Comparative Biochemistry and Physiology, Part B 157 (2010) 310–316

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

Regulation of liver glutamate dehydrogenase by reversible phosphorylation in a hibernating mammal

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ARTICLE INFO

Article history:
Received 1 June 2010
Received in revised form 23 July 2010
Accepted 25 July 2010
Available online 30 July 2010

Keywords:
Amino acid metabolism
Ground squirrel hibernation
Metabolic rate depression
Phosphoprotein staining
Reversible protein phosphorylation
Spermophilus richardsonii

ABSTRACT

Glutamate dehydrogenase (GDH) is a key enzyme that links amino acid and carbohydrate metabolism in cells. Regulation is likely most important when organisms are confronted with extreme stresses such as the low environmental temperatures and lack of food associated with winter. Many small mammals, such as Richardson's ground squirrels, *Spermophilus richardsonii*, cope with these conditions by hibernating. Animals enter long periods of profound torpor where metabolic rate is greatly suppressed, body temperature drops to near-ambient and all metabolic needs must be met from fixed internal body stores of fuels. To investigate how GDH is regulated under these conditions, kinetic properties of GDH were analyzed in liver from euthermic and torpid squirrels, revealing significant differences in V_{max}, K_m glutamate, K_a ADP and inhibition by urea between the two forms of GDH. These data suggested an activation of the glutamate-oxidizing activity of GDH in the hypometabolic state. Subsequent experiments suggested that the molecular basis of the kinetic differences was a change in the protein phosphorylation state of GDH between euthermia and torpor. Specifically, liver GDH appears to be dephosphorylated and activated when animals transition into torpor and this may serve to promote amino acid oxidation to contribute to energy production and gluconeogenesis. This is the first study to show that mammalian liver GDH can be regulated by reversible phosphorylation, providing an important new regulatory mechanism for GDH control.

1. Introduction

Glutamate dehydrogenase (GDH; E.C. 1.4.1.3) plays a key role in metabolism, acting as the primary link between carbohydrate and amino acid metabolism. This resident of the mitochondrial matrix catalyzes the NAD(P)$(H)^+$-linked interconversion of L-glutamate to α-ketoglutarate and ammonium ion. In the oxidative deamination direction, GDH gates entry into the Krebs cycle by carbon skeletons derived from several amino acids (glutamate, glutamine, arginine, proline, histidine) and in the opposite direction is the first step in the synthesis of several amino acids. Furthermore, reversible transamination reactions involving glutamate are the primary way that amino groups are introduced onto carbon skeletons to synthesize amino acids and, oppositely, the primary way of deaminating most amino acids. This makes glutamate and GDH that synthesizes it extremely important. Hence, regulatory control of GDH is critical and, GDH is well-known to be regulated by multiple allosteric modifiers (reviewed in Smith and Stanley, 2008). The enzyme from bacteria and yeast is also subject to reversible phosphorylation control (Hemmings, 1978, 1981; Uno et al., 1984; Lin and Reeves, 1994) but this form of posttranslational regulation of GDH has not previously been demonstrated in mammalian organs.

Reversible phosphorylation often mediates major changes in the activity states of enzymes, frequently providing virtual on–off control, and triggering major changes in metabolic flux through many pathways. Perhaps the most extreme example of a need for strong, global controls on intermediary metabolism in mammalian systems can be found in the phenomenon of hibernation. During the hibernating season, animals enter long periods of deep cold torpor with features including a strong suppression of all physiological processes (e.g. rates of breathing, heartbeat, and kidney filtration), a suppression of mitochondrial respiration in some organs (notably liver), a reprioritization of ATP-expensive metabolic functions in cells (e.g. ion pumping, transcription, translation, among others), a reorganization of fuel metabolism, and a reduction in core body temperature (Tb) to as low as 0–5 °C (Zatman, 1984; McArthur and Milsom, 1991; and reviewed in Carey et al., 2003; Geiser, 2004; Storey and Storey, 2004; Staples and Brown, 2008). Metabolic rate in deep torpor is often only 1–5% of the resting rate at euthermic Tb (36–38 °C), and is strictly controlled and coordinated via the regulation of gene expression and enzyme function (reviewed in Storey, 1997; Carey et al., 2003; Storey and Storey, 2004;). Reversible phosphorylation of enzymes and functional proteins is a major regulatory mechanism that mediates the suppression and reprioritization of metabolic reactions when animals enter hypometabolic states and

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doi:10.1016/j.cbpb.2010.07.005

widespread control by this mechanism has been demonstrated in hibernating species for glycolytic enzymes (Brooks and Storey, 1992), ion motive ATPases (MacDonald and Storey, 1999), ribosomal proteins (van Breukelen et al., 2004), and transcription factors (reviewed in Storey, 1997; Carey et al., 2003), among others.

We reasoned that the transition from active euthermia to cold torpor would be a time where strong stable control of GDH should be expressed. Such control could contribute both to the overall suppression of metabolic rate and also to a reorganization of fuel metabolism to favor glutamate oxidation, thereby allowing amino acid catabolism to contribute to energy needs during torpor. Indeed, in a previous study, Thatcher and Storey (2001) documented significant stable differences in the kinetic and physical properties of purified liver GDH from of euthermic versus hibernating Richardson's ground squirrels (*Spermophilus richardsonii*), but without identifying the mechanism responsible. The present study demonstrates that ground squirrel GDH is regulated by reversible protein phosphorylation and that changes in phosphorylation state occur in deep torpor that make significant changes to enzyme properties. This study provides the first example of reversible phosphorylation control of mammalian GDH.

2. Materials and methods

2.1. Animals

Protocols for holding and experimentation with Richardson's ground squirrels (*S. richardsonii*) were as reported previously (Thatcher and Storey, 2001). Briefly, ground squirrels were captured in the summer near Calgary, Alberta and housed in the animal care facility at the University of Calgary. The animals were kept in separate cages at a room temperature of 22 °C with a fall photoperiod (10 h light:14 h dark). Following an 8-week feeding period to optimize their body lipid reserves, some squirrels were transferred into a 4 °C cold room in constant darkness. Food was withheld in the cold room but water was available. Squirrels were allowed to enter hibernation and after one month of torpor cycles, animals were sampled in their last cycle after a minimum of 2 days of constant torpor (rectal temperature 6.4–7.4 °C); the minimum length of continuous torpor was confirmed visually by monitoring the disturbance of sawdust bedding. Euthermic squirrels, maintained and fed at 22 °C throughout, were also sampled at this time; all euthermic animals were alert and active and had rectal temperature between 36–37 °C. All squirrels were euthanized by decapitation. Tissues were immediately excised, frozen in liquid nitrogen, and then shipped to Carleton University on dry ice where they were stored at −80 °C.

2.2. Preparation of liver extracts

Samples of frozen liver were homogenized 1:5 w:v using a Polytron homogenizer in buffer A that contained 50 mM Tris–HCl (pH 8.0), 10 mM 2-mercaptoethanol, 10% v:v glycerol and inhibitors of protein kinases (2.5 mM EDTA, 2.5 mM EGTA) and protein phosphatases (25 mM β-glycerophosphate [β-GP]) with a few crystals of phenylmethylsulfonyl fluoride (PMSF) added just prior to homogenization. Initial tests showed that 25 mM sodium fluoride (NaF), another protein phosphatase inhibitor, decreased recoverable GDH activity and therefore was not used. Homogenates were centrifuged for 30 min at 13,500 *g* at 5 °C and then the supernatant was decanted and held on ice until use. GDH activity in these extracts was fully stable over 24 h at 4 °C, but after 48 h, ~50% of activity was lost.

2.3. Enzyme assay

GDH was assayed spectrophotometrically at 340 nm using an MR5000 microplate reader and Biolinx 2.0 software. Optimum assay conditions were 1.5 mM $NAD(P)^+$, 50 mM L-glutamate and 50 mM Tris–HCl buffer, pH 8.0 in a total volume of 200 μL with 5 μL of liver extract used per assay. Activity was measured as mU/mg soluble protein. K_m values for substrates were determined under optimal cosubstrate concentrations. Analysis of the effects of nucleotide activators and inhibitors (GTP, ATP, ADP) was carried out at sub-optimal substrate concentrations of 12.5 mM L-glutamate and 0.5 mM $NAD(P)^+$.

2.4. Incubations to stimulate endogenous protein kinase and phosphatase activities

Incubations that stimulated the activities of endogenous protein kinases or protein phosphatases were used to determine whether torpor-responsive changes in GDH properties could be due to phosphorylation or dephosphorylation of the enzyme. Aliquots of liver extracts were mixed 1:2 v:v with the appropriate incubation stock solution, designed so that after dilution, the final concentrations were as indicated below. All incubations included a basic incubation buffer B (50 mM Tris–HCl, 10% v:v glycerol, 10 mM β-mercaptoethanol, pH 8.0), with additions as follows.

(A) Control Incubations (denoted as STOP): buffer B plus 2.5 mM EDTA, 2.5 mM EGTA and 25 mM β-GP.
(B) Stimulation of endogenous protein kinase activities: buffer B plus 5 mM Mg ATP, 30 mM β-GP and either (1) 1 mM cAMP to stimulate protein kinase A (PKA); (2) 1 mM cGMP to stimulate protein kinase G (PKG); (3) 1.3 mM $CaCl_2$ + 7 μg/mL phorbol myristate acetate (PMA) to stimulate protein kinase C (PKC); (4) 1 mM AMP to stimulate AMP-dependent protein kinase (AMPK); or (5) 1 U of calmodulin + 1.3 mM $CaCl_2$ to stimulate calcium–calmodulin kinase activity (CaMK).
(C) Stimulation of endogenous protein phosphatase (PPase) activities: buffer B plus (1) 2.5 nM okadaic acid + 2 mM EDTA + 2 mM EGTA + 5 mM Na_3VO_4 to optimize protein phosphatase type 1 (PP1) action; (2) 5 mM $MgCl_2$ + 5 mM $CaCl_2$ + 5 mM Na_3VO_4 + 1 μM okadaic acid for stimulation of protein phosphatase 2B and 2C (PP2B + PP2C); (3) 1 μM okadaic acid + 5 mM $CaCl_2$ + 2 mM EDTA + 5 mM Na_3VO_4 for protein phosphatase type 2B (PP2B); and (4) 1 μM okadaic acid + 5 mM $MgCl_2$ + 2 mM EGTA + 5 mM Na_3VO_4 for protein phosphatase type 2C (PP2C). Note that okadaic acid at the low concentration (2.5 nM) effectively inhibits PP2A in the PP1 assay but that the higher (1 μM) okadaic acid concentration is used in the PP2B and PP2C assays to inhibit both PP1 and PP2A; sodium orthovanadate is present in all assays as a tyrosine phosphatase inhibitor.

Ground squirrel liver extracts were incubated for 4 h at ~4 °C. Following incubation, K_m glutamate was determined under optimum assay conditions with NAD^+ as the cofactor.

2.5. ProQ Diamond phosphoprotein staining

GDH from liver of euthermic and torpid ground squirrels was partially purified by affinity chromatography using a method (GTP-agarose chromatography) that worked well for ground squirrel GDH previously (Thatcher and Storey, 2001). Liver samples were homogenized in buffer A and the extracts (~2 mL) obtained after centrifugation were applied to a GTP-agarose column (2.5 × 1 cm) equilibrated in homogenization buffer A. Once the extract entered the column, the column was washed with ~10 mL of buffer A to remove any unbound material prior to elution of GDH with a gradient of 0–1 M KCl in buffer A; fractions of 0.75 mL were collected and assayed for maximal GDH activity with NAD^+ as the coenzyme (as described earlier). The five fractions with the most activity were pooled and soluble protein content was quantified using the Coomassie blue dye-binding method.

Aliquots of the pooled fractions were then mixed 1:1 v:v with SDS loading buffer (100 mM Tris buffer, pH 6.8, 4% w:v SDS, 20% v:v glycerol, 0.2% w:v bromophenol blue, 10% v:v 2-mercaptoethanol) and then boiled for 5 min, cooled on ice and frozen at −20 °C.

Aliquots containing 0.5 μg of protein were added to the wells of a 10% SDS-PAGE gel. The gel was run at 180 V for 45 min in running buffer containing 25 mM Tris-base, 250 mM glycine, and 0.1% w:v SDS. The gel was then washed twice in fixing solution (50% v:v methanol, 10% v:v acetic acid) and left overnight in this solution at ~4 °C. The following day, the gel was washed three times with ddH_2O for 10 min each and then stained with ProQ Diamond phosphoprotein stain (Invitrogen, Eugene, OR, USA) for 90 min. During staining the gel container was covered with tin foil to prevent light from reaching the light-sensitive stain; the gel also remained covered for the next steps. Following staining, the gel was destained by washing twice with ProQ Diamond destaining solution (20% v:v acetonitrile, 50 mM sodium acetate, pH 4) for 30 min each time. The gel was then washed three times with ddH_2O for 5 min each. Fluorescent bands on the gel were visualized using the ChemiGenius Bioimaging System (Syngene, Frederick, MD, USA) and intensities were quantified using the associated GeneTools software.

An identical gel was run in parallel with the aforementioned gel, and after electrophoresis proteins were stained for 20 min with Coomassie blue (25% w/v Coomassie Brilliant Blue R in 50% v/v methanol, 7.5% v/v acetic acid) and destained for 10 min with destaining mix (60% v/v methanol, 20% v/v acetic acid in ddH_2O). GDH band intensities from ProQ Diamond chemiluminescence were normalized against the corresponding Coomassie brilliant blue stained band to normalize for any variations in sample loading.

2.6. Ion exchange chromatography of GDH

Liver extracts were prepared from euthermic and torpid squirrels essentially as above but with a modified homogenization buffer (buffer C): 25 mM MES [2-(N-morpholino) ethanesulfonic acid], 5 mM 2-mercaptoethanol, 1.25 mM EDTA, 1.25 mM EGTA, 5% v:v glycerol, 12.5 mM β-GP, pH 6. Aliquots of 200 μL supernatant were applied to 3 × 1.8 cm (h × d) columns of CM^- cellulose equilibrated in buffer C. Columns were washed with ~10 mL of buffer C to remove any unbound material and then GDH was eluted with a 0–2 M gradient of KCl in buffer C. Fractions of 350 μL were collected and assayed for maximal GDH activity. A euthermic liver extract was incubated with commercial acid phosphatase (Sigma Chemical Company; Catalog number P-0157) in an attempt to stimulate dephosphorylation of euthermic GDH. The extract was incubated overnight at 4 °C in a 1:2 v:v ratio with the incubation solution (25 mM MES pH 6.0, 5 mM 2-mercaptoethanol, 5% v:v glycerol, 10 mM $MgCl_2$, 5 mM EDTA, and 1 U of acid phosphatase). After incubation the solution was applied to a CM^- cellulose column and the procedure that followed was identical to that used in obtaining the original GDH elution profiles.

2.7. Physical stability of GDH

GDH structural stability was assessed by examining its susceptibility to urea denaturation. Enzyme extracts were incubated with varying concentrations of urea (0.5–6.6 M) for 24 h prior to assaying under standard conditions for maximal GDH activity. C_{50} values (the concentration of urea that reduced activity by 50%) were determined using the Kinetics program.

2.8. Data, statistics and protein determination

Enzyme activity was analyzed with a Microplate Analysis Program (Brooks, 1994) and kinetic parameters were determined using the Kinetics v.3.5.1 program (Brooks, 1992). Data are expressed as mean ± SEM from independent determinations on separate preparations of enzyme. Statistical testing used the Student's t-test. Soluble protein concentration was determined by the Coomassie blue dye-binding method using the BioRad prepared reagent with bovine serum albumin as the standard.

3. Results

Ground squirrel liver GDH could use either NAD^+ or $NADP^+$ as its cofactor with near equal facility. Indeed, the $NADP^+$-linked activity was 82% of the NAD^+-linked activity for the glutamate-utilizing direction when GDH from liver of euthermic animals was analyzed at 23 °C. The pH optimum of the glutamate-oxidizing reaction using either NAD^+ or $NADP^+$ was determined to be 8.0, but ~95% of activity remained over the range from pH 7.5–9.0.

3.1. Kinetic analysis of GDH using NAD^+ as the cofactor

The properties of liver GDH were analyzed from ground squirrels in two states: euthermic animals with rectal Tb of 36–37 °C (E-GDH) and animals in hibernation sampled after a minimum 2 days of cold torpor with a rectal Tb of 6–7 °C (H-GDH). Kinetic properties of E-GDH and H-GDH were significantly different in a manner that also depended on the nicotinamide cofactor that was used. Table 1 shows the properties of E-GDH and H-GDH assayed at room temperature with NAD^+ as the cofactor. The K_m glutamate of E-GDH was 4.2 ± 0.4 mM, significantly higher ($P<0.05$) by 24% than the value for H-GDH. GDH K_m values for NAD^+ did not change between the two physiological states but the maximum activity of H-GDH was 44% higher than E-GDH ($P<0.05$).

3.2. Kinetic analysis with $NADP^+$ as the cofactor

Assays with $NADP^+$ as the cofactor also showed significant differences in kinetic parameters between E-GDH and H-GDH. Table 2 shows that the K_m glutamate was nearly 80% lower for H-GDH, as compared with E-GDH. However, contrary to the kinetics with NAD^+ as the cofactor, K_m $NADP^+$ was 78% higher and V_{max} was 37% lower ($P<0.05$) for H-GDH when compared to E-GDH.

3.3. Effectors of GDH

GDH is affected by a number of metabolites that act as allosteric modifiers and analysis of some of these showed significant differences between E-GDH and H-GDH that depended on the cofactor being used. With NAD^+ as the cofactor, K_a ADP of H-GDH was 54% lower than E-GDH (Table 1). Furthermore, under these conditions, ADP activation of H-GDH was 3.5 fold but just 1.6 fold for E-GDH.

Table 1
Comparison of liver GDH kinetic parameters from euthermic and hibernating ground squirrels, *S. richardsonii*, using NAD^+ as the cofactor.

	E-GDH	H-GDH
K_m glutamate (mM)	4.2 ± 0.4	3.2 ± 0.2[a]
K_m NAD^+ (mM)	1.17 ± 0.08	1.21 ± 0.08
V_{max} (mU/mg)	16 ± 2	23 ± 2[a]
K_a ADP (μM)	50 ± 10	23 ± 7[a]
ADP fold activation	1.61 ± 0.03	3.5 ± 0.4[a]
C_{50} Urea (M)	3.8 ± 0.1	3.14 ± 0.05[a]
I_{50} GTP (μM)	1.8 ± 0.2	1.8 ± 0.2

Data are means ± SEM, n = 3–4 determinations on liver extracts from different individual animals. K_m values were determined at optimal cosubstrate concentrations. K_a and I_{50} values were determined at suboptimal substrate concentrations (defined in Materials and methods).

[a] Significantly different from the corresponding euthermic value using a two-tailed Student's t-test, $P<0.05$.

Table 2
Comparison of liver GDH kinetic parameters from euthermic and hibernating ground squirrels using $NADP^+$ as the cofactor.

	E-GDH	H-GDH
K_m glutamate (mM)	2.8 ± 0.2	0.6 ± 0.1[a]
K_m $NADP^+$ (mM)	0.41 ± 0.03	0.73 ± 0.06[a]
V_{max} (mU/mg)	13.2 ± 0.8	8.3 ± 0.5[a]
K_a ADP (μM)	21 ± 1	14 ± 3[a]
ADP fold activation	1.9 ± 0.1	4.4 ± 0.5[a]
C_{50} urea (M)	3.4 ± 0.2	2.76 ± 0.02[a]

Data are means ± SEM, n = 3–4 determinations on liver extracts from different animals. Other information as in Table 1.

Similar to the situation with the NAD^+-linked enzyme, H-GDH assayed with $NADP^+$ showed a K_a ADP that was 33% lower than that of E-GDH (Table 2). Moreover, H-GDH showed a significantly greater fold activation (4.4 fold) by ADP than did E-GDH (1.9 fold).

The effects of two high-energy molecules (GTP and ATP) on GDH activity were assessed with NAD^+ as the cofactor. ATP, tested at concentrations up to 10 mM, had no significant effect on liver GDH from either state. However, GTP was a strong inhibitor with an I_{50} value of just 1.8 μM for both E-GDH and H-GDH (Table 1).

3.4. Reversible phosphorylation of GDH

To determine if ground squirrel liver GDH was subject to reversible phosphorylation and whether this could account for the stable kinetic differences between euthermic and torpid states, *in vitro* incubation studies were conducted in which crude liver extracts were incubated with activators/stimulators of specific endogenous protein phosphatases or protein kinases. The effects of these treatments on K_m glutamate was then analyzed (Figs. 1 and 2). Incubation conditions that inhibited both kinases and phosphatases (STOP) resulted in a high K_m for E-GDH and a significantly lower one for H-GDH, similar to the data in Table 1 but with somewhat lower absolute values. The incubations that facilitated protein phosphatase action lead to 28–50% decreases ($P<0.05$) in the K_m glutamate for E-GDH (Fig. 1). Stimulation of PP1 activity had no effect but incubation under conditions that stimulated either PP2B or PP2C significantly reduced the K_m glutamate of E-GDH but without affecting H-GDH. However, when both PP2B + PP2C were stimulated an additive effect was seen on the K_m glutamate of E-GDH, reducing it to about one-half of the value under STOP conditions. Furthermore, stimulation of both phosphatases suppressed the K_m glutamate of H-GDH by 24% as compared to its corresponding STOP condition (Fig. 1).

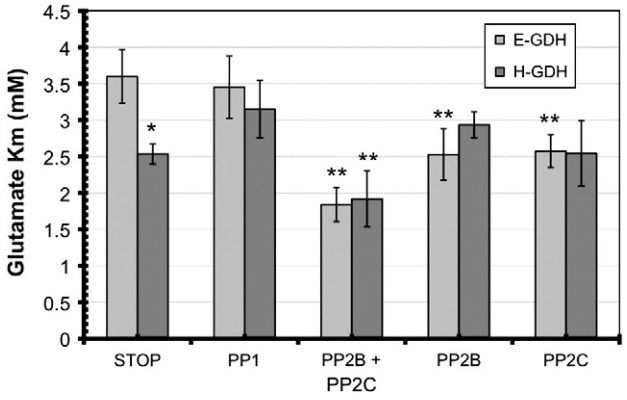

Fig. 1. Effects of *in vitro* incubations to stimulate the activities of endogenous protein phosphatases on K_m glutamate of *S. richardsonii* liver GDH. Crude extracts were incubated for 4 h before assay at 23 °C. Data are means ± SEM, n = 3 determinations on liver extracts from different animals. * - Significantly different from E-GDH as determined by the Student's t-test, $P<0.05$; ** - significantly different from the corresponding 'STOP' condition, $P<0.05$.

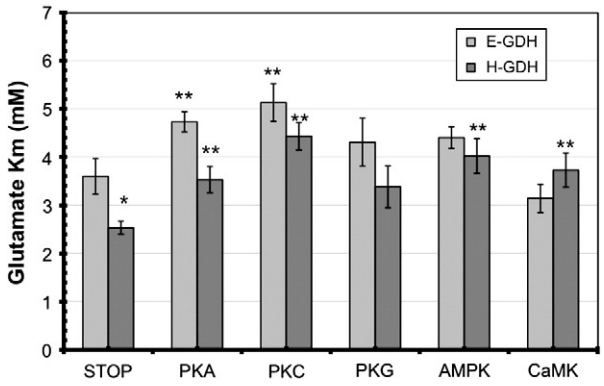

Fig. 2. Effects of *in vitro* incubations to stimulate the activities of endogenous protein kinases on K_m glutamate for liver GDH. Other information as in Fig. 1.

In general, the incubations that stimulated the action of endogenous protein kinases had the opposite effect, producing significant increases ($P<0.05$) in K_m glutamate compared to the STOP condition (Fig. 2). For E-GDH, the stimulation of PKA or PKC resulted in a 31 and 42% increase, respectively, in K_m glutamate compared to the control situation. Similarly, actions of endogenous protein kinases on hibernator GDH resulted in 44–76% increases in the K_m glutamate. Hibernator GDH responded to stimulation by PKA, PKC, AMPK and CaMK.

Altering the phosphorylation state of an enzyme changes its net charge and allows for the separation of high- and low-phosphate forms of an enzyme by ion exchange chromatography. Fig. 3 shows three elution profiles (run independently but graphed together) from a CM^- cellulose column using a linear 0–2 M KCl gradient and featuring: (a) E-GDH, (b) H-GDH, and (c) E-GDH treated by incubation with commercial acid phosphatase. Liver extracts from euthermic ground squirrels showed a single, sharp peak of GDH activity early in the gradient. By contrast, the enzyme from torpid animals eluted in two peaks, a broad minor peak eluting near the E-GDH peak and a major peak eluting at a higher salt concentration. Treatment of euthermic extracts with commercial acid phosphatase caused a major shift in the elution position of GDH so that the enzyme eluted at the same salt concentration as the major peak of H-GDH. This provided strong evidence that E-GDH is the phosphorylated form and that the major peak of H-GDH is a dephosphorylated enzyme.

The difference in phosphorylation state between GDH from euthermic and torpid conditions was also investigated after partial purification of GDH via GTP-agarose chromatography followed by electrophoresis on SDS-PAGE. The resulting gel was stained with ProQ

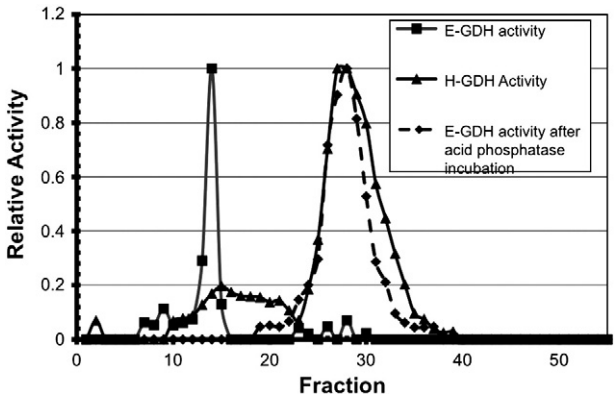

Fig. 3. CM^- cellulose elution profiles for ground squirrel liver GDH comparing E-GDH, H-GDH, and E-GDH after incubation with acid phosphatase. The enzyme was eluted with a 0–2 M KCl gradient in buffer C at pH 6. The elution profiles shown are representative of n = 3 total trials (each using extracts from different animals) that gave comparable results (data not shown).

Diamond phosphoprotein stain which detects proteins that are phosphorylated. Having partially purified GDH using its strong affinity for the GTP-agarose column, most other proteins were removed prior to electrophoresis and purified commercial bovine liver GDH (Sigma) confirmed the location of GDH subunits (~50 kDa). Quantification of band intensities on the stained gel showed that the relative intensities of GDH bands in samples from euthermic liver were significantly stronger by 30% ($P<0.05$) compared with the phosphoprotein stained intensity of H-GDH (Fig. 4).

3.5. GDH stability

One way to characterize structural differences between enzymes is to assess their susceptibility to denaturation by urea. Liver extracts from both euthermic and torpid squirrels were incubated with varying concentrations of urea (0.5–6.6 M), and then GDH activity was measured and C_{50} values for the reduction in GDH activity were calculated. Tables 1 and 2 show that when assayed with either cofactor, the C_{50} urea was significantly different when comparing the two enzyme forms. In all cases, H-GDH was more susceptible to urea denaturation (lower C_{50} value than E-GDH).

4. Discussion

Although the oxidation of endogenous lipid reserves constitutes the majority of the fuel used for energy production during mammalian hibernation, amino acids derived from tissue proteins can also be significant fuels (Yacoe, 1983; Wang, 1988). Furthermore, gluconeogenesis from amino acids contributes to the production of glucose that remains a preferred or necessary fuel for selected cell types. Thus, GDH has a central role to play in channeling the carbon skeletons of several amino acids into the tricarboxylic acid cycle for use as either an aerobic fuel or a gluconeogenic substrate during torpor. Indeed Muleme et al. (2006) found no significant difference in respiration rates of isolated liver mitochondria oxidizing glutamate or palmitoyl carnitine between torpid, arousing and summer active ground squirrels indicating the importance of these fuels in torpor whereas respiration with pyruvate was strongly suppressed in mitochondria from torpid animals, in line with the known inhibition of pyruvate dehydrogenase in the hypometabolic state (Storey, 1997).

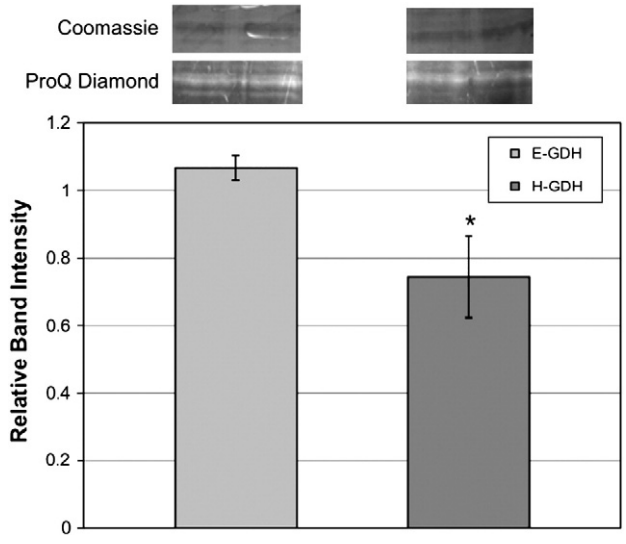

Fig. 4. Relative phosphorylation state of partially purified liver E-GDH and H-GDH as assessed from band intensities on a SDS-PAGE gel stained with ProQ Diamond phosphoprotein stain. The histogram shows mean ± SEM, n = 4 determinations on liver extracts from different animals. Two representative bands from the ProQ Diamond and Coomassie blue stained gels are shown above the histogram. * - Significantly different from the mean relative band intensity of E-GDH using the Student's t-test, $P<0.05$.

This present study shows that liver GDH from euthermic and torpid Richardson's ground squirrels displays markedly different properties suggesting active regulation between the two states and that these differences are linked with a significant change in the phosphorylation state of the enzyme.

Previous studies with bacterial and yeast systems have reported phosphorylation of GDH (Hemmings, 1978; Hemmings, 1981; Uno et al., 1984; Lin and Reeves, 1994) and a recent study of mammalian GDH demonstrated posttranslational modification of the enzyme by ADP-ribosylation, catalyzed by Sir2, in pancreatic beta cells (Haigis et al., 2006). Thatcher and Storey (2001) first reported evidence for structural and kinetic differences between liver GDH isolated from euthermic versus torpid ground squirrels. In particular, size exclusion chromatography on HPLC showed a difference in native molecular mass between the two forms; mean values for hexameric GDH were 335 ± 5 kDa for the euthermic enzyme and 320 ± 5 kDa for GDH from animals in deep torpor versus 331 ± 5 kDa for bovine liver GDH (all $n = 3$). The difference in mass between the two ground squirrel forms could be due, at least in part, to higher numbers of phosphorylated residues on E-GDH.

The present study further explored this idea and provides the first good evidence that mammalian GDH is subject to reversible phosphorylation. Three lines of evidence support this proposal. Firstly, differentially phosphorylated forms of proteins can be separated on ion exchange columns due to their differences in charge. Chromatography of ground squirrel liver GDH on a carboxymethylcelluose column showed that E-GDH eluted from the cation exchange column at a lower salt concentration than did the major form in torpid animals (Fig. 3). Furthermore, incubation of E-GDH with acid phosphatase caused a shift in the GDH elution pattern to a higher salt concentration that overlapped with the elution position of the major peak of H-GDH. Since the removal of phosphate groups from an enzyme would decrease the negative surface charge and lead to later elution off the negatively charged CM column, these results indicate that E-GDH is the higher phosphate form.

Secondly, the ProQ Diamond phosphoprotein stain directly analyzes the relative amount of bound phosphate associated with bands of purified E- and H-GDH. This confirmed the presence of a greater phosphate content associated with E-GDH; the mean relative band intensity of H-GDH was 30% lower than that of the E-GDH when equal amounts of protein were compared (Fig. 4). This shows that liver GDH from euthermic versus torpid animals are the high and low phosphate forms, respectively.

Finally, *in vitro* incubations of liver extracts under conditions that promoted either phosphorylation or dephosphorylation resulted in significant changes in the K_m glutamate of GDH that mimicked the kinetic differences between E- and H-GDH. Thus, protein phosphatase action by PP2B and/or PP2C was highly effective in reducing the K_m glutamate of E-GDH to a value that mimicked the K_m of H-GDH (Fig. 1). By contrast, K_m of H-GDH was not affected by stimulation of any of the phosphatases individually but in incubations that stimulated both PP2B + PP2C a small reduction in K_m glutamate was seen. This susceptibility of E-GDH to phosphatase action further supports the idea that this is the high phosphate form *in vivo*. Furthermore, the response of E-GDH to incubations that stimulated either PP2B or PP2C argues that the enzyme may be controlled *in vivo* by more than one protein phosphatase and/or protein kinase. It is interesting to note that MacDonald and Storey (2007) found that PP2C activity increased significantly during hibernation in liver of Richardson's ground squirrels. Thus, PP2C may be a good candidate for the enzyme that dephosphorylates GDH *in vivo* when ground squirrels enter torpor.

The *in vitro* incubations that stimulated protein kinases also supported the proposal that reversible phosphorylation regulates ground squirrel liver GDH. *In vitro* incubations that stimulated protein kinases led to significant increases in K_m glutamate for both E- and H-

GDH (Fig. 2). Stimulation of PKA or PKC elevated the K_m of E-GDH by 31 and 42%, respectively, whereas stimulation of PKA, PKC, AMPK or CaMK all affected H-GDH, raising K_m glutamate by 44–76%. Hence, H-GDH responded to more protein kinases and also showed a greater percentage increase in K_m when treated with protein kinases. This argues that H-GDH is the lower phosphate form. However, the fact that protein kinases could alter the kinetics of E-GDH indicates that the enzyme was not maximally phosphorylated in the euthermic state or that it was phosphorylated only at selected sites by one or more of the multiple protein kinases that can modify the enzyme.

Recent studies have investigated the role of some protein kinases in ground squirrel hibernation. For instance, Horman et al. (2005) found that liver AMPK was much more active in euthermia compared to torpor. Thus, it is plausible that AMPK phosphorylates GDH *in vivo* when ground squirrels exit torpor thereby helping to re-establish normal euthermic functioning. Similarly, PKA activity increased rapidly upon arousal from torpor in brown adipose tissue of ground squirrels (MacDonald and Storey, 1998); a similar occurrence in liver could also implicate PKA in GDH control during arousal. Additional evidence for the regulation of GDH by reversible phosphorylation comes from an analysis of mammalian GDH sequences to search for putative phosphorylation motifs in their amino acid sequences. Analysis of human, mouse, and rat GDH using PhosphoSite (http://www.phosphosite.org) showed peptide sequences of recognized tyrosine and serine phosphorylation sites, as well as, for human GDH, putative phosphorylation sites for PKA, CaMK and other kinases.

The change in the phosphorylation state of GDH between euthermic and hibernating conditions is clearly linked with the change in the K_m glutamate between the two states (Figs. 1, 2). It is also interesting to note that the generally lower K_m values for glutamate in the torpid state would favor the use of glutamate as a substrate in torpor and are well-matched with *in vivo* glutamate levels in liver of ground squirrels. Thus, Serkova et al. (2007) reported levels of 0.4–0.6 μmol/g for glutamate in liver of *S. tridecemlineatus* during entrance into torpor and late torpor whereas we measured mean values of 1.9 ± 0.6 and 1.5 ± 0.3 μmol/g in *S. lateralis* liver from euthermic and late torpor states, respectively (T.A. Churchill and K.B. Storey, unpublished data). Other kinetic differences between E- and H-GDH were also identified and these could also be a consequence of the change in phosphorylation state. For example, V_{max} changed significantly between euthermic and torpid states for activities measured at 23 °C. This contrasts with results for Djungarian hamsters where daily torpor had no effect on the maximal activities of GDH in eight tissues (Heldmaier et al., 1999). Another difference between E- and H-GDH was the relative use of NAD^+ versus $NADP^+$ in the glutamate-oxidizing reaction. This difference was highlighted by looking at the V_{max}/K_m $NAD(P)^+$ ratio, also known as enzyme efficiency, for each enzyme form. The calculated enzyme efficiencies, shown in Table 3, indicated that cofactor preference changed between the two states. At room temperature, E-GDH showed a >2-fold higher enzyme efficiency with $NADP^+$ as the cofactor as compared to enzyme efficiency with NAD^+. This is consistent with data for bovine liver GDH (a nonhibernating mammal) that show a preference for $NADP^+$ as the cofactor when glutamate was being oxidized (Male and Storey, 1982). $NADP^+$ preference in euthermic situations could fit the enzyme role in processing excess dietary glutamate without committing the α-ketoglutarate that is produced into NAD^+-linked oxidation by the Krebs cycle. It is important to note that changes in cofactor preference may reflect a physical difference between E- and H-GDH, that may be one result of posttranslational modification during torpor.

ADP is a well-known activator of mammalian GDH, and acts by causing a conformational change in the enzyme that decreases product affinity and thereby increases reaction rate (Smith et al., 2001). In all cases in this study, GDH exhibited a K_a ADP between 14–50 μM (well within the range of physiological levels of ADP in liver of ground squirrels), with H-GDH being more sensitive to ADP activation regardless of the cofactor used (Table 1 and 2; Churchill et al., 1996; Serkova et al., 2007). Thus, ADP could play an important role in regulating GDH activity *in vivo* under both euthermic and torpid conditions. Measured liver ADP levels during euthermia and torpor exceed the K_a ADP values measured and thus the difference in fold activation of GDH by ADP may be more physiologically relevant during hibernation. If this is the case, H-GDH displayed higher fold activation by ADP with either cofactor when compared to the euthermic condition. This likely indicates that H-GDH is more responsive to changes in the energy state of the cell which may better tailor enzyme function for a role in fuel metabolism during torpor.

Protein stability, measured here as a function of urea denaturation, can also be an indicator of a physical difference between two forms of an enzyme. In all cases, E-GDH showed a higher C_{50} urea than H-GDH which indicates that the euthermic enzyme was more stable to denaturing conditions (Table 1 and 2). These differences may also derive from different levels of phosphorylation of GDH in the two states. A greater sensitivity by H-GDH to urea denaturation may mean that this enzyme form has a more flexible structure that, although more sensitive to denaturants, is better designed for function at low temperatures. By contrast, a more rigid structure of E-GDH would better suit function at the higher Tb in euthermia.

Table 3
The enzyme efficiency, represented as V_{max}/K_m, for GDH in crude extracts from liver of euthermic and hibernating ground squirrels. The ratios were calculated using the maximal velocity and the K_m for NAD^+ or $NADP^+$.

	E-GDH	H-GDH
V_{max}/K_m with NAD^+ (mU/mg/mM)	14 ± 2	19 ± 2
V_{max}/K_m with $NADP^+$ (mU/mg/mM)	32 ± 3	11 ± 1

5. Conclusion

The data presented above indicate that GDH from euthermic ground squirrels undergoes dephosphorylation when animals transition into torpor. The low phosphate H-form of GDH typically showed greater affinity for its substrates as compared to the high phosphate E-GDH. Regulation of GDH by reversible phosphorylation could be adaptive for ground squirrels in torpor since it could alter poise of the enzyme (amino acid synthesis versus breakdown), and contribute to regulating glutamate oxidation as a hepatic fuel or a gluconeogenic substrate during torpor.

Acknowledgments

Thanks to J.M. Storey for editorial commentary on the manuscript and T. Forrest for help with bioinformatic analysis. The research was supported by a discovery grant from the Natural Sciences and Engineering Research Council of Canada (OPG 6793) to K.B.S. and by an NSERC CGS-M postgraduate scholarship to R.B.

References

Brooks, S.P.J., 1992. A simple computer program with statistical tests for the analysis of enzyme kinetics. Biotechniques 13, 906–911.
Brooks, S.P.J., 1994. A program for analyzing enzyme rate data obtained from a microplate reader. Biotechniques 17, 1155–1161.
Brooks, S.P.J., Storey, K.B., 1992. Mechanisms of glycolytic control during hibernation in the ground squirrel *Spermophilus lateralis*. J. Comp. Physiol. B 162, 23–28.
Carey, H.V., Andrews, M.T., Martin, S.L., 2003. Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature. Physiol. Rev. 83, 1153–1181.
Churchill, T.A., Simpkin, S., Wang, L.C., Green, C.J., Williams, S.R., Busza, A.L., Fuller, B.J., 1996. Metabolic effects of cold storage on livers from euthermic and hibernating Columbian ground squirrels. Cryobiology 33, 34–40.
Geiser, F., 2004. Metabolic rate and body temperature reduction during hibernation and daily torpor. Annu. Rev. Physiol. 66, 239–274.

Haigis, M.C., Mostoslavsky, R., Haigis, K.M., Fahie, K., Christodoulou, D.C., Murphy, A.J., Valenzuela, D.M., Yancopoulos, G.D., Karow, M., Blander, G., Wolberger, C., Prolla, T.A., Weindruch, R., Alt, F.W., Guarente, L., 2006. SIRT4 inhibits glutamate dehydrogenase and opposes the effects of calorie restriction in pancreatic beta cells. Cell 126, 941–954.

Heldmaier, G., Klingenspor, M., Werneyer, M., Lampi, B.J., Brooks, S.P., Storey, K.B., 1999. Metabolic adjustments during daily torpor in the Djungarian hamster. Am. J. Physiol. 276, E896–E906.

Hemmings, B.A., 1978. Phosphorylation of NAD-dependent glutamate dehydrogenase from yeast. J. Biol. Chem. 253, 5255–5258.

Hemmings, B.A., 1981. Reactivation of the phospho form of the NAD-dependent glutamate dehydrogenase by a yeast protein phosphatase. Eur. J. Biochem. 116, 47–50.

Horman, S., Hussain, N., Dilworth, S.M., Storey, K.B., Rider, M.H., 2005. Evaluation of the role of AMP-activated protein kinase and its downstream targets in mammalian hibernation. Comp. Biochem. Physiol. B 142, 374–382.

Lin, H.P., Reeves, H.C., 1994. *In vivo* phosphorylation of $NADP^+$ glutamate dehydrogenase in *Escherichia coli*. Curr. Microbiol. 28, 63–65.

MacDonald, J.A., Storey, K.B., 1998. cAMP-dependent protein kinase from brown adipose tissue: temperature effects on kinetic properties and enzyme role in hibernating ground squirrels. J. Comp. Physiol. B 168, 513–525.

MacDonald, J.A., Storey, K.B., 1999. Regulation of ground squirrel Na^+K^+ ATPase activity by reversible phosphorylation during hibernation. Biochem. Biophys. Res. Commun. 254, 424–429.

MacDonald, J.A., Storey, K.B., 2007. The effect of hibernation on protein phosphatases from ground squirrel organs. Arch. Biochem. Biophys. 468, 234–243.

Male, K.B., Storey, K.B., 1982. Regulation of coenzyme utilization by bovine liver glutamate dehydrogenase: investigations using thionicotinamide analogues of NAD and NADP in a dual wavelength assay. Int. J. Biochem. 14, 1083–1089.

McArthur, M.D., Milsom, W.K., 1991. Changes in ventilation and respiratory sensitivity associated with hibernation in Columbian (*Spermophilus columbianus*) and gold-mantled (*Spermophilus lateralis*) ground squirrels. Physiol. Zool. 64, 40–59.

Muleme, H.M., Walpole, A.C., Staples, J.F., 2006. Mitochondrial metabolism in hibernation: metabolic suppression, temperature effects, and substrate preferences. Physiol. Biochem. Zool. 79, 474–483.

Serkova, N.J., Rose, J.C., Epperson, L.E., Carey, H.V., Martin, S.L., 2007. Quantitative analysis of liver metabolites in three stages of the circannual hibernation cycle in 13-lined ground squirrels by NMR. Physiol. Genomics 31, 15–24.

Smith, T.J., Stanley, C.A., 2008. Untangling the glutamate dehydrogenase allosteric nightmare. Trends Biochem. Sci. 33, 557–564.

Smith, T.J., Peterson, P.E., Schmidt, T., Fang, J., Stanley, C.A., 2001. Structures of bovine glutamate dehydrogenase complexes elucidate mechanism of purine regulation. J. Mol. Biol. 307, 707–720.

Staples, J.F., Brown, C.L., 2008. Mitochondrial metabolism in hibernation and daily torpor: a review. J. Comp. Physiol. B 178, 811–827.

Storey, K.B., 1997. Metabolic regulation in mammalian hibernation: enzyme and protein adaptations. Comp. Biochem. Physiol. A 118, 1115–1124.

Storey, K.B., Storey, M.J., 2004. Metabolic rate depression in animals: transcriptional and translational controls. Biol. Rev. Camb. Philos. Soc. 79, 207–233.

Thatcher, B.J., Storey, K.B., 2001. Glutamate dehydrogenase from liver of euthermic and hibernating Richardson's ground squirrels: evidence for two distinct enzyme forms. Biochem. Cell Biol. 79, 11–19.

Uno, I., Matsumoto, K., Adachi, K., Ishikawa, T., 1984. Regulation of NAD-dependent glutamate dehydrogenase by protein kinases in *Saccharomyces cerevisiae*. J. Biol. Chem. 259, 1288–1293.

van Breukelen, F., Sonenberg, N., Martin, S.L., 2004. Seasonal and state-dependent changes of eIF4E and 4E-BP1during mammalian hibernation: implications for the control of translation during torpor. Am. J. Physiol. Regul. Integr. Comp. Physiol. 287, 349–353.

Wang, L.C.H., 1988. Mammalian hibernation: an escape from the cold. In: Gilles, R. (Ed.), Advances in Comparative and Environmental Physiology. Springer-Verlag, Heidelberg, pp. 1–45.

Yacoe, M.E., 1983. Adjustments in metabolic pathways in the pectoralis muscles of the bat, *Eptesicus fuscus*, related to carbohydrate sparing during hibernation. Physiol. Zool. 56, 648–658.

Zatman, M.L., 1984. Renal and cardiovascular effects of hibernation and hypothermia. Cryobiology 21, 593–614.