Suspended animation: the molecular basis of metabolic depression

KENNETH B. STOREY
Institute of Biochemistry and Department of Biology, Carleton University, Ottawa, Ont., Canada K1S 5B6

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An impressive array of organisms is capable of radically depressing basal metabolic rate and entering a hypometabolic state characterized by a marked reduction of many normal physiological functions. Environmental cues are often the trigger: low oxygen, low temperature, or lack of water, for example. Entry into a hypometabolic state does not, apparently, involve major biochemical reorganization but appears, instead, to result from molecular controls operating at a level "above" that of allosteric regulation of enzymes and "below" that of gene expression. The mechanisms involved are widely applicable to the coordinated inactivation of many cellular processes. Studies of anaerobiosis in marine molluscs provide the most complete information on the molecular mechanisms involved in metabolic rate depression. Glycolytic rate depression in the marine whelk involves (*i*) covalent modification of key regulatory enzymes (e.g., phosphofructokinase, pyruvate kinase) via enzyme phosphorylation to produce less active enzyme forms, (*ii*) dissociation of enzymes from complexes bound to the subcellular particulate fraction to disrupt pathway flux, and (*iii*) decreased levels of fructose-2,6-bisphosphate, a potent activator of phosphofructokinase, to help limit the anabolic uses of carbohydrate in the depressed state. Continuing studies are demonstrating the universality of these mechanisms as the basis of metabolic depression, including involvement in mammalian hibernation and anoxia tolerance in goldfish and turtles.

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Un grand nombre d'organismes sont capables de réduire de façon remarquable leur taux de métabolisme de base et de se maintenir dans un état d'hypométabolisme caractérisé par une forte réduction de plusieurs des fonctions métaboliques normales. Des paramètres de l'environnement agissent souvent comme déclencheurs : concentrations faibles d'oxygène, basses températures, pénuries d'eau. L'éntrée en hypométabolisme n'implique pas, semble-t-il, une réorganisation biochimique majeure, mais résulte plutôt du fait que les contrôles moléculaires agissent à un niveau «supérieur» à celui de la régulation allostérique des enzymes et «inférieur» à celui de l'expression des gènes. Les mécanismes impliqués s'appliquent aussi à l'inactivation coordonnée de nombreux processus cellulaires. Des études de l'anaérobie chez des mollusques marins mettent bien en évidence les mécanismes moléculaires sous-jacents à la réduction du métabolisme. La réduction du taux de la glycolyse chez les buccins marins suppose (*i*) la modification covalente des enzymes régulateurs clés (p.e., la phosphofructokinase, la pyruvate kinase) par phosphorylation des enzymes, ce qui les rend moins actifs, (*ii*) la dissociation des enzymes des complexes reliés à la fraction particulaire subcellulaire, ce qui rompt les voies de passage des flux et (*iii*) la réduction des concentrations de fructose-2,6-biphosphate, un puissant activateur de la phosphofructokinase, ce qui limite l'utilisation anabolique des hydrates de carbone durant les périodes de métabolisme réduit. Des études courantes mettent en lumière l'universalité de ces mécanismes comme base de la réduction métabolique, par exemple au cours de l'hibernation chez les mammifères et au cours de la manifestation de la tolérance à l'anoxie chez les poissons rouges et les tortues.

[Traduit par la revue]

Metabolic depression as a survival strategy

Although alien to the physiology of man, a facility for metabolic depression occurs widely in nature. Very often, escape to a hypometabolic, or even ametabolic, state is a mechanism for eluding harsh environmental conditions (e.g., low oxygen, low temperature, low water availability). As such, metabolic rate depression is a key factor in facultative anaerobiosis, anhydrobiosis, hibernation, estivation, torpor, and diapause. In addition, metabolic depression characterizes the quiescent life stages of many organisms (e.g., eggs, cysts, seeds, and spores) (Hochachka and Guppy 1987; Busa and Nuccitelli 1984).

Metabolic rate depression allows organisms to economize on fuels and energy reserves in the face of environmental stresses of unpredictable duration. For example, Wang (1978) has calculated for ground squirrels that the metabolic depression of hibernation saves 88% of the energy that would otherwise be utilized to remain euthermic throughout the winter season. Likewise, oxygen consumption in estivating lungfish decreases by 50 to 85% (Swan et al. 1968; Lahiri et al. 1970). Respiratory rate in diapausing insects is one-quarter to one-third that of nondiapausing individuals at the same life stage, while a 20-fold metabolic rate reduction has been measured during winter dormancy of adult insects (Wigglesworth 1972). The well-developed anoxia tolerance of many species is also based upon metabolic rate depression in the anoxic state. *Mytilus edulis*, for example, shows an anoxic metabolic rate only 5 to 10% of the normoxic rate (Famme et al. 1981; Shick et al. 1983). Similarly, diving turtles combat low oxygen by lowering metabolic rate to 15% of the standard metabolic rate in air (Jackson 1968). In the extreme, a virtual ametabolic state can be achieved; thus, in response to dehydration, *Artemia* cysts reduce metabolic rate to the point where no metabolic activity is detectable below a water content of 0.1 g/g cysts (Clegg 1981).

Although the triggers stimulating entry into a hypometabolic state are many, the consequences for cellular metabolism are similar. Various physical processes (e.g., feeding, movement) are curtailed, physiological functions (e.g., heart beat, breathing, or ventilation rate) are reduced, nonessential cellular functions (e.g., anabolism, growth) are reduced or suspended, and essential metabolic functions must be brought into a new balance. It would not be surprising, therefore, to find that the molecular mechanisms controlling metabolic depression are conserved across phylogenetic lines, as are the very well-studied events and controls surrounding metabolic activation (as during muscle exercise).

Mechanisms of metabolic depression

For some species, entry into a hypometabolic or ametabolic

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state is a once-in-a-lifetime event, such as the obligatory diapause of many insect species. Here, hypometabolism may be just one facet of a massive developmental reorganization. More commonly, however, individuals that are otherwise in a steady state experience multiple periods of hypometabolism with entry into (and reversal of) the depressed state occurring over short time scales. For example, gill-breathing intertidal invertebrates face twice-daily anoxic exposures, hummingbirds experience daily torpor, and hibernating small mammals undergo cycles of hibernation and arousal throughout the winter. Of note is the fact that even though the metabolic depression of hibernation may be quantitatively equal to that expected from Q_{10} considerations, based on the drop in body temperature, regulatory mechanisms are still required to shut down cellular metabolism and to reestablish balances between metabolic reactions with differing temperature coefficients. The regulatory controls used for metabolic depression must, therefore, meet certain criteria by being (*i*) rapidly initiated, (*ii*) easily reversed, (*iii*) applicable to many cellular processes, (*iv*) readily coordinated, and (*v*) responsive, possibly through second messengers, to external stimuli. In general, control must be "above" the level of allosteric regulation (which deals with specific regulation of individual enzymes or proteins) and "below" the level of gene expression (to facilitate rapid transitions into and out of the depressed state). Coordination of cellular processes is the key to survival. Imbalances in the rates of metabolic processes can rapidly lead to irreversible damage.

The dangers inherent in an improperly controlled hypometabolic state are readily observed when the phenomenon is imposed upon nontolerant species (Siesjo 1981; Hansen 1985; Hochachka 1986). Such is the case when mammalian organs are exposed to hypothermia or ischaemia. Both of these phenomena disrupt the balance between ATP-producing, ATP-utilizing, and passive processes in the cell. The most injurious effects apparently arise from membrane depolarization caused by an imbalance between energy-driven ion pumps (which are strongly affected in an energy-limited state) and ion movement through ion-specific channels. When imbalances in Na^+ and K^+ influx−efflux reach a critical level of depolarization, voltage-dependent Ca^{2+} channels are activated, leading to an uncontrolled influx of Ca^{2+} into the cell. A variety of Ca^{2+}-stimulated disruptions ensue, most notably membrane destruction via Ca^{2+}-activated phospholipases. In mammalian brain, for example, irreparable harm occurs after only a few minutes of ischaemia.

Facultative metabolic depression must, therefore, involve coordinated regulation to reduce or inactivate nonessential processes while maintaining and (or) reestablishing the balance between essential processes. The molecular mechanisms involved must be widely applicable to many cellular functions. Energy availability can help integrate the rates of many processes in an energy-starved hypometabolic state; thus, a decline in the rates of ATP production during anoxia due to the loss of oxidative phosphorylation with no compensation (no Pasteur effect) by glycolysis would necessarily restrict ATP-utilizing processes in the depressed state. However, rates of passive processes escape energy-linked control and these too must be regulated during the metabolic transition so that a balance is maintained between all essential processes.

Our initial interest in the molecular mechanisms of metabolic depression arose from studies of anaerobic metabolism in marine molluscs, in particular the control of glycolysis during anoxia. Alternative routes of fermentative ATP production

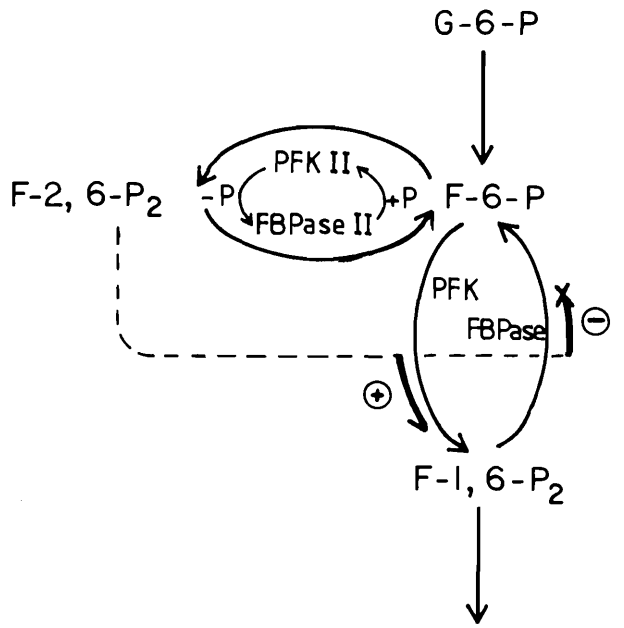

FIG. 1. Fructose-2,6-bisphosphate metabolism. F-2,6-P_2 activates phosphofructokinase and inhibits fructose-1,6-bisphosphatase. Synthesis and degradation of the compound is controlled by a single enzyme protein that exhibits 6-phosphofructo-2-kinase (PFK-II) activity in its phosphorylated form and fructose-2,6-bisphosphatase (FBPase-II) activity in its dephosphorylated form.

improve ATP yield in the anoxic state (succinate or propionate synthesis yields 5 or 7 mol ATP per mol glucose-1-phosphate fermented as well as an associated aspartate catabolism), compared with anaerobic glycolysis which forms lactate as the product, but cannot replace the ATP yield of oxidative phosphorylation. No Pasteur effect is seen (de Zwaan and Wijsman 1976; Gade 1983) and, indeed, *M. edulis* shows an inhibition of glycolysis (reverse Pasteur effect) during anoxia (Ebberink and de Zwaan 1980). Long-term survival in the absence of a Pasteur effect is only possible, then, if ATP demand is severely reduced in the anoxic state. Such is the case: calorimetry shows a 10- to 20-fold depression of metabolic heat production in anoxic *M. edulis* (Famme et al. 1981; Shick et al. 1983). To date we have identified three molecular mechanisms key to the metabolic depression associated with molluscan facultative anaerobiosis (Storey 1985*a*). One is specific to glycolysis but restricts the use of carbohydrate as an anabolic substrate; this is reduced levels of fructose-2,6-bisphosphate. The other two mechanisms, also key to glycolytic control, have wide-ranging potential for control of numerous cellular reactions. These are (*i*) covalent modification of enzymes and proteins and (*ii*) enzyme association with the particulate fraction of the cell. Our most recent studies show that these mechanisms also underlie metabolic depression in other situations, notably anoxia tolerance in vertebrates (goldfish, turtles) and hibernation in small mammals.

Fructose-2,6-bisphosphate control of carbohydrate catabolism

Fructose-2,6-bisphosphate (F-2,6-P_2) is a potent activator of phosphofructokinase from many sources (Fig. 1) (Hers and van Schaftingen 1982). Levels of the bisphosphate in mammalian tissues are altered under a variety of conditions (stimulation by various hormones, starvation, anoxia, changes in

glucose availability) and, as such, the action of the compound focuses the effects of diverse stimuli on the rate-controlling enzyme of glycolysis (Hue 1983). The enzyme 6-phosphofructo-2-kinase, which produces F-2,6-P_2, is a phosphoprotein and is regulated by cAMP and Ca^{2+}–calmodulin dependent covalent modification. Studies of the effects of starvation and ischaemia–anoxia on mammalian organs show that F-2,6-P_2 content decreases as a result of these stresses (e.g., F-2,6-P_2 falls to less than 10% of control levels within 10 min during anoxic incubation of hepatocytes; Hue 1982). F-2,6-P_2 apparently acts as an indicator of the abundance of glucose and its regulatory effects on phosphofructokinase control the use of carbohydrate for biosynthetic purposes during times of plentiful energy. The compound is withdrawn when carbohydrate must be conserved. The prediction for natural metabolic depression is, therefore, that tissue F-2,6-P_2 levels would be decreased in the hypometabolic state as a mechanism of restricting carbohydrate use. Such is, indeed, the case.

Our initial studies addressed anaerobiosis in marine molluscs. F-2,6-P_2 content is strongly reduced in the soft (biosynthetic) tissues (e.g., mantle, gill, hepatopancreas) of the oyster, mussel, and periwinkle within the first 3 h of anoxia (Storey 1985*b*). In the mantle of *Ostrea edule*, for example, content of the bisphosphate was 433 pmol/g wet weight in controls, and 83 and 12 pmol/g after 3 and 24 h anoxia, respectively. Comparable values for hepatopancreas were 806, 110, and 20 pmol/g. Tissues of the whelk, *Busycotypus canaliculatum*, also showed rapid and dramatic changes in F-2,6-P_2 during anoxia (K. Storey, unpublished data). For example, F-2,6-P_2 content in ventricle dropped to 0.6% of control values with a $t_{1/2}$ of only 1 h, while in kidney and gill, levels fell to 2.5 and 3.5% of control values with $t_{1/2}$ values of 2.5 and 1.5 h, respectively.

Such dramatic changes to the content of the most potent allosteric effector of the most important regulatory enzyme of glycolysis have extremely important consequences both for glycolysis and for metabolism as a whole: (*i*) removal of F-2,6-P_2 leaves phosphofructokinase (and glycolytic rate) responsive to cellular energy requirements alone (via AMP and ATP allosteric effects) during anoxia; (*ii*) strongly reduced levels of the activator, plus the resulting loss of the very powerful synergistic interactions between F-2,6-P_2 and AMP (Storey 1985*c*), greatly reduces phosphofructokinase activity and glycolytic rate, in line with the absence of a Pasteur effect during anoxia in marine molluscs; and (*iii*) carbohydrate use for purposes other than ATP production (e.g., biosynthesis) is strongly depressed, contributing to the general metabolic depression of anaerobiosis.

Further studies are indicating that modulation of F-2,6-P_2 content in tissues is an important mechanism of metabolic control in other instances of facultative hypometabolism. Metabolic depression accompanying anoxia tolerance in goldfish and hibernation in small mammals both depend on modulation of F-2,6-P_2 content in selected tissues as a means of glycolytic rate control (Storey 1987*a*, 1987*b*).

Goldfish (*Carassius auratus*) are known to survive complete anoxia for 16 h at 20°C or for several months at 0°C (Blazka 1958; van den Thillart 1982). Estimates of anoxic metabolic rate are 20–30% of normoxic rates determined by calorimetry and one-third of normal rates based on the sum of accumulated end products plus the depletion of ATP, phosphagen, and endogenous O_2 reserves (van den Thillart 1982). Our studies of goldfish showed that anoxia (15 h in N_2- followed by 9 h in CO-bubbled water) had more diverse effects on contents of F-2,6-P_2 in goldfish tissues than was seen for marine molluscs. Content was depressed in liver, spleen, and gill to levels 10, 25, and 59%, respectively, of control values (Storey 1987*a*). For liver, as the central site for interconversions of dietary carbohydrate, lipid, and amino acids, this is probably a critical response in terms of depressing the anabolic use of carbohydrate and preserving glycogen reserves for their function in supplying anaerobic substrate (blood glucose) to all organs during anoxia. Content of the bisphosphate in red and white skeletal muscle, as in adductor and foot muscles of molluscs (Storey 1985*b*), remained relatively constant throughout anoxia, while in brain and heart, F-2,6-P_2 content increased 3.5-fold during anoxia. The response in brain and heart is in line with the relative activation of glycolysis required to maintain the critical functions of these specific organs during anoxia (Shoubridge and Hochachka 1983; Storey 1987*a*).

Hibernation in small mammals is a situation in which oxygen-based metabolic rate is radically depressed. As a model animal for hibernation studies, we chose the meadow jumping mouse, *Zapus hudsonius*, a profound hibernator that spends about 7 months of the year in hibernation without feeding during periodic bouts of arousal (Whitaker 1972). Tissue levels of F-2,6-P_2 were measured in control mice, and in short-term (less than 24 h) and long-term (5 to 8 days) hibernators. Tissue contents of the bisphosphate were not altered during short-term hibernation, suggesting that manipulation of F-2,6-P_2 is not involved in inducing hibernation. However, the metabolic adjustments involved in establishing long-term homeostasis in the closed system of the depressed state included manipulation of F-2,6-P_2 contents in some tissues. Long-term hibernators showed significantly reduced levels of F-2,6-P_2 in brain, heart, and fat pad (72, 26, and 33% of control values, respectively) but not in liver or skeletal muscle (Storey 1987*c*). In liver, however, a modulation of phosphofructokinase by probable enzyme phosphorylation (see next section) reduces the effectiveness of F-2,6-P_2 as an enzyme activator in the hibernator (Storey 1987*b*); the overall effect is still a reduction in liver phosphofructokinase activity in the hypometabolic state.

Overall, then, these studies show an involvement of F-2,6-P_2 as one of the controlling elements in metabolic depression. Modulation of the levels of F-2,6-P_2 is apparently a key factor when metabolic depression must include a decrease in glycolytic rate and a restriction of carbohydrate use for purposes other than ATP production.

Enzyme and pathway control by covalent modification

As a molecular mechanism of metabolic depression, covalent modification via protein phosphorylation or dephosphorylation reactions holds great potential. Advantages of covalent modification for the widespread control of metabolism include the following: (*i*) covalent modification can produce major changes in the activities of enzymes and other proteins, (*ii*) the time frame in which covalent modification operates is short, (*iii*) the mechanism is widely applicable to the control of diverse cellular proteins and enzymes, and (*iv*) regulation of kinases and phosphatases by intracellular messengers (cAMP, Ca^{2+}) and extracellular hormones is in place in all animal cells.

Our investigations of covalent modification as a mechanism of metabolic depression began with anoxia tolerance in marine

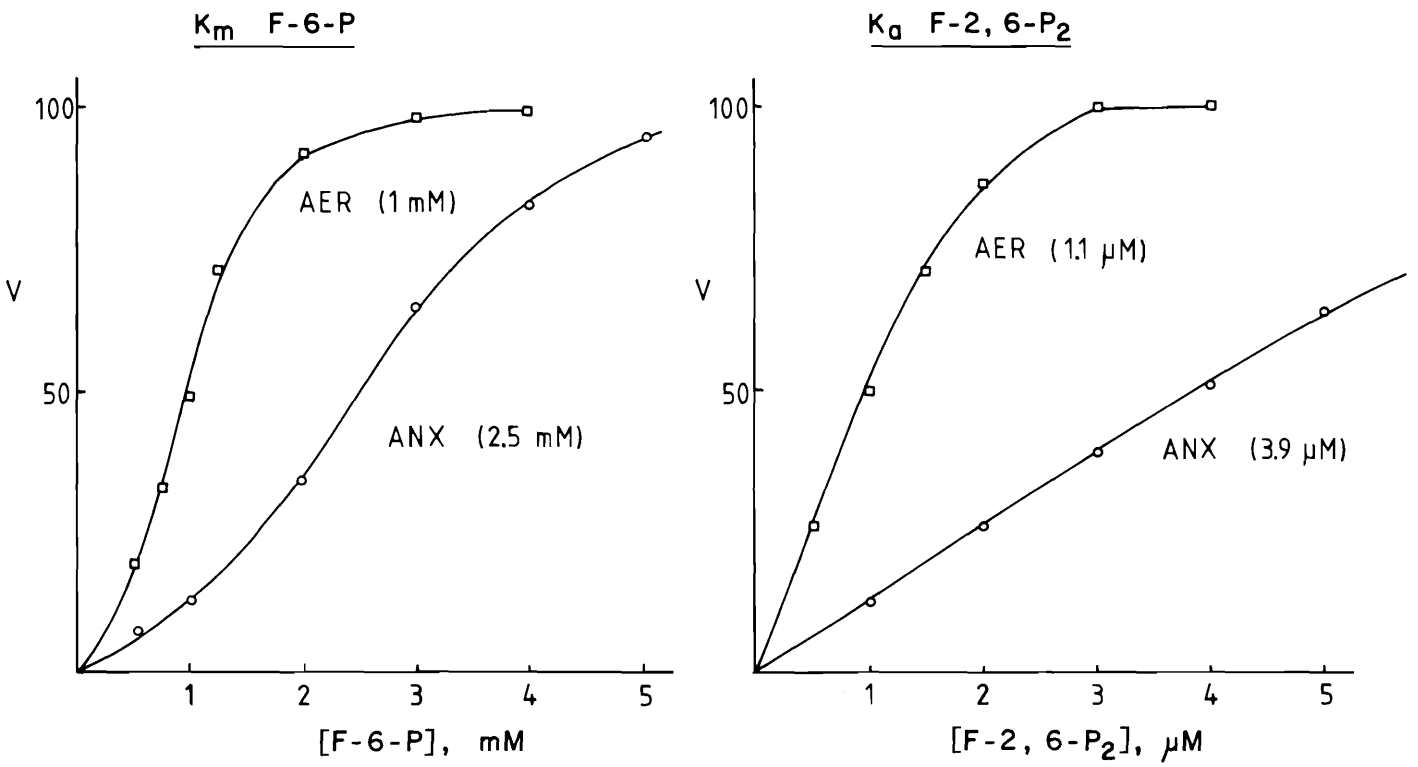

FIG. 2. Effect of anoxia-induced covalent modification on kinetic properties of phosphofructokinase from whelk foot muscle. AER, aerobic; ANX, anoxic enzyme forms. Affinity for F-6-P is shown in the presence of physiological concentrations of F-2,6-P_2, 0.26 and 0.04 nmol/g wet weight for aerobic and anoxic foot muscle, respectively. Data modified from Storey (1984).

molluscs. Anoxia-induced phosphorylations of phosphofructokinase and pyruvate kinase, producing less active enzyme forms, proved to be key to the control of anaerobic glycolysis in all tissues of the whelk (Storey 1985*a*). Furthermore, we found substantial evidence that protein phosphorylation was widespread during anoxia; studies of ^{32}P incorporation showed that extracts of anoxic radular retractor muscle contained high amounts of covalently bound phosphate in many protein fractions (Plaxton and Storey 1984*b*). It is highly probable, therefore, that covalent modification is widely used to depress the functions of a variety of cellular proteins during anoxia.

Phosphofructokinase

Allosteric control of phosphofructokinase is primarily responsible for the anoxia-induced activation of glycolysis known as the Pasteur effect (Ramaiah 1974). Changes in the levels of adenylates, phosphagen, and inorganic phosphate in tissues of facultative anaerobes during anoxia should produce an allosteric activation of phosphofructokinase, just as they do in mammalian tissues (Ebberink and de Zwaan 1980). However, the absence of a Pasteur effect and the depression, instead, of phosphofructokinase activity and glycolytic rate in facultative anaerobes appears to be due to the overriding effects of an anoxia-induced covalent modification of phosphofructokinase, coupled to a radical decrease in tissue F-2,6-P_2 content (Storey 1985*a*). Phosphofructokinase isolated from foot muscle of anoxic (21 h in N_2-bubbled seawater) whelks showed substantially different kinetic properties than that from aerobic controls including: (*i*) reduced effects by activators, NH_4^+, and F-2,6-P_2 (for F-2,6-P_2, both K_a and the effect on $S_{0.5}$ for F-6-P were altered), (*ii*) increased substrate inhibition by ATP, and (*iii*) reduced affinity for Mg^{2+} (Fig. 2) (Storey 1984). All of these alterations would produce a less active enzyme form during anoxia. These same kinetic differences characterize the phosphorylated, compared with the dephosphorylated, form of phosphofructokinase in mammalian tissues (Foe and Kemp 1982; Sakakibara and Uyeda 1983). Treatment of the anoxic enzyme variant from foot muscle with alkaline phosphatase restored kinetics typical of the aerobic form, providing further evidence that covalent modification is the mechanism underlying phosphofructokinase inactivation during anoxia (Storey 1984).

Pyruvate kinase

Anoxia-induced covalent modification of pyruvate kinase produces less active enzyme forms of all three tissue-specific isozymic forms found in the whelk (Plaxton and Storey 1984*a*, 1985*a*, 1985*b*). Kinetic and electrophoretic properties of the aerobic versus anoxic enzyme forms are those characteristic of the dephosphorylated and the phosphorylated forms, respectively, of mammalian L-type pyruvate kinase (Engstrom 1978). By use of ^{32}P incorporation we have demonstrated conclusively that the anoxic enzyme form in radular retractor muscle is a phosphoprotein containing O-phospho-L-threonine residues (Plaxton and Storey 1984*b*).

Anoxia-induced phosphorylation of pyruvate kinase produces a much less active enzyme form via modification of several enzyme properties. Compared with the aerobic form, the anoxic variant of pyruvate kinase from radular retractor showed (*i*) lower maximal activity (39 versus 116 U/g wet weight), (*ii*) greatly reduced affinity for phosphoenolpyruvate ($S_{0.5}$ 12-fold higher), (*iii*) greatly reduced activation by F-1,6-P_2 (K_a 26-fold higher), and (*iv*) extremely potent inhibition by L-alanine (K_i 490-fold lower) (Fig. 3) (Plaxton and Storey 1984*a*). Similar kinetic differences characterized the aerobic and anoxic forms of hepatopancreas pyruvate kinase in

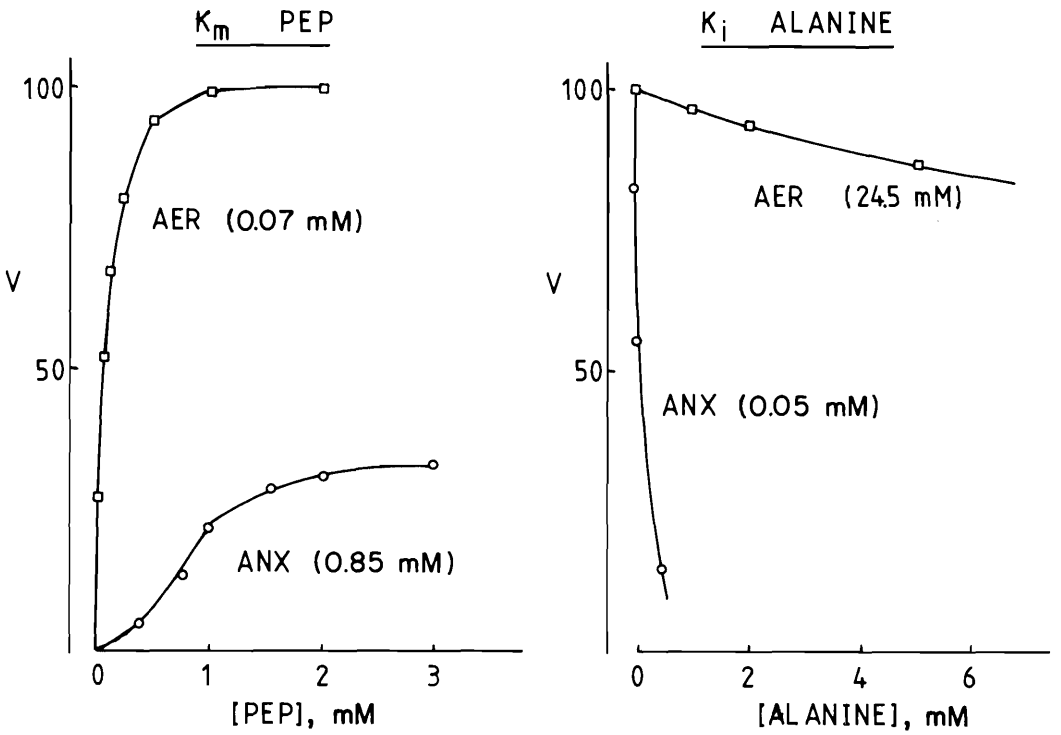

FIG. 3. Effect of anoxia-induced covalent modification on some kinetic properties of pyruvate kinase from whelk radular retractor muscle. AER, aerobic; ANX, anoxic enzyme forms. Relative V_{max} values are 100:34 for AER:ANX. Data modified from Plaxton and Storey (1984*a*).

the whelk (Plaxton and Storey 1985*b*).

The effects of protein phosphorylation on pyruvate kinase in the marine mollusc not only are important to glycolytic rate depression during anoxia but also regulate the so-called "phosphoenolpyruvate branch point" to switch the product of glycolysis from alanine in the early hours of anoxia to succinate during long-term anaerobiosis. Phosphorylation of pyruvate kinase, along with rising levels of alanine (a potent inhibitor of the phosphoenzyme), cuts off carbon flow through the enzyme and diverts carbon, instead, via phosphoenolpyruvate carboxykinase into succinate. Obviously, since pyruvate kinase is active during early anoxia to permit alanine production, covalent modification of the enzyme must be a gradual event. In the whelk, we have found that half-times for enzyme phosphorylation (based on measured changes to K_m phosphoenolpyruvate and I_{50} L-alanine) were 1 h in ventricle and 8 h in foot and hepatopancreas (R. Whitwam and K. Storey, unpublished data). Not surprisingly, these patterns parallel the depletion of aspartate (the substrate for succinate synthesis in early anoxia) and also mirror changes in tissue F-2,6-P_2 contents, suggesting that phosphofructokinase and pyruvate kinase are regulated in concert. Because F-2,6-P_2 levels are themselves regulated via phosphorylation–dephosphorylation control of 6-phosphofructo-2-kinase, it is probable that an anoxia signal (or signals) acts via intracellular second messengers (cAMP, Ca^{2+}) to regulate a variety of protein phosphorylations in the cell and coordinate an anoxia-induced metabolic depression.

Regulation by covalent modification in other systems

Continuing studies on other model systems indicate that enzyme and protein regulation via covalent modification will prove to be a universal mechanism of metabolic depression. Control of glycolysis during anoxia in goldfish and turtles and during hibernation in *Z. hudsonius* all involve phosphorylation-mediated enzyme inactivation.

In goldfish, kinetic changes indicative of covalent modification of glycogen phosphorylase and pyruvate kinase occur under anoxia in various organs. In liver, anoxia resulted in a 50% decrease in the content of the phosphorylated *a* form of glycogen phosphorylase (Storey 1987*a*) and produced changes in phosphofructokinase and pyruvate kinase kinetics (e.g., for pyruvate kinase, K_m for phosphoenolpyruvate increased 2-fold and I_{50} L-alanine decreased by 50%) indicative of enzyme inactivation by phosphorylation (Rahman and Storey 1987). Results for most other organs, except red and white skeletal muscle, were similar. A similar response appears to characterize anoxia tolerance in diving turtles; our initial studies have shown a reduction in the content of glycogen phosphorylase *a* and kinetic evidence of phosphofructokinase phosphorylation in various tissues in the diving animal (S. Brooks and K. Storey, unpublished data).

Covalent modification as a control mechanism in metabolic depression also extends to hibernating mammals. We have recently evaluated liver glycolysis in hibernating *Z. hudsonius*. The three regulatory enzymes, glycogen phosphorylase, phosphofructokinase, and pyruvate kinase, all showed kinetic changes indicative of a covalent modification during hibernation (Storey 1987*b*). For glycogen phosphorylase the amount of active, phosphorylated enzyme decreased 7-fold in mice during hibernation for 5 to 8 days. Phosphofructokinase from liver of hibernators was less activated by F-2,6-P_2 (K_a 2.5-fold higher than controls) and more strongly inhibited by ATP and magnesium citrate (I_{50} values 4- and 3.7-fold lower than controls, respectively), such changes indicating enzyme phosphorylation. Pyruvate kinase also showed changes consistent with enzyme phosphorylation during hibernation; the enzyme from liver of hibernating mice was less activated by F-1,6-P_2 (K_a 4.4-fold higher) and more strongly inhibited by L-alanine

(I_{50} 6.3-fold lower) than that in control liver. Metabolic depression and the reorganization of metabolism in liver during hibernation appear, therefore, to involve covalent modification of regulatory enzymes to reduce glycolytic flux.

Covalent modification via phosphorylation–dephosphorylation reactions is obviously a powerful mechanism for glycolytic control during facultative hypometabolism. It is also a mechanism that is widely applicable to the control of many enzymes or proteins. Examples of rate-limiting enzymes controlled by reversible phosphorylations are now many, including glycogen synthase, triglyceride lipase, pyruvate dehydrogenase, acetyl-CoA carboxylase (Cohen 1980). Thus, an appropriate signal, translated through second messengers and protein kinase or phosphatase actions, can effectively depress the activities of multiple metabolic pathways. Undoubtedly, continuing research will produce many more examples of reversible phosphorylations in the control of metabolic depression. One promising area for research is the regulation of membrane ion channel proteins. The numbers or the activity level of functional ion-specific channels must be reduced in the hypometabolic state to reduce ion flux down concentration gradients in line with the reduced capacities of ATP-driven ion pumps in the anoxic state. Covalent modification offers an effective method for the rapid inactivation of channel proteins coordinated with the depression of numerous other metabolic processes.

Metabolic control through enzyme–particle associations

The third molecular mechanism that we have associated with metabolic depression is enzyme association–dissociation with the particulate fraction of the cell. The old concept of the cytoplasm viewed enzymes and metabolites as randomly distributed with flux through pathways requiring substrate–product transfer via diffusion from one enzyme to another. The modern concept is of a much more organized environment. So-called "soluble" enzymes form associations, apparently physiologically relevant, with each other, with structural proteins, with glycogen particles, and with membrane fractions (Knull 1978; Moses 1978; Wilson et al. 1982). Well-documented examples include aldolase and creatine kinase associations with actin in muscles, and hexokianse associations with the mitochondrial membrane (Knull et al. 1973; Westrin and Backman 1983). The microcompartmentation of metabolism that results from such associations can provide an effective means of enzyme and pathway control by (*i*) channeling substrates and products between consecutive enzymes in a pathway and (*ii*) altering enzyme kinetic properties by conformational changes on binding (Arnold and Pette 1970; Masters 1985). Studies on mammalian muscle have shown that metabolic situations that increase glycolytic flux (e.g., tetanic contraction, anoxia, ischaemia) also increase the proportions of several glycolytic enzymes associated with the particulate fraction of the cell (Knull et al. 1973; Clarke et al. 1984). For example, a Pasteur effect during ischaemia in mammalian heart is facilitated by a 2.3-fold increase in the amount of phosphofructokinase bound to particulate fractions and a 3-fold increase in aldolase bound (Clarke et al. 1984). Thus, modulation of the physical location of enzymes, particle-bound or soluble, appears to be an additional and powerful form of metabolic regulation.

As a mechanism of metabolic depression, manipulation of enzyme–particle interactions holds great promise. If enzyme–enzyme and enzyme–particle associations can promote organized and rapid carbon flow through metabolic pathways, then the opposite occurrence, enzyme dissociation from multienzyme complexes or from interactions with subcellular components, should effectively depress pathway flux. We investigated the effect of anoxia on the organization of glycolysis in the whelk using two different techniques. The technique of Clarke et al. (1984) uses rapid homogenization in a buffer containing high sucrose to stabilize enzyme–particle associations. Soluble versus particle-bound enzymes are then separated by centrifugation. Results for whelk ventricle are shown in Fig. 4. All eight glycolytic enzymes examined showed a significant reduction in the percentage associated with the particulate fraction as a result of anoxia stress (Plaxton and Storey 1986). The effects were most dramatic for hexokinase, a decrease from 44.5 to 14% bound, and aldolase, a decrease from 41 to 11% bound. With the exception of lactate dehydrogenase and alanopine dehydrogenase, the phenomenon was reversed during aerobic recovery. A second technique, that of Ross and Hultin (1980), uses centrifugation at 60 000 × *g*, to force soluble enzymes (in a press juice) out of ventricle strips, leaving bound enzymes within the tissue. The technique also found a significant increase in the percentages of hexokinase, aldolase, and pyruvate kinase in the soluble fraction of the cell during anoxia (Plaxton and Storey 1986).

Enzyme control by reversible binding to subcellular structures or by reversible associations into active enzyme complexes has powerful potential for the control of many facets of metabolic depression. Key areas that deserve future examination include (*i*) regulation of membrane ion channel densities and (*ii*) control of forward (aerobic) versus reverse (anoxic) functioning of the tricarboxylic acid cycle in anoxia-tolerant marine molluscs.

Coordinated control of metabolic depression

Using glycolysis as a model system, we have identified key molecular mechanisms underlying metabolic rate depression in animals capable of facultative hypometabolism. These mechanisms are summarized in Fig. 5. High flux through glycolysis is promoted by the formation of an enzyme complex (glycolytic particle) bound to a subcellular framework, containing active enzyme forms (for phosphofructokinase and pyruvate kinase these are the dephosphorylated forms). Levels of key allosteric activators are high, as are enzyme sensitivities to these activators (e.g., dephosphorylated forms of phosphofructokinase and pyruvate kinase are much more sensitive to F-2,6-P_2 and F-1,6-P_2 activation, respectively, than are phosphorylated forms). Reduced pathway flux is achieved by the opposite means: covalent modifications produce less active enzyme forms, activators are withdrawn, and enzyme complexes dissociate. Interactions between these mechanisms are probable and provide key questions for future research. How does enzyme phosphorylation affect enzyme–enzyme associations or enzyme binding to particulate fractions? Do allosteric effectors inhibit or enhance the action of protein kinases?

Obviously, long-term survival in the hypometabolic state requires a coordinated reduction in the rates of all cellular processes during the metabolic transition. These same molecular mechanisms are equally applicable, and indeed admirably suited, to the widespread control of many cellular functions including various ATP-utilizing (e.g., anabolic pathways) and membrane (e.g., ion pumps, ion-specific channels) processes. Continuing studies will have to test the universality of reversible phosphorylations and enzyme-binding associations in the

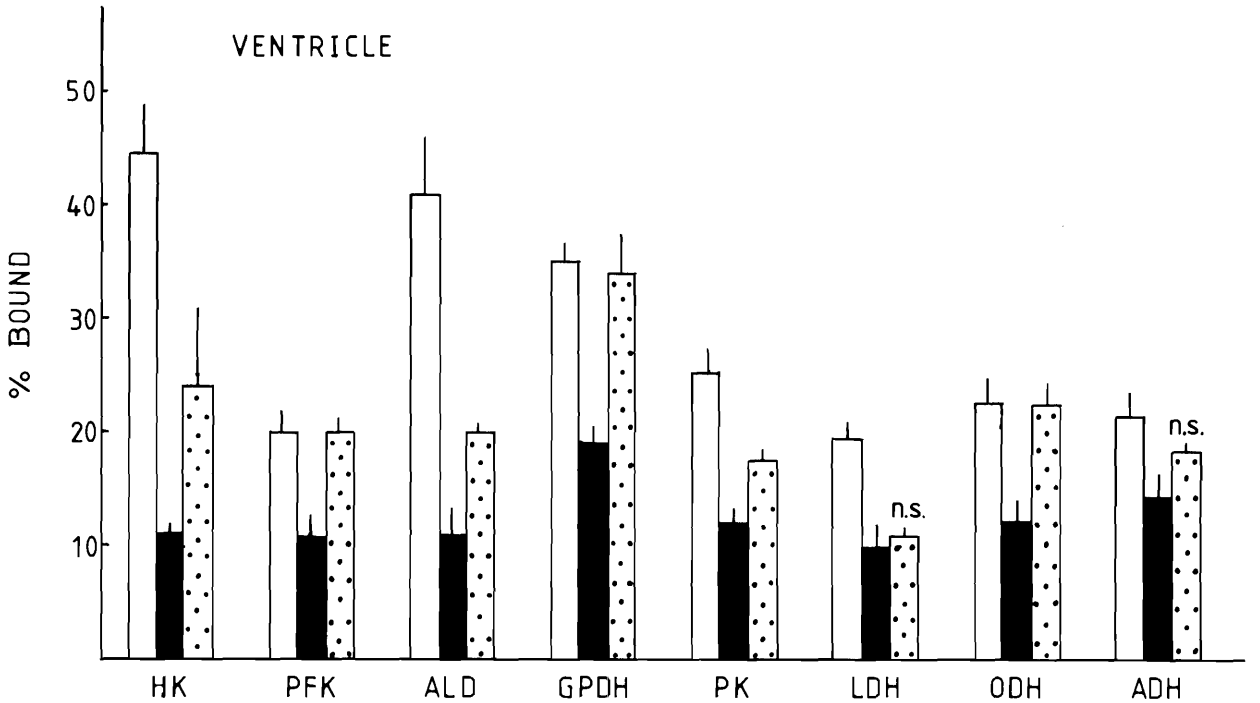

FIG. 4. Percentage of enzyme activity associated with the particulate fraction in whelk ventricle under three conditions: □, aerobic; ■, 21 h anoxia in N_2-bubbled seawater; □, 2 h aerobic recovery from anoxia. Separation of enzyme activities into soluble and bound fractions was by the method of Clarke et al. (1984) in which homogenization in a high sucrose medium is used to preserve enzyme associations with subcellular particles. Data are means ± SEM; $n = 4$ for control and anoxic, $n = 2$ for recovery. Enzymes are hexokinase (HK), phosphofructokinase (PFK), aldolase (ALD), glycerol-3-phosphate dehydrogenase (GPDH), pyruvate kinase (PK), lactate dehydrogenase (LDH), octopine dehydrogenase (ODH), and alanopine dehydrogenase (ADH). All values for aerobic controls and aerobic recovery are significantly different from the corresponding anoxic value ($p < 0.05$) except for two recovery values indicated n.s. (not significant). Data from Plaxton and Storey (1986).

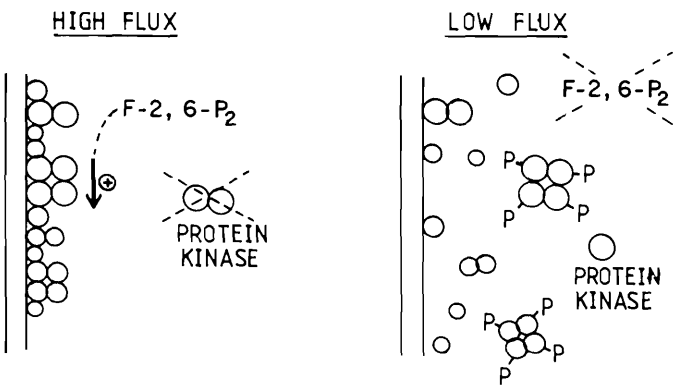

FIG. 5. Molecular mechanisms for metabolic depression. Reduced flux through glycolysis results from loss of F-2,6-P_2 activation of phosphofructokinase, phosphorylation inactivation of regulatory enzymes, and dissociation of enzymes from an organized bound state.

control of other areas of metabolism.

Having discussed the probable molecular mechanisms of metabolic depression in animals, a final question must be asked. What are the intracellular and extracellular signals that initiate and coordinate metabolic depression? A few suggestions can be offered at the present time. At an extracellular level, hormones are involved in the depression of aerobic metabolic rate to produce the hypometabolic states characteristic of insect diapause, lungfish estivation, and mammalian hibernation (Yamashita and Hasegawa 1984; Swan et al. 1981; Heller et al. 1986). Hormonal involvement in anoxia-induced metabolic depression is unclear, as tissues of marine molluscs show the same metabolic responses when exposed to anoxia in vivo or in vitro (de Zwaan 1983; Korycan 1984). Thus, loss of oxygen itself probably triggers the cellular response either directly or through the action of intracellular second messengers.

At the intracellular level, at least three factors can be identified that have a high probability of involvement in metabolic depression. These are cAMP, Ca^{2+}, and pH. Ca^{2+} and cAMP have widely documented involvements in the stimulation of protein phosphorylation (Cohen 1980) and are very likely the signals responsible for coordinating the covalent modification of numerous cellular enzymes and proteins during entry into the hypometabolic state. Intracellular pH changes are often correlated with metabolic rate transitions (Busa and Nuccitelli 1984). The transition from dormancy to active metabolism in many systems (bacterial and yeast spores, protozoan and *Artemia* cysts), the activation of egg metabolism as a result of fertilization, and even the activation of metabolism preceding mitosis all are accompanied by an increase in intracellular pH. Oppositely, entry into a hypometabolic state is often accompanied by decreased cellular pH. In the extreme, the transition from aerobic development to anaerobic dormancy in *Artemia* cysts is accompanied by an intracellular pH shift from 7.9 to 6.3 (Busa et al. 1982). During anaerobiosis in marine molluscs, intracellular pH falls 0.1 to 0.4 units (Ellington 1983*a*, 1983*b*). Both estivating lungfish and hibernating mammals develop respiratory acidosis (in hibernators not all tissues are involved) (DeLaney et al. 1977; Malan et al. 1985). However, acidification during hypometabolism develops over the long term, so that a pH drop cannot be the proximate signal for metabolic depression. However, as Busa and Nuccitelli (1984)

suggest, pH shifts can provide a "metabolic context" which influences various cellular events. For example, pH alters the ratio of free versus particle-bound glucose-6-phosphate dehydrogenase in sea urchin eggs (Aune and Epel 1978). Sustained acidification in the hypometabolic state may thus help to preserve the new steady state by creating an environment that favours, for example, enzyme dissociation from a particle-bound state or the activity of protein kinases over protein phosphatases.

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