Effects of hibernation on multicatalytic proteinase complex in thirteen-lined ground squirrels, Spermophilus tridecemlineatus

ARTICLE *in* MOLECULAR AND CELLULAR BIOCHEMISTRY · APRIL 2005

Impact Factor: 2.39 · DOI: 10.1007/s11010-005-6341-5 · Source: PubMed

2 AUTHORS:

Ashley Woods
Cornell University
10 PUBLICATIONS **159** CITATIONS
SEE PROFILE

Kenneth B. Storey
Carleton University
712 PUBLICATIONS **16,139** CITATIONS
SEE PROFILE

Molecular and Cellular Biochemistry **271:** 205–213, 2005.

Effects of hibernation on multicatalytic proteinase complex in thirteen-lined ground squirrels, *Spermophilus tridecemlineatus*

Ashley K. Woods and Kenneth B. Storey

Institute of Biochemistry and Department of Biology, College of Natural Sciences, Carleton University, 1125 Colonel By Drive, Ottawa, Ontario, Canada

Received 24 September 2004; accepted 16 November 2004

Abstract

Multicatalytic proteinase complex (MCP) was studied in skeletal muscle of the hibernating ground squirrel, *Spermophilus tridecemlineatus*. MCP was partially purified using a S-400 gel filtration column and Centricon concentrating devices and assayed fluorometrically using three AMC-labeled substrates. K_m and V_{max} values were determined for each substrate with no significant differences between the enzyme from euthermic versus hibernating animals when assayed at 23 °C. However, properties of MCP from euthermic and hibernating ground squirrels were differentially affected by low assay temperature (8–10 °C) and also differed from the mouse enzyme, the data indicating that ground squirrel MCP is better suited for low temperature function. MCP preferentially degrades oxidatively-damaged proteins and quantification of protein carbonyl content showed that the level of oxidatively-damaged protein in skeletal muscle decreased by >75% during hibernation suggesting a continuing role for the MCP in the torpid state. (Mol Cell Biochem **271:** 205–213, 2005)

Key words: proteasome, protein degradation, carbonyl proteins, metabolic rate depression, oxidative damage

Introduction

Conditions of cold temperature and/or low food supply stimulate a variety of mammalian species to enter a hypometabolic state for periods ranging from brief torpor bouts of a few hours to 6–9 months of winter hibernation [1, 2]. Metabolic rate depression results from a strong reduction in net ATP turnover achieved by coordinated suppression of the rates of both energy-producing and energy-consuming processes while maintaining a relatively constant adenylate energy charge. Suppression of the rates of multiple cell functions occurs including enzyme activities, ion channels, protein synthesis, protein degradation and gene expression often via the mechanism of reversible protein phosphorylation [3].

One of the major energy costs to cells is protein turnover, the synthesis and degradation of cellular proteins. Estimates of the proportion of cellular ATP turnover devoted to protein synthesis vary from 12 to 80% depending on species, tissue and metabolic state [4]. Not surprisingly, rigorous suppression of this energy expensive process is a key component of hypometabolic states, including mammalian hibernation [5, 6]. Inhibitory control over protein synthesis has been traced to at least two ribosomal proteins: the eukaryotic initiation factor 2 and the eukaryotic elongation factor 2 [5, 7]. For example, phosphorylation of eIF2 on the α subunit inhibits the ability of this factor to bind and deliver the initiating methionine residue to the assembling translation complex. Predictably, a decrease in protein synthesis in the cells of hibernators should be accompanied by a comparable decrease in protein degradation so that cellular protein content remains largely unchanged during torpor and cells can return to normal euthermic functions as soon as the animal

Address for offprints: Kenneth B. Storey, Institute of Biochemistry and Department of Biology, College of Natural Sciences, Carleton University, 1125 Colonel By Drive, Ottawa, Ontario, Canada K1S 5B6 (E-mail: kenneth_storey@carleton.ca)

rewarms. Indeed, a strong reduction in protein turnover has been demonstrated in several hypometabolic systems [3].

Under normal metabolic conditions, many cellular processes including protein metabolism, gene transcription and the cell cycle are governed by the activation and inactivation of proteins and their subsequent degradation. Cells have several strategies for protein degradation. The major proteases in mammalian cells are lysosomal cathespsins, calpains and the proteasome [8]. Membrane bound proteins and cell surface proteins are mainly degraded by the lysosome [9], whereas the bulk of cytosolic proteins (80–90%) are tagged and sent to the proteasome for degradation [10].

The proteasome is a large barrel-structured protein complex capable of degrading old, damaged or oxidized proteins into peptides averaging 7–9 amino acids in length that can then be further broken down and recycled in the cell [10]. The basic form of the 20S proteasome is a cylindrical 700 kDa structure with four rings of seven subunits. The two outer rings consist of alpha subunits while the inner rings house the beta subunits that are responsible for the catalytic activity. The proteasome has within it three major types of protease activities: tryptic, chymotryptic and peptidylglutamyl-peptide hydrolytic activity. The 20S proteasome degrades many proteins and peptides but preferentially degrades oxidatively damaged proteins in an ubiquitin and ATP independent fashion [11].

Alternative forms of the proteasome are present in the cell. The 26S proteasome (MW 2000 kDa) is composed of the native barrel of the 20S proteasome with two 19S regulatory caps on either end [11]. The 19S regulatory caps contain a ring of six ATPases that use energy from ATP hydrolysis to catalyze the unfolding of the incoming protein into the 20S barrel. Most of the substrates of the 26S proteasome undergo a tagging process of ubiquitination where polymers of ubiquitin are added in sequence to a damaged protein destined for the 26S proteasome. Recognition sites on the 19S regulatory cap help to remove the ubiquitin chains and recycle this valuable signal protein [10]. Although the 26S proteasome is capable of degrading proteins in a non-ubiquitinated fashion, the predominant targets are poly-ubiquitinated proteins [10, 11]. Van Breukelen and Carey [12] showed that liver and gut levels of ubiquitin-conjugated protein rose by 2–3-fold during hibernation in ground squirrels (as compared with summer active levels) and suggested that this was best explained by an inhibition of proteolysis during torpor, a process that is generally highly sensitive to temperature reduction in mammalian cells. However, direct measurement of proteasome activity has not previously been undertaken for a hibernating species.

The present study measured the activity of the proteasome in skeletal muscle of euthermic versus hibernating thirteen-lined ground squirrels using peptides that target the individual protease activities of the proteasome. Protein carbonyl content was also measured in muscles to assess the amount of oxidative damage to cellular proteins during hibernation.

Materials and methods

Animals

Thirteen-lined ground squirrels, *Spermophilus tridecemlineatus*, were obtained from TLS Research (Bartlett, IL) in early autumn 2001 and 2002 and transported to the NIH (Bethesda, MD) where they were held in the animal hibernation facility in the laboratory of Dr. J.M. Hallenbeck (National Institute of Neurological Disorders and Stroke). The squirrels were housed individually in a room with an ambient temperature of 21 °C and a 12 h:12 h light:dark cycle, and were fed standard rodent diet and water *ad libitum*. Once the animals had completed the pre-hibernation phase characterized by maximizing their body lipid reserves and a rapid weight gain from 130–180 g up to 220–240 g, the animals were placed in a dark room with temperatures of 5–6 °C to induce hibernation. Some animals were maintained at 21 °C and sampled as euthermic controls. Animals that had been hibernating for 2–5 days, as well as controls, were sacrificed by decapitation and tissues were excised, immediately frozen in liquid nitrogen and transported to Ottawa where they were stored in a −80 °C freezer until use. Samples of mouse (*Mus musculus*) skeletal muscle were provided from the Carleton University Animal Care facility and were stored at −80 °C until use.

Tissue homogenization and preparation

Samples of frozen hind leg skeletal muscle were powdered 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 presence of chelating agents (EDTA, EGTA) that inhibit protein kinases and NaF that inhibits protein phosphatases allows the native phosphorylation state of the MCP to be maintained during tissue extraction. Samples were centrifuged at 9000 × g for 15 min at 4 °C and the supernatant was removed. In most cases, a measured volume of extract was then centrifuged through a Centricon (Amicon) concentrating device to remove small molecular weight proteins from the sample (large proteins are held back in the retentate). After preliminary tests with Centricon-100 and Centricon-500 devices (molecular weight cutoff 100 or 500 kDa, respectively) centrifugation through Centricon-500 was used for standard preparation of the proteasome. Centricons were centrifuged at 5000 × g at 4 °C until sample volume was reduced by half. Retentate in the Centricon was

then washed with the addition of 2 volumes of homogenizing buffer followed by re-centrifugation, again until volume was reduced to about half of the original starting volume. The final retentate volume was then carefully measured and adjusted back to be equal to the original volume with homogenization buffer.

Assay conditions

Enzyme activity was assayed fluorometrically using the method described by Rogers and Dean [13]. 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 with samples 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 8 °C; the instrument was chilled overnight and before each assay, the microplate filled with assay mixture was pre-equilibrated in the incubator for ~15 min until the temperature in a blank well (filled with buffer) stabilized at 8 °C, as determined by a thermister in the well. After the assay was completed, the temperature in blank well was measured again; temperature rose by 1–1.4 °C due to heat generation by the machine during the run.

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 (pmole/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 with statistical significance determined as $P < 0.05$.

Partial purification of multicatalytic proteinase

A Sephacryl-400 gel filtration column (90 × 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). Tissues were 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.

Carbonyl assay for total protein oxidative damage

The protocol was modified from Reznick and Packer [14]. Tissue extracts 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. Samples were measured on a Multiscan Spectrum spectrophotometer at 380 nm with an absorption coefficient of 22,000 M^{-1} cm^{-1}. Protein content was determined based on a standard curve of bovine serum albumin ranging from 0.25 to 2 mg/mL in guanidine hydrochloride read at 280 nm. Carbonyl content was measured as nmol/mg protein.

Native gel electrophoresis and silver stain

Native polyacrylamide gel electrophoresis was used to analyze peak column fractions; 4% acrylamide gels were run for 2 h at 100 V using the Bio-Rad Mini Protean apparatus. Gels were silver stained by the method outlined by Blum *et al.* [15].

Results

Partial purification of the proteasome by Centricon concentrating devices

Figure 1 shows the effect of partial purification of *S. tridecemlineatus* skeletal muscle proteasome activity by use of Centricon concentrators. Activity of the proteasome in crude supernatant was relatively low but could be increased substantially by separating the proteasome from lower molecular weight metabolites and proteins using either Centricon-100 (MW cutoff 100 kDa) or Centricon-500 (MW cutoff 500 kDa) devices. Separated from lower molecular weight subcellular components using the Centricon-500, proteasome activity in muscle extracts was nearly doubled. Centricon-500 treatment was used subsequently for the standard preparation of enzyme extracts for kinetic studies. The effect of Centricon-500 treatment on activity suggested that possible endogenous inhibitors of the proteasome were being removed in the filtrate. Therefore, additional tests were performed on the filtrate in an effort to determine the nature of these potential low molecular weight inhibitors. Samples of the Centricon-500 filtrate were treated in two ways: (a) passage through a small column of Sephadex G-50 column to separate protein versus metabolite fractions, or (b) boiled for 10 min to denature proteins. When added back to the partially purified proteasome in the Centricon-500 retentate, samples of untreated filtrate inhibited enzyme activity. However, neither the boiled filtrate nor the G-50 eluate affected proteasome activity when added back to the proteasome-containing retentate. The lack of inhibition by the boiled sample suggests that the inhibitor is a peptide or protein and the lack of inhibition by the G-50 eluate suggests that the peptide/protein inhibitor must have a molecular weight less than 50 kDa (and hence was retained on the column).

Centricon partial purification could also remove low molecular weight proteases that could cleave the substrate peptides and potentially yield false results for proteasome activity. As an internal check, several samples of Centricon retentate were incubated with a potent proteasome inhibitor MG132 prior to assay. In the presence of the inhibitor, activity was zero indicating that all activity in the uninhibited assay was due to the proteasome and not to other proteases. MG132 is also an inhibitor of lysosomal proteases and calcium dependent protease, but these proteases have low molecular weights and would be removed by the Centricon filtration.

Separation by Sephacryl-400 gel filtration

A 1:5 w:v muscle extract, prepared as stated above, was fractionated on a Sephacryl-400 column. After a void volume of 50 mL was eluted, fractions of 1 mL were collected and assayed using Z-LLE-AMC peptide to determine the native molecular mass of the proteasome. The proteasome complex eluted in a single peak corresponding to 900 ± 12 kDa

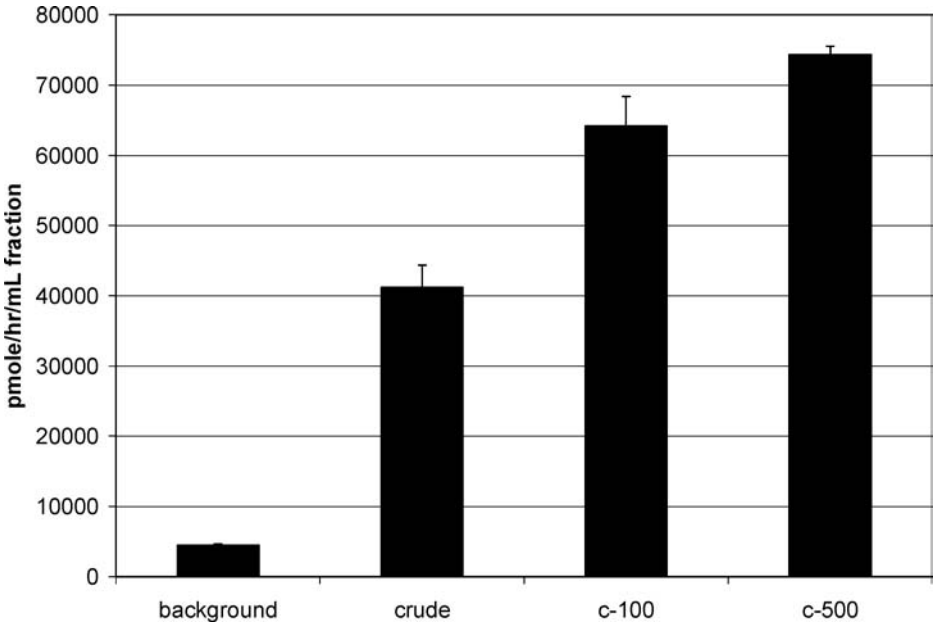

Fig. 1. Effect of partial purification using Centricon-100 or Centricon-500 filters on ground squirrel skeletal muscle proteasome activity. Activity was assayed at 23 °C with 100 μM Z-LLE-AMC as the substrate and is expressed as pmole AMC produced per h per mL, all retentate volumes having been adjusted back to equal the original volume added to the Centricon. In this way, activities are comparable between treated and untreated samples. Values are means $\pm$ S.E.M., $n = 3$.

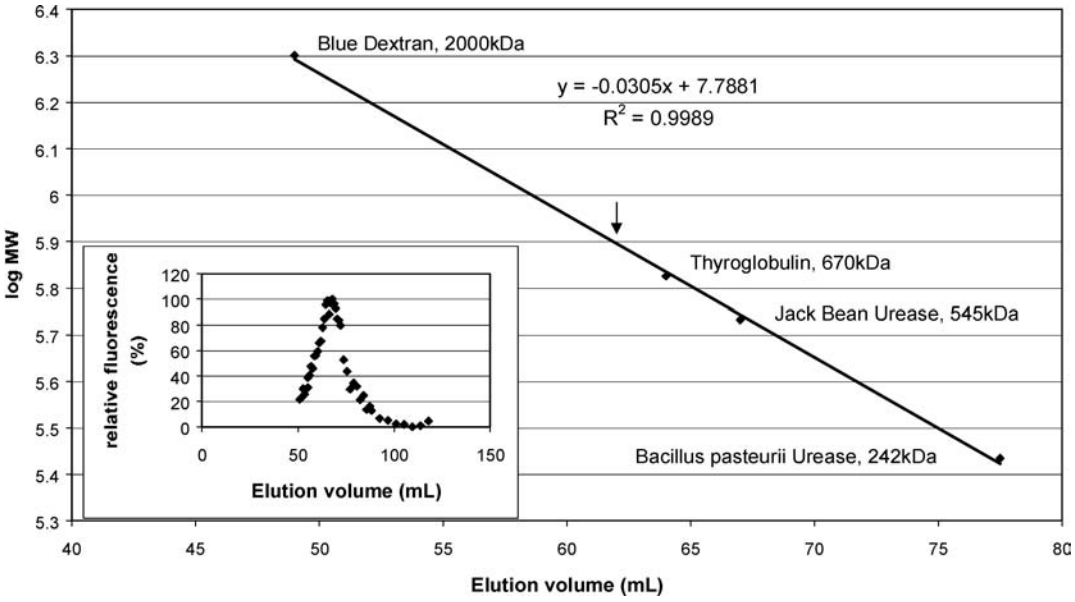

Fig. 2. Elution profile for the proteasome off Sephacryl-S400 and standard curve relating log molecular weight and elution volume for four protein standards. The elution position of the 20S proteasome is shown by an arrow on the standard curve. Activity in the fractions is expressed relative to activity in the peak fraction that is set to 100.

(Fig. 2). Peak fractions from the column were pooled and concentrated and then separated on a native polyacrylamide gel. After silver staining a single strong band was seen that migrated with a higher molecular mass than the 700 kDa thyroglobulin standard (data not shown). Hence, both size separation techniques are consistent with 20S form of the proteasome.

Assays of proteasome activity in euthermic and hibernating ground squirrels

K_m and V_{max} values for the proteasome were measured at 23 °C in skeletal muscle extracts from euthermic and hibernating ground squirrels after partial purification by Centricon-500 filtration. Table 1 shows values for each of the peptide substrates: Z-LLE-AMC, Z-ARR-AMC and Suc-LLVY-AMC. The proteasome showed distinctly different V_{max} and K_m values with each of the substrates, highlighting the different protease activities of the proteasome complex. However, for each individual substrate, no significant differences were seen for either substrate affinity or maximal activity between the enzyme from euthermic and hibernating animals.

Temperature effects on substrate K_m values

The effects of temperature on the K_m values for each of the three substrates of the proteasome were evaluated at 23 °C and at 8 °C, the cold temperature being similar to hibernating body temperature. Assays were performed on muscle extracts from euthermic and hibernating ground squirrels and additionally on mouse skeletal muscle extracts for comparison to a non-hibernating mammal. The effects of temperature were mixed among the peptidase activities (Table 2). Assays at low temperature resulted in a significant increase in the K_m value (2.6–3 fold) for the peptide Z-LLE-AMC corresponding to the peptidyl-glutamyl peptide hydrolytic activity in all three cases. However, K_m values for the peptides measuring the tryptic and chymotryptic activities were either decreased or unaltered when assayed in the cold. The K_m values for Z-ARR-AMC of the proteasome from euthermic and hibernating muscle decreased to 19–24% of the euthermic value whereas the corresponding

Table 1. Proteasome activity and K_m values with three substrates for the muscle enzyme from euthermic and hibernating ground squirrels

	Peptide substrate		
	Z-LLE-AMC	*Z-ARR-AMC*	*Suc-LLVY-AMC*
K_m (μM peptide)			
Euthermic	59.0 ± 4.9	20.7 ± 3.1	73.5 ± 4.5
Hibernating	60.4 ± 3.8	18.6 ± 1.9	70.6 ± 3.8
V_{max} (pmol/h/μg protein)			
Euthermic	16.3 ± 1.5	7.84 ± 0.6	5.30 ± 0.7
Hibernating	16.8 ± 1.9	9.52 ± 1.8	6.80 ± 0.9

Note. Assays were conducted at 23 °C and values are means ± S.E.M., $n = 3$.

Table 2. Effects of assay temperature on K_m values for the three substrates of the proteasome in skeletal muscle extracts from euthermic and hibernating ground squirrels and the non-hibernator mouse

	Z-LLE-AMC		*Z-ARR-AMC*		*Suc-LLVY-AMC*	
	23 °C	8–10 °C	23 °C	8–10 °C	23 °C	8–10 °C
Euthermic	59.0 ± 4.9	176 ± 29.6*	20.7 ± 3.1	3.94 ± 1.9*	73.5 ± 4.6	48.9 ± 4.1*
Hibernator	60.4 ± 3.8	159 ± 25.9*	18.6 ± 1.9	4.49 ± 1.1*	70.6 ± 3.8	86.7 ± 19.6
Mouse	75.1 ± 3.1	195 ± 16.4*	15.7 ± 0.5	14.9 ± 2.3	87.1 ± 15.1	66.9 ± 11.6

Note. Data are means ± S.E.M., $n = 6$. The temperature range for the cold assay represents initial (8 °C) versus highest final (10 °C) temperature measured in microplate wells; the slight warming during the 45 min assay is due to the mechanical activity microplate reader.
*Significantly different from the K_m value at 23 °C as assessed by the Student's *t*-test, $P < 0.05$.

K_m for the mouse proteasome was unaltered at low temperature. The K_m for SUC-LLVY-AMC decreased significantly by 34% for the euthermic enzyme at low assay temperature but neither the hibernator nor the mouse enzymes showed significantly altered K_m values for this substrate.

Enzyme maximal activity was also determined at two temperatures (Fig. 3). The activity of the ground squirrel enzyme at 8 °C was significantly reduced to ∼30% of the value at 23 °C; this corresponded to a Q_{10} value of 2.03. However, the mouse enzyme utilizing either Z-ARR-AMC or SUC-LLVY substrate was more strongly affected by low temperature with activity reduced to 13–19% of the value at 23 °C.

Carbonyl content

The level of protein carbonyls is a measure of oxidative damage to cellular proteins. Carbonyl levels were assayed in muscle extracts from both euthermic and hibernating squirrels ($n = 6$). Levels decreased significantly from 0.567 ± 0.118 nmole carbonyl/mg protein in euthermic squirrels to

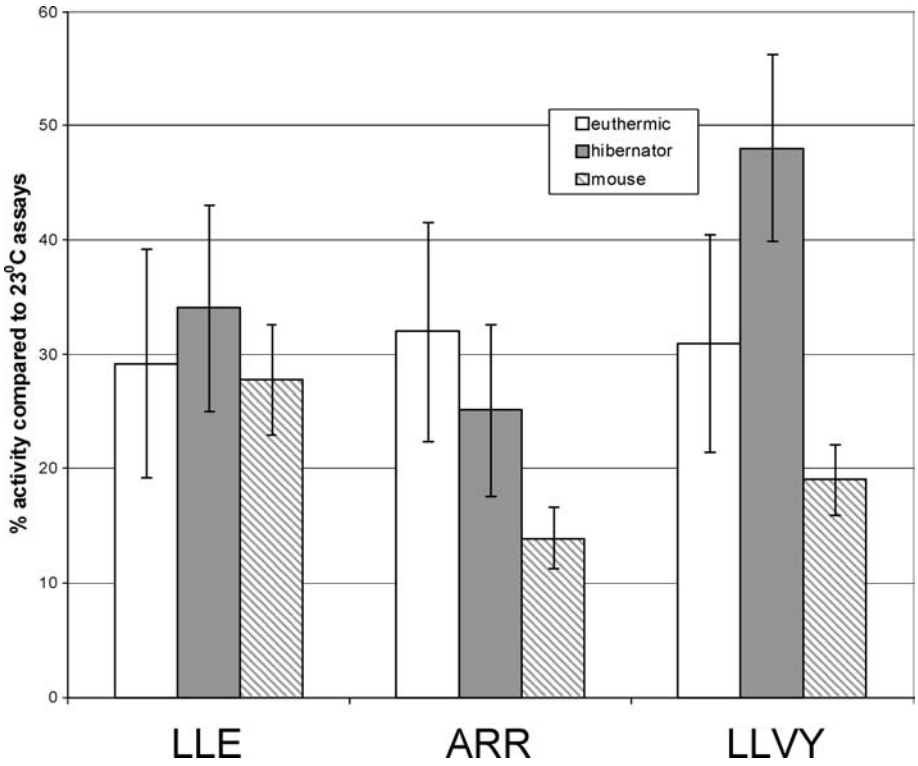

Fig. 3. Effect of temperature on the V_{max} activity of the proteasome in skeletal muscle extracts from euthermic and hibernating ground squirrels as well as the non-hibernator mouse. Shown are the relative activities at 8 °C compared with 23 °C; values are mean ± S.E.M., $n = 6$.

0.125 ± 0.029 nmole carbonyl/mg protein in hibernating squirrels.

Discussion

The present study assessed proteasome activity in skeletal muscle from euthermic and hibernating ground squirrels at both warm and cold assay temperatures in an effort to elucidate the mechanism of regulation of the proteasome employed during hibernation. Protein carbonyl content of muscle was quantified to provide a measure of oxidative damage to proteins during torpor and as a possible indirect assessment of proteasome activity.

The use of a membrane filtration device for the partial purification of the proteasome was first described by Rogers and Dean [13]. In mirroring their purification step in the present study, we found that the activity of the proteasome increased after low molecular weight cellular constituents were removed by Centricon filtration. This suggested that low molecular weight proteins, metabolites or ions might be inhibiting the proteasome in crude extracts and subsequent attempts to characterize the inhibitor suggested that a low molecular weight protein was involved. Since there were no significant differences in the maximal activities of the proteasome in filtered extracts from euthermic versus hibernating conditions, it is possible that hibernation-related changes in the levels of small molecular weight inhibitors of the proteasome may be the means of suppressing proteasome activity during torpor.

Fractionation of the ground squirrel muscle proteasome by column gel filtration revealed one peak of activity and a native gel of the pooled peak fractions revealed a single band. Both were consistent with a molecular mass of ∼900 kDa which is indicative of the 20S form of the proteasome. Subsequent assay of the peak column fractions in the presence of ATP, to stimulate the 26S proteasome, or low concentrations of SDS, to stimulate the 20S proteasome also suggested that activity was the 20S proteasome (data not shown).

Kinetic analysis used peptide substrates to evaluate the three different proteolytic activities of the proteasome. Parameters of substrate affinity (K_m) and activity (V_{max}) gave unique results for each peptide. However, no significant differences between the kinetic parameters of the proteasome from euthermic versus hibernating animals were found (Table 1). All extracts and assays were conducted in the presence of additives that inhibited endogenous kinases and phosphatases so that any differences in the phosphorylation state of the proteasome between the two physiological states would be maintained. Some studies have shown that there are phosphorylation sites on the proteasome specifically on the C8 and C9 subunits [16] that are phosphorylated by casein kinase II. Casein kinase II is also known to copurify with the proteasome [17] and, hence, phosphorylation could be a mechanism of proteasomal regulation. Since there were no significant changes in the kinetic parameters of the proteasome in euthermic versus hibernating conditions, this argues that changes in the phosphorylation state of the proteasome are not likely to be a mechanism of regulation of proteolytic suppression during entry into torpor.

Although there were no significant changes in K_m and V_{max} values for the proteasome from euthermic and hibernating samples when assayed at 23 °C, assay temperature had substantial effects on K_m values for the different substrates. When assayed at 8–10 °C, compared with 23 °C, the K_m for Z-LLE-AMC increased significantly (i.e. substrate affinity decreased) for both the euthermic and hibernating enzyme as well as for the mouse proteasome. Conversely, the K_m for Z-ARR-AMC decreased significantly at low temperature for the euthermic and hibernating enzyme but not for the mouse proteasome. K_m for SUC-LLVY-AMC was temperature independent for the hibernator and mouse enzymes but decreased significantly for the euthermic enzyme. The differential effects of temperature on the K_m values for the different substrates undoubtedly arises due to temperature effects on the different types of weak bonds that contribute to binding each of the substrates [18] and may suggest that enzyme preference for different physiological protein substrates could change at the low body temperature of the hibernating state.

Maximal activity of the proteasome with each of the substrates was suppressed by low temperature. With a 15 °C reduction in assay temperature, the activity of the proteasome in ground squirrel extracts decreased by ∼70% (Fig. 3); the calculated Q_{10} associated with this was 2.03, a value consistent with the general effect of temperature on the reaction rates of most enzymes. However, mouse proteasome activity was more strongly inhibited at low temperature (reduced to just 13–19% of the value at 23 °C). This suggests that the protein from the hibernating species is better suited for function over a wide range of environmental temperatures. Retention of function of proteins over a wide temperature range can be achieved by making minor alterations in amino acid sequence [19].

Low tissue perfusion rates during torpor-arousal cycles can be a source of oxidative damage to tissues. Torpor is not continuous over the winter months but is interspersed with brief arousal periods where body temperature is rapidly returned to euthermic values before another torpor bout commences. Ischemia-reperfusion injury may occur in areas where blood flow is not fully restored before elevations in body temperature [20]. Skeletal muscle may be susceptible to ischemic-reperfusion injury during hibernation as it is one of the first areas of the squirrel where blood flow is minimized. Quantification of carbonyl proteins is a very sensitive and widespread means of estimating oxidative damage to proteins and we predicted that carbonyl protein levels would

rise in hibernation. However, contrary to the expectations for a system that could show ischemia-induced damage, the level of oxidatively modified proteins actually decreased in hibernation. These results for protein carbonyls in skeletal muscle could be interpreted in two ways: (a) the proteasome remains active in degrading oxidatively damaged proteins during hibernation, and (b) oxidative damage to proteins is actually reduced in muscle during hibernation. In support of the former, it is well known that the 20S proteasome selectively degrades oxidatively damaged proteins through recognition of hydrophobic surface patches [21] and this function may continue during torpor to avoid the accumulation of damaged proteins. The latter possibility contrasts with the results of another study that showed elevated levels of oxidative damage products of lipids (conjugated dienes) in intestine of hibernating ground squirrels as compared with summer active animals sampled several weeks before hibernation [22]. It is also generally believed that hibernation and/or the arousal process (which is accompanied by a huge increase in oxygen consumption) must cause oxidative stress because several studies have documented enhanced levels of antioxidant defenses (both metabolites such as ascorbate and enzymes) during hibernation (summarized in [20]). However, if antioxidant defenses are elevated prior to hibernation, then the accumulation of oxidative damage products during torpor or arousal could be minimized or even suppressed below normal, an outcome that could be beneficial for maintaining the viability of cells/tissues over long weeks of continuous torpor. These issues of oxidative stress and antioxidant defense during hibernation are far from resolved and require more study to sort out a variety of issues including organ-specific responses, assessment of oxidative damage to different types of macromolecules (e.g. lipid, protein, DNA), and analysis of the patterns of changes in oxidative damage products and antioxidant defenses over the course of torpor-arousal cycles.

Van Breukelen and Carey [12] found an increase in the level of protein-ubiquitin conjugates during hibernation and suggested a storage function for these conjugates so they may be broken down when euthermia resumes. Ubiquitin-dependent protein degradation via the 26S proteasome requires ATP input and, hence, this activity would be selectively suppressed as part of the overall metabolic rate depression of the hibernating state resulting in the accumulation of ubiquitin conjugates. In contrast, protein degradation by the 20S proteasome is a more energetically favorable pathway and could continue to function during hibernation to serve a housekeeping purpose for elimination of oxidatively damaged proteins.

Acknowledgments

Thanks to J.M. Hallenbeck for providing the samples of ground squirrel tissues and to J.M. Storey for critical review of the manuscript. Research support to KBS is from a discovery grant from the Natural Sciences and Engineering Research Council of Canada and the Canada Research Chairs program. AKW holds an Ontario Graduate Scholarship.

References

1. Wang LCH, Lee TF: Torpor and hibernation in mammals: Metabolic, physiological, and biochemical adaptations. In: M.J. Fregley, C.M. Blatteis (eds). Handbook of Physiology: Environmental Physiology. Oxford University Press, New York, 1996, Section 4, Vol. 1, pp. 507–532
2. Storey KB, Storey JM: Mammalian hibernation: Biochemical adaptation and gene expression. In: K.B. Storey (ed). Functional Metabolism: Regulation and Adaptation. Wiley-Liss, Hoboken, USA, 2004, pp. 443–471
3. Storey KB, Storey JM: Metabolic rate depression in animals: Transcriptional and translational controls. Biol Rev 79: 207–233, 2004
4. Fuery CJ, Withers PC, Guppy M: Protein synthesis in the liver of *Bufo marinus*: Cost and contribution to oxygen consumption. Comp Biochem Physiol A 119: 459–467, 1998
5. Frerichs KU, Smith CB, Brenner M, DeGracia DJ, Krause GS, Marrone L, Dever TE, Hallenbeck JM: Suppression of protein synthesis in brain during hibernation involves inhibition of protein initiation and elongation. Proc Natl Acad Sci 95: 14511–14516, 1998
6. Hittel D, Storey KB: The translation state of differentially expressed mRNAs in the hibernating thirteen-lined ground squirrel (*Spermophilus tridecemlineatus*). Arch Biochem Biophys 401: 244–254, 2002
7. Chen Y, Matsushita M, Nairn AC, Damuni Z, Cai D, Frerichs KU, Hallenbeck JM: Mechanisms for increased levels of phosphorylation of elongation factor-2 during hibernation in ground squirrels. Biochemistry 40: 11565–11570, 2001
8. Shringarpure R, Grune T, Davies KJ: Protein oxidation and 20S proteasome-dependent proteolysis in mammalian cells. Cell Mol Life Sci 58: 1442–1450, 2001
9. Doherty FJ, Dawson S, Mayer RJ: The ubiquitin-proteasome pathway of intracellular proteolysis. Essays Biochem 30: 51–63, 2002
10. Orlowski M, Wilk S: Catalytic activities of the 20S proteasome, a multicatalytic proteinase complex. Arch Biochem Biophys 383: 1–16, 2000
11. Orlowski M, Wilk S: Ubiquitin-independent proteolytic functions of the proteasome. Arch Biochem Biophys 415: 1–5, 2003
12. Van Breukelen F, Carey HV: Ubiquitin conjugate dynamics in the gut and liver of hibernating ground squirrels. J Comp Physiol B 172: 269–273, 2002
13. Rogers KJ, Dean RT: Assessment of proteasome activity in cell lysates and tissue homogenates using peptide substrates. Int J Biochem Cell Biol 35: 716–727, 2003
14. Reznick AZ, Packer L: Oxidative damage to proteins: Spectrophotometric method for carbonyl assay. Enzymology 233: 357–363, 1994
15. Blum H, Beier H, Gross HJ: Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8: 93–99, 1987
16. Castaño JG, Mahillo E, Arizti P, Arribas J: 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: 3782–3789, 1995
17. Ludemann R, Lerea KM, Etlinger JD: Copurification of casein kinase II with 20S proteasomes and phosphorylation of a 30-kDa proteasome subunit. J Biol Chem 268: 17413–17417, 1993
18. Hochachka PW, Somero GN: Biochemical Adaptation. Princeton University Press, Princeton, NJ, 1984

19. Somero GN: Protein adaptation to temperature and pressure: Complementary roles of adaptive changes in amino acid sequence and internal milieu. Comp Biochem Physiol B 136: 577–591, 2003
20. Carey HV, Andrews MT, Martin SL: Mammalian hibernation: Cellular and molecular responses to depressed metabolism and low temperature. Physiol Rev 83: 1153–1181, 2003
21. Grune T, Merker K, Sandig G, Davies KJA: Selective degradation of oxidatively modified protein substrates by the proteasome. Biochem Biophys Res Commun 305: 709–718, 2003
22. Carey HV, Frank CL, Seifert JP: Hibernation induces oxidative stress and activation of NF-κB in ground squirrel intestine. J Comp Physiol B170: 551–559, 2000