Protein degradation in mammalian hibernator, Spermophilus lateralis

Vanja Velickovska

University of Nevada, Las Vegas

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PROTEIN DEGRADATION IN MAMMALIAN HIBERNATOR, *SPERMOPHILUS LATERALIS*

by

Vanja Velickovska

Bachelor of Sciences
Goteborg University
2000

Master of Science
Goteborg University,
2002

A dissertation submitted in partial fulfillment
of the requirements for the

Doctor of Philosophy Degree in Biological Sciences
School of Life Sciences
College of Sciences

Graduate College
University of Nevada, Las Vegas
August 2008
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VANJA VELICKOVSKA

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Examination Committee Chair

Dean of the Graduate College
ABSTRACT

Protein Degradation in Mammalian Hibernators, *Spermophilus lateralis*

by

Vanja Velickovska

Dr. Allen Gibbs, Examination Committee Chair
Associate Professor of School of Life Sciences
University of Nevada, Las Vegas

Protein synthesis is virtually non-existent during hibernation in golden-mantled ground squirrels. In the presence of negligible protein synthesis continued protein degradation would disrupt the maintenance of homeostasis and the survival of the animal. Earlier investigation showed that ubiquitin conjugate concentrations increase 2-3 fold during hibernation, which would seemingly suggest an increase of protein degradation. In this study I examined protein degradation *per se* as a function of the temperatures experienced by the hibernator and demonstrated that protein degradation is virtually absent at low temperatures. A new method was developed to determine the rates of ubiquitylation as a function of temperature, and it was demonstrated that ubiquitylation continues at 30% of maximum even at 0° C. The quality of the ubiquitylation was also examined and revealed no significant change between the torpor states. These results may explain the increase in ubiquitin conjugates during hibernation as a simple accumulation event. Contrary to the common belief that hibernators are *perfectly* adapted for hibernation, this study demonstrates that this may not be correct. The apparent mismatch to the temperature effects between protein degradation and the levels
of ubiquitin conjugates demonstrates that hibernating animals may employ the temperature, albeit not perfectly, to down-regulate important homeostatic processes. The consequences of this mismatch may be the reason for the existence of the interbout arousals.
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**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Acetyl CoA</td>
<td>Acetyl coenzyme A</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>AMC</td>
<td>7-amido-methylcoumarin</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine Monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>ChTL</td>
<td>Chymotrypsin like peptidase activity</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-dithiobisnitrobenzoic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>DUB</td>
<td>Deubiquitylating enzymes</td>
</tr>
<tr>
<td>E1</td>
<td>Ubiquitin activating enzyme</td>
</tr>
<tr>
<td>E2</td>
<td>Ubiquitin conjugating enzyme</td>
</tr>
<tr>
<td>E3</td>
<td>Ubiquitin ligase enzyme</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene-diamine-tetra-acetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmatic Reticulum</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>IBA</td>
<td>Interbout Aroused Squirrels</td>
</tr>
<tr>
<td>K63</td>
<td>Ubiquitin lysine residue 63</td>
</tr>
<tr>
<td>MVB</td>
<td>Multivesicular bodies</td>
</tr>
<tr>
<td>N2</td>
<td>Nitrogen gas (liquid)</td>
</tr>
<tr>
<td>NaF</td>
<td>Sodium Fluoride</td>
</tr>
<tr>
<td>PGPH</td>
<td>Peptidyl-glutamyl peptide hydrolases</td>
</tr>
<tr>
<td>PPh</td>
<td>Pyrophosphate</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>Rat</td>
<td><em>Rattus norvegicus</em></td>
</tr>
<tr>
<td>SA</td>
<td>Summer Active Squirrels</td>
</tr>
<tr>
<td>T</td>
<td>Torpid Squirrels</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans Golgi Network</td>
</tr>
<tr>
<td>TL</td>
<td>Trypsin-like peptidase activity</td>
</tr>
<tr>
<td>TTBS</td>
<td>Tween Tris buffered saline</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>V-ATPase</td>
<td>Vacuolar Adenosine Triphosphatase pump</td>
</tr>
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University of Nevada, Las Vegas.
CHAPTER 1

GENERAL INTRODUCTION

Mammalian hibernation

During exposure to harsh environmental conditions, such as cold or food deprivation, a diversity of mammals may employ hibernation (Carey et al. 2003; van Breukelen and Martin 2002). Golden-mantled ground squirrels (Spermophilus lateralis) employ hibernation during winter months. This particular strategy involves explicitly coordinated and controlled processes of all metabolic functions involving thermal, chemical and neuronal factors in order to maintain homeostasis. Two major bouts characterize hibernation: torpor bout and interbout arousals (Fig. 1-1). Typically, a torpor bout consists of entrance, maintenance and arousal from torpor. During entrance into torpor, a drastic decrease of heart rate occurs from the normal 200-300 beats per minute down to 3-5 beats per minute (Zatzman 1984). Respiration is drastically reduced from the normal 100-200 breaths per minute down to irregular 4-6 breaths per minute with long periods of apnea, as the body temperature of the hibernator approaches the ambient temperature, which can be as low as -2.9°C (Barnes 1989; McArthur and Milsom 1991). Renal function is either greatly reduced or ceases altogether (Zatzman 1984). The metabolic rate is severely reduced to as low as 1% of the usual active rates (Wang and Lee 2000). Overall, all physiological functions during torpor are maintained at minimum. Depending on temperature, species and season, torpor bouts
Figure 1-1.
Body temperature during a typical torpor cycle for golden-mantled ground squirrels (*Spermophilus lateralis*). Temperature sensitive radiotelemeters were implanted into the animal to allow for a precise determination of torpor bouts. Basic states of torpor that are studied in this dissertation are as indicated: Torpid = torpor bout (~75% of completed cycle), IBA = interbout aroused (euthermic in between bouts of torpor). For better understanding the terms entrance and arousal of torpor are indicated: Entrance = going from euthermic toward torpor bout, process usually taking 12-20 h, Arousal = arousing from torpor as body temperature increases to euthermic values (2-4 h). Not shown here are SA = summer active, non-hibernating ground squirrels caught during the summer, used as a seasonal control. The temperature of the environmental chamber where hibernating animals were kept was 4° C.
may last from 1-3 weeks (Buck and Barnes 2000). There are usually 15 bouts of torpor per year.

Arousal from torpor is a rather volatile process demanding 2-4 hours involving stimulation of major energy production processes. For example, non-shivering thermogenesis in the brown adipose tissue is used to reach eutheremic temperatures. These brief interruptions from the torpor bout (interbout arousals) usually last 12-20h, after which both body temperature and metabolic rates return to torpid values.

Hibernating golden-mantled ground squirrels store fat during the summer before entering the hibernation season. Their body weight doubles from the spring, when they emerge from hibernation, until the end of the summer. They do not ingest food during the winter; rather they rely on the products of lipid hydrolysis, mainly fatty acids and glycerol, obtained from their fat reserves in the white adipose tissue. However, even with their enormous fat storage, hibernating squirrels would not survive without food intake for 5-8 months if they were euthermic (Carey et al. 2003). Their greatly depressed metabolism saves energy and enables them to survive the hibernation season. The total energy saved during the entire hibernation season would be as much as 90% if it were not for the interbout arousals (reviewed in Wang and Lee 2000). These interbout arousals account for up to 70% of the energy consumed during the hibernation season. Squirrels’ ability to hibernate depends on these brief returns to euthermic temperatures (reviewed in Carey et al. 2003). The exact function of these interbout arousals remains to be elucidated, but their energetic cost suggests that these interruptions are very important for the hibernating animals. Many hypotheses have been postulated in an attempt to explain those arousals, ranging from the elimination of metabolic wastes, to stocking up on fuel reserves, or to readjusting neuronal circuits (Martin et al. 1993; Heller and Ruby 2004).
On a cellular level, energetically expensive biochemical processes must be coordinated to adjust for energy requirements and to ensure that metabolic fuel will last throughout the whole hibernation season. Not surprisingly, all cellular metabolic processes need to be strongly depressed during torpor. Metabolic rates during torpor can be as low as 1% of the active rates. In some tissues, protein synthesis accounts for 20-40%, while protein degradation takes 4% of the total energy cost in euthermic organisms (e.g. Schmidt et al. 1991; Rolfe and Brown 1997). It is natural to expect down-regulation of both protein synthesis and degradation in order to maintain cellular homeostasis during hibernation. Indeed, protein synthesis during hibernation represents only 0.13-0.5% of the rates of active squirrels (Zhegunov et al. 1988; van Breukelen and Martin 2001). In the near absence of protein synthesis during hibernation, it is to be expected that protein degradation must be depressed as well to maintain cellular homeostasis.

Surprisingly, earlier investigations revealed an increase of ubiquitin conjugates during torpor (van Breukelen and Carey 2002). An increase in ubiquitin conjugates is usually linked with an increase in protein degradation (Munro and Pelham 1985; van Breukelen and Hand 2000). Continued protein degradation in the near absence of protein synthesis could lead to loss of important protein pools during hibernation and serve as a possible trigger for interbout arousals, so that under euthermic conditions such proteins can be restored. To my knowledge, no investigation has revealed significant loss of proteins during torpor. Thus, one purpose of these studies was to examine whether protein degradation per se might be severely depressed during the cold temperatures typically experienced by torpid animals.
Ubiquitin-mediated protein degradation

Protein degradation in mammalian cells has two major pathways. One is ubiquitin-mediated proteolysis, which is responsible for degrading 80-90% of all cytosolic proteins. Ubiquitin-mediated proteolysis is a rather complex process that involves several energy dependent steps to conjugate ubiquitin molecule to target proteins. The ubiquitin-tagged proteins are then degraded by the 26S proteasome (Fig 1-2). The other pathway involves degradation in the lysosomes.

Ubiquitin is a well-conserved protein present in all eukaryotes made of 76 amino acids. It has seven lysine residues, which likely are involved in conjugation with target proteins (Pickart 2004). The attachment of ubiquitin to the target protein occurs through a well-controlled pathway that involves at least three enzymes: E1, E2 and E3. The first step is an energy dependent step that is catalyzed by the ubiquitin-specific activating enzyme E1, which activates the C-terminus of ubiquitin (G-76). An intermediate step involves the transfer of ubiquitin from E1 to a conjugating enzyme E2. Then the ubiquitin is transferred to the target protein by E3, a ligase. The interaction between E2 and E3 determines the choice of proteins for degradation (reviewed in Hershko and Ciechanover 1998, Weissman 2001). Proteins can be either attached to one ubiquitin molecule (monoubiquitylation), or they can be attached to several ubiquitin molecules (polyubiquitylation; Pickart 2004). Lysine residue K48 of the ubiquitin is mainly involved in formation of polyubiquitin chains in many nuclear, cytosolic and endoplasmic reticulum (ER) membrane proteins and targets them for subsequent degradation by the 26S proteasome (Chau et al. 1989; Pickart 2004; Pickart and Rhaasi 2005; Thrower et al. 2000). Lysine residue K63 is primarily involved in monoubiquitylation.
During conjugation, ubiquitin is attached to a target protein through the activity of three enzymes in an energy dependent manner. Once ubiquitylated, the target protein can be ubiquitylated with additional ubiquitin molecules. As more ubiquitin molecules are attached to a target protein, the probability that the protein will be degraded by the activity of the 26S proteasome increases. After protein degradation, ubiquitin molecules are recycled (Pickart 2004).
Monoubiquitylation is involved in the regulation of many processes in the cell such as: histone regulation, endocytosis, virus budding, lysosomal degradation, protection of DNA and ribosomes during stress but not degradation by the 26S proteasome (reviewed in Hicke 2001b). Similar to phosphorylation and dephosphorylation of cellular proteins, ubiquitylation is tightly controlled by the activity of deubiquitylating enzymes (DUB). The DUBs are cysteine proteases that specifically cleave the bond between ubiquitin and the conjugated proteins. The DUB family includes over 200 enzymes that have been divided into five distinct subfamilies, based on sequence similarities and likely mechanisms of action (Amerik and Hochstrasser 2004)

A closer look at the machinery of the 26S proteasome reveals a structure that is quite large (ca 2.5 MDa), which consists of a 20S protease barrel, where all proteolytic activities occur and two 19S caps on each side of the 20S barrel, which serve as a lid and control the entrance of proteins for degradation (Fig 1-3). The 20S protease is approximately 700 kDa and is the actual site for protein degradation. The barrel is composed of α and catalytically active β subunits. Three well-characterized activities are recognized in the 20S protease: trypsin-like peptidase activity that targets basic residues; chymotrypsin-like peptidase activity that targets hydrophobic residues; and caspase-like or peptidyl-glutamyl peptide hydrolases (PGPH) that targets acidic residues (Orlowski and Wilk 2000). In my dissertation research, I examined all three proteolytic activities of the 20S protease.

Lysosomal degradation system

Discovered in 1955 by de Duve, lysosomes were first recognized biochemically, as vacuolar structures encapsulating numerous enzymes used to break down proteins, lipids,
Figure 1-3.
Schematic view of the 26S proteasome. The 26S proteasome is made of three major parts: 19S regulatory caps that are involved in recognizing ubiquitylated proteins and a 20S protease barrel where the actual proteolysis occurs. Three well characterized peptidase activities inside the 20S barrel are: (1) trypsin-like peptidase activity that cleaves after basic residues; (2) chymotrypsin-like peptidase activity, that cleaves after hydrophobic residues; and (3) peptidyl-glutamyl peptide hydrolyse or caspase-like peptidase activity that cleaves after acidic residues (Orlowski and Wilk 2000).
nucleotides and polysaccharides. Lysosomal proteolysis is important in the turnover of membrane proteins and large organelles such as mitochondria, as well as the turnover of monoubiquitylated proteins (Hicke and Dunn 2003). It is generally accepted that the optimal environment for those hydrolases is low pH (4-5.5). The acidity of the lysosomes is maintained by a vacuolar ATPase pump that uses ATP to pump hydrogen ions into the lysosome. Lysosomes are very dynamic organelles, since they are part of a more complex system that involves carrier vesicles from the plasma membrane, the Golgi complex and the endoplasmatic reticulum (ER). There are several pathways to make lysosomes: (1) the endocytotic pathway that includes formation of vesicles or primary endosomes from the plasma membrane, which are then processed through the Golgi complex, thus continuing as secondary endosomes that eventually become lysosomes; (2) the autophagocytotic pathway that includes the formation of a membrane in the ER that wraps itself around bigger organelles (e.g. mitochondria); (3) the phagosomatic pathway, which is actually an enormous endosome that involves transfer of materials from the Golgi (Fig 1-4). At least three different stages can be recognized: early endosomes that usually have pH values of 6.0-6.5; late endosomes, also known as multivesicular bodies (MVB) with more acidic intralumenal pH values of about 5.5-6.0; and lysosomes with the most acidic intralumen, with pH values ranging from 4.0-5.5 (Castino et al. 2003; Hicke and Dunn 2003; Katzmann et al. 2002). In addition, early and late endosomal compartments are pleiomorphic and represent a dynamic network of tubular and vesicular compartments, while lysosomes are usually circular organelles of about 0.5 μm (Castino et al. 2003). Two major hypotheses have been proposed to explain the transfer of material between vacuoles and lysosomes. The first is a "kiss and run" hypothesis, which involves the transient and limited fusion
between two organelles (e.g. lysosomes and late endosomes). The second hypothesis is the fusion of two organelles to form a transient organelle that eventually becomes a lysosome (Luzio et al. 2000). However, the most reliable way to characterize a lysosome is biochemically (reviewed in Castino et al. 2003 and Luzio et al. 2000).

Lysosomal degradation is of specific interest in this study, because of recent evidence that monoubiquitylation on plasma membrane proteins stimulates lysosomal degradation (Hicke 2001a; Cuervo 2004; Dupre et al. 2001; Hicke and Dunn 2003). Not only does ubiquitin serve as an internal and endosomal sorting signal, but it also serves as a signal that regulates trafficking of endocytic proteins between Golgi and endosomes (Hicke and Dunn 2003). Overall, the function of both monoubiquitylation of membrane proteins and lysosomal degradation seems to be connected.

Previous investigations in rat models have revealed that there is no lysosomal acidification below 18°C (Roederer et al. 1987; Ahlberg et al. 1985). Since hibernators experience low body temperature, I developed an experimental method to investigate the ability of the lysosomes to acidify in vitro, under the range of temperatures usually experienced during hibernation.

Lysosomes contain a variety of hydrolases that have optimal activity at low pH values (4-5.5), such as lipases, nucleases, hydrolases, and proteases (cathepsins). The cathepsins represent the largest and the most common group (Turk et al. 2001). The focus of this study is on the cathepsins, since they are responsible for protein degradation. I investigated 6 cathepsins that are the most abundant proteases in lysosomes, cathepsins B, H, D, E, S and L (Turk et al. 2001). While these all exhibit proteolytic activity, there are some important functional differences.
Figure 1-4
A schematic view of three pathways to make a lysosome: (1) At the plasma membrane endocytosis produces early endosomes that mature with the fusion of Golgi vesicles (through the Trans Golgi Network TGN) and became late endosomes that later transform into lysosomes. (2) A similar process occurs in phagocytosis, but in this case huge endosomes are formed. (3) Autophagosomes are formed in a different way, membrane parts that come from ER actually wrap themselves around organelles that need to be digested.
Cathepsins B, H, S and L belong to a family of papain enzymes, referring to their similar amino acid sequences and folds (Turk et al. 2001). Cathepsin B is a carboxypeptidase, which also performs endopeptidase activity (Turk et al. 2001). Recent reports suggest that cathepsin B functions at neutral pH values (Sloane et al. 1990; Moin et al. 1992). In addition, inactivation of cathepsin B in mice livers decreases apoptotic injury induced by ischemia/reperfusion (Ben-Ari et al. 2005). Previous studies in hibernators have revealed the possibility that they may experience ischemia/reperfusion injuries during entrance into torpor (Milsom et al. 1999; Zimmer and Milsom 2001). The question that arises is whether cathepsin B activity is reduced during entrance and torpor. I hypothesized that the activity of cathepsin B needs to be reduced during torpor, to provide protection from ischemia/reperfusion injuries in hibernators.

Cathepsin H acts both as endopeptidase and as aminopeptidase and has been demonstrated to be upregulated at the level of transcription during winter in hibernating golden-mantled ground squirrels (Epperson et al. 2002). Investigators have tied the increase of cathepsin H activity to an increase in surfactant protein B in the lungs of the neonatal mice, thereby ensuring their survival (Ueno et al. 2004). This surfactant protein B prevents damage of the lungs during prolonged periods of inactivity (e.g. apnea, Ueno et al. 2004). It should be noted that hibernators do experience long periods of apnea during torpor. I hypothesized that the activity of cathepsin H should be increased considering the possibility that increase in cathepsin H activity might be closely related to survival of hibernators during torpor.

Cathepsin S and L have been described as being very similar in structure and function (Bohley and Seglen 1992; Kirschke and Wiederanders 1994). I differentiated
between those two enzymes using the specific inhibitor for cathepsin L, NapSul-Ile-Trp-CHO (Yasuma et al. 1998). Previous investigators have recognized cathepsin L as very unstable at neutral pH values (reviewed in Turk et al. 1999). Although it is present in the liver, cathepsin S is more active in lymph nodes and spleens and is mainly involved with the inflammatory system and macrophages (Kirschke et al. 1989). Its activity remains significant at neutral pH values (Chapman et al. 1997). Hibernating ground squirrels’ inflammatory system is mainly dormant during torpor (Maniero 2002; Prendergast et al. 2002). I hypothesized that the activity of both cathepsins S and L should be reduced during torpor.

Cathepsin D and E are members of the aspartyl peptidase family, together with pepsin and renin. Cathepsin D is important because it activates the majority of the lysosomal enzymes (Conner and Richo 1992). Cathepsin D is probably the major player in the lysosomal digestive activity (Metcalf and Fusek 1993; Diment et al. 1988). To my knowledge, no investigation has revealed continued activity of either cathepsin D or E at higher pH (7 and above). I hypothesized that activities of both cathepsins D and E should be reduced during torpor.

The scope of the dissertation

Mammalian hibernators experience severely depressed metabolic rates during torpor, to as low as 1% of the rates in active animals. With the reduced metabolic rate, major metabolic processes, such as protein synthesis and protein degradation must be reduced as well to maintain cellular homeostasis. Indeed, earlier investigations demonstrated that protein synthesis is reduced during torpor (Zhegunov et al. 1988; van Breukelen and Martin 2002). To my knowledge, no previous study has demonstrated the fate of protein
degradation in hibernators. I decided to investigate whether protein degradation per se is depressed during torpor. In chapter 2, I examine the activity of the 26S proteasome at the level of the 20S core protease by developing an in vitro assay. This assay involved substrates that release fluorescence as an indirect measurement of the activity of the 20S protease. The three major proteolytic activities of the 26S proteasome were investigated as well in order to observe if all of them are equally depressed at low temperatures.

Previous research demonstrated that ubiquitin conjugates increase 2-3 fold during torpor in hibernating golden-mantled ground squirrels (van Breukelen and Carey 2002). The 26S proteasome is almost non-functional at low temperature. I hypothesized that the increase of ubiquitin conjugates might simply be an accumulation effect. Consequently, the actual process of ubiquitylation was investigated in chapter 3. I developed a new method to measure the process of ubiquitylation in vitro at the temperatures experienced by the hibernators. I found that ubiquitylation continues at moderate levels even at 0°C. However, the increase of ubiquitin conjugates could represent a problem for the hibernating animal, once it returns to euthermicity, thus activating the function of the 26S proteasome. The question was how hibernating squirrels deal with this potential problem. One possibility was that monoubiquitylation might occur more often than polyubiquitylation. Monoubiquitylated proteins are not degraded by the 26S proteasome. In chapter 3, I examined the proportion of monoubiquitylation vs. polyubiquitylation during different states of the torpor. In contrast, if ubiquitylation occurs unchecked during torpor one would expect much higher levels of ubiquitylated proteins than the 2-3 fold that were observed previously (van Breukelen and Carey 2002). To elucidate why ubiquitylation does not
continue unchecked during torpor, I investigated the possibility of substrate (ubiquitin) limitation in chapter 3.

Lysosomal degradation is the other major aspect of proteolysis, besides 26S proteasome degradation, that needs to be addressed in the context of hibernation. The optimal pH for the majority of the hydrolases inside the lysosomes is low pH (4-5.5). The acidity of the lysosome is maintained by the activity of a vacuolar ATPase pump that uses ATP to pump hydrogen ions into the lysosomes. Previous studies have demonstrated that there is no acidification below 18°C (Roederer et al. 1987; Ahlberg et al. 1985). Considering the enormous change of temperature during hibernation, I examined the ability of the isolated lysosomes from hibernating and non-hibernating animals to acidify in chapter 4. An isolation technique was performed and an in vitro assay was developed in order to elucidate this question. In addition, it is generally accepted that the optimal pH for lysosomal hydrolases is low (4-5.5). If acidification of the lysosome does not occur during hibernation, would that limit the function of the lysosomal proteases? In addition, will the effect of temperature alone reduce the activities of the cathepsins? Several cathepsins' activities were investigated in chapter 4.

Understanding how hibernators manage to control a vital component of cellular homeostasis, such as protein degradation, during conditions of severe metabolic depression, will give insight in the overall mechanisms that are involved in hibernation. Although the study of humans and hibernators may seem unrelated, hibernators can represent an excellent model for studies on ischemia, bone and muscle dystrophy, obesity and issues associated with transplantation of organs. Understanding the basic processes that underlie hibernation
can lead to better understanding and significant insight into the development of new therapies for variety of human diseases.
References


macrophage endosomes. J Biol Chem. 263: 6901-6907


associated with hibernation in Columbian (Spermophilus columbianus) and golden-mantled (Spermophilus lateralis) ground squirrels. Physiol Zool 64: 940-959


van Breukelen F, Martin SL (2001) Translational initiation is uncoupled from elongation at 18 C during mammalian hibernation. Am J Physiol Regul Integr Comp Physiol 281: R1374-R1379


CHAPTER 2

PROTEOLYSIS IS DEPRESSED DURING TORPOR IN HIBERNATORS AT THE LEVEL OF THE 20S CORE PROTEASE

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Abstract

Protein synthesis is depressed during mammalian hibernation in concordance with metabolic demands. In the absence of significant protein synthesis, continued proteolysis would rapidly deplete protein pools. Since ubiquitin-dependent proteolysis is implicated in the turnover of most regulatory proteins, we examined the fate of this system during hibernation. Ubiquitin-dependent proteolysis consists of two major steps: (1) the tagging of a protein substrate by ubiquitin and (2) the protein substrate's subsequent degradation by the 26S proteasome. An earlier study revealed a two to threefold elevation of ubiquitin conjugate concentrations during hibernation: an unexpected result that seemingly would suggest increased proteolytic activity. A more likely explanation for these data would be that proteolysis per se was depressed and that the increased levels of ubiquitylated proteins reflect an inability to degrade tagged proteins. We employed an assay based on the cleavage of fluorogenic substrates to address the well-characterized proteolytic activities of the proteasome. All activities show little to no activity at temperatures associated with deep torpor. Coordinated depression of proteolytic activities by low temperature supports the hypothesis that the increased levels of ubiquitylated proteins during hibernation are explained by a net accumulation due to an inability to degrade the tagged proteins.
Introduction

Many mammals employ hibernation or a state of torpor as a means for energy conservation (for reviews, see Carey et al. 2003; van Breukelen and Martin 2002). Torpor is characterized by a periodic lowering of body temperature from approximately 37 °C to as low as -2.9 °C and a reduction in metabolic rate to as low as 1% of the basal rate. Hibernation results in an energy conservation of as much as 90% across the season. Energy savings could be even greater if torpor remained uninterrupted throughout the winter. However, torpor is interrupted by brief interbout arousals wherein body temperature and metabolic rate return to the values typically seen in an active animal. These interbout arousals account for 70% of the energy consumed during the hibernation season. While the exact function of the interbout arousal has not been fully elucidated, this energetically-costly expenditure suggests that survivability or the ability to hibernate depends on these brief returns to warmer temperatures. A possible function for the interbout arousals is that they allow for homeostatic physiological processes that do not occur in the torpid state, such as the turnover of protein pools. Maintenance of protein pools is the result of a balance between protein synthesis and degradation.

In some tissues, protein synthesis and degradation account for as much as 20–40 and 4%, respectively, of oxygen consumption (e.g. Schmidt et al. 1991; Rolfe and Brown 1997). Since metabolic rate is severely reduced during torpor, it is natural to expect downregulation of both protein synthesis and proteolysis. Earlier data show the acute downregulation of translation during torpor (e.g. Frerichs et al. 1998; Chen et al. 2001; van Breukelen and Martin 2001, 2004 and references therein). In the absence of new protein synthesis, continued proteolysis would result in depletion of key enzymes and may compromise an
animal’s ability to arouse from torpor. Therefore, we also expect a depression of proteolysis during torpor.

Several proteolytic pathways exist in mammalian cells. However, ubiquitin-mediated proteolysis is responsible for degrading virtually all regulatory proteins (Ciechanover et al. 1984; Rock et al. 1994). Ubiquitin is a highly conserved 76 amino acid polypeptide. Conjugation with a polyubiquitin chain targets the protein for degradation by the 26S proteasome. The 26S proteasome is made of two major parts: a 19S regulatory cap that is involved in recognizing ubiquitylated proteins and a 20S protease barrel where the actual proteolysis occurs (reviewed in Pickart and Cohen 2004). The 20S core protease consists of four seven membered rings—two central β rings and two distal α rings.

Proteolytic activity appears to be restricted to the β catalytic sites. Three well-characterized peptidase activities inside the 20S barrel are: (1) trypsin-like peptidase activity (TL) that cleaves after basic residues, (2) chymotrypsin-like peptidase activity (ChTL) that cleaves after hydrophobic residues, (3) peptidyl-glutamyl peptide hydrolase (PGPH) or caspase-like peptidase activity that cleaves after acidic residues (Orlowski and Wilk 2000).

An earlier study of ubiquitylation during hibernation revealed a two to threefold elevation of ubiquitin conjugate concentrations during torpor: an unexpected result that seemingly would suggest increased proteolytic activity (van Breukelen and Carey 2002). Since protein pools are not depleted during torpor, a more likely explanation for these data would be that proteolysis per se was depressed rather than the conjugation process.

The increased levels of ubiquitylated proteins likely reflect an accumulation of tagged proteins that are not able to be degraded. In that earlier paper, we were unable to confirm this hypothesis due to technical difficulties. Here, we demonstrate that protease
activity would be depressed at the low temperatures typical of torpor.

Materials and methods

Golden-mantled ground squirrels (Spermophilus lateralis) were trapped in summer. Some squirrels were killed directly for seasonal controls (summer active, SA). Other squirrels were implanted with temperature-sensitive radiotelemeters (Mini Mitter Co., Sun River, OR, USA) as described previously (Martin et al. 1999). Animals were sacrificed following 80% of the expected torpor bout (7 days in torpor, late torpor, LT) or when euthermic between torpor bouts (interbout aroused, IBA). Body temperature during torpor was 5 °C. Livers were removed, snap frozen in liquid N₂, and stored at -80 °C until use. Rat liver was also used as an additional nonhibernator control.

Frozen liver was pulverized in liquid N₂ and homogenized in five volumes of 50 mM Tris–HCl, pH 8.0; 0.1 mM EDTA; and 1 mM 2-mercaptoethanol. Lysates were centrifuged at 20,000-g for 1 h at 4 °C to remove cellular debris. Supernatant protein concentrations were determined by a modified Lowry assay.

Proteolytic activity was estimated by following the release of a fluorogenic compound linked to specific amino acid sequences. Typical assay conditions were as follows unless noted otherwise. In a 100 µl reaction, there was 100 µg of protein; 100 mM Tris–HCl, pH 8.0; 0.0475% SDS; and 40 µM of substrate. The following substrates were used: succinyl-leu-tyr-7-amido-4-methylcoumarin (suc-LY-AMC; Boston Biochemical) which is a suitable substrate for the general peptidase activity of the proteasome; succinyl-leu-leu-val-tyr-AMC (suc- LLVY-AMC; Boston Biochemical) which is a suitable substrate for the chymotrypsin-like activity of the proteasome; t-butyloxycarbonyl-leu-arg-arg-AMC
(boc-LRR-AMC; Affiniti Research Products) which is a suitable substrate for the trypsin-like activity of the proteasome; and benzyloxy carbonyl-leu-leu-glu-β napthylamide (z-LLE-bNA; Boston Biochemical) which is a suitable substrate for the peptidyl-glutamyl peptide hydrolase activity. Typical substrate concentrations for z-LLE-bNA were 0.4 µM final concentration, and an additional 10 mM MgCl₂ was added to these reactions to support PGPH activity. Parallel samples were supplemented with 50 µM MG-115 (Boston Biochemical) a potent inhibitor of proteasome activity.

All reactions were performed for 30 min at the indicated temperature (see figure legends). Reactions were stopped with the addition of 0.3 ml of 1% SDS and 1 ml of 0.1 M borate buffer, pH 9.1. Following addition of 1 ml of water, sample fluorescence was determined at excitation/emission wavelengths of 365/440 nm for AMC and 340/425 nm for bNA. Under our typical assay conditions, maximal reaction rates ranged from 1 pmol to 2.5 pmol of AMC liberated per 100 µg protein in a 30 min assay. Day to day variation in the extent of AMC liberation precluded the meaningful inclusion of absolute data in all graphs. As a result, all data are expressed as a percentage of maximal liberation or in arbitrary fluorescence units.

Results and discussion

During hibernation, there is negligible protein synthesis (values are as low as 0.13% of normothermic rates; Zhegunov et al. 1988). A typical protein has a half-life of 20–30 h (Rogers et al. 1986; Dice 1987). If proteolysis were to continue as a function of normal metabolic activities, these proteins would be depleted to 20% of active levels in just 45–70 h. To date, no study has revealed significant loss of protein pools during torpor. Here, we
show that the low body temperatures experienced by hibernators would depress proteolysis to the extent required to preserve protein levels throughout the torpor bout.

Proteolytic capacity was not different in animals sampled at different points of the hibernation cycle (Fig. 2-1). Proteolytic activity based on the cleavage of succinyl-lysine-tyrosine (sucLY) from 7-amido-4-methylcoumarin (AMC) was estimated in hepatic lysates containing equal amounts of total protein following incubation at 37 °C for 30 min. There were no significant differences between lysates independent of source (ANOVA; P>0.05). Linear rates were maintained for 30 min in these assays (data not shown). A significant reduction in proteolytic capacity from LT animals would be consistent with an active mechanism, e.g. a phosphorylation event, for depressing proteolysis. No such mechanism is indicated based on these data. Rather, measurement of general 20S core proteolytic activity as a function of assay temperature indicates little to no activity at cold temperatures (Fig. 2-2). Therefore, the cold body temperatures that a hibernator would experience would passively depress proteolysis to virtually negligible values. Furthermore, hibernators do not appear specialized in the ability to downregulate protein degradation in that rats exhibit the same thermal sensitivity (Fig. 2-2). The core of the 26S proteasome consists of a barrel like 20S core protease with three distinct proteolytic activities (reviewed in Pickart and Cohen 2004). These activities are associated with degrading distinct pools of proteins/peptides. There are an estimated several hundred thousand to millions of peptides in an average cell (Schrader and Schulz-Knappe 2001). In order to avoid preferential degradation of a given peptide class, the activities of the three major proteases would need to be coordinated during torpor. Differential activity would result in a net depletion or accumulation of a given class of peptides.
Figure 2-1. Proteolytic capacity (activity per 100 µg of protein) in hepatic lysates from golden-mantled ground squirrels representing late torpor (~7 days in torpor, late torpor, LT), when euthermic between torpor bouts (interbout aroused, IBA), or freshly caught in summer (summer active, SA). Lysates were incubated in the presence of Suc LY-AMC, a substrate used for the estimation of generalized 20S protease activity and incubated at 37 °C for 30 min. Data represent means ± SE, n=3 for each of two animals for each state. There were no significant differences, ANOVA, $P > 0.05$. 
We examined the distinct activities through use of substrates specific for each activity. Chymotrypsin-like peptidase activity (Fig. 2-3), trypsin-like peptidase activity (Fig. 2-4), and peptidyl-glutamyl-peptide-hydrolase activity (Fig. 2-5) were all depressed at the low assay temperatures typical of hibernator body temperatures. It is generally accepted that the chymotrypsin-like activity is the rate limiting activity for degrading proteins under normal circumstances (e.g. Kisselev et al. 1999). It should be noted that the approach that we employed here does not actually address ubiquitin-dependent proteolysis per se. Rather, the small peptide substrates enter the 20S core protease independent of ubiquitylation. Larger proteins are usually degraded by the 26S proteasome following conjugation with ubiquitin. Our earlier work found ubiquitin conjugate concentrations were increased two to threefold during hibernation (van Breukelen and Carey 2002).

Ubiquitin conjugate concentrations are generally correlated with increased protein degradation (Munro and Pelham 1985; van Breukelen and Hand 2000). However, the data presented here would indicate that there is little to no 20S core proteolytic activity at the low temperatures typical of torpor. Thus, proteins that are conjugated to ubiquitin would not be appreciably degraded if they were to enter the 20S core protease. Furthermore, one might expect that the cold temperatures would also affect the actual entry of ubiquitylated proteins into the proteasome. It is generally accepted that the function of the 19S regulatory cap of the 26S proteasome is to chaperone ubiquitylated proteins into the complex. If low temperature affects this chaperoning activity in an expected manner, then one might expect reduced entry of substrate proteins into the proteasome thereby reducing total proteolytic processing even further. The data presented here are in agreement with data from other systems.
Figure 2-2.
Proteolytic activity as a function of assay temperature. Hepatic lysates from LT, IBA, and SA squirrels and rats were incubated in the presence of Suc-LY-AMC, a substrate used for the estimation of generalized 20S protease activity. Incubation temperatures were 0, 5, 10, 15, 20, 25, 30 and 37 °C. Squares represent lysates that were supplemented with 50 μM MG115, a potent inhibitor of the 20S protease. Circles represent lysates without inhibitors. Values represent means ± SE, n=3. Data shown represent a typical result for an individual animal. Similar results were obtained using lysates derived from different animals.
Figure 2-3.
Chymotrypsin-like peptidase activity as a function of assay temperature. Experimental details were as in Fig 2-2 except that suc-LLVY-AMC was used as the substrate.
Figure 2-4.
Trypsin-like peptidase activity as a function of assay temperature. Experimental details were as in Fig 2-2 except that boc-LRR-AMC was used as the substrate.
Figure 2-5.
Peptidyl-glutamyl peptide hydrolase activity as a function of assay temperature. Experimental details were as in Fig 2-2 except that z-LLE-βNA was used as the substrate. An additional 10 mM MgCl₂ was added to these reactions to support activity.
Proteolysis is extremely temperature sensitive; ubiquitin-mediated protein degradation in rabbit reticulocytes virtually ceased below 27 °C (Muller et al. 1980). Additional studies on antigen processing (an ubiquitin mediated proteolytic process; reviewed in Rock and Goldberg 1999) demonstrated a block below 18 °C (Harding and Unanue 1990). Protein degradation in cultured mammalian cells decreased with a Q10>4 and the rate of degradation at 6 °C was <2% of the rate at 37 °C (Hough and Rechsteiner 1984). In the latter study, the marked temperature dependence of degradation appeared to be related to decreased proteolytic processing through the 26S proteasome rather than ubiquitylation. Nonubiquitin mediated pathways may also be acutely temperature sensitive; lysosomal acidification and function in nonhibernators is blocked at low temperatures (Ahlberg et al. 1985; Roederer et al. 1987).

In summary, a significant consequence of the depression of protein synthesis during torpor is that protein degradation must also be depressed. Continued degradation of proteins could jeopardize the ability of the hibernator to return to the euthermic state by depleting critical protein pools. Our data support the view that hibernators restrict ubiquitin-mediated proteolysis to the interbout arousal when body temperatures are warm with the concomitant high protein synthesis rate. However, hibernators downregulate ubiquitin-mediated proteolysis in a rather unconventional way: ubiquitin conjugates remain high throughout the torpor bout but the low body temperature associated with torpor severely reduces protein degradation per se. These data support the hypothesis that one possible function for the interbout arousal is to allow for replenishment of protein pools.
References


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van Breukelen F, Sonenberg N, Martin S (2004) Seasonal and state dependent changes of

CHAPTER 3

UBIQUITYLATION OF PROTEINS IN LIVERS OF HIBERNATING GOLDEN-MANTLED GROUND SQUIRRELS, *SPERMOPHILUS LATERALIS*

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The contribution of the authors to this paper are as following: Velickovska V did all the work and wrote the manuscript; van Breukelen F provided laboratory space and equipment.
Abstract

Rodent hibernators experience low core body temperature (as low as -2 °C) and reduced metabolic rates during hibernation. Concordant with energetic constraints, protein synthesis is negligible during torpor. To maintain pools of key regulatory proteins, proteolysis must be depressed as well. Ubiquitin-dependent proteolysis consists of two major steps: (1) ubiquitylation or tagging of a protein substrate by ubiquitin and (2) the protein substrate's subsequent degradation by the 26S proteasome. Earlier, we demonstrated that the low temperatures typical of torpor virtually arrest proteolytic processing. Here, we demonstrate that in vitro ubiquitylation still continues at greater than 30% of maximal rates at temperatures as low as 0 °C. Continued ubiquitylation in the presence of severely depressed proteolysis may explain the previously observed 2- to 3-fold increase of ubiquitin conjugates during torpor. We determined if there is a qualitative change in the type of ubiquitylation e.g., monoubiquitylation vs. polyubiquitylation that occurs during torpor. We found no bias for monoubiquitylation in any state of the torpor cycle. We further determined that substrate limitation of free ubiquitin is not limiting ubiquitylation during torpor. We conclude that while the cold temperatures of torpor may limit proteolysis in accordance with metabolic demands, continued ubiquitylation may result in increased ubiquitin conjugate concentrations that must be processed upon arousal.
Introduction

A variety of mammals use hibernation presumably as a means for energy conservation (reviewed in [6,33]). Hibernation is not static. Hibernators oscillate between (1) torpor where body temperature (Tb) may approach that of ambient temperature to as low as \(-2\,^{\circ}\text{C}\) [3,11] and metabolic rate may be as low as 1% of the active rate [38] and (2) a short interbout arousal where animals restore body temperature and metabolic rates to euthermic values. A torpor bout may last from 1 to 3 weeks depending on time of year, species, and ambient temperature. Concordant with a depressed metabolic rate, protein synthesis is depressed to as low as 0.13–0.5% of active levels ([39,34] and references therein).

Continued proteolysis in the absence of significant protein synthesis would lead to a net depletion of protein pools. Therefore, it follows that there should be a coordination of both protein synthetic and degradative processes in order to maintain homeostatic balance.

There are several proteolytic pathways in the mammalian cell. Ubiquitin-mediated proteolysis appears to be responsible for degrading virtually all regulatory proteins; estimates are as high as 80–90% of cytosolic proteins are degraded by ubiquitin-mediated proteolysis [7,26]. Ubiquitin-mediated proteolysis consists of two major events: (1) ubiquitylation or the tagging of a protein substrate by ubiquitin and (2) the subsequent degradation of the ubiquitylated protein by the 26S proteasome (reviewed in [21,22]).

Typically, conjugation of a polyubiquitin chain with a protein targets that protein for degradation by the 26S proteasome [21,22,36]. An examination of ubiquitin conjugate concentrations during torpor revealed a 2- to 3-fold elevation over values of active squirrels depending on tissue type [31]. This finding would seemingly suggest increased proteolytic activity, as an increase in ubiquitin conjugates usually correlates with increased proteolysis.
However, no study to our knowledge has demonstrated widespread depletion of protein pools during torpor and we found that activity through the 26S proteasome would be reduced to negligible values at the cold temperatures typical of torpor [35]. Even moderate ubiquitylation in the presence of severely limited proteolysis would result in an accumulation of ubiquitin conjugates during torpor. Here, we developed a method to measure the active process of ubiquitylation in liver lysates.

Ubiquitin may be conjugated to a protein through either a monoubiquitylation event or a polyubiquitylation chain. Although polyubiquitylation usually serves as a strong signal for degradation, monoubiquitylation events may be involved in such activities as DNA and ribosomal preservation [37,16–18]. One possibility is that the increased levels of ubiquitin conjugates that are found in torpid squirrels represent an increase in monoubiquitylated proteins rather than polyubiquitylated proteins. Here, we determined an index of monoubiquitylated proteins vs. polyubiquitylated proteins as a function of torpor state.

The concentrations of ubiquitin conjugate increased 2- to 3-fold but not more in torpid squirrels [31]. A possible limiting factor to continued ubiquitylation might be substrate limitation e.g., free ubiquitin. We measured the concentrations of free ubiquitin present at different states of the torpor cycle.

Materials and methods

Animals

Adult golden-mantled ground squirrels (Spermophilus lateralis) were captured during the summer from southern Nevada and California. Some animals were sacrificed immediately as a seasonal control (summer active). Temperature sensitive radiotelemeters [20,32].
were implanted as described previously in order to allow for precise determination of torpor status [19]. Animals were housed in an environmental chamber at 4 °C and allowed to hibernate. Torpid body temperature (Tb) was 5 °C. In addition to summer active squirrels, winter squirrels representing torpid and interbout aroused animals were sampled. Torpid animals were sacrificed when Tb was approximately 5 °C. Interbout aroused animals were sacrificed when euthermic between torpor bouts. Rats were used as an additional non-hibernator control where indicated. Squirrels were sacrificed by CO₂ asphyxiation except for torpid animals. Torpid animals were killed by decapitation because of their low respiratory rates. Livers were removed and snap frozen in liquid nitrogen and stored at -80 °C until use.

**Ubiquitylation rates**

An assay was developed to measure the rates of ubiquitylation as a function of temperature. Frozen liver was pulverized in liquid N₂, and homogenized in 3 volumes of 10 mM HEPES, pH 7.8, 5 mM MgCl₂, and 10 mM KCl. Cellular debris was removed by centrifuging the lysates at 20,000g for 30 min at 4 °C. Lysates were ATP depleted by supplementing them with 20 mM 2-deoxyglucose and incubating at 25 °C for 30 min. In order to remove free ATP and reduce levels of endogenous ubiquitin, lysates were passed across two sequential Sephadex G-50 desalting columns [15]. These columns remove smaller molecular weight molecules below 10,000 Da (ubiquitin is 8360 Da). The Sephadex G-50 used in the spin columns was pre-equilibrated to 100 mM Hepes, pH 7.8, 5 mM MgCl₂, and 10 mM KCl. The resulting lysates were frozen at -80 °C until use. Lysate protein concentrations were determined by a modified Lowry protein assay.

The ubiquitylation reaction volumes totaled 50 μl and contained 75 μg of liver
protein; an ATP regenerating system consisting of 45 mM creatine phosphate, 5 mM ATP, 0.1 mM inorganic pyrophosphate, and 8 ng creatine phosphokinase; 0.5 mM dithiothreitol (DTT) and 50 µM MG115 (specific 26S proteasome inhibitor; Bio mol International). The reaction was initiated with 5 µg biotinylated ubiquitin (Biomol International) and allowed to proceed for 1 h at the indicated temperatures (0, 5, 10, 15, 20, 25, 30, and 37 °C). Reactions were stopped by adding 100 µl of 50 mM Tris–HCl, pH 8.3, 20% glycerol, 2% SDS, and 0.4 M β-mercaptoethanol. Ubiquitylation was determined by following incorporation of the biotinylated ubiquitin with western blotting. SDS–PAGE was performed using a 12% polyacrylamide gel. After transfer to PVDF membrane, blots were incubated in the presence of 10 µg of streptavidin conjugated to horseradish peroxidase and visualized using ECL+ on a Molecular Dynamics Typhoon multivariable imager (GE Life Sciences). Ubiquitylation as a function of temperature was determined for three animals from each state (summer active, torpid, and interbout aroused) and for rat as a non-hibernator control. Preliminary western blotting using lysates that were not supplemented with biotinylated ubiquitin revealed interference by endogenous biotinylated proteins as indicated by bands whose intensity did not vary between lysates supplemented with biotinylated ubiquitin and control lysates. As a result, we restricted analyses to portions of the western blot where endogenous biotin was not present e.g., we analyzed ubiquitylated proteins between 20 and 75 kDa molecular weight.

**Mono vs. polyubiquitylation**

An index of mono vs. polyubiquitylation as a function of torpor state was determined using a competitive antibody technique. Frozen livers were pulverized in liquid N₂ and homogenized in 3 volumes of 50 mM Tris–HCl, pH 8.3, 20% glycerol, 2% SDS, and 0.4 M
β-mercaptoethanol. Lysates were cleared of cellular debris by centrifugation at 20,000g for 30 min at 4 °C. Protein concentrations of the supernatant were determined by a modified Lowry protein assay. Dot blots were performed using 60 or 90 µg of protein from livers of summer active, torpid, and interbout aroused squirrels (n = 3 animals from each state).

These concentrations of protein are below the manufacturer's indicated binding capacity of the membrane (Sequi-Blot PVDF; Bio-Rad; binding capacity is 170–200 lg/cm2). The dot blot membranes were blocked overnight with 3% nonfat dried milk in a solution of 25 mM Tris–HCl, pH 7.5 and 145 mM NaCl (TBS) with the addition of 0.1% Tween 20 (TTBS).

The membranes were washed in TBS, washed twice in TTBS and washed once more in TBS. The dot blots were incubated overnight with monoclonal antibodies to conjugated ubiquitin (Biomol international; CosmoBio). Antibody clone FK1 belongs to the IgM subclass of immunoglobulins and recognizes only polyubiquitylated proteins [5,12–14]. Clone FK2 belongs to the IgG subclass of immunoglobulins and recognizes both mono and polyubiquitylated proteins [5,13,14,27–29]. Simultaneous exposure to both of these antibody clones should reveal relative contributions of mono and polyubiquitylated proteins to the overall ubiquitin conjugate pool.

Analogous assays utilizing competitive antibody approaches with both IgG and IgM antibodies were performed previously (e.g., [25]). Both FK1 and FK2 were added simultaneously in a concentration of 1:1000 in a solution of 1% nonfat dried milk in TTBS. Blots were washed in TBS, twice in TTBS, and once more in TBS before incubating the membranes with secondary antibodies. The secondary antibodies were: (a) goat anti-mouse IgM conjugated with AlexaFluor 594 (red) and (b) goat anti-mouse IgG conjugated with AlexaFluor 488 (green; Invitrogen). Both secondary antibodies were added at a
concentration of 1:10,000 in a solution of 1% bovine serum albumin (BSA) in TTBS.

Following visualization on a Molecular Dynamics Typhoon multivariable imager, an index of the relative contributions of monoubiquitylation and polyubiquitylation was determined. The index value was determined by dividing the green signal (FK2) by the red signal (FK1). A higher value is indicative of relatively increased monoubiquitylation.

Concentrations of free ubiquitin

Free ubiquitin was measured by western blotting. Frozen liver was pulverized in liquid N$_2$ and homogenized in 3 volumes of 50 mM Tris–HCl, pH 8.3, 20% glycerol, 2% SDS, and 0.4 M β-mercaptoethanol. Lysates were cleared of cellular debris by centrifugation at 20,000g for 30 min at 4° C. Protein concentrations of the supernatant were determined by a modified Lowry protein assay. SDS–PAGE was performed using a 20% resolving gel. Lanes were loaded with 70 μg of total protein from summer active, torpid, and interbout aroused animals (n = 3 from each state) or varying concentrations of free ubiquitin (0–150 ng; Sigma Chemical). Following transfer to PVDF membrane, the membranes were blocked overnight with 3% nonfat dried milk in a solution of TTBS. The membranes were washed once in TBS, twice in TTBS, and once more in TBS and then incubated with polyclonal antibody that recognizes free ubiquitin (a gift from Dr. Art Haas). The antibody was diluted 1:1000 in a solution of 3% nonfat dried milk in TTBS. Secondary antibody incubation was performed at a 1:3000 dilution in 1% BSA in TTBS for 1 h using a goat anti-rabbit IgG antibody conjugated to horseradish peroxidase (Sigma Chemical Co). Blots were washed as above. ECL+ was used to visualize the samples on a Molecular Dynamics Typhoon multivariable imager. A standard curve was constructed using the varying concentrations of free ubiquitin. The correlation coefficient ($r^2$) of the standard
curve was 0.99. The standard curve was used to calculate the concentrations of free ubiquitin in different states of the torpor cycle.

Results and discussion

Ubiquitylation continues at a moderate rate even at the lowest temperatures typical of torpor; ubiquitylation at 0 °C was greater than 30% of maximal rates (Fig. 3-1). Previously, we demonstrated that cold temperatures reduce 26S proteasome activity to negligible values [35]. In the presence of negligible 26S proteasome activity, continued ubiquitylation would lead to an accumulation of ubiquitylated proteins. Concordant with that expectation, ubiquitin conjugates increase 2- to 3-fold during torpor [31].

An accumulation of 2- to 3-fold higher concentrations of ubiquitin conjugates may have significant physiological consequences for the hibernating animal. Upon arousal, ubiquitin conjugate concentrations are restored to values typical of active animals [31]. A significant question is how this restoration is accomplished.

Proteins that are conjugated to ubiquitin may either enter into the degradation process with the proteasome or they may be deubiquitylated in an editing type fashion [1,8]. Does the reduction in ubiquitin conjugate concentrations upon arousal represent proteolytic processing or deubiquitylation of proteins? If the elevated ubiquitin conjugates found during torpor are degraded upon arousal when permissive body temperatures are available, then there may be a significant energetic outlay. Increased proteolysis would necessitate increased de novo protein synthesis to maintain protein pools. Indeed, protein synthesis is 30% higher during the interbout arousal over summer values [39]. These data suggest compensatory protein synthesis when euthermic body temperatures are restored.
Figure 3-1.
Ubiquitylation in hepatic lysates as a function of temperature. Hepatic lysates from summer active, torpid and interbout aroused squirrels and rats were supplemented with biotinylated ubiquitin and incubated for 30 min at indicated assay temperatures. (a) Western blot analyses were performed. An area of the blot that did not contain endogenous biotin was used for analysis (20-75 kDa). (b) Western blots were quantified. Data represent means ± SE, n=3 animals for each state. Data were shifted along the axis of abscissa to allow for greater clarity.
An interesting observation is that ubiquitylation in ground squirrels appears to be more temperature sensitive than in rats (Fig. 3-1). Although speculative at best, and based on only two species comparisons, it is possible that the increased thermal sensitivity represents an evolutionary transition toward greater control of ubiquitylation in hibernators. However, additional species comparisons would need to be done before any significant conclusion could be made.

The increase in ubiquitin conjugates may not necessarily represent proteins targeted for degradation. One possible solution to reduce the increased ubiquitin conjugate levels might be the activity of deubiquitylating enzymes (DUBs) (for review, see [1,8]). These enzymes catalyze the cleavage of the bond between ubiquitin and its target proteins. At least 140 DUBs belonging to several subfamilies have been described based on either genetic similarity (such as ubiquitin-specific processing protein groups, UBP), and/or the mechanism of action (such as ubiquitin carboxy-terminal hydrolases, UCH) [1,8]. It is tempting to speculate that there may be a role for DUBs during torpor or arousal in recycling proteins that were conjugated to ubiquitin so as to avoid degradation. Our initial efforts to measure DUB activity in crude lysates of hibernators failed. Additional efforts to use purified DUBs are underway.

Ubiquitylation during torpor may be qualitatively distinct from the active states i.e., there may be more monoubiquitylation than polyubiquitylation during torpor. Polyubiquitylated proteins are typically destined for degradation [21,22,36]. However, monoubiquitylated proteins are generally considered to be poor substrates for the 26S proteasome [23]. Instead, monoubiquitylation is usually associated with stable proteins during stressful periods such as oxidative stress and ischemia [4,16,18]. We hypothesized
that perhaps, proteins are preferentially monoubiquitylated during the stressful period of torpor. This monoubiquitylation may help maintain protein integrity. However, the data presented here reveal no bias for monoubiquitylation over polyubiquitylation in torpid animals (Table 3-1).

Concentrations of ubiquitin conjugated proteins increase only 2 to 3-fold but not more during torpor. The total ubiquitin pool in the cell is represented by the sum of both free ubiquitin and ubiquitin conjugate concentrations [2]. If ubiquitylation continued in the cold, one eventuality would be the depletion of free ubiquitin pools. Free ubiquitin concentrations were determined at different states of the torpor cycle. We found no depletion of the free ubiquitin pools (Fig. 3-2). Rather, free ubiquitin concentrations in the liver of squirrels represent 93–95% of the total ubiquitin pool (Table 3-2). Thus, a 2- to 3-fold increase in ubiquitylated proteins represents only a small fraction of the available free ubiquitin. Depletion of free ubiquitin does not seem to be a factor in limiting the increase of ubiquitin conjugate concentrations during torpor. However, the limited accumulation of ubiquitin-conjugated proteins implies that some other factor(s) besides temperature and substrate limitation of ubiquitin must be involved in the regulation of ubiquitylation during torpor.

A depleted ATP pool may limit ubiquitylation. During hibernation, there may be a 2-fold reduction of ATP [9,10]. The hydrolysis of ATP yields a myriad of adenylate metabolites including adenosine, ADP, AMP, hypoxanthine, inosine, and inosine monophosphate [24,30]. In the embryos of the brine shrimp, *Artemia franciscana*, active ATP hydrolysis led to the accumulation of an unidentified metabolite(s) that restricted both ubiquitylation and protein degradation [32]. Despite a screening of likely candidates, the
* A higher value represents increased monoubiquitylation. Values represent means ± SE, n = 3 animals. ANOVA revealed no significant difference p ≥ 0.05.

Table 3-1.

Index of monoubiquitylation for different states of the torpor cycle

<table>
<thead>
<tr>
<th>Trial</th>
<th>Summer Active</th>
<th>Interbout Aroused</th>
<th>Torpid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial 1 (60 μg protein)</td>
<td>1.2794 ± 0.049*</td>
<td>1.1606 ± 0.029</td>
<td>1.2545 ± 0.015</td>
</tr>
<tr>
<td>Trial 2 (90 μg protein)</td>
<td>1.3533 ± 0.062</td>
<td>1.1900 ± 0.089</td>
<td>1.3088 ± 0.016</td>
</tr>
</tbody>
</table>
Figure 3-2.
Concentrations of free ubiquitin in liver of squirrels from different states of the torpor cycle: summer active, torpid and interbout aroused. Values represent means ± SE, n=3 animals for each state. ANOVA revealed no significant difference p > 0.05.
<table>
<thead>
<tr>
<th></th>
<th>[Ubiquitin conjugates]</th>
<th>[Free ubiquitin]</th>
<th>[Ubiquitin conjugates]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/µg of protein *</td>
<td>pmol/µg of protein</td>
<td>% of total ubiquitin pool</td>
</tr>
<tr>
<td>Summer Active</td>
<td>0.0347 ± 0.004</td>
<td>0.6676 ± 0.026</td>
<td>4.9%</td>
</tr>
<tr>
<td>Torpid</td>
<td>0.0606 ± 0.003</td>
<td>0.7597 ± 0.066</td>
<td>7.4%</td>
</tr>
<tr>
<td>Interbout Aroused</td>
<td>0.0430 ± 0.004</td>
<td>0.7538 ± 0.058</td>
<td>5.4%</td>
</tr>
</tbody>
</table>

Values represent means ± SE, n = 3 animals for each state. * Data taken from van Breukelen and Carey 2002.

Table 3-2.

Concentrations of ubiquitin conjugates and free ubiquitin from different states of the torpor cycle in livers of ground squirrels
precise metabolite was not identified [32]. It should be noted that this inhibition was found when concentrations of ATP were sufficient to support full proteolytic activity in the absence of active ATP hydrolysis. In the hibernator, it is possible that limited ATP hydrolysis could result in the accumulation of this metabolite(s) as well. This hypothesis must be considered in light of our in vitro ubiquitylation data (Fig. 3-1). In these experiments, ubiquitylation did not cease even at the lowest temperatures typical of torpor. We may have avoided accumulation of this hypothetical metabolite. The same ATP regenerating system was used as in the *Artemia* experiments and inhibition was only observable when ATP hydrolysis overwhelmed the system.

A successful and prolonged metabolic depression like hibernation requires coordination between anabolic and catabolic processes in order to maintain homeostasis. In the face of limited protein synthesis, hibernators must restrict proteolysis. Indeed, proteolytic processing of the 26S proteasome is virtually arrested at the cold temperatures typical of torpor [35]. However, ubiquitylation of proteins still continues at these temperatures at greater than 30% of maximal rate (Fig. 3-1). This mismatch between proteolysis and ubiquitylation may result in an accumulation of ubiquitin conjugates as is observed during hibernation [31]. However, we suspect that ubiquitylation does not occur unchecked during hibernation. The increase in ubiquitin conjugated proteins is limited to 2- to 3-fold despite very high concentrations of free ubiquitin (Fig. 3-2) i.e., substrate limitation of ubiquitin does not restrict ubiquitylation. Other factors might limit ubiquitylation during torpor such as ATP hydrolysis or, perhaps, the activity of deubiquitylating enzymes. Further experimentation is required to understand the roles of these processes in regulating protein degradation. Concordant with what is required for a successful and prolonged metabolic
depression, protein synthesis and degradation are reduced during torpor. However, these processes are not precisely coordinated. Consequently, there is an accumulation of ubiquitylated proteins. Taken together, these data suggest that hibernators exploit cold temperatures, albeit not perfectly, to downregulate critical homeostatic processes. It is possible that the function of the interbout arousal could be to rectify the accumulative effects of physiological mismatches like the one described here.
References


CHAPTER 4

EFFECTS OF HibernATION ON LYSOSOMAL ACIDIFICATION AND CATHEPSIN ACTIVITY IN GOLDEN-MANTLED GROUND SQUIRRELS, SPERMOPHILUS LATERALIS

Abstract

Protein synthesis is remarkably depressed during hibernation in golden-mantled ground squirrels, Spermophilus lateralis. Continued proteolytic function without the presence of significant protein synthesis should rapidly deplete vital protein pools. In order to maintain homeostasis of the protein pools, protein degradation must be depressed as well. Two major proteolytic systems are present in the mammalian cell: the ubiquitin-mediated and the lysosomal proteolytic systems.

Previously, I have demonstrated that ubiquitin-mediated proteolysis is virtually non-existent at the low temperatures experienced by hibernators. Here, I investigated the ability of the lysosomal proteolytic system to function during torpor. I examined the ability of the lysosomes to acidify at a range of temperatures usually experienced during hibernation. Absence of acidification might increase pH inside the lysosome to cytosolic pH values and reduce the ability of lysosomal cathepsins to function at conditions experienced during hibernation. The proteolytic activities of the cathepsins might also be inhibited during hibernation as a result of the low temperatures. I observed that cathepsin activities are
reduced both as a consequence of the higher pH and of the low temperatures typical for the
torpor bout.

Introduction

Golden-mantled ground squirrels may employ hibernation or a state of torpor to
conserve energy during the winter season (for reviews see Carey et al. 2003; van Breukelen
and Martin 2002). During hibernation, the body temperature (Tb) can decrease from 37\degree C
to as low as -2.9\degree C, and metabolic rate can be reduced to as low as 1% of the active basal
rate (Barnes 1989; Wang and Lee 2000). Torpor bouts are interrupted with brief arousals,
when both Tb and metabolic rate return to euthermic values. Energy conservation
throughout the hibernation season can be as high as 90% compared to similar intervals when
the animals are not hibernating (reviewed in Wang and Lee 2000). If it were not for the
brief arousals between torpor bouts, the energy savings would be even higher. Taken
together, these interbout arousals can cost the animals as much as 70% of their stored energy
resources (Wang 1979). One possibility to explain these arousals is that the hibernating
animal is progressively depleted of critical gene products during the torpor bout and that
arousals are induced by the deficiency of particular proteins (Martin et al. 1993). During
torpor bouts, protein synthesis is largely depressed (0.13-0.5% of active rates; Zhegunov et
al. 1988; van Breukelen and Martin 2001). However, during the interbout arousal protein
synthesis increases up to 30% when compared to non-hibernating summer active ground
squirrels (Zhegunov et al. 1988).

To maintain the homeostasis of the hibernator, protein synthesis and protein
degradation have to be in balance. Accordingly, in the absence of significant protein
synthesis, protein degradation has to be reduced as well. Two major proteolytic pathways have been described in the mammalian cell: ubiquitin-mediated proteolysis and lysosomal proteolysis. Estimates are that ubiquitin-mediated proteolysis is responsible for degrading 80-90% of cytosolic proteins in mammals (Ciechanover et al. 1984; Rock et al. 1994). Previous research has demonstrated that ubiquitin-mediated proteolysis is actually absent at the low temperatures experienced during hibernation (Velickovska et al. 2005). Lysosomal proteolysis represents the other major proteolytic system in the mammalian cell.

Lysosomes are acidic subcellular structures that contain large numbers of acid hydrolases of diverse specificities and, as such, are able to degrade most macromolecules in the cell. The acidity of the lysosome is maintained by the activity of a vacuolar ATPase (V-ATPase), which utilizes ATP to pump hydrogen ions into the lysosome. Several separate transport mechanisms can provide substrates for degradation to the lysosomes: phagosomic, autophagocytotic, microautophagocytotic and crinophagocytotic delivery systems (Castino et al. 2003). Lysosomes are generally small (0.5 μm) spherical organelles, but not always. A more reliable way to confirm the presence of the lysosome is with biochemical markers (Luzio et al. 2003; de Duve and Wattiaux 1966). As a biochemical marker specific for the lysosomes, I chose to measure N-acetyl-β-D-glucosaminidase activity (Barret and Heath 1977; Bouquelet et al 1980) because it is a straightforward method that can produce a linear rate of activity.

Lysosomal protein degradation may be an especially important factor in the turnover of membrane proteins (Hicke and Dunn 2003, Pickart 2004). However, previous researchers have demonstrated that there is no acidification below 18°C in other mammalian systems (Roederer et al. 1987; Ahlberg et al. 1985). Here, I developed an *in vitro* system to examine
the ability of the lysosomes to acidify at temperatures usually experienced by the hibernating animal. I demonstrated that the lysosomes were unable to acidify at low temperatures.

The lysosomes encapsulate numerous hydrolases used to break down proteins, lipids, nucleotides and polysaccharides. The most numerous and largest group of the lysosomal hydrolases are the proteases or cathepsins (reviewed in Turk et al. 2001). The activities of the majority of the hydrolases within the lysosomes are optimal at low pH (de Duve and Wattiaux 1966). However, if there is no acidification at low temperatures, how will this affect the activity of the cathepsins during hibernation? I investigated if the combined effects of high pH (due to possible absence of acidification) and low temperature could completely inhibit the activity of the cathepsins during torpor. In this study, 6 cathepsins were investigated: cathepsins B, D, E, H, L and S. These particular cathepsins were chosen due to their abundance and their ability to function even at high pH values. In general, the activities of the cathepsins were lower under conditions usually experienced by the hibernators. Some activities were reduced as a result of the low temperatures (cathepsins L and S). Other activities decreased because of the high pH values (cathepsins H and D). Taken together, these data demonstrate that lysosomal degradation is generally reduced during torpor. Hibernating animals likely use the combined effects of low temperature and the absence of acidification to reduce proteolysis during torpor.

Materials and methods

Animals

Golden-mantled ground squirrels, *Spermophilus lateralis*, were captured during the summer from California and Southern Nevada. Some of the newly captured animals were
killed as a seasonal control (Summer Active, SA). The rest of the animals were implanted with temperature sensitive radiotelemeters as described previously (Martin et al. 1999) to allow for precise determination of the torpor bouts. The squirrels were kept in an environmental chamber at 4°C and allowed to hibernate. Animals were killed either when $T_b$ was ~5°C (Torpid, T) or when euthermic, in between torpor bouts (Interbout Aroused, IBA). In general, animals were sacrificed by CO$_2$ asphyxiation. The torpid squirrels were decapitated because of their low respiratory rates. Livers were removed and immediately frozen in liquid N$_2$ and stored at -80°C for later use. Rat livers were used as an additional non-hibernator control.

Isolation of liver lysosomes

Lysosomes were prepared following an isolation protocol using a self-generated iodixanol gradient as described previously (Graham et al. 1994). Briefly, frozen livers were pulverized in liquid N$_2$ and homogenized in 4 volumes of the following buffer: 40% glycerol, 10 mM KCl, 1 mM EDTA, 10 mM HEPES pH 7.4. Lysates were centrifuged at 3,000xg for 10 min at 4°C. The supernatant was saved and the pellet was resuspended in 0.25 M sucrose, 1 mM EDTA and 10 mM HEPES, pH 7.4. Supernatants from both centrifugations were pooled before centrifuging again at 17,000xg for 15 min at 4°C. This isolation protocol allows for isolation of a membrane fraction that might contain mitochondria and peroxisomes in addition to the lysosomes (Graham et al 1994). To reduce contamination by mitochondria, peroxisomes and endoplasmatic reticuli, CaCl$_2$ [8 mM] was added to the pellet to swell mitochondria and endoplasmatic reticulum and reduce their density (Arai et al. 1991; van Dyke 1993). CaCl$_2$ is absorbed into the mitochondria and peroxisomes and causes swelling as a result of increased ion concentration (Arai et al. 1991;
Nicholls and Brand 1980; Brustovetsky et al. 2003; van Dyke 1993). Lysosomes do not absorb Ca^{2+} (Arai et al. 1991). The suspension was incubated for at least 15 min before centrifugation at 5,000xg for 10 min at 4°C. Finally, the pellet was resuspended with 20% iodixanol, 0.25 M sucrose, 1 mM EDTA and 10 mM HEPES, pH 7.4, before centrifugation at 350,000xg for 2 h at 4°C. The gradients were fractionated in 1 ml fractions using an ISCO flow cell (Isco, Inc. Nebraska, USA). All fractions were centrifuged again in 0.25 M sucrose and 10 mM HEPES, pH 7.4 at 30,000xg for 10 min at 4°C, to avoid potential interaction of the iodixanol with the later enzymatic assays. The fractions were aliquoted before being frozen at -80°C for later analysis.

Visualization and biochemical confirmation of the lysosomes

Lysosomes appear spherical under the microscope (de Duve and Wattiaux 1966; Luzio et al. 2003). The lipid dye, Sudan Black B was used to determine the presence of spherical organelles under the microscope as described (Carson 1990; Crookham et al. 1991). Briefly, in 100 ml of propylene glycol, 0.7 g of Sudan Black B was dissolved and later filtered to remove the excess dye. A small volume of the lysosomal fractions was stained for 15 min with the Sudan Black B solution (1:1). It was post-stained in 85% propylene glycol (3:1 propylene glycol to Sudan Black B solution) for 3 min, and then rinsed in distilled water and observed under the microscope.

I used Sudan Black B to confirm that the fractions contained spherical organelles. However, to verify that the spherical organelles observed under the microscope were indeed lysosomes, N-acetyl-β-glucosaminidase activity was measured (a well known biochemical marker for lysosomes; Barret and Heath 1977). The enzyme β-glucosaminidase is present in the lysosome, but is absent from mitochondria and peroxisomes. In brief, a 50 µl fraction of
the lysosomal lysate was incubated with 950 μl of 0.3 M citrate buffer, pH 4 and 30 nM of 4-methylumbelliferone-N-acetyl-β-D-glucosaminide for 45 min at 37°C. The reaction was stopped by the addition of 3 ml 0.5 M bicarbonate buffer, pH 9.9. A standard curve was made using known concentrations of 4-methylumbelliferone ranging from 1.2 nM to 12 nM.

Latency and purity of the isolated lysosomes

To ensure that the lysosomes isolated were functionally intact, an assay was developed to determine the integrity (latency) of the lysosomes. The assay allows for a comparison between activity of the N-acetyl-β-glucosaminidase in intact lysosomes and lysosomes that had their membrane disrupted by the detergent, Triton X-100. If the activity of the N-acetyl-β-glucosaminidase is higher in the detergent treated lysosomes but not in the intact lysosomes, that is an indicator of the preserved integrity of the lysosomes. When the lysosomes are intact, N-acetyl-β-glucosaminidase activity is low, because the membrane prevents access to the substrate. Once the membrane of the lysosome is disrupted by the Triton X-100, N-acetyl-β-glucosaminidase enzyme has clear access to the substrate and its activity increases. The integrity of the lysosomes is high when there is a high difference between intact and detergent treated lysosomes (Bergmann et al. 2004; Schutt et al 2002; van Dyke 1993). Briefly, the latency of the lysosomes was expressed as:

\[
\text{((B-glucosaminidase activity + detergent) - (B-glucosaminidase activity))} \times 100% \\
\frac{[(B-glucosaminidase activity + detergent)]}{[(B-glucosaminidase activity + detergent)]}
\]

Only fractions with the highest latency (>90%) were used in further experiments. The fractions that showed the highest amount of intact lysosomes were also examined for contamination by other organelles such as peroxisomes and mitochondria (Graham et al
Possible contamination of peroxisomes was investigated as described before (van Lente and Pepoy 1990). Briefly, this coupled-enzyme method is based on the peroxidic reaction between hydrogen peroxide, ethanol and catalase. In the presence of hydrogen peroxide, catalase transforms ethanol into acetaldehyde. Oxidation of acetaldehyde by the activity of aldehyde dehydrogenase produces acetate and in the same time transforms NAD+ into NADH. NADH absorbance can be measured at 340 nm. In short, in a reaction volume of 1 ml, the activity of the enzyme catalase, specific for peroxisomes, was determined by adding final concentrations of 0.1 units/ml aldehyde dehydrogenase, 150 mM ethanol, 1 mM NAD+ and 30 μl of the lysosomal fractions. The absorbance of NADH was measured at 340 nm for at least 2 min to allow for determination of activity.

Mitochondrial contamination was assessed as described earlier (Wickler 1981). Briefly, a reaction mixture containing 100 mM Tris pH 7.5, 2.5 mM EDTA, 0.2 mM acetyl Coenzyme A (acetyl-CoA), 0.5 mM oxaloacetate, 0.1 mM 5,5'-dithiobis-nitrobenzoic acid (DTNB) and 30 μl of lysosomal fractions was used. The formation of citrate from acetyl CoA and oxaloacetate is catalyzed by citrate synthase, an enzyme found in mitochondria, with CoA-SH as a byproduct. DTNB reacts with the SH group from CoA-SH and forms 2-nitro-5-thiobenzoate anion (TNB²⁻), a product with intense yellow color that can be quantified spectrophotometrically at 412 nm. Absorption at 412 nm was monitored for at least 2 min allowing detection of a possible mitochondrial contamination. As negative controls, fractions without lysosomes were utilized. Pre-homogenization fractions were employed as positive control for both peroxisomes and mitochondria. Due to the particular density gradient used for the lysosomal isolation, and the addition of CaCl₂, there was no significant contamination by other organelles. Marker enzymes for both mitochondria and
peroxisomes demonstrated less than 0.3 % activity when compared to the pre-
homogenization lysate that served as positive control (Appendix figures A-2, A-3, A-4 and
A-5).

**Acidification assay**

I investigated the ability of the lysosomes to acidify in the range of temperatures
usually experienced by hibernating ground squirrels (0, 5, 10, 15, 20, 25, 30 and 37°C).
Protein concentrations for each lysate were determined by a modified Lowry method and
then adjusted to 100 μg/μl (Peterson 1977). Lysosomes were added to the previously
temperature-adjusted solution: 80 mM HEPES, pH 7.4, 10 mM KCl, 5 mM MgCl₂, and an
ATP regenerating system (5 mM ATP, 45 mM creatine phosphate, 0.1 mM inorganic
pyrophosphate and creatine phosphokinase [8 ng]). The lysates were incubated at specific
temperatures for 1 h. Acridine orange [10 μM] was added after incubation, and fluorescence
was measured at excitation wavelength 493 nm, emission wavelength 520 nm. Acridine
orange is a metachromatic weak base and has been utilized before as an acidification
detector in isolated lysosomes, endocytotic granules, synaptic vesicles as well as whole cells
(Bowman et al. 1988; Gluck et al. 1992; Tabb et al. 1992; Hartinger and Jahn 1993;
Dell’Antone et al. 1995). In its unprotonated form, acridine orange is freely permeable
through membranes and is fluorescent. Once it becomes protonated it will concentraate in the
acidic compartment where it will dimerize and became non-fluorescent (Zoccarato et al.
1999). As negative controls, samples without lysosomes were utilized. Two inhibitors were
also included: NaF [10 mM] and bafilomycin C1 [6.4 μM]. NaF is a general inhibitor of all
ATPases (Murphy and Coll 1992), while bafilomycin C1 is a specific inhibitor of the V-
ATPase that has no effect on other ATPases (Bowman et al. 1988).
Cathepsin activity

Cathepsin activities were measured both as a function of pH (5.4; 6.0; 6.6; 7.2; 7.4;) and as a function of temperatures experienced during torpor (0, 5, 10, 15, 20, 25, 30 and 37°C). Each cathepsin hydrolyzes specific peptide sequences. Synthetic peptides with an attached fluorophore were used to measure cathepsin activity. Upon hydrolysis, the fluorescent compound (AMC, pNA) is released and is measurable at its specific wavelength (general activity, Z-FR-AMC, excitation 380, emission 460 nm; cathepsin B activity, Z-RR-AMC, excitation 365, emission 440 nm; cathepsin H activity, Arg-pNA emission 410 nm; cathepsins S and L activity, Z-VVR-AMC excitation 355, emission 450 nm; and cathepsins D and E Mca-peptide excitation 328, emission 393 nm). The assay conditions to estimate activities of the cathepsins were as follows: in a 100 μl reaction there were 1 mg/ml of lysosomal protein, 0.2 mM Tris-maleate buffer at specific pH with 5 mM MgCl₂, 10 mM KCl, 20 mM DTT, 20 mM EDTA. Tris-maleate buffer was chosen mainly because of its physiological pH range for our assays (pH 5.2-8.0), as well as its sensitivity to the temperature. pH was measured both before and after the addition of the lysosomes to allow for monitoring of the possible temperature effect on the pH change of the lysates. The substrates and inhibitors used in this study are listed in Table 4-1.

Before incubation, 0.1% Triton X-100 [0.625 M] was added to the lysosomes. The lysates were incubated for at least 1h at each experimental temperature and pH value. Parallel samples were supplemented with the specific inhibitors mentioned before (NaF and bafilomycin C1). Two negative controls were employed: samples with previously boiled lysosomes (boiling for 5 min at 100° C) and samples that had 10 μl of concentrated HCl [0.1 M] added before the incubation (high concentrations of HCl were used to precipitate
<table>
<thead>
<tr>
<th>Cathepsins</th>
<th>Substrates</th>
<th>Inhibitors</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>General</td>
<td>Z-FR-AMC</td>
<td></td>
<td>25 μM</td>
</tr>
<tr>
<td>B</td>
<td>Z-RR-AMC</td>
<td></td>
<td>25 μM</td>
</tr>
<tr>
<td>S &amp; L</td>
<td>Z-VVR-AMC</td>
<td>NapSul-Ile-Trp-CHO (L)</td>
<td>25 μM; 25 μM</td>
</tr>
<tr>
<td>H</td>
<td>Arg-pNA</td>
<td></td>
<td>25 μM</td>
</tr>
<tr>
<td>D &amp; E</td>
<td>Mca-Peptide</td>
<td>Pepstatin A</td>
<td>25 μM; 25 μM</td>
</tr>
</tbody>
</table>

Table 4-1
List of specific substrates and inhibitors for measuring cathepsins activities
proteins). The reaction was stopped by the addition of HCl. The reaction mixtures were immediately centrifuged at 10,000g for 10 min at 4°C. Sample fluorescence was measured as indicated previously. Calibration curves were determined by allowing the lysosomal mixture to incubate at optimal temperature and pH and testing activity at different time points (5 min, 10 min, 15 min, 30 min, 60, 120 min). The linearity was never below \( r^2 = 0.98 \) (Appendix figures A-6 and A-7).

Results

Lysosomal acidification was absent at the low temperatures experienced by the hibernating ground squirrels (Fig 4-1). The ability to acidify drops to negligible levels below 20°C, and is consistent with previous reports using rat lysosomes (Roederer et al. 1987). Lysosomal acidification did not differ between ground squirrels in different states of torpor, and it did not differ between squirrels and rats (Fig 4-1).

The ability of the lysosomes to acidify is likely due to the activity of the V-ATPase, as this ability plummets in the presence of bafilomycin C1 (Fig 4-1). The integrity of the lysosomes is preserved during torpor in dormice (Malatesta et al. 2001; Malatesta et al. 2002). Likely, the integrity of the lysosomes is also maintained during torpor in golden-mantled ground squirrels. It is possible that the inactivity of the V-ATPase alone leads to decrease of acidification. As a consequence of the low temperatures, lysosomal acidification was expected to decrease gradually. However, in Fig 4-1, there is an abrupt reduction in acidification below 20°C. This suggests other mechanisms beside the passive effect of low temperatures.
Figure 4-1.
Acidification. Hepatic lysosome-enriched lysates from Torpid (T), Interbout Aroused (IBA) and Summer Active (SA) squirrels and rats (Rat) were incubated in the presence of acridine orange, an indicator used to estimate the pH (493/520 nm). Incubation temperatures were 0, 5, 10, 15, 20, 25, 30 and 37°C. Filled circles (•) represent torpid squirrels, filled squares (■) represent interbout aroused squirrels, empty circles (○) represent summer active squirrels and empty squares (□) represent rats. Filled triangles (▲) represent lysates that were supplemented with NaF [10 mM], a potent general inhibitor of all ATPase pumps. Empty triangles (△) represent lysates that were supplemented with bafilomycin C1 [6.4 μM], a potent specific inhibitor for vacuolar ATPases. Crosses (+) represent negative control lysates that were boiled before the incubation. Values represent 2 animals of each state (T, SA, IBA) and rat and 3 samples of each animal. Data were shifted along the x-axis to allow for better clarity.
The cellular pH of hibernators can vary from 7.24 during euthermia to 7.44 during torpor (Kreienbuhl et al. 1976; Malan 1982). Hibernators experience severe reduction in breathing and long periods of apnea during entrance into torpor (Milsom et al. 1999; Zimmer and Milsom 2001). Reduction in oxygen consumption before the temperature decrease and the possible increase of CO$_2$ production during torpor in the blood, could lead to a decrease in cellular pH likely due to the respiratory acidosis. However, compensating mechanisms possibly prevent fluctuations of the intracellular pH, due to the presence of substances with high buffering capacity such as protein imidazole buffers (Hochachka and Somero 1984). Other possibilities could involve Na$^+$/H$^+$ pumps or Ca$^{2+}$ pumps that probably compensate for the increase of H$^+$ ions in the cell.

It is likely that the hydrolases in the lysosomes would be affected not only by the low temperatures but also by the possible increase of the pH, considering that the intracellular pH measured in torpor was 7.44 (Kreienbuhl et al. 1976; Malan 1982). Cathepsin activity was investigated both as a function of pH and as a function of temperature. General cathepsin activity was examined over a range of pH values at 37°C and at 5°C (Fig 4-2A). Not surprisingly, the activity was highest at low pH and at 37°C (Fig 4-2A). At 5°C, the activity was reduced when compared to the activity at 37°C. The general activities were also assayed as a function of temperature at two different pH values (Fig 4-2B). In general, these results demonstrate that general cathepsin activity is reduced at the low temperatures and at increased pH (Fig 4-2).

However, the general cathepsin activity cannot tell which particular cathepsins are more or less active at a range of temperatures and pH values. I investigated cathepsins B, D, E, H, L and S in this study, mainly because of their abundance and their activities at neutral
Figure 4-2.
General cathepsin activity as indicated by the release of AMC, was measured both as function of pH and temperature. Hepatic lysates from T (○), IBA (■) and SA (○) squirrels and rats (□) were incubated in the presence of Z-FR-AMC, a substrate used to estimate generalized cathepsin activity with fluorescence measurable at 380/460 nm. The black triangles (▲) represent lysosomes that were boiled for 5 min at 100°C before incubation, to serve as negative controls. Incubation pH values were: 5.4, 6.0, 6.6, 7.0, 7.4. Incubation temperature was 0, 5, 10, 15, 20, 25, 30 and 37°C. The general cathepsin activity was estimated as a function of pH at A) 37°C and B) 5°C. The general cathepsin activity estimated as a function of temperature at C) low pH (5.2-5.7) and D) high pH (7.1-7.7). Values represent means ± SE, 2 animals of each state and rat and 3 lysates from each animal. Data were shifted along the x-axis to allow for better clarity.
pH values (Turk et al 2001). In particular, previous work demonstrated that cathepsin B is more active at higher and more neutral pH values (Moin et al 1992; Turk et al 1994; Sloane et al. 1990). I compared the activity of cathepsin B as a function of pH at 37°C and at 5°C (Fig 4-3A). In this study, cathepsin B remained active at pH values as high as 7.4 (Fig.4-3). The activity of cathepsin B is affected by low temperature and increased pH (Fig. 4-3B). However, cathepsin B activity is preserved to at least 40% of its activity when measured under optimal conditions, such as low pH and high temperature, in this in vitro study (Fig 4-3).

Cathepsin D is considered the primary lysosomal aspartic cathepsin, and it is widely distributed in almost all mammalian cells (Tang and Wong 1987; Metcalf and Fusek 1993; Diment et al. 1988). Cathepsin D is of particular importance because it is the primary activator of almost all enzymes inside the lysosome (Conner and Richo 1992; Saftig et al. 1995). Cathepsin D is responsible for transforming the proenzymes into active enzymes by hydrolysis. In this study, the activities of cathepsin D and E were severely depressed at a pH above 7 (Fig 4-4). Cathepsin D and E were primarily insensitive to low temperature effects at low pH; however little activity was measured at pH values above 7 (Fig 4-4). The substrate used for these experiments was recognized only by cathepsin D and cathepsin E (Yasuda et al. 1999). It should be mentioned that cathepsin E is considered as a non-lysosomal protease, since it is primarily present in the early and late endosomes but not always in lysosomes (Fowler et al. 1995).

Cathepsin H is a lysosomal cysteine peptidase of the papain family together with cathepsins B, S and L. In this study, cathepsin H was not affected by the temperature (Fig 4-5A). Cathepsin H activity is still present at pH values around 7, yet its activity drops
Figure 4-3.
Cathepsin B activity as a function of pH and temperature. Experimental details were as in Figure 4-2 except that Z-RR-AMC was used as a substrate. Cathepsin B activity was estimated as a function of pH at A) 37°C and B) 5°C. The activity of cathepsin B was estimated as a function of temperature at C) Low pH (5.2-5.7) and D) High pH (7.1-7.7). Data were shifted along the x-axis to allow for better clarity.
Figure 4-4.
Cathepsins' D and E activities as a function of pH and temperature. The activities of cathepsins D and E were estimated as a function of both pH and temperature. Experimental details were identical as in Figure 4-2 except that 7-methoxycoumarin-4-yl-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys (dinitrophenyl)-D-Arg-NH₂ (McA peptide) was used as a substrate. Pepstatin A (∨), an inhibitor of both cathepsin D and E was also used. Low pH (5.2-5.7), high pH (7.1-7.7). Data were shifted along the x-axis to allow for better clarity.
Figure 4-5.
Activity of cathepsin H as a function of pH and temperature
The activity of cathepsin H was estimated in the presence of Arg-pNA substrate. Experimental details were exactly the same as in Figure 4-2. Low pH (5.2-5.7), high pH (7.1-7.7). Data were shifted along the x-axis to allow for better clarity.
A tremendously once the pH values exceed 7 (Fig 4-5A). The highest activity of cathepsin H was at low pH values and at temperatures around 20-25 °C (Fig 4-5B). Both cathepsins S and L belong to the family of papain cysteine proteases and are involved in intracellular protein degradation, and they shared the same fluorescent substrate (Bohley and Seglen 1992; Kirschke and Wiederanders 1994). Application of NapSul-Ile-Trp-CHO, a specific inhibitor of cathepsin L but not of cathepsin S made it easier to distinguish between the activities of the two cathepsins (Yasuma et al. 1998; Fig 4-6). It has been demonstrated that cathepsin L is quite unstable at neutral pH values (Turk et al. 1999). Cathepsin S has been described to be more stable at neutral pH than cathepsin L (Kirschke et al. 1989). My results were in agreement with Kirschke et al., demonstrating that cathepsin S is relatively unaffected by pH and still exhibits activity near neutral pH (Fig 4-6A). In the presence of a specific inhibitor of cathepsin L, the activity of cathepsin S is more noticeable (Fig 4-6A). Low temperature affects the activities of both cathepsins. No significant activity of cathepsin S was measured at either low or high pH at temperatures below 10°C (Fig. 4-6B). Cathepsin L was more active at low pH and high temperature, yet no activity was measured at low temperatures (Fig 4-7).

Discussion

The ability to acidify the lysosomes dropped abruptly below 20° C (Fig 4-1). It appears that the acidification drop is common both for the non-hibernating rat and the hibernating squirrels, suggesting that the temperature effect alone could not be responsible for reducing acidification and that there might be a possible involvement of active suppression mechanism in the hibernators. The application of the two inhibitors, NaF as a
Figure 4-6
The activity of cathepsin S as a function of pH and temperature.
The activity of cathepsin S was estimated in the presence of a specific substrate Z-VVR-AMC both as a function of pH and temperature. Experimental details were exactly the same as in Figure 4-2 except for the involvement of NapSul-Ile-Trp-CHO, a specific inhibitor of cathepsin L (∇). Data were shifted along the x-axis to allow for better clarity.
Figure 4-7
The activity of cathepsin L as a function of pH and temperature.
Activity of cathepsin L was estimated in the presence of a specific substrate Z-VVR-AMC, as the difference between total cathepsin (S+L) activity, minus activity in the presence of a specific inhibitor of cathepsin L, NapSul-Ile-Trp-CHO. Experimental details were exactly the same as in Figure 4-2. Data were shifted along the x-axis to allow for better clarity.
general inhibitor of all ATPase pumps and bafilomycin C1 as a specific inhibitor of V-ATP pumps, suggests that the acidification of the lysosomes could be entirely due to the employment of the V-ATPase pumps. In the absence of acidification, lysosomes could eventually lose H+ ions and reach pH of the cytosol. The cytosolic pH of hibernators is around pH 7.44 (Kreienbuhl et al. 1976; Malan 1982). The activities of the lysosomal cathepsins were investigated at a range of temperature and at a range of pH values experienced by the hibernators. Activities of lysosomal cathepsins were reduced, although not totally diminished, at low temperatures and increased pH. Some cathepsins’ activities were reduced as a result of low temperature (cathepsins S and L; Fig 4-6), while others seem to be not affected by it (cathepsin D and H; Fig 4-4 and 4-5). In addition, increased pH values affect some cathepsins (cathepsins D and H; Fig 4-4 and 4-5) more than they affect others (cathepsin B; Fig 4-3).

Inactive cathepsin B has been connected with decreased apoptotic injury caused by ischemia/reperfusion injuries in mice (Ben-Ari et al. 2005). Earlier investigations suggest that hibernators could experience ischemia/reperfusion injuries during entrance into torpor (Zimmer and Milsom 2001; Zimmer and Milsom 2002). It is likely that other factors, such as stefins, might be involved in controlling the activity of cathepsin B during torpor. Interestingly, stefins are effective, specific, intracellular inhibitors of cathepsin B concentrated outside the lysosomes (Turk et al. 1994; Turk et al. 2002).

Cathepsin H is upregulated transcriptionally during winter in hibernating golden-mantled ground squirrels (Epperson et al. 2002). In addition, other studies have connected increase of cathepsin H activity with the increase in surfactant protein B in lungs of neonatal mice, thereby ensuring their survival (Ueno et al. 2004). The highest activity for cathepsin
H measured in this study was at 20-25°C at low pH. Considering that the hibernators experience extended periods of apnea and reduced frequency of breathing during torpor (Milsom et al. 1999, Zimmer and Milsom 2001), it is tempting to speculate that cathepsin H might be involved in protecting hibernators with a similar mechanism as it protects the neonatal mice. However, additional research is necessary, especially for the activity of cathepsin H in lungs during torpor.

In addition, cathepsin S occurs in higher activities in lymph nodes and spleens and is thought to be primarily involved with the inflammatory system and active macrophages (Kirschke et al. 1997). It is interesting to note that deficiency of cathepsin S may protect from hypoxia-induced lung injury in neonates (Hirakawa et al. 2007). Again, it is tempting to speculate that the same mechanism could be involved in hibernators as in neonates. Further research investigating the activity of cathepsin S in spleen or in lungs might elucidate more aspects and clarify the role of cathepsin S in other tissues.

The combined effects of low temperature and increased pH had strong effects on the majority of the cathepsins by severely reducing their activities. However, if the low temperature effect is the only one considered during torpor, then there might be other factors that could prevent the activity of the cathepsins. One possible factor is the availability of protein substrates. Previous research had demonstrated that low temperatures inhibit transport between ER and Golgi apparatus in mammalian systems (Saraste and Kuismanen 1984; Saraste et al. 1986; Tartakoff 1986). In addition, the Golgi apparatus and ER seem to disappear during torpor and reappear during arousal in dormice (Malatesta et al 2002; Malatesta et al 2001). However, the integrity of the lysosomes seems to be preserved during torpor (Malatesta et al. 2002). It is possible that the overall transport toward the lysosomes
can actually cease during torpor. Thus, if there are no available substrates during torpor, there could be no lysosomal protein degradation. Other factors could involve the possibility of specific cathepsin inhibitors on a cellular level, such as stefins, as suggested previously (Turk et al. 1994).

In summary, during torpor, there is negligible protein synthesis (Zhegunov et al. 1988; van Breukelen and Martin 2002). In the presence of insignificant protein synthesis, continued protein degradation could deplete important protein pools and endanger survival of the hibernators. Thus, protein degradation needs to be reduced during torpor. Of the two major proteolytic systems present in the mammalian cell, ubiquitin-mediated proteolysis is severely depressed during torpor (Velickovska et al. 2005). Here, we demonstrate that lysosomal degradation is also reduced during torpor. Temperature affects the acidification of the lysosomes and acidification plummets below 20°C. The strongly reduced acidification is probably due to the inactivity of the V-ATPase below 20°C. I also investigated the activities of several lysosomal cathepsins as a function of both temperature and pH.

It is interesting to note that some cathepsin activities were sensitive to low temperatures, while others appeared not to be affected by it. In addition, some cathepsins were strongly inhibited, while others were only slightly inhibited at high pH values. Figure 4-8 summarizes the effects of hibernation on lysosomal activity. My data support the view that hibernators are employing the passive effects of temperature to reduce activities, such as protein degradation, that might be harmful if continued during torpor. It should be mentioned that rats and active squirrels are not different in any of these assays. This suggests that the mechanism for lysosomal protein degradation employed by the hibernators
Figure 4-8
Effects of hibernation on lysosomal proteolytic degradation. This model represents a comparison between active (SA or IBA) and passive (torpid) golden-man-tled ground squirrels and summarizes the data presented here.
might not be different from the mechanism employed in other euthermic mammals such as rats and that elucidation of the general mechanisms employed by hibernators could provide more important information about the mechanisms involved in other non-hibernating mammals such as rat or humans. However, my results do not contradict possible involvement of active suppressors. Hibernators could serve as excellent model in revealing the mechanisms of protein degradation and in particular, lysosomal protein degradation.
References


Barnes BM (1989) Freeze avoidance in a mammal: body temperature below 0°C in an Arctic hibernator. Science 244:1593–1595


Bergmann M, Schutt F, Holz FG, Kopitz J (2004) Inhibition of the ATP-driven proton pump in RPE lysosomes by the major lipofuscin fluorophore A2-E may contribute to the pathogenesis of age-related macular degeneration. FASEB J 18: 562-564


86
protein degradation demonstrated in the mammalian cell cycle mutant ts85. Cell 
37:57-66

Conner GE, Richo G (1992) Isolation and characterization of a stable activation 
intermediate of the lysosomal aspartyl protease cathepsin D. Biochemistry 31: 1142-
1147

Anatech, 2nd Ed.


phygostigmine accumulates in acidic compartments of the cell. Biochim Biophys 
Acta 1270: 137-141

macrophage endosomes. J Biol Chem. 263: 6901-6907

Epperson LE, Martin SL. (2002) Quantitative assessment of ground squirrel RNA levels in 
multiple stages of hibernation. Physiol Genomics 10: 93-102

Letters 366: 72-74

Gluck S, Kelly S, Al-Awqati Q (1992) The proton translocating ATPase responsible for 


Willey and Sons Ltd: Chicheste http://www.els.net

Hartinger J, Jahn R (193) An anion binding site that regulates the glutamate transporter of 

Hicke L, Dunn R (2003) Regulation of membrane protein transport by ubiquitin and 

Hirakawa H, Pierce RA, Bingol-Karakoc G, Karamslan C, Weng M, Shi GP, Saad A, 
deficiency confers protection from neonatal hyperoxia-induced lung injury. Am J 
Respir Crit care Med 176: 778-785

87


88


van Breukelen F, Martin SL (2001) Translational initiation is uncoupled from elongation at 18 C during mammalian hibernation. Am J Physiol Regul Integr Comp Physiol 281:R1374-R1379


Yasuda Y, Kageyama T, Akamine A, Shibata M, Kominami E, Uchiyama Y, Yamamoto 90


Zimmer MB, Milsom WK (2001) Effects of changing ambient temperature on metabolic, heart, and ventilation rates during steady state hibernation in golden – mantled ground squirrels (Spermophilus lateralis). Physiol Biochem Zool74(5); 714-723

CHAPTER 5

GENERAL DISCUSSION

As a response to unfavorable conditions, such as cold and/or food deficit, golden-mantled ground squirrels employ hibernation. Hibernation is not static. It oscillates between two states: the torpid state, when the body temperature is reduced to the ambient temperature to as low as -3°C and metabolic rate drops to as low as 1% of the active rate; and the interbout aroused state, when both body temperature and metabolic rates return to euthermic levels between the torpor bouts. As a consequence of the greatly reduced metabolic rate, protein synthesis, as an important process, is reduced to 0.13-0.5% of the active rates (Zhegunov et al. 1988, van Breukelen and Martin 2001). An average half-life of a protein is 20-40 hours (Rolfe and Schmidt 1984). If protein degradation were not reduced during hibernation, the squirrels' protein pool would be depleted to 20% in just 45-70 hours. Depletion of important protein pools would lead to atrophy and death. Yet, torpor bouts can last up to 3 weeks. In order to preserve the homeostatic balance, protein synthesis and protein degradation need to be in balance. Thus, protein degradation has to be depressed during torpor. Unexpected results were revealed in a previous study where ubiquitin conjugates actually increase 2-3 fold during torpor (van Breukelen and Carey 2001) suggesting an increase in protein degradation (Munro and Pellman 1984). To elucidate more aspects of this conundrum, I investigated protein degradation per se using a newly developed method in chapter 2. My results clearly indicated that protein degradation is
depressed as a result of the low temperatures experienced during torpor. I did not observe any evidence for active suppression of the activity of the 20S protease during the cold temperatures typical of torpor. No significant difference was detected between the animals from different states (Chapter 2). In addition, I found no evidence of active suppression, since there was no difference in proteolytic capacity between the different states of the torpor bout in squirrels and rats (Figure 2-1). Considering that there are from several hundred thousand to several million peptides (Schrader and Schulz-Knappe 2001), I also investigated if all major proteolytic activities of the 20S protease are equally depressed at low temperatures. As described in chapter 2, all proteolytic activities of the 20S protease were equally depressed at cold temperatures (Figures 2-2; 2-3; 2-4; 2-5). Moreover, the hibernator does not seem to be unique in depressing proteolytic activities at low temperature, since rats exhibit the same phenomenon.

Although work in chapter 2 revealed that there is no protein degradation at the low temperatures experienced during torpor, the 2-3 fold increase of ubiquitin conjugates (van Breukelen and Carey 2001) was still unexplained. It seemed logical to expect that the 2-3 fold increase of ubiquitin conjugates might simply be due to accumulation effects. Thus, in chapter 3, I developed a method to measure the process of ubiquitylation per se. I observed that ubiquitylation continues at 30% of its maximum rate even at 0° C (Figure 3-1). So, it seems that ubiquitin conjugates increase as a result of accumulation. They are the result of absent protein degradation and continued ubiquitylation. The increase of ubiquitin conjugates during torpor could represent a serious problem for the hibernator once protein degradation is restored at euthermic temperatures. One can speculate that because of the increase of protein degradation during arousal, an increase in protein synthesis might be
necessary during the interbout arousals when the animal returns to euthermicity. Indeed, there is a 30% increase in protein synthesis during interbout arousal when compared to the summer active ground squirrels (Zhegunov et al. 1988). Hibernators seem to control proteolysis at the level of the 26S proteasome, but ubiquitylation seems to be more difficult to control. There are differences in ubiquitylation rates between rats and ground squirrels (Figure 3-1). Although based on comparison of only two species, and in need of more research, these differences suggest that there might be a tendency for hibernators to lower their ubiquitylation rates during low temperatures.

One possible mechanism to further reduce the possible negative effect of the accumulated ubiquitin conjugates might be involvement of deubiquitylating enzymes (DUB) that can break the bond between the ubiquitin and the target proteins. This could prevent possible excessive degradation of proteins during arousal. Our attempts to detect the activity of DUB in crude lysates during different states of torpor were unsuccessful (Appendix, Fig. A-1). Perhaps due to the strong presence of cathepsin activity, even in the presence of potent cathepsin inhibitors, DUB activity was undetectable (personal communication with Dr. Keith Wilkinson).

Another possibility to reduce the potential damaging effects of the accumulated ubiquitin conjugates could be the quality of ubiquitylation. Ubiquitin molecules can be tagged to target proteins in different ways. Certainly, there might be many intermediate steps of regulation that determine if a protein is going to be monoubiquitylated or polyubiquitylated. If most proteins during torpor are monoubiquitylated, then they will not be degraded once the animal rewarms back to euthermic temperatures. Previous researchers have connected monoubiquitylated proteins with being involved in regulatory processes in
the cell, and with stable proteins during stressful conditions such as ischemia and oxidative stress (Bergink et al. 1989; Hicke 2001; Lee et al. 2005). I speculate that rather than being polyubiquitylated and degraded, perhaps proteins are monoubiquitylated during torpor. However, the data in chapter 3 (Table 3-1) revealed that there is no preference for monoubiquitylation over polyubiquitylation between the animals in different states of torpor.

It was interesting to note that with a 30% continuance of the ubiquitylation at the lowest temperature, there is still only a 2-3 fold increase of ubiquitin conjugates during torpor. One would expect a much greater increase in ubiquitin conjugates, if ubiquitylation were to continue unchecked during torpor. In fact, if ubiquitylation continued at 50% of maximum throughout the torpor bout, free ubiquitin would be depleted while ubiquitin conjugates would increase dramatically. However, this is not the case. One possibility could be substrate limitation. Ubiquitylation may simply stop if no more substrates (e.g. free ubiquitin) are left in the cell. Thus, I examined the levels of free ubiquitin. Surprisingly, the levels of free ubiquitin comprise 92-97% of the total ubiquitin pool in the cell (Figure 3-2). In addition, the 2-3 fold increase in ubiquitin conjugates represents only a 5-7% change in the total ubiquitin pool (Table 3-2). Therefore, free ubiquitin is not the limiting factor for ubiquitylation.

Another limiting factor, to be considered in future research, might be depleted ATP pools. Previous researchers have demonstrated a 2-fold reduction of ATP during torpor. Various metabolites can be present as a result of ATP hydrolysis (Pilwein et al. 1987; van Bilsen et al. 1989). In addition, earlier research has revealed that active ATP hydrolysis leads to possible accumulation of an unidentified metabolite that is responsible for stopping both ubiquitylation and proteasomal degradation (van Breukelen and Hand
2000). It is possible that similar mechanisms might be employed by hibernators to further reduce ubiquitylation.

Another possibility for reduced ubiquitylation during torpor might be the behavior of the activating enzyme of ubiquitin, E1. The tagging of protein with ubiquitin is a rather controlled process, not random under normal circumstances. Previous researchers have demonstrated that even when E1 is totally absent, ubiquitylation can still occur, although in a more non-specific, random manner (Tongaonkar et al. 1999). There are reasons to believe that E1 might not function well in cold temperatures (personal communication with Dr. Art Haas). I suspect that E1 in ground squirrels might behave the same way. Once temperatures start to decrease, there might be a spurious tagging of proteins with ubiquitin. The outcome would be carelessly tagged proteins that are ready for degradation, but not supposed to be degraded. It is possible that, although ubiquitylation appears not be efficiently controlled by the hibernators, there might be a tendency to lower it.

In chapter 4, I investigated lysosomal function at conditions usually experienced by the hibernators. Two major aspects can be considered to examine the activity of the lysosomes at cold temperatures experienced by the hibernators. One is to investigate the ability of the lysosomes to acidify. Lysosomes maintain their acidity due to the activity of the vacuolar ATPase (V-ATPase), which employs ATP to pump hydrogen ions into the lysosome (van Dyke 1993). The other aspect is the ability of the cathepsins, hydrolytic proteases found in the lysosomes, to function at conditions experienced during hibernation.

Ability to acidify plummets as the temperature drops below 20 °C (Figure 4-1) and it appeared that there is no difference between a non-hibernator (rat) and hibernators.
(squirrels), suggesting a possible common mechanism to reduce acidification. The application of two potent inhibitors (NaF, a general inhibitor of all ATPases in the mammalian cell, and bafilomycin C1, a specific inhibitor of only V-ATPase) make it clear that due to the inactivity of the V-ATPase, acidification of the lysosomes declines below 20°C. This result is in agreement with previous studies in rat lysosomes that have found absence of acidification at low temperatures (Roederer et al. 1987; Ahlberg et al. 1985). In the absence of acidification, it is possible that lysosomes could reach cytoplasmic pH values. In the case of the hibernators, their cytoplasmic pH reaches 7.44 during torpor (Kreienbuhl et al. 1976; Malan 1982).

The activities of the different cathepsins investigated here varied under conditions experienced by the hibernators. Several cathepsins were chosen for investigation here, based on their abundance and for some, their ability to function at high pH. The general cathepsin activity was measured and it was reduced at the combined effect of high pH and low temperature (Figure 4-2). However, it was unclear what cathepsins in particular could still be active at high pH and low temperature. Cathepsin B activity was examined in these studies. Previous research demonstrated that cathepsin B is active at high pH values (Moin et al. 1992; Turk et al. 1994; Sloane et al. 1990). Indeed, cathepsin B retained at least 40% of its activity at the highest pH and lowest temperature (Figure 4-3). However, inactive cathepsin B was demonstrated to prevent ischemia induced injuries by reducing apoptosis (Ben-Ari et al. 2005). It seems that hibernators could benefit from non-functional cathepsin B during torpor. Considering that hibernators probably do experience ischemia during entrance into torpor, when their heart rate drops from 200-300 beats/minute to 2-3 beats/minute, before their body temperature drops (Milsom et al. 1999; Zimmer and Milsom...
one would expect to observe reduced activity of cathepsin B.

Activities of cathepsins D and E were remarkably decreased by pH values above 7.2. Although it seemed that temperature had major effect, cathepsin D and E activities were severely reduced at pH above 7 (Figure 4-4). Cathepsin D is considered the primary cathepsin since it activates almost all proteases in the lysosome (Conner and Richo 1992; Saftig et al. 1995). Activity of cathepsin H was still present at low pH, and unaffected by temperature (Figure 4-5). The highest activity of cathepsin H was measured at temperatures around 20 °C at low pH (Figure 4-5). Interestingly, cathepsin H is upregulated at the transcription level during winter in hibernating golden mantled ground squirrel (Epperson et al. 2002). It is tempting to speculate that increased activity of cathepsin H might serve as protectant from lung injury, since it activates a protein that reduces injury when the lungs are not active in neonates (Ueno et al. 2004). However, it will be more appropriate to look specifically for the activity of cathepsin H in lungs to determine more precisely its involvement during torpor.

Activity of cathepsin S was generally affected by the low temperature (Figure 4-6). Cathepsin S was more active at neutral pH, yet it was reduced by the low temperature (Figure 4-6). I was able to distinguish between cathepsin S and cathepsin L activity by the employment of a specific inhibitor for cathepsin L, NapSul-Ile-Trp-CHO (Yasuma et al. 1988). Cathepsin L was generally more active at lower pH and reduced at low temperature and high pH (Figure 4-7). L and S were generally decreased both by lower temperature and increased pH.

In general, the majority of the cathepsins' activities were reduced either as a result of low temperature, and/or by increased pH. The activity of cathepsin B was
somewhat lowered by temperature, yet the combined effect of low temperature and high pH value reduced the activity to only 40% of the maximum activity. Previous research indicated that reduced activity of cathepsin B might give more protection during stressful conditions, such as those experienced during hibernation. As a result, there might be other ways to reduce activity of cathepsin B. It is interesting to note that the integrity of the lysosomes is consistent during torpor (Malatesta et al. 2001; Malatesta et al. 2002). In addition, low temperatures inhibit vacuolar transport between Golgi and ER (Saraste and Kuismanen 1984; Saraste et al. 1986; Tartakoff 1986). Thus, the transport between Golgi and lysosomes could cease at the cold temperatures experienced by hibernators. It is likely that, even if cathepsin B remains active, if there is no access to proteins, no degradation can occur. In addition, the existence of intracellular cathepsin inhibitors, stefins (Turk et al. 1994), just outside the lysosomes, indicates that additional control of lysosomal cathepsins might be necessary to more precisely control activity of the cathepsins in general.

In summary, the data presented here indicate that overall protein degradation is reduced at conditions experienced during torpor. The 26S proteasome activity is severely reduced during torpor. Acidification of lysosomes is also remarkably decreased during torpor. Activities of most of the cathepsins are reduced either as a consequence of lower temperature or of increased pH values during torpor. The unsynchronized effects utilized during torpor between protein degradation and continued ubiquitylation, and between lysosomal acidification and remaining activity of cathepsin B, might have unwanted consequences for the hibernator that need to be resolved upon arousal. Indeed, the 30% increase in protein synthesis during interbout arousal implies that there might have been loss of proteins during arousal and that this needs to be adjusted before the animal returns to
torpor. Hibernators are utilizing the effects of low temperature, although not perfectly, to lower their metabolism. However, the consequences of this imperfection need to be resolved before the animal reenters torpor to ensure survival. It would be interesting to determine if one of the functions of interbout arousals might be to correct the consequences from the sustained proteolytic activities during arousal.
References


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Zimmer MB, Milsom WK (2001) Effects of changing ambient temperature on metabolic, heart, and ventilation rates during steady state hibernation in golden – mantled ground squirrels (Spermophilus lateralis). Physiol Biochem Zool74(5); 714-723
Figure A-1
Measuring the activity of the Deubiquitylating enzymes (DUB) in crude liver lysates. UCHL3 is a deubiquitylating enzyme and it was added in the lysates as a control. No activity was registered in the assay. A probable cause could be the activity of other enzymes (cathepsins) that might degrade the DUB before they can be activated.
Figure A-2
Latency, Citrate Synthase activity and Catalase activity of fractions collected from torpid squirrels
(A) The integrity (latency) of the lysosomes was calculated by measuring their activity in the presence and in the absence of Triton X-100 (explained in the methods in chapter 4). (B) Citrate synthase activity was measured at 412 nm to allow for detection of mitochondria. (C) Catalase activity was determined at 340 nm to allow for detection of peroxisomes. The fractions tested are represented with the number in order from the lightest to the heaviest. The negative control is represented by 0, where lysosomes were boiled for 5 min at 100 °C. PC represents pre-homogenization samples to allow for better overview of the enrichment of lysosomes and seducement of mitochondria and peroxisomes. Values represent 3 samples from one animal.
Figure A-3
Latency, Citrate Synthase activity and Catalase activity of fractions collected from squirrels in between torpor bouts when euthermic.
Experimental details were exactly as in figure A-2. Values represent 3 samples from one animal.
Figure A-4
Latency, Citrate Synthase activity and Catalase activity of fractions collected from summer active squirrels Experimental details were exactly as in figure A-4. Values represent 3 samples from one animal
Figure A-5
Latency, citrate synthase activity and catalase activity of fractions collected from rats as an example of non-hibernator.
Experimental details were exactly as in figure A-4. Values represent 3 samples from one animal.
The linearity for each fluorescent peptide was investigated at 37°C and pH 5.4 at their specific excitation and emission wavelengths. Samples were measured at indicated time points. Experimental details were as follows: Torpid animals (T •), Interbout aroused animals (IBA ■), Summer Active (SAO) and Rat (□). Values represent 3 samples from each animal.
Figure A-7
Linearity for fluorescent peptide for cathepsin H activity.
Experimental details are same as in Figure A-2 except that the linearity was measured at 410 nm, specific for Arg-pNA, the fluorescent peptide for cathepsin H activity.
VITA

Graduate College
University of Nevada, Las Vegas
Vanja Velickovska

Local Address:
1116 Healing Waters Lane
North Las Vegas, NV, 89031

Degrees:
Bachelor of Science, Genetics, 2000
Goteborgs University

Master of Science, Cell Biology, 2002
Goteborgs University

Publications:
Velickovska Vanja, Lloyd Bryan P, Qureshi Safter, van Breukelen Frank. 2005:
Proteolysis is depressed during torpor in hibernators at the level of the 20S core
protease; Journal of Comparative Physiology B; V 175 329-335

Utz Jenifer, Velickovska Vanja, Shmereva Anastasia, van Breukelen Frank. 2007:
Temporal and temperature effect on the maximum rate of rewarming from
hibernation; Journal of Thermal Biology 2007 Jul; 32(5); 276-281

Velickovska Vanja and van Breukelen Frank. 2007: Ubiquitylation of proteins in
livers of hibernating golden-mantled ground squirrels, Spermophilus lateralis;
Cryobiology; 55(3); 230-5. Epub 2007 Aug 24

Van Breukelen Frank, Pan Peipei, Rausch Candice M, Utz Jenifer and
Velickovska Vanja. 2007: Homeostasis on hold: implication of imprecise
coordination of protein metabolism during mammalian hibernation; In press

Velickovska Vanja. 2008: Effects of hibernation on lysosomal acidification and
cathepsin activity in golden-mantled ground squirrels, Spermophilus lateralis; In
review

Professional presentations:
Van Breukelen Frank and Velickovska Vanja. Physiological mismatches in protein
degradation and their implications for coordinated metabolic suppression during
mammalian hibernation. Federation of Allied Societies for Experimental Biology;
San Francisco, Ca Abstract: The FASEB Journal, 20(4) Late Breaking Abstract
Supplement: LB107. 2006
Velickovska Vanja, Lloyd Bryan, Qureshi Safter and van Breukelen Frank. Proteolysis is depressed during torpor in hibernators at the level of the 26S proteasome. Federation of Allied Societies for Experimental Biology; San Diego, CA Abstract: the FASEB Journal, 19(4): A675. 2005

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Dissertation Examination Committee:
Chairperson: Dr. Allen Gibbs, Ph.D.
Committee Member: Dr. Andrew Andres, Ph.D.
Committee Member: Dr. Jeffery Quingxi Shen, Ph.D.
Graduate Faculty Representative: Dr. Ronald Gary, Ph.D.