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A hydrogen peroxide safety valve: The reversible phosphorylation of catalase from the freeze-tolerant North American wood frog, *Rana sylvatica*

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ABSTRACT

Background: The North American wood frog, *Rana sylvatica*, endures whole body freezing while wintering on land and has developed multiple biochemical adaptations to elude cell/tissue damage and optimize its freeze tolerance. Blood flow is halted in the frozen state, imparting both ischemic and oxidative stress on cells. A potential build-up of H_2O_2 may occur due to increased superoxide dismutase activity previously discovered. The effect of freezing on catalase (CAT), which catalyzes the breakdown of H_2O_2 into molecular oxygen and water, was investigated as a result.

Methods: The present study investigated the purification and kinetic profile of CAT in relation to the phosphorylation state of CAT from the skeletal muscle of control and frozen *R. sylvatica*.

Results: Catalase from skeletal muscle of frozen wood frogs showed a significantly higher V_{max} (1.48 fold) and significantly lower K_m for H_2O_2 (0.64 fold) in comparison to CAT from control frogs (5 °C acclimated). CAT from frozen frogs also showed higher overall phosphorylation (1.73 fold) and significantly higher levels of phosphoserine (1.60 fold) and phosphotyrosine (1.27 fold) compared to control animals. Phosphorylation via protein kinase A or the AMP-activated protein kinase significantly decreased the K_m for H_2O_2 of CAT, whereas protein phosphatase 2B or 2C action significantly increased the K_m.

Conclusion: The physiological consequence of freeze-induced CAT phosphorylation appears to improve CAT function to alleviate H_2O_2 build-up in freezing frogs.

General significance: Augmented CAT activity via reversible phosphorylation may increase the ability of *R. sylvatica* to overcome oxidative stress associated with ischemia.

1. Introduction

The North American wood frog, *Rana sylvatica*, can remarkably overwinter in a frozen state, in which as much as 70% of its total body water is frozen into extracellular ice [1]. Overwintering imposes many hardships on the wood frog during the frozen state including the fact that cells are exposed to ischemic conditions as a result of the complete loss of cardiac function and blood flow [1,2]. *R. Sylvatica* are able to withstand ischemia stress imposed by seasonal freezing through (i) metabolic rate depression to diminish overall energy demands; (ii) maintaining elevated levels of antioxidants and antioxidant enzymes; (iii) increasing the total antioxidant capacity by increasing the activity of select antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidise (GPx), glutathione-S-transferase (GST), catalase (CAT), and glutathione reductase (GR) [3–5]. It is apparent that antioxidant defenses are critical for entry and exit from the frozen state to deal with changes in oxygen consumption and, ultimately, the generation of reactive oxygen species (ROS). The importance of antioxidant enzymes for freeze tolerance stems from the build-up of ROS during, and upon exit from, ischemic events [6].

Abbreviations: Akt, Protein kinase B; AMPK, Adenosine monophosphate-activated protein kinase; cAMP, Cyclic adenosine monophosphate; CAMK, Calmodulin dependent kinase; Cdc2, Cyclin dependent kinase 1; Cdk5, Cyclin dependent kinase 5; cGMP, Cyclic guanidine monophosphate; DEAE+, Diethylaminoethyl cellulose; DEPC, Diethylpyrocarbonate; DTT, Dithiothreitol; EDTA, Ethylene diamine tetraacetic acid; EGFR, Epidermal growth factor receptor tyrosine kinase; EGTA, Ethylene glycol tetraacetic acid; Erk1, Mitogen-activated protein kinase 3; GSK3, Glycogen synthase kinase 3; H_2O_2, Hydrogen peroxide; K_m, Michaelis–Menten constant; KPi, Potassium phosphate; Lck, Lymphocyte-specific protein tyrosine kinase; MAPK, Mitogen-activated protein kinase; NBT, Nitrotetrazolium blue; PDGFR, Platelet-derived growth factor receptor kinase; PKA, Protein kinase A; PKC, Protein kinase C; PKG, Protein kinase G; PMSF, Phenylmethylsulfonyl fluoride; PP1, Protein phosphatase 1; PP2A, Protein phosphatase 2A; PP2B, Protein phosphatase 2B; PP2C, Protein phosphatase 2C; PVDF, Polyvinylidene difluoride; ROS, Reactive oxygen species; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBST, Tris buffered saline with Tween; T_m, The temperature at which half of the protein is unfolded; V_{max}, Maximal velocity.

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Previously, [5] have shown that in the wood frog, *Rana sylvatica*, muscle SOD activity seems to be boosted in the mitochondria, which could lead to the increased formation of H_2O_2. Formation of H_2O_2 in animal tissues can arise directly from enzymatic sources, specifically a number of different oxidases including xanthine oxidase, monoamine oxidases, and amino acid oxidases; however, H_2O_2 formation due to SOD activity is considered to be the major cellular source [7,8]. Although O_2^- can dismutate spontaneously, SOD catalyzes this reaction at much higher rates. The mitochondria are a major site of H_2O_2 formation, due in large part to the superoxide formed via complexes I and III of the electron transport system [9–11]. Specifically, complex III releases O_2^- into the intermembrane space, while complex I releases O_2^- into the inner mitochondrial matrix space where MnSOD produces H_2O_2 [7,9,10].

It is generally accepted that H_2O_2 is generated in a site-specific manner and the local concentration of H_2O_2 is modulated by its generation and subsequent removal. The diffusion of H_2O_2 away from the site of production and across membranes creates an H_2O_2 gradient [12]. Furthermore, the sites of H_2O_2 removal, via CAT in the peroxisomes, GPx and peroxiredoxins in the cytosol and other organelles further drives this diffusion gradient from the site of production to the site of removal [12–14]. Under normal cellular concentrations, peroxiredoxins and peroxidases seem to play a much larger role in catalyzing the reduction of H_2O_2 in comparison to other enzymatically driven reduction reactions. This is in large part due to the significantly higher affinity for H_2O_2 by the peroxiredoxins [15]. Peroxiredoxins are estimated to be responsible for the reduction of greater than 90% of the mitochondrial and cytosolic H_2O_2 under normal cellular conditions [16,17]. However, peroxiredoxins, like most peroxidase driven reactions, have limited flux and are inactivated via H_2O_2 reaction with cysteine thiolate in peroxiredoxins in the face of elevated levels of H_2O_2 [18–20]. This means that under conditions such as ischemia/reperfusion, peroxiredoxins would likely be inactive, and CAT would play a much larger role in the reduction of H_2O_2 [21,22]. The exact mechanism by which CAT decomposes H_2O_2 is very complex; however, it can be simplified as follows:

$$H_2O_2 + \text{Fe(III)–CAT} \rightarrow H_2O + O = \text{Fe(IV)–CAT}^+ \; H_2O_2 + H_2O + O = \text{Fe(IV)–CAT}^+ \rightarrow H_2O + \text{Fe(III)–CAT} + O_2$$

The reduction of very high levels of H_2O_2 via CAT would benefit *R. sylvatica* greatly, as elevated H_2O_2 during ischemia/reperfusion has been demonstrated to overwhelm delicate balances in cellular H_2O_2 distribution, maintained by compartmentalized peroxidases, often triggering apoptosis under oxidative stress [23]. Although it is unclear how H_2O_2 stimulates apoptosis, it is clear that CAT is important in humans and traditional animal models; however, less is known about its role in disease states, or its role aiding survival in the freezing frog. This study presents the first investigation of the potential method of regulation of CAT in the leg muscle of *R. sylvatica*, comparing control and frozen states, and provides evidence of CAT regulation by reversible protein phosphorylation during freezing, including the possibility of multiple phosphorylation states in vitro.

2. Materials and methods

2.1. Chemicals

All biochemicals were from BioShop (Burlington, ON, Canada) with a few exceptions; hydrogen peroxide (H_2O_2) was from Caledon Labs (Ontario, Canada), the Cibacron blue column was from Affiland (Ans, Belgium), hydroxyapatite Bio-Gel® HTP Gel column was from BioRad (Hercules, CA), and potassium phosphate, monobasic was from J. T. Baker Chemical Company (London, UK).

2.2. Animals

Male wood frogs were collected from the Ottawa area and, upon capture, washed in a tetracycline bath. Frogs were sampled as previously described in Dawson et al. [5].The frogs were then placed in containers containing damp sphagnum moss and held at 5 °C for 1 week. The frogs were separated into different groups. Control frogs were sampled directly from the 5 °C group. Frozen frogs were placed in closed boxes with damp paper towel lining the bottom and placed in an incubator set at −3 °C. The frogs were allowed to cool during a 45 min period to allow the body temperature of the frogs to cool to below −0.5 °C. Ice nucleation was achieved through skin contact with ice crystals formed on the wet paper towel. The frozen group of frogs was kept under these conditions for 24 h. Both control and frozen frogs were euthanized by pithing and tissues were quickly excised and frozen in liquid N_2. All tissue samples were stored at −80 °C until later 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 hind leg skeletal muscle 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, 10 mM β-mercaptoethanol, 5% *v/v* glycerol, and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Homogenates were then centrifuged at 13,500 × *g* at 4 °C and the supernatant collected for use in protein purification.

2.4. Purification of CAT

A hydroxyapatite column was equilibrated in homogenization buffer A. A 3 mL aliquot of frog muscle extract was applied to the column (1.5 × 2 cm) and washed with 20 mL of buffer A to remove unbound proteins. CAT was eluted from the hydroxyapatite column with a linear gradient of 0–3.5 M KCl in buffer A. Fractions of 900 μL were collected and 10 μL from each fraction was assayed to detect CAT activity. The top 7 fractions of peak CAT activity were pooled and diluted 10-fold with buffer A. The diluted fractions were applied to a Cibacron blue column (1.5 × 10 cm) pre-washed with 30 mL of buffer A. The Cibacron blue column was then washed with 30 mL of buffer A to remove unbound protein. Bound proteins were eluted with a linear gradient of 0–2 M KCl in buffer A. Fractions of 450 mL were collected and 10 μL from each fraction was assayed to detect CAT activity. The top 14 fractions of peak CAT activity were pooled and diluted 10-fold with buffer A. The diluted fractions were applied to a $DEAE^+$ column (1.5 × 20 cm) pre-washed with 50 mL of buffer A. The $DEAE^+$ column was then washed with 50 mL of buffer A to remove unbound protein. Bound proteins were eluted with a linear gradient of 0–1 M KCl in buffer A. Fractions of 900 mL were collected and 10 μL from each fraction was assayed to detect CAT activity, the top 12 fractions were pooled for control CAT, while the top 9 fractions were pooled for frozen CAT. The purity of CAT was determined by combining aliquots of samples 2:1 v:v with 2× 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), boiling for 5 min, and then running 30 μL samples from each purification step on SDS-PAGE, as described in Section 2.6.

2.5. Kinetic assays

CAT was assayed using a modified version of the method of Aebi [24]. Assay conditions were 50 mM KPi buffer (pH 7.2), 40 mM H_2O_2, and 10 μL enzyme preparation. One unit of enzyme activity is the amount that reduces 1 μmol of H_2O_2 per minute at 25 °C. The amount of H_2O_2 was measured at 240 nm in a Thermo Labsystems Multiskan