Available online at www.sciencedirect.com
ScienceDirect
Cryobiology 53 (2006) 297–309
CRYOBIOLOGY
www.elsevier.com/locate/ycryo

Stress-induced activation of the AMP-activated protein kinase in the freeze-tolerant frog *Rana sylvatica* ☆

Mark H. Rider [a,*], Nusrat Hussain [a], Sandrine Horman [a], Stephen M. Dilworth [b], Kenneth B. Storey [c]

[a] *Hormone and Metabolic Research Unit, Christian de Duve Institute of Cellular Pathology, University of Louvain Medical School, Avenue Hippocrate, 75, B-1200 Brussels, Belgium*
[b] *Tumour Cell Biology, Department of Metabolic Medicine, Imperial College Faculty of Medicine, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK*
[c] *Institute of Biochemistry, Department of Biology, Carleton University, 1125 Colonel By Drive, Ottawa, Ont., Canada K1S 5B6*

Received 1 April 2006; accepted 4 August 2006
Available online 14 September 2006

Abstract

Survival in the frozen state depends on biochemical adaptations that deal with multiple stresses on cells including long-term ischaemia and tissue dehydration. We investigated whether the AMP-activated protein kinase (AMPK) could play a regulatory role in the metabolic re-sculpting that occurs during freezing. AMPK activity and the phosphorylation state of translation factors were measured in liver and skeletal muscle of wood frogs (*Rana sylvatica*) subjected to anoxia, dehydration, freezing, and thawing after freezing. AMPK activity was increased 2-fold in livers of frozen frogs compared with the controls whereas in skeletal muscle, AMPK activity increased 2.5-, 4.5- and 3-fold in dehydrated, frozen and frozen/thawed animals, respectively. Immunoblotting with phospho-specific antibodies revealed an increase in the phosphorylation state of eukaryotic elongation factor-2 at the inactivating Thr56 site in livers from frozen frogs and in skeletal muscles of anoxic frogs. No change in phosphorylation state of eukaryotic initiation factor-2α at the inactivating Ser51 site was seen in the tissues under any of the stress conditions. Surprisingly, ribosomal protein S6 phosphorylation was increased 2-fold in livers from frozen frogs and 10-fold in skeletal muscle from frozen/thawed animals. However, no change in translation capacity was detected in cell-free translation assays with skeletal muscle extracts under any of the experimental conditions. The changes in phosphorylation state of translation factors are discussed in relation to the control of protein synthesis and stress-induced AMPK activation.

Keywords: Metabolic rate depression; AMPK; Energy stress; mTOR; eEF2; eEF2K; p70S6K; 4E-BP1; Ribosomal protein S6

☆ The work was supported by the Interuniversity Attraction Poles Program–Belgian Science Policy (P5/05), the Directorate General Higher Education and Scientific Research, French Community of Belgium, the Fund for Medical Scientific Research (Belgium), the EXGENESIS Integrated Project (LSHM-CT-2004-005272) from the European Commission and a Natural Sciences and Engineering Research Council of Canada Grant (#6793).
* Corresponding author. Fax: +32 2 764 7507.
E-mail address: rider@horm.ucl.ac.be (M.H. Rider).

0011-2240/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.cryobiol.2006.08.001

The present study explores the responses of AMPK and multiple downstream targets of its action in liver and muscle of wood frogs in order to determine the role of this central regulator of cellular energy metabolism in mediating metabolic events that aid natural freeze tolerance. In parallel, we analyzed changes in the activity (phosphorylation states) of various translation factors in order to assess global protein synthesis activity. Our analysis includes the effects of freezing and thawing on these parameters as well as the effects of two component stresses of freezing, anoxia and dehydration.

Freezing is one of the most serious environmental stresses faced by living organisms and is lethal for the vast majority. However, a variety of organisms that live in cold environments have perfected mechanisms for freeze tolerance. Freezing survival depends on adaptations that address the multiple stresses caused by freezing, such as potential physical damage to tissues by ice crystals, dehydration and cell volume reduction due to the exit of cellular water into extracellular ice, and long-term anoxia/ischaemia caused by the freezing of blood plasma. Biochemical adaptations that support natural freeze tolerance include the accumulation of high levels of carbohydrate cryoprotectants, the production of ice-nucleating proteins, defenses against anoxia and oxidative stresses, alterations in gene expression, and metabolic rate depression [39,40,43]. Carbohydrate metabolism and energy production are central issues during freezing. All organs must deal with long-term anoxia/ischaemia, relying on endogenous glycogen reserves and anaerobic glycolysis (ending in lactate) to sustain cell viability. Glycogen reserves are also mobilized to synthesize the high concentrations of cryoprotectants that are needed to regulate cell volume and protect macromolecules during the freeze. For example, in wood frogs (*Rana sylvatica*), freezing triggers a massive hyperglycemia that raises glucose in core organs to levels as high as 150–300 mM compared with concentrations around 5 mM in unfrozen controls [38]. This requires several alterations to the normally tight homeostatic control of glucose levels that is typically displayed by vertebrates.

The AMP-activated protein kinase (AMPK) is a highly conserved eukaryotic serine/threonine protein kinase that acts not only as a sensor of cellular energy status but also plays a critical role in systemic energy balance [14,20,21]. AMPK is a heterotrimer consisting of a catalytic α subunit and two regulatory subunits, β and γ. Each subunit has multiple isoforms (α1, α2, β1, β2, γ1, γ2, γ3) giving twelve possible combinations of holoenzyme with different tissue distributions and subcellular localizations. AMPK is activated via phosphorylation by upstream AMPK kinases (AMPKKs) [20,47]. The activating phosphorylation site is Thr172 in the T-loop of the α-subunit and phosphorylation at this site is both sufficient and necessary for AMPK activation. Upstream activating kinases phosphorylating Thr172 have been identified as the Peutz-Jeghers Syndrome protein LKB1 and calcium/calmodulin-dependent protein kinases [20,47]. AMPK is activated by changes in the intracellular AMP:ATP ratio, as occurs under anoxia or other stresses, and the binding of AMP to the γ-subunits is thought to somehow induce a conformational change that allows α-subunit Thr172 phosphorylation by LKB1. Once activated, AMPK inhibits ATP-consuming processes and stimulates ATP-producing pathways [14,21,20]. For example, fatty acid synthesis is inhibited via the AMPK-mediated phosphorylation and inactivation of acetyl-CoA carboxylase (ACC). As a consequence, malonyl-CoA concentrations fall which stimulates fatty acid oxidation, thereby contributing to the maintenance of intracellular ATP levels. In addition, AMPK activation stimulates glycolysis by increasing glucose uptake in skeletal muscle [30] and heart [36] and by activating heart 6-phosphofructo-2-kinase [27].

Protein synthesis and the Na^+/K^+-ATPase are the two most prominent consumers of ATP in mammalian cells [7,46]. Peptide chain elongation consumes at least 4 equivalents of ATP for each peptide bond synthesized and is inhibited via the phosphorylation of eukaryotic elongation factor-2 (eEF2) at Thr56 by a highly specific Ca^{2+} and calmodulin-dependent kinase called eEF2 kinase (eEF2K) [32]. AMPK activation leads to the inhibition of eEF2 by phosphorylating and activating eEF2K [5,16,17]. This promotes inhibition of energy-expensive protein synthesis under low cellular energy conditions. Control of eEF2K is also exerted by a variety of other upstream protein kinases including p70S6K [44]. p70S6K phosphorylates and inactivates eEF2K and the mammalian target of rapamycin (mTOR) lies upstream of p70S6K and has been proposed to act as an ATP sensor in cells [11]. Thus, a reduction in ATP levels and a decrease in mTOR signalling could lead to an increase in eEF2K activity and the subsequent phosphorylation of eEF2. In addition, AMPK activation inhibits p70S6K [3,12,22] possibly via phosphorylation of the tuberous sclerosis complex (TSC1–TSC2) [19]. TSC2 acts as a GAP (GTPase activating protein) for the small GTPase Rheb (Ras-homologue enriched in brain) thereby inactivating