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Creatine kinase regulation by reversible phosphorylation in frog muscle

Christopher A. Dieni [a], Kenneth B. Storey [b,*]

[a] Department of Chemistry, The Pennsylvania State University, University Park, PA 16802, United States
[b] Institute of Biochemistry and Department of Chemistry, Carleton University, 1125 Colonel By Drive, Ottawa, Ontario, Canada K1S 5B6

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ABSTRACT

Creatine kinase (CK) was analyzed from skeletal muscle of wood frogs, *Rana sylvatica*, a species that survives natural whole body freezing during the winter months. Muscle CK activity increased by 35% and apparent K_m creatine decreased by 29% when frogs froze. Immunoblotting analysis showed that this activity increase was not due to a change in total CK protein. Frog muscle CK was regulated by reversible protein phosphorylation; *in vitro* incubations with ^{32}P-ATP under conditions that facilitated the actions of various protein kinases (PKA, PKG, PKC, CaMK or AMPK) resulted in immunoprecipitation of ^{32}P-labeled CK. Furthermore, incubations that stimulated CaMK or AMPK altered CK kinetics. Incubation under conditions that facilitated protein phosphatases (PP2B or PP2C) reversed these effects. Phosphorylation of CK increased activity, whereas dephosphorylation decreased activity. Ion-exchange chromatography revealed that two forms of CK with different phosphorylation states were present in muscle; low versus high phosphate forms dominated in muscle of control versus frozen frogs, respectively. However, CK from control versus frozen frogs showed no differences in susceptibility to urea denaturation or sensitivity to limited proteolysis by thermolysin. The increased activity, increased substrate affinity and altered phosphorylation state of CK in skeletal muscle from frozen frogs argues for altered regulation of CK under energy stress in ischemic frozen muscle.

1. 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 ADP, and *vice versa*. As an enzyme responsible for buffering energy reserves, CK is found in tissues with high and/or fluctuating ATP demands such as skeletal muscle, heart, and brain (Wallimann et al., 1992). For example, CK constitutes ~20% of the total soluble protein of skeletal muscle (Lipskaya, 2001). Two cytosolic isozymes of CK are present in organ-specific dimers: an MM-CK homodimer in skeletal muscle, a BB-CK homodimer in brain, and an MB-CK heterodimer in heart (McLeish and Kenyon, 2005). The main role of CK in skeletal or cardiac muscle is to maintain energy homeostasis at sites of high ATP turnover (typically as a result of contraction). In this function, CK uses the phosphagen reservoir, PCr, to buffer fluctuations in the intracellular ATP/ADP ratio (McLeish and Kenyon, 2005). In brain, CK-BB has been implicated in maintaining high levels of ATP for ATP-driven ion homeostasis and neurotransmitter trafficking (Wallimann and Hemmer, 1994). Mitochondrial CK isozymes also exist; these are octomers, with Mi*u*-CK being ubiquitous whereas Mi*s*-CK is present in sarcomeric mitochondria (McLeish and Kenyon, 2005). The discovery of mitochondrial isozymes gave rise to the idea of a phosphotransfer network wherein a Cr/PCr shuttle, involving mitochondrial and cytosolic CK isozymes, moves high energy phosphate between sites of ATP synthesis (mitochondria) and ATP consumption (Dzeja and Terzic, 2003; McLeish and Kenyon, 2005).

Possible mechanisms for the regulation of CK under energy stress have been studied, and protein phosphorylation is known to be a post-translational regulatory mechanism. For example, AMP-dependent protein kinase (AMPK) phosphorylation has variable effects on CK both *in vitro* and in differentiated muscle cells (Ponticos et al., 1998; Ingwall, 2002). Inhibition of protein kinase C (PKC) also decreased CK activity in brain (Chida et al., 1990a,b). Calcium–calmodulin dependent protein kinase (CaMK) phosphorylates CK in some cases but had no apparent effect on activity (Singh et al., 2004). Protein kinase A (PKA), protein kinase G (PKG), PKC and AMPK also phosphorylate skeletal muscle CK in ground squirrels, with PKA, PKG, and PKC all affecting activity (Abnous and Storey, 2007).

Winter survival for several species of terrestrially-hibernating amphibians and reptiles ectothermic relies on freeze tolerance, the ability to endure the conversion of 65–70% of total body water into extracellular ice. To survive, animals strongly suppress their energy-consuming physiological needs and undergo a series of metabolic changes that provide cryoprotection to their cells (Crerar et al., 1988; Layne et al., 1989; Storey et al., 1992; Costanzo et al., 1993; King et al., 1993; Swanson et al., 1996; Storey and Storey, 2004). One consequence of freezing is the interruption of heart beat and breathing that causes anoxia and ischemia, and places an energy stress on organs that includes a drop in ATP levels (Storey, 1987). A mechanism is needed to stabilize cellular energetics and help maintain viability in skeletal

* Corresponding author. Tel.: +1 613 520 3678; fax: +1 613 520 3749.
E-mail address: kenneth_storey@carleton.ca (K.B. Storey).

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muscle during long term freezing. The pool of PCr and appropriate regulation of CK could accomplish this; indeed, consumption of PCr fully sustained the adenylate pool of skeletal muscle in wood frogs (*Rana sylvatica*) frozen for 4 days but organs without significant phosphagen reserves (e.g. liver) showed a large decline in adenylates over the same time (Storey and Storey, 1984). The present study analyzes the mechanisms of CK regulation that could be involved in enzyme control with respect to the energetic needs for freezing survival, using as our model skeletal muscle CK from the freeze-tolerant wood frog.

2. Materials and methods

2.1. Animals and biochemicals

Male wood frogs (*Rana sylvatica*, 5–7 g body mass) were captured from spring breeding ponds in the Ottawa area. Animals were washed in a tetracycline bath, and placed in plastic containers with damp sphagnum moss at 5 °C for 2 weeks prior to experimentation. Control frogs were sampled directly from this condition. For freezing exposure, frogs were placed in closed plastic boxes with damp paper toweling on the bottom, and put in an incubator set at −3 °C. A 45 min cooling period was allowed during which the body temperature of the frogs cools to below −0.5 °C (the equilibrium freezing point of wood frog body fluids) and nucleation is triggered due to skin contact with ice crystals formed on the paper toweling (Storey and Storey, 1985). Subsequently, timing of a 24 h freeze exposure began. Both control and experimental frogs were sacrificed by pithing, followed by rapid dissection, and freezing of tissue samples in liquid nitrogen. Frozen tissues were stored at −80 °C until use. Conditions for animal care, experimentation, and euthanasia were approved by the university animal care committee in accordance with guidelines set down by the Canadian Council on Animal Care. All biochemicals and coupling enzymes were purchased from Sigma Chemical Co. (St. Louis, MO) or Boehringer–Mannheim (Montreal, PQ).

2.2. Preparation of tissue extracts

Frozen muscle samples were mixed 1:10 w:v in ice-cold buffer A (50 mM MOPS, 2 mM EDTA, 2 mM EGTA, 50 mM β-glycerophosphate, 10 mM β-mercaptoethanol, pH 7.0), with a few crystals of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) added immediately prior to homogenization with a Polytron homogenizer at 50% of full power. The concentrations of EDTA/EGTA and β-glycerophosphate have been tested in our *in vitro* assays and are sufficient and appropriate to inhibit endogenous protein kinases and phosphatases under the conditions used in our incubations. The activities of the target enzymes in our studies stay constant during the length of time used in our studies, indicating that the level of PMSF is sufficient to allow for complete enzyme survival. Homogenates were centrifuged in a Hermle Z360K centrifuge for 30 min at 10,000 ×*g* and the supernatant was removed and stored on ice.

2.3. Enzyme assays and determination of kinetic parameters

Enzymes were assayed by continuous monitoring of absorbance at 340 nm in a 200 µl final volume using a Dynatech MR5000 microplate reader. Optimal assay conditions for CK were determined to be 50 mM Tris buffer (pH 8.6), 10 mM Mg^{2+}·ATP (stock solutions contained $MgCl_2$ and ATP in a 1:1 molar ratio), 12.5 mM creatine, 4 mM phosphoenolpyruvate (PEP), 0.225 mM NADH, 2.5 mM $MgCl_2$, 1 U each of pyruvate kinase (PK) and lactate dehydrogenase (LDH), and 10 µl of enzyme extract diluted 20-fold immediately prior to assay. Initial studies optimized the amount of enzyme used, assessed blank activity (minus creatine) which was subtracted if present, and demonstrated that a linear rate of NADH oxidation was maintained over at least 5 min. One unit (U) of CK activity is defined as the amount that consumed 1 µmol of NADH per min at 25 °C. Enzyme kinetic constants were determined using a nonlinear least-squares regression computer program (Brooks, 1992); substrate affinity data were fitted to Michaelis–Menten plots.

2.4. In vitro incubations to stimulate endogenous kinases and phosphatases

To assess the effect of reversible phosphorylation on CK, incubations were prepared under conditions that facilitated the activities of selected endogenous protein kinases or protein phosphatases. Homogenization (1:5 w:v in buffer A) and centrifugation were as described above. The supernatant was removed and then aliquots were mixed with 2 vol of an appropriate incubation buffer. Each buffer contained 50 mM MOPS, pH 7.0, and 10 mM β-mercaptoethanol with the following additions:

(A) *"Stop" incubations:* 50 mM β-glycerophosphate, 2 mM EDTA and 2 mM EGTA to inhibit both protein kinase and protein phosphatase activities.
(B) *Promotion of endogenous protein kinases:* 5 mM Mg·ATP, 50 mM β-glycerophosphate and either (1) 1 mM cAMP to stimulate PKA; (2) 1 mM cGMP to stimulate PKG; (3) 1.3 mM $CaCl_2$+7 µg/mL phorbol myristate acetate to stimulate PKC; (4) 1 mM AMP to stimulate AMPK; (5) 1 U of calmodulin (Sigma P1431)+1.3 mM $CaCl_2$ to stimulate CaMK; or (6) all of these components to stimulate all kinases together.
(C) *Promotion of endogenous protein phosphatases:* (1) 5 mM $MgCl_2$ and 5 mM $CaCl_2$ to promote the activities of all protein phosphatases. Other conditions contain inhibitors of selected protein phosphatases thereby isolating and promoting the activities of the stated protein phosphatases. These included (2) to promote PP1 activity: 2.5 nM okadaic acid, 5 mM Na_3VO_4, 2 mM EDTA and 2 mM EGTA; (3) to promote PP1+PP2A: 30 mM Na_3VO_4, 2 mM EDTA and 2 mM EGTA; (4) to promote PP2B: 5 mM $CaCl_2$, 2 mM EDTA, 1 µM okadaic acid and 5 mM Na_3VO_4; (5) to promote PP2C: 5 mM $MgCl_2$, 2 mM EGTA, 1 µM okadaic acid, 5 mM Na_3VO_4 and 1 nM cypermethrin.

Samples were incubated overnight at 4 °C. Following incubations, all samples were diluted 20-fold in ice-cold buffer A assayed under optimal conditions.

To confirm that the treatments used to stimulate protein kinases actually resulted in the phosphorylation of CK, tissue extracts (1:5 w:v) were prepared in buffer A as above. Extracts were then treated to fully dephosphorylate CK. To do this, extracts were first centrifuged through small columns of Sephadex G-50 (equilibrated in 50 mM MOPS, pH 7.0, 10 mM β-mercaptoethanol) to remove low molecular weight effectors. The eluant was collected and $MgCl_2$ and $CaCl_2$ were added to final concentrations of 5 mM each to facilitate the activities of endogenous protein phosphatases. Samples were incubated at 4 °C overnight and then incubations designed to stimulate specific protein kinases were set up as above but this time with the additional presence of 10 µCi radiolabeled γ-^{32}P-ATP. After overnight incubation, 60 µl of insoluble protein A (IPA) (Sigma, P7155) was added to each sample, incubated overnight, and then samples were centrifuged at 2000 ×*g* and the supernatant was removed; this treatment removed proteins that bind non-specifically to IPA. Samples were then incubated with 1 µg of MM-CK goat polyclonal IgG (Santa Cruz) for one h and then 60 µl IPA was added and incubated overnight to form CK–antibody–IPA complexes. Pellets were collected by centrifugation at 2000 ×*g* following by washing 7 times with 100 µl 50 mM Tris buffer, pH 7.5. Aliquots (5 µl) of the suspension were spotted on P81 paper and exposed to a phosphorscreen. The radioactivity in each spot was quantified using a Personal Molecular Imager-FX (BioRad).