Available online at www.sciencedirect.com
ScienceDirect
Archives of Biochemistry and Biophysics 468 (2007) 234–243
ABB
www.elsevier.com/locate/yabbi

# The effect of hibernation on protein phosphatases from ground squirrel organs

Justin A. MacDonald [a,*], Kenneth B. Storey [b]

[a] Department of Biochemistry and Molecular Biology, University of Calgary, 3330 Hospital Drive NW, Calgary, Alta., Canada T2N 4N1
[b] Institute of Biochemistry and Department of Biology, Carleton University, 1125 Colonel By Drive, Ottawa, Ont., Canada K1S 5B6

Received 7 August 2007, and in revised form 1 October 2007
Available online 13 October 2007

**Abstract**

Protein phosphorylation has been identified as a reversible mechanism for the regulated suppression of metabolism and thermogenesis during mammalian hibernation. The effects of hibernation on the activity of serine/threonine and tyrosine protein phosphatases (PP1, PP2A, PP2C and PTPs) were assessed in five organs of Richardson's ground squirrel. Each phosphatase subfamily responded differently during torpor, and each showed organ-specific patterns of activity changes. The distribution of PP1 catalytic subunit (PP1c) isoforms ($\alpha$, $\delta$, $\gamma 1$) was assessed in five organs, and changes in the subcellular distribution of PP1 were observed during hibernation in liver and muscle. For example, in muscle, cytosolic PP1 content increased and myofibril-associated PP1 decreased during torpor. PP1c from ground squirrel liver was purified to homogeneity and characterized; temperature effects on PP1c maximal activity suggested that temperature had little or no effect on relative dephosphorylation potential at low temperatures. However, nucleotide inhibition of PP1c by ATP, ADP and AMP was much weaker at 5 °C compared with 37 °C assay temperatures. PP2A activity decreased in three organs (brown adipose, kidney, brain) during hibernation whereas PP2C activity was increased in liver and brain. PTPs were assessed using both a general substrate (ENDpYINASL) and a substrate (DADEpYLIPQQG) specific for PTPs containing the SH2-binding site; both revealed hibernation-associated changes in PTP activities. Changes in protein phosphatase activities suggest the relative importance of these modules in controlling metabolic function and cellular processes during mammalian hibernation.


*Keywords:* Mammalian hibernation; Torpor; *Spermophilus richardsonii*; Phosphorylation; Protein phosphorylation; Protein phosphatase; PP1; PP2A; PP2C

Winter hibernation allows small mammals to minimize metabolic energy costs at a time when a scarcity of food and cold environmental temperatures endanger normal life. By hibernating, animals can reduce their energy requirements by at least 90% and survive for many months while slowly catabolizing body lipid reserves [1]. While hibernating, metabolic rate drops to low levels, frequently to as little as 1–2% of the normal resting rate of euthermic animals, and core body temperature falls to near ambient (often to 0–5 °C) [2]. Research in recent years has demonstrated that metabolic rate depression during hibernation is an active, controlled process that includes the coordinated suppression of metabolism [3], the reorganization of various cellular functions [4–6], and the induction of selected genes and processes to provide long term stability in the dormant state [7,8]. One of the biochemical regulatory mechanisms that figures prominently in metabolic suppression during hibernation is reversible protein phosphorylation [9,10]. The reversible phosphorylation of enzymes of carbohydrate and lipid catabolism has been well studied in hibernation [11–13] and provides a means for the rapid change in metabolic flux that underlies both the transition into torpor and the high rates of thermogenesis that drive re-warming of the body during arousal. Protein phosphorylation is dynamic and is modulated by the action of protein kinases and protein phosphatases. In recent studies, we have studied protein kinases in mammalian hibernation [14–18].

* Corresponding author. Fax: +1 613 520 4389.
*E-mail address:* kenneth_storey@carleton.ca (K.B. Storey).

0003-9861/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2007.10.005

Organ-specific changes in kinase activities and protein kinase functional adaptation to the temperature range (i.e., 2–37 °C) that characterizes euthermia and torpor were investigated. However, comprehensive studies on protein phosphatases from mammalian hibernators have yet to be conducted.

Protein phosphatases play key roles in regulating diverse physiological events such as cell division, metabolism, motility, transcription and translation. Protein tyrosine phosphatases (PTPs)[1] catalyze the dephosphorylation of proteins on tyrosine residues. The PTPs are broadly classified as both membrane receptor and non-receptor PTP families. The protein serine/threonine phosphatases are classified into three broad categories, namely type-1 phosphatase (PP1), several type-2 phosphatases (including PP2A, PP2B, and PP2C), and additional PP2A-like phosphatases (such as PP4, PP5, PP6, and PP7) [19]. Several holoenzyme forms of PP1 are known. These contain the PP1 catalytic subunit (PP1c) associated with different regulatory subunits: glycogen-bound PP1 consists of PP1c plus a glycogen binding subunit in muscle ($PP1G_M$) [20] and $PP1G_L$ in liver [21], myosin-associated PP1 consists of PP1c bound to a myosin targeting subunit [22], and nuclear PP1 consists of PP1c associated with NIPP-1 (nuclear inhibitor of PP1) [23]. The role of each regulatory subunit type is to target PP1c to a specific subcellular location and to modulate PP1c activity toward a selective pool of substrates. For example, glycogen-associated PP1 is a key regulator of glycogen metabolism, being the major phosphatase that dephosphorylates the enzymes of glycogen degradation (i.e., glycogen phosphorylase) and synthesis (i.e., glycogen synthase) [20].

Members of the type-2 phosphatases are subdivided on the basis of their metal ion requirements: spontaneously active (PP2A), $Ca^{2+}$/calmodulin-dependent (PP2B), and $Mg^{2+}$-dependent (PP2C) [19]. Research has revealed prominent roles for PP2A in cell cycle regulation, translation initiation, apoptosis, cell morphology and development [24]. Several holoenzyme forms of PP2A have been characterized with the core enzyme consisting of a 36 kDa catalytic subunit (PP2Ac) complexed with a regulatory A subunit. This core dimer can also be associated with additional B subunits. Unlike the PP1 and PP2A phosphatases, members of the PP2C family are monomeric, lacking regulatory subunits. The PP2Cs contribute to the generalized maintenance of stress-induced protein kinase (SAPK) cascades in an inactive state under non-stressed conditions [25,26]. SAPKs are activated by various extracellular stimuli, including environmental stresses and inflammatory cytokines.

The present study explores the tissue-specific responses of protein phosphatases, PP1, PP2A, PP2C and PTPs, during hibernation in Richardson's ground squirrels in order to determine the role of these key regulators of reversible protein phosphorylation. In addition, PP1c from ground squirrel liver was characterized to address whether its intrinsic properties or its regulation by other factors modulate PP1 activity in a temperature-dependent fashion to affect desirable metabolic changes during hibernation.

## Materials and methods

### Animals and chemicals

Adult Richardson's ground squirrels, *Spermophilus richardsonii*, were obtained in the foothills of the Rocky Mountains near Calgary, Alta. All animals were individually housed at the animal care facility of the University of Calgary, Calgary, Alta. and maintained at 22 °C on a fall (10L:14D) photoperiod. Ground squirrels were induced to hibernate as previously described [17]. Control animals were housed at 22 °C prior to sacrifice. Tissues from both euthermic and hibernating squirrels were immediately excised, immersed in liquid nitrogen, and subsequently stored at −80 °C.

[$\gamma$-$^{32}$P]ATP (3000 Ci/mmol) was obtained from New England Nuclear (Montreal, PQ). Okadaic acid was from Calbiochem (La Jolla, CA) and microcystin–agarose was from Upstate Biotechnology Inc. (Lake Placid, NY). All other chemicals, column materials, and enzymes were purchased either from Sigma or Boehringer Mannheim. Rabbit polyclonal antibodies recognizing the carboxy-terminus of rat $\alpha$, $\delta$, or $\gamma$1 PP1c isozymes were a kind gift of Dr. Emma Villa-Moruzzi (University of Pisa, Italy). Goat anti-rabbit antibody coupled to horseradish peroxidase was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The ECL kit and reflection autoradiography film were from Mandel Scientific (Guelph, ON). $^{32}$P-labeled glycogen phosphorylase a was prepared as described previously [27].

### Assay of "active" and "total" PP1 activity

PP1 activity was assayed with $^{32}$P-labeled glycogen phosphorylase a (GPa) substrate, one unit (U) of activity being defined as the amount of enzyme that catalyzes the release of 1 nmol $^{32}$P-phosphate per min at 23 °C. Unless otherwise indicated, the standard assay mixture contained in a 50 μL volume: 20 mM Tris–HCl, pH 7.0, 0.1 mM EGTA, 10 mM β-mercaptoethanol, 5 mM theophylline, and 2.5 nM okadaic acid. Assays were initiated by the addition of $^{32}$P-labeled GPa and terminated by the addition of 100 μL ice-cold 25% (w/v) trichloroacetic acid/10 mM phosphoric acid. After incubating in an ice bath for 5 min, all samples were centrifuged and then 100 μL of each supernatant was removed and counted in an LKB 1900CA liquid scintillation counter. Two control assays were included and $^{32}$P released under these conditions was subtracted as a blank: (1) zero time control, assays were stopped immediately by the addition of TCA/phosphoric acid solution; and (2) minus enzyme control, buffer replaced the enzyme extract. Both controls gave similar results.

Activities were measured both in concentrated tissue extracts (1:3 w/v homogenization) and after pre-treatment of tissue extracts with trypsin. Measurement of PP1 activity in concentrated tissue extracts provides an approximate measure of "active" phosphatase at physiological concentrations of modulating proteins and other factors [28]. However, whereas PP1c is relatively resistant to trypsinolysis, pre-treatment with trypsin removes regulatory proteins that would otherwise inhibit phosphatase activity and so gives a measure of "total" phosphatase present [29]. In this procedure, frozen tissue samples were homogenized with 2–3 volumes of 50 mM imidazole–HCl, pH 7.4, containing 0.25 M sucrose, 4 mM EDTA and 0.5 mM dithiothreitol. Homogenates were centrifuged at 1000*g* for

[1] *Abbreviations used:* PTPs, protein tyrosine phosphatases; PP1, type-1 phosphatase; PP2A, PP2B, and PP2C, type-2 phosphatases; PP1c, PP1 catalytic subunit; PMSF, phenylmethylsulfonyl fluoride; TPCK, tosylphenylchloroketone; PEG, polyethylene glycol; PVDF, polyvinylidene difluoride; AMPK, AMP-activated protein kinase.