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# Regulation of tail muscle hexokinase in the anoxia-tolerant freshwater crayfish, Orconectes virilis

Neal J. Dawson[1]* and Kenneth B. Storey[1]

## Abstract

Hexokinase (HK)(E.C.2.7.1.1) is the enzyme responsible for catalyzing the first step of glucose metabolism, the phosphorylation of glucose to glucose-6-phosphate (G6P). The present study investigated HK from tail muscle of the freshwater crayfish Oconectes virilis exploring changes to kinetic properties, phosphorylation levels and structural stability between two forms of the enzyme (aerobic control and 20 h anoxic). Evidence indicated that HK was converted to a low phosphate form under anoxia. ProQ Diamond phosphoprotein staining showed a 39% higher bound phosphate content on aerobic HK compared with the anoxic form, yet treatment of aerobic HK with treatments that stimulated the activities of different endogenous protein phosphatases (stimulating PP1+PP2A, PP2B, and PP2C) yielded no significant changes in kinetic parameters. By contrast, investigation of the stability and bound fractions of aerobic verses anoxic HK yielded stark differences in both susceptibility to urea denaturation and subsequent proteolytic cleavage, as well as a decrease in the amount of enzyme in the bound state. The physiological consequence of anoxia-induced HK dephosphorylation may be to stabilize and release HK during anoxia, increasing the glycolytic capacity of the animal.

## Background

Hexokinase (HK)(E.C.2.7.1.1) is the enzyme responsible for catalyzing the first step of glucose metabolism, the phosphorylation of glucose to glucose-6-phosphate (G6P):

$$\text{D-Glucose} + \text{ATP} \rightarrow \text{D-Glucose-6-phosphate} + \text{ADP} + \text{H}^+$$

Glucose is a key source of energy for living organisms and is delivered by the blood to all organs of the body. Once transported into cells, glucose is rapidly phosphorylated by HK to form G6P and this allows the sugar to be directed into many different pathways such as; glycolysis to produce ATP, the pentose phosphate pathway to form NADPH and various sugar phosphates, or glycogen as a fuel storage [1]. There have been 4 isozymes reported in mammals, HK I-IV, which are found in different tissues and locations within the cell [2,3]. HK IV, otherwise known as glucokinase, is liver-specific and is primarily responsible for storing excess sugar into glycogen reserves; it has a high $K_m$ for glucose. HK I-III all share a similar molecular weight (~100 kDa) and much lower $K_m$ values for glucose (< 1 mM). HK I-III have been separated by ion exchange chromatography and isoelectric focusing [2,3]. HK isozymes I-III can bind to the outer membrane of mitochondria via an association with the porin that is located on the outer surface of the mitochondria [4]. The isozymes exhibit different kinetic parameters such as their substrate affinities for ATP and glucose, as well as their susceptibility to product inhibition by G6P [1].

Crayfish have a significant capacity for long term survival under anoxic conditions by switching to anaerobic glycolysis as their primary ATP-generating pathway and buffering lactate accumulation by $Ca^{2+}$ release from their carapace [5]. Past studies on the regulation of glycolytic enzymes have demonstrated that, in cancer, a delicate interaction between the transcription factors MYC and HIF cause a differential expression of HK II [6]. As MYC levels decrease and HIF expression increase under low oxygen conditions, HK is upregulated. In addition to altered amounts of HK protein under low oxygen conditions, HK could also be regulated by posttranslational or allosteric mechanisms to alter its activity and/or function under high versus low oxygen conditions. Given the well-developed anaerobic capacity of crayfish, this model would be a good one in which to assess the effects of anoxia on HK regulation, particularly in light of the results from previous work showing anoxia-responsive regulation of other key enzymes including arginine kinase and glutamate dehydrogenase in crayfish muscle [7,8].

Previous studies in our lab have shown that one of the mechanisms of HK regulation in response to stress is reversible protein phosphorylation. HKI and II from the skeletal muscle of hibernating ground squirrels [9] and HK from the skeletal muscle of freeze-tolerant frogs [10] was shown to be regulated by reversible phosphorylation. Given the role of HK in gating glucose entry into glycolysis and the particular importance of HK to energy metabolism under anoxic conditions, as well as the evidence from previous studies of differential regulation of HK in response to stress, the current work investigates the regulation of HK during anoxia in the tail muscle of *O. virilis*.

## Methods

### Experimental animals and tissue sampling

Freshwater crayfish, *Orconectes virilis*, were obtained from local area bait shops in Ottawa, Ontario. Crayfish are placed in freshwater tubs (10 L of aerated water) and acclimated to 15°C for 7 d in incubators. Animals were then separated into two groups. One group was maintained as above (normoxic conditions), whereas the second group were transferred into plastic tubs with closed lids that were fitted with two

Correspondence: ndawson@connect.carleton.ca
[1] Institute of Biochemistry & Department of Biology, Carleton University, 1125 Colonel By Drive, Ottawa, Ontario, Canada.

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ports, one to accommodate a nitrogen gas bubbler, and the other to vent gas (anoxic conditions). These tubs had been previously bubbled with nitrogen gas for 45 min before introducing the crayfish and then nitrogen bubbling was continued throughout a 20 h anoxia exposure (final oxygen content in the water was <1 torr). This model may well mimic the natural experiences of the crayfish when they are exposed to hypoxic and anoxic waters during different seasons. For sampling, animals were killed by severing the head and then tail muscle was quickly harvested, immediately frozen in liquid nitrogen, and stored at -70°C.

**Chemicals**

Chemicals, biochemicals, chromatography media and coupling enzymes were from Sigma Chemical Co. (St. Louis, MO) whereas primary and secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) and ProQ Diamond Phosphoprotein stain was from Invitrogen (Eugene, OR).

**Preparation of Tissue Extracts**

Samples of frozen tail muscle or hepatopancreas were homogenized 1:5 w:v in ice-cold buffer A: 50 mM Tris buffer, pH 7.5 containing 50 mM NaF, 2.5 mM EGTA, 2.5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Other tests showed that β-glycerophosphate yielded worse HK activity compared with NaF and that good long term stability of muscle HK over time was not altered in the presence/absence of additional protease inhibitors. Tail muscle homogenates were centrifuged at 13 500 x g at 4°C and the supernatant was decanted; both the supernatant and the pellet were held on ice until use.

To assess the kinetic properties of membrane bound HK, pellets from crude extracts were resuspended in buffer A, recentrifuged, and the supernatant was removed. This was repeated three times to ensure that there was no contaminating cytosolic HK. The resulting pellet was then resuspended in 1 mL of homogenization buffer and HK was assayed in the pellet fraction.

**Sephadex G-50 filtration of crude extracts**

Low molecular weight metabolites and ions were found to interfere with both the purification and kinetic assay of HK. These were removed by Sephadex G-50 gel filtration. A 5 cm column of Sephadex G-50 in a syringe barrel was equilibrated in buffer B (12.5 mM Tris-HCl buffer pH 9.0, 25 mM NaF, 2.5 mM EGTA, 2.5 mM EDTA, 10 mM b-mercaptoethanol) and centrifuged at 500 g in a bench-top centrifuge for 2 min to remove excess buffer. Then a 500 µL aliquot of tail muscle extract was applied to the column and centrifuged again. The resulting eluant was collected.

**DEAE ion exchange chromatography**

Ion exchange chromatography was used to purify both control and anoxic forms of HK. Muscle extracts were prepared 1:5 w:v in buffer B. An aliquot of crude extract was applied to a DEAE Sephadex G50 column (1.5 cm x 5 cm) equilibrated in the buffer mentioned above. The column was washed with this same buffer and then eluted with a linear KCl gradient (0-1 M) in the same buffer and peak fractions were pooled.

**Cibacron Blue chromatography**

The peak fractions from the DEAE Sephadex G50 column were pooled and applied to a Cibacron Blue 3GA column (1.5 cm x 5 cm) and eluted under the same conditions as the DEAE column. After both the DEAE and Cibacron Blue steps, the aforementioned spun column protocol was used to remove excess salt from the preparation before assay or application of the enzyme to the next column. Peak fractions were pooled. The purity of HK at each step was checked by running samples on SDS-PAGE (as described for Western blotting) with Coomassie blue staining.

**HK assay**

HK activity was measured as the rate of ADP production coupled to an assay system with glucose-6-phosphate dehydrogenase. Optimal assay conditions for HK were found to be 50 mM Tris buffer pH 7.5, 10 mM Mg.ATP, 10 mM glucose, 10 mM $MgCl_2$, 1.5 mM NADP and 1 unit G6PDH. Enzyme activity was assayed with a Thermo Labsystems Multiskan spectrophotometer at an absorbance of 340 nm. Data was analyzed using Kinetics v.3.5.1 program [11]. One unit of HK activity is defined as the amount that utilizes 1 µmol of glucose per minute at 23°C.

Protein concentrations in tail muscle extracts were determined using the Coomassie blue dye-binding method with the BioRad prepared reagent and bovine serum albumin as a standard.

**In vitro Incubation to Stimulate Protein Kinases and Phosphatases**

Samples of tissue extracts, prepared as previously described, were filtered through a G50 spun column equilibrated in buffer A. Aliquots of the filtered supernatants were incubated for 12 hours at 4°C with specific inhibitors and stimulators of protein kinases and phosphatases, as described in Macdonald and Storey [12]. Each aliquot was mixed 1:2 v:v with the appropriate solutions to stimulate either protein kinases or phosphatases. Each solution was prepared in a incubation buffer (50 mM Tris, 10% v:v glycerol, 30 mM β-mercaptoethanol, pH 7.5) and the following three incubation conditions were used:

(a) STOP conditions: 2.5 mM EGTA, 2.5 mM EDTA and 30 mM β-glycerophosphate.

(b) Stimulation of endogenous kinases: 5 mM Mg-ATP, 30 mM β-glycerophosphate,1 mM cAMP (to stimulate PKA), 1 mM cGMP (for PKG), 1.3 mM $CaCl_2$ +7 µg/mL PMA (for PKC), 1 mM AMP (for AMPK), and 1 U of calf intestine calmodulin + 1.3 mM $CaCl_2$ (for CaMK).

(c) Stimulation of endogenous phosphatases: 5 mM $CaCl_2$ and 5 mM $MgCl_2$.

β-glycerophosphate was used instead of NaF during incubations, as NaF caused precipitation and ultimately failure of the incubation procedure. After incubation, low molecular weight metabolites and ions were removed from the extracts by centrifugation for 2 min at 2000 rpm through small spun columns of Sephadex G50 equilibrated in buffer A.

**Western blotting of HK**

Tail muscle samples were gently homogenized (1:3 w/v) with a

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ground glass homogenizer in buffer D (25 mM Tris, pH 7.6, 25 mM NaCl, 100 mM sucrose, 1% w/v SDS), centrifuged 13 500 x g at 4°C and the supernatant was removed. Soluble protein concentration was measured by the Coomassie blue dye binding method. Tail muscle extracts 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-mercapotethanol) and subsequently boiled for 5 min and stored at -20°C until used. Lanes of 10% SDS-PAGE gels were loaded with 20 µg of protein and electrophoresis was carried out at 180 V for 45 min in running buffer (0.05 M Tris, 0.5 M glycine, 0.05% w/v SDS). Proteins were transferred to polyvinylidene difluoride membranes at 70 V overnight. Membranes were then blocked with 5% non-fat dried milk in Tris-buffered saline containing Triton-X (TBST) for 1h and washed three times with TBST. Membranes were then incubated with primary antibody (1:3000 dilution) against muscle HKII (HK goat polyclonal IgG) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 10 ml of TBST overnight at 4°C. After washing with TBST, membranes were incubated with anti-goat IgG secondary antibody (1:3000 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1h and then washed. Immunoreactive bands were visualized by enhanced chemiluminescence and visualized on the ChemiGenius Bioimaging System (Syngene, Frederick, MD). Band intensities were quantified using GeneTools software. Coomassie blue staining was used to confirm equal loading of the lanes and to standardize immunoblotting band intensities.

**ProQ Diamond Phosphoprotein Staining**

HK was purified from tail muscle of both normoxic and anoxic crayfish as described above using affinity chromatography. The top three fractions were pooled, and protein levels in the pooled fractions were quantified using the Coomassie blue dye-binding method. Aliquots of the pooled fractions were then prepared for electrophoresis as above. Aliquots containing 0.5 µg protein were loaded in each well of a 10% SDS-PAGE gel and electrophoresis was carried out as above. The gel was removed and washed in fixing solution (50% v/v methanol, 10% v/v acetic acid) twice for 10 min, then left in fixing solution overnight at ~4°C followed by 3 washes with $ddH_2O$ for 10 min. The gel was then stained with ProQ Diamond Phosphoprotein stain for 90 min and washed. The gel was covered with aluminum foil during staining (and for the remainder of the protocol) to prevent the photosensitive stain from interacting with light. After staining, bands on the gel were visualized using the ChemiGenius to assess the relative intensities of the fluorescent bands. The fluorescence of the bands was quantified using the accompanying GeneTools software. Due to background on the gels which caused distortion or interference in quantifying bands, gels were subsequently washed in ProQ Diamond destaining solution (20% v/v acetonitrile, 50 mM sodium acetate, pH 4) for 45 min, washed 3 times in $ddH_20$ for 10 minutes, and reimaged.

**Pulse Proteolysis of HK**

Crayfish tail muscle tissue was homogenized in buffer A as previously described, without the inclusion of PMSF. To assess the possibility of structural/conformational changes in HK between normoxic and anoxic conditions, the susceptibility of HK to denaturation by urea was evaluated. Aliquots of 20 µl of crude muscle extracts were incubated with different concentrations of urea for 24 h at 4°C in 100 µl of buffer A without PMSF. After incubation, HK was subjected to pulse proteolysis as outlined by Park and Marqusee [13] to cleave unfolded denatured enzyme. To do this, thermolysin (stock prepared in 50 mM Tris, pH 8.0, 2.5 M NaCl, 10 mM $CaCl_2$) was added to a final concentration of 0.40 mg/ml and samples were incubated for 10 min (incubation time was optimized in initial tests). The reaction was then quenched by adding 18 µl of 50 mM EDTA, pH 8.0. Samples were prepared for Western blotting as above and band intensities were quantified to reveal the amount of folded native HK remaining. From this, $C_{50}$ values were calculated, representing the amount of urea that reduced the amount of folded protein by one-half.

## Results

**Optimization of experimental conditions**

The various components in the homogenization buffers, and assay conditions were evaluated prior to determining kinetic parameters of HK from crayfish tail muscle. It was determined that the inclusion of EGTA and EDTA to the homogenization buffer did not reduce the recoverable activity of HK in the tail muscle of crayfish in either the control or anoxic conditions. The inclusion of the protein phosphatase inhibitor, β-glycerolphosphate, significantly decreased the recoverable activity in comparison to NaF which showed negligible changes in activity. Therefore, NaF, EGTA and EDTA were included in the standard homogenizing buffer. The pH optimum for HK was determined to be pH 7.5 and activity remained fairly stable over a large pH range (7.0-9.0).

**HK Purification**

The purification scheme for tail muscle HK from crayfish is shown in Table 1. Two affinity columns, $DEAE^+$ Sephadex and Cibacron Blue, were used. Tail muscle HK was purified 10.1 fold with an overall yield of 53.2%. The final specific activity of enzyme was 14.46 U/mg protein. The success of each purification step was assessed by electrophoresis on an SDS-PAGE gel stained with Coomassie blue staining (Figure 1). The end result of the purification showed that the enzyme was purified to homogeneity as there is only one strong band corresponding to HK at the correct molecular weight of ~100 kDa [2,3]. The same purification protocol was used to purify HK from anoxic crayfish tail muscle.

**HK Kinetics**

Kinetic parameters of tail muscle HK were assessed to determine any differences between control and anoxic forms. Kinetic parameters changed significantly in response to anoxia. As compared to control conditions, the HK $K_m$ for glucose increased by 36 % in anoxic muscle and, although $K_m$ ATP did not change, ATP kinetics showed a shift from a sigmoidal velocity versus [ATP] relationship to Michaelis Menten kinetics (h=2.06 for control, h=1.33 for anoxic) (Table 2). Furthermore, HK $V_{max}$ increased by 2.74 fold under anoxia.

Muscle HK is known to bind to mitochondria and as a result, some percentage of muscle HK will undoubtedly remain in the pellet after

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**Table 1**. A typical purification of HK from tail muscle of control crayfish

| | Total Protein (g) | Total activity (U) | Specific Activity (U/mg) | Fold purification | Yield (%) |
|---|---|---|---|---|---|
| Crude extract | 1.925 | 1382.45 | 1.44 | - | 100 |
| DEAE+ Sephadex | 0.554 | 1003.08 | 1.81 | 2.57 | 72.6 |
| Cibacron Blue | 0.051 | 735.86 | 14.46 | 20.52 | 53.2 |

**Table 2**. Comparison of kinetic parameters of purified HK from tail muscle of anoxic and control crayfish assayed at 23°C. Data are means ± SEM, n = 4 preparations of HK from muscle of different crayfish.
*- Significantly different from the corresponding control value using the Student's t-test, P<0.05.

| | Control | Anoxic |
|---|---|---|
| $V_{max}$ (U/g wet weight) | 19.34 ± 0.58 | 52.9 ± 1.53* |
| $K_m$ Glucose (mM) | 0.11 ± 0.01 | 0.15 ± 0.01* |
| $K_m$ ATP (mM) | 0.36 ± 0.02 | 0.32 ± 0.01 |
| Hill Coefficient | 2.06 ± 0.03 | 1.33 ± 0.07* |
| $C_m$ Urea (M) | 4.48 ± 0.36 | 5.97 ± 0.51* |

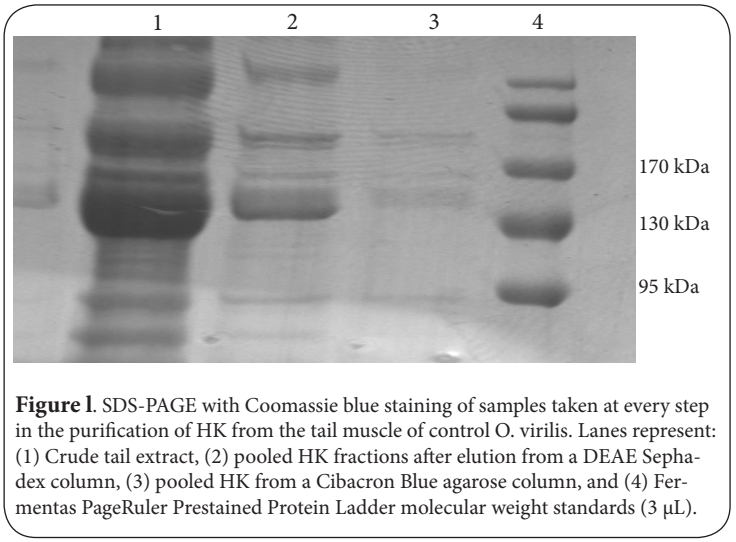


**Figure 1**. SDS-PAGE with Coomassie blue staining of samples taken at every step in the purification of HK from the tail muscle of control O. virilis. Lanes represent: (1) Crude tail extract, (2) pooled HK fractions after elution from a DEAE Sephadex column, (3) pooled HK from a Cibacron Blue agarose column, and (4) Fermentas PageRuler Prestained Protein Ladder molecular weight standards (3 µL).

homogenization and centrifugation. A change in the amount of bound HK between control and anoxic states could potentially be the cause of the above-mentioned large increase in HK activity in soluble extracts under anoxic conditions. After removal of soluble HK in the supernatant after the first centrifugation, the pellet was resuspended, recentrifuged and washed three times (the washes were added to the soluble fraction) and then the pellet was resuspended a final time and activity of HK in the bound fraction and the soluble fraction was assessed. Figure 2 shows that there was a significant decrease (P<0.05) in the amount of bound HK activity during anoxia (11.3% of total ) as compared to control aerobic conditions (30.9% bound).

**HK protein levels**

Western blotting was used to evaluate the relative amount of HK protein in tail muscle from control versus anoxic crayfish. Antibodies detecting mammalian HK crossreacted with the crayfish protein and showed one strong band at the expected molecular weight of ~100 kDa for HK [2,3]. A comparison of control versus anoxic conditions showed a significant increase in HK protein content of 43% under anoxia (Figure 3).

**Phosphorylation State of HK**

High and low phosphate forms of enzymes can be resolved using ion exchange chromatography due to a change in net charge of an enzyme arising from the addition of negatively charged phosphate groups onto an enzyme when it is phosphorylated. Figure 4 shows the elution patterns for both aerobic control and anoxic HK activity after chromatography on a DEAE-Sephadex column with elution using a 0-1 M KCl gradient. HK from aerobic control crayfish muscle eluted in one large broad peak between the 45$^{th}$ and 58$^{th}$ fractions, whereas the anoxic form of HK eluted earlier on the salt gradient between the 35$^{th}$ and 47$^{th}$ fractions. This indicates a substantial change in the net charge state of the enzyme between control and anoxic conditions, probably due to a change in the amount of covalently bound phosphate on the protein.

To test whether or not reversible phosphorylation was the mechanism by which HK kinetic parameters had changed, the top 3 fractions from the control and anoxic peaks eluted off the above DEAE columns were pooled and aliquots containing equal amounts of soluble protein were loaded onto gels and subjected to SDS-PAGE, followed by staining with ProQ Diamond phosphoprotein stain. HK was easily identified since the extract was previously purified, and a strong band was seen ~100 kDa. Phosphoprotein staining showed a strong 39% decrease in the relative band intensity for the purified anoxic HK when compared to the control condition (p<0.05) (Figure 5).

**In vitro Incubation to Stimulate Protein Kinases and Phosphatases**

To investigate the possibility of phosphorylation as a regulatory mechanism for HK in crayfish tail muscle, crude enzyme from both control and anoxic muscle were incubated under conditions that stimulated either endogenous protein kinases or protein phosphatases and compared with incubations where both kinases and phosphatases were inhibited (denoted STOP). Results for the STOP condition showed a significant difference (p<0.05) in the $K_m$ of glucose for HK between the control and anoxic conditions and these STOP conditions served as the basis for evaluating the effects of incubations that promoted the activities of total endogenous protein phosphatases (PP1+PP2A, PP2B, PP2C) and total endogenous

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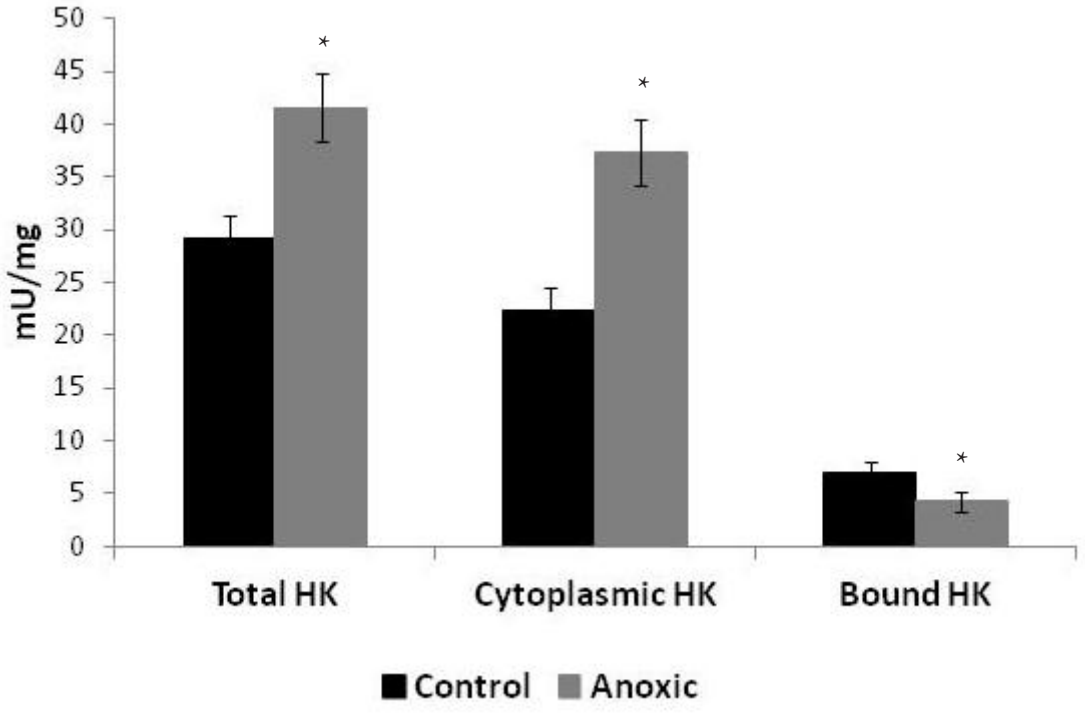


**Figure 2**. Distribution of HK between cytoplasmic and bound states in tail muscle extracts of control and anoxic crayfish. Total HK is cytoplasmic HK + bound HK. Data are expressed in milliunits per mg (mU/mg) protein, means + SEM, n= 3. *- Significantly different from the corresponding control value, $P < 0.05$.

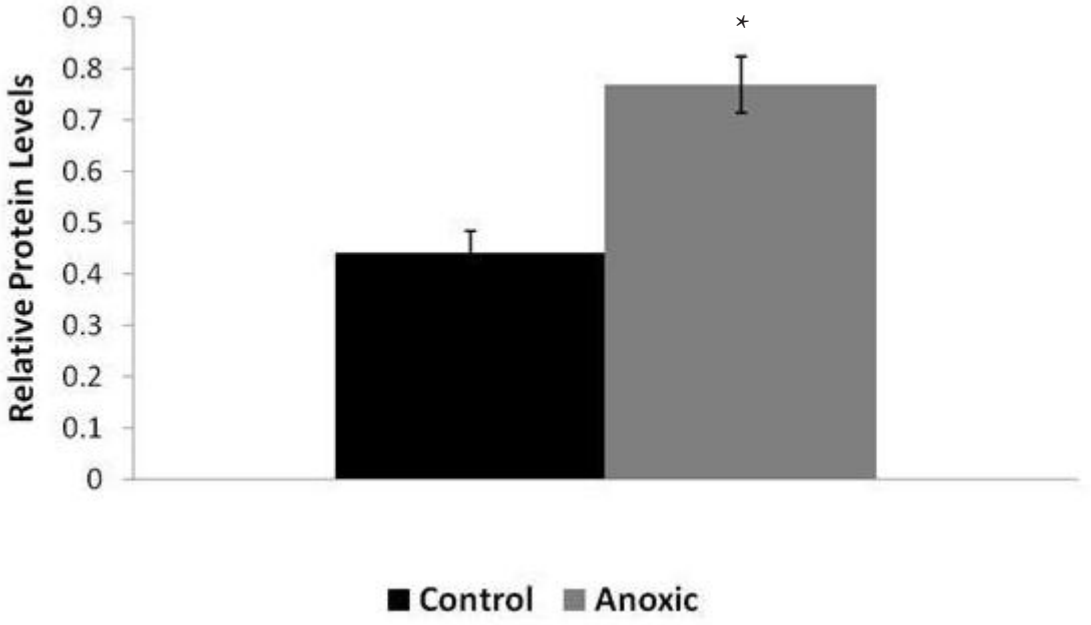


**Figure 3**. Western blotting analysis of the amount of HK protein in the tail muscle of O. virilis showing upregulation of protein amount from control to anoxic conditions. Data are means + SEM, n=3. Coomassie blue staining was used to standardize the amount of protein in each band. * - Significantly different from the corresponding control value via the Student's t-test, $p<0.05$.

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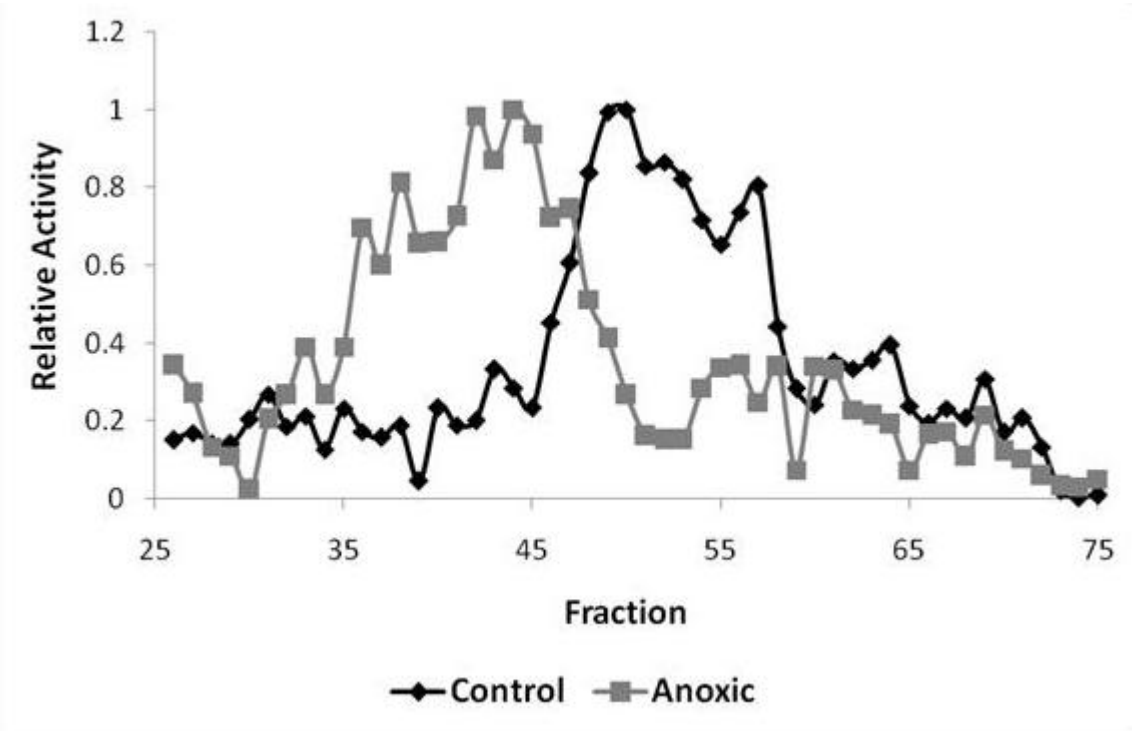


**Figure 4**. DEAE+ Sephadex elution profiles for HK activity from tail muscle from normoxic and anoxic crayfish. HK was eluted using a 0-500 mM KCl gradient in column buffer at pH 9.0. Activity profiles are expressed relative to activity in the peak tube. Control and anoxic extracts were run separately and then activity profiles were superimposed in one graph. The data are representative of n=3 trials on separate preparations of enzyme..

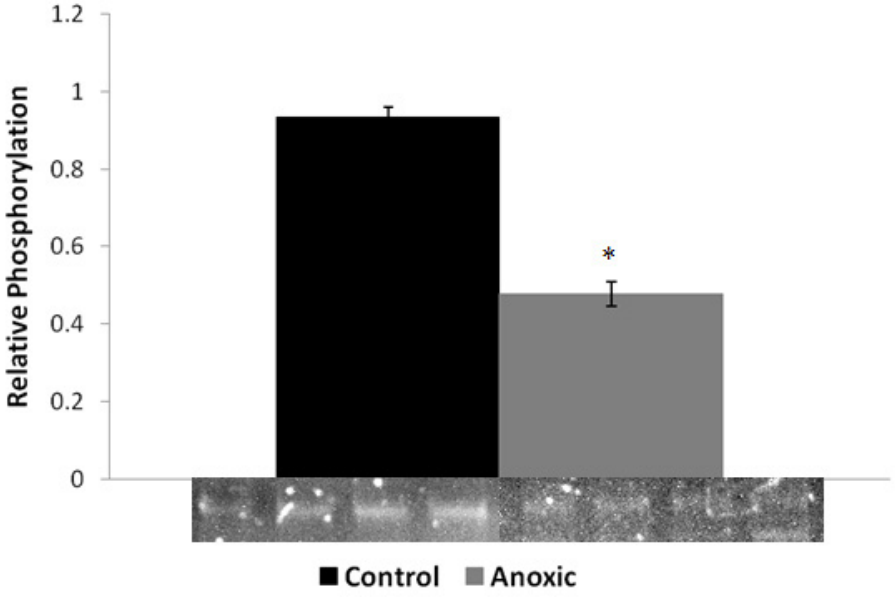


**Figure 5**. A comparison of the relative phosphorylation state of control and anoxic HK from the tail muscle of O. virilis using Pro-Q diamond phospho-staining. Data are means + SEM, n=4. Coomassie blue staining was used to standardize the amount of protein in each band. * - Significantly different from the corresponding control value via the Student's t-test, $p<0.05$.

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protein kinases (AMPK, PKA, PKC, PKG, CaMK). However, neither total phosphatase nor total kinase stimulations significantly altered the $K_m$ for glucose of either control or anoxia HK as compared to the corresponding STOP conditions (Figure 6).

**Structural Stability of Crayfish Tail Muscle HK**

The structural stability of HK was evaluated by testing the enzyme sensitivity to urea denaturation followed by proteolysis with thermolysin to degrade unfolded denatured enzyme. The amount of native protein remaining was then assessed by Western blotting. The calculated $C_m$ value (concentration of urea that results in 50% loss of folded HK) was 5.97 M urea for HK from anoxic tail muscle, which was significantly higher in comparison to the mean value of 4.48 M urea for HK from control tail muscle ($P<0.05$) (Table 2; Figure 7).

## Discussion

During exposure to severe hypoxia or anoxic conditions, organisms that have evolved to tolerate such exposures, cope with oxygen limitation by entering a hypometabolic state where ATP demand and metabolic fuel use are greatly reduced. By doing so, they greatly extend the time that body fuel reserves can support survival and, in the case of oxygen deprivation, lower metabolic rate to a level that can be supported by the ATP output of anaerobic pathways (chiefly glucose or glycogen catabolism by glycolysis ending in lactate). Of particular interest is the regulation of those pathways responsible for fuel catabolism and ATP generation. Most anoxia tolerant species show a switch from aerobic metabolism that is probably primarily based on lipid catabolism to anaerobic metabolism that must be based primarily on carbohydrate fermentation. Many invertebrates have boosted their capacity for anaerobic ATP production by making use of additional fermentable substrates (some amino acids) and linking the output of glycolysis (pyruvate) into further reactions that yield extra ATP while producing end products including succinate, propionate and acetate [14]. However, this is not true of crustaceans, so their survival relies on anaerobic glycolysis alone with lactate as the end product. Some enhancements improve the capacity for anaerobic ATP production (e.g. large stores of glycogen fuel, methods for buffering lactate production) and metabolic rate depression lowers the ATP demand. Muscle tissue is a prime candidate for requiring a basal level of metabolism under anoxia to maintain basal cell function, to sustain the transmembrane ion gradients that support contraction by tail muscle should the animal need to flee danger, and to remain prepared for recovery from the anoxic state.

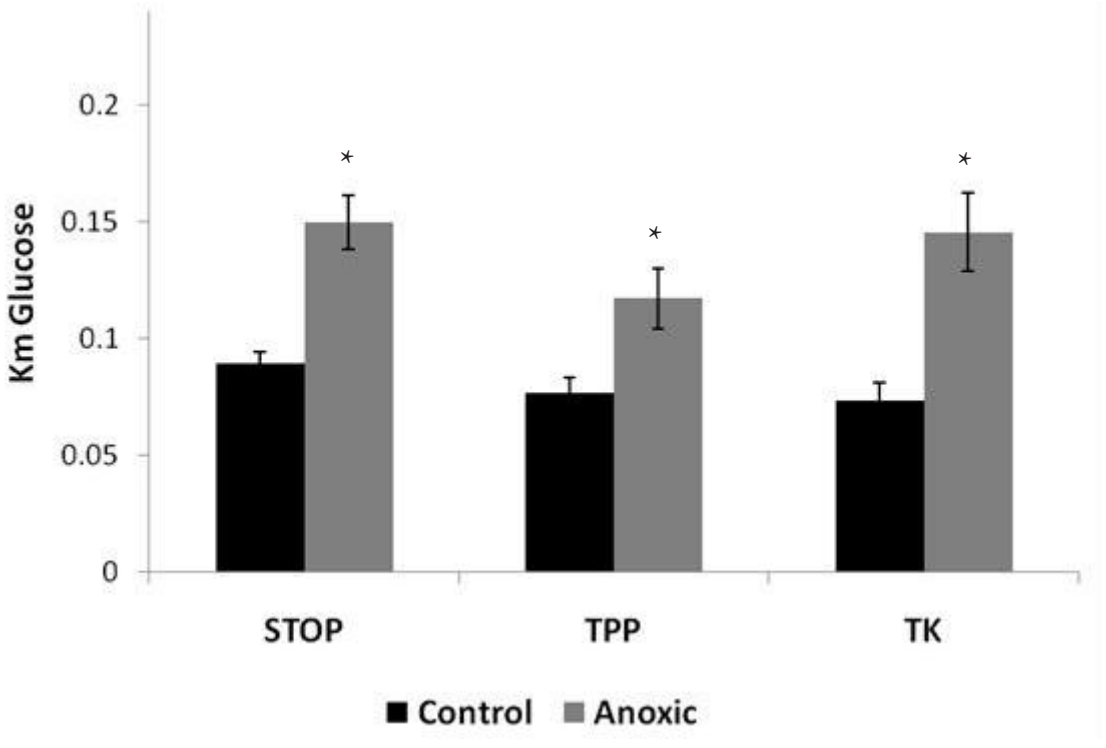


**Figure 6**. Effects of in vitro incubations that stimulated the activities of endogenous protein kinases or protein phosphatases on the Km of glucose of HK from O. virilis tail muscle extracts. Crude extracts were incubated at 4°C for 24 h before assay. Data are means + SEM, n=3. Stop conditions contained inhibitors of all protein kinases and phosphatases. Other incubations stimulated total phosphatases (TPP) or total kinases (TK). * - Significantly different from the corresponding control value via the Student's t-test, $p<0.05$.

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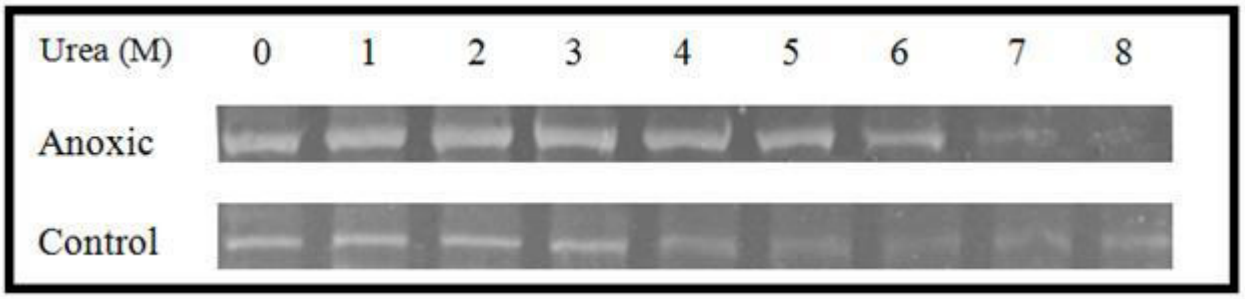


**Figure 7**. Stability of HK from crude extracts of tail muscle. Samples were incubated for 24 h at 4°C with different concentrations of urea and then treated with pulse proteolysis (10 min incubation with thermolysin) to digest denatured HK. Western blotting was then used to measure the amount of remaining folded HK. Figure shows representative results from a total of 3 independent experiments.

HK is the first enzyme involved in the breakdown and use of glucose as a fuel; therefore, the regulation of HK is vital to understanding carbohydrate metabolism during anoxia. At first glance, the kinetic parameters of HK in the tail muscle of the freshwater crayfish, *O. virilis*, seem to indicate that HK is less active during the anoxic state, since there is a significant (~36%) increase in the $K_m$ glucose in transitioning from the control to anoxic state (Table 2). However, a large 2.74-fold increase in the maximal activity of HK also occurs during anoxia. This dramatic increase in enzyme activity is indicative of a major change in the regulation of HK. Such a change in maximal activity could be based on allosteric regulation, transcriptional or translational regulation, post-translational modifications, or changes in the distribution of HK between free and bound states. In previous studies, increased activity of HK has been observed during hypoxia or anoxia in many different species and tissues such as; anoxic rat heart muscle [15], hypoxic pancreatic or liver cancer cells [16,17], anoxic plant seedlings [18], as well as anoxic maize roots [19].

Western blotting was implemented to determine if the large, 2.74, increase in HK enzymatic activity during anoxia was due to a change in HK protein expression. The results showed a significant increase in the amount of HK protein in muscle under anoxic conditions in comparison to control conditions (Figure 3). An up-regulation of HK gene expression to produce more HK protein under low oxygen conditions is conducive to a potential enhancement of glycolytic potential [6], which agrees with the needs of an anoxic crayfish. In the same study, a cooperative activation of both HKII and pyruvate dehydrogenase kinase 1 (PDK1) (which inhibits pyruvate dehydrogenase to shut down pyruvate use by mitochondria) were shown to occur in hypoxic cancer cells, ultimately resulting in an increase in the glycolytic conversion of glucose to lactate. These results are indicative of an increase in anaerobic metabolism during low oxygen events and this also seems to be the case for crayfish muscle.

Although there is strong evidence to support a translational regulation of HK in crayfish muscle in response to anoxia, this does not account for the changes in kinetic parameters discovered in the anoxic state. Therefore, further exploration into the regulation of HK is required. Kinetic parameters can often be manipulated via posttranslational modification of an enzyme, often by reversible phosphorylation. Through the use of ion exchange chromatography, two distinct elution profiles were seen for HK from anoxic and control muscle samples, suggesting that there are two forms of crayfish muscle HK that differ in their charge state (Figure 4). The enzyme from anoxic tail muscle eluted from DEAE Sephadex at a lower ionic strength than did the control form of HK. Since phosphorylation increases the negative charge on an enzyme, leading to stronger binding to DEAE and later elution on a salt gradient, these results suggest that anoxic HK is present primarily in a low phosphate form, whereas the control form is present mostly in a high phosphate form. To determine if the ion exchange data above was truly the result of a change in phosphorylation state, ProQ diamond phosphoprotein staining was used. Samples of DEAE Sephadex purified HK from both anoxic and control conditions were separated on SDS-PAGE and then tested using ProQ diamond phosphoprotein staining. Figure 5 shows a greater level of phosphorylation of control HK as compared with the anoxic condition. This confirms the ion exchange data and allows the conclusion that HK is subject to anoxia-responsive changes in its phosphorylation state with the anoxic versus control forms being the low versus high phosphate forms, respectively.

Further exploration of this change in phosphorylation state, and the impact on HK kinetic parameters was explored. Incubations which stimulated total phosphatase and total kinase activity were utilized to elucidate any potential changes in kinetic parameters, which mirror a change in the phosphorylation of the enzyme. Data obtained from incubations exploring the change in the $K_m$ for glucose in tail muscle HK showed no significant changes despite the use of incubations that stimulated either kinase or phosphatase activity (Figure 6). This suggests that the kinetic change in the affinity of HK for glucose noticed during anoxia is not a result of the kinases and phosphatases targeted in this study.

Another parameter that was explored in both anoxic and normoxic states was the stability of HK. The ability of yeast HK to bind glucose is dependent on a conformational change in the enzyme [20]. Therefore, changes in the stability of HK may arise due to post-translational modification such as reversible phosphorylation.

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Structural analysis of HK by urea denaturation and pulse proteolysis was explored. Results showed that HK from control muscle was more susceptible to conformational denaturation by urea as assessed by the subsequent destruction of denatured protein by thermolysin (Figure 7). Phosphorylation of an enzyme introduces a large negative charge, which may affect the structural arrangement of an enzyme, either making it more or less stable [21], and could possibly be the control mechanism for this apparent stabilization of HK.

Crayfish muscle HK also showed a significant decrease in the percentage of bound HK versus soluble cytosolic HK during anoxia (Figure 2). Phosphorylation can often affect the subcelluar location of an enzyme [22,23,24]. Studies have shown that bound HK (associated with mitochondrial porins) prefers to use ATP generated in the mitochondria rather than cytosolic ATP [25]. Since generation of ATP by mitochondria is essentially halted during anoxia, this source of ATP for HK would be wiped out. This would result in a need for HK to use ATP generated by glycolysis in the cytosol, probably requiring a release of HK from binding interactions and resulting in an increased amount of HK in the soluble cytoplasmic fraction.

## Conclusion

The regulation of crayfish tail muscle HK during anoxia seems to be a complex chain of events. The activity and protein content of HK increases during anoxia, as does the amount of soluble HK (released from mitochondrial binding), the enzyme undergoes dephosphorylation and shows altered kinetic properties and an increase in the susceptibility to urea denaturation. All of these factors could readjust HK for a key role in optimizing glycolytic energy production under anoxic conditions. Changes in the phosphorylation state of tail muscle HK in anoxia may be responsible for a variety of the anoxia-induced effects on HK properties. Thus, the aerobic control enzyme is less phosphorylated, less stable, and has a higher percentage of bound enzyme, whereas the less phosphorylated anoxic form of HK is more stable, yet has less bound enzyme.

**Competing Interest**
Authors declare that they have no competing interests.

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