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Vertebrate freezing survival: Regulation of the multicatalytic proteinase complex and controls on protein degradation

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Abstract

The wood frog, *Rana sylvatica*, survives weeks of whole body freezing during winter hibernation, expressing numerous metabolic adaptations that deal not only with freezing but with its consequences including organ ischemia and cellular dehydration. The present study analyzes the 20s multicatalytic proteinase (MCP) complex from skeletal muscle to determine how protein degradation is managed in the ischemic frozen state. MCP was partially purified and assayed fluorometrically using three AMC-labeled substrates to compare multiple states: control (5 °C acclimated), 24 h frozen at −2.5 °C, 4 or 8 h thawed at 5 °C, 8 h anoxia, and 40% dehydration. MCP from frozen frogs showed significantly different K_m and V_{max} values compared with controls; e.g., K_m Z-LLE-AMC increased by 45% during freezing and 52% under anoxia whereas V_{max} decreased by 40%. After thawing, K_m was restored and V_{max} rose by 2.2-fold. Incubations promoting protein kinase or phosphatase action on MCP showed that phosphatase treatment strongly increased V_{max} implicating reversible phosphorylation in MCP regulation during freeze–thaw. Western blotting showed a 36% decrease in MCP protein in muscle from frozen frogs. The 20s MCP preferentially degrades oxidatively-damaged proteins and evidence of impaired function during freezing came from a 1.4-fold increase in protein carbonyl content in muscle and liver during freezing. Ubiquitin and ubiquitin conjugate levels were unchanged in muscle but changed markedly in liver during freeze–thaw.

Keywords: Proteasome; Metabolic suppression; Ubiquitin conjugation; Protein carbonyl; *Rana sylvatica*; Cryopreservation

1. Introduction

The wood frog, *Rana sylvatica*, is one member of a small group of terrestrially-hibernating anurans that can survive whole body freezing during the winter months. Freezing survival requires a range of physiological and biochemical adjustments to deal with the stressful consequences of the conversion of ~65% of total body water into extracellular ice [1]. These include adjustments that deal with (a) long term organ ischemia/anoxia during freezing, (b) major changes in cell volume as water moves out of cells to freeze in extracellular ice masses, (c) ice propagation through extracellular spaces, and (d) interruption of physiological functions including breathing, heart beat and skeletal muscle movement. Adaptations that aid survival include the accumulation of high concentrations of glucose as a cryoprotectant (levels in core organs rise from 1–5 μmol/g in controls to 150–300 μmol/g in frozen frogs) [1], the up-regulation of selected genes [2], and a reorganization of metabolic priorities to suppress those that are unnecessary in the frozen state in order to conserve energy for critical vital functions [3]. Identification of the mechanisms that support freezing survival in natural systems has important applications for the design of medical cryopreservation technologies for use with transplantable tissues and organs.

Under stressful conditions, many organisms enter a hypometabolic state by coordinating the suppression of energy-producing and energy-consuming processes to readjust their metabolism to a much lower rate of ATP turnover [3]. A major energy-consuming process in cells is protein synthesis, which can use 12–80% of cellular ATP output depending on tissue type and metabolic state [4]. Consequently, protein synthesis is typically strongly reduced when organisms enter a hypometabolic state and a primary mechanism involved in translation inhibition is the phosphorylation of various ribosomal initiation and elongation factors and/or the proteins that regulate these

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factors [3]. If protein synthesis is suppressed during hypometabolism, it stands to reason that the opposing metabolic function, protein degradation, must also be suppressed in a coordinated fashion in order to maintain protein homeostasis in cells.

The proteasome, or multicatalytic proteinase (MCP) complex, is responsible for 80–90% of protein degradation in cells [5] and, therefore, should be a primary target of regulation aimed at altering the rate of protein degradation. The proteasome is a large barrel-like structure that houses three main protease activities: tryptic, chymotryptic and peptidylglutamyl-peptide hydrolytic activity. These activities hydrolyze incoming proteins into peptides of 7–9 amino acids in length that are then further broken down by other cellular proteases [5]. Two main forms of the proteasome occur. The base form of the MCP is the 20s proteasome (700 kDa) which has four rings of seven subunits. The outer rings consist of alpha subunits whereas the inner rings are composed of beta subunits that are responsible for the catalytic activity of the proteasome. Two alpha subunits can be phosphorylated and have been shown affect the activity of the proteasome [6]. The 20s proteasome preferentially degrades oxidatively damaged proteins and small peptides in an ubiquitin and ATP independent fashion [7].

The larger 26s proteasome (2000 kDa) is composed of the native 20s barrel with 19s regulatory caps added at either end of the cylinder. This form is involved in the ubiquitin pathway of protein degradation. Damaged proteins are often tagged with long chains of ubiquitin and the 26s proteasome is responsible for the recognition of these polyubiquitinated proteins that are destined for degradation. Within the 19s regulatory caps are recognition sites for the identification of ubiquitin and the recycling of this valuable signal protein [5]. ATPases in the 19s cap help to catalyze the unfolding of the incoming protein so that it may enter the protease barrel of the 20s proteasome.

The present study assesses the effects of freeze/thaw and related stresses (dehydration, anoxia) on the activity and properties of the wood frog 20s proteasome. We also analyzed the content of protein carbonyls in muscle and liver to determine whether oxidatively damaged proteins accumulate during freezing and measured the levels of ubiquitin and ubiquitin conjugates to provide an indication of 26s proteasomal function in the frozen state.

2. Materials and methods

2.1. Animals

Male wood frogs (*Rana sylvatica*) were collected from breeding ponds in the Ottawa area during early April. Animals were rinsed in a tetracycline bath and housed at 5 °C in plastic boxes containing damp sphagnum moss for 1–2 weeks before use. Control frogs were sampled from this condition, sacrificed by pithing and tissues were rapidly dissected out, frozen in liquid nitrogen, and stored at −80 °C until use. For freezing exposure, frogs were transferred to closed plastic boxes with moist paper toweling on the bottom and then placed in an incubator at −2.5 °C. Under these conditions, frogs cool and begin freezing after ~45 min due to nucleation by contact with ice forming on the paper toweling. Some frogs were sampled after 24 h frozen, whereas others were returned to 5 °C and sampled after 4 or 8 h of thawing.

Experimental dehydration studies were carried out as previously described [8], with frogs held at 5 °C in glass desiccators separated from silica gel by a 1-cm layer of sponge. Initial body water content of frogs prior to dehydration was 0.81±0.02 g H_2O/g body mass (mean±S.E.M., n=6). The rate of water loss was 0.5–1% total body water lost per hour. Once animals had lost 40% of total body water, they were sacrificed as above. For experimental anoxia exposure frogs were placed in closed plastic jars sitting in crushed ice. The jars had two syringe ports in the lid to introduce and vent gas and had been flushed with 100% nitrogen gas for 15 min prior to introducing the frogs. After frogs were added, flushing with N_2 was continued for a further 15–20 min and then the ports were capped, followed by sealing the lid and ports with a layer of parafilm. A piece of damp paper toweling (wetted with deoxygenated distilled water) on the bottom of jars ensured that the frogs did not dehydrate during the experiment. Jars were then returned to the 5 °C incubator for an 8 h anoxia exposure; our previous work showed that wood frogs readily survive at least 48 h under these conditions [9]. Subsequently, jars were replaced in the crushed ice and N_2 gassing was reconnected while frogs were quickly sampled.

2.2. Preparation of tissue extracts

Samples of frozen hind leg thigh muscle were pulverized under liquid nitrogen using a mortar and pestle and then 100 mg of tissue was mixed with 1 mL of homogenization buffer (50 mM Tris–HCl, pH 7.8, 10 mM β-mercaptoethanol, 5 mM EDTA, 5 mM EGTA, 50 mM NaF) and homogenized using a Polytron homogenizer. The addition of chelating agents (EDTA, EGTA) inhibits endogenous protein kinases, whereas NaF inhibits protein phosphatases; these additions stabilize the native phosphorylation state of the MCP during tissue extraction. Samples were centrifuged at 9000×g for 15 min at 4 °C and the supernatant was removed. In most cases (with the exception of incubation studies), a measured volume of extract was then centrifuged through a Microsep (Pall) concentrating device (molecular weight cutoff 300 kDa) to remove small molecular weight proteins from the sample (large proteins are held back in the retentate). Concentrating devices were centrifuged at 7500×g at 4 °C until the sample volume had decreased to half. Homogenization buffer was then added to wash the sample, and the sample was recentrifuged. Final retentate was adjusted to the original volume with homogenization buffer.

2.3. Assay conditions

Enzyme activity was assayed fluorometrically using the method of Rogers and Dean [10]. Release of the fluorophore AMC from substrate-AMC conjugates was quantified using a Victor 1420 Multilabel counter (Perkin Elmer). All peptides were prepared in 3:1 v:v DMSO:water as higher amounts of DMSO could inhibit proteasome activity. The peptide substrates measured the three main proteinase activities of the proteasome: Suc-LEU-LEU-VAL-TYR-AMC (Sigma Chemical Co.) for chymotryptic activity, Cbz-ALA-ARG-ARG-AMC (Sigma Chemical Co.) for tryptic activity, and Cbz-LEU-LEU-GLU-AMC (Dalton Peptides) for the peptidyl-glutamyl-peptide-hydrolytic activity. Assays were conducted with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Preliminary studies optimized enzyme amount and the linearity of the reaction over time at both high and low assay temperatures; from this, 45 min was chosen as the standard reaction time. Inhibitor experiments were carried out using a potent proteasome inhibitor, MG132 (Sigma Chemical Co.), at a final assay concentration of 50 μM; samples were incubated with the inhibitor for 20 min prior to assay. Assays were performed in 96 well microplates with a total volume of 200 μL and standard reaction conditions: 50 mM Tris, pH 7.8, 20 mM KCl, 0.5 mM magnesium acetate and substrate varying from 0 to 200 μM.

To conduct low temperature assays the fluorometer was placed in a cold incubator set at an air temperature of ~4 °C and chilled overnight. Before each assay, the microplate filled with assay mixture was pre-equilibrated in the incubator for ~15 min. Due to heat generated by the fluorometer, the temperature in assay wells stabilized at 8 °C, as measured by a thermister placed in a buffer-filled well. After the assay was completed, the temperature in blank wells was measured again and had typically risen by 1–1.4 °C due to heat generation during the run.

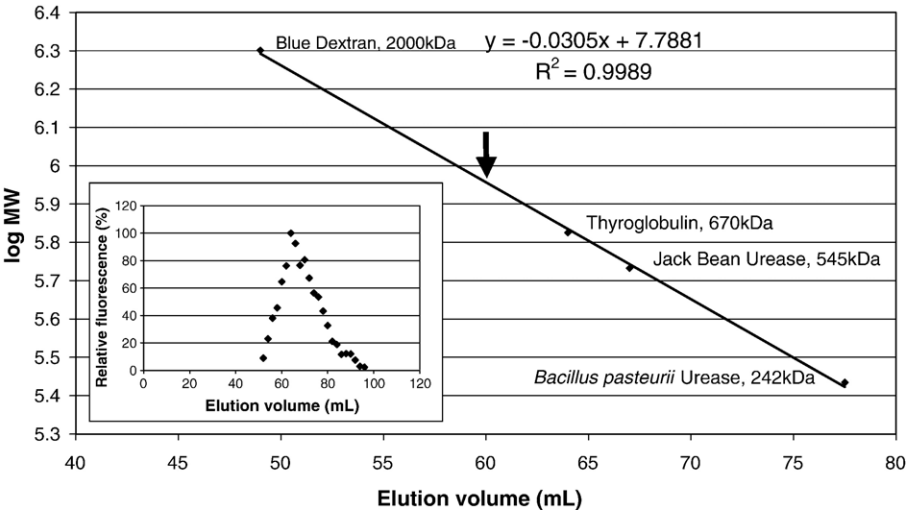

Fig. 1. Elution profile for the 20s proteasome off Sephacryl S400 and standard curve relating log molecular weight and elution volume for four protein standards. The elution position of the proteasome is shown by an arrow on the standard curve. Activity in the fractions is expressed relative to activity in the peak fraction which is set to 100.

2.4. Incubation studies to assess endogenous kinases and phosphatases effects on MCP

For these experiments only, muscle extracts were prepared in 50 mM Tris–HCl, pH 7.8 containing 10 mM β-mercaptoethanol and were not spun through Microsep centrifugal devices; this allowed all small molecular weight endogenous kinases and phosphatases to be maintained in the extract preparation. To test the effects on MCP of stimulating either endogenous protein kinases or protein phosphatases, aliquots of enzyme extract were incubated for 4 h at 4 °C under the following conditions: (a) to stimulate protein kinases additions to the buffer were 5 mM ATP, 5 mM $MgCl_2$, 1 mM cyclic 3′5′ adenosine monophosphate (cAMP), 1 mM cyclic 3′5′ guanosine monophosphate (cGMP), 1.3 mM $CaCl_2$, 1 μg/mL phorbol 12-myristate-13-acetate (PMA), and 30 mM NaF, or (b) to stimulate protein phosphatases additions to the buffer were 5 mM $MgCl_2$ and 5 mM $CaCl_2$.

2.5. Protein determination and statistics

Protein concentration was determined using the Coomassie blue dye-binding method and the Bio-Rad prepared reagent. Enzyme activities were measured as AMC production over time (pmol/h/μg protein) based on a standard curve constructed with 0–15 μM of the AMC fluorophore. Data were analyzed using a two-tailed Student's *t*-test or analysis of variance followed by the Student–Newman–Keuls test.

2.6. Partial purification of MCP

A Sephacryl-400 gel filtration column (90 cm×2.5 cm) was equilibrated in 25 mM Tris–HCl, pH 7.5 buffer containing 2 mM EDTA, 2 mM β-mercaptoethanol, 10% v:v glycerol and 0.04% w:v sodium azide. The column was calibrated using high molecular weight standards: blue dextran (2000 kDa), thyroglobulin (670 kDa), Jack Bean urease (545 kDa), and *Bacillus pasteuri* urease (242 kDa). Skeletal muscle was homogenized 1:5 w:v in homogenization buffer, centrifuged at 9000×*g* and then the supernatant was loaded onto the column. A flow rate of 0.35 mL/min was obtained and 1 mL fractions were collected. Fractions were assayed with Z-LLE-AMC peptide.

2.7. Carbonyl assay for total protein oxidative damage

The protocol was modified from Reznick and Packer [11]. Tissue extracts of liver and muscle were prepared 1:10 or 1:20 w:v in homogenization buffer and centrifuged for 15 min at 10,000×*g*. An aliquot of 500 μL of sample was added to 500 μL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) (dissolved in 2 M HCl). HCl both denatures the protein and allows the sample to be derivatized with DNPH. Blanks contained a 500-μL sample aliquot added to 500 μL of 2 M HCl. Both samples and blanks (run in duplicate in all cases) were then subjected to the following protocol. All were vortexed every 10 min for 1 h and then the reaction was stopped by the addition of 500 μL of 30% TCA to precipitate protein. After a 10-min incubation on ice, samples were centrifuged at 11,000×*g* for 15 min. Supernatants were decanted and pellets were washed in 1 mL of 1:1 v:v ethanol:ethylacetate. After a second centrifugation at 11,000×*g* for 15 min, the supernatant was discarded and the pellets were washed three more times. Finally, pellets were solubilized in 6 M guanidine hydrochloride and incubated at 37 °C for 30–60 min. Remaining insoluble material was removed by a final centrifugation at 11,000×*g* for 10 min. Sample absorbance was measured on a Multiscan Spectrum spectrophotometer at 380 nm with an absorption coefficient of 22,000 $M^{-1}\cdot cm^{-1}$. Protein

Table 1
Proteasome maximal activity and K_m values for three substrates for the skeletal muscle enzyme from wood frogs given experimental freeze/thaw, anoxia or dehydration exposures in vivo

Substrate	Condition	V_{max} (pmol/h/μg protein)	K_m (μM)
Z-LLE-AMC	Control	62.7±3.9	126.0±12.9
	Frozen 24 h	37.8±3.5 [a]	183.2±17.8 [a]
	Thaw, 4 h	135.4±8.6 [a,b]	150.0±12.7
	Thaw, 8 h	110.2±11.2 [a,b,c]	123.0±9.6 [b]
	Anoxia, 8 h	40.8±4.1 [a]	192.8±11.9 [a]
	40% Dehydrated	30.8±3.8 [a]	119.7±9.9
Z-ARR-AMC	Control	20.6±4.7	10.6±0.7
	Frozen 24 h	17.8±0.7	7.7±0.76 [a]
Suc-LLVY-AMC	Control	60.8±14.3	157.6±10.2
	Frozen 24 h	4.5±0.2 [a]	87.2±4.9 [a]

In vivo freezing was at −2.5 °C; all other conditions were at 5 °C. Assays used three peptides representing the different hydrolytic activities of the proteasome: Z-LLE-AMC (peptidyl-glutamyl-peptide-hydrolytic activity), Z-ARR-AMC (tryptic activity), and Suc-LLVY-AMC (chymotryptic activity). Assays were conducted at 23 °C and values are means±S.E.M., n=4 independent determinations on muscle extracts from different animals.

[a] Significantly different from the corresponding control value, as determined using ANOVA followed by the Student–Newman–Keuls test, $P<0.05$.

[b,c] Significantly different from the 24 h frozen and 4 h thawed values, respectively, $P<0.05$.

Table 2
Effects of assay temperature on K_m values and proteasome activity in skeletal muscle extracts from control and frozen wood frogs

	Assay at 23 °C	Assay at 8 °C
Control muscle		
K_m Z-LLE-AMC (μM)	135.5±13.8	84.5±5.3 [a]
V_{max} (pmol/h/μg protein)	62.7±7.8	38.3±2.0 [a]
Frozen Muscle		
K_m Z-LLE-AMC (μM)	192.5±19.5	59.3±3.96 [a]
V_{max} (pmol/h/μg protein)	43.4±7.0	19.0±2.0 [a]

Data are means±S.E.M., n=6.
[a] Significantly different from the corresponding values at 23 °C as assessed by the Student's t-test, P<0.05.

content was determined from a standard curve of bovine serum albumin, 0.25–2.0 mg/mL in guanidine hydrochloride assayed at 280 nm. Carbonyl content was measured as pmol/mg protein.

2.8. Western blotting of ubiquitin conjugates

Frozen tissue samples of skeletal muscle and liver were homogenized (1:5 w/v) in homogenization buffer containing 20 mM Tris, pH 7.8, 150 mM NaCl, 10 mM β-glycerophosphate, 1 mM EDTA, 1% w/v Triton X-100, 1 mM NaF and 1 mM EGTA; a small aliquot of protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), was added just prior to homogenization. Samples were centrifuged at 10,000×g for 15 min at 4 °C. The supernatant was collected and the protein concentration was determined using the BioRad protein assay with bovine serum albumin as a standard. Samples were then diluted to the desired concentration in sample buffer containing 100 mM Tris–HCl (pH 6.8), 4% SDS, 20% v/v glycerol, 5% v/v β-mercaptoethanol, and 0.2% w/v bromophenol blue. Samples were boiled for 5 min, cooled and then frozen at −20 °C until use.

SDS-polyacrylamide gel electrophoresis was carried out using 12% acrylamide gels and the BioRad Mini-PROTEAN 3 system. A 20-μg aliquot of soluble protein was loaded into each well and gels were run at 180 V until the desired separation of colored molecular weight markers (Kaleidoscope, BioRad) was achieved. Proteins were blotted onto polyvinylidene difluoride (PVDF) membranes (Pall) by wet transfer in cold 25 mM Tris pH 8.5, 192 mM glycine and 20% v/v methanol. Transfer was carried out at 300 mA for ~90 min.

PVDF membranes were incubated in blocking solution composed of TBST buffer (10 mM Tris pH 7.5, 150 mM NaCl, 0.05% v/v Tween-20) and 5% non-fat milk for 30–60 min at room temperature. The blocking solution was then discarded and the membrane was incubated in fresh TBST containing primary antibody, diluted according to manufacturer's instructions (1:1000 v/v). Membranes were incubated overnight at 4 °C to allow primary antibody to bind. Rabbit polyclonal antibodies for anti-ubiquitin and ubiquitin conjugates (Biomol) and anti-20s proteasome (CalBioChem; detecting the α1-subunit) were used. After incubation with primary antibody, membranes were washed with TBST in three washes of 5 min each. Membranes were then incubated with secondary HRP-linked goat anti-rabbit IgG antibody (1:2000 v/v) in TBST for 1.5 h at 4 °C. After incubation the membranes were washed again in TBST for 3 washes of 5 min. Protein detection used an enhanced chemiluminescence assay with Super Signal West Pico Chemiluminescent Substrate (PIERCE). Band densities were quantified using the ChemiGenius Bio Imaging System (SynGene) and GeneTools software (SynGene). To confirm that a consistent amount of protein was added to each well, membranes were subsequently stained with Coomassie blue and rescanned. Selected strong bands that showed constant intensities between samples (and were not near the MW of the protein of interest) were chosen as control bands. They were quantified by densitometry and used to normalize the intensity of the immunoreactive bands between lanes. A minimum of four independent isolations was performed per treatment.

3. Results

3.1. Enzyme preparation and Sephacryl-400 gel filtration

Initial tests showed that partial purification of the MCP using Microsep concentrating devices to remove lower molecular weight proteins resulted in an ~80% increase in enzyme activity (data not shown); hence, this procedure was routinely used for most preparations. To confirm that no other proteases that were capable of using the three peptide substrates remained in the Microsep treated retentate, several samples were incubated with the potent MCP inhibitor MG132 prior to assay. In the presence of the inhibitor, no protease activity was detected in the retentate.

Fractionation of a muscle extract on Sephacryl-400 was used to determine the native molecular mass of the proteasome. The frog muscle proteasome complex eluted in a single peak

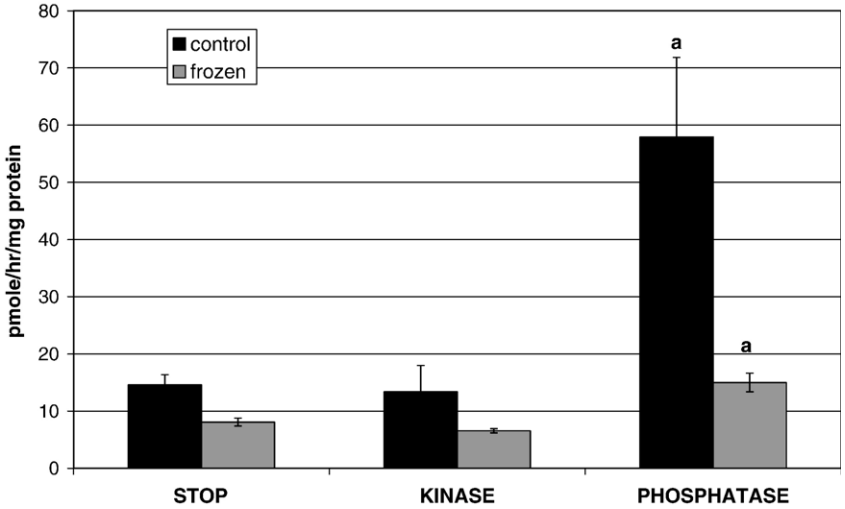

Fig. 2. The effects of incubations that stimulated the activities of endogenous kinases and phosphatases on the activity of the 20s proteasome in skeletal muscle extracts from control and 24 h frozen wood frogs. Data are mean±S.E.M., n=4 independent determinations on muscle samples from different animals. [a] Significantly different from the corresponding control (STOP) and kinase incubations, as determined using ANOVA followed by the Student–Newman–Keuls test, P<0.005.

Table 3
Protein carbonyl content as a measure of oxidatively damaged proteins in skeletal muscle and liver of control, frozen (24 h at −2.5 °C) and thawed (4 h at 5 °C) frogs

	Carbonyl content, pmol/mg protein	
	Muscle	Liver
Control	422±56	435±40
Frozen, 24 h	601±56[a]	616±55[a]
Thawed, 4 h	298±17[b]	399±29[b]

Data are means±S.E.M., n=6.
[a] Significantly different from the corresponding control value, as determined using ANOVA followed by the Student–Newman–Keuls test, P<0.05.
[b] Significantly different from the corresponding 24 h frozen value P<0.05.

corresponding to molecular mass of 830 kDa (Fig. 1). Pooled peak fractions from the Sephacryl S400 column were run on a native polyacrylamide gel and a single protein band was seen at a molecular weight somewhat greater than the 669-kDa band of the thyroglobulin marker, again consistent with the expected size of the 20s proteasome (data not shown). ATP is needed for the proper functioning of the 26s proteasome, whereas SDS is a known stimulator of the 20s proteasome [10]. Increased activity of the frog proteasome was noted when the assay mixture contained 0.02% w:v SDS, but not 0.5 mM ATP (concentrations recommended in ref. 10], confirming that the activity detected was the 20s proteasome.

3.2. Effects of freezing, anoxia or dehydration exposures in vivo on skeletal muscle MCP

Freezing exposure resulted in significant changes in the K_m values of the proteasome for all of its peptide substrates. K_m Z-LLE-AMC was 45% higher for MCP from frozen muscle, compared with controls, whereas K_m values for Z-ARR-AMC and Suc-LLVY-AMC decreased by 27 and 46%, respectively, during freezing (Table 1). Thawing reversed the effect of freezing on the K_m Z-LLE-AMC, K_m dropping over time to reach control values after 8 h thawed. Anoxia exposure also stimulated a strong increase in the K_m Z-LLE-AMC which rose by 52%, mimicking the effects of freezing on the enzyme. However, experimental dehydration in vivo had no effect on the K_m Z-LLE-AMC. Table 1 also shows that whole animal freezing exposure reduced the maximal activity of the proteasome in muscle when assayed with either Z-LLE-AMC or Suc-LLVY-AMC; activity in frozen muscle was just 60% and 7%, respectively, of the corresponding control activity. Thawing for 4 h increased the V_{max} Z-LLE-AMC by 2.2-fold compared with the control value (a 3.6-fold increase over the value for frozen muscle) but the value declined back towards control levels after 8 h thawing. Both anoxia and dehydration exposures significantly suppressed V_{max} to 65 and 49% of the control value, respectively.

3.3. Temperature effects on substrate K_m value and V_{max} values

Maximal activity and substrate affinity for Z-LLE-AMC were determined at warm (23 °C) and cold (8 °C) temperatures for MCP from skeletal muscle of control and frozen frogs (Table 2). In all cases, kinetic parameters were significantly reduced in assays at 8 °C as compared with 23 °C. The K_m value for MCP from control muscle decreased by 37% when assayed at 8 °C, whereas the decrease in K_m was 69% for muscle MCP from frozen frogs; hence, substrate affinity increased at low

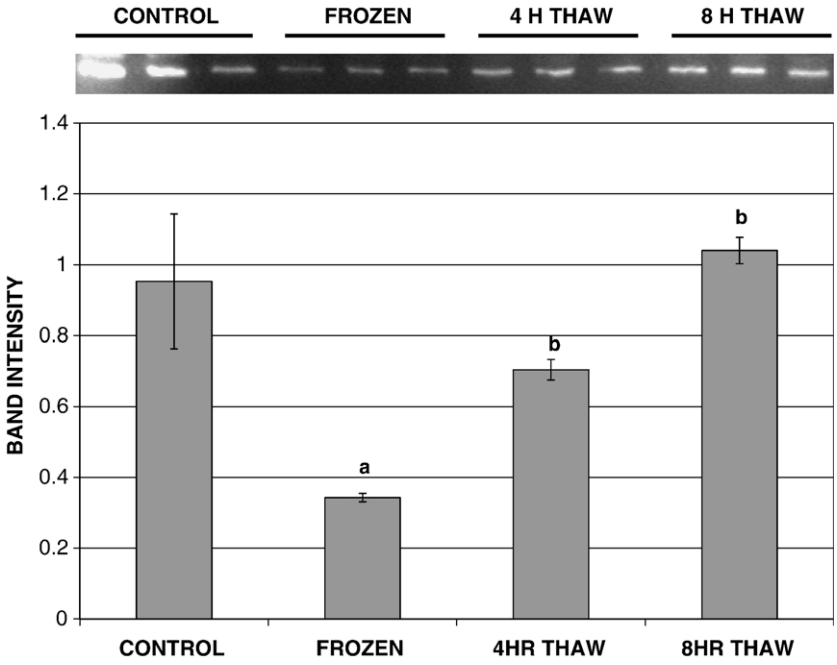

Fig. 3. Western blot analysis detecting the amount of α1-subunit of the 20s proteasome in skeletal muscle sampled from control, 24 h frozen, 4 h thawed and 8 h thawed wood frogs. Representative blots are shown along with histograms showing normalized band intensities. Data in histograms are means±S.E.M., n=4 separate determinations on independent tissue extracts. [a]Significantly different from the corresponding control value, as determined using ANOVA followed by the Student–Newman–Keuls test, P<0.05; [b]significantly different from the 24 h frozen value, P<0.05.

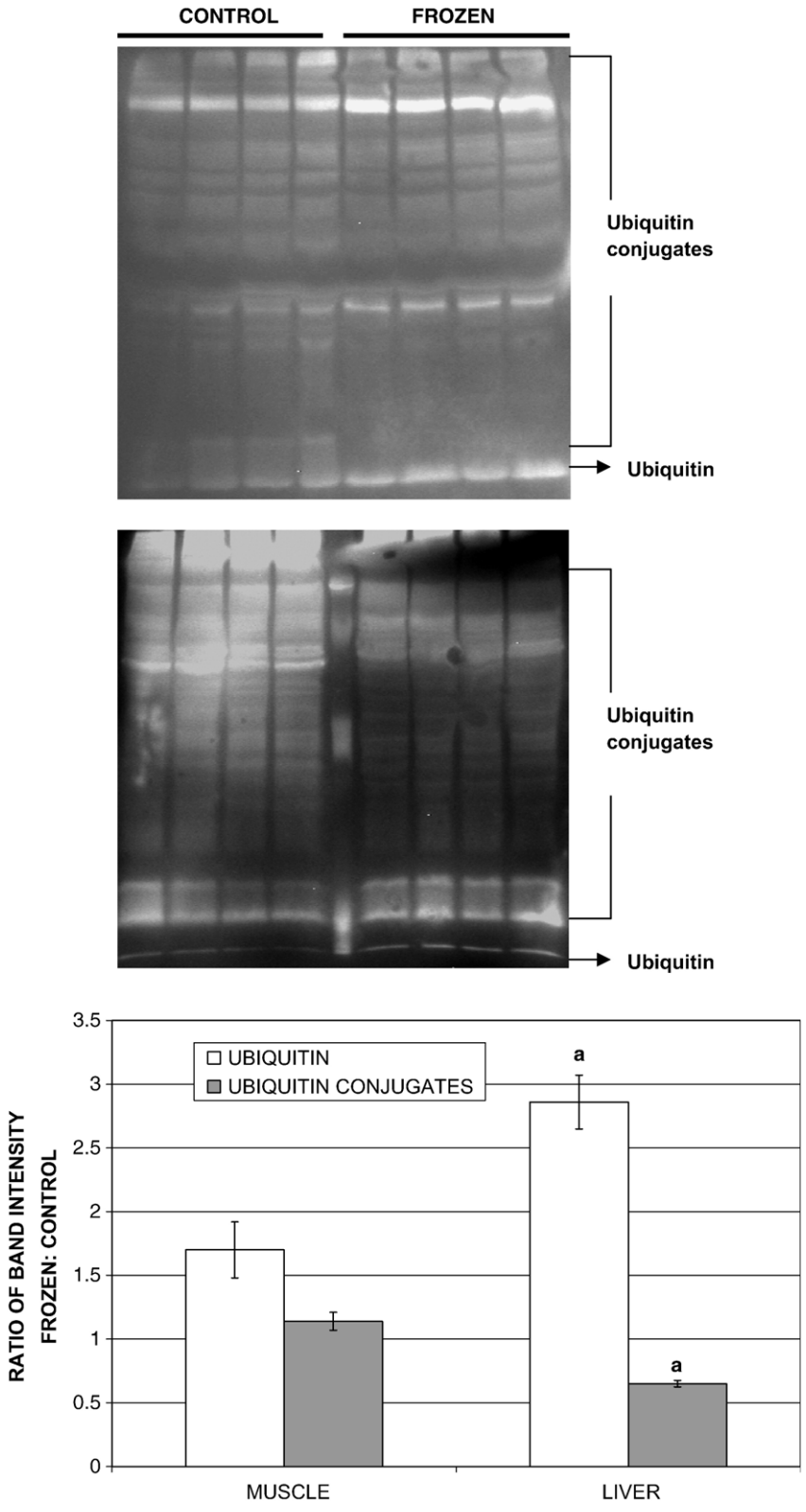

Fig. 4. Western blot analysis of the levels of free ubiquitin and ubiquitin conjugates in muscle (upper blot) and liver (lower blot) extracts from control and frozen frogs. Ubiquitin was quantified as the intensity of the single band at 8 kDa. Ubiquitin conjugates were quantified by summing the intensities of bands at higher molecular weights (within the brackets shown). On the liver blot a molecular weight marker lane (Kaleidoscope broad range prestained protein markers, BioRad) separates the four control lanes (left) from the four frozen (right) lanes. Immunoreactive band intensities in each lane were normalized against corresponding Coomassie blue stained bands in these lanes and then mean normalized band densities (±S.E.M., n=4 independent extracts) were calculated for control versus frozen samples. Significance testing compared these two groups and then histograms were constructed showing the frozen:control ratio. [a]Mean control and frozen values are significantly different, as determined by the Student's t test, $P<0.05$.

temperature. Maximal activity decreased at the lower assay temperature and calculated Q_{10} values were 1.42 and 1.86 for the enzyme from control and frozen frog muscle, respectively.

3.4. Effects of stimulating endogenous kinases and phosphatases on proteasome activity

Enzyme activity in crude muscle extracts from control and 24 h frozen frogs was measured after in vitro incubations to stimulate the activities of endogenous protein kinases and protein phosphatases that might covalently modify the proteasome. The lower V_{max} values for untreated enzyme in Fig. 2, as compared with Table 2, are attributable to the absence of the Microsep filtration step in the preparation of the extracts. Stimulation of the activities of endogenous kinases by the addition of Mg.ATP and second messengers of protein kinases A, G and C did not significantly affect the activity of the proteasome in extracts from either control or frozen muscle. However, stimulation of phosphatase activities by incubation with Mg^{2+} and Ca^{2+} resulted in a marked increase in proteasome activity in extracts from both control and frozen muscle; activity increased by roughly 4- and 2- fold, respectively. Notably, proteasome activity in extracts from frozen muscle increased to the activity seen in untreated control extracts.

3.5. Carbonyl content

Protein carbonyls form as a result of oxidative damage and, hence, the carbonyl content of tissues is a measure of oxidative stress under different experimental conditions. The amount of damaged protein increased significantly by 1.4-fold in both muscle and liver from frozen frogs, as compared with controls (Table 3). However, carbonyl content returned to control values within 4 h of thawing in both tissues.

3.6. Western blot analysis

Changes in the amount of the 20s proteasome in skeletal muscle were evaluated over a freeze–thaw cycle using Western blotting with an antibody recognizing the α1-subunit of the 20s proteasome. A marked decrease in the amount of proteasome protein occurred during freezing; the mean amount in muscle from 24 h frozen frogs was just 36% of the amount detected in controls (Fig. 3). However, the amount of proteasome complex in muscle increased again after thawing.

An antibody detecting ubiquitin and ubiquitin-conjugates was used to examine the effects of freezing on the ubiquitin tagging system, which is upstream from protein degradation. Free ubiquitin was visualized as a prominent 8 kDa band (Fig. 4). Ubiquitin conjugates of proteins show up as multiple bands because the tagged proteins are of many different molecular weights and the number of ubiquitin moieties bound on each protein type can also vary. Hence, ubiquitin conjugates were quantified by summing the densities of multiple bands outlined in brackets in Fig. 4. In muscle, both free ubiquitin and the amount of ubiquitin conjugates remained constant during freezing. By contrast, liver pools of free ubiquitin were significantly elevated (by about 2.8-fold) during freezing whereas the levels of ubiquitin conjugates were reduced during freezing by about 30% as compared with control liver.

4. Discussion

The proteasome from wood frog skeletal muscle eluted off the S400 gel filtration column in a single peak corresponding to a molecular mass of ~800 kDa which indicated that it was the 20s proteasome; native polyacrylamide gel electrophoresis of the S400 peak fractions and the effects of ATP and SDS on proteasome activity also supported this designation. Kinetic assessment of the frog proteasome showed expression of the three characteristic proteolytic activities: tryptic, chymotryptic and peptidylglutamyl peptide hydrolytic activities. Significant changes in substrate affinity, K_m, were found for all three peptides when the proteasome from muscle of frozen frogs was compared with the enzyme from control 5 °C-acclimated frogs. Substrate affinity for Z-LLE-AMC decreased (K_m increased) whereas affinity for Z-ARR-AMC and Suc-LLVY-AMC increased in muscle from frozen frogs (Table 1). The significant increase in K_m Z-LLE-AMC under frozen conditions was reversed when frogs thawed. In vivo anoxia exposure had the same effect as freezing on K_m Z-LLE-AMC whereas dehydration had no effect. V_{max} of the MCP with Z-LLE-AMC also decreased in both frozen and anoxic muscle (and also during dehydration) (Table 1). These data suggest that the anoxia/ischemia caused by tissue freezing may be the underlying trigger for the modification of proteasome properties in frozen frogs. Various other metabolic and gene expression responses to freezing in wood frog tissues have been linked either to anoxia stress or dehydration stress as the underlying trigger [1,2].

The proteasome is known to undergo covalent modification by phosphorylation at three sites: the alpha subunits 7 and 3 in the core 20s barrel and the ATPase subunit S4 in the 19s regulatory cap [6]. Indeed, protein phosphorylation is important in the assembly of the 19s regulatory cap(s) to form the 26s proteasome [6]. Stress-induced stable changes in the K_m and V_{max} values of enzymes are often the result of covalent modification. Changes in proteasome kinetic properties after stimulation of protein kinase or phosphatase activities would provide presumptive evidence that the addition or removal of phosphate groups from the MCP might be the cause of the freeze- and anoxia-responsive changes in MCP properties. A significant increase in MCP activity occurred when samples were incubated under conditions that stimulated endogenous phosphatase activities (Fig. 2). This suggests that protein phosphorylation may be the mechanism responsible for modifying MCP activity and substrate affinity under stress conditions (freezing, anoxia) whereas enzyme dephosphorylation could underlie the large increase in MCP activity seen during thawing (Table 1). Such posttranslational modification could also alter the priorities of MCP action towards different protein substrates in the frozen state; i.e., note that Table 1 shows differential effects (up, down, no change) of freezing on the K_m and V_{max} of each of the three MCP proteolytic activities.

In particular, priority may be given to tryptic activity in the frozen state since Z-ARR-AMC kinetics showed no significant change in V_{max} and a decrease in K_m (i.e., increased affinity) in muscle from frozen animals. Posttranslational modification producing a strong increase in MCP activity during thawing could also be critical for orchestrating a rapid degradation of damaged proteins that accumulated over time when frogs were frozen.

The protein kinases and phosphatases that mediate frog MCP responses during freeze/thaw remain to be determined. Casein kinase II is known to phosphorylate the proteasome subunits alpha 7 and 3 [12] and, indeed, this kinase is known to copurify with the proteasome [13]. Unfortunately, this enzyme cannot be stimulated singly in incubations to determine whether it is responsible for the freeze-responsive changes in frog MCP. A cGMP-dependent phosphorylation site has also been found in the alpha 3 subunit [14].

Proteasome V_{max} activity was reduced significantly during freezing when peptidylglutamyl-peptide hydrolytic and chymotryptic activities were assessed (activities fell to 60 and 9% of control values, respectively) but the tryptic activity was not significantly affected (Table 1). As suggested above, these unequal effects of freezing exposure on the three activities of the proteasome could result from a freeze-responsive covalent modification of the enzyme. However, another factor could be responsible for reduced activities of the proteasome in muscle of frozen frogs and that is a decrease in the amount of proteasome protein present. Indeed, the results of Western blotting support this idea and show a significant decrease in the amount of 20s proteasome, as assessed using antibodies against the α1-subunit, between control and frozen states (Fig. 3). However, the amount of proteasome protein rose again when frogs were thawed. Hence, both an increase in proteasome protein content (Fig. 3) and a posttranslational modification (dephosphorylation) of the enzyme (Fig. 2) could be responsible for the very large the increase in proteasome activity in muscle after thawing (Table 1).

Temperature also had a significant effect on both K_m and V_{max} values for the MCP from muscle of control and frozen frogs. The K_m values were significantly reduced in low temperature (8 °C) assays as compared with 23 °C. This shows a higher affinity of the proteasome for substrates at lower temperatures which is perhaps not unexpected since the animals used in the present study were collected just after they emerged from winter hibernation and they were maintained in the lab at 5 °C. An MCP optimized for function in vivo at near 0 °C temperatures would be appropriate over the winter months. Maximal activity of the proteasome showed an expected decrease at 8 °C as compared with 23 °C with a calculated Q_{10} for this enzyme of 1.42 and 1.86 for the enzyme from control and frozen frog muscle, respectively.

During whole animal freezing, vital functions including breathing and heartbeat cease. Blood circulation is cut off when plasma freezes and tissue oxygen levels are depleted. An ischemic state is maintained until thawing allows the reperfusion of organs. In mammalian systems, reperfusion damage is common after ischemia and is caused by a burst of oxygen-free radicals formed when blood flow is re-established. However, studies with wood frogs have shown that this species maintains high constitutive activities of antioxidant enzymes, apparently to deal with the oxidative stress that occurs during thawing [15]. Furthermore, levels of glutathione and activities of some antioxidant enzymes increase in frog organs during freezing; e.g., glutathione peroxidase activity rose significantly in skeletal muscle [15]. As a result, when two indices of oxidative damage to lipids were assessed, no changes were found over the course of freeze/thaw exposure [15]. However, the measurements of carbonyl content in the present study show that oxidatively damaged proteins accumulated in muscle while frogs were frozen but these were rapidly removed after thawing. This accumulation might arise because of enhanced oxidative damage to proteins during freezing, but equally could be due to an inhibition of MCP function in the ischemic, energy-limited frozen state. The MCP catabolizes oxidatively damaged proteins through recognition of hydrophobic surface patches [16]. Hence, carbonyl proteins could accumulate in frozen frogs due to a reduced ability to catabolize them in the frozen state. This explanation also tallies with the observation that carbonyl protein levels in muscle were reduced to below control levels within 4 h of thawing.

In a mammalian form of hypometabolism (hibernation), Van Breukelen and Carey [17] found an increase in ubiquitin conjugates during torpor in gut and liver of hibernating ground squirrels. The present data for wood frogs found no change in ubiquitin conjugates in muscle during freezing but levels in liver fell by 30%. This shows that there are tissue specific differences between frog organs in their response to freeze/thaw. Skeletal muscle and skin, being peripheral tissues, are the first tissues to freeze and their metabolism is likely arrested very quickly. Hence, unchanged levels of ubiquitin and ubiquitin conjugates in muscle are perhaps not unexpected if protein degradation processes are rapidly halted. The carbonyl protein data suggest that oxidatively damaged proteins continue to accumulate throughout the freeze so the lack of change in ubiquitin conjugate levels suggests a regulated suppression of the ubiquitin tagging process in frozen muscle. Ubiquitin tagging involves three enzymes (E1, E2, E3) and ATP is required for the activation of ubiquitin to bind to E1. This ATP requirement may mean that ubiquitin tagging is too energetically expensive to be maintained under stress conditions and so is shut down. The 20s proteasome is much more efficient at degrading oxidatively damaged proteins than the ubiquitin tagged 26s proteasome pathway [18], and therefore ubiquitin pathways may be turned off in frozen frogs but the 20s proteasome activity may be retained to deal with oxidatively damaged proteins. The data from liver show elevated ubiquitin and decreased ubiquitin conjugates during freezing. Again, this could also be due to the regulation of ubiquitin tagging enzymes E1–E3. Liver is the last organ to freeze in the frog and, during the several hours that it takes a frog to freeze, liver metabolism is highly active in both cryoprotectant synthesis and the production of freeze-responsive proteins. It is possible that during this time, existing ubiquitin conjugates continue to be degraded but ATP limitation and/or specific controls on E1–E3

halt the formation of new conjugates. The net result of this would be elevated free ubiquitin levels in liver of 24 h frozen frogs coupled with reduced conjugates. Future studies should look into the tissue specific regulation of ubiquitin enzymes E1–E3 to confirm these hypotheses.

Freeze-responsive alterations in muscle protein degradation machinery may also have a role in helping to restore the physiological function of muscle after thawing. Freezing in nontolerant organisms results in a variety of cellular damage that frequently triggers apoptosis after thawing and prevents recovery of muscle function. Even wood frogs require some time for the restoration of muscle contractile function after freezing [1]. Twitch responses by hind legs take 4 h or more to reappear after thawing [19] and, compared with control frogs, previously frozen frogs still showed a 40% reduction in locomotor endurance during treadmill exercise even 96 h after thawing [20]. Hence, full recovery of muscle function may take several days and may be linked with both the clearance of damaged proteins by the MCP (and by the 26s proteasome once cellular ATP availability is again high) and with the de novo synthesis of new contractile proteins when biosynthetic machinery is reactivated.

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References

[1] K.B. Storey, J.M. Storey, Physiology, biochemistry and molecular biology of vertebrate freeze tolerance: the wood frog, in: E. Benson, B. Fuller, N. Lane (Eds.), Life in the Frozen State, CRC Press, Boca Raton, 2004, pp. 243–274.

[2] K.B. Storey, Strategies for exploration of freeze responsive gene expression: advances in vertebrate freeze tolerance, Cryobiology 48 (2004) 134–145.

[3] K.B. Storey, J.M. Storey, Metabolic rate depression in animals: transcriptional and translational controls, Biol. Rev. 79 (2004) 207–233.

[4] C.J. Fuery, P.C. Withers, M. Guppy, Protein synthesis in the liver of *Bufo marinus*: cost and contribution to oxygen consumption, Comp. Biochem. Physiol., A 119 (1998) 459–467.

[5] M. Orlowski, S. Wilk, Catalytic activities of the 20 S proteasome, a multicatalytic proteinase complex, Arch. Biochem. Biophys. 383 (2000) 1–16.

[6] A.J. Rivett, S. Bose, P. Brooks, K.I. Broadfoot, Regulation of proteasome complexes by γ-interferon and phosphorylation, Biochimie 83 (2001) 363–366.

[7] M. Orlowski, S. Wilk, Ubiquitin-independent proteolytic functions of the proteasome, Arch. Biochem. Biophys. 415 (2003) 1–5.

[8] T.A. Churchill, K.B. Storey, Metabolic responses to dehydration by liver of the wood frog, *Rana sylvatica*, Can. J. Zool. 72 (1994) 1420–1425.

[9] C.P. Holden, K.B. Storey, Second messenger and cAMP-dependent protein kinase responses to dehydration and anoxia stresses in frogs, J. Comp. Physiol., B 167 (1997) 305–312.

[10] K.J. Rodgers, R.T. Dean, Assessment of proteasome activity in cell lysates and tissue homogenates using peptide substrates, Int. J. Biochem. Cell Biol. 35 (2003) 716–727.

[11] A.Z. Reznick, L. Packer, Oxidative damage to proteins: spectrophotometric method for carbonyl assay, Enzymology 233 (1994) 357–363.

[12] J.G. Castaño, E. Mahillo, P. Arizti, J. Arribas, Phosphorylation of C8 and C9 subunits of the multicatalytic proteinase by casein kinase II and identification of the C8 phosphorylation sites by direct mutagenesis, Biochemistry 35 (1995) 3782–3789.

[13] R. Ludemann, K.M. Lerea, J.D. Etlinger, Copurification of casein kinase II with 20S proteasomes and phosphorylation of a 30-kDa proteasome subunit, J. Biol. Chem. 268 (1993) 17413–17417.

[14] G.G. Mason, K.B. Hendil, A.J. Rivett, Phosphorylation of proteasomes in mammalian cells. Identification of two phosphorylated subunits and the effect of phosphorylation on activity, Eur. J. Biochem. 238 (1996) 453–462.

[15] D.R. Joanisse, K.B. Storey, Oxidative damage and antioxidants in *Rana sylvatica*, the freeze tolerant wood frog, Am. J. Physiol. 271 (1996) R545–R553.

[16] T. Grune, K. Merker, G. Sandig, K.J.A. Davies, Selective degradation of oxidatively modified protein substrates by the proteasome, Biochem. Biophys. Res. Commun. 305 (2003) 709–718.

[17] F. Van Breukelen, H.V. Carey, Ubiquitin conjugate dynamics in the gut and liver of hibernating ground squirrels, J. Comp. Physiol., B 172 (2002) 269–273.

[18] T. Grune, T. Reinheckel, K.J.A. Davies, Degradation of oxidized proteins in mammalian cells, FASEB J. 11 (1997) 526–534.

[19] J.R. Layne, M.C. First, Resumption of physiological functions in the wood frog (*Rana sylvatica*) after freezing, Am. J. Physiol. 261 (1991) R134–R137.

[20] J.T. Irwin, J.P. Costanzo, R.E. Lee, Postfreeze reduction of locomotor endurance in the freeze-tolerant wood frog, *Rana sylvatica*, Physiol. Biochem. Zool. 76 (2003) 331–338.