HOAJ Biology
ISSN 2050-0874

HOAJ
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# Regulation of liver glutamate dehydrogenase from an anoxia-tolerant freshwater turtle

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## Abstract

**Background:** Freshwater turtles, Trachemys scripta elegans, are one of the few vertebrate species capable of surviving prolonged periods without oxygen. Anoxic survival requires numerous physiological and biochemical changes, including a drastic reduction in metabolic rate, a cessation of oxygen-based metabolism, and a suppression of urea-synthesis. Given this state, the purpose of this study was to investigate the possible regulation of liver glutamate dehydrogenase (GDH), a key enzyme in both nitrogen and carbohydrate metabolism, when these freshwater turtles transition from normoxic to anoxic conditions.

**Methods**: GDH was purified to electrophoretic homogeneity using a combination of blue-agarose and GTP-agarose chromatography. Subsequent kinetic analysis of GDH derived from the liver of both control (normoxic) and 20 h anoxic turtles was performed spectrophotometrically. ProQ Diamond phosphoprotein staining was used to determine if GDH was present in differently phosphorylated forms between normoxic and anoxic states, and in vitro incubations with alkaline and acid phosphatases were used to determine if changes in phosphorylation state resulted in kinetic changes.

**Results:** Kinetic studies revealed that the anoxic form of GDH was significantly less active than the aerobic control, as well as more susceptible to pH-induced inactivation, and GTP inhibition. ProQ Diamond phosphoprotein staining indicated that anoxic liver GDH was significantly less phosphorylated than control GDH. Subsequent stimulation of exogenous alkaline and acid phosphatase activity significantly lowered the Km α-ketoglutarate for control GDH to a value similar to the value found for anoxic GDH in its native state.

**Conclusion:** We conclude that effector molecules, tissue acidification and reversible phosphorylation act in concert to suppress GDH during anoxia in accordance with general metabolic rate depression and the overall shutdown of oxygen-based metabolism.

## Background

Glutamate dehydrogenase (GDH; E.C. 1.4.1.3) is an enzyme present in the mitochondrial matrix that catalyzes the reversible $NAD(P)^+$-linked oxidative deamination of L-glutamate to α-ketoglutarate and ammonium ion. In the glutamate-oxidizing reaction (denoted the forward reaction) GDH is capable of shuttling the carbon skeletons of several amino acids (glutamate, glutamine, arginine, proline, histidine) into the Krebs cycle in the form of α-ketoglutarate where they can contribute to energy production or gluconeogenesis. Alternatively, in the glutamate-synthesizing reaction (denoted the reverse reaction) GDH is able to produce glutamate for use in protein synthesis or in transamination reactions to produce other amino acids. GDH's role in such important cellular processes suggests the need for regulation, and indeed, this enzyme has been found to be regulated allosterically by common nucleotide cofactors (AMP, ADP, ATP, and GTP; reviewed in [1]) as well as by reversible phophorylation in bacteria, yeast, and most recently, in the liver of Richardson's ground squirrels during winter hibernation [2-6]. Although known to be regulated, the control of amino acid synthesis/degradation through GDH has yet to be investigated during prolonged oxygen deprivation.

Those organisms that can endure environments under low oxygen (hypoxia) or no oxygen (anoxia) conditions require extensive biochemical, behavioral and physiological changes. These changes typically include sluggish movements, decreased blood pressure, heart rate, and renal function [7]. Large scale metabolic changes are also needed to adjust to long term oxygen deprivation, and this could possibly include: (i) compiling large reserves of fermentable fuels; (ii) utilizing strategies to buffer or excrete anaerobic metabolic end products that are typically acidic; (iii) utilizing alternative anaerobic routes of substrate fermentation that are linked to enhanced ATP output; (iv) developing good antioxidant defenses in preparation for the reintroduction of oxygen to the body; (v) up-regulating genes that aid anoxia survival, and most importantly, (vi) a strong reduction of metabolic rate [8,9]. Metabolic rate depression is essential due to the large reduction in ATP production by fermentative pathways in comparison to oxidative metabolism. With this reduction in ATP output during anoxia, there is typically a coordinated reduction in ATP consuming processes, such as protein synthesis, protein degradation, gluconeogenesis, urea synthesis, and ion motive ATPases [10].

Freshwater turtles from the *Trachemys* and *Chrysemys* genera are among the few vertebrate species that are capable of surviving extended periods of anoxia. The red-eared slider, *Trachemys scripta elegans*, is a major model for studies of anoxia tolerance, and is the animal investigated in this study. During the winter these turtles can remain submerged in cold water for 4-5 months to escape freezing air temperatures. While submerged, these red-eared sliders can absorb a sufficient amount of $O_2$ in cold water to drive their metabolic needs [11], however, as the dissolved oxygen levels become reduced in

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doi: 10.7243/2050-0874-1-3

ice-locked lakes and rivers and anoxic conditions emerge, these turtles become facultative anaerobes. In order to survive without oxygen *T. s. elegans* typically suppress their metabolic rate to approximately 10-20% of the corresponding aerobic rate at the same temperature [12]. More specifically, hepatocytes isolated from *Chrysemys picta bellii* demonstrated a 90% reduction in metabolic rate under anoxic conditions when compared to hepatocytes in a normoxic state [13]. During anoxia these turtles rely completely on glycolysis for energy and have not developed alternative fermentative pathways for increased ATP production as is seen in many other anoxia tolerant species [14]. As a result, the turtles have developed mechanisms to buffer against severe acidosis, as tissue acidification is inevitable due to ATP hydrolysis outweighing glycolytic ATP production (i.e. there will be a net production of H+ in the cell)[15].

The inability of the Krebs cycle and the ETC to participate in energy production suggests that it may be necessary to regulate the enzymes that feed these processes during anoxia. With this in mind, it was hypothesized that liver GDH would be suppressed upon long-term exposure of freshwater turtles to an anoxic environment. The present study analyzes liver GDH purified from *T. s. elegans* comparing and contrasting glutamate-oxidizing and glutamate-synthesizing reaction kinetics for the enzyme from control and 20 h anoxic animals, as well as responses of each enzyme form to cellular metabolites, changes in pH, and reversible phosphorylation.

## Methods

### Animals

Adult red-eared slider turtles, *T. s. elegans*, were obtained from Wards Natural Science, Mississauga, Ontario during the winter months and maintained in tanks of dechlorinated water at 7°C for three weeks prior to experimentation. Turtles had access to deep water, a small platform, and food in the form of trout pellets, lettuce and egg shells. Control (normoxic) turtles were sampled directly from the tanks, whereas anoxia was imposed by submerging turtles in sealed tanks filled with deoxygenated water (previously bubbled with 100% nitrogen gas) at 4°C. A wire mesh placed below the surface of the water prevented the turtles from surfacing. Although forced submergence is thought to cause stress for turtles at room temperature, turtles submerged in cold water (~3°C) are typically quiescent and seldom surface to breathe [11,16]. Thus, covering their containers at this point mimics what they would experience in winter environments where eventually ice forms and prevents resurfacing. Turtles used in the present study were sampled after 20 h of anoxic submergence. All turtles were killed by decapitation, via a protocol approved by the university Animal Care Committee and meeting the guidelines of the Canadian Council on Animal Care. Organs were quickly dissected out, frozen in liquid nitrogen and transferred to a -80°C freezer for storage.

### Preparation of tissue extracts and GDH purification

Samples of frozen liver were homogenized 1:5 w:v in a homogenization buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM 2-mercaptoethanol, 10% glycerol and inhibitors of protein kinases (2.5 mM EDTA, 2.5 mM EGTA) and protein phosphatases (25 mM β-GP) with a few crystals of the serine protease inhibitor phenylmethylsulphonyl fluoride (PMSF) added just prior to homogenization. Initial tests showed that 25 mM NaF, another protein phosphatase inhibitor, decreased GDH activity over time and was not used in any assays. Homogenates were centrifuged for 30 minutes at 13,500 x g at 5°C and the supernatant was decanted and held on ice until purification columns could be prepared.

Purification of GDH began with the application of a 1.5 mL aliquot of tissue extract to a Blue-agarose column (2.5 x 1.8 cm h x d equilibrated in homogenization buffer). The column was then rinsed with 10 mL of homogenization buffer to remove unbound material. Turtle liver GDH was then eluted by a linear salt gradient of 0-0.5 M KCl. Fractions of ~750 µL were collected by a Gilson FC203B Fraction Collector. GDH activity was determined by assaying each fraction under optimal conditions (as determined for the crude extract in the forward direction). The five most active fractions were then pooled and held on ice until a GTP-agarose column was prepared.

The GTP-agarose column (2.8 x 1.2 cm h x d) was equilibrated in homogenization buffer and a 2 mL aliquot of the pooled eluant from the Blue-agarose column was then applied. The column was washed with 10 mL of homogenization buffer to remove any unbound material, and then GDH was eluted with a 0-1 M linear KCl gradient. Fractions were assayed using the optimal conditions for GDH, and the five most active fractions were pooled and held on ice until use in kinetic assays.

### GDH Assay

GDH activity was assayed spectrophotometrically at 340 nm using a Thermo Labsystems Multiskan spectrophotometer. All assays were performed at room temperature and the optimal assay conditions for purified turtle liver GDH in the forward direction were 50 mM L-glutamate, 1.5 mM $NAD^+$, 0.5 mM Mg-ADP, and 50 mM Tris-HCl buffer, pH 8.0 in a total volume of 200 µL with 25 µL of purified extract used per assay. However, without Mg-ADP, the optimum concentration of L-glutamate was 15 mM. In the reverse reaction, the optimal concentrations of substrates were 1 mM α-ketoglutarate, 100 mM $NH_4Cl$, 0.1 mM NADH, 0.5 mM Mg-ADP, 50 mM HEPES buffer, pH 7.2 with 10 µL of purified liver extract used in each assay. Enzyme activity was measured in U/mg of soluble protein.

$K_m$ values for substrates were determined at optimal co-substrate concentrations. Analysis of the effects of activators and inhibitors was carried out at sub-optimal substrate concentrations (for the forward reaction 2 mM L-glutamate, and 0.5 mM $NAD^+$; for the reverse reaction 0.2 mM α-ketoglutarate, 0.05 mM NADH, and 40 mM $NH_4Cl$).

### ProQ Diamond phosphoprotein staining

The phosphorylation state of purified liver GDH from control and 20 h anoxic turtles was assessed by ProQ Diamond phosphoprotein staining. The five fractions, originating from the blue-agarose column, with the most activity were pooled and soluble protein content was

doi: 10.7243/2050-0874-1-3

quantified using the Coomassie blue dye-binding method. Aliquots of the pooled fractions were then mixed 1:1 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 minutes, cooled on ice and frozen at -20°C until use.

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 minutes in running buffer containing 25 mM Tris-base, 250 mM glycine, and 0.1% SDS. The gel was then washed twice in fixing solution (50% v:v methanol, 10% v:v acetic acid) for ten minutes each time, and then left overnight in the fixing solution at 4˚C. The following day the gel was washed with $ddH_2O$ three times for 10 minutes each time, and the ProQ Diamond Phosphoprotein stain (Invitrogen, Eugene, OR) was poured over the gel and allowed to sit with continuous motion for 90 minutes. During staining the container holding the gel was covered with tin foil to prevent light from interacting with the light-sensitive stain. The gel remained covered for the remaining steps. Following staining, the gel was washed three times with $ddH_2O$ for 5 minutes each. The ChemiGenius Bioimaging System (Syngene, Frederick, MD) was used to visualize the fluorescence intensity of the bands on the gel and the associated GeneTools software was used for quantification.

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, 7.5% v/v acetic acid) and destained for 10 min with destaining solution (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. The band corresponding to GDH was identified using the commercially purified bovine liver GDH (Sigma) that was run on the gel with the control and anoxic turtle samples.

Effect of alkaline and acid phosphtase activities on GDH activity GDH purified from control liver was incubated with commercial alkaline or acid phosphatases (Sigma) in an attempt to stimulate dephosphorylation of GDH. Purified tissue extracts were incubated for ~24 hours at 4°C in a 1:2 ratio with the incubation solution, designed so that following dilution, the final concentrations were as indicated below. All incubations contained a basic incubation buffer (50 mM HEPES, 10% v:v glycerol, 10 mM β-mercaptoethanol, pH, 7.2) with additions as follows.

Control Incubations (also denoted as STOP): incubation buffer plus 2.5 mM EDTA, 2.5 mM EGTA and 25 mM β-GP.

Alkaline or Acid Phosphatases: incubation buffer plus 7.5 mM $MgCl_2$, 3.75 mM EDTA with either 30 U of alkaline phosphatase or 0.6 U of acid phosphatase.

Following incubation, the $K_m$ α-ketoglutarate (in the absence of ADP) under optimum assay conditions was determined.

### Effect of pH on the $V_{max}$ ratio of purified GDH

Purified GDH was assayed in both the forward and reverse directions (under optimal assay conditions) at pH 6.6 and pH 7.4. All of these reactions were done with and without 0.5 mM ADP. $V_{max}$ ratios were calculated by dividing the activity of the forward reaction by the activity of the reverse reaction.

### GDH structural stability

To assess the possible structural differences between GDH from control and anoxic states, crude extracts were exposed to various levels of urea (0 - 4 M), guanidine hydrochloride (GnHCl; 0 - 4 M), or KCl (0 – 2.5 M). Crude turtle liver extracts were prepared as described above (prior to any GDH purification procedures), and 30 µL of extract were incubated with the various concentrations of the aforementioned molecules in a 200 µL final volume. Incubations were performed at room temperature (22°C) for 1 h prior to assay under optimal conditions for GDH in the forward direction. $C_{50}$ (the concentration of denaturant that reduced activity by 50%) and $K_a$ values were subsequently determined.

### Data, statistics and protein determination

Enzyme activity was analyzed with a Microplate Analysis Program [17] and the kinetic parameters were determined using the Kinetics v.3.5.1 program [18]. Data are expressed as mean ± SEM from multiple independent determinations on separate preparations of enzyme. The statistical testing used in this study was the unpaired Student's t-test ($P < 0.05$). Protein concentration in extracts was determined by the Coomassie blue dye-binding method using the BioRad prepared reagent and bovine serum albumin as the standard.

### Chemicals and Biochemicals

All biochemicals were from Sigma Chemical Company with a few exceptions. Urea and glycerol were obtained from Fisher Biotech Company; NH4Cl was from Mallinckrodt Co.; NAD+ was from Boehringer Mannheim; $CaCl_2$ was purchased from J.T. Baker Chemical Company; okadaic acid was from CalBiochem; NaF was from Fisher Scientific, and β-glycerol phosphate was purchased from BIOSHOP.

## Results

### GDH purification

The purification scheme for liver GDH from control turtles is depicted in Table 1. Using a combination of two affinity columns (Blue-agarose and GTP-agarose) GDH was purified 17-fold with an overall yield of 41%. The specific activity of the final purified control enzyme was 0.157 U/mg (forward direction). The effectiveness of each step in the purification process was assessed by SDS-polyacrylamide gel electrophoresis with Coomassie blue staining (Figure 1). After purification of GDH by GTP-agarose a single band was seen on the gel, which indicates that the enzyme was purified to homogeneity. The same purification procedure was used to purify GDH from liver of 20 h anoxia-exposed turtles.

### GDH substrate kinetics

The optimum pH for purified GDH varied depending on the direction

doi: 10.7243/2050-0874-1-3

**Table 1**. Purification of GDH from the liver of control T .s. elegans

| Purification Step | Total Protein (mg) | Total Activity (U) | Specific Activity (U/mg) | Fold Purification | % Yield |
|---|---|---|---|---|---|
| Supernatant | 0.30 | 0.0028 | 0.0093 | - | 100 |
| Blue-Agarose | 0.0074 | 0.0011 | 0.147 | 16 | 78 |
| GTP-Agarose | 0.0017 | 0.00061 | 0.360 | 39 | 41 |

of the reaction. The forward reaction displayed a broad activity peak with ~90% of the activity being retained from pH 8-10. Activity dropped below 50% of the maximum activity below pH 7 and above pH 10.5. For the reverse reaction, the activity peak was also very broad, and ~90% of the maximal activity was retained from pH 7.0-9.5. Above pH 10.5, GDH activity dropped below 50% of the maximal activity (data not shown).

GDH was purified from liver of both aerobic control and anoxic turtles and kinetic properties of the purified enzyme were assessed in both the forward (glutamate-utilizing) and reverse (glutamate-synthesizing) directions. In the forward direction, several kinetic parameters changed significantly ($p<0.05$) between control and 20 h anoxic turtles. For instance, the $K_m$ glutamate was ~69% higher and $V_{max}$ 39% lower for the anoxic enzyme when compared to control GDH. Similarly, in the reverse direction, the $K_m$ α-ketoglutarate of purified anoxic GDH was 45% lower and $V_{max}$ 70% lower ($p<0.05$) in comparison to the same value for aerobic control GDH (Table 2).

### Effects of cellular metabolites on GDH activity

The effect of various cellular metabolites on GDH activity (at sub-optimal substrate concentrations) was determined for both the forward and reverse reactions of GDH. In the forward direction, ADP acted as an activator of control and 20 h anoxic GDH; increasing activity by 70% and 63%, respectively. However, both the $K_a$ and the fold activation of the enzyme were not significantly different between the two conditions (Table 3). That being said, ADP was able to alter other kinetic parameters that were assessed in its presence. For instance, for both control and 20 h anoxic GDH the $K_m$ glutamate increased significantly in the presence of ADP as contrasted with $K_m$ glutamate without ADP. Also, $K_m$ $NAD^+$ for anoxic GDH decreased by 54% ($p<0.05$) in the presence of ADP as compared to assays without ADP (Table 2).

Although the kinetic values changed when assayed with and without ADP, these changes were not uniform for both control and anoxic GDH. For instance, $K_m$ glutamate in the presence of 0.5 mM ADP was ~45% higher for anoxic, compared with control GDH. Alternatively, the $K_m$ $NAD^+$ for anoxic GDH in the presence of ADP was significantly lower ($p<0.05$) as compared to the normoxic value. Also, the anoxic GDH maximal activity with ADP was 42% lower than the control $V_{max}$ (Table 2).

Unlike the situation for the forward reaction, control and anoxic GDH displayed different sensitivities to activation by ADP while assayed

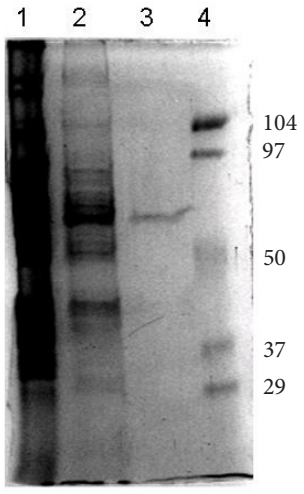


**Figure 1: Purification of turtle liver GDH**
SDS-PAGE with Coomassie blue staining of samples taken at every step in the purification of GDH from the liver of control T. s. elegans. Lanes represent: (1) Crude liver extract, (2) pooled GDH fractions after elution from a blue-agarose column, (3) pooled GDH from a GTP-agarose column, and (4) Biorad Kaleidoscope molecular weight standards (3 µL). Molecular weight standards from top to bottom are phosphorylase B, 104 kDa; bovine serum albumin, 97 kDa; ovalbumin, 50 kDa; carbonic anhydrase, 37 kDa; and soybean trypsin inhibitor, 29 kDa.

**Table 2. Comparison of the kinetic parameters of purified liver GDH from control and 20 h anoxic turtles.**

| Kinetic Parameter (No ADP) | Control | 20 h Anoxic |
|---|---|---|
| $K_m$ Glutamate (mM) | 0.71 ± 0.05 | 1.20 ± 0.07[a] |
| $K_m$ $NAD^+$ (mM) | 0.49 ± 0.03 | 0.50 ± 0.04 |
| $V_{max}$ (U/mg) | 0.215 ± 0.005 | 0.131 ± 0.004[a] |
| $K_m$ α-ketoglutarate (mM) | 0.128 ± 0.005 | 0.070 ± 0.002[a] |
| $K_m$ $NH4^+$ (mM) | 39 ± 1 | 44 ± 3 |
| $V_{max}$ (U/mg) | 2.10 ± 0.03 | 0.62 ± 0.01[a] |
| **Kinetic Parameter (with 0.5 mM ADP)** | | |
| $K_m$ Glutamate (mM) | 1.73 ± 0.08[b] | 2.5 ± 0.1[ab] |
| $K_m$ $NAD^+$ (mM) | 0.45 ± 0.03 | 0.23 ± 0.02[ab] |
| $V_{max}$ (U/mg) | 0.365 ± 0.008[b] | 0.213 ± 0.005[ab] |
| $K_m$ α-ketoglutarate (mM) | 0.24 ± 0.01[b] | 0.27 ± 0.05[b] |
| $K_m$ $NH4^+$ (mM) | 18 ± 2[b] | 29 ± 3[ab] |
| $V_{max}$ (U/mg) | 2.80 ± 0.04[b] | 1.10 ± 0.02[ab] |

Note: GDH was assayed in both the glutamate-oxidizing and glutamate-synthesizing directions (data separated by a single horizontal line), both with and without ADP (data separated by a double horizontal line). Data are means ± SEM, n = 3-4 independent determinations on enzyme preparations from separate turtle liver samples. Km values were determined at optimal co-substrate concentrations. a-Significantly different from the corresponding control value using the unpaired Student's t-test, $P<0.05$. b-Significantly different from the corresponding value without ADP using the unpaired Student's t-test, $P<0.05$.

doi: 10.7243/2050-0874-1-3

in the reverse direction; anoxic $K_a$ ADP being significantly higher than the control value (Table 3). Additionally, reverse reaction kinetic values were also affected by the presence of 0.5 mM ADP. For instance, the $K_m$ α-ketoglutarate and $V_{max}$ increased significantly ($p<0.05$) for both control and anoxic GDH upon the addition ADP, while the $K_m$ $NH_4^+$ decreased significantly ($p<0.05$; Table 2). As with the situation for the forward reaction, the changes in kinetic values were not always uniform for both control and anoxic GDH assayed in the presence of ADP; $K_m$ $NH_4^+$ was 38% higher for anoxic GDH in comparison to the corresponding control value (Table 2).

In addition to studies involving the activator ADP, the effects of high energy molecules, such as ATP and GTP, on the forward reaction of GDH were also investigated. ATP had very little effect on GDH activity up to 10 mM, whereas GTP at low micromolar concentrations strongly inhibited liver GDH from both control and 20 h anoxic turtles. Anoxic GDH was significantly ($p<0.05$) more sensitive to GTP inhibition when compared to the control condition (~80% drop in $I_{50}$ during anoxia).

Some non-nucleotide molecules were also able to effect GDH activity *in vitro*. Both leucine and lactate were activators of GDH activity, however, the response varied for control and anoxic forms of GDH. The $K_a$ leucine was significantly ($p<0.05$) smaller for anoxic GDH as compared to control, whereas $K_a$ lactate was significantly higher for anoxic GDH when compared to the control condition (Table 3). Pyruvate on the other hand showed no effect on GDH activity *in vitro* (data not shown).

**Reversible phosphorylation of GDH**

Stable kinetic changes between an enzyme from two states is often the result of posttranslational modification and most commonly reversible phosphorylation. To determine if this mechanism was responsible for the kinetic changes seen between GDH from control and 20 h anoxic turtles, partially purified enzyme extracts were run on an SDS-PAGE gel and stained with ProQ Diamond phosphoprotein stain. This stain showed a nearly 40% decrease in the relative band intensity ($p<0.05$) for the purified anoxic GDH when compared to the control condition (Figure 2).

To further investigate the possibility of phosphorylation as a regulatory mechanism for turtle GDH during anoxia, purified GDH from control turtles was incubated with commercial alkaline or acid phosphatases and $K_m$ α-ketoglutarate values were then assessed. Incubations without any added phosphatases (denoted STOP) showed $K_m$ α-ketoglutarate values comparable to that seen for control GDH in its native state (Table 2). Incubation of purified control GDH with commercial alkaline or acid phosphatase decreased the $K_m$ α-ketoglutarate by ~46% ($p<0.05$) in comparison to the STOP condition (Figure 3).

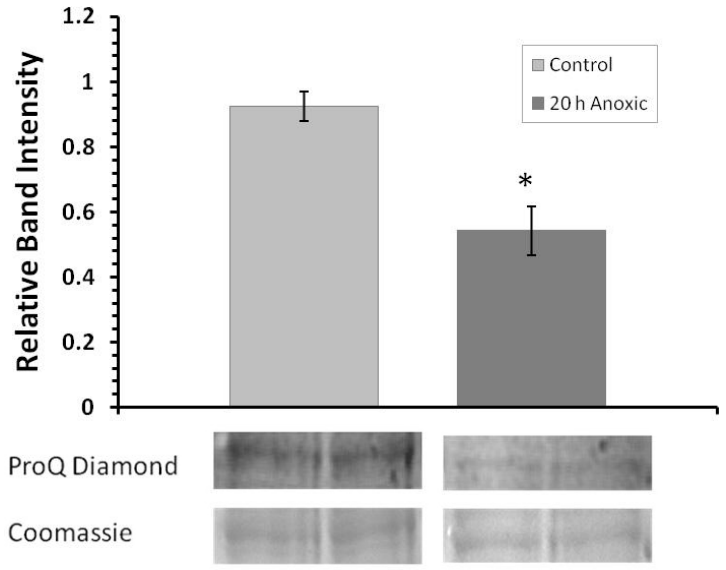


**Figure 2. ProQ Diamond phosphoprotein staining of purified GDH**
The relative level of purified liver GDH phosphorylation from control and 20 h anoxic T. s. elegans as assessed on a SDS-PAGE gel stained with ProQ Diamond phosphoprotein stain. Sample bands are included below each bar on the histogram. Data are means ± SEM, n=3 independent determinations on enzyme preparations from separate turtle liver samples. * - Significantly different from controls using the unpaired Student's t-test ($p<0.05$).

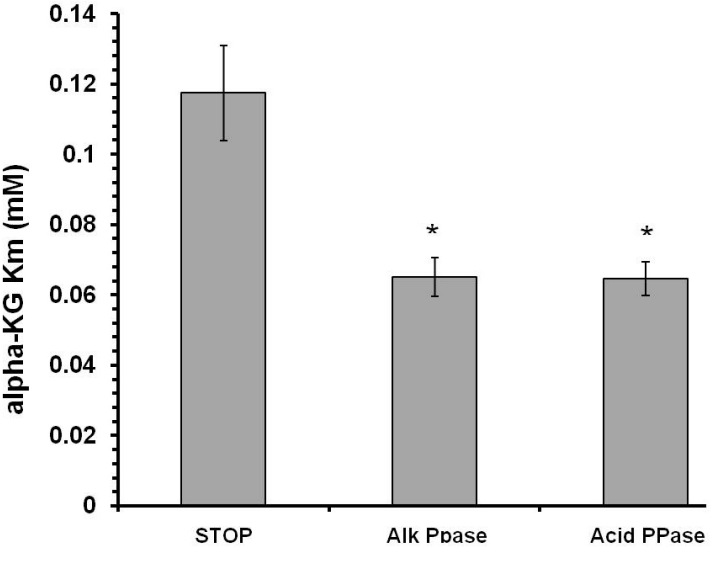


**Figure 3. Dephosphorylation of control GDH by commercial phosphatases**
Effects of in vitro incubations to stimulate the activities of exogenous alkaline and acid phosphatases on Km α-ketoglutarate for liver GDH from control T. s. elegans. Purified extracts were incubated for ~24 h before assay at 23°C. Data are means ± SEM, n=3 independent determinations on enzyme preparations from separate turtle liver samples. *-Significantly different from the corresponding STOP value via the unpaired Student's t-test, $p<0.05$

doi: 10.7243/2050-0874-1-3

**Table 3. The effect of common cellular metabolites on purified liver GDH from control and 20 h anoxic turtles.**

| Kinetics | Control | 20 h Anoxic |
|---|---|---|
| (Glutamate-oxidizing direction) | | |
| Ka ADP (μM) [Fold Activation] | 28 ± 7 [1.6 ± 0.1] | 37 ± 5 [1.67 ± 0.05] |
| Ka AMP (mM) [Fold Activation] | 0.34 ± 0.6 [1.52 ± 0.06] | No effecta |
| I50 GTP (μM) | 0.59 ± 0.07 | 0.109 ± 0.006a |
| Ka Leucine (μM) [Fold Activation] | 68 ± 8 [2.08 ± 0.08] | 30 ± 5a [2.4 ± 0.1] |
| Ka Lactate (mM) [Fold Activation] | 1.3 ± 0.3 [1.30 ± 0.02] | 3.5 ± 0.4a [1.5 ± 0.1] |
| Kinetics | | |
| (Glutamate-synthesizing direction) | | |
| Ka ADP (μM) [Fold Activation] | 17 ± 4 [1.77 ± 0.04] | 40 ± 5a [1.84 ± 0.06] |

Note: Assays were performed in the glutamate-oxidizing and glutamate-synthesizing reactions as indicated. Data are means ± SEM, n = 3 independent determinations on enzyme preparations from separate turtle liver samples. Ka and I50 values were determined at sub-optimal substrate concentrations (as defined in the Materials and Methods). a-Significantly different from corresponding control value using the unpaired Student's t-test, P<0.05.

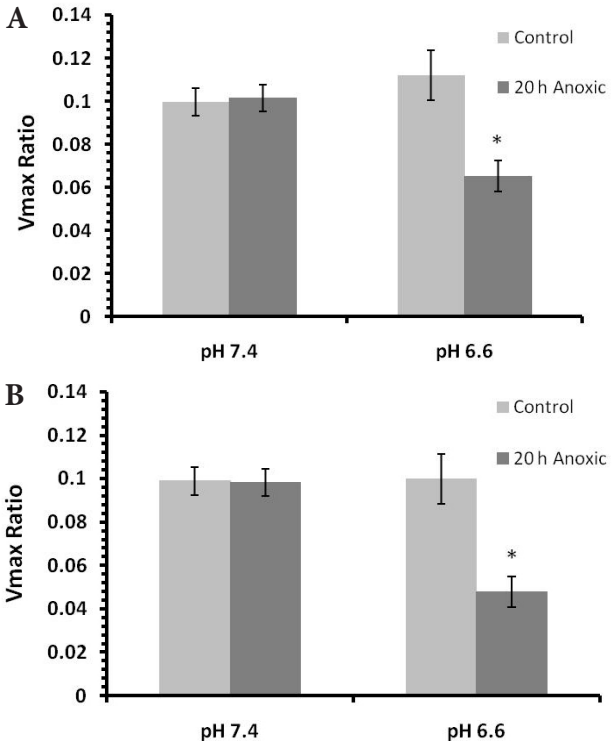


**Figure 4. Vmax ratios (forward reaction/reverse reaction) of purified control and 20 h anoxic GDH at pH 7.4 and 6.6**
(**A**) The Vmax ratios without ADP being added to the assays.
(**B**) The Vmax ratios with 0.5 mM ADP being added to the assays. Data are means ± SEM, n=3 independent determinations on enzyme preparations from separate turtle liver samples. *-Significantly different from the control value at the same pH and significantly different from the corresponding value at normoxic pH (7.4) using the Student's t-test ($p<0.05$).

### Effect of pH on GDH activity

The data presented above was gathered for assays conducted at the optimal pH for the forward and reverse reactions. However, a key consequence of anoxia in all systems is a gradual decrease in intracellular pH as anoxia is prolonged and acidic end products accumulate (pH can drop to 6.6 in some cases) [19]. Anoxia tolerant animals use mechanisms to minimize the pH decrease but it still occurs. Therefore, it is important to understand how enzyme function changes under the high versus low pH conditions of the aerobic versus anoxic states. To analyze this, the maximal velocities of both the forward and reverse reactions of purified aerobic and anoxic GDH were measured at two pH values: pH 7.4 representing normoxic conditions and pH 6.6 representing anoxic conditions. Ratios of activity in the forward versus reverse directions were then calculated for $V_{max}$ values measured in the absence versus presence of ADP. Under the normoxic pH conditions control and anoxic GDH $V_{max}$ ratios were essentially the same, both with and without ADP being used in the reaction. However, under anoxic pH conditions, anoxic GDH $V_{max}$ ratio was nearly 50% of the control GDH $V_{max}$ ratio (Figure 4A and B). The decrease in the anoxic GDH $V_{max}$ ratio is due only to the decrease in forward reaction velocity, with the reverse reaction velocity remaining the same at both pH 7.4 and 6.6 (Data not shown).

### GDH stability

Structural variation between enzymes can be elucidated by determining the effects of urea, GnHCl, and KCl on enzyme activity. Both control and 20 h anoxic GDH (crude samples) were incubated with various concentrations of the above molecules (concentrations found in the Materials and Methods) and $K_a$ and $C_{50}$ values were determined where possible. All three molecules affected GDH, however, the responses between control and anoxic GDH were often different. For instance, the $C_{50}$ urea was 25% higher for anoxic GDH as compared to control. Furthermore, the $K_a$ GnHCl and $K_a$ KCl for anoxic GDH was 19% and 48% lower, respectively, when compared to the corresponding control condition.

## Discussion

Dramatic fluctuations in environmental oxygen levels have forced some organisms to develop the capacity for long-term anoxia tolerance. Adaptations to decreased oxygen availability include the expression and suppression of various genes, accumulation of fermentable fuels,

doi: 10.7243/2050-0874-1-3

alternative routes for anaerobic metabolism, and most importantly, metabolic rate depression [20]. A champion of vertebrate facultative anaerobes are species of freshwater turtles, including *T. s. elegans*, which can survive at least 3 months of full anoxia in cold water. During anaerobiosis these turtles shut down oxygen-based metabolism and rely completely on glycolysis for energy production [14]. Such drastic changes to cellular metabolism necessitates some form of regulation for carbohydrate, fatty acid and amino acid oxidation pathways. Glutamate dehydrogenase, a key enzyme in amino acid metabolism that forms the gateway for several amino acids (e.g. glutamate, glutamine, proline, arginine, and histidine) to enter aerobic metabolism, may be an important regulatory site during anoxia in freshwater turtles. The present study shows that purified liver GDH from control and 20 h anoxic turtles has markedly different kinetics, responses to cellular metabolites, denaturants, and pH, and is present in different phosphorylation states.

Phosphorylation of GDH was first reported in bacterial and yeast systems [2-5], and was later found to be a regulatory mechanism in Richardson's ground squirrels during hibernation [6], and freshwater crayfish as they transitioned into an anoxic state [21]. Regulation of enzyme activities during anaerobiosis through reversible phosphorylation is not uncommon for the freshwater turtles, and is likely an important part of overall metabolic rate depression. Examples include regulation of turtle liver glycogen phosphorylase, phosphofructokinase, and pyruvate kinase by phosphorylation under anoxia [22].

There are two lines of evidence that support GDH regulation by reversible phosphorylation during anoxia. Firstly, ProQ Diamond phosphoprotein stain was used to quantify relative phosphate content for control and 20 h anoxic GDH. Staining results indicated that anoxic GDH was significantly less phosphorylated than GDH from control turtles (Figure 2). Secondly, incubation of the purified control enzyme with commercial alkaline or acid phosphatase confirmed that control GDH was phosphorylated, and that dephosphorylation stimulated a significant decrease in $K_m$ α-ketoglutarate to a value similar to that of the anoxic enzyme (Figure 3). These results support the idea that GDH is regulated by phosphorylation when *T. s. elegans* transitions into the anoxic state; the control form of GDH being significantly more phosphorylated than the anoxic form.

Although the relevant research into the activities of various protein kinases and phosphatases during turtle anaerobiosis is not extensive, work conducted on other anoxia-tolerant animals may provide insight into possible candidates that could act on GDH *in vivo*. For instance, in the hepatopancreas (liver-like organ) of the anoxia-tolerant crayfish, protein phosphatase 1 and 2C remain just as active after long (20 h) anoxia exposures as was seen in control animals. Furthermore, protein phosphatase 2A increased in activity during long anoxia exposures in the hepatopancreas of these crayfish [23]. The activity of these phosphatases during anoxia could suggest a role for these enzymes in the dephosphorylation and inactivation of metabolic enzymes, which is essential for metabolic rate depression during anaerobiosis. It is important to note, however, that protein phosphatase 1 activity in the turtle liver was suppressed during anoxia and remained low during long anoxic exposures [24]. Thus, PP1 is unlikely to be responsible for the dephosphorylation of GDH during anoxia in *T. s. elegans*.

In addition to the change in $K_m$ α-ketoglutarate, there are several other kinetic parameters that change between control and anoxic states that support a structural difference between the two enzyme forms and suggest a decreased role for GDH during anoxia. For instance, without ADP in the assays, $K_m$ glutamate increases, and $V_{max}$ decreases (for both forward and reverse reactions) significantly during anoxia (Table 2). Furthermore, in the presence of ADP, there are significant increases in $K_m$ glutamate, and $K_m$ $NH_4^+$, as well as substantial decreases in forward and reverse reaction maximal velocities for anoxic GDH when compared to control GDH (Table 2). Taken together this suggests a substantial decrease in both forward and reverse reaction activity *in vivo* during anoxia.

Shutting down GDH during anoxia corresponds well with the metabolic changes that occur during anoxia in the freshwater turtle. Besides reduced liver glycolytic activity during anaerobiosis [14], the TCA cycle slows down substantially and oxidative phosphorylation via the ETC ceases. Without these major metabolic pathways functioning to produce ATP, the breakdown of glutamate to α-ketoglutarate for energy would be unnecessary and uneconomical. Further evidence supporting diminished glutamate oxidation via GDH stems from the known ammonia levels and the degree of ureogenesis and gluconeogenesis in the fully anoxic turtles. Ammonia levels in the anoxic turtles are known to stay constant at fairly low concentrations of 0.13 mM in the blood plasma [16]. Moreover, during anoxia freshwater turtles suppress urea and glucose synthesis in the liver by 70% and 100%, respectively [10]. Thus, it stands to reason that if the metabolic pathways that utilize the products of glutamate oxidation are switched off under anoxic conditions that the forward reaction of GDH is suppressed as well.

The constant low-millimolar concentrations of ammonia in the anoxic turtle, not only indicates a slowing of ammonia production, but also a lack of ammonia utilization during anaerobiosis. This observation is consistent with the decreased activity of the ammonia-utilizing/glutamate-producing reaction of GDH seen in this study. Reduced glutamate synthesis seems intuitive since it is well established that protein synthesis is almost completely switched off during anoxia, and thus amino acid synthesis should also be suppressed [10,25].

Also supporting the idea that GDH activity is reduced during anoxia is the enzyme's response to effector molecules. For instance, Table 3 shows that purified anoxic GDH is much more sensitive to GTP inhibition than control GDH. Furthermore, purified anoxic GDH is significantly less sensitive to activation by ADP, AMP, and lactate when compared to control GDH. This decreased sensitivity to adenosine nucleotides and lactate, and increased sensitivity to GTP likely corresponds to decreased GDH activity under anoxia as compared to normoxic conditions.

The majority of the kinetic properties assessed were consistent with a less active form of GDH under anoxia, but there are some deviations

doi: 10.7243/2050-0874-1-3

from this trend. The first to be considered is the substantial decrease in the $K_m$ α-ketoglutarate during anaerobiosis (Table 2). However, the apparent activation of GDH that this could offer may be inconsequential *in vivo* due to two factors. First, estimates from rat hepatocytes under normoxic conditions indicate that mitochondrial α-ketoglutarate concentrations are approximately 0.3 μmol/g dry weight (~0.06 mM) [26], however, this number decreases dramatically during anoxia. For instance, Bowman [27] reported a 90% drop in α-ketoglutarate concentration during anoxia in rat heart. He proposed that the large decrease in α-ketoglutarate was the result of a decrease in the $NAD^+$: NADH ratio which consequently inhibited isocitrate dehydrogenase (i.e. NADH inhibits through product inhibition). Furthermore, the typical ammonia concentrations found during anoxia are low whereas the $K_m$ $NH_4^+$ is so high that the ability of GDH to synthesize glutamate is very likely limited by ammonia levels. Taken together it is unlikely that the significant decrease in $K_m$ α-ketoglutarate has any physiological significance during anaerobiosis in the freshwater turtle.

Similar to the case with α-ketoglutarate, the $K_m$ $NAD^+$ in the presence of ADP was significantly reduced during anoxia when compared to the normoxic condition (Table 2). It is important to note that without added ADP, the $K_m$ $NAD^+$ did not change between control and anoxic conditions. Thus, it appears that ADP binding to purified GDH caused a conformational change in the enzyme that increased its affinity for $NAD^+$ during anoxia. Again, the physiological significance of this increased affinity is limited due to the typical mitochondrial concentration of $NAD^+$ (4-6 mM in mitochondria of rat hepatocytes) [28]. At these $NAD^+$ concentrations, GDH would be saturated with the cofactor, potentially making the actual $K_m$ values irrelevant *in vivo*. However, the kinetic difference identified here as well as other kinetic changes listed above do indicate a structural difference between control and 20 h anoxic GDH.

To further support the idea of GDH existing in two different physical forms between control and anoxic conditions, protein stability was assessed through the effects of common denaturants urea and guanidine hydrochloride, as well as KCl. In all cases, these molecules elicited a different response in control and 20 h anoxic GDH activity. For instance, $C_{50}$ urea for anoxic GDH was 25% higher when compared with the control condition. Furthermore, $K_a$ GnHCl and $K_a$ KCl were 19% and 48% lower, respectively, for anoxic GDH as compared to control GDH (Table 4). Although the concentrations at which all of these effector molecules alter GDH activity are well above physiological concentrations, the different effects between control and anoxic conditions support the existence of two forms of GDH between the two states.

Anaerobic metabolism typically leads to tissue acidification, and low pH conditions will affect the functioning of all metabolic enzymes [20]. Changes in pH alter the charge on amino acids and cause changes in enzyme-substrate affinities as well as enzyme conformation [29]. Figure 4 shows $V_{max}$ ratios (forward reaction $V_{max}$/reverse reaction $V_{max}$) for turtle liver GDH at pH 6.6 and 7.4. For the high-phosphate control form of GDH, the $V_{max}$ ratios with and without ADP did not change significantly between anoxic and normoxic conditions. However, for the low-phosphate anoxic form of GDH, acidification led to a strong decrease in the $V_{max}$ ratio. This decrease was the result of a significant decrease in the forward reaction $V_{max}$ at anoxic pH values. A similar effect was seen when the assays of the anoxic enzyme were performed in the presence of ADP. The negative effect of acidification on the forward reaction of anoxic GDH coincides with the fact that under anoxia shuttling carbon skeletons into the Krebs cycle would be uneconomical. The significant differences between $V_{max}$ ratios for control and anoxic GDH are further evidence that GDH exists in two distinct enzyme forms.

**Table 4. Structural stability of crude liver GDH from control and 20 h anoxic turtles as assessed by susceptibility to urea, GnHCl, and KCl effects.**

| | Control | | 20 h Anoxic | |
|---|---|---|---|---|
| | Ka (M) [Fold Activation] | $C_{50}$ (M) | Ka (M) [Fold Activation] | $C_{50}$ (M) |
| Urea | n.a. | 0.91 ± 0.03 | n.a. | 1.14 ± 0.07a |
| GnHCl | 0.58 ± 0.03 [2.7 ± 0.2] | 1.76 ± 0.04 | 0.47 ± 0.02a [2.16 ± 0.04a] | 0.47 ± 0.02a [2.16 ± 0.04a] |
| KCl | 2.5 ± 0.3 [2.4 ± 0.2] | n.a. | 1.3 ± 0.1a [2.25 ± 0.04] | n.a. |

Note: Assays were performed in the forward direction. Data are means ± SEM, n = 3 independent determinations on crude enzyme preparations from separate turtle liver samples. Ka and C50 values were determined at optimal substrate concentrations. a-Significantly different from corresponding control value using the unpaired Student's t-test, P<0.05. n.a. is not applicable.

## Conclusions

This paper indicates that GDH purified from control versus 20 h anoxic liver has different kinetic properties, and in general, the anoxic form of GDH was less active in both the forward and reverse directions. Via ProQ Diamond phosphoprotein staining and specific protein phosphatase incubations it appears that the transition to a less active GDH during anoxia occurs through reversible phosphorylation of the enzyme, the control form being a high phosphate enzyme (that is susceptible to the action of protein phosphatases) and the anoxic form being a low phosphate form. In addition to phosphorylation, other significant factors may be involved in regulating GDH activity *in vivo*. This includes the action of several energy molecules (AMP, ADP, and GTP) and the acidification of tissues during anoxia. Several of these factors appear to act in concert to decrease GDH activity in an effort to prevent both the uneconomical build-up of Krebs cycle intermediates and wasteful synthesis of amino acids during anoxia. The overall suppression of GDH activity identified here coincides with overall metabolic rate depression observed during anoxia in the freshwater turtle.

doi: 10.7243/2050-0874-1-3

**Competing Interests**

The Author declare that they not have any competing interests.

**Author's Contributions**

RAVB performed all of the experimental work for this study as well as all of the analysis of the results. KBS conceived the study and gave substantial contributions to the design of the study. All authors have read and approved the final manuscript.

**Acknowledgments**

Thanks to J.M. Storey for editorial commentary on the manuscript. The research was supported by a discovery grant from the Natural Sciences and Engineering Research Council of Canada (OPG 6793) to KBS and by an NSERC CGS-M postgraduate scholarship to RAVB.

**Article history**

Received: 17-Jan-2012 Revised: 25-Feb-2012 Re-revised: 17-Mar-2012
Accepted: 20-Mar-2012 Published: 02-Apr-2012

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**Citation:**
Bell R A and Storey K B: **Regulation of liver glutamate dehydrogenase from an anoxia-tolerant freshwater turtle**. *HOAJ Biology* 2012, **1**:3. http://dx.doi.org/10.7243/2050-0874-1-3