attached to the threonine residue; different sizes of AFGPs repeat the tripeptide from 4 (2.6 kDa) to 55 (34 kDa) times. The lower panel shows the region of the trypsinogen gene from which the 9 nucleotide segment coding for the tripeptide was taken; this region spans the boundary between the first intron (thin boxes) and the second exon (thick boxes). The segment was iteratively repeated to produce tandem repeats in the AFGP gene (in this case 41) with retention of the 5′ and 3′ regions; white segments between the AFGP coding regions represent proteolytic cleavage sites. The 5′ signal sequence is key for secreting the AFGP into the digestive tract where the antifreeze coats ingested ice crystals. [The lower panel is reproduced from J. M. Logsdon and W. F. Doolittle (1997). *Proc Nat Acad Sci U.S.A.* **94**:3485–3487. Used with permission.]

of gene/protein evolution. AFGPs consist of a family of isoforms of different sizes (2.7 to 34 kDa) that are all composed primarily of a simple glycotripeptide repeat $(\text{Thr-Ala/Pro-Ala})_n$ with galactose-*N*-acetylgalactosamine attached to each Thr (Fig. 14.12). How this odd structure came to be was unknown until researchers began to address the structure of the notothenioid AFGP genes. Each gene encodes a large polyprotein precursor with multiple AFGP molecules; for example, the first gene that was characterized coded for a polyprotein of 46 AFGP molecules (44 of isoform 8 and 2 of isoform 7) linked in direct tandem by a 3-residue spacer that was the posttranslational cleavage site. Notably, the creation of a direct tandem linkage is a highly efficient way of "quickly" constructing a new gene under intense selective pressure and arranging for the synthesis of copious quantities of antifreeze from a single mRNA. The clue to the origin of this strange gene came when the flanking sequence downstream from the 3′ stop codon was analyzed. A search in GenBank showed ~80% homology with the C terminus of a fish trypsinogen, the precursor of the gut protease, trypsin. Then, when the AFGP and trypsinogen genes from notothenioid pancreas were isolated and sequenced, it became clear that the former clearly evolved from the latter. The 5′ and 3′ ends of the trypsinogen gene were conserved in the AFGP gene (94 and 96% identical, respectively) with the 5′ end contributing the secretory signal sequence needed to allow AFGP secretion into extracellular spaces (e.g., blood plasma, lumen of the gut). Studies showed that the AFGP coding region evolved by repeated duplication and rearrangement of a 9-bp (base pair) segment crossing the first exon–intron boundary of the trypsinogen gene. Hence, unlike the type II AFPs, the notothenid AFGP did not arise by divergence of a preexisting protein. Instead, a new gene was created from a very small DNA segment of the trypsinogen gene, part of which was noncoding. Interestingly, the genome of the notothenids actually contains a "historical" record of the events of AFGP evolution. Searches have revealed a gene that encodes for a protease with a Thr-Ala-Ala element, a gene that encodes for both a protease and an antifreeze, and other genes that encode for just antifreezes.

A surprising twist occurred, however, when researchers began to characterize the AFGP genes of northern cod. Although the protein sequences of notothenid and cod AFGPs are virtually identical, the gene for cod AFGP bears no resemblance at all to the trypsinogen gene and clearly has a very different evolutionary origin. Furthermore, the AFGP genes from the two types of fish use different codons for the amino acids of the functional tripeptide and are constructed with very different intron–exon organ-

ization and spacer sequences so that very different processing of polyprotein precursors must occur. Therefore, evolution of the cod and notothenioid AFGP genes were independent events, and they represent excellent examples of convergent evolution where the same protein product arose from two different genomic origins.

ADAPTIVE CONTROL OF TRANSLATION

Other opportunities for adaptive responses at the gene/protein level exist via controls on translation as well as controls on protein degradation. Our treatment here will be brief because general aspects of translational control are discussed in Chapter 7, whereas Chapters 15 and 16 provide multiple illustrations of the adaptive regulation of protein translation that occurs during metabolic rate depression in anoxia-tolerant and hibernating animals. The little that is known about adaptation responses of proteolytic mechanisms is also discussed in Chapter 15.

In most cases, an increase in the rate of transcription of a gene automatically leads to a comparable increase in the rate of translation of the mRNA transcript and a proportional rise in the levels of the protein. Because of this and because of the relatively short half-life of most mRNA transcripts, changes in the number of mRNA transcripts of a given gene are typically good indicators of both a change in the rate of gene transcription and a change in the rate of synthesis of the encoded protein. Hence, techniques that quantify mRNA levels in control versus stressed states (e.g., Northern blotting, reverse transcription-polymerase chain reaction, cDNA array screening) are widely used with good accuracy to predict whether a stress has altered the rate of transcription and translation of a given gene and its protein (Fig. TB14.2).

However, there are several opportunities for differential controls that unlink transcription and translation. A temporal separation can occur resulting, for example, in mRNA up-regulation in response to a stress but a delay in protein synthesis until the recovery period after the stress is removed (e.g., see Chapter 16 for the example of the organic cation transporter regulation in mammalian hibernation). In other cases mRNA transcripts may be stabilized and held constitutively in an untranslatable form by the binding of specific regulatory proteins that are only released in response to a specific signal/stress (e.g., see Chapter 15 for the example of ferritin regulation).

Multiple modes of translational control can contribute to biochemical adaptation. These include global controls that increase or decrease the overall rate of translation and specific controls that affect the translation of individual types of mRNA transcripts. Among the mechanisms that will be discussed in the next chapters are:

1. Changes in the proportion of ribosomes in translationally active polysome versus translationally silent monosome fractions and the differential distribution of mRNA species between these two pools.
2. Differential transcript stabilization by multiple mechanisms including transcript binding to poly A binding proteins.
3. Regulation of mRNA entry, ribosome assembly, and peptide elongation by reversible protein phosphorylation control over the function of ribosomal initiation and elongation factors.
4. Mechanisms for circumventing the normal mode of transcription initiation (m^7G-capped-dependent initiation) by the presence of an internal ribosome entry site (IRES) in the mRNA transcripts of selected genes. The presence of an IRES allows the continued transcription of certain mRNA species under situations where the transcription of the majority of transcripts is blocked.

The present chapter has described many of the types of mechanisms that can be used for biochemical adaptation. The following three chapters explore the use of these mechanisms in greater detail in dealing with specific situations: oxygen limitation (Chapter 15), winter survival of small mammals by hibernation (Chapter 16), and survival at subzero temperatures by enduring the freezing of body fluids (Chapter 17).

SUGGESTED READING

General Principles of Biochemical Adaptation

Hochachka, P. W., and Somero, G. N. (2002). *Biochemical Adaptation: Mechanism and Process in Physiological Evolution*. Oxford University Press, Oxford, UK. *Principles of biochemical adaptation; easy to read and illuminating.*

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Storey, K. B. (2002). Life in the slow lane: Molecular mechanisms of estivation. *Comp Biochem Physiol A* **133**:733–754. *Principles of metabolic regulation that allow organisms to enter a hypometabolic or dormant state including enzyme responses, reversible phosphorylation control, signal transduction, and role of urea as an enzyme protectant during desiccation. An integrated example of how the mechanisms of biochemical adaptation discussed in this chapter are put together to achieve long-term survival of organisms under stress.*

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Willmer, P., Stone, G., and Johnston, I. (2000). *Environmental Physiology of Animals*. Blackwell Science, Oxford, UK. *Comprehensive textbook dealing with physiological adaptations to many of the same environmental stresses as are dealt with at the biochemical level in the present chapter and the subsequent three chapters.*

Specific Topics

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Sullivan, D. T., MacIntyre, R., Fuda, N., Fiori, J., Barrilla, J., and Ramizel, L. (2003). Analysis of glycolytic enzyme co-localization in *Drosophila* flight muscle. *J Exp Biol* **206**:2031–2038. *Current ideas about and latest techniques for investigating enzyme association with the muscle myosin motor.*