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Free-radical first responders: The characterization of CuZnSOD and MnSOD regulation during freezing of the freeze-tolerant North American wood frog, *Rana sylvatica*

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ABSTRACT

Background: The North American wood frog, *Rana sylvatica*, is able to overcome subzero conditions through overwintering in a frozen state. Freezing imposes ischemic and oxidative stress on cells as a result of cessation of blood flow. Superoxide dismutases (SODs) catalyze the redox reaction involving the dismutation of superoxide ($O_2^{-\bullet}$) to molecular oxygen and hydrogen peroxide.
Methods: The present study investigated the regulation of CuZnSOD and MnSOD kinetics as well as the transcript, protein and phosphorylation levels of purified enzyme from the muscle of control and frozen *R. sylvatica*.
Results: CuZnSOD from frozen muscle showed a significantly higher V_{max} (1.52 fold) in comparison to CuZnSOD from the muscle of control frogs. MnSOD from frozen muscle showed a significantly lower K_m for $O_2^{-\bullet}$ (0.66 fold) in comparison to CuZnSOD from control frogs. MnSOD from frozen frogs showed higher phosphorylation of serine (2.36 fold) and tyrosine (1.27 fold) residues in comparison to MnSOD from control animals. Susceptibility to digestion via thermolysin after incubation with increasing amount of urea (C_m) was tested, resulting in no significant changes for CuZnSOD, whereas a significant change in MnSOD stability was observed between control (2.53 M urea) and frozen (2.92 M urea) frogs. Expressions of CuZnSOD and MnSOD were quantified at both mRNA and protein levels in frog muscle, but were not significantly different.
Conclusion: The physiological consequence of freeze-induced SOD modification appears to adjust SOD function in freezing frogs.
General significance: Augmented SOD activity may increase the ability of *R. sylvatica* to overcome oxidative stress associated with ischemia.

1. Introduction

The North American wood frog, *Rana sylvatica*, is a unique terrestrial frog that overwinters in a whole animal frozen state, in which as much as 65–70% of the frog's total body water is converted into extracellular ice [1]. In a frozen state, cells are exposed to ischemic conditions as a result of a cessation in cardiac function and blood flow [1,2]. Wood frogs cope with freezing/ischemic defense by (a) employing metabolic rate depression to reduce overall energy demands, (b) sustaining elevated constitutive levels of antioxidants and antioxidant enzymes in their tissues and (c) increasing the total antioxidant capacity by increasing select antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase, glutathione-S-transferase, catalase, and glutathione reductase [3,4]. It is apparent that antioxidant defenses are critical for entry and exit from hypometabolism to deal with changes in oxygen consumption and generation of reactive oxygen species (ROS), such as during wood frog freezing.

Abbreviations: ALS, amyotrophic lateral sclerosis; AMPK, 5′ adenosine monophosphate-activated protein kinase; C_m, the denaturant concentration at which half of the protein is unfolded; CuZnSOD, copper and zinc containing SOD; $DEAE^+$, diethylaminoethyl cellulose; DEPC, diethylpyrocarbonate; DTT, dithiothreitol; FeSOD, iron containing superoxide dismutase; K_m, Michaelis–Menten constant; IMAC, immobilized metal ion affinity chromatography; IP_3, inositol trisphosphate; KPi, potassium phosphate; MAPK, mitogen activated protein kinase; MnSOD, manganese containing superoxide dismutase; NBT, nitrotetrazolium blue; NiSOD, nickel containing superoxide dismutase; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; PMSF, phenylmethylsulfonyl fluoride; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; PP2C, protein phosphatase 2C; PVDF, polyvinylidene difluoride; ROS, reactive oxygen species; TBST, Tris buffered saline with Tween; V_{max}, maximal velocity

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The importance of antioxidant enzymes for freeze tolerance stems from the buildup of ROS during, and upon exit from, ischemic events [5]. Superoxide dismutases (SODs, E.C. 1.15.1.1) are crucial enzymes involved in the mitigation of oxidative damage by catalyzing the disproportionation of superoxide ($O_2^{-\bullet}$) into oxygen and hydrogen peroxide. Specifically, SOD catalyzes the following reactions:

$$\text{M (ox)-SOD} + O_2^{-\bullet} \rightarrow \text{M (red)-SOD} + O_2$$

$$\text{M (red)-SOD} + O_2^{-\bullet} + 2H^+ \rightarrow \text{M (ox)-SOD} + H_2O_2$$

(where M = Cu(II/I) or Mn(II/I)).

$O_2^{-\bullet}$ is one of the main reactive oxygen species found ubiquitously across tissues and organisms. There are several forms of SOD found in nature, these unique metalloproteins are identified through their metal cofactors: 1) copper and zinc containing SOD (CuZnSOD) is typically found in the cytosol of nearly all eukaryotic cells; 2) manganese SOD (MnSOD) is found in the mitochondria or peroxisomes, or 3) nickel (NiSOD) or iron (FeSOD) containing SODs are typically found in prokaryotes, protists and select eukaryotes [6–9]. Of the many forms of SOD, only CuZnSOD and MnSOD are known to exist in frogs [10–13]. It has been suggested that the cytoplasmic form, CuZnSOD, acts like a buffer against the buildup of intracellular $O_2^{-\bullet}$, whereas the mitochondrial form, MnSOD, plays a pivotal role in the disproportionation of the large amount of $O_2^{-\bullet}$ generated by the electron transport chain housed within the mitochondria [14,15]. CuZnSOD and MnSOD have been sequenced from the African clawed frog, *Xenopus laevis*, and exist as homodimers with molecular weights of approximately 32 kDa and 46 kDa, respectively [10,13]. Studies have shown the importance of functional CuZnSOD as many disease states can be attributed to mutations in the *sod1* gene [16]. The role of MnSOD in disease states is less characterized as knockout experiments involving *sod2* are lethal [14,17–19].

SODs have been widely studied and characterized from traditional animal models (human, rat mouse), however, less is known about the role of SODs in aiding survival of the freeze tolerant frog. Reversible protein phosphorylation continues to emerge as an increasingly common method of posttranslationally modifying and regulating enzymes within freeze-tolerant animals. There is evidence to support the posttranslational modification of enzymes such as protein kinases, phosphatases, and metabolic enzymes via reversible phosphorylation as a potential control mechanism for altering enzymatic activity in *R. sylvatica* in response to freezing in the muscle [20–28]. This manuscript presents the first investigation of the potential regulation of CuZnSOD and MnSOD separately in the leg muscle of *R. sylvatica* comparing control and frozen states, and provides evidence for post-translational modification as the mechanism of enzyme regulation to aid successful overwintering of the frog.

2. Materials and methods

2.1. Chemicals

All biochemicals were from BioShop (Burlington, ON, Canada) with a few exceptions; xanthine and xanthine oxidase were from Sigma (St. Louis, MO), immobilized metal ion affinity chromatography (IMAC) chelating fast flow column was from Pharmacia (Uppsala, SE), and potassium phosphate, monobasic was from J.T. Baker Chemical Company (London, UK).

2.2. Animals

Male wood frogs were obtained from the Ottawa area and were washed in a tetracycline bath before being placed in plastic containers with damp sphagnum moss at 5 °C for one week. Control frogs were sampled from this condition. Frogs were exposed to freezing conditions for 24 h as previously described [29,30]. Control and frozen frogs were euthanized by pithing. Muscle tissues were quickly excised and flash frozen in liquid N_2. All tissue samples were stored at −80 °C until use. The Carleton University Animal Care Committee, in accordance with the Canadian Council on Animal Care guidelines, approved all animal handling protocols used during this study.

2.3. Preparation of muscle tissue lysates for protein purification

For protein purification, samples of frozen muscle tissue were homogenized 1:5 w/v in ice-cold homogenizing buffer A [20 mM potassium phosphate (KPi) buffer, pH 7.2, containing 15 mM β-glycerophosphate, 1 mM EGTA, 1 mM EDTA, 5% v/v glycerol and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Homogenates were then centrifuged at 13,500 × *g* at 4 °C for 30 min and the supernatants collected for use in protein purification.

2.4. Preparation of muscle tissue lysates for determination of relative protein levels

Samples of muscle tissue were crushed under liquid nitrogen and then homogenized 1:2.5 w/v in homogenizing buffer B (20 mM HEPES, pH 7.5, 200 mM NaCl, 0.1 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4, 10 mM β-glycerophosphate) with a few crystals of PMSF and 1 μL/mL Sigma protease inhibitor cocktail (104 mM AEBSF, 80 μM Aprotinin, 4 mM Bestatin, 1.4 mM E-64, 2 mM Leupeptin, 1.5 mM Pepstatin A). Samples were homogenized on ice with a Polytron PT1000 homogenizer and centrifuged at 4 °C for 15 min at 10,000 × *g*. Soluble protein concentrations were assessed using the BioRad protein assay with bovine serum albumin (BSA) used as the standard curve according to the manufacturer's instructions. All samples were adjusted to 10 μg/μL by adding a calculated small volume of homogenizing buffer B. Aliquots of samples were then mixed 1:1 v/v with 2× SDS loading buffer (100 mM Tris-base, 4% w/v SDS, 20% v/v glycerol, 0.2% w/v bromophenol blue, 10% v/v 2-mercaptoethanol). Final sample concentrations were 5 μg/μL. Proteins were denatured by placing the tubes in boiling water for 5 min. Samples were stored at −80 °C until use.

2.5. Purification of CuZnSOD and MnSOD

A 3 mL aliquot of crude supernatant was applied to a $DEAE^+$ column (1.5 cm × 20 cm), previously equilibrated with 50 mL of homogenization buffer A. The $DEAE^+$ column was then washed with 50 mL of buffer A to remove unbound protein. SOD was eluted from the $DEAE^+$ column with a linear gradient of 0–1 M KCl in homogenization buffer A. Fractions of 1.24 mL were collected and 5 μL from each fraction was assayed to detect SOD activity (see Kinetic assays section below for methodology). The fractions of peak SOD activity were pooled and applied to Sephadex G-50 gel buffer exchange columns equilibrated with buffer C (20 mM KPi buffer, pH 7.2 containing 15 mM β-glycerophosphate, 5% v/v glycerol). A 5 cm column of Sephadex G-50 in a syringe barrel was equilibrated in buffer C and centrifuged at 500 × *g* in a bench-top centrifuge for 2 min to remove excess buffer. A 500 μL aliquot of SOD eluant was applied to each buffer exchange column and centrifuged again. The resulting eluant was collected and applied to an IMAC column pre-charged with 1 mg/mL $CuSO_4 \cdot H_2O$ (1.5 cm × 10 cm), equilibrated in homogenization buffer C. The copper chelate affinity column was then washed with 50 mL of homogenization buffer C to remove unbound protein. Bound proteins were eluted with a linear gradient of 0–1 M KCl in homogenization buffer A. Fractions of 1.24 mL were collected and 10 μL from each fraction was assayed to detect SOD activity. Fractions containing the highest SOD activity from the copper chelate affinity column were pooled and used for further