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Glucose-6-phosphate dehydrogenase regulation during hypometabolism

Christopher J. Ramnanan, Kenneth B. Storey *

Institute of Biochemistry and Department of Biology, College of Natural Sciences, Carleton University, 1125 Colonel By Drive, Ottawa, Ont., Canada K1S 5B6

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Abstract

Glucose-6-phosphate dehydrogenase (G6PDH) from hepatopancreas of the land snail, *Otala lactea*, shows distinct changes in properties between active and estivating (dormant) states, providing the first evidence of pentose phosphate cycle regulation during hypometabolism. Compared with active snails, G6PDH V_{max} increased by 50%, K_m for glucose-6-phosphate decreased by 50%, K_a Mg·citrate decreased by 35%, and activation energy (from Arrhenius plots) decreased by 35% during estivation. DEAE–Sephadex chromatography separated two peaks of activity and in vitro incubations stimulating protein kinases or phosphatases showed that peak I (low phosphate) G6PDH was higher in active snails (57% of activity) whereas peak II (high phosphate) G6PDH dominated during estivation (71% of total). Kinetic properties of peaks I and II forms mirrored the enzyme from active and estivated states, respectively. Peak II G6PDH also showed reduced sensitivity to urea inhibition of activity and greater stability to thermolysin protease treatment. The interconversion of G6PDH between active and estivating forms was linked to protein kinase G and protein phosphatase 1. Estivation-induced phosphorylation of G6PDH may enhance relative carbon flow through the pentose phosphate cycle, compared with glycolysis, to help maintain NADPH production for use in antioxidant defense.
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Glucose-6-phosphate dehydrogenase (G6PDH) catalyzes the rate-limiting step in the pentose phosphate pathway by oxidizing glucose-6-phosphate to 6-phosphogluconate and at the same time reducing $NADP^+$ to generate NADPH. The pentose phosphate pathway has multiple important functions in cells including: (i) production of pentose phosphates for nucleotide biosynthesis and 3–7 carbon sugars or sugar phosphates for many other uses; and (ii) production of reducing equivalents in the form of NADPH for a huge range of biosynthetic functions and for antioxidant defense [22]. Recent studies have shown that G6PDH has a critical role in cell growth, particularly in providing the NADPH needed for maintenance of redox regulation [32] and that G6PDH inhibition contributes to H_2O_2-induced cell death [33]. One of the newly recognized mechanisms for controlling G6PDH activity is reversible protein phosphorylation. Recent studies have documented G6PDH inhibition by cAMP-dependent protein kinase (PKA) mediated phosphorylation in mammalian systems including aortic endothelial cells, kidney cortex, and macrophages [11,37,38]. Another study also reported epidermal growth factor receptor tyrosine kinase mediated phosphorylation of Baker's yeast and bovine adrenal G6PDH [21]. However, although these studies show that G6PDH maximal activity is suppressed by phosphorylation, almost nothing is known to date about the effects of phosphorylation on the kinetic and regulatory parameters of the enzyme or how G6PDH phosphorylation state in cells responds to various external environmental stresses.

The land snail, *Otala lactea*, is native to the seasonally arid lands of the Mediterranean region. During the dry season, this pulmonate gastropod enters estivation, a dormancy that is a response to water and/or food limitation and often occurs at high environmental temperatures. Although

* Corresponding author. Fax: +1 613 520 2569.
E-mail address: kenneth_storey@carleton.ca (K.B. Storey).

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alien to man, the ability to enter a hypometabolic or dormant state in response to environmental stress is quite common among invertebrate and lower vertebrate species. Several species of land snails, including *O. lactea*, have been studied as invertebrate models of estivation (for review, see [4,7,24,29]). Estivating animals show behavioral, physiological, and biochemical adaptations that support prolonged water and energy conservation. Two of the important features of estivation are: (a) strong metabolic rate depression (O_2 consumption is typically <20% of normal) that maximizes the time that animals can survive using endogenous fuel reserves, and (b) good antioxidant defenses to protect cells from oxidative injuries over long-term dormancy [15,30]. Indeed, although oxygen consumption is significantly reduced in estivation, and the generation of oxyradicals in tissues is generally proportional to oxygen consumption, the activities of a variety of antioxidant enzymes are elevated during estivation in *O. lactea* [14]. This seems to serve two purposes [15]: (a) to allow animals to cope with a large increase in oxyradical formation associated with arousal from dormancy, when oxygen is reintroduced to the system in large amounts, and (b) to deal with intermittent sharp increases in tissue oxygenation (and oxyradical formation) brought about by discontinuous breathing patterns during estivation. The latter results because, to minimize water loss during dormancy, the pneumostome opens only intermittently for a rapid exchange of CO_2 and O_2 in the lung [2,3]. For antioxidant defenses to be elevated during estivation, it follows that pools of reducing power must be available.

In recent years, reversible phosphorylation control over the activities of key metabolic enzymes and functional proteins has been shown to be a primary mechanism of metabolic control across the animal kingdom [9,10,29,30]. Coordinate suppression of key loci in carbohydrate catabolism (e.g., glycogen phosphorylase, phosphofructokinase [PFK], pyruvate kinase, and pyruvate dehydrogenase), ion pumping (NaK-ATPase, and CaATPase), and protein synthesis (selected initiation and elongation factors) is achieved by this mechanism not only during estivation but also in other natural states of hypometabolism such as hibernation, anaerobiosis, diapause, etc. Control of the pentose phosphate cycle has never before been evaluated in a species that undergoes profound metabolic rate depression. On the one hand, suppression of G6PDH activity could be predicted so that pentose phosphate cycle activity is reduced in concert with the general suppression of carbohydrate catabolism. On the other hand, maintenance or perhaps even enhancement of NADPH supply is key to sustaining antioxidant defenses during long-term dormancy. The present study examines G6PDH regulation in the estivating land snail, *O. lactea*. We show that the enzyme is regulated by reversible phosphorylation mediated by either PKA- or cGMP-dependent protein kinase (PKG), with the phosphorylated form dominating during estivation. The high phosphate (estivating) form displays kinetic properties (e.g., reduced K_m G6P) that could favor G6P use by the pentose phosphate cycle during dormancy to help sustain antioxidant defenses.

Materials and methods

Animals. Snails were imported from Morocco and purchased from a retail source. In the laboratory, snails were held in plastic containers lined with damp paper towels and fed shredded carrots and cabbage (sprinkled with crushed chalk) every 2–3 days. After one month, estivation was induced in one group of snails by placing them in a container with dry paper towels and no food, while active snails were maintained under the same conditions as previously. After 10 days, active and estivating snails were sacrificed, and foot muscle and hepatopancreas were rapidly dissected out, immediately frozen in liquid nitrogen, and stored at −70 °C until use.

Sample preparation. Frozen tissue samples were homogenized (1:5 w:v) in ice-cold buffer A containing 25 mM imidazole, pH 7.5, 10% v:v glycerol, 10 mM 2-mercaptoethanol, 25 mM NaF, 2 mM EDTA, and 2 mM EGTA; initial trials optimized NaF, EDTA, and EGTA concentrations with respect to amount of recoverable activity. A few crystals of the protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), were added at the time of homogenization. After centrifugation at 10,000*g* for 20 min, the supernatant was removed and held on ice.

Partial purification of G6PDH. A 500 μL aliquot of supernatant was added to a 2 cm × 1.8 cm (h × d) column of DEAE–G25 Sephadex (equilibrated in buffer A at pH 8.0) and then the column was washed with 10 mL of the same buffer to remove unbound proteins. G6PDH was eluted with a 0–2 M gradient of NaCl in buffer A and 200 μL fractions were collected.

G6PDH assay. Optimum assay conditions for G6PDH were 1 mM $NADP^+$, 5 mM G6P, and 25 mM imidazole, pH 7.8. Reactions were initiated by adding 10 μL of crude extract or 100 μL of column fractions to a 200 μL total reaction volume in the microplate well. Activity was monitored at 340 nm using a MR5000 microplate reader and Biolinx 2.0 software (kinetic mode, reading interval = 12 s) with Microplate Analysis (MPA) and Kinetics 3.51 computer programs used to analyze the data. Activities are reported in mU/mg protein. Soluble protein content was quantified using the Coomassie blue dye binding method and the Bio-Rad prepared reagent with a standard curve of bovine serum albumin.

In vitro incubations to stimulate endogenous kinases and phosphatases. To assess the effects of phosphorylation state on G6PDH activity, tissue extracts were prepared without NaF/EDTA/EGTA in the homogenization buffer and subsequently incubated under conditions that promoted either protein phosphorylation or protein dephosphorylation (protocol modified from [28]). Incubation times were 4 h at room temperature when assessing G6PDH in crude extracts or 12 h when assessing fractions from DEAE–Sephadex. Enzyme samples were mixed 1:2 v:v with buffer B (25 mM imidazole, pH 7.5, 10% v/v glycerol, and 10 mM 2-mercaptoethanol), containing one of the following additions.

(*A*) *Control incubations*: 25 mM NaF, 2 mM EDTA, and 2 mM EGTA to inhibit all phosphatase and kinase activities.

(*B*) *Stimulation of endogenous protein kinase activities*: 5 mM Mg·ATP, 25 mM NaF, and either (1) 1 mM cAMP to stimulate PKA; (2) 1 mM cGMP to stimulate PKG; (3) 1.3 mM $CaCl_2$ + 7 μg/mL phorbol myristate acetate to stimulate PKC; (4) 1 mM AMP to stimulate AMPK; or (5) 1 U of calmodulin activity/incubation tube + 1.3 mM $CaCl_2$ to stimulate CaMK.

(*C*) *Stimulation of endogenous protein phosphatases* (*PPase*): (1) for total PPase activity: 5 mM $CaCl_2$ + 5 mM $MgCl_2$; (2) for PP1: 2.5 nM okadaic acid (inhibits PP2A) + 2 mM EDTA + 2 mM EGTA; (3) for PP1 + PP2A: 2 mM EDTA and 2 mM EGTA; (4) for total PPase minus PP1/PP2A: 1 μM okadaic acid + 5 mM $CaCl_2$ + 5 mM $MgCl_2$; (5) for PP2B: 1 μM okadaic acid + 5 mM $CaCl_2$ + 2 mM EDTA; (6) for PP2C: 1 μM okadaic acid + 5 mM $MgCl_2$ + 2 mM EGTA; (7) for full dephosphorylation: incubation with 1 U calf intestinal alkaline phosphatase (AP) + 5 mM $MgCl_2$ + 5 mM $CaCl_2$.

After incubation, all samples were desalted by low-speed centrifugation through small columns of G25 Sephadex equilibrated in buffer B followed by assay under optimum conditions.

Pulse proteolysis of partially purified G6PDH. To assess changes in the structural stability of the two peaks of G6PDH activity eluted from the DEAE column, the enzyme was subjected to urea-dependent denaturation and subsequent thermolysin-mediated proteolysis, methods adapted from [23,31]. Hepatopancreas extracts were prepared as previously in buffer A but without the addition of PMSF, centrifuged, and the supernatant was fractionated by DEAE–G25 Sephadex chromatography, as described above. Peak fractions were pooled and 50 μL aliquots of each peak were incubated with 100 μL of a urea solution made up in buffer A. After 24 h incubation at room temperature, extracts were treated with 10 μL of 10 mg/mL thermolysin (Sigma; stock prepared with 2.5 M NaCl and 10 mM $CaCl_2$). Thermolysin activity was stopped after 10 min by the addition of 20 μL of 50 mM EDTA (pH 8.0). Enzyme protein content was measured by Western blotting. Parallel experiments were performed to determine enzyme activity as a function of urea concentration. Parameters of protein unfolding and activity inhibition due to urea were calculated using the Kinetics program.

Western blotting. Samples were mixed 1:1 v:v with freshly prepared 2× SDS–PAGE loading buffer (100 mM Tris–HCl, pH 6.8, 4% w:v SDS, 20% v:v glycerol, 0.2% w:v bromophenol blue, and 10% v:v 2-mercaptoethanol), boiled for 5 min, and then immediately cold-snapped and stored until use at −20 °C. Aliquots (20 μL) were loaded into wells of SDS–polyacrylamide gels (12% resolving gel, 5% stacking gel) using a Hamilton syringe. Samples were electrophoresed at 200 V for ~1 h in 1× running buffer (5× buffer contains 15.1 g Tris–base, 94 g glycine, and 5 g SDS per liter, pH 8.0) until the dye front reached the bottom of the gel.

Proteins were wet-transferred to PVDF membrane using a current of 300 mA for 1.5 h at 4 °C and the Bio-Rad Mini Trans-Blot Cell apparatus. Transfer buffer contained 25 mM Tris–base (pH 8.8), 192 mM glycine, and 20% v:v methanol, chilled to 4 °C. Membranes were blocked with 1% nonfat dried milk in Tris-buffered saline containing Tween 20 (TBST: 10 mM Tris–base, pH 7.0, 150 mM NaCl, and 0.1% Tween 20) for 0.5 h at 4 °C. The membranes were then incubated with G6PDH primary antibody purchased from Sigma (rabbit anti-G6PDH raised against antigen from *Saccharomyces cerevisiae*, stock prepared following manufacturer's directions, and diluted 1:1000 v:v in TBST) overnight at 4 °C. Unbound primary antibody was removed with 3 × 5 min washes with TBST and then the membrane was incubated with HRP-conjugated anti-rabbit secondary antibody (Santa Cruz Biotechnology, diluted 1:2000 v:v in TBST) for 2–3 h at room temperature, followed by 3 × 5 min washes in TBST. Proteins were visualized using Western Lightning Chemiluminescence Plus reagents (NEN, Perkin Elmer) following manufacturer's protocols. The luminol (substrate for HRP) and the oxidizing reagents were mixed 1:1 v:v on the membrane for 1 min and the ECL signal was detected using a Syngene (Bio-Rad) after an exposure of 15 min. Band densities were quantified using GeneTools software (v3.00.02). Bio-Rad Kaleidoscope pre-stained markers were run in selected lanes and used to assess the molecular mass of the test protein.

Arrhenius analysis. Enzyme assays were performed under V_{max} conditions over the temperature range 4–45 °C. Activation energy (E_a) was determined in kJ/mol for linear portions of the relationship. For temperature control, the MR5000 microplate reader was equilibrated to the appropriate temperature in a Precision 815 low temperature incubator. Assay plates were also equilibrated in the incubator for ~10 min before reactions were initiated by the addition of enzyme.

Results

Optimization of experimental conditions

Inclusion of EDTA and EGTA in homogenizing buffer did not affect the recoverable G6PDH activity in hepatopancreas extracts from either active or estivating snails whereas inclusion of NaF substantially improved recoverable activity. However, use of alternate phosphatase inhibitors (β-glycerophosphate, Na_3OV_4) reduced recoverable activity. Thus, NaF, EDTA, and EGTA were used in the standard homogenizing buffer. Fig. 1 shows the pH optima of G6PDH from active and estivated snails; the optimum for the enzyme from estivated snails was pH 7.8 compared with a slightly higher value of 8.0 for active snails (although activity for the enzyme from active snails at pH 7.8 was not significantly different from that at pH 8.0). Throughout the study, standard assays were performed at pH 7.8.

Kinetic parameters

The maximal activity of G6PDH in crude extracts from hepatopancreas of estivating snails was 1.5-fold higher than the value in active snails (Table 1). Velocity versus substrate concentration curves for hepatopancreas G6PDH are shown in Fig. 2. The enzyme displayed sigmoidal kinetics with respect to $NADP^+$ concentration, with Hill coefficients ranging between 1.8 and 2.4; the same relationship was seen with both crude extracts and partially purified preparations after DEAE–G25 Sephadex chromatography. However, the $S_{0.5}$ $NADP^+$ did not change between active and estivated states (Table 1). G6PDH displayed hyperbolic kinetics with respect to G6P concentration and significantly different K_m values between active and estivated states. K_m G6P values for the enzyme in crude extracts from estivated snails decreased to 54% of the value for G6PDH from active snails, indicating substantially higher affinity for this substrate in the estivating animal. Snail G6PDH was activated by Mg^{2+}-citrate and the K_a for citrate also decreased during estivation to a value that was 65% of the K_a for active animals.

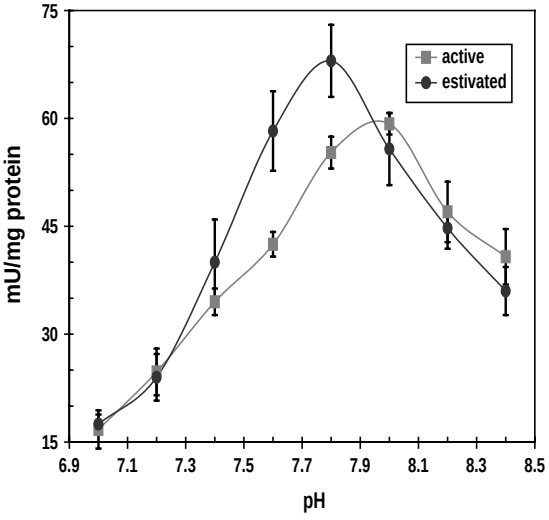

Fig. 1. Determination of optimal pH for G6PDH activity from hepatopancreas of active and estivated *O. lactea*. Optimal assay conditions were used at 22 °C with pH of the imidazole adjusted to the values shown. Data are means ± SEM, $n = 4$.

Table 1
Kinetic parameters for G6PDH from hepatopancreas of active and 10 d estivated *O. lactea* and for peaks I and II G6PDH separated by DEAE chromatography

	Active	Estivated	Peak I	Peak II
V_{max} (mU/mg)	54 ± 5	79 ± 8*	316 ± 42	552 ± 52**
K_m G6P (mM)	1.41 ± 0.30	0.76 ± 0.26*	1.68 ± 0.48	0.62 ± 0.33**
$S_{0.50}$ $NADP^+$ (mM)	0.21 ± 0.05	0.19 ± 0.04	0.25 ± 0.08	0.19 ± 0.08
K_a Mg^{2+}-citrate (mM)	2.6 ± 0.5	1.7 ± 0.4*	3.1 ± 0.8	1.4 ± 0.5**
E_a (kJ/mol)	31.4 ± 6.6	26.2 ± 5.1	37.0 ± 4.3	24.2 ± 4.9**
I_{50} urea (M)	ND	ND	4.4 ± 0.2	5.3 ± 0.3**
C_m urea (M)	ND	ND	4.3 ± 0.1	5.2 ± 0.3**

Assays were conducted at 22 °C and data are means ± SEM ($n = 3$ for C_m and I_{50} for urea, $n = 4$ for all other parameters). I_{50} is the concentration of urea that reduces enzyme activity by 50%. C_m is the concentration of urea required to unfold 50% of the test protein. Kinetic constants were determined at optimal concentrations of ions or cosubstrates. Peak I and peak II parameters were assessed for DEAE–G25 Sephadex elution profile peaks recovered from active snail extracts. Significance testing used Student's *t* test.

* Significantly different from the corresponding value for active snails, $P < 0.05$.

** Significantly different from the corresponding peak I value, $P < 0.05$. ND, not determined for these preparations.

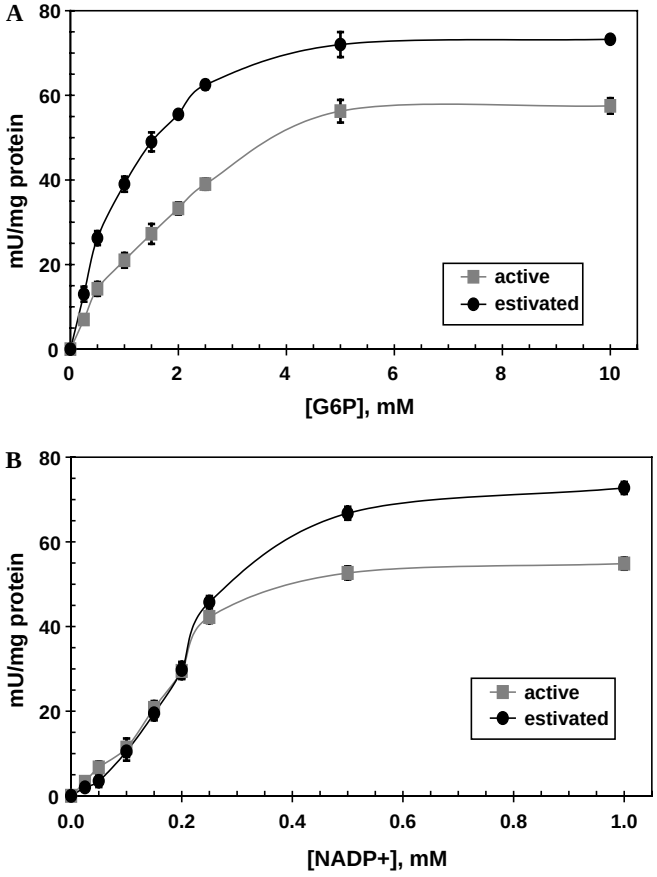

Fig. 2. Velocity versus substrate concentration for G6PDH activity from hepatopancreas of active and estivated *O. lactea*. (A) Activity as a function of G6P concentration at 1 mM NADP. (B) Activity as a function of NADP concentration at 5 mM G6P. Results are for assays on crude extracts; data are means ± SEM, $n = 4$.

In vitro incubations to assess changes in G6PDH activity as a result of phosphorylation state

Aliquots of crude extracts of hepatopancreas from active and 10 d estivated snails were incubated under conditions that stimulate the activities of endogenous protein kinases or protein phosphatases and the effects on G6PDH maximal activity were determined. Fig. 3A shows that stimulation of PKA or PKG activities in crude extracts from active snails produced 1.4-fold increases in G6PDH activity, raising V_{max} to the activity seen during estivation. Stimulation of other kinases failed to alter G6PDH activity in extracts from active snails, and none of the kinase treatments had any effect on G6PDH from estivated snails. Conversely, G6PDH V_{max} activity in preparations from active snails was not affected by any of the treatments that stimulated endogenous protein phosphatase activities, nor was activity affected by incubation with AP (Fig. 3B). However, stimulation of several phosphatase classes reduced G6PDH activity in extracts from estivated snails. This included stimulation of total serine/threonine phosphatase activity, PP1-type activity, and PP1/PP2A-type activity; G6PDH V_{max} fell to 68%, 72%, and 77%, respectively, of the V_{max} of G6PDH in control incubations. Incubation with AP also strongly reduced G6PDH activity to 46% of that in the control condition.

Ion exchange chromatography of high and low phosphate forms of G6PDH

DEAE–G25 Sephadex chromatography resolved two peaks of G6PDH activity from crude hepatopancreas extracts of both active and estivated snails. Peak I consistently eluted at 0.6–1.0 M NaCl and peak II eluted at 1.1–1.6 M NaCl (Fig. 4). A typical elution profile for G6PDH activity from active snails is seen in Fig. 4A; peak I dominated in active snails containing 57 ± 5% ($n = 3$) of the total activity compared with 43 ± 5% in peak II. The elution pattern changed markedly when extracts from estivated snails were resolved. The amount of activity in peak II increased greatly to 78 ± 7% ($n = 3$) of total activity recovered from the column (Fig. 4B).

In vitro treatments that stimulated selected kinases and phosphatases strongly altered the elution profile of G6PDH on the ion exchange column. When hepatopancreas extracts from active snails were incubated for 12 h under

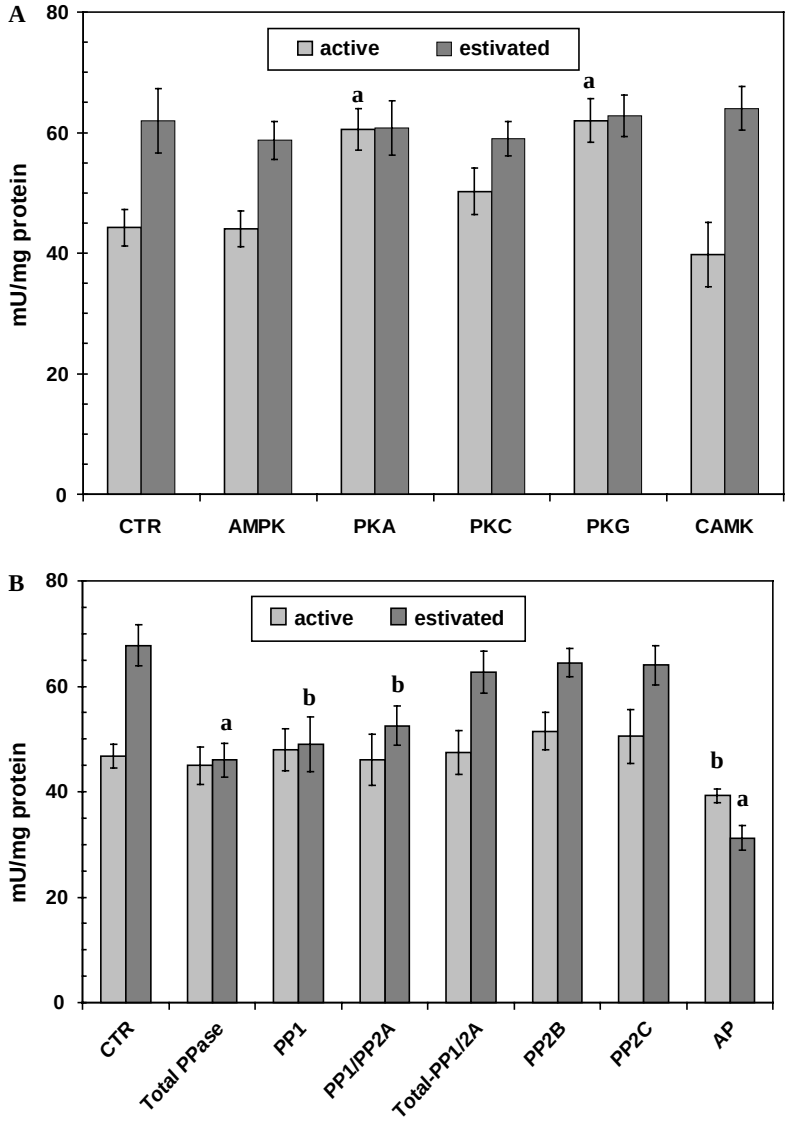

Fig. 3. Effects of in vitro incubations to stimulate the activities of (A) protein kinases or (B) protein phosphatases on the maximal activity of G6PDH from *O. lactea* hepatopancreas. Crude extracts were incubated for 4 h before assay at 22 °C. Data are means ± SEM, $n = 4$ independent determinations. (a) Significantly different from the corresponding control value (no additions) using Student's t test, $P < 0.01$; (b) $P < 0.05$.

conditions that stimulated endogenous protein kinase A, the G6PDH activity profile shifted to resemble the pattern seen during estivation with 71 ± 6% ($n = 3$) of total activity in peak II (Fig. 4C). Conversely, when extracts from estivated snails were incubated for 12 h under conditions that promoted endogenous PP1 activity, the peak profile shifted to resemble the elution profile of G6PDH in active snails with the greater proportion of activity in peak I (56 ± 7%, $n = 3$) (Fig. 4D). Incubation of extracts from active snails under conditions that stimulated PKG resulted in the shift of 100% of activity into a single peak, eluting at 1.2–1.6 M NaCl, consistent with peak II activity from the other conditions (Fig. 4E). Finally, when extracts from estivated snails were incubated with AP, only one peak of activity was found, eluting at 0.6–1.0 M NaCl and consistent with peak I G6PDH in the other profiles (Fig. 4F).

Kinetic properties were assessed for the two pooled peaks of G6PDH activity from active snail extracts (Table 1). As compared with the peak I enzyme, peak II G6PDH had a significantly lower K_m G6P (37% of the peak I value) and a significantly lower K_a for Mg·citrate (45% of the peak I value). The properties of the peak I enzyme mimicked those of the enzyme from active snails whereas peak II G6PDH showed properties very similar to those of the enzyme from estivated snails.

The response of G6PDH to temperature was also evaluated. Arrhenius plots were linear from 4 °C to ~30 °C (Fig. 5) and calculated activation energies are shown in Table 1. The E_a for peak II G6PDH was significantly lower than the value for the peak I enzyme and resembled the value for the enzyme from estivated snails whereas the peak I value was similar to the value for active snails.

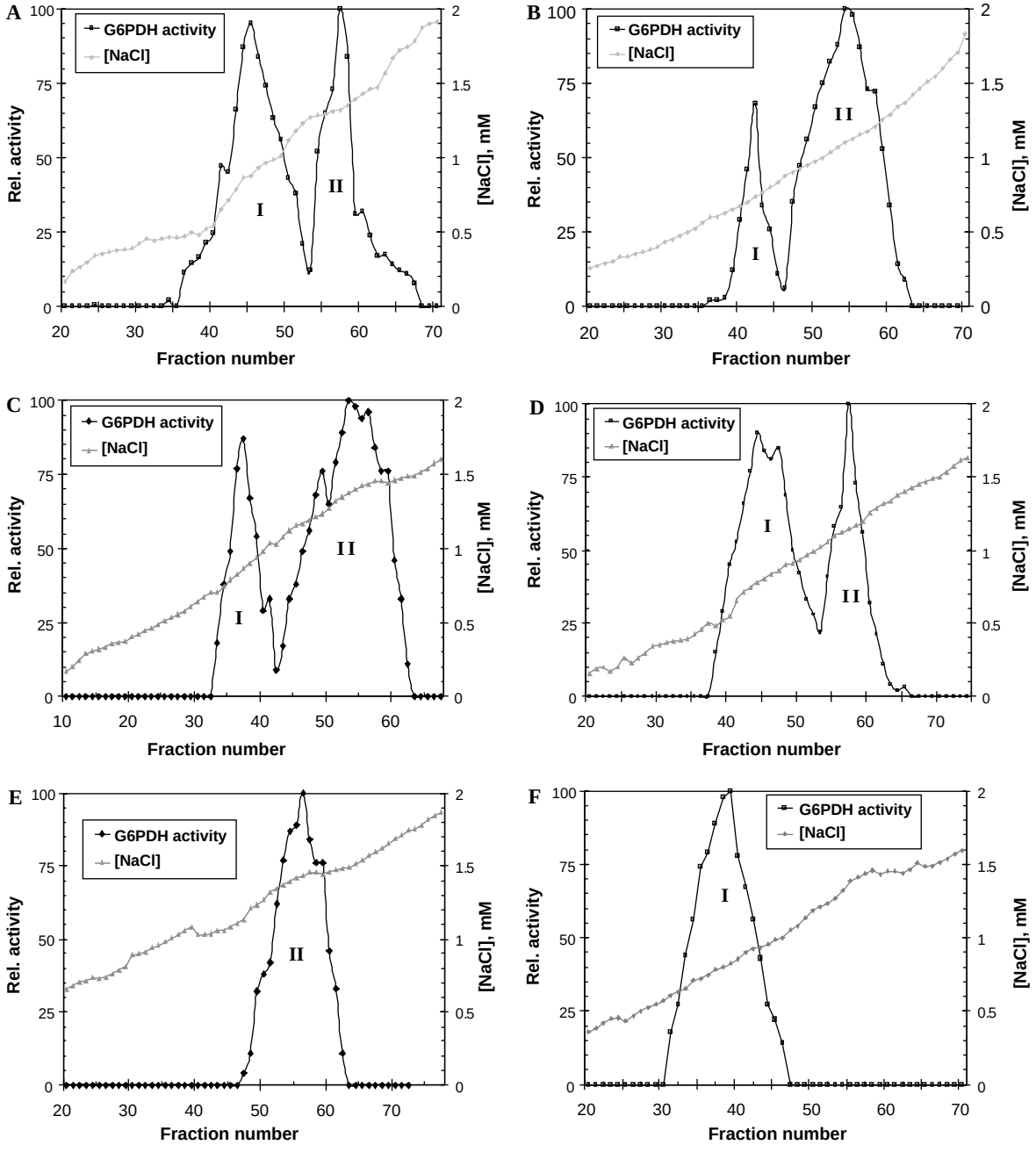

Fig. 4. DEAE–G25 Sephadex elution profiles for G6PDH activity from hepatopancreas of active and estivating *O. lactea*. Profiles are representative of three or more independent determinations for each condition. Activities are expressed relative to the highest activity fraction. Peaks I and II eluted at lower and higher NaCl concentrations, respectively. (A) G6PDH elution profile of untreated hepatopancreas extracts of active snails, (B) G6PDH from untreated hepatopancreas extracts of estivating snails, (C) G6PDH from active snails after 12 h incubation to stimulate endogenous PKA, (D) G6PDH from estivating snails after 12 h incubation to stimulate endogenous PP1, (E) G6PDH from active snails after 12 h incubation to stimulate endogenous PKG, and (F) G6PDH from estivating snails after 12 h incubation with alkaline phosphatase. Details of incubations are in the Materials and methods.

The structural integrity of enzymes can be changed by covalent modification (or by the presence of allosteric modifiers) making a protein more or less susceptible to denaturation by urea and subsequent proteolytic attack on the denatured (unfolded) enzyme by thermolysin. Hence, the pulse proteolysis technique is an effective way to determine whether there are structural or conformational differences in a protein between two different physiological states. Pulse proteolysis of resolved peak I and peak II G6PDH populations was conducted with thermolysin following incubation of the enzymes with different concentrations of urea to achieve different degrees of protein denaturation. The results revealed that peak II G6PDH had a significantly higher resistance to unfolding and denaturation by urea, relative to peak I enzyme (Fig. 6). This was evident both from the I_{50} value (the amount of urea required to inhibit 50% of activity) and from the C_m value (the amount of urea required to unfold 50% of the protein, rendering it

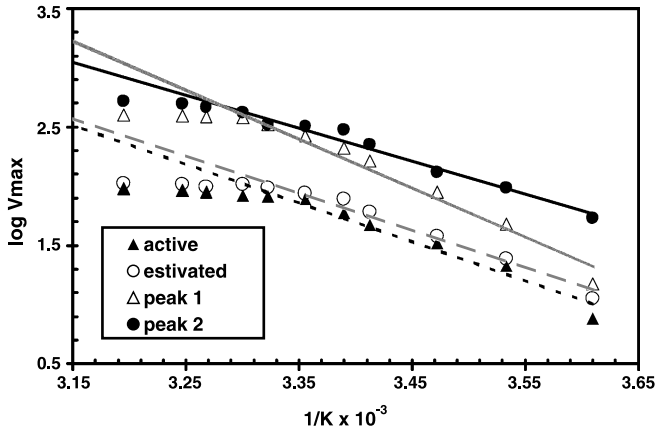

Fig. 5. Representative Arrhenius plots for *O. lactea* hepatopancreas G6PDH activity comparing the enzyme from crude extracts of active and estivated snails as well as the peaks I and II fractions from DEAE–G25 Sephadex separations. Activities were measured under optimal conditions between 4 and 45 °C.

susceptible to thermolysin proteoloysis) (Table 1). Both values were 4.3–4.4 M urea for the peak I enzyme and 20% higher at 5.2–5.3 M for peak II G6PDH.

Discussion

G6PDH from the hepatopancreas (liver-like organ) of the terrestrial gastropod *O. lactea* is subjected to reversible phosphorylation and the data indicate that this mechanism underlies the estivation-induced changes in activity and kinetic/thermal properties of the enzyme as well as its susceptibility to urea denaturation. This is the first time that phosphorylation control of a pentose phosphate cycle enzyme has been implicated as a component of metabolic rate depression in a system of natural hypometabolism and suggests that pentose phosphate cycle flux is another of the metabolic functions that are targeted for coordinated control in animals switching between active and torpid states. It will be interesting to determine whether G6PDH and the pentose phosphate pathway are also regulated in other tissues and other species during hypometabolism. Preliminary studies of foot muscle and mantle G6PDH in *O. lactea* showed no change in the V_{max} or substrate kinetics during estivation, and a study of another estivating snail, *Helix aspersa*, reported no change in G6PDH V_{max} but kinetic properties were not examined [24]. The central role of the liver or liver-like organs of animals in biosynthesis, antioxidant defense, and xenobiotic detoxification may mean that regulation of G6PDH in hepatopancreas is particularly important and specifically targeted for regulatory control during hypometabolism.

Several of the properties of hepatopancreas G6PDH were significantly different between active and estivated states. Notably, the K_m for G6P was significantly lower during estivation and mirrored the value for the peak II high phosphate form of the enzyme. Hence, it is apparent that estivation triggers the phosphorylation of hepatopancreas G6PDH and elevates its affinity for hexose phosphate

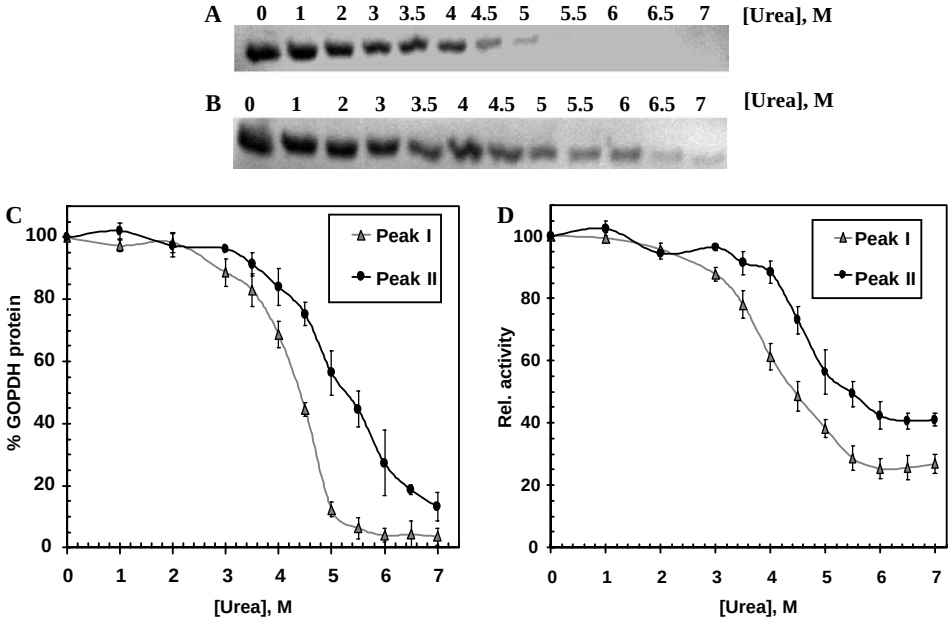

Fig. 6. Structural unfolding and enzymatic inhibition by urea of peak I and II G6PDH from active snails. Pooled enzyme from each peak was denatured overnight with different concentrations of urea and then either treated with thermolysin or assayed under optimal conditions. (A) Representative Western blot detecting the ~60 kDa band of G6PDH protein from pooled peak I samples and showing the amount of G6PDH protein remaining after proteolysis as a function of urea concentration (0–7 M). (B) Comparable Western blots for G6PDH protein from pooled peak II samples. (C) Mean relative G6PDH protein content remaining after overnight incubation with urea and subsequent proteolysis with thermolysin; data are means ± SEM, $n = 3$. (D) Mean relative G6PDH activity in pooled peak I and pooled peak II samples from active snails as a function of overnight incubation with different concentrations of urea; data are means ± SEM, $n = 3$.

substrate. A previous study found that G6P substrate levels were reduced by >67% in hepatopancreas of estivating snails, compared with active animals [8], so the increase in substrate affinity of G6PDH during estivation could help to realign enzyme activity with substrate availability. Interestingly, *O. lactea* hepatopancreas PFK, the key regulatory enzyme of glycolysis, showed the opposite response during estivation. PFK affinity for its substrate, fructose-6-phosphate, was significantly reduced during estivation (the enzyme also showed reduced sensitivity to its activator, fructose-2,6-bisphosphate) and these changes in enzyme properties were again traced to phosphorylation of the enzyme [36]. Glycolysis and the pentose phosphate cycle are two of the main pathways competing for G6P in cells and estivation-responsive changes in the properties of their primary regulatory enzymes (PFK and G6PDH) suggest that carbohydrate flux through the pentose phosphate cycle may be relatively enhanced during estivation compared with carbohydrate flow through glycolysis. Citrate inhibition of both enzymes was also interesting; the high phosphate, estivating form of both G6PDH and PFK showed stronger inhibition by Mg·citrate [36]. Citrate accumulation typically signals high rates of lipid oxidation and provides feedback inhibition of PFK to spare carbohydrate use (for energy production or for lipid synthesis) under these circumstances. Citrate inhibition of G6PDH could similarly signal that NADPH supply for lipid synthesis is not needed and stronger citrate inhibition of the estivating form of the enzyme is consistent with the net catabolic state of the animals; biosynthetic processes are very strongly suppressed during hypometabolism [30].

G6PDH from active and estivating snails also showed significantly different activation energies and differed in the extent of urea-dependent protein unfolding as evidenced both by urea inhibition of activity and susceptibility to thermolysin treatment. These differences all argue for a difference in protein conformation between active and estivating states which undoubtedly derives from differences in the amount of covalently bound phosphate on the enzyme in the two states. Both the C_m and the I_{50} value for urea were significantly higher for the peak II (phosphorylated) enzyme, indicating a more stable conformation compared with the peak I form. Although this result derives from physical differences in the phosphorylation state of the protein, it may also have some physiological relevance. Urea is one of the nitrogenous end products that accumulates in snail tissues during estivation [1,25] and estivation is also associated with a progressive dehydration of tissues which results in elevated ionic strength and osmolality of body fluids. Thus, an enzyme form that is less sensitive to the denaturating effects of urea would be better suited for function under the cytoplasmic conditions that prevail during long term estivation.

Analysis of the effects of protein kinases and protein phosphatases on snail G6PDH was consistent with the identification of the peak I enzyme that dominates in active snails as the dephosphorylated (or low phosphate) form and the peak II enzyme as the phosphorylated (or high phosphate) form. Interestingly, the phosphorylated (estivating) form of hepatopancreas G6PDH seems to be the more active form (V_{max} is higher and K_m G6P lower than the dephosphorylated form) whereas studies of mammalian G6PDH have shown that PKA-mediated phosphorylation inhibits G6PDH activity [11,37,38]. The incubation studies indicated that both PKA and PKG could phosphorylate hepatopancreas G6PDH and mimic the effects of estivation on G6PDH maximal activity and its elution profile off DEAE–Sephadex (Figs. 3 and 4). Stimulation of other protein kinases (PKC, CaMK, and AMPK) was ineffective. One or both of these kinases could mediate the estivation-induced response in vivo. Phosphorylation of PFK and pyruvate kinase in hepatopancreas extracts was also stimulated by incubation with Mg·ATP plus the second messengers of PKA, PKG, and PKC [35,36]. However, another study showed that changes in PKA activity and levels of cAMP and inositol 1,4,5-trisphosphate in *O. lactea* hepatopancreas were not consistent with either PKA or PKC being the kinases involved in mediating estivation-induced phosphorylation of pyruvate kinase [6]. Indeed, PKG has long been known to have an central role in molluscan signal transduction [17] and has been shown to catalyze the phosphorylation of PFK and pyruvate kinase from marine mollusks during anoxia-induced metabolic rate depression [5,7,18]. The strong response by *O. lactea* G6PDH to incubation with cGMP, a conversion of 100% of activity to the phosphorylated peak II form (Fig. 4), implies that PKG may similarly be the protein kinase responsible for estivation-induced enzyme phosphorylation in land snails.

Incubation with AP fully converted hepatopancreas G6PDH to the peak I form, confirming that this peak contains the dephosphorylated enzyme. Dephosphorylation of G6PDH was also evident in incubations that stimulated endogenous (i) total serine/threonine phosphatase activity; (ii) PP1 activity; or (iii) PP1 and PP2A activity. These three conditions all have PP1 in common and this suggests that PP1 may be the endogenous phosphatase responsible for G6PDH dephosphorylation during the arousal of snails out of estivation.

Estivation in *O. lactea* includes oxidative stress. Although net oxygen consumption is reduced in estivating *O. lactea* to 9–28% of that in active snails [16,2], the animals must cope with irregular rapid increases in oxygen concentration at intervals during dormancy. The pneumostome, the specialized oriface leading to the lung, only opens intermittently during estivation for 2–3 short breathing periods per hour [3]. Oxygen uptake can increase 5-fold during these brief periods where the snail hyperventilates and releases CO_2 [2], and brief bursts of oxyradical generation can be associated with this pattern of discontinuous oxygen consumption. Furthermore, a burst of ROS generation accompanies arousal from estivation; this has been confirmed from measurements of the levels of lipid

peroxidation products which rise rapidly to peak within the first few minutes of arousal [14]. To deal with oxidative stress during estivation, a variety of antioxidant mechanisms are enhanced during estivation in *O. lactea* [14,15]. Activities of catalase, superoxide dismutase, glutathione peroxidase, and glutathione-*S*-transferase were generally elevated in hepatopancreas and other tissues during estivation [29]. Similarly, glutathione peroxidase activity increased by 4- to 5-fold in tissues of estivating *H. aspersa* [24]. Glutathione peroxidase and glutathione-*S*-transferase use reduced glutathione as their substrate and reduced glutathione is supplied by the NADPH-dependent glutathione reductase reaction. NADPH is also used to regenerate reduced thioredoxin for peroxidiredoxin-mediated antioxidant activity [12] and provides the reducing power for the detoxification and xenobiotic transformation reactions catalyzed by the cytochrome P450 family of enzymes [13]. Hence, NADPH supply is essential for antioxidant defense and the estivation-responsive changes to G6PDH, the rate-limiting reaction in the pentose phosphate pathway that supplies the bulk of cellular NADPH, would enhance G6PDH function in this critical supporting role in antioxidant defense. Notably, G6PDH activity is elevated in a variety of systems, ranging from yeast to humans, in response to oxidative stress [20,27,34] whereas inhibited and/or reduced G6PDH activity has been correlated with reduced antioxidant defense capacities, ROS-related cellular damage, and ROS-induced cell death [19,26,33].

In summary, the present study shows that G6PDH from *O. lactea* hepatopancreas is a phosphoprotein and that entry into the hypometabolic state of estivation results in a strong increase in the fraction of phosphorylated G6PDH. The properties of phosphorylated G6PDH are consistent with a more active enzyme which may favor enhanced carbon flow through the pentose phosphate cycle during estivation to sustain NADPH production for antioxidant defense. The data provide the first demonstration of coordinated regulation of the pentose phosphate cycle between active and hypometabolic states, and implicate PKG and PP1 as the protein kinase and phosphatase activities responsible for G6PDH regulation.

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