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Regulation of skeletal muscle creatine kinase from a hibernating mammal

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Abstract

Control over skeletal muscle energetics is critical in hibernation to sustain viability over weeks of cold torpor and to support shivering thermogenesis during arousal. Creatine kinase (CK)[1] has a key role in muscle energetics and this study analyzes muscle CK from ground squirrels, *Spermophilus richardsonii*. CK activity was ~20% lower during hibernation than in euthermia, as was CK protein whereas CK mRNA was reduced by ~70%. Hibernator CK showed reduced affinity for ATP and creatine, compared with euthermic CK. Incubations that promoted endogenous protein kinase or phosphatase action, coupled with ion exchange chromatography to separate high and low phosphate forms, showed that soluble CK from euthermic squirrels was a mix of phosphorylated and dephosphorylated forms whereas only phospho-CK was detected in hibernating animals. High and low phosphate CK forms had different affinities for ATP and creatine substrates but did not differ in stability to urea denaturation. About 20–25% of CK was bound to the insoluble fraction of muscle and bound CK showed different kinetic responses to kinase and phosphatase treatments.

Keywords: Torpor; Muscle energy metabolism; Reversible protein phosphorylation; Temperature; Myosin binding; *Spermophilus richardsonii*

Introduction

Creatine kinase (CK; EC 2.7.3.2) catalyzes the transfer of a phosphate group from ATP to creatine (Cr) to produce phosphocreatine (PCr) and *vice versa*. CK is found in tissues with high and/or fluctuating energy demand like muscle, heart, brain, spermatozoa and photoreceptor cells of the retina [1]. For example, the enzyme constitutes ~20% of the total soluble protein of skeletal muscle [2]. For many years it was believed that the main role of CK was maintenance of energy homeostasis at sites of high ATP turnover. In this function, the CK/PCr system acts as a reservoir of high energy phosphate to buffer fluctuations in the intracellular ATP/ADP ratio [3]. Discovery of a mitochondrial CK isoform added the further idea of a Cr/PCr shuttle to move high energy phosphate between sites of ATP synthesis (mitochondria) and ATP consumption (e.g. muscle fibrils) [3].

CK has three organ-specific cytoplasmic isozymes: an MM-CK homodimer in skeletal muscle, a BB-CK homodimer in brain, and a MB-CK heterodimer in heart. In addition, two mitochondrial isozymes exist as octamers: the ubiquitous Mi*u*-CK and the sarcomeric Mi*s*-CK [3]. Changes in the activity of CK can have an effect on available ATP, the energy currency of the cell. Both transcriptional and posttranscriptional mechanisms have been studied as possible mechanisms for the regulation of CK under energy stress. Ponticos et al. [4] showed that the AMP-dependent protein kinase (AMPK) inhibited creatine kinase by protein phosphorylation in vitro and in differentiated muscle cells. However, Ingwall [5] showed that under low pO_2 CK velocity fell by 2–4-fold, whereas the increase in AMPK activity was modest; hence, other mechanisms may also be involved. Chida et al. [6,7] found that inhibition of protein kinase C (PKC) can decrease CK activity.

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[1] *Abbreviations used:* CK, creatine kinase; Cr, creatine; PCr, phosphocreatine; PKC, protein kinase C.

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The goal of the present study was to analyze the possible mechanisms of regulation that could be involved in CK control during the phenomenon of mammalian hibernation.

Hibernation is the winter survival strategy for a variety of small mammals. To survive, animals strongly suppress their energy-consuming physiological functions and enter a state of cold torpor. During hibernation, body temperature (T_b) falls to near 0 °C, heart beat is reduced from its normal 200–300 to just 3–5 beats/min, respiration falls from 100–200 to 4–6 breaths/min, and all other physiological functions are similarly slowed [8]. Metabolic rate is typically reduced to only 1–5% of the corresponding rate in euthermia [9]. Hibernators maintain a viable energy status despite the very low T_b that for most mammals would cause lethal hypothermia.

Hibernation is a highly regulated state. Specific controls are applied to accomplish a wide range of actions including (a) a strong suppression of overall metabolic rate, (b) differential organ responses, (c) differential suppression of individual cellular processes to reorganize cell priorities for energy use, (d) differential gene/protein expression to put in place proteins/enzymes that aid hibernation, and (e) differential regulation of enzymes to resculpt metabolic functions and aid survival at near 0 °C T_b values. Studies in our lab have examined the regulation of hibernation at a variety of levels including hibernation-responsive gene expression [10], the role of reversible protein phosphorylation in overall metabolic rate depression and differential control of enzymes [11,12], and the individual regulation of metabolic enzymes at high and low temperatures [13–16]. Maintenance of viable cellular energy status during torpor is important. Total adenylate levels in organs typically fall by about one-third during torpor but adenylate energy charge is maintained [11,15]. Differential regulation of AMP deaminase and adenylate kinase from prairie dog skeletal muscle has been reported with respect to the high and low T_b values that characterize euthermia versus hibernation [15]. Specific regulation of other enzymes involved in energy metabolism probably also contributes to the control of cellular energetics during torpor.

The present study evaluates skeletal muscle CK from Richardson's ground squirrels, *Spermophilus richardsonii*, assessing the enzyme in euthermic and hibernating states with respect to protein and mRNA levels, activity and kinetic parameters, reversible phosphorylation, and stability against denaturants to determine how the enzyme is regulated when the animal enters torpor.

Materials and methods

Animals

Protocols for care and handling of Richardson's ground squirrels, *S. richardsonii*, were essentially as reported previously [14,16]. Animals were captured in the late summer near Calgary, Alberta. All animals were individually housed in rat cages with free access to food and water at 22 °C and on an autumn (10 h light:14 h dark) photoperiod. After 8 weeks under this regime, half of the animals continued to be maintained under these control (euthermic) conditions. The others were moved into a 4 °C cold room that was maintained in darkness; free access to water was maintained but food was removed. Squirrels were allowed to enter torpor and animals were sampled after 2 days of continuous torpor (rectal temperature of 5–8 °C). Euthermic animals were sampled on the same day. Both hibernating and euthermic animals were killed by decapitation and tissues were immediately excised, frozen in liquid nitrogen and then stored in −80 °C.

Preparation of tissue extracts

Samples of thigh skeletal muscle were homogenized 1:12.5 w:v in 50 mM Tris buffer, pH 8.0 containing 50 mM β-glycerol phosphate, 2 mM EDTA, 2 mM EGTA and 10 mM β-mecapthoethanol with a few crystals of the protease inhibitor, phenylmethylsulfonyl fluoride (PMSF) added just prior to homogenization. Homogenates were centrifuged at 25,000 g for 30 min and then the supernatant was removed and stored on ice. For kinetic studies, samples of supernatant were diluted 20 times with cold homogenization buffer. To study the kinetic properties of CK bound to the particulate fraction, pellets were re-suspended in homogenization buffer, recentrifuged, and the supernatant was removed. This washing procedure was repeated three times to make sure there was no unbound CK left. The final pellet was then resuspended in 2 ml of homogenization buffer and CK properties were assayed using aliquots of the well-mixed suspension. In selected cases, the final pellets were re-suspended in 150 mM KCl in homogenization buffer in order to release bound CK so that maximal activity could be quantified in bound soluble fractions to determine the percentage of total CK that was bound.

Enzyme assay

CK activity (creatine + ATP → phosphocreatine + ADP) was measured at 340 nm using a Multiskan Spectrum microplate reader to measure the rate of ADP production in a coupled assay system with pyruvate kinase (PK) and lactate dehydrogenase (LDH). Optimal assay conditions were determined to be 50 mM Tris buffer, pH 8.4, 5 mM MgATP, 10 mM creatine, 20 mM $MgCl_2$, 100 mM KCl, 4 mM PEP, 0.225 mM NADH, and 1 U/assay each of LDH and PK. Routine assays were run at 25 °C and used 5 μl of diluted supernatant. $S_{0.5}$ values for creatine and MgATP were determined at constant co-substrate concentrations, 5 mM MgATP or 10 mM creatine. All ATP stock solutions were prepared as a 1:1 molar mix with $MgCl_2$.

Incubations to promote protein phosphatase or kinase activities

Samples of supernatant or pellet extracts were incubated for 4 h at 25 °C with inhibitors or stimulators of protein kinases or protein phosphatases as described previously [11]. To assess protein kinase effects, tissue extracts were prepared as above. To stimulate protein kinases, samples of supernatant were incubated in 50 mM Tris buffer pH 8.4 containing 5 mM MgATP, 50 mM β-glycerol phosphate, and 5 mM Na_3VO_4 with the further addition to stimulate specific kinases of 1 mM cAMP for PKA, 1.3 mM $CaCl_2$ and 7 μg/mL phorbol myristate acetate for PKC, 1 mM cGMP for PKG, or 1 mM AMP for AMP-dependent protein kinase. To assess protein phosphatase effects, tissue extracts were prepared in 50 mM Tris–HCl buffer pH 8.0, 10 mM β-mecapthoethanol (with PMSF added just before homogenization). To promote protein phosphatase action, samples were incubated with 5 mM $MgCl_2$ and 5 mM $CaCl_2$ (for total protein phosphatases) with the further addition of specific inhibitors to assess the effects of individual phosphatase types: (a) to assess Ser/Thr phosphatase effects, the incubation also included 5 mM Na_3VO_4 to inhibit tyrosine phosphatases, (b) to assess protein phosphatase type 1 (PP1), the incubation also included 0.5 nM okadaic acid (to inhibit PP2A), 2 mM EDTA and 2 mM EGTA, (c) to assess protein phosphatase type 2B and 2C, the incubation contained 1 μM okadaic acid, 5 mM $CaCl_2$ and 2 mM EDTA for PP2B or 1 μM okadaic acid, 5 mM $MgCl_2$ and 2 mM EGTA for PP2C. To test