Purification and characterization of NADP-isocitrate dehydrogenase from skeletal muscle of *Urocitellus richardsonii*

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Abstract

NADP-dependent isocitrate dehydrogenase (NADP-IDH, EC 1.1.1.42) catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate with the concomitant production of NADPH. NADPH plays important roles in many biosynthesis pathways, maintenance of proper oxidation–reduction balance, and protection against oxidative damage. This present study investigated the dynamic nature of NADP-IDH during hibernation by purifying it from the skeletal muscle of Richardson’s ground squirrel (*Urocitellus richardsonii*) and analyzing its structural and functional changes in response to hibernation. Kinetic parameters of purified NADP-IDH from euthermic and hibernating ground squirrel skeletal muscle were characterized at 22 °C and 5 °C. Relative to euthermic muscle, -NADP-IDH in hibernating muscle had a higher affinity for its substrate, isocitrate at 22 °C, whereas at 5 °C, there was a significant decrease in isocitrate affinity. Western blot analysis revealed greater serine and threonine phosphorylation in hibernator NADP-IDH as compared to euthermic NADP-IDH. In addition, Bioinformatic analysis predicted the presence of 18 threonine and 21 serine phosphorylation sites on squirrel NADP-IDH. The structural and functional changes in NADP-IDH indicate the ability of the organism to reduce energy consumption during hibernation, while emphasizing increased NADPH production, and thus antioxidant activity, during torpor arousal cycles.

Keywords NADP-dependent isocitrate dehydrogenase · Enzyme kinetics · Hibernation · Post-translational modification · *Urocitellus richardsonii*

Introduction

Hibernation is a well-known phenomenon which enables many mammals to withstand harsh winter conditions. During hibernation, they typically enter a hypometabolic state to reduce their metabolic expenditure in extreme conditions. This ‘energy saving’ state is characterized by suppression of metabolic rate and lowering of body temperature to conserve energy [1]. Richardson’s ground squirrels (*Urocitellus richardsonii*) are obligate hibernators that possess the ability to drop their metabolic rate by approximately 95% relative to their euthermic state [2, 3]. Overall, this allows these mammals to conserve about 88% of their energy [2–4]. The most obvious vital changes during hibernation are the reduction of heart rate, breathing rate and body temperature, along with the biochemical adaptations that mediate the entry, maintenance, and exit from a hypometabolic state [4, 5]. During hibernation, there is a notable change in fuel source, with hibernating mammals switching from carbohydrates as a main source for energy to the oxidation of stored triglycerides in adipose tissue [6, 7]. The marked changes in metabolic demand during hibernation is strictly controlled and coordinated by the regulation of gene expression and enzyme function [8–10]. The major regulatory mechanism that mediates the entry into the hypometabolic state is reversible phosphorylation of enzymes and proteins [11, 12].

NADP-dependent isocitrate dehydrogenase (NADP-IDH, EC 1.1.1.42) is a highly regulated enzyme in the tricarboxylic acid cycle, which is a crucial pathway in energy metabolism and biosynthesis. It catalyzes the oxidative decarboxylation of isocitrate into alpha-ketoglutarate with the concomitant production of NADPH [13]. The enzyme is thus dependent on isocitrate, NADP, and an additional metal
cofactor, Mg$^{2+}$, to carry out the alpha-ketoglutarate producing reaction. NADPH is known for acting as an important cofactor for various metabolic processes [14]. As contribution of NADP-IDH to NADPH production is significant, it is possible that the regulation of NADP-IDH enzymatic activity may have an indirect effect on the metabolism of animals during hibernation. NADPH is often necessary in various antioxidant reactions making the regulation of its production critically important, as this can be a key for the survivability of ground squirrels and other organisms undergoing hibernation. This present study explores the regulation of NADP-IDH in hibernating *U. richardsonii* skeletal muscle. Assessments on the kinetic activity of the enzyme were explored in addition to determination of phosphorylation status in the euthermic versus hibernating state as a potential mechanism to facilitate any observed kinetic changes. Bioinformatic analysis of the enzyme was performed to observe the viability of phosphorylation sites, as well as to determine potential enzyme structure, sequence similarity, and size.

**Materials and methods**

**Animal treatments**

Richardson’s ground squirrels (*Urocitellus richardsonii*) were caught in the summer near Calgary, Alberta. They were then held at the animal care facility at the University of Calgary. The ground squirrels were caged with free access to food and water at 22 °C for 8 weeks on a natural autumn photoperiod (10 h light: 14 h dark). Following this period, half of the animals were sampled as control (euthermic) and the other half of the animals were moved to dark room maintained at 4 °C with water, but no food (hibernating). Subsequently, animals from these conditions were sampled after 2 days of continuous torpor (rectal temperature 5–8 °C). Both hibernating and euthermic animals were killed by decapitation and tissues were immediately excised, frozen in liquid nitrogen, and then stored at −80 °C. All protocols described above for animal holding and hibernation had prior approval by the university animal care committee and met guidelines for the Canadian Council on Animal Care. The protocols used for care and handling of Richardson’s ground squirrels, *U. richardsonii*, were reported previously [15, 16].

**Preparation of tissue extracts**

Samples of frozen skeletal muscle tissue of *U. richardsonii* were homogenized in a 1:10 w:v in ice cold homogenization buffer A (25 mM K$_2$PO$_4$, 2 mM EDTA, 2 mM EGTA, 15 mM β-glycerophosphate, 10 mM β-mercaptoethanol, 10% v:v glycerol, pH 6.0) in addition to a few crystals of phenylmethylsulphonyl fluoride (PMSF). Samples were homogenized using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY, USA) and centrifuged in a pre-chilled Eppendorf 5810 R tabletop centrifuge (22331 Hamburg, GER) for 30 min at 13,500 × g (4 °C). The supernatant (crude homogenate) was collected and held on ice until further use.

**NADP-isocitrate dehydrogenase purification**

Crude skeletal muscle homogenate was initially applied to a CM$^+$ Sephadex column previously equilibrated with 30 mL of buffer A. Unbound protein was removed from the column with 30 mL wash of buffer A. The enzyme was then eluted from the column with a 20 mL bump of 45 mM isocitrate solution made in buffer A. Using an automated fraction collector (Gilson Medical Electronics, Inc., Middleton, WI, USA), 1.6 mL fractions of column eluant were collected. From each fraction, 10 µL was taken and assayed for NADP-IDH activity. Peak activity fractions were pooled together, increased to pH 7.0 and diluted 10:1 v:v with buffer B (25 mM imidazole, 2 mM EDTA, 2 mM EGTA, 15 mM β-glycerophosphate 10 mM β-mercaptoethanol, 10% v:v glycerol, pH 7.0). The diluted sample was then applied to a Cibacron blue column that had been previously equilibrated with 30 mL of buffer B. NADP-IDH was eluted with 0–2 M KCl gradient made in buffer B and collected in 1.6 mL fractions. Aliquots of 10 µL were taken to access NADP-IDH activity from each fraction. Fractions with the highest activity were pooled together. The salt was subsequently removed from the final sample with use of the Sephadex G-25 medium (Cat# G25150-10G, Sigma-Aldrich). For every 500 µL of enzyme sample, 5 mL of beads equilibrated with 5 mL of buffer B were used and centrifuged at 12, 500 × g for 1 min. The final sample was held at 4 °C until further use. This procedure was observed for both euthermic and hibernating samples. Protein concentrations were determined using Coomassie blue G-250 dye-binding reagent (Bio-Rad, Hercules, CA, USA) as instructed by the manufacturer using bovine serum albumin as a standard [17].

**NADP-isocitrate dehydrogenase enzyme assay**

NADP-IDH activity was measured as the rate of production of NADH by measuring absorbance at 340 nm using a Multiskan spectrophotometer (Thermo Scientific, Waltham, MA, USA). Optimal assay conditions were found to be 35 mM Tris (pH 7.2), 2.5 mM isocitrate, 0.8 mM NADP, 0.5 mM Mg$^{2+}$, and 10 µL of purified enzyme in a 200 µL total volume. Assays were initiated through the addition of NADP, and assays were run at room temperature (22 °C). For kinetic analysis, $K_m$ values for NADP, isocitrate and Mg$^{2+}$ were determined at constant, saturating co-substrate...
concentrations. $I_{50}$ values were determined by the addition of increasing concentration of denaturant molecules (urea, KCl) to the purified enzyme. The kinetic analysis of all three substrate’s $K_m$ values was also performed at 4 °C to simulate hibernating temperature conditions. Kinetic data were analyzed using the Kinetics v.3.5.1 program [18].

**Gel electrophoresis and staining**

Purified NADP-IDH samples were separated on 10% SDS-PAGE gels via electrophoresis for 55 min at 180 V. Gels were then stained with Coomassie Blue dye (0.25% w:v brilliant blue R, 7.5% v:v acetic acid, 50% v:v methanol) for 1 h. Adequately stained gels were then immersed in destain solution (10% v:v acetic acid, 25% v:v methanol) until distinct bands were visualized. Gels were then visualized and imaged using a ChemiGenius Bio-Imaging system with GeneSnap software (Syngene, Frederick, MD).

**Western blotting**

Skeletal muscle NADP-IDH PTMs were analyzed using Western blotting. In brief, samples of purified NADP-IDH were concentrated 8 x using an Amicon® Ultra Filter Centricron (Millipore). For both euthermic and hibernating preparations, concentrated samples were 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, and 10% v:v 2-mercaptoethanol) and boiled for 5 min. SDS resolving gel (10% v:v acrylamide, 400 mM Tris [pH 8.8], 0.1% w:v SDS, 0.2% w:v ammonium persulfate [APS], 0.04% v:v TEMED) were prepared with 5% stacking gel (5% acrylamide, 190 mM Tris [pH 6.8], 0.1% w:v SDS, 0.15% w:v APS, 0.1% v:v TEMED). Samples were loaded onto these gels and separated electrophoretically in SDS-PAGE running buffer (25 mM Tris [pH 8.5], 190 mM glycine, and 0.1% w:v SDS) for 45 min at 180 V. Following electrophoresis, proteins on the gel were electroblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore) by wet transfer for 90 min at 160 amps.

After protein transfer, PVDF membranes were washed three times with Tris buffered saline (TBS: 100 mM Tris, 140 mM NaCl [pH 7.6]) containing 0.05% Tween-20 (TBST) for 5 min each wash. Following this initial washing, membranes were blocked for 10 min with 2.5% skim milk before primary antibodies (diluted 1:1000 v:v in TBST) were applied for overnight incubations at 4 °C on a rocker. Primary antibodies included: (1) rabbit anti-phosphoserine (Cat. #618100, Invitrogen, Carlsbad, CA, USA); (2) rabbit anti-phosphothreonine (Cat. #718200, Invitrogen, Carlsbad, CA, USA); (3) mouse anti-phosphotyrosine (Cat. #615800, Invitrogen, Carlsbad, CA, USA). Following primary antibody incubation, membranes were washed three times with TBST for 5 min each and incubated with either a 1:8000 v:v dilution of goat anti-rabbit IgG-peroxidase secondary antibody (Cat. #APA007P, BioShop Canada Inc., Burlington, ON, Canada) or 1:8000 v:v dilution of anti-mouse IgG-peroxidase secondary antibody (Cat. #APA005P.2, BioShop Canada Inc., Burlington, ON, Canada) depending on the primary antibody. All phospho-antibody probed membranes were incubated in their respective secondary antibody dilution for 40 min at room temperature. Blots were washed with TBST (3 x 5 min) prior to visualization on the ChemiGenius Bioimaging System (Syngene, Frederick, MD, USA). Observed band intensities were quantified using the GeneTools software (Syngene, Frederick, MD, USA). Subsequent staining of visualized membranes in Coomassie blue occurred in order to standardize immunoblot band intensities.

**Bioinformatics**

The NADP-IDH sequence of the 13-lined ground squirrel, *Ictidomys tridecemlineatus* was taken as it is the closest sequenced relative of the Richardson’s ground squirrel. For determination of molecular weight and isoelectric point, the ExPASy Compute pI/Mw bioinformatics tool was used (http://web.expasy.org/compute_pi/). ClustalW2 was used to observe the conservation of NADP-IDH amino acid residues across different animals (https://www.ebi.ac.uk/Tools/msa/clustalw2/). The NADP-IDH sequence from *I. tridecemlineatus* was aligned with rabbit (*Oryctolagus cuniculus*), humans (*Homo sapiens*) that do not undergo hibernation and frog (*Xenopus laevis*) that can experience significant dehydration stress (Larkin et al., 2007). Potential phosphorylation sites on the NADP-IDH amino acid sequence were predicted using NetPhos 3.1 server (http://web.expasy.org/compute_pi/). The NADP-IDH sequence from *I. tridecemlineatus* was aligned with rabbit (*Oryctolagus cuniculus*), humans (*Homo sapiens*) that do not undergo hibernation and frog (*Xenopus laevis*) that can experience significant dehydration stress (Larkin et al., 2007). Potential phosphorylation sites on the NADP-IDH amino acid sequence were predicted using NetPhos 3.1 server (https://services.healthtech.dtu.dk/service.php?NetPhos-3.1) [19]. Finally, protein structure of NADP-IDH was predicted using SWISS-MODEL (https://swissmodel.expasy.org/), a protein structure homology-modelling server [20–22].

**Data and statistical analysis**

All enzyme assays were analyzed using a Microplate analysis program (MPA) [23], while kinetic parameters were determined using a nonlinear least squares regression computer program Kinetics v.3.5.1 [18]. RBioplot statistical software was used in data analysis to generate graphs and perform Student’s t-test or analysis of variance (ANOVA) combined with a Tukey’s post-hoc test [24]. Data were determined to be statistically different between euthermic and torpid groups when the p < 0.05.
Results

Purification of NADP-IDH from the skeletal muscle of euthermic and hibernating U. richardsonii

NADP-IDH was successfully purified from skeletal muscle of euthermic and hibernating U. richardsonii. Purification was performed by two chromatography steps in succession: (1) CM⁺ column (enzyme eluted via isocitrate bump), and (2) a Cibacron blue affinity column (elution with 0–2 M KCl) (Table 1). Elution of the euthermic and hibernating NADP-IDH from the CM⁺ Sephadex column resulted in a 3.2- and sixfold purification, respectively (Table 1). Finally, elution of the euthermic and hibernator enzyme from the Cibacron blue column using a KCl gradient resulted in a 5.9- and 8.8-fold purification, respectively (Table 1). The final purification resulted in specific activities of 0.84 U/mg for euthermic NADP-IDH and 0.61 U/mg, for hibernator NADP-IDH (Table 1). SDS-PAGE and Coomassie Blue was used to visually assess the purification of NADP-IDH. The purified enzyme had a molecular weight of 46 kDa (Fig. 1).

| Step                  | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Fold purification | Yield (%) |
|-----------------------|--------------------|--------------------|--------------------------|-------------------|-----------|
| **Euthermic**         |                    |                    |                          |                   |           |
| Crude                 | 14.8               | 2.08               | 0.14                     | –                 | –         |
| CM sephadex           | 2.10               | 0.96               | 0.46                     | 3.2               | 46.2      |
| Cibacron blue         | 0.59               | 0.49               | 0.84                     | 5.9               | 23.6      |
| **Hibernating**       |                    |                    |                          |                   |           |
| Crude                 | 22.5               | 1.56               | 0.069                    | –                 | –         |
| CM Sephadex           | 1.76               | 0.74               | 0.42                     | 6.0               | 47.3      |
| Cibacron blue         | 0.58               | 0.36               | 0.61                     | 8.8               | 22.9      |
Multiple kinetic parameters of purified skeletal muscle NADP-IDH were compared between euthermic and hibernating states (Table 2). There were significant differences in the Km values for isocitrate between euthermic and hibernating ground squirrel at 22 °C and 5 °C. The Km values for isocitrate and NADP at 22 °C for the hibernating form was decreased by 85% (5.5 ± 0.6 µM) and by 83% (1.2 ± 0.1 µM), respectively, from their respective euthermic values. Following the above trend, the Km value for NADP at 5 °C showed a decrease of 74% (3.6 ± 0.4 µM) compared to euthermic value of NADP-IDH (14 ± 3 µM) at 5 °C. Interestingly, at 5 °C Km value for isocitrate showed fourfold increase in hibernating form (24 ± 3 µM) of NADP-IDH as compared to its euthermic NADP-IDH (6 ± 0.4 µM). The Km Mg²⁺ values at 22 °C and 5 °C showed 2-fold (19 ± 0.8 µM) and three-fold (81 ± 4 µM) increase, respectively, in hibernating form of NADP-IDH as compared to their corresponding euthermic values (Table 2). The effects of inhibition by general enzyme denaturants were determined for euthermic and hibernating NADP-IDH at standard conditions (pH 7.2, 22 °C). A significant increase in KCl I₅₀ values was observed in hibernating NADP-IDH (0.85 ± 0.01 M) when compared to the corresponding euthermic value (0.68 ± 0.04 M) (Table 2). No significant differences were observed between euthermic (4.21 ± 0.08 M) and hibernating (4.54 ± 0.16 M) urea I₅₀ values (Table 2).

### Reversible post-translational modifications of skeletal muscle NADP-IDH.

Immunoblotting was used to assess differences in post-translational phosphorylation on euthermic and hibernating NADP-IDH enzyme. Overall phosphorylation of threonine and serine residues of the hibernating NADP-IDH enzyme increased 3.7- and 4.6-fold, respectively, when compared to the corresponding euthermic enzyme (Fig. 2).

### Bioinformatic analysis

Analysis of the predicted 13-LGS NADP-IDH amino acid sequence (NCBI Reference Sequence: XP_005342105.1) via the phosphorylation site predict server NetPhos3.1 (https://services.healthtech.dtu.dk/service.php?NetPhos-3.1) [19] indicated 18 threonine, 21 serine and 10 tyrosine sites (Fig. 3). The isoelectric point (pI) and molecular weight of the enzyme were determined to be 6.34 and 46.46 kDa, respectively. Sequence alignments between I. tridecemlineatus, human, rabbit, and frog NADP-IDH was determined. With respect to the primary structure of the enzyme, the amino acid sequence seems to be highly conserved across mammals (squirrels, human and rabbit) (Fig. 4). Finally, SWISS-MODEL was used to generate NADP-IDH structure (Fig. 5).

### Discussion

Various metabolic adaptations and regulatory controls are required to withstand metabolic rate depression, including reorganization of metabolic fuel and differential expression of genes/proteins [25]. Most importantly, and of particular interest to this study, are the ways in which mammals coordinate their entry into arousal from torpor and sustain its metabolic function at low body temperature without causing hypothermia. Huge reserves of triglycerides act as a primary fuel for most organs during hibernation. Strict metabolic rate depression and allowing the body temperature to fall to near ambient temperatures during torpor bouts, allows hibernators to save huge amounts of energy as compared to euthermic animals (Ruf & Geiser, 2015). Richardson’s ground squirrel is additionally faced with oxidative stress during hibernation, particularly during arousals in which oxygen consumption increases drastically [9, 27]. In many instances of hypometabolism, an increase in antioxidant enzyme activity is observed to prepare for imminent oxidative stress upon arousal and preserve current cell integrity during periods of reduced repair [28–31]. NADP-IDH often contributes significantly to the NADPH pool required.
for reductive fatty acid biosynthesis. This enzyme provides NADPH for maintenance of proper oxidation–reduction balance and protection against oxidative damage [32].

NADP-IDH was purified to electrophoretic homogeneity from Richardson’s ground squirrel skeletal muscle through a combination of ion-exchange and affinity chromatography (Fig. 1). The apparent molecular weight (~ 46 kDa) determined by SDS-PAGE corresponds well with the molecular weights reported for the same enzyme through bioinformatics analysis. Kinetic analysis of NADP-IDH was assessed at high and low temperatures. Many significant changes in parameters were observed when comparing euthermic and

Fig. 2 Quantification of post-translational modifications of purified euthermic and hibernating skeletal muscle NADP-IDH from U. richardsonii. Fold change in phosphorylation of purified enzyme samples is shown. Chemiluminescence signal intensities were standardized to protein amount, and the value for hibernating NADP-IDH was expressed relative to the control value that was set to 1. Data are mean ± SEM, n = 6 for purified enzyme samples. *—indicates significant differences from the corresponding euthermic value determined by a student’s t-test, p < 0.05
hibernating samples. The Km values of isocitrate and NADP were both significantly reduced in hibernating squirrels at 22 °C as compared to euthermic control squirrels (Table 2). A decrease in Km values is suggestive of an increased enzyme affinity and, in turn, enzyme activity in the hibernating state. Increased enzyme activity is indicative of elevated NADPH production in hibernating squirrels. This increased NADPH presence could contribute to enhanced antioxidant activity and overall oxidative stress defense in the organism while in the hypometabolic state. Previous studies done by Vucetic et al., 2013 [33], on the impact of hibernation on antioxidant defences in the European ground squirrel (Spermophilus citellus) showed increase in antioxidant defence enzyme protein expressions in the hibernating state. Similar study was also done by Allan & Storey on a molecular level, in which they analysed the expression downstream antioxidant target, NF-κB in skeletal muscle of hibernating ground squirrels (Ictidomys tridecemlineatus) [34]. During

Fig. 3 Predicted phosphorylation sites on threonine, serine, and tyrosine residues of NADP-IDH for thirteen-lined ground squirrels (I. tridecemlineatus) as a reference for Richardson’s ground squirrels. (A) Location of predicted phosphorylation residues in the NADP-IDH sequence. In the above figure, a period (.) indicates the residue is predicted not to be phosphorylated as the score is below the threshold value (0.500). Residues with a score above threshold (0.500) that are predicted to be phosphorylated are denoted with either an S, T or Y. (B) Diagram depicting calculated scores for each residue in relation to the threshold line below
hibernation, the expression of NF-κB was increased, particularly in early torpor and arousal suggesting increased antioxidant defense [34]. Additionally, in this study, increased expression of manganese dependent superoxide dismutase in early torpor and increased expression of heme oxygenase 1 in early arousal was also noted [34]. One more study suggested that there was a significant increase in MafK affirming an increase in relative Nrf2 and catalase levels seen in

Fig. 4 Multiple alignment of the deduced amino acid sequence of thirteen-lined ground squirrels (I. tridecemlineatus) as a reference for Richardson’s ground squirrels. Comparison of sequence is to three non-hibernating organisms, two mammals (rabbit and human) and one amphibian. Asterisks (*) denote amino acid residues that are conserved amongst all species; colons (:) denote the conversion between amino acid residues of strongly similar properties; periods (.) denote the conversion between residues of weakly similar properties. Additionally, amino acid differences from the I. tridecemlineatus sequence are highlighted for each the frog (blue), rabbit (red) and human (green) sequences. Amino acid residues unique to only ground squirrels are highlighted in orange boxes. Accession numbers for the frog, rabbit and human NADP-IDH sequences are XP_018094513.1, XP_017198555.1 and NP_001269316.1, respectively
arousal. These results seen during arousal correspond to a surge in oxygen consumption, which causes increased reactive oxygen species production [31]. Overall, this is also contributing to the idea that ground squirrels increase oxidative stress defense during hibernation to cope with increased oxygen fluctuations during periodic arousals and to preserve cells during reduced repair periods in hypometabolism.

Interestingly, when observing the same Km kinetic parameters at a reduced temperature (5 °C), the increase in enzyme affinity in the hibernating state is no longer observed. In fact, both isocitrate and Mg2+ Km values were increased in the hibernating state when compared to their respective euthermic counterparts (Table 2). Such results are more indicative of a decrease in enzyme affinity in the hibernating state at the cooler temperature. Perhaps, reduced NADP-IDH activity in the hibernating state would allow energy conservation in ground squirrels during hypometabolism. Reduced metabolic rate and the suppression of unnecessary processes during stages of reduced oxygen, food, and water consumption has been well studied in the past [8, 35, 36]. In hibernators, evidence has been presented that global suppression of ATP-expensive processes such as transcription, translation, and growth stimulated by the Akt transcription factor takes place in order to facilitate energy conservation during hibernation [37–39]. Therefore, decreased activity of NADP-IDH in ground squirrel skeletal muscle at reduced temperatures could be the result of an assortment of energy conservation mechanisms. The conflicting results of Km values of NADP-IDH at 22 °C and 5 °C are indicative of more complex regulation of this enzyme. During hibernation, the ground squirrel reduces its core body temperature to near ambient temperatures and can remain at approximately 5 °C for prolonged bouts of torpor [2]. However, the squirrels do not always remain in a state of torpor throughout hibernation and instead cycle back to normal body temperature and metabolic rate periodically through interbout arousal periods [4, 9]. During torpor at colder temperatures, there is a decrease in oxidative stress. When moving to periods of arousal, the immediate phase of high oxygen consumption puts the hibernating squirrel at risk of increased mitochondrial reactive oxygen species (ROS) production [27, 40]. Thus, enzyme activity is likely being controlled in Richardson’s ground squirrel based on antioxidant need by regulating NADP-IDH not only according to euthermic or hibernating state, but also according to temperature. Altogether, this study explored the idea that at low temperatures, when oxidative stress is low and energy conservation needs are high, NADP-IDH can be downregulated. Yet, at increased temperatures and increased ROS threat during interbout arousal periods, NADPH production becomes more essential to cell survivability and is thus upregulated. Previous studies of enzymes from ground squirrels have also documented significant changes in the kinetic properties of enzymes in hibernating versus euthermic states and in high versus low assay temperatures. These include creatine kinase [41], hexokinase [42], pyruvate kinase [43] and citrate synthase [44] (all well-known regulatory enzymes) from skeletal muscle of Richardson’s ground squirrels. A significant increase in I50 value of KCl at room temperature was observed in hibernating as compared to euthermic squirrel (Table 2). Increased KCl tolerance could mean that the hibernating form of NADP-IDH has increased chemical stability in Richardson’s ground squirrels. A similar phenomenon was observed in the SERCA (sarcoendoplasmic reticulum Ca2+-ATPase) enzyme of turtles which undergoes anoxia, another form of hypometabolic stress [45].

The differences observed in the kinetic properties of NADP-IDH purified from euthermic and hibernating skeletal muscles may be explained by structural differences. Phosphorylation levels of serine, threonine, and tyrosine residues were therefore assessed by western blotting. There was a significant increase in phosphorylation on serine and threonine residues of NADP-IDH in the hibernating animal was significantly more phosphorylated...
[43]. In another study, immunoblotting showed that hibernator muscle glycerol-3-phosphate dehydrogenase had a higher phosphoserine content than in euthermic controls [3, 46]. Protein phosphorylation is an important post-translational modification that can quickly and reversibly alter the properties of metabolic enzymes [47]. Phosphorylation of NADP-IDH during hibernation could thus also be responsible for the kinetic differences observed in the present study. However, further analysis of how phosphorylation effects the activity of NADP-IDH in Richardson's ground squirrel hibernation is required to support these findings.

To complement the experimental studies on U. richardsonii NADP-IDH, bioinformatics tools were used to analyze the NADP-IDH protein sequence from the thirteenc-lined ground squirrel, a closely related hibernator with a sequenced genome (Fig. 3). This analysis was aimed at identifying sites of post-translational modifications that may play a role in stabilizing enzyme structure and enhancing enzyme functionality in euthermic versus hibernating states. Bioinformatics results further confirmed the presence of potential threonine and serine residues which can likely be phosphorylated in hibernating NADP-IDH enzyme. (Fig. 3). The addition of phosphate groups to these serine or threonine amino acid residues could play a role in modifying ground squirrel skeletal muscle functionality at high (euthermic) versus low (hibernating) body temperatures.

**Conclusion**

Therefore, this study demonstrates the regulation of the NADP-IDH in euthermic and hibernating Richardson’s ground squirrel. Purified NADP-IDH exhibited distinct kinetic properties in response to hibernation compared to the enzyme purified from euthermic animals. These differences are likely due to the phosphorylation of hibernating form NADP-IDH. The hibernating form of NADP-IDH from the skeletal muscles of Richardson's ground squirrels maintain better functionality at 22 °C than 5 °C when compared to their respective euthermic NADP-IDH. In high temperature hibernation periods (i.e., interbout arousals), the increased influx of oxygen could lead to increased threat of ROS damage, leading to increased NADP-IDH activity to mitigate resultant oxidative damage. However, in low temperature hibernation conditions and subsequently low oxidative damage periods, energy conversation is favoured and NADP-IDH activity is decreased accordingly. The data presented here reinforce the fact that small hibernators such as Richardson's ground squirrels are confronted with oxidative stress over torpor-arousal cycles and must up-regulate their antioxidant defenses to prevent oxidative injuries whenever required.

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**Author contributions** IAM and KBS conceptualized and designed the project and IAM conducted all experiments. Data analysis and assembly of the manuscript were carried out by AV, IAM, and KBS.

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**Data availability** The data are available on reasonable request.

**Declarations**

**Conflict of interest** The authors declare no conflict of interest.

**Ethical approval** All protocols for animal care and treatment had prior approval by the Carleton University Animal Care Committee and met guidelines for the Canadian Council on Animal Care.

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