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THE COVALENT MODIFICATION OF
PHOSPHOFRUCTOKINASE AND PYRUVATE KINASE
IN THE HYPOMETABOLIC PULMONATE
OTALA LACTEA

by

ROSS EDWARD WHITWAM, B.Sc.

A thesis submitted to the
Faculty of Graduate Studies and Research
in partial fulfillment of the requirements
for the degree of Master of Science

Dept. of Biology
Carleton University
Ottawa, Ont.

25 July 1988

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ABSTRACT

Pyruvate kinase (PK) and phosphofructokinase (PFK) from 3 tissues of the desert pulmonate snail Otala lactea are covalently modified during both dormancy and anaerobiosis. The covalent modifications were evinced by changes in the kinetic constants of the enzymes. For all three tissues, the kinetic changes in PK during dormancy were nearly, but not quite completely, identical to those during anaerobiosis. The kinetic changes in the PFK isozymes during dormancy were for the most part dissimilar to those during anaerobiosis, but each PFK isozyme showed at least one identical kinetic change under both stresses. During both dormancy and anoxia the kinetic changes in foot muscle PK and mantle PK are consistent with an enzymatic inactivation, which probably serves to help depress the metabolic rate of the stressed snails. The kinetic changes in hepatopancreas PK are consistent with an enzymatic activation, which may serve to funnel glycolytic carbon into fatty acid biosynthesis during dormancy and anoxia, despite an overall reduction in the snail's metabolic rate. The kinetic changes in PFK in all 3 tissues under both stresses were generally small and were not clearly poised in the direction of either activation or inactivation of PFK. The

physiological relevance of the covalent modifications to PFK may be in some area not investigated.

The dormancy-induced kinetic changes in foot muscle PK's I_{50} for MgATP and hepatopancreas PK's $S_{0.5}$ for PEP were slow to be initiated in both tissues (>12 hours), were quickly reversed in foot muscle (<10 minutes), and were slowly reversed in hepatopancreas (between 3 and 24 hours). The anoxia-induced kinetic changes in hepatopancreas PK's $S_{0.5}$ (PEP) were reversed after 5 minutes of normoxia.

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LIST OF ABBREVIATIONS AND SYMBOLS

A ₃₄₀	- Optical absorbance at 340 nm
ADP	- Adenosine 5'-diphosphate
Ala	- L-Alanine
AMP	- Adenosine 5'-monophosphate
ATP	- Adenosine 5'-triphosphate
CAK	- cAMP-dependent protein kinase
CAMP	- Adenosine 3',5'-monophosphate
CGMP	- Guanosine 3',5'-monophosphate
CIT	- Citrate
CoA	- Coenzyme A
d	- Days
DHAP	- Dihydroxyacetone phosphate
EDTA	- Ethylenediaminetetraacetate
EGTA	- [Ethylenbis(oxyethylenitrilo)]-tetraacetate
F ₂₆ P ₂	- Fructose-2,6-bisphosphate
F6P	- Fructose-6-phosphate
FBPase-2	- Fructose-2,6-bisphosphatase
FDP	- Fructose-1,6-bisphosphate
Fructose-1,6-P ₂	- Fructose-1,6-bisphosphate
Fructose-2,6-P ₂	- Fructose-2,6-bisphosphate
GAP	- Glyceraldehyde 3-phosphate
GDP	- Guanosine 5'-diphosphate
α-GP	- α-Glycerophosphate

GTP	- Guanosine 5'-triphosphate
h	- Hours
I ₅₀	- Inhibitor concentration producing 50% reduction in enzyme velocity
K _a	- Activator concentration producing 50% of maximal enzyme activation
kDa	- Kilodalton
K _i	- Inhibitor-enzyme dissociation constant
m	- Minutes
Mg ₂ Cit	- Dimagnesium citrate
MR	- Metabolic rate
N ₂	- Nitrogen gas
NAD ⁺ , NADH	- Nicotinamide-adenine dinucleotide in oxidised and reduced forms
NADP ⁺ , NADPH	- Nicotinamide-adenine dinucleotide phosphate in oxidised and reduced forms
n _h	- Hill coefficient, or slope of Hill plot
NH ₄ ⁺	- Ammonium ion
OXA	- Oxaloacetate
PEP	- Phosphoenolpyruvate
PFK	- Phosphofructokinase
Phe	- L-Phenylalanine
pH _i	- Intracellular pH
P _i	- Inorganic phosphate
pI	- Isoelectric point
PK	- Pyruvate kinase
PMSF	- Phenylmethylsulfonyl fluoride

pO_2	- Partial pressure of oxygen
Pro	- L-Proline
$S_{0.5}$	- Substrate concentration producing 50% of maximal enzyme velocity
SEM	- Standard error of the mean
v	- Enzyme velocity in the absence of any enzyme effectors
v_i	- Enzyme velocity in the presence of inhibitors
V_{max}	- Maximum enzyme velocity

INTRODUCTION

Metabolic depression is a common strategy used to withstand environmental extremes that would otherwise prove deadly to metabolically active organisms. It is seen in a great variety of animals across many phyla. The stresses known to induce hypometabolic states in receptive animals include lack of food, low temperatures, high temperatures, aridity, and hypoxia. In these situations an animal living its life to the hilt is apt to have its energy or water stores rapidly depleted, resulting in quick death.

What, then, is involved in metabolic depression? Basically, the animal either slows down or stops all together most of its normal metabolic and physical activities (Storey, 1988; Wang and Wolowyk, 1988). Movement is usually curtailed. Heart rate and breathing are decreased. In homeotherms, body temperature is often allowed to fall to the level of the ambient temperature. Non-essential (largely biosynthetic) metabolic pathways are shut down. Essential (usually energy-producing) pathways are slowed down. In situations where the animal is also in danger of desiccation, an habitat is chosen where water loss will be minimised before metabolic rate (MR) depression occurs.

Taken together, these adjustments serve to decrease energy utilisation, energy production, and, where warranted, evaporative water loss. Placed in a situation where the animal is in danger of depleting its endogenous energy stores and water content, hypometabolism permits the animal to extend its survival time long enough to wait out the return of conditions in which fuel and water can be replenished.

For instance, Hochachka and Somero (1985) have calculated that the anoxia-tolerant sea mussel Mytilus edulis has sufficient glycogen reserves to survive anaerobically (i.e., using only glycolysis to generate ATP) for only 3 days at its normoxic metabolic rate. But because Mytilus depresses its metabolic rate some 20-fold during anaerobiosis, its glycogen reserves are theoretically capable of sustaining its life for some sixty days (if no other factors were limiting).

In many cases the environmental stresses that induce hypometabolism in susceptible animals are recurring but variable in timing. Protopterus annectens, an African lungfish, enters hypometabolic aestivation only when the swamps in which they live dry out during arid stretches (Greenwood, 1987). Littoral marine molluscs, such as clams and mussels, must depress their metabolic rate in response to hypoxia any time they are cut off from the ocean during low tide. Many birds undergo a daily period of torpor, characterised by a decreased MR, lasting only a few hours

(Wang and Wolowyk, 1988). The small mammals which hibernate (ground squirrels, hedgehogs, etc.) appear to regulate this hypometabolic state by the combination of an internal clock and external triggers (photoperiod, etc.) (Pengelley and Asmundson, 1974); but it is not a continuous state. The hibernator regularly (every few days or every few weeks, depending on the species) revives for short periods, re-establishing its high non-hibernating MR, and then resumes hibernating hypometabolically (Malan, 1988).

This kind of hypometabolism, then, is quickly responsive to the animal's changing circumstances. It is capable of first being rapidly initiated and then being rapidly reversed, both in apparent response to external stimuli. This seems to rule out an elaborate genetic rearrangement as the root mechanism behind hypometabolism; for such a mechanism would be both excessively time-consuming (in light of the documented rapidity of the metabolic state transitions) and excessively energy-consuming (considering the regularity with which hypometabolic states occur in some animals).

Yet a great deal of co-ordination is needed among the various pathways that are to be either slowed down or shut down during hypometabolism. Imbalances in the rates of metabolic processes can have devastating consequences.

For instance, one of the most crucial balances to be maintained is the balance between ATP utilisation and ATP production, particularly as it pertains to the maintenance

of cellular ionic gradients (Hochachka, 1986). These gradients are disrupted when ATP is lacking. Due to ion channels in the plasma membrane, K^+ ions are continually leaking out of the cell while Na^+ ions are accumulating inside. If ATP production is insufficient to power the active transport of K^+ ions back inside the cell, and of Na^+ ions back outside the cell, membrane depolarisation quickly occurs. This, in turn, leads to a number of destructive events, including the opening of voltage-dependent Ca^{2+} channels and a host of subsequent Ca^{2+} -stimulated disruptions (such as the activation of Ca^{2+} -dependent phospholipases and proteases which destroy the cellular milieu).

The ability to maintain cellular ionic gradients during extended periods of low ATP production may be the key ability that allows stress-tolerant animals to reduce their MR to cope with the stress, and yet still survive.

Research is just beginning into the question of how hypometabolic animals maintain their cellular ionic gradients. Preliminary results indicate that, instead of increasing the efficiency of ATPase pumps in the face of low ATP availability, passive ion channels are "blocked", to decrease leaks and thereby lower the need for energy-intensive pumping (Wang and Wolowyk, 1988; Hochachka *et al.*, 1987; Harris *et al.*, 1986). The mechanism(s) by which ionic flux through these channels are blocked is not yet known, but an elegant system would incorporate the same or a

similar mechanism as that used to block metabolic flux through biochemical pathways. A common mechanism would go a long way towards ensuring that depressed MR is co-ordinated with depressed requirements for ATP in at least one highly significant area.

The search for mechanisms behind hypometabolism in general have progressed a fair bit farther than those for specific "channel blockers". As discussed above, the regulation probably does not involve extensive genetic reorganisation via the degradation of existing enzymes and the synthesis of a whole new set with altered properties better suited to the new metabolic state. This, however, does not rule out a less elaborate genetic response to help induce co-ordinated hypometabolism.

Hochachka (1988) has recently hypothesised that stress proteins (heat shock proteins, cold shock proteins, and glucose-regulated proteins) might play a significant role in reducing ATP utilisation under the same circumstances which result in a reduced MR. However, no one has ever bothered to look for stress proteins in animals that are actually hypometabolic, so the idea remains highly conjectural.

Acidosis appears to play a key regulatory role in depressing the MR of anoxic brine shrimp embryos. This has lead to speculation that it may regulate hypometabolism in other animals as well.

Embryos of Artemia salina, the brine shrimp, become almost ametabolic under anoxic conditions. Catabolism of

carbohydrates and lipids is undetectable even after months of dormancy (Ewing and Clegg, 1969), and MR drops to about 2% of its normoxic value (Hand and Gnaiger, 1988).

Accompanying this metabolic state transition is an extreme acidification which drops intracellular pH (pH_i) from 7.9 to as low as 6.3 (Busa *et al.*, 1982).

This acidification is closely correlated with the drop in MR. When aerobic embryos are artificially acidified (pH_i 7.9 to pH_i 6.8), a dormant state is induced comparable to that of anaerobic dormancy (Busa and Crowe, 1983). And when dormant anaerobic embryos are artificially alkalised with ammonium chloride, metabolic rate (as measured by heat dissipation) increases five-fold to approximately 10% of aerobic values (Hand and Gnaiger, 1988).

Because acidification clearly plays a regulatory role in depressing the MR of Artemia, it has been proposed that it might also serve to depress the MR of other stress-tolerant animals (Busa, 1985; Malan, 1988; DeZwaan and Thillart, 1985). The value of using intracellular pH changes as a regulator of metabolic state transitions is that the proton is an ubiquitous effector of enzymes. By altering the concentration of this single metabolite, virtually all areas of cellular metabolism can be affected. This would allow the kind of concerted metabolic regulation necessary for an orderly transition into and out of hypometabolism.

A certain amount of acidosis accompanies most hypometabolic states. During anoxia, acidic end-products accumulate in the absence of oxidative metabolism; and during aerobic dormancy CO_2 accumulates during the hypercapnia that often characterises decreased respiration. This acidosis has been clearly shown to occur in the blood of hypometabolic animals (see DeZwaan and van den Thillart, 1985; and Malan, 1988 for references). However, there have been far fewer studies on the true intracellular pH of these animals. It would be premature to identify acidosis as a regulatory mechanism in the absence of such studies.

Another control system that can regulate a broad spectrum of metabolic activities is the reversible covalent modification of proteins via phosphorylation and dephosphorylation. The terminal-phosphate group from ATP can be transferred to receptive proteins by protein kinases and removed by phosphoprotein phosphatases. Phosphorylation /dephosphorylation seems to act on many regulatory enzymes as an "on-off" switch. In either its high- or low-phosphate form (it differs with different enzymes) the enzyme is "off". That is to say, it can be entirely non-functional, or it can function at a much lower efficiency than in its "on" state, due to the increased effects of inhibitors, decreased effects of activators, decreased affinities for its substrates and cofactors, or all of the above. In its "on" state, then, the enzyme is optimally poised to perform its metabolic function.

] A particularly elegant example of enzyme regulation by phosphorylation/dephosphorylation involves the enzyme responsible for controlling levels of fructose-2,6-bisphosphate (fructose-2,6- P_2) a potent activator of glycolysis (it acts upon PFK; see Uyeda *et al.*, 1982).

A single bifunctional enzyme catalyses both the synthesis of fructose-2,6- P_2 from F6P and ATP (6-phosphofructo-2-kinase/PFK-2 activity), and its degradation to F6P and P_i (fructose-2,6-bisphosphatase/FBPase-2 activity). In its dephosphorylated form, FBPase-2 activity is low compared to PFK-2 activity and the enzyme preferentially synthesises fructose-2,6- P_2 . But in its phosphorylated form, PFK-2 activity is inhibited, FBPase-2 activity is stimulated, and the enzyme preferentially degrades fructose-2,6- P_2 (Hue and Rider, 1987; El-Maghrabi, 1982). Phosphorylation/dephosphorylation in PFK-2/FBPase-2, then, serves to prevent futile cycling.

In eumetabolic animals enzyme phosphorylation /dephosphorylation seems to be an ubiquitous way of favouring a single direction at a time in bidirectional pathways. It also serves to integrate the various metabolic functions of a cell or tissue so that they are all working towards a single end.

For instance, in adipose tissue phosphorylation activates hormone-sensitive lipase, while it inhibits rate-limiting enzymes of triglyceride biosynthesis such as acetyl-CoA carboxylase and glycerol phosphate acyl

Figure 1. The pathways of lipid metabolism and the effects of phosphorylation on the rate-limiting enzymes of those pathways.

Enzymes inactivated by phosphorylation:

(1) acetyl-CoA carboxylase

(2) glycerol phosphate acyl transferase

Enzymes activated by phosphorylation:

(3) hormone-sensitive lipase

Adapted from Hardie (1985).

triglyceride

3

L+

fatty acid

acetyl-CoA

phospholipid

phosphatidate

monoacylglycerol phosphate

2*

fatty acyl-CoA

fatty acid

malonyl-CoA

1*

acetyl-CoA

transferase (Hardie, 1985). Dephosphorylation has the reverse effects. Thus a signal that activates protein kinases in adipose tissue ensures that lipids are efficiently catabolised, while a signal that activates protein phosphatases ensures that lipids are efficiently anabolised (see Figure 1).

In metabolically more complicated tissues a number of distinct biochemical pathways will be under similar control (phosphorylation/dephosphorylation of the rate-limiting enzymes). Activation of kinases or phosphatases will activate a number of pathways and inhibit a number of others. The general principle seems to be that anabolic pathways are co-ordinated with one another in the opposite direction of co-ordinated catabolic pathways.

Different protein kinases are responsive to different second messengers in the cell such as cAMP, cGMP, and Ca^{2+} (Walsh, 1987). Therefore extracellular signals, such as hormones, can trigger widespread protein phosphorylations. Different protein phosphatases are regulated to varying degrees by second messengers (Ca^{2+}), phosphorylation/dephosphorylation, polyamines, and thermostable inhibitor proteins (Fisher *et al.*, 1987). That a number of different kinases and phosphatases exist, each of which is regulated by different means and each of which is specific to a different group of proteins, allows an extra level of control in the regulation of metabolism. Not all the

phosphorylated proteins in a cell need be always in the same state of phosphorylation.

Membrane ion channels are also known to be regulated by phosphorylation/dephosphorylation (Hescheler *et al.*, 1987; Lazo and Velasco, 1987; Reuter, 1987). This one system of control, then, can integrate a wide variety of metabolic functions.

In mammals, control of carbohydrate metabolism via enzyme phosphorylation is achieved largely through cAMP-dependent protein kinases. Among the enzymes known to serve regulatory roles in the control of carbohydrate metabolism which are also phosphorylated by cAMP-dependent protein kinase are glycogen phosphorylase, glycogen synthase, phosphofructokinase, pyruvate kinase, pyruvate dehydrogenase, and isocitrate dehydrogenase (Krebs, 1985). This thesis will look at two of these enzymes from a pulmonate snail--phosphofructokinase and pyruvate kinase--with the goal of determining whether their covalent modification (phosphorylation) plays any role in depressing the snails' MR during hypometabolic interludes.

Phosphofructokinase or PFK (ATP:D-fructose-6-phosphate-1-phosphotransferase, E.C. 2.7.1.11) catalyses the reaction

fructose-6-phosphate + MgATP^{1-}

--> fructose-1,6-bisphosphate + MgADP^{-2} + H^+

The first committed step of glycolysis, it is thought also to be the rate-limiting step of glycolysis, regulation of its catalytic rate largely regulating the rate of glycolysis (Uyeda, 1979).

PFK is responsive to both pH and a large number of metabolite effectors. Its pH optimum is usually around pH 8. At physiological pH's even small changes in pH can alter its activity significantly. Among its important inhibitors are ATP, citrate, and phosphoenolpyruvate. Its activators include AMP, ADP, NH_4^+ , P_i , and fructose-2,6- P_2 .

Fructose-2,6- P_2 is the most potent known activator of PFK, and regulation of its intracellular levels (controlled by phosphorylation/dephosphorylation reactions) seems to be an important regulator of PFK activities, and hence glycolytic flux (Hue and Bartons, 1985; Hue and Rider, 1987).

PFK is also affected by its aggregation state. In mammals its smallest active aggregate appears to be an homotetramer, but these tetramers also associate with one another (Uyeda, 1979). The highly aggregated forms of the enzyme may have different kinetic properties than the mere tetramers (Reinhart, 1980; Bosca *et al.*, 1985). In the highly crowded intracellular milieu, it is likely that the enzyme is found largely in its polymeric form (Reinhart and Lardy, 1980).

With the large number and varied nature of PFK's effectors, it is possible that its control does not extend

beyond the allosteric level in most cases. Nonetheless, a number of studies have shown that in eumetabolic mammals (usually rats or rabbits are used in the experiments), PFK is reversibly phosphorylated, and that its phosphorylation state might play a role in its regulation.

PFK from most mammalian tissues can be isolated in various states of phosphorylation. Skeletal muscle PFK from resting or non-active rats can have as little as 0.15 moles P_i /mole subunit (Foe and Kemp, 1982), or as much as 0.70 moles P_i /mole subunit (Hofer and Furst, 1974), though most research groups have found P_i contents closer to the lower value (see Kemp and Foe, 1983 for references). When muscle begins contracting, the phosphate content of PFK goes up. In contracting rabbit muscle, the phosphate content of PFK increases to about 1.0 mole P_i /mole subunit from about 0.54 mole P_i /mole subunit in the resting muscle (Hofer, 1985). Some research groups have found both high- and low-phosphate forms of PFK within individual muscle preparations (Uyeda et al., 1978).

Rat liver contains PFK in both high- and low-phosphate states. In hepatocytes incubated with [^{32}P]-phosphate, the high-phosphate state has about 0.8 moles P_i /mole subunit, and the low-phosphate state has about 0.3 moles P_i /mole subunit (Furya and Uyeda, 1980). No phosphate-free PFK is found in either muscle or liver.

In mammalian muscle, PFK phosphorylation is stimulated under conditions in which the glycolytic rate is also

stimulated (Hofer, 1985). This has lead to the assumption that the phosphorylation of mammalian skeletal muscle PFK must somehow activate the enzyme. But the mechanism by which this activation occurs is uncertain. Native high-phosphate forms of PFK from muscle preparations do not show any significant in vitro kinetic differences from their low-phosphate counterparts (Kemp and Foe, 1983). Nor does phosphorylation appear to affect the rate of the enzyme's proteolytic degradation (Hofer, 1985), as has been suggested is the case with fructose-1,6-bisphosphatase, L-type pyruvate kinase, and several others (Rivett, 1986). And attempts to correlate muscle PFK's phosphorylation state with its aggregation state have been contradictory, some groups finding phosphorylation stabilises PFK polymers (Uyeda et al., 1978), others finding phosphorylation induces depolymerisation (Hussey et al., 1977).

Of course, dilute in vitro experiments might not correctly duplicate the crowded intracellular milieu. Under in vivo conditions physiological relevant changes to the enzyme might occur. Or perhaps the wrong enzyme parameters are being checked in vitro. For instance, Luther and Lee (1986) found that not only does phosphorylated rabbit muscle PFK have a greater affinity for F-actin than does unphosphorylated PFK, but also that phospho-PFK, in the presence of actin, is significantly less sensitive to inhibition by ATP than its unphosphorylated counterpart

under the same conditions (K_i 's for ATP are 2.8 ± 1.1 mM and 1.5 ± 1.1 mM, respectively.)

In mammalian livers, the effects of phosphorylation are more clear-cut. Glucagon, which in the liver stimulates the cAMP-dependent phosphorylations of phosphorylase kinase, glycogen phosphorylase, and glycogen synthase (activating glycogen's hydrolysis to glucose), also causes the incorporation of additional phosphate into low-phosphate forms of rat liver PFK (Kagimoto and Uyeda, 1979). The high-phosphate PFK has kinetic properties consistent with an inactivation of the enzyme (Sakakibara and Uyeda, 1983). Thus, when glycogenolysis in the liver is stimulated, glycolysis is blocked and the liver dumps its glucose into the blood. Additional glucose can be synthesised along the gluconeogenic pathway while glycolysis remains blocked.

The high-phosphate PFK has a decreased affinity for F6P, shows more inhibition by ATP and citrate, and is less activated by AMP and fructose-2,6-P₂, though none of the changes in its kinetic constants (K_m 's, I_{50} 's, K_a 's) are greater than about 50% (Sakakibara and Uyeda, 1983).

The phosphorylation states of native PFK from mammalian adipose tissue have never been determined. However, adrenaline triggers an increase in the glycolytic flux of rat adipose tissue (Saggerson and Greenbaum, 1970; Halperin and Denton, 1969) without a concomitant increase in the levels of fructose-2,6-P₂, the usual allosteric effector used to activate PFK (Rider and Hue, 1985). This suggests

that adrenaline is activating PFK non-allosterically, possibly through phosphorylation.

Purified PFK from rat adipose tissue can be phosphorylated in vitro by the catalytic subunit of cAMP-dependent protein kinase (CAK) (Sale and Denton, 1985). The kinase causes 0.86 ± 0.26 moles P_i /mole enzyme tetramer to be incorporated into white adipose PFK, and 0.60 ± 0.13 moles P_i /mole tetramer into brown adipose PFK. The initial, pre-kinase treatment, phosphorylation states of the purified enzymes were not determined. The phosphorylated enzymes show slightly less inhibition by ATP, and the phosphorylated white adipose PFK shows an increase in its affinity for fructose-2,6- P_2 of about 50%.

In vitro phosphorylation studies of mammalian PFK use cAMP-dependent protein kinase almost exclusively, reflecting the consensus that this is the enzyme responsible for phosphorylating PFK in vivo. There are a number of rationales backing up this belief: 1) CAK is responsible for phosphorylating the other regulatory phospho-enzymes of carbohydrate metabolism (Cohen, 1980; Fisher, 1987); 2) Glucagon, which can cause in vivo PFK phosphorylations in liver, acts to increase intracellular cAMP levels; and catecholamines, which can cause in vivo phosphorylations in adipose tissue or muscle, can act to increase intracellular cAMP; 3) PFK phosphorylated in vitro by CAK has the same phosphorylation site as PFK phosphorylated in vivo (Kemp *et al.*, 1981); 4) CAK is one of the only protein kinases which

has been found able to phosphorylate mammalian PFK in vitro (there are three others).

Mammalian PFK is a poor substrate for cAK (Cohen, 1978). Its K_m for rabbit muscle PFK is $230 \mu M$, which is an order of magnitude higher than the enzyme's in vivo concentration (Kemp et al., 1981). Thus if this kinase truly is responsible for phosphorylating PFK, it must act at a slow rate, much slower than it acts upon its other substrates.

Also able to phosphorylate PFK in vitro are phosphorylase kinase, phospholipid-dependent protein kinase C, and Ca^{2+} /calmodulin-dependent protein kinase. Like cAK, phosphorylase kinase has a low affinity for PFK and is an inefficient phosphorylating agent (Hofer, 1985). The K_m of protein kinase C for rabbit muscle PFK is within the enzyme's physiological range, but the presence of substrates and activators (F6P, FDP, AMP, fructose-2,6- P_2) severely inhibits the reaction, making its physiological significance suspect (Nettelblad et al., 1986). Ca^{2+} /calmodulin-dependent protein kinase phosphorylates rat liver PFK at the same site as cAK, and at a comparable rate, suggesting it may be an alternative phosphorylating agent available to the liver (Mieskes et al., 1987).

While a fair amount of ambiguity remains about the nature, purpose, and regulation of mammalian PFK phosphorylation, the phosphorylation of mammalian pyruvate

kinase (PK), the other regulatory enzyme of glycolysis, is generally well understood.

Pyruvate kinase (ATP:pyruvate phosphotransferase, E.C. 2.7.1.40) catalyses the last reaction of glycolysis:

In mammals, PK exists in 3 known isozymic forms (Ibsen et al., 1975). M-type PK is found in striated muscles as the only PK isozyme present, and in the brain as the major isozyme. L-type PK is found only in the liver (as the major isozyme) and the kidney (as the minor isozyme). K-type PK is the major isozymic component of kidney PK, the minor isozymic component of liver and brain PK, the only isozymic component of heart muscle, spleen, adipose tissue, and virtually all other tissue, PK.

The K- and M-type isozymes of PK in mammals are uncomplicated enzymes. They show Michaelis-Menten kinetics with respect to both PEP and ADP, are inhibited by only ATP and phenylalanine, and are at best only slightly activated by their single activator fructose-1,6-P₂ (FDP) (Munday et al., 1980).

Mammalian L-type PK, on the other hand, exhibits co-operative kinetics with respect to PEP (Michaelis-Menten kinetics with respect to ADP), is sensitive to a number of inhibitors, including ATP, AMP, and alanine, and is highly

sensitive to activation by FDP (Munday et al., 1980).

Neither M-type nor K-type PK from mammals has ever been shown to be phosphorylated in vivo, but L-type PK has (Engstrom, 1980).

PK has long been identified as a regulatory enzyme--its reaction is unidirectional and non-equilibrium, and a change in its substrate and product levels is noticeable immediately after metabolic changes. The differing properties of the PK isozymes are suited to their respective regulatory roles in the tissues in which they are found.

Muscle cells are involved almost exclusively in carbohydrate catabolism, so M-type PK need not be subject to a complex set of controls. It need simply respond to the ATP requirements of the cell. Hence its hyperbolic kinetics provide rapid enzymatic flux except in the presence of high concentrations of ATP. All the sensitive tuning of glycolytic flux occurs at PFK.

The K-type isozyme of PK, like the M-type, is found in non-gluconeogenic tissues, and so likewise needs only the minor controls of its Michaelis-Menten kinetics.

Liver cells, in contrast, are involved in a great variety of metabolic activities, both catabolic and anabolic. Control of metabolism at all its loci (PK included) is of necessity much more finely tuned.

The phosphorylation of mammalian L-type PK is thought to primarily serve the function of switching liver carbohydrate metabolism from glycolytic to gluconeogenic.

The maximum rate of PK-directed breakdown of PEP to pyruvate is 25X the maximum rate of gluconeogenic synthesis of PEP from pyruvate (Munday *et al.*, 1980). Therefore, in the absence of regulatory controls to shut down PK activity, a net flow up the glycolytic pathway (i.e. gluconeogenesis) would be impossible. Thus L-type PK is inactivated by phosphorylation.

There is little doubt that the *in vivo* phosphorylation of L-type PK is catalysed by cAK in response to glucagon-stimulation of the liver (Engstrom, 1980). Phospho-PK has a decreased affinity for PEP, is more strongly inhibited by ATP and alanine, and is less strongly activated by FDP, than dephosphorylated PK (Engstrom, 1980). One mole of phosphate/mole subunit is incorporated into this homotetramer (Ljungstrom *et al.*, 1976; Ekman *et al.*, 1976; Ljungstrom and Ekman, 1977).

The role of phosphorylation/dephosphorylation in the control of PFK and PK during hypometabolism has not been extensively studied. Yet preliminary indications are that it does play a part in depressing MR.

The role of enzyme phosphorylation/dephosphorylation in eumetabolic mammals appears to be limited to regulating transitions between different metabolic pathways. Certainly this is true in the case of PK and PFK. But in mammals capable of depressing their MR, enzyme phosphorylation/dephosphorylation may be additionally used to regulate transitions between metabolic states; and in lower animals

capable of depressing their MR, enzyme phosphorylation/dephosphorylation may have been entirely co-opted for just that purpose.

During hibernation, both PFK and PK from the liver of the meadow jumping mouse Zapus hudsonius show kinetic changes consistent with mammalian PFK and PK phosphorylation, though the enzymes's actual phosphorylation states were not examined (Storey, 1987b). PFK shows decreased activation by fructose-2,6-P₂ (K_a increases), and increased inhibition by ATP and citrate (I₅₀'s increase). PK shows decreased activation by FDP, and increased inhibition by alanine. In its hypometabolic hibernating state, then, liver PFK and PK from Zapus are likely to be inactivated, and liver glycolytic flux thereby restricted--as is expected in hypometabolic states.

During anoxia, goldfish survive by depressing their metabolic rate to as little as 20% of its normoxic value (van den Thillart et al., 1976). PFK and PK from all goldfish tissues except red and white muscle showed kinetic changes during anoxia consistent with an inactivation of the enzymes (inhibitor effects increased; activator effects decreased) (Rahman and Storey, 1988). And while white and red muscle PFK and PK were not altered kinetically during anoxia, the pI values for the enzymes from these tissues increased during anoxia; a similar increase was seen in the pI values of liver PK and PFK. In the liver the kinetic changes were shown to be the result of enzyme

phosphorylation. That similar kinetic or pI changes occurred in the isozymes from the other tissues suggests that PFK and PK were being phosphorylated in those tissues also.

The anoxia-tolerant marine gastropod Buyscotypus canaliculatum phosphorylates PFK in its foot muscle in response to anoxia (Storey, 1984). The phosphorylated enzyme is less sensitive to fructose-2,6-P₂ and NH₄⁺ activations, and more sensitive to ATP inhibition. PFK isozymes from the other tissues of this animal (radula retractor muscle, liver, kidney, gills) also show kinetic changes to anoxia, suggesting that they too are phosphorylated (R. Whitwam, unpublished results).

PK from B. canaliculatum is phosphorylated in all tissues, including muscle, during anoxia (Plaxton and Storey, 1984a, 1984b, 1985a, 1985b).

In anoxia-tolerant marine invertebrates, the inactivation of PK by phosphorylation serves not only to aid PFK slow flux through glycolysis, but also to improve the energy yield of what little anaerobic glycolysis there is occurring (see Figure 2). When PK is inactivated, PEP is taken up by phosphoenolpyruvate carboxykinase and converted to oxaloacetate. Oxaloacetate then enters a series of reverse Kreb's cycle reactions, yielding succinate and an additional ATP. In some marine invertebrates, succinate is further metabolised to propionate, yielding a second ATP (Kruetzer et al., 1985). By not channelling PEP simply into

Figure 2. If PK is inactivated, the energy yield of anaerobiosis can be increased by channelling PEP into a series of reverse Kreb's cycle reactions. Marine invertebrates using such a pathway will either accumulate succinate or propionate, depending on the species. Dotted arrows show forward direction of Kreb's cycle.

pyruvate and then lactate, marine invertebrates increase their ATP yield during anaerobiosis by either 2 ATP (end-product is succinate) or 4 ATP (end-product is propionate) per glucose molecule. By increasing the energy efficiency of their fermentative consumption of carbohydrate stores, marine invertebrates decrease the rate at which they deplete those stores. This extends even further the time the animal is able to survive hypometabolically.

This thesis will examine hypometabolism in a land invertebrate, the pulmonate snail Otala lactea (Gastropoda, Pulmonata, Helicidae). Pulmonates are fairly rare among experimental animals in that they will enter hypometabolic states in response to either of two distinct environmental stresses--anoxia or aridity/starvation--both of which can be easily imposed under lab conditions.

While the ecological relevance of anoxia-tolerance in land snails is suspect, the threat of starvation and desiccation is commonplace. Pulmonates commonly spend a large part of their lives in a dormant state because food and water are lacking (Comfort, 1957). When this quiescent state occurs in the summer, as is common with desert snails like Otala, it is termed aestivation; when it occurs in the winter, it is termed hibernation. But for most snails the state can be induced year-round in the lab, any time food and water are withheld. So following the example of Herreid (1977), when discussing this quiescent state in snails only the term "dormancy" will be used. This is to emphasise the

fact that it is nutritional considerations, not the time of year, which are triggering the metabolic state transition."

During dormancy, pulmonates withdraw into their shell, sealing the aperture with their mantle, and depress their metabolic rate. Evaporative water loss in the dormant snail is prevented because not only is the snail's calcium carbonate shell impermeable to water, but so is the only living tissue the snail exposes to the environment, its mantle (Machin, 1975). While dormant, the snail will secrete an epiphragm from its mantle. This is a dry membrane covering the aperture made up of a mucoprotein matrix and crystalline calcium carbonate (Campion, 1961; Block, 1971). While largely impermeable, the epiphragm has small distinct regions which are fully permeable and allow respiratory gas exchange, as well as water loss (Barnhart, 1983). Over long periods of dormancy, the snail may secrete a series of epiphragms.

Snails generally remain aerobic throughout dormancy, though their oxygen consumption decreases due to their hypometabolic state (Coles, 1968, 1969; Schmidt-Nielsen et al., 1971; Burky et al., 1972; Herreid, 1977). Reports do exist, however, of snails which are anaerobic during dormancy (Meenakshi, 1964).

Oxygen consumption in active *Q. lactea* is $80 \mu\text{L} \cdot \text{hr}^{-1} \cdot \text{g}^{-1}$. In dormant snails it drops as low as $5 \mu\text{L} \cdot \text{hr}^{-1} \cdot \text{g}^{-1}$, though about once a day the rate increases to as much as $33 \mu\text{L} \cdot \text{hr}^{-1} \cdot \text{g}^{-1}$ for several hours (Herreid, 1977; Barnhart,

1987). After epiphragm secretions, bursts in oxygen consumption are much less frequent (Barnhart, 1987).

Using oxygen consumption as a measurement, then, MR in Q. lactea is decreased 2.5- to 16-fold during dormancy.

The metabolic strategies used by pulmonates during dormancy have not, for the most part, been extensively worked out. Some facts, however, have been established. For instance, during dormancy snails deplete their glycogen stores to meet their energy requirements. The lipid stores of Capaea nemoralis remain constant over several months of dormancy while glycogen is depleted (van der Horst and Zandee, 1973). The dormant giant African snail, Achatina achatina, likewise slowly depletes its hepatopancreas glycogen stores while its free fatty acid content remains constant (Umezuike and Iheanacho, 1983).

Some anabolism continues to take place in dormant snails. Using radioactive precursors, van der Horst et al. (1974) found that lipid biosynthesis was taking place in the hepatopancreas of dormant C. nemoralis. Similar patterns of radiolabel incorporation into lipids were found whether 1-¹⁴C-acetate, 2-¹⁴C-pyruvate, D-¹⁴C-glucose-UL, or L-¹⁴C-alanine-UL was used as the precursor. Phospholipids were the major biosynthetic product (60-70%), while free fatty acids, diglycerides, and triglycerides made up the remainder (about 10% each). The rate of lipid biosynthesis in the dormant animals was markedly less than that in active snails. The amount of radiolabelled product after 7 days of

dormancy was equivalent to the amount of radiolabelled product seen after less than 30 minutes of eumetabolic activity (van der Horst, 1973; van der Horst *et al.*, 1974).

After one month of dormancy, haemolymph pH of *O. lactea* decreases from pH 7.8 to 7.4; after 18 months, haemolymph pH is down to pH 7.0 (Barnhart, 1986a). Because haemocyanin in *O. lactea* shows a reverse Bohr shift (Barnhart, 1986b), this acidification allows the haemocyanin to load more oxygen at a time when pO_2 values are low.

Haemolymph acidification might also reflect a general intracellular acidification of *O. lactea* during dormancy, though this possibility has never been tested. Certainly, when extracellular pH is experimentally reduced in the marine bivalve *Mytilus edulis*, intracellular pH is also reduced (Lindinger *et al.*, 1984). But, on the other hand, pulmonate neurons have been shown to regulate pH_i independently of extracellular pH (Thomas, 1982). The question of intracellular acidification during dormancy is unresolved.

The metabolic rearrangements which occur in pulmonates during anoxia are no better elucidated than those which occur during dormancy. Anaerobic glycolysis probably provides the principle means of generating energy for the anoxic snail (von Brand *et al.*, 1950), as it does with most other anoxic facultative anaerobes (see Hochachka and Somero, 1985). During anaerobiosis in *Helix pomatia*, succinate and D-lactate accumulate in both the haemolymph

and the hepatopancreas; propionate does not accumulate (Wieser, 1981).

An interesting feature of anaerobiosis in pulmonates, apparently unique to them, is that anoxia stimulates the biosynthesis of fatty acids in the hepatopancreas, perhaps serving to re-oxidise glycolytically-produced NADH (van der Horst, 1974). The acetyl CoA for this purpose is apparently obtained from pyruvate that has been diverted away from LDH, which in snails is readily inhibited by pyruvate (K_i is 10 mM) (Storey, 1976). Obviously, not all the available pyruvate is being diverted towards fatty acid synthesis if D-lactate and succinate are nonetheless accumulating.

Pulmonates show a remarkable tolerance to anoxia (von Brand, 1944) that belies its seeming irrelevance to their lives. Dormancy, in most species, is not in itself an anaerobic process; so any exposure to hypoxia must be the result of environmental conditions. It is conceivable that snails in temperate locales might experience occasional hypoxia if their living quarters were flooded by rain or melting snow in Spring; but for desert snails, such as Otala, even this possibility seems remote. Certainly, the ancestral marine gastropods from which the pulmonates evolved must have required anoxia-tolerance if they were to survive intertidal lifestyles. Perhaps the pulmonates retained this ability to survive anoxia even after evolving into land animals away from tides. While anoxia-tolerance was not greatly required, the same mechanism for depressing

their MR proved useful in surviving repeated threats of starvation and desiccation. That anoxia-tolerance persists in snails suggests that it may come in handy occasionally-- though perhaps only once a lifetime for most snails.

This study on Otala lactea will provide the opportunity to compare and contrast an aspect of the hypometabolism resulting from anoxia with that of the hypometabolism resulting from dormancy.

This study examines PK and PFK from three tissues of O. lactea during dormancy and anaerobiosis. There is evidence from all three tissues that both enzymes are modified after either stress. The relevance of these modifications to the snail's hypometabolic state is discussed, and the similarities between the modifications induced by anoxia and those induced by dormancy are discussed in light of a possible evolutionary relationship between anoxia-tolerance and starvation/aridity-tolerance.

METHODS AND MATERIALS

Chemicals and Animal

All biochemicals and coupling enzymes were obtained from Sigma Chemical Co., Boehringer Mannheim Corp., and JT Baker Co. Distilled, deionised water was used throughout.

Snails, Otala lactea, were provided by Chris Barnhart from an introduced population at Playa del Rey in Los Angeles County, California. While being stored in the lab, the snails were usually kept in their dormant state, dry without food. Every three weeks they were aroused with water and fed damp paper towels, cabbage, and CaCO_3 (blackboard chalk.) Arousal usually lasted only a few days before the snails once again entered dormancy.

All snails used for experiments were awoken from dormancy by feeding and were regularly spritzed with water to ensure continual arousal for at least two days. Fully aroused snails were easily distinguished from dormant snails by their extended heads and tentacles.

Control (active) snails were sacrificed directly from the aroused population.

Anoxic snails were taken from the aroused population and placed in a jar containing damp paper towels and cabbage. The jars were then filled with N_2 gas and sealed with a screw-on lid and 3 layers of parafilm (one beneath

the lid, two outside the lid). A wire mesh barrier prevented the snails from climbing more than half-way up the jar. Under anoxic conditions the snails would usually distend themselves out of their shells and press out flat on the walls of the jar. They never ate the cabbage provided.

Dormancy was induced in the aroused snails by placing them in dry containers. In the dry environment the snails would withdraw into their shells (the preliminary stage of dormancy) within a couple of hours.

For the time course of dormancy and recovery, length of dormancy was timed from the moment aroused snails (active for at least 2 days) were placed in a fully aerated dry jar without food or water. Length of recovery was timed from the moment the snails, who had been dormant for 22 days, were given food and water. At each time point of recovery, only the 3 or 4 most active snails were taken.

Tissue Preparation

All tissues were dissected out of the snails within one minute of killing the animal and then frozen in liquid nitrogen. Frozen tissues were stored at -60°C until use. Tissues were homogenised in 1:5 (w/v) homogenisation buffer containing 100 mM NaF, 5mM EDTA, 5 mM EGTA, 0.1 mM PMSF, 30 mM 2-mercaptoethanol, 40% glycerol in 50 mM imidazole (pH 7.0 for PK, pH 7.4 for PFK.) Foot and mantle muscles were homogenised in a Polytron PT 10 sonicating homogeniser. Hepatopancreas was homogenised in an Ultra-Turrax

homogeniser. The homogenates were centrifuged for 20 minutes at 16,000 X.g in a Sorvall RC-5B refrigerated centrifuge at 5°C. The resultant supernatant was passed through a 5 mL column of fine Sephadex G-25 equilibrated in 40 mM imidazole (pH 7.0 for PK, pH 7.4 for PFK), 5 mM EDTA, 15 mM 2-mercaptoethanol, and 40% glycerol for PK or 20% glycerol for PFK. The 5 mL columns were spun in a desk-top centrifuge at top-speed for 1 minute. The filtrate was used as the enzyme extract.

Enzyme Assays

PK activity was determined by coupling pyruvate formation to NADH utilisation with excess LDH, according to the scheme

PFK activity was determined by coupling FDP formation to NADH utilisation with excess aldolase, TPI, and aGPDH, according to the scheme

NADH utilisation for both enzymes was recorded on a Gilford 240 recording spectrophotometer as the decrease in A_{340} over time.

PK from all three tissues (foot muscle, mantle muscle, hepatopancreas) was assayed in the presence of 50 mM imidazole (pH 7.0), 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM NADH, and 1 u/mL LDH (dialysed for hepatopancreas assays only.) PEP and MgADP were added as described in Results. Final cuvette volume was 1 mL.

PFK from foot and mantle muscles was assayed in the presence of 50 mM imidazole (pH 7.0), 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM NADH, 0.5 u/mL aldolase, 0.5 mM TPI, 2 u/mL a-GPDH (all coupling enzymes dialysed). F6P and MgATP were added as described in Results. Final cuvette volume was 1 mL.

PFK from hepatopancreas was assayed in the presence of 50 mM imidazole (pH 7.0), 30 mM KCl, 3 mM $MgCl_2$, 0.1 mM NADH, 0.5 u/mL aldolase, 0.5 u/mL TPI, 2 u/mL a-GPDH (all coupling enzymes dialysed). F6P and MgATP were added as described in Results. Final cuvette volume was 1 mL.

All assays (PK and PFK) were initiated by the addition of enzyme homogenates.

One unit of enzyme activity is defined as the amount of enzyme that catalyses the utilisation of 1 umole of NADH per minute at 20°C.

Calculations and Statistics

To obtain kinetic constants observed data were fitted by computer to the appropriate equation using the Gauss-Newton minimising algorithm for non-linear regression. The program was supplied by Steven Brooks.

All $S_{0.5}$, n_h , and V_{max} values were obtained from the Hill equation (Cornish-Bowden, 1979).

I_{50} was defined as the concentration of inhibitor required to reduce enzyme activity (at set substrate concentrations) to one-half of its value in the absence of inhibitor. I_{50} values were calculated from a fitted plot of v/v_1 vs. $[I]$ in the manner of Job et al. (1978).

K_a was defined as the concentration of activator required to produce one half of the maximal activation achieved by the enzyme (at set substrate concentrations) in the presence of saturating amounts of the activator. K_a values were calculated from a fitted sigmoidal curve of V_a vs. $[A]$.

Statistical significance was determined by student's t test (Mendenhall, 1983).

RESULTS

PK and PFK from Otala lactea were modified in all 3 tissues studied--foot muscle, mantle, and hepatopancreas--during both dormancy and anoxia. That is to say, both enzymes in all three tissues showed kinetic changes in response to the stresses.

Foot Muscle

The properties of PK and PFK from Otala lactea foot muscle are shown in Table 1 and Table 2.

The kinetic changes in foot muscle PK brought about by dormancy were identical, both in direction and magnitude, to those brought about by anoxia. In both situations, affinity for PEP decreased ($S_{0.5}$ increased), and inhibition by L- alanine and MgATP increased (I_{50} 's decreased).

In active snails, $S_{0.5}(\text{PEP})$ was $62 \pm 1 \mu\text{M}$. During dormancy $S_{0.5}(\text{PEP})$ increased to $89 \pm 8 \mu\text{M}$, while during anoxia it increased to $94 \pm 7 \mu\text{M}$. These two values are both significantly different from the active value by student's t test ($p < .005$), but they are not significantly different from one another. Similarly, the I_{50} for MgATP was $8.0 \pm .4 \text{ mM}$ during dormancy, and was $8.2 \pm .6 \text{ mM}$ during

Table 1. Kinetic properties of PK from the foot muscle of *O. lactea*. Snails were active for 2 days, dormant 22 days, or anoxic for 45 hours.

	Active	Dormant	Anoxic
$S_{0.5}(\text{ADP})$ (mM)	$0.34 \pm .01$	$0.40 \pm .03^*$	$0.35 \pm .03$
n_h	$1.30 \pm .02$	$1.28 \pm .07$	$1.14 \pm .07$
$S_{0.5}(\text{PEP})$ (μM)	62 ± 1	$89 \pm 8^{**}$	$94 \pm 7^{**}$
n_h	$1.62 \pm .06$	$1.43 \pm .06$	$1.38 \pm .07$
$V_{\max}$ (units/ gm wet wt.)	13 ± 1	$17.6 \pm .7^*$	16 ± 2
$I_{50}(\text{Ala})$ (mM)	$5.0 \pm .5$	$2.1 \pm .2^{**}$	$3.3 \pm .3^*$
$I_{50}(\text{MgATP})$ (mM)	$13.9 \pm .7$	$8.0 \pm .4^{**}$	$8.2 \pm .6^{**}$

Results are means of 4-6 determinations.

$S_{0.5}(\text{ADP})$ values were determined at 3 mM PEP. $S_{0.5}(\text{PEP})$ values were determined at 3 mM ADP. $V_{\max}$ values were determined at 3 mM PEP and 3 mM ADP. I_{50} values were determined at 0.1 mM PEP and 3 mM ADP.

*Significantly different from "active" value, $p < .05$.

**Significantly different from "active" value, $p < .005$.

Table 2. Kinetic properties of PFK from the foot muscle of *O. lactea*. Snails were active for 2 days, dormant for 22 days, or anoxic for 45 hours.

	Active	Dormant	Anoxic
$S_{0.5}(\text{F6P})$ (mM)	$3.32 \pm .15$	$2.36 \pm .41^*$	$2.28 \pm .17^*$
n_h	$2.19 \pm .08$	$1.79 \pm .27$	$1.68 \pm .15^*$
$S_{0.5}(\text{F6P}) + 6 \text{ mM NH}_4^+$ (mM)	$1.47 \pm .11$	$0.86 \pm .19^*$	$1.22 \pm .10^*$
n_h	$1.93 \pm .11$	$1.55 \pm .16^*$	$2.07 \pm .09$
$I_{50}(\text{MgATP})$ (mM)	$7.0 \pm .3$	$6.5 \pm .3$	$6.2 \pm .5$
$I_{50}(\text{Mg}_2\text{Cit})$ (mM)	$11.4 \pm .7$	$11.4 \pm .8$	$8.4 \pm .8^*$
$K_a(\text{AMP})$ (mM)	$0.42 \pm .03$	$0.28 \pm .01^*$	$0.68 \pm .02^*$
$K_a(\text{F}_{26}\text{P}_2)$ (μM)	$3.40 \pm .17$	$2.17 \pm .33^*$	$3.81 \pm .11^*$
$K_a(\text{PEP})$ (μM)	49 ± 3	$74 \pm 2^*$	59 ± 2

Results are means $\pm$ SEM of 3-6 determinations.

$S_{0.5}(\text{F6P})$ values were determined at 3 mM MgATP. I_{50} values were determined at 2 mM F6P and 3 mM MgATP. K_a values were determined at 0.4 mM F6P and 3 mM MgATP.

*Significantly different from "active" value; $p < .05$.

anoxia; both are significantly lower than the active value of $13.9 \pm .7 \text{ mM}$ ($p < .005$), but are not statistically distinguishable from one another.

The I_{50} for L-alanine decreased from $5.0 \pm .5 \text{ mM}$ in active snails to $2.1 \pm .2 \text{ mM}$ in dormant snails, but decreased to only $3.3 \pm .3 \text{ mM}$ in anoxic snails. The anoxic value is significantly different from the dormant value, but the direction of the change from the control value, and at least the general magnitude of that change, are the same for both hypometabolic states.

Overall, the kinetic changes in PK seen during dormancy and anoxia are consistent with an inactivation of the enzyme during those hypometabolic interludes.

Both dormancy and anoxia produced statistically significant kinetic changes in foot muscle PFK, but the changes were not identical for the two situations.

PFK's affinity for F6P increased some 30% under both stresses: $S_{0.5}(\text{F6P})$ dropped from $3.3 \pm .15 \text{ mM}$ in active snails to $2.36 \pm .41 \text{ mM}$ in dormant snails, and to $2.28 \pm .17 \text{ mM}$ in anoxic snails. This was the only kinetic parameter whose change in dormancy was identical to its change in anoxia. $S_{0.5}(\text{F6P})$ in the presence of 6 mM NH_4^+ decreased farther in dormant snails (to $0.86 \pm .19 \text{ mM}$) than in anoxic snails (to only $1.22 \pm .10 \text{ mM}$). The I_{50} for citrate changed in anoxia (decreasing to $8.4 \pm .8 \text{ mM}$ from $11.4 \pm .7 \text{ mM}$), but not in dormancy; whereas the K_a for PEP

increased in dormancy, but did not change in anoxia. During dormancy, the K_a 's for AMP and fructose-2,6-P₂ both decreased; but during anoxia, these two values increased.

The general trend of the kinetic changes during dormancy seems to be towards activation of PFK--affinity for substrate (F6P) and for two activators (AMP and fructose-2,6-P₂) increased. However, none of the changes was greater than 30%.

There was no clear-cut trend to the kinetic changes during anoxia. Affinity for F6P increased, indicative of enzyme activation; but at the same time, the I_{50} for citrate decreased, and the K_a 's for AMP and fructose-2,6-P₂ increased, indicative of enzyme inactivation. Again, the magnitude of the changes was not large.

Mantle

The properties of PK and PFK from *Q. lactea* mantle are shown in Table 3 and Table 4.

Mantle PK showed statistically significant kinetic changes during both dormancy and anoxia in all the parameters tested except V_{max} . $S_{0.5}(ADP)$ increased to the same extent under both conditions (from $0.34 \pm .01$ mM during activity to $0.42 \pm .02$ mM during dormancy, and to $0.44 \pm .02$ mM during anoxia.) $S_{0.5}(PEP)$ increased from 41 ± 1 μ M in active snails to only 75 μ M in dormant snails, but increased to 100 ± 10 μ M in anoxic snails. Likewise, the I_{50} for

Table 3. Kinetic properties of PK from the mantle of *O. lactea*. Snails were active for 2 days, dormant for 22 days, or anoxic for 45 hours.

	<u>Active</u>	<u>Dormant</u>	<u>Anoxic</u>
$S_{0.5}(\text{ADP})$ (mM)	$0.34 \pm .01$	* $0.42 \pm .02^*$	$0.44 \pm .02^*$
n_h	$1.22 \pm .04$	$1.22 \pm .08$	$1.54 \pm .03^*$
$S_{0.5}(\text{PEP})$ (μM)	41 ± 1	$75 \pm 1^*$	$100 \pm 10^*$
n_h	$1.08 \pm .04$	$1.47 \pm .11^*$	$1.40 \pm .09^*$
V_{max} (units/ gm wet wt.)	8 ± 1	12 ± 2	10 ± 2
$I_{50}(\text{Ala})$ (mM)	$4.6 \pm .4$	$8.0 \pm .6^*$	$2.5 \pm .2^*$
$I_{50}(\text{MgATP})$ (mM)	$15.0 \pm .6$	$10.0 \pm .9^*$	$5.3 \pm .3^*$

Results are means $\pm$ SEM.

$S_{0.5}(\text{ADP})$ values were determined at 3 mM PEP. $S_{0.5}(\text{PEP})$ values were determined at 3 mM ADP. V_{max} values were determined at 3 mM PEP and 3 mM ADP. I_{50} values were determined at 0.1 mM PEP and 3 mM ADP.

*Significantly different from "active" value, $p < .005$.

Table 4. Kinetic properties of PFK from the mantle of *Q. lactea*. Snails were active for 2 days, dormant for 22 days, or anoxic for 45 hours.

	Active	Dormant	Anoxic
$S_{0.5}$ (F6P) (mM)	$0.31 \pm .03$	$1.94 \pm .19^{**}$	$1.71 \pm .28^{**}$
n_h	$1.51 \pm .10$	$1.71 \pm .13$	$1.79 \pm .14$
$S_{0.5}$ (F6P) + 6mM Pi (mM)	$0.30 \pm .01$	$1.60 \pm .03^{**}$	$0.52 \pm .05^{**}$
n_h	$1.36 \pm .06$	$2.43 \pm .44^*$	$2.00 \pm .11^*$
$S_{0.5}$ (F6P) + 6 mM NH_4^+ (mM)	$0.22 \pm .01$	$1.11 \pm .11^{**}$	$1.35 \pm .17^{**}$
n_h	$1.35 \pm .11$	$1.64 \pm .24$	$1.53 \pm .15$
$S_{0.5}$ (F6P) + 5 μM F_{26}P_2 (mM)	$0.17 \pm .02$	$0.39 \pm .04^{**}$	$0.25 \pm .01^{**}$
n_h	$1.28 \pm .08$	$1.54 \pm .19$	$1.31 \pm .05$
I_{50} (MgATP) (mM)	$12.7 \pm .7$	$7.6 \pm .6^{**}$	$7.9 \pm .2^{**}$
I_{50} (Mg ₂ Cit) (mM)	>15	>15	>15
K_a (PEP) (μM)	67 ± 5	67 ± 6	72 ± 5
K_a (F_{26}P_2) (μM)	$2.18 \pm .48$	$1.75 \pm .36$	$4.15 \pm .22^{**}$

Results are means $\pm$ SEM or 3-6 determinations.

$S_{0.5}$ (F6P) values were determined at 3 mM MgATP. I_{50} values were determined at 2 mM F6P and 3 mM MgATP. K_a values were determined at 0.4 mM F6P and 3 mM Mg ATP.

*Significantly different from "active" value, $p < .01$

**Significantly different from "active" value, $p < .005$

MgATP decreased in both conditions, but it dropped further under anoxic conditions than it did under dormant conditions--to $5.3 \pm .3$ mM as opposed to $10.0 \pm .9$ mM. The I_{50} for L-alanine actually rose in dormant snails--from $4.6 \pm .6$ mM to $8.0 \pm .6$ mM--but decreased in anoxic snails--to $2.5 \pm .2$ mM.

Generally, the kinetic changes in PK during both dormancy and anoxia were consistent with an enzyme inactivation. In the anoxic snails, however, the extent of the kinetic changes was larger.

The kinetic changes seen in mantle PFK during dormancy and anoxia were also consistent with an inactivation of the enzyme. Both conditions produced kinetic changes in consistently parallel directions, but the magnitude of the changes again sometimes varied with the different conditions.

The changes in $S_{0.5}(\text{F6P})$ and $I_{50}(\text{MgATP})$ were identical in both anoxia and dormancy. $S_{0.5}(\text{F6P})$ increased 6-fold; $I_{50}(\text{MgATP})$ decreased from $12.7 \pm .7$ mM (active snails) to $7.6 \pm .6$ mM (dormant snails) or $7.9 \pm .2$ mM (anoxic snails). In the presence of 6 mM NH_4^+ , $S_{0.5}(\text{F6P})$ again increased 6-fold in both conditions; but in the presence of 6 mM phosphate, $S_{0.5}(\text{F6P})$ increased 5.3-fold in dormant snails, but increased only 1.7-fold in anoxic snails. In the presence of $5 \text{ } \mu\text{M}$ fructose-2,6-P₂, $S_{0.5}(\text{F6P})$ again increased in both conditions, but increased to a greater extent during

dormancy (130% increase vs. only a 50% increase during anoxia). The K_a for fructose-2,6-P₂ increased only in the anoxic snails.

Hepatopancreas

The properties of PK and PFK from hepatopancreas of Q. lactea are shown in Table 5 and Table 6.

The kinetic changes seen in hepatopancreas PK during dormancy are identical to those seen during anoxia. With the exception of the change in $S_{0.5}(\text{ADP})$ (an increase) and the change in activation by FDP (eliminated all together) the kinetic changes are indicative of an enzymatic activation. $S_{0.5}(\text{PEP})$ decreases 3-fold. V_{max} increases from $3.5 \pm .6$ units/g wet weight in active snails, to $5.7 \pm .7$ units/g wet weight in dormant snails, and to $8.0 \pm .8$ units/g wet weight in anoxic snails. $I_{50}(\text{MgATP})$ increases 2.5-fold. I_{50} for L-alanine quadruples.

Fructose-1,6-P₂ activates PK from active snails with a K_a of $4.0 \pm .4 \mu\text{M}$, but it has no effect on PK from dormant or anoxic snails at concentrations up to 5 mM.

A number of metabolites were tested for their effects on hepatopancreas PK: Most had little effect on enzyme velocity, but 5 mM proline reduced the activity of PK from active snails by 45%. The inhibitory effect was not as

Table 5. Kinetic properties of PK from the hepatopancreas of *Q. lactea*. Snails were active for 2 days, dormant for 22 days, or anoxic for 45 hours.

	Active	Dormant	Anoxic
$S_{0.5}^*$ (ADP) (mM)	$0.25 \pm .01$	$0.38 \pm .01^{**}$	$0.34 \pm .02^{**}$
n_h	$1.94 \pm .13$	$1.29 \pm .08^{**}$	$1.51 \pm .05^*$
$S_{0.5}$ (PEP) (mM)	$0.57 \pm .03$	$0.20 \pm .03^{**}$	$0.17 \pm .02^{**}$
n_h	$1.84 \pm .15$	$1.41 \pm .05^*$	$1.37 \pm .11^*$
V_{max} (units/ gm wet wt.)	$3.5 \pm .6$	$5.7 \pm .7^*$	$8.0 \pm .8^{**}$
I_{50} (MgATP) (mM)	$4.7 \pm .5$	$13 \pm 1^{**}$	$11.8 \pm .8^{**}$
I_{50} (Ala) (mM)	$0.97 \pm .05$	$3.9 \pm .7^{**}$	$4.3 \pm .7^{**}$
K_a (FDP) (μ M)	$4.0 \pm .4$	$>>5$ mM	$>>5$ mM
Relative activities			
control	100	100	100
+ 10 mM P_i	123 ± 7	130 ± 12	106 ± 11
+ 10 mM NH_4^+	113 ± 2	111 ± 5	108 ± 3
+ 5 mM Pro	55 ± 2	$74 \pm 8^*$	$116 \pm 8^{**}$
+ 5 mM α GP	82 ± 2	88 ± 5	118 ± 5
+ 5 mM Phe	98 ± 2	155 ± 14	92 ± 3

Results are means $\pm$ SEM of 3-6 determinations.

$S_{0.5}$ (ADP) values were determined at 3 mM PEP. $S_{0.5}$ (PEP) values were determined at 3 mM ADP. V_{max} values were determined at 3 mM ADP and 3 mM PEP. I_{50} values were determined at 1.0 mM PEP and 3 mM ADP. K_a (FDP) values and relative activity values were determined at 0.5 mM PEP and 3 mM ADP. Enzyme velocities in the presence of effectors ("Relative activities") are expressed relative to controls (no effectors) set at 100.

*Significantly different from "active" value, $p < .05$.

**Significantly different from "active" value, $p < .005$.

Table 6. Kinetic properties of PFK from the hepatopancreas of O. lactea. Snails were active for 2 days, dormant for 22 days, or anoxic for 45 hours.

	<u>Active</u>	<u>Dormant</u>	<u>Anoxic</u>
$S_{0.5}(\text{F6P})$ (mM)	$2.39 \pm .24$	$3.15 \pm .14^*$	$3.08 \pm .25^*$
n_h	$2.29 \pm .23$	$2.26 \pm .15$	$2.58 \pm .14$
$S_{0.5}(\text{F6P})$ + 5 mM NH_4^+ (mM)	$1.56 \pm .09$	$1.81 \pm .10$	$1.67 \pm .02$
n_h	$2.15 \pm .06$	$2.86 \pm .37$	$2.75 \pm .19^*$
$I_{50}(\text{MgATP})$ (mM)	$1.86 \pm .19$	$1.88 \pm .09$	$1.65 \pm .17$
$I_{50}(\text{Mg}_2\text{Cit})$ (mM)	>15	>15	$7.37 \pm .37^{**}$
$K_a(\text{P}_i)$ (mM)	$0.21 \pm .03$	$0.30 \pm .01^*$	$0.26 \pm .02$
$K_a(\text{AMP})$ (mM)	$0.30 \pm .01$	$0.23 \pm .01^{**}$	$0.27 \pm .02$
$K_a(\text{F}_{26}\text{P}_2)$ (μM)	$0.70 \pm .09$	$0.55 \pm .05$	$2.31 \pm .34^{**}$
$K_a(\text{PEP})$ (μM)	42 ± 3	$30 \pm 1^*$	$30 \pm 3^*$

Results are means $\pm$ SEM of 4-10 determinations.

$S_{0.5}$ values were determined at 0.3 mM MgATP. I_{50} values were determined at 4 mM F6P and 0.3 mM MgATP. K_a values were determined at 0.5 mM F6P and 0.3 mM MgATP, except $K_a(\text{P}_i)$ which was determined at 2 mM F6P and 0.3 mM MgATP.

*Significantly different from "active" value, $p < .05$.

**Significantly different from "active" value, $p < .005$.

great on PK from dormant snails (26% reduction in activity) and was not present at all in PK from anoxic snails.

The kinetic changes in hepatopancreas PFK during dormancy were not entirely the same as those during anoxia. During both dormancy and anoxia, $S_{0.5}(\text{F6P})$ increased to the same value ($3.15 \pm .14 \text{ mM}$ in dormancy; $3.08 \pm .25 \text{ mM}$ in anoxia), and $K_a(\text{phosphate})$ decreased to the same value. But during dormancy, $K_a(\text{Pi})$ increased marginally and $K_a(\text{AMP})$ decreased marginally, while neither changed at all during anoxia. Likewise, $I_{50}(\text{Mg}_2\text{citrate})$ decreased substantially, and $K_a(\text{fructose-2,6-P}_2)$ increased 3-fold, during anoxia, but did not change at all during dormancy.

The kinetic changes in PFK during anoxia are clearly consistent with enzyme inactivation--inhibition by citrate increased dramatically and activation by fructose-2,6-P₂ decreased dramatically, while all the other kinetic changes were not large. The kinetic changes seen during dormancy were not clearly poised in either direction--activation or inactivation--and were all small changes.

Time course

In active snails, the $I_{50}(\text{MgATP})$ of foot muscle PK is $13.9 \pm .7 \text{ mM}$; after 22 days of dormancy the value drops to $8.0 \pm .4 \text{ mM}$. Figure 3 shows a time course detailing the pattern of this kinetic change during entry into, and recovery from, dormancy. For the first 12 hours after food and water had been withheld (time 0 of dormancy), foot

Figure 3. A time course of the change in I_{50} (MgATP) from foot muscle PK of *Q. lactea* during dormancy and recovery from dormancy. Recovery was initiated after 22 days of dormancy by giving the snails food and water.
Abbreviations: m = minutes; h = hours; d = days.

muscle PK's I_{50} (MgATP) remained at its "active" level. By 48 hours, the value dropped to its "dormant" level. Ten minutes after food and water had been re-introduced, the value returned to active levels. It was at about this time that the snails began to emerge from their shells.

A similar time course of dormancy and recovery is shown in Figure 4 for hepatopancreas PK, except the parameter being followed is $S_{0.5}$ (PEP). $S_{0.5}$ (PEP) remained at its high "active" value even 12 hours after the snails had been placed in a dry environment without food. By 48 hours, however, $S_{0.5}$ (PEP) had dropped to its "dormant" level. After food and water had been given to the dormant snails, it took somewhere between 3 and 24 hours for $S_{0.5}$ (PEP) to return to its "active" level. The snails were physically active long before this. They began crawling around and feeding in less than half an hour.

It is not apparent from Figure 3 and Figure 4, but neither foot muscle PK's I_{50} nor hepatopancreas PK's $S_{0.5}$ (PEP) showed any intermediate values between "dormant" and "active" values during the time course. No matter what time point was sampled, the kinetic values obtained fell into a bimodal distribution--either close to the average dormant value, or close to the average active value. The points on the time course which appear intermediate are actually averages of values from both states. For instance, after 10 minutes of recovery from dormancy the average of the hepatopancreas PK $S_{0.5}$ (PEP) values was $0.28 \pm .08$ mM.

Figure 4. A time course of the change in $S_{0.5}$ (PEP) from hepatopancreas PK of *Q. lactea* during dormancy and recovery from dormancy. Recovery was initiated after 22 days of dormancy by giving the snails food and water.
Abbreviations: m = minutes; h = hours; d = days.

This was an average of 3 dormant values (0.16, 0.14, 0.25 mM) and 1 active value (0.55 mM).

The description given above of the 2 time courses was, therefore, a description only of the general trend in snails. Generally, $S_{0.5}(\text{PEP})$ from hepatopancreas PK took >3 hours to return to its active levels after dormancy; but in individual cases, recovery might occur after only 10 minutes.

The recovery of hepatopancreas PK from anoxia was also investigated. Snails were left for 5 minutes in the air after 48 hours of anoxia. PK from the hepatopancreas of these animals showed kinetics identical to control snails (active for 2 days). $S_{0.5}(\text{PEP})$ was $0.90 \pm .08$ mM, and there was activation by fructose-1,6- P_2 .

DISCUSSION

Hypometabolism in O. lactea, whether brought on by dormancy or by anaerobiosis, is characterised by changes in the kinetic properties of PFK and PK, two key regulatory enzymes of glycolysis. These kinetic changes were probably brought on by covalent modification as all metabolite effectors were removed from the enzyme preparations by gel filtration. Enzyme phosphorylation/dephosphorylation is commonly used by hypometabolic animals to alter their regulatory enzymes (Storey, 1988) and this may well be the mechanism employed by O. lactea, though detailed studies into the mechanism of the kinetic changes were not undertaken.

In the enzymes of eumetabolic mammals, the kinetic effects of phosphorylation on PK are clear cut and serve an easily identifiable role (Engstrom, 1980); the kinetic effects of phosphorylation on PFK are more ambiguous and their purpose is less well understood (Kemp and Foe, 1983; Hofer, 1985; Sakakibara and Uyeda, 1983). In hypometabolic O. lactea, the situation is somewhat analogous; the kinetic changes seen in PK follow a consistent pattern while those seen in PFK are more ambiguous.

(In both foot muscle PK (Table 1) and mantle PK (Table 2), the kinetic changes brought about by dormancy are virtually identical to those brought about by anoxia. The

two stresses cause $S_{0.5}(\text{PEP})$ to increase and the I_{50} 's for MgATP and alanine to decrease (except in dormant mantle, where the $I_{50}(\text{Ala})$ of PK increases). These changes are consistent with an inactivation of *Q. lactea* PK.

In marine invertebrates, PK is often phosphorylated during anoxia to produce an inactive variant of the enzyme (Storey, 1988; Holwerda et al., 1983). This causes PEP to accumulate and allows PEP to be diverted through PEPCK into a series of reverse Krebs's cycle reactions, generating additional ATP (see Figure 2). In *Q. lactea* the kinetic changes in PK during anoxia might serve a similar purpose. Except the same kinetic changes are seen in *Q. lactea* PK during dormancy. Dormancy in these snails is an aerobic process (Barnhart, 1987); there is no need to divert PEP away from pyruvate production as pyruvate can be channelled into the Krebs's cycle. Channelling PEP through PEPCK into a series of reverse Krebs's cycle reactions only makes sense in the absence of oxygen, when the Krebs's cycle proper is blocked up and won't turn.

Maximising the energetic efficiency of carbohydrate break-down is an important aspect of hypometabolism. In anoxia, this means diverting PEP away from PK and the subsequent production of lactate, and towards PEPCK and the subsequent production of alternative anaerobic end-products such as succinate. During aerobic dormancy, though, it means allowing PEP to enter the Krebs's cycle via PK. The similar kinetic changes in foot muscle and mantle PK during

both anoxia and dormancy suggest that a similar control strategy is being employed at this regulatory locus during both conditions. If PEP were being diverted towards the production of alternative anaerobic end-products during anoxia, then a similar (highly inefficient) re-routing would be taking place during aerobic dormancy.

Of course, *O. lactea* are desert snails, and probably rarely encounter hypoxic conditions in their natural environment. It seems more probable that the kinetic changes in foot muscle and mantle PK are geared towards maximum energy efficiency during aerobic dormancy, a more physiological relevant stress. In this case, PK is probably not "shut off" by these changes, simply slowed down. The alteration of PK's kinetic properties serve to help reduce flux through glycolysis in general, consistent with the overall depression in MR.

This view is supported by data from preliminary metabolite studies on *O. lactea* foot muscle, which show that during anoxia the major anaerobic end-product is D-lactate (levels increase 50-fold over control levels) while succinate increases only 2-fold (T. Churchill and K. Storey, unpublished data). During dormancy, no anaerobic end-products accumulate. Therefore, during both dormancy and anoxia, PEP still passes through foot muscle and mantle PK, though at a reduced rate due to the altered kinetics of the enzyme.

In contrast to the kinetic changes in mantle and foot muscle PK under the same circumstances, the kinetic changes in hepatopancreas PK during dormancy and anoxia were consistent, not with an inactivation, but with an activation of the enzyme. This is in apparent contradiction to the general role of enzyme modification during hypometabolism--to shut off or slow down MR. Q. lactea is the first hypometabolic animal shown to activate an PK isozyme during hypometabolism.

But pulmonate snails are unique among hypometabolic animals in another way as well. During hypometabolic interludes they synthesise fatty acids.

During dormancy, the snails maintain their lipid content at constant levels while synthesising new lipids in the hepatopancreas at a low rate, presumably just fast enough to replace lipids lost through the natural cellular turn-over of biomacromolecules (van der Horst and Zandee, 1973; van der Horst et al., 1974). During anoxia, the snails greatly increase free fatty acid synthesis in the hepatopancreas, perhaps to re-oxidise glycolytically-generated NADH (van der Horst, 1974).

The activation of hepatopancreas PK during hypometabolism, then, is consistent with the role of the hepatopancreas PK in lipid biosynthesis. By increasing the rate of production of pyruvate, the rate of production of acetyl CoA is also increased by simple mass action. Acetyl CoA can then be used in fatty acid biosynthesis.

During both anoxia and dormancy, the hepatopancreas of the snail is hypometabolic along with the rest of the animal. Carbohydrate utilisation in the hepatopancreas must be slowed. As PK is activated, the slowing of flux down glycolysis must be accomplished at other loci, possibly including PFK. But at the same time as glycolysis is slowed down, and the concentrations of later glycolytic intermediates are reduced, fatty acid biosynthesis must continue. By activating PK, enough pyruvate can still be channelled into the pyruvate dehydrogenase complex to allow the low level of fatty acid biosynthesis occurring during dormancy, or the high level of fatty acid biosynthesis occurring during anoxia, to proceed.

If hepatopancreas PK is activated, it can be in no way involved in the reduction of glycolytic flux necessary for hypometabolic survival. This, then, must largely be under the control of PFK.

The kinetic changes seen in hepatopancreas PFK during anoxia are indeed consistent with an inactivation of the enzyme--citrate begins to exert inhibitory effects and fructose-2,6-P₂ is a much more inefficient activator; the other kinetic changes are proportionally much smaller, and are probably not significant enough by themselves to alter PFK velocity to any great extent. However, the kinetic changes seen in hepatopancreas PFK during dormancy are all small, and none seems significant enough to effect large changes in PFK velocity. Yet glycolytic flux in the

hepatopancreas is probably greater in anoxia than dormancy, as fatty acid biosynthesis is stimulated in the former and is inhibited in the latter. The kinetic changes recorded for hepatopancreas PFK cannot alone account for needed metabolic adjustments during anoxia and dormancy. That kinetic changes occurred at all during dormancy and anoxia indicates that some kind of covalent modification of PFK was indeed taking place. But the significance of the modifications may not have been entirely in the kinetic parameters examined. The situation may be analogous to the phosphorylation of mammalian muscle PFK--phosphorylation is known to happen; it correlates (in mammalian muscle) with an activation of the enzyme; but the consequences of the phosphorylation on the enzyme's properties are not known (Kemp and Foe, 1983; Hofer, 1985).

Perhaps the covalent modification of Q. lactea PFK produces significant changes (changes consistent with an enzymatic inactivation) in some aspect of the enzyme's properties not examined in this study. Some possibilities include PFK's properties in vivo (which might not be accurately reflected by the dilute in vitro assay conditions), enzyme binding to particulate fractions in the cell, or subunit associations/dissociations.

PFK from Q. lactea foot muscle and mantle, like PFK from Q. lactea hepatopancreas, sometimes showed kinetic changes during anoxia or dormancy consistent with enzymatic inactivation (such as in mantle during anoxia), and

sometimes showed only small, physiologically insignificant changes. However, in all the situations glycolytic flux required shutting down. It seems likely, then, that in the mantle and foot muscle, just as in the hepatopancreas, the physiological significance of the covalent modification was in some area not detected or not examined.

Two time courses were plotted of the entry into and recovery from a hypometabolic state--one for Q. lactea foot muscle and one for Q. lactea hepatopancreas. Of course, the time courses looked only at PK and followed only dormancy, so they do not provide the complete picture; nonetheless, some interesting insights are revealed. For instance, the covalent modification of PK during dormancy appears to be largely an "on/off" process. There were no partial kinetic changes detected, only values corresponding to the active state or to the dormant state. This indicates that there were no partial covalent modifications of Q. lactea PK. On the other side of the coin is PK from the anoxia-tolerant sea mussel Mytilus edulis, which, during the initial stages of anoxia and during the initial stages of recovery from anoxia, did show intermediate activity ratios ($V_{0.05 \text{ mM PEP}} / V_{1 \text{ mM PEP}}$) between the enzyme's normoxic and fully anoxic values (Holwerda et al. ., 1984).

Generally, Q. lactea does not enter dormancy until somewhere between 12 and 48 hours after food and water have been withheld. This is not surprising as re-arranging its

metabolism so as to depress MR is a drastic step for the snail, and not a step to take frivolously. The snail no doubt will take time to fully explore its environment, ensuring that no food or water is present before resigning itself to hypometabolism.

After 22 days of dormancy, recovery in the foot muscle is very quick. In less than 10 minutes foot muscle PK has resumed its "active" kinetics. The foot muscle is required to move the snail about; since its metabolism is probably shut down during dormancy (PK certainly is inactivated), it is logical that the foot muscle would be among the first tissues to return to its "active" metabolic mode, as it is required to transport the snail to the coveted food and water.

Recovery in the hepatopancreas, on the other hand, is much slower--at least as measured by PK's return to "active" kinetics. The hepatopancreas is involved in comparatively complicated metabolic processes during dormancy (shutting down some pathways, but continuing others such as fatty acid biosynthesis). It is to be expected that it would require more complicated metabolic readjustments before returning to its typical "active" metabolism.

In view of this explanation, however, it is surprising to note that after 48 hours of anoxia, hepatopancreas PK returns to "active" kinetics in just 5 minutes. Of course, the two stresses produce metabolic responses that are not at all identical (for instance, fatty acid biosynthesis slows

down during dormancy but it speeds up during anoxia), so it is not to be expected that their post-stress metabolic readjustments would be identical.

Protein phosphorylation is known to regulate PK and PFK in various hypometabolic animals during anoxia--including a fellow gastropod to Q. lactea, the channelled whelk, Busycotypus canaliculatum (Storey, 1988). It is possible that protein phosphorylation is also responsible for the kinetic changes seen in hypometabolic Q. lactea. Certainly, the rapid reversibility of the kinetic changes (hepatopancreas PK returns to active kinetics 5 minutes after the termination of anoxia, and foot muscle PK returns to active kinetics 10 minutes after the termination of dormancy) is consistent with a phosphorylation/dephosphorylation mechanism.

The kinetic changes seen in PK and PFK from Q. lactea tissues during dormancy were not identical to those seen during anoxia. However, in every tissue at least one kinetic parameter of each enzyme was altered identically during the two stresses, and at least one kinetic parameter was altered dissimilarly. This suggests that a similar mechanism is responsible for the covalent modification of the enzymes during the two stresses, but that the mechanism does not operate identically in the two cases.

If phosphorylation/dephosphorylation is indeed responsible for the kinetic changes, the precise nature of

the phosphorylations must somehow differ in the two stresses. The extent of the phosphorylations might differ; or perhaps in one of the stresses additional sites on the enzymes are phosphorylated by a second protein kinase; or perhaps different protein kinases altogether are triggered by the different stresses, so completely different sites on the enzymes are phosphorylated. In any event, the similarities and differences in the kinetic changes under the two stresses will remain an intriguing paradox until further studies are undertaken.

CONCLUSIONS

The desert snail Otala lactea covalently modifies both PK and PFK in its foot muscle, mantle, and hepatopancreas while depressing its MR in response to either anoxia or dormancy. The kinetic changes consistently shared some features in common, indicating a similar underlying mechanism responsible for the covalent modifications. Judging from past studies on hypometabolic animals, this mechanism is likely to be enzyme phosphorylation/dephosphorylation.

O. lactea in its natural desert environment is frequently subject to periods of aridity and starvation, which induce dormancy, but likely only rarely, if ever, encounters anoxia. However, the gill-breathing littoral marine gastropods from which pulmonates evolved were frequently subject to anoxia when trapped between tides. Pulmonates have maintained the ability to tolerate anoxia and have additionally developed the ability to tolerate aerobic dormancy.

In marine gastropods, anoxia tolerance is characterised by, among other things, MR depression and enzyme phosphorylations. In O. lactea, dormancy is likewise characterised by MR depression and the covalent modification

of enzymes. That the covalent modification of PK and PFK during dormancy is similar to, and in some features identical to, the covalent modification of PK and PFK during anoxia suggests that the ancestral pulmonate may have co-opted its mechanisms for anoxia-tolerance for the new problem of aridity/starvation-tolerance, with the result that two widely different stresses are dealt with by Q. lactea in a very similar manner.

The kinetic changes in PFK from Q. lactea hepatopancreas, foot muscle, and mantle during both anoxia and dormancy sometimes shared kinetic changes consistent with the enzymatic inactivation expected during these hypometabolic states (such as in the mantle during anoxia). But they often showed only small, seemingly insignificant (though statistically real) kinetic changes. The true physiological relevance of these kinetic changes may be in some area not explored.

The kinetic changes in PK from Q. lactea foot muscle and mantle during dormancy and anoxia are consistent with an enzymatic inactivation. Inactivating PK in these tissues probably helps serve to depress glycolytic flux as part of the overall MR reduction occurring in the hypometabolic animal.

The kinetic changes in PK from Q. lactea hepatopancreas during anoxia and dormancy serve to produce a more active variant of the enzyme. This enzymatic alteration helps ensure that PEP continues to be channelled into acetyl CoA

Figure 5. Carbohydrate metabolism in the
hepatopancreas of Otala lactea during
hypometabolism (dormancy or anoxia).
ENZYMES: (1) PFK; (2) PK.
(3) Unknown mechanism.

synthesis, even during a period of reduced glycolytic flux. Acetyl CoA is then used in fatty acid biosynthesis (see Figure 5).

During dormancy, fatty acid biosynthesis in the hepatopancreas probably serves only to maintain the snails lipid stores, which do not change over even months of dormancy (van der Horst and Zandee, 1973). During anoxia, fatty acid biosynthesis may serve the additional role of re-oxidising glycolytically-produced NADH, though why such an energetically-expensive pathway would be used for such a purpose in times of limited energy-generation is a mystery. Fatty acid biosynthesis during anoxia may well serve additional, as yet unknown, roles important to anoxic hypometabolism.

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