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Properties of Pyruvate Dehydrogenase from the Land Snail, Otala lactea: Control of Enzyme Activity during Estivation

Properties of Pyruvate Dehydrogenase from the Land Snail, *Otala lactea:* Control of Enzyme Activity during Estivation

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Abstract

The kinetic properties and regulation of pyruvate dehydrogenase (PDH) activity during estivation and recovery were examined in foot muscle and hepatopancreas of the terrestrial snail, Otala lactea *(Muller) (Pulmonata, Helicidae). During estivation, the percentage of PDH in the active* a *form (PDH*a*) decreased from a control value of 98% to an estivating value of approximately 60% in both foot and hepatopancreas but the time course for changes in PDH*a *activity was different for the two tissues. Foot PDH*a *steadily decreased from the initiation of estivation until 20 h; after this time, the levels of PDH*a *showed no further change. Hepatopancreas PDH*a *levels did not decrease until 20 h after the initiation of estivation, after which time they rapidly dropped to reach a new, constant, estivating level by 30 h. Recovery curves for both tissues showed that PDH*a *had reached control levels 1 h after the reintroduction of food and water. Kinetic parameters of PDH*a *were also examined. The Michaelis constant value (K_m) for pyruvate was 274 μM (foot) or 164 μM (hepatopancreas) with a maximal activity of 0.195 IU/g wet weight for foot and 0.120 IU/g wet weight for hepatopancreas. The Hill coefficient for both enzymes was not significantly different from 1.0, which demonstrates that the dehydrogenase activity was not cooperative with respect to pyruvate. Michaelis constants for CoA were 6.4 μM and 20.4 μM for foot and hepatopancreas, respectively. Both enzymes showed maximal activity under alkaline conditions with pH optima occurring at 8 (foot) and 7.5 (hepatopancreas).*

Introduction

Pulmonate land snails show well-developed metabolic adaptations to wide variations in environmental extremes because of a poor ability to elude

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these conditions. For example, snails estivate in response to low water availability, hibernate in response to cold temperatures, and survive periods of anaerobiosis during low oxygen availability (Machin 1975; Livingstone and de Zwaan 1983). The common strategy underlying all of these responses is a metabolic rate depression during which energy expenditures are reduced to only 5%–30% of the corresponding amounts in active snails (Herreid 1977; Shick, de Zwaan, and de Bont 1983; Barnhart and McMahon 1987). Although the biochemical mechanisms underlying this metabolic rate depression were initially identified in anoxic marine snails (see Storey 1988*a*), these strategies are now proving to be universal. Recent studies of anoxic and estivating *Otala lactea* help to illustrate this point. Measurements of changes in the concentrations of glycolytic intermediates in tissues of *O. lactea* over time suggested a decreased glycolytic rate after 22 d of estivation (Churchill and Storey 1989). Other studies also showed an overall metabolic rate depression by 2 d of estivation (Barnhart and McMahon 1987). Subsequent experiments revealed three general mechanisms for controlling glycolytic rate depression during estivation: (1) a modification of the activity state of regulatory enzymes by reversible phosphorylation of enzyme subunits (Whitwam and Storey 1990, 1991), (2) enzyme and pathway control by the reversible association of enzymes with cellular particulate matter (Brooks and Storey 1990), and (3) control of carbohydrate utilization via fructose 2,6-bisphosphate regulation of phosphofructokinase (Brooks and Storey 1990). These mechanisms act to specifically control key regulatory enzymes to reorganize metabolism permitting a rapid entry into, and arousal from, dormancy (Storey 1985, 1988*a*; Hochachka and Guppy 1987). These same mechanisms have also been shown to control glycolysis in anoxia-tolerant whelks (Storey 1984, 1988*a*, 1988*b*; Plaxton and Storey 1985, 1986), goldfish (Rahman and Storey 1988), and turtles (Brooks and Storey 1988, 1989) as well as in small hibernating mammals (Storey 1987).

During the dry summer months, land snails estivate to avoid desiccation stress. At this time, they retreat into their shells and secrete a water impermeable epiphragm over the shell opening (Barnhart 1983). During dormancy heart rate falls, water loss declines and metabolic rate falls to levels approximately sixfold lower than active values (Herreid 1977). Studies of the physiology of estivation in *O. lactea* revealed that the estivating animals also experience hypercapnia, hypoxia, and respiratory acidosis as a result of prolonged periods of apnea (Barnhart 1986*a*, 1986*b*; Barnhart and McMahon 1987). The most recent studies have implicated hypercapnia as the triggering agent in metabolic rate depression (Barnhart and McMahon 1988).

The present study examines the role of pyruvate dehydrogenase (PDH) in coordinating metabolic depression during estivation. The PDH complex

is situated in the inner mitochondrial membrane and catalyzes the oxidative decarboxylation of pyruvate to CO_2 and acetyl-CoA. As such, it directly regulates the entry of carbohydrate into the citric acid cycle. In mammalian systems, PDH activity is controlled by reversible phosphorylation to interconvert active and inactive enzyme forms. The relative amounts of active (PDH*a,* dephosphorylated) and inactive (PDH*b,* phosphorylated) forms are controlled through the effects of activators and inhibitors that act primarily on the PDH-kinase enzyme. PDH-kinase can be reversibly (1) activated by Mg^{2+}, acetyl-CoA, NADH, and fatty acids (compounds that increase with an increasing cellular energy status) or (2) inhibited by ADP, pyruvate, and thiamine pyrophosphate (compounds that decrease with increasing cellular energy status). The PDH-phosphatase is apparently activated by Mg^{2+} and Ca^{2+} and inhibited by NADH (Wieland, Patzelt, and Lofeler 1972; Reed and Yeaman 1975; Denton et al. 1987). Mammalian studies have also shown that the enzyme may be regulated through changes in hormone levels, PDH*a* increasing in response to the addition of adrenaline, vasopressin, insulin or high calcium, and PDH*a* decreasing in response to addition of Ca^{2+} binding substances (such as ruthenium red; Denton et al. 1987). Reversible phosphorylation thus serves to link PDH activity to the energy state of the cell. The present study investigates the role of reversible phosphorylation of snail foot and hepatopancreas PDH in mediating the depression of aerobic metabolism observed during the early stages of estivation (Barnhart and McMahon 1987).

Material and Methods

Chemicals and Animals

All biochemicals and coupling enzymes were purchased from Sigma Chemical, St. Louis, or Boehringer Mannheim, Montreal. *Otala lactea* were provided by C. Barnhart from an introduced population in Los Angeles County. Animals were held in the laboratory at 22°C in covered plastic containers lined with paper towels. Snails were fed cabbage and dusted with shaved chalk every 15–25 d. Active snails were identified 16 h after the introduction of food and water. Control animals were sampled immediately from the active population. Estivating snails were obtained by placing active snails in a glass jar without food or water but with ample aeration. These animals were allowed to reenter a dormant state and were sampled after 8, 24, 32, and 48 h and after 18 d. In order to measure the PDH activity during recovery from estivation, 18-d estivating animals were placed in glass jars lined with

moist paper towels containing fresh cabbage leaves. The animals were liberally sprayed with a fine mist of dechlorinated water.

Measurement of PDH Activity

Pyruvate dehydrogenase activity was measured using a modification of method 3 from Elnageh and Gaitonde (1988). Foot muscle and hepatopancreas from freshly killed *O. lactea* were each homogenized 1:3 (wt:vol) with ice-cold sample buffer (50 mM potassium phosphate, pH 7.8, 1 mM β-mercaptoethanol, 1 mM potassium EDTA, and 0.1% [wt/vol] Triton X-100). The homogenates were then frozen at −80°C for 0.5 h, thawed to ice-cold temperature, and assayed immediately for PDH activity. This activity represented the percentage of enzyme in the active *a* form. In order to obtain the total PDH activity of the homogenate, 100-μL samples were incubated with 4 μL of 250 mM $MgCl_2$ plus 25 mM $CaCl_2$ at room temperature for 40 min. These short incubation times are important because of the instability of the enzyme at room temperature (fig. 1). Total PDH activity is defined as the activity in the sample after the incubation period.

Pyruvate dehydrogenase activity was monitored by following the reduction of *p*-iodonitrotetrazolium violet (INT) at 500 nm with a Gilford model 250 spectrophotometer at 21° ± 1°C. The reaction mixture, in a final volume of 1.0 mL, contained 50 mM Tris-HCl (pH 7.8), 0.5 mM EDTA, 0.2% (wt/vol) Triton X-100, 2.5 mM NAD^+, 0.2 mM coenzyme A, 1 mM $MgCl_2$, 0.1 mM oxalate, 1 mg of bovine serum albumin (Cohn fraction V), 0.6 mM INT, 5 IU of lipoamide dehydrogenase, 0.4 mM thiamine pyrophosphate (all components were optimized for assay of *O. lactea* PDH). The reaction was initiated by addition of an appropriate volume of foot or hepatopancreas homogenate and allowed to proceed for approximately 5 min. During this time the background rate was measured. The activity of PDH was then measured after addition of 10 mM pyruvate (20 μL of 0.5 M) to the cuvette and following the change in absorbance. The observed PDH activity was obtained by subtracting the background rate from that in the presence of pyruvate. The extinction coefficient for reduced INT at 500 nm is 15.4×10^3 cm^{-1} (Owens and King 1975).

Kinetic characterization of PDH was performed with crude enzyme homogenates that had been treated with $CaCl_2$ and $MgCl_2$ to catalyze the complete conversion to the active *a* form. Data were analyzed using a nonlinear least-squares regression fitted to the Hill equation. The effects of neutral salts and metabolites on activity were determined at pH 7.8 using a pyruvate concentration that gave a velocity 60% of V_{max} and maximal concentrations of all other assay components. The activity at various pH values was deter-

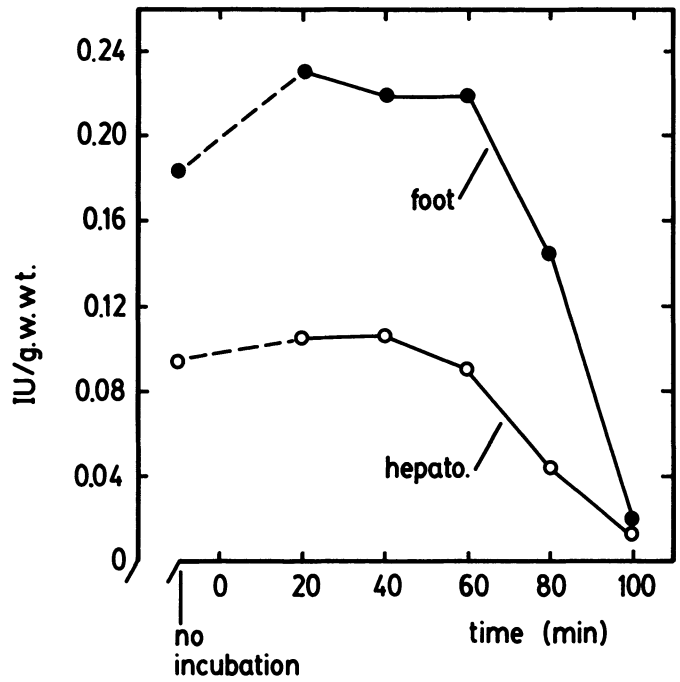

Fig. 1. Time course for the conversion of PDH into the active a *form in vitro. Total PDH activity in foot (closed circles) and hepatopancreas (open circles) was measured as a function of incubation time. Samples (100 µL aliquots) were incubated at 21° ± 0.5° C in the presence of 20 mM $MgCl_2$ and 2 mM $CaCl_2$ for various times, and the homogenate was assayed for PDH activity.*

mined by using maximal concentrations of all assay components and by substituting 20 mM 2-[H-Cyclohexylamino]ethane sulfonic acid (CHES) plus 20 mM 3-[H-Morpholino]propane sulfonic acid (MOPS) plus 20 mM Tris for 50 mM Tris-HCl in the assay buffer. This buffer mix was adjusted to specified pH values using KOH prior to assay of the enzyme. Maximal enzyme activity in the presence of the buffer mix (used for pH profiles) was identical to that measured with the standard Tris-HCl buffer system at pH 7.8.

Results

Effect of Estivation on Overall PDH Activity In Vivo

The percentage of total PDH activity in the active *a* form, measured at various times after the removal of food and water, is shown in figure 2. In foot

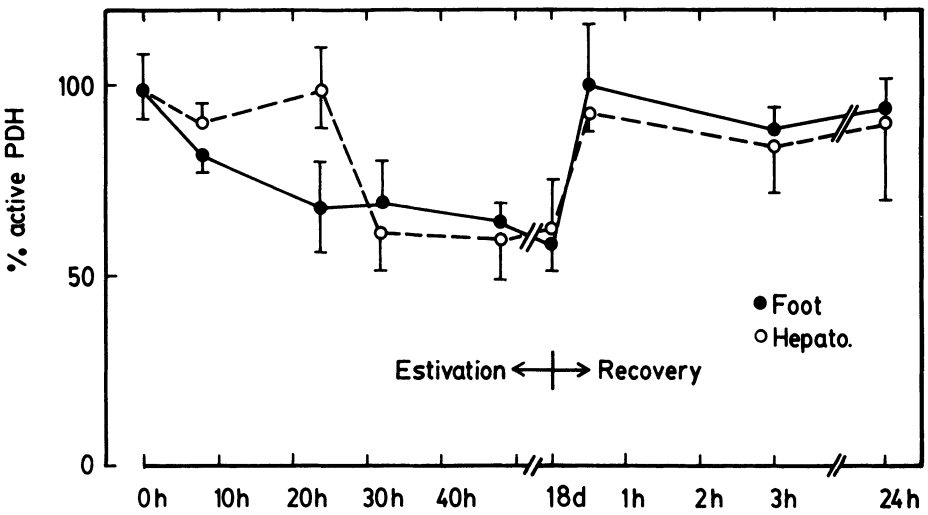

Fig. 2. Percentage of pyruvate dehydrogenase in the active a *form during estivation (0–18 d) and recovery (0–24 h) from estivation in foot (closed circles) and hepatopancreas (open circles). Values are means ± SEM (N = 4).*

muscle, PDH*a* gradually decreased during estivation from 100% for control animals (0 h estivation) to 65% after 20 h estivation. After this time, no further change in the percentage of PDH in the *a* form was observed. In hepatopancreas, PDH*a* activity was not significantly different from control values until 20 h after the initiation of estivation. After this time, a rapid decrease in the percentage *a* was observed over the following 10 h to give a final value of 60% PDH*a* by 30 h estivation. After 30 h, no further decreases in PDH*a* activity were observed up to 18 d after initiation of estivation.

The PDH*a* activity was also measured during recovery from estivation. The data of figure 2 show that the percentage of PDH*a* increased rapidly after addition of food and water to estivating animals in both foot and hepatopancreas tissues. One hour after the addition of food and water, the percentage of PDH*a* had returned to control values and no further change was observed after this point. Total PDH activity remained constant during estivation and recovery at 0.195 IU/g wet weight in foot and at 0.120 IU/g wet weight in hepatopancreas (see table 1).

Pyruvate Dehydrogenase Kinetic Parameters

It is possible to separate the overall PDH reaction into three separate enzyme-catalyzed steps that are carried out by three different enzyme subunits. These

TABLE 1
*Kinetic constants for foot and hepatopancreas PDH*a

Parameter	Foot	Hepatopancreas
K_m pyruvate (μM)	274 ± 28	164 ± 25
Hill coefficient pyruvate	1.3 ± .3	1.0 ± .3
K_m CoA (μM)	6.4 ± .6	20.4 ± 2.2
Total activity (IU/g · W · wt) ...	.195 ± .003	.120 ± .003

Note. Values are $\bar{X} \pm$ SEM ($N = 4$) except total activity ($N = 16$). Hill coefficients were not significantly different from 1.00 as determined by the one-tailed Student's *t*-test.

three different types of PDH subunits catalyze (1) a decarboxylase step, (2) a CoA-transfer step, and (3) an oxidation/reduction step, with substrate transferred between subunits by means of a dihydrolipoamide arm attached to the core subunit. The kinetic parameters for the decarboxylase and CoA-transfer steps of PDH are listed in table 1. Foot muscle had a 1.7-fold lower affinity for pyruvate than did the enzyme from hepatopancreas: the foot muscle K_m value was 274 ± 28 μM as opposed to 164 ± 25 μM for hepatopancreas. In both cases, the enzyme kinetic patterns were not sigmoidal with respect to pyruvate (Hill coefficients were not significantly different from 1.0 as determined with Student's *t*-test). Foot and hepatopancreas K_m values for CoA were also different with the enzyme from foot muscle showing a 3.2-fold greater affinity for CoA than PDH from hepatopancreas. Total enzyme activity was 1.63-fold higher in foot tissue as compared to hepatopancreas.

Tables 2 and 3 report the effect of various metabolites and neutral salts on overall PDH activity from foot and hepatopancreas. Table 2 shows that increasing concentrations of aspartate, ADP, ATP, GTP, potassium phosphate, and KCl inhibited PDH. Aspartate and ADP were more effective inhibitors of the hepatopancreas enzyme when compared with the enzyme from foot: the aspartate concentration of inhibitor that reduces velocity by 50% (I_{50}) value was 4.7-fold lower and the ADP I_{50} was 3.3-fold lower in hepatopancreas as compared to foot muscle. Inhibition by triphosphate compounds was apparently nonphysiological since the I_{50} values were approximately fourfold higher than cellular concentrations (ATP $\approx$ 1 mM; Churchill and Storey 1989). Overall PDH activity was also inhibited by increasing neutral salt concentrations with I_{50} values of 50–55 mM for both KCl and potassium phosphate. Since the ionic strength of a potassium phosphate solution is

TABLE 2
Effect of inhibitors on PDHa activity from foot and hepatopancreas: I_{50} values

Compound	I_{50} Value (mM)	
	Foot	Hepatopancreas
Aspartate . . .	13.1 ± 3.4	2.8 ± .3
ADP	11.1 ± 1.4	3.4 ± .1
ATP	4.2 ± .7	4.2 ± .6
GTP	5.1 ± 2.1	ND
KP_i	49.7 ± 3.0	56.8 ± 10.1
KCl	55.9 ± 2.1	55.3 ± 15.2

Note. I_{50} represents the concentration that inhibits control (no effector) activity by 50%. Values are $\bar{X}$ ± SEM (N = 3). ND, not determined.

approximately 2.4 times greater than the ionic strength of a KCl solution of the same molarity at pH 7.8, the fact that KCl and potassium phosphate inhibit at similar total salt concentrations suggests that the degree of inhibition depends primarily on the anion concentration.

Table 3 shows the effect of amino acids, citric acid cycle intermediates, glycolytic intermediates, and fermentative end products on the dehydrogenase activity. In general, addition of these compounds did not affect the activity of either foot or hepatopancreas PDH, although adenosine inhibited hepatopancreas PDH to a small degree. Figure 3*A* and 3*B* shows pH profiles for the enzyme from foot muscle and hepatopancreas, respectively. Both enzymes showed an alkaline pH optimum with maximal foot-muscle PDH activity occurring at pH 8.0 and maximal hepatopancreas PDH activity occurring at pH 7.5.

Discussion

The importance of aerobic respiration during estivation in *Otala lactea* was demonstrated by (1) an estivating respiratory quotient between 0.9 and 1.0 (Barnhart 1986*b*; Barnhart and McMahon 1987) and (2) a lack of accumulation of fermentative products such as D-lactate, succinate, and L-alanine (Churchill and Storey 1989). Both these facts are consistent with an amino

TABLE 3
Effect of metabolites on PDHa activity from foot and hepatopancreas

Compound	Percentage of Control Activity	
	Foot	Hepatopancreas
Alanine	99 ± 7	130 ± 7
Glutamate	112 ± 20	91 ± 17
Citrate	100 ± 10	92 ± 15
Succinate	64 ± 5	96 ± 10
Fructose 1,6-bisphosphate	100 ± 3	110 ± 30
Phosphoenolpyruvate	98 ± 6	115 ± 11
L-lactate	ND	123 ± 6
α-glycerol phosphate	94 ± 3	98 ± 15
Adenosine	95 ± 13	65 ± 12
AMP	ND	93 ± 20

Note. Percentage of control values were measured at 20 mM concentration of the effector. Values are means ± SEM ($N = 3$). ND, not determined.

acid and carbohydrate oxidation-based metabolism for estivating *O. lactea* (see also Bishop, Ellis, and Burcham 1983). The role of PDH in controlling respiration rates during estivation in *O. lactea* foot muscle and hepatopancreas is best understood when compared with previous data on metabolic rates determined from oxygen consumption measurements. During the first 6–8 h of estivation, rates of oxygen uptake declined rapidly to about 70% of control values in *O. lactea* and remained at this value until 15 h of estivation (Barnhart and McMahon 1988). Subsequent measurement of metabolic rates in *O. lactea* showed that after 2 wk of estivation, the metabolic rate had dropped to only 15% of that in active animals (Barnhart and McMahon 1987). Changes in the percentage of foot muscle PDH in the active *a* form also occurred within this time frame. For example, figure 1 shows that after 8 h estivation the percentage of PDH*a* had decreased from 98% ± 9% to 82% ± 5%, and PDH*a* had further dropped to 68% ± 12% by 24 h estivation (values are means ± SEM, $N = 4$). The close correlation between oxygen consumption and PDH activity in vivo suggests that these two parameters are intimately linked during estivation. This link may result directly from PDH regulation of glycolytic carbon entry into the citric acid cycle; PDH catalyzes the oxidative decarboxylation of pyruvate to give CO_2,

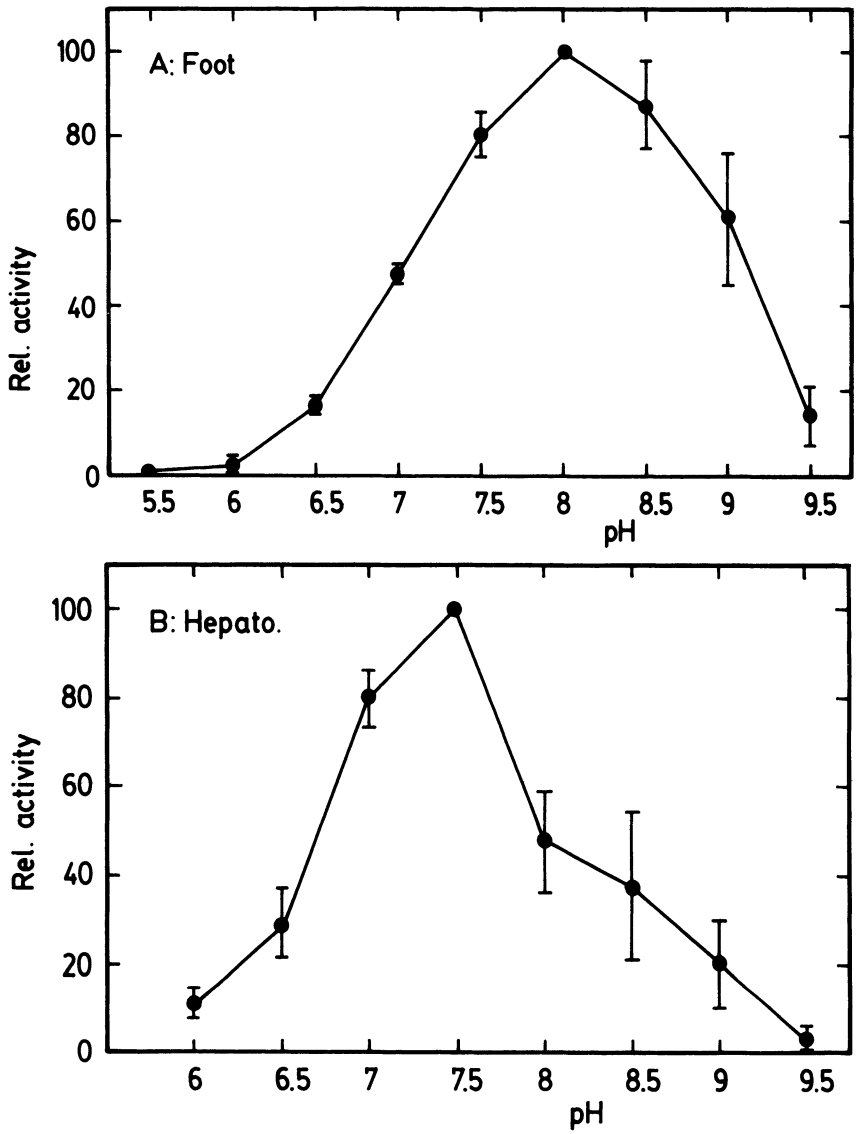

*Fig. 3. Profiles of activity vs. pH for PDH*a *from foot and hepatopancreas. Relative activity compared to pH 8 (foot,* A*) or pH 7.5 (hepatopancreas,* B*) was measured as a function of the pH of the medium. Assay buffer was 20 mM Tris (pKa = 8.1), 20 mM MOPS (pKa = 7.2), and 20 mM CHES (pKa = 6.5). The pH was adjusted to the appropriate value prior to assay.*

acetyl-CoA, and NADH. In foot muscle, therefore, PDH may be the key to the regulation of oxygen consumption rates during estivation through a direct control of the availability of the mitochondrial substrate acetyl-CoA.

The percentage of PDH*a* in hepatopancreas, on the other hand, did not decrease until approximately 20 h after the initiation of estivation. The time course for changes in hepatopancreas PDH activity was, therefore, similar to that measured for changes in *O. lactea* pyruvate kinase from foot muscle, hepatopancreas, and mantle (Whitwam and Storey 1990) and to that measured for changes in glycogen phosphorylase activity, fructose 2,6-bisphosphate concentrations, and glycolytic enzyme binding (Brooks and Storey 1990). The longer time course for hepatopancreas PDH-activity changes illustrates potential differences in tissue activity during the early stages of estivation. Foot muscle is retracted into the mantle during entry into dormancy and remains inactive behind the mantle collar throughout the dormant period. Accordingly, the percentage of active PDH in foot muscle decreased rapidly during early estivation and remained low up to 18 d after initiation of estivation. In contrast, hepatopancreas may continue to be active in the assimilation of ingested food for several hours; hepatopancreas PDH was active until 20 h after the start of estivation. Tissue differences may also reflect differences in citric acid cycle rates since PDH activity is intimately linked to citric acid cycle function (Walajtys, Gottesman, and Williamson 1974; Wan et al. 1989).

Recovery time courses for PDH from both foot and hepatopancreas showed that PDH activity increased dramatically within the first hour after the introduction of food and water. This time course for PDH recovery corresponded with that observed for increased rates of oxygen uptake after addition of water to estivating *O. lactea* (Herreid 1977) and with that for changes in pyruvate kinase kinetic constants during recovery from estivation in *O. lactea* foot and hepatopancreas (Whitwam and Storey 1990). These data further illustrate the importance of covalent modifications during estivation since awakening of *O. lactea* involves an immediate reactivation of these enzymes.

The data of tables 1–3 describe kinetic properties of PDH*a* from foot muscle and hepatopancreas. *Otala lactea* PDH*a* K_m values for pyruvate were 3–5 fold higher than those measured for bovine heart PDH (Paxton et al. 1986) and pigeon breast (Sanadi 1963). The K_m values for coenzyme A indicate that both enzymes have a high affinity for coenzyme A. Similar to PDH enzymes from mammalian sources, *O. lactea* PDH was inhibited by several metabolites, ATP, and relatively low salt concentrations (Wieland 1983; Paynter et al. 1985), but anaerobic end products did not affect the enzyme activity (table 3). Both PDH isozymes also showed an alkaline pH optima of either 7.5 (hepatopancreas) or 8 (foot). These results, coupled with measurements of (1) metabolite concentrations and (2) the time course for hemolymph pH changes during estivation suggest that overall PDH activity is not controlled by changes in the concentration of its effectors during

short term estivation (≤ 3 d). This was indicated by small changes in the levels of metabolite effectors of PDH (Churchill and Storey 1989) and small changes in pH values during early stage estivation (3 d; Barnhart 1986*a*). In addition, increases in PDH inhibitor concentrations over long-term estivation (1 m) are not large enough to effect PDH activity in either foot or hepatopancreas (Churchill and Storey 1989), although a 0.44-unit decrease in hemolymph pH, which occurs after 1 mo of estivation, is sufficiently large to affect total PDH activity assuming that hemolymph pH changes reflect changes in tissue pH (Barnhart 1986a). These studies indicate, therefore, that the major regulation of *O. lactea* PDH activity over short- and long-term estivation is via reversible covalent modification to decrease the amount of active *a* enzyme, increase the amount of inactive *b* enzyme, and, consequently, decrease overall PDH activity.

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