A molecular approach to the concerted action of kinases involved in energy homoeostasis

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Abstract

One of the most important duties of a cell is energy homoeostasis. Several kinases, including AMP-activated protein kinase (AMPK), creatine kinase and adenylate kinase, are involved in the immediate response to stress, resulting in energy depletion. Here, we present our view of events preceding the downstream processes mediated by AMPK and leading to reduced energy expenditure and increased energy production. Unfortunately, AMPK is very poorly defined at the molecular level. Thus a procedure for production of AMPK in milligram amounts is presented which will greatly facilitate the functional and structural characterization of this protein kinase.

Cellular energy homoeostasis: maintenance of energy state by creatine kinase (CK) and adenylate kinase (AK) isoenzymes

A fundamental principle in multicellular organisms is the strict maintenance of stable concentrations of intracellular oxygen [1] and ATP [2], the universal energy currency of biological systems, as well as the tight regulation of energy utilization with energy supply. Upon activation of excitable cells, such as skeletal and cardiac muscle, or brain and nerve cells, ATP turnover rates may increase by several orders of magnitude within seconds, but [ATP] remains remarkably stable and ATP/ADP ratios, as well as ATP/AMP ratios, are maintained as high as possible to guarantee optimal efficiency for cellular ATPases that are at work to perform a multitude of energy-dependent cellular activities, such as muscle contraction, cell motility and ion pumping [3].

ATP homoeostasis and maintenance of high ATP/ADP and ATP/AMP ratios are facilitated by the action of two well-known enzyme systems, working as very fast and efficient energy safeguards. First, CKs, efficiently regenerating ATP at the expense of phosphocreatine (PCr) by the following reaction [4,5]:

$$PCr + ADP \rightarrow ATP + Cr$$

where Cr is creatine, and second, AK, reconverting two ADP molecules into one ATP and one AMP [6]. These two enzymes, working together in an intricate subcellular energy distribution network or circuit [4,7], temporally and, due to their subcellular microcompartmentation, spatially buffer subcellular ATP levels [8–11].

Key words: adenylate kinase, AMP-activated protein kinase, creatine kinase, energy homoeostasis.

Abbreviations used: AMPK, AMP-activated protein kinase; AK, adenylate kinase; CK, creatine kinase; Cr, creatine; PCr, phosphocreatine.

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A common denominator of many diseases, like cardiac insufficiency, cardiac hypertrophy as well as most of the neurodegenerative pathologies, is a generally lowered cellular PCr/ATP ratio, indicating a lowered energy state of cells and tissues. This is often accompanied by elevated calcium levels, leading to chronic calcium overload with its host of negative consequences on cell function and viability [12].

Sensing and signalling of compromised cellular energy states by AMPK

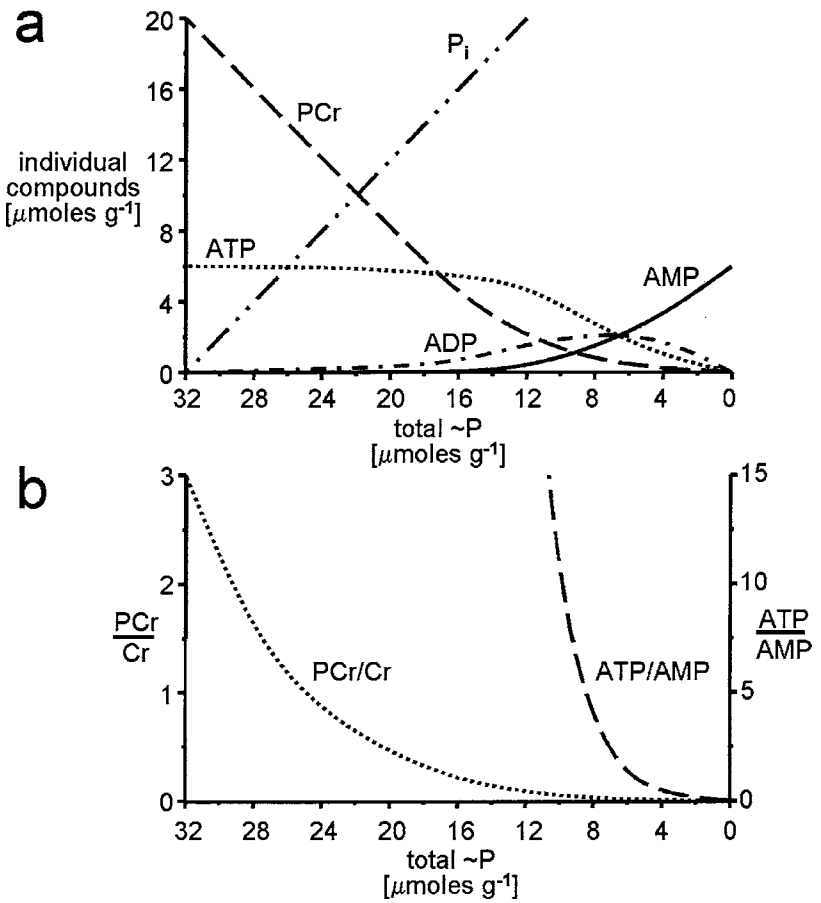
A lowered energy state may be induced experimentally in animal models by blocking Cr uptake into Cr-containing tissues, e.g. by feeding β-guanidinopropionic acid, which competitively inhibits Cr uptake and thus reduces total Cr levels, as well as the PCr/ATP ratio in muscle and brain [13,14]. Alternatively one can create transgenic knock-out animals that no longer express CK isoenzymes [15] or AK [16].

Lowering high-energy phosphates in the cell or eliminating the enzymes directly involved in high-energy phosphate metabolism both result in a compromised energy state, which becomes physiologically relevant at high workloads [13,17]. Interestingly, in skeletal and cardiac muscle, both of these interventions lead to the induction of similar compensatory mechanisms [14,15,18,19] to counteract and compensate for the deficit in cellular energy state. As a common denominator, these phenotypes are characterized by a dramatic upregulation of mitochondrial proliferation [15,19,20], as well as enhanced glucose uptake [13,21,22] that can lead to elevated glycogen accumulation [15]. In the case of β-guanidinopropionic acid administration, the increase in mitochondrial volume is accompanied by significant overexpression of mitochondrial CK [19,23], which can also be observed in patients with a variety of myopathies [24,25].

Now, an important question arises of how cells that are energetically compromised, e.g. by ischaemia, nutritional

Figure 1 | **PCr and adenylate pool sizes during utilization of high-energy phosphates**

(**a**) Concentrations of PCr, adenosine phosphates (ATP, ADP and AMP) and P_i calculated from CK and AK kinetic constants (data featured originally in [60]). (**b**) PCr/Cr and ATP/AMP ratios calculated from values shown in (**a**).



deprivation, oxidative damage, heat shock or chronic calcium overload, are able to (i) sense a lowered energy state, (ii) transmit this signal into activation of compensatory pathways and (iii) re-programme gene transcription for long-term compensation to counteract such low-energy states. In other words, there must be cellular signals relevant to energy stress that are able to induce compensatory reactions or even genetic re-programming of the cells to avoid potentially dangerous energy depletion [20] that eventually may lead to cell death.

In a fast-twitch glycolytic skeletal muscle, which is rich in PCr (up to 30 mM or more, depending on the muscle and the species [4]), cell activation is accomplished first by the use of PCr to keep ATP levels constant (Figure 1). Only after PCr is reduced by 70–80% is ATP concentration lowered, in parallel with the appearance of ADP and, later on, AMP, both globally accumulating in appreciable amounts only at a rather late stage of cellular energy stress (Figure 1a).

Thus the three most important signals, which upon cell activation show up sequentially by their time of manifestation, are (i) lowered PCr/Cr, (ii) lowered ATP/ADP and ATP/AMP ratios and (iii) chronically elevated Ca^{2+} levels, if the energetics of calcium pumps are compromised. As a matter of fact, a lowered cellular energy charge first affects the sarcoplasmic/endoplasmic reticulum Ca^{2+} pumps that are energetically highly demanding and thus depend on a high local ATP/ADP ratio [26]. CK in the vicinity of these Ca^{2+} pumps guarantees efficient regeneration of local ATP levels and a high local ATP/ADP ratio [27], and the fact that CK-null mutant transgenic mice show a clear phenotype with altered calcium homoeostasis and muscle relaxation [15,17,28]. These are convincing indications that the CK system is crucially involved in the energetics of calcium homoeostasis [3]. The manifold effects of elevated calcium levels [29], among others, leading to increased expression of GLUT4 glucose transporter [30,31] and, by activation of the Ca^{2+}/calmodulin-dependent kinase (CaMK) system, to an induction of mitochondrial proliferation [32], are being studied in many research laboratories worldwide.

However, the mechanisms by which cells may respond to lowered PCr/Cr and ATP/AMP ratios have only recently been fully appreciated after it became obvious that the enzyme AMP-activated protein kinase (AMPK) can be activated

not only by a lowered ATP/AMP ratio [33], but by a lowered PCr/Cr ratio [34], i.e., by the appearance of AMP and by increased levels of Cr within the cell (Figure 1a). In terms of the changes in high-energy phosphate levels upon cell activation, the decrease in the PCr/Cr or PCr/ATP ratio represents the first signal seen by cells that are being energetically challenged (Figure 1b). Thus the high-energy-phosphate-utilizing enzymes, CK and AK, are intricately linked to the AMPK system in that the substrates or products of these two enzymes can activate the latter protein kinase. Interestingly enough, activation of AMPK leads to very similar compensatory reactions, as mentioned for the experimental animal models at lowered energy state, e.g. to up-regulation of glucose transport (GLUT4) [35–37], as well as to increased mitochondrial capacity [38]. Therefore, it seems entirely conceivable that some of the same compensatory events seen with Cr depletion or with CK- or AK-null mutant mice are in fact mediated by lowered PCr/Cr and/or ATP/AMP ratios through activation and downstream signalling of AMPK.

The CK/AMPK connection

AMPK is a member of a metabolite-sensing protein kinase family that is found in all eukaryotes [39]. Experimental work over the last decade has highlighted the key role of the AMPK cascade in maintaining high ATP/ADP and ATP/AMP ratios [39,40]. Moreover, PCr also allosterically inhibits AMPK at concentrations that lie within the physiological range [34]. Since PCr in muscle and some other cells acts as a short-term energy reservoir for fast regeneration of ATP [3], this fits very well into the above energy sensor concept. However, the recent discovery that the muscle isoform of CK is phosphorylated and thereby inactivated by approx. 60% by AMPK [34] has unexpectedly added a new aspect to the CK/AMPK connection. This seems to represent more than just substrate monitoring, but rather involves metabolic regulation of CK via the low-energy sensor AMPK.

Many aspects of this new regulatory principle need to be elucidated and some questions about its physiological significance still need to be answered. So far, our understanding of the role of CK was that this enzyme is fully active when immediate energy replenishment is most necessarily needed. One may ask the question of why AMPK should inactivate CK at times when, due to high-energy consumption, ATP is badly needed? This certainly would only make sense in physiological terms at a point when the PCr pools would already be empty; that is, to prevent the backward reaction of CK to consume scarce ATP for resynthesis and replenishment of PCr and the PCr pool, respectively. Based on this assumption, a model summarizing this line of thinking is given in Figure 2.

The scheme in Figure 2 also shows the role of mitochondria in producing PCr. Although, we and others have shown that the PCr/Cr shuttle is an efficient energy-transport system that is in operation preferentially at high workloads [41] (symbolized by a broken arrow in Figure 2), we would certainly expect ATP also to diffuse directly to the cytosol. However, since the outer membrane is a diffusion barrier for nucleotides [42] this pathway may be less effective (symbolized by dotted arrows in Figure 2).

Approaching molecular characterization of AMPK

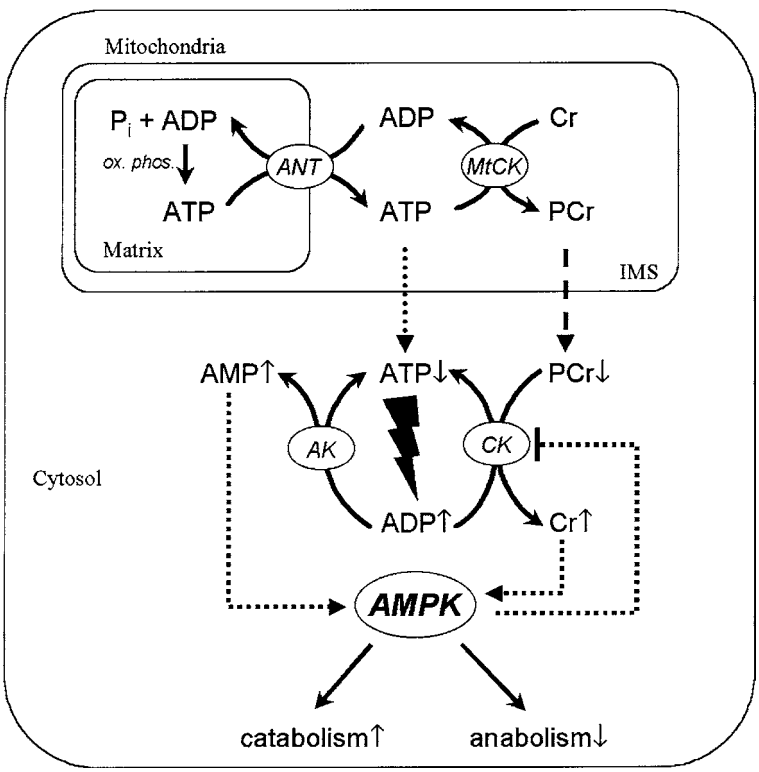
Over the last decades, our laboratory has been involved in elucidating the CK system and investigating the astonishing cell protective effects of Cr supplementation linked with several aspects of metabolic regulation (for recent reviews see [5,43–45]). Hence, our current interest in AMPK is not only based on the direct link between CK and AMPK, but also on the role of AMPK as a master switch for metabolic regulation. A detailed biochemical and biophysical characterization of AMPK would be likely to open new avenues in the field of cellular energy metabolism. Our expertise in molecular characterization of CKs by overexpression of functional protein in *Escherichia coli* prompted us to develop such a heterologous system for AMPK. *E. coli* is the most widely used expression host [46]. It combines numerous advantages, including low cost, ease of use, the possibility of automation and metabolic labelling of the produced proteins with selenomethionine or $^{15}N/^{13}C$ for X-ray crystallography or NMR studies, respectively [47]. In eukaryotic cells, AMPK is expressed in different isoforms (see below) and purification of AMPK from tissues therefore always results in a mixture of isoforms. Homologues of AMPK are also found in fungi and plants, but *E. coli* is not expected to contain any relatives of AMPK, which makes this bacterium an ideal host for heterologous production of the complex.

AMPK is known to exist as a heterotrimeric complex comprising α, β and γ subunits. In mammals, each subunit is encoded by two or three genes giving rise to subunit isoforms ($\alpha1$, $\alpha2$, $\beta1$, $\beta2$, $\gamma1$, $\gamma2$, $\gamma3$) that combine to a maximum of 12 heterotrimeric AMPK isoenzymes [48–50]. All previous attempts to produce large amounts of heterotrimeric AMPK in bacteria have failed and therefore some technical problems need to be addressed. Our first notion was that co-expression of all subunits in a single cell might be necessary to obtain correctly folded AMPK, since co-translational subunit–subunit interaction may assist in proper folding and assembly of the complex enzyme. Co-expression in *E. coli* was the most challenging problem, because there is no commercial expression system available that ensures the overexpression of three different polypeptides at the same time and, ideally, to the same stoichiometric level. However, it is widely known that bacteria transcribe polycistronic messengers from specific genes leading to simultaneous translation of several proteins. We adapted this simple idea to the overexpression of AMPK and constructed tricistronic vectors as shown in Figure 3 (D. Neumann, A. Woods, D. Carling, T. Wallimann and U. Schlattner, unpublished work).

Given the fact that the initial reports on AMPK date back to 1973 [52,53] and that no high-level expression system is

Figure 2 | The Cr–PCr shuttle and its effect on allosteric regulation of AMPK

ATP is generated by oxidative phosphorylation inside mitochondria. This ATP is exported by the adenine nucleotide transporter (ANT) to the intermembrane space (IMS), where it is transphosphorylated on to Cr by mitochondrial CK (MtCK) to give PCr and ADP. ADP is then re-imported via ANT into the matrix and PCr diffuses to the cytosol. Cellular stress leads to increased ATP consumption, but immediate energy demand is buffered through PCr and the action of cytosolic CKs. If PCr is used up, Cr and ADP levels will rise and the latter is interconverted into AMP and ATP by the action of AK. Since AMP and Cr both result in allosteric activation of AMPK, this would inactivate CK and preserve the highly compromised ATP pool until it is replenished by glycolysis and/or oxidative phosphorylation. Subsequently, if ATP replenishment by ATP-synthetic pathways is guaranteed, AMP levels are bound to drop and the inhibition of AMPK on CK should be alleviated, thus facilitating recharging of the PCr pool by the CK reaction.



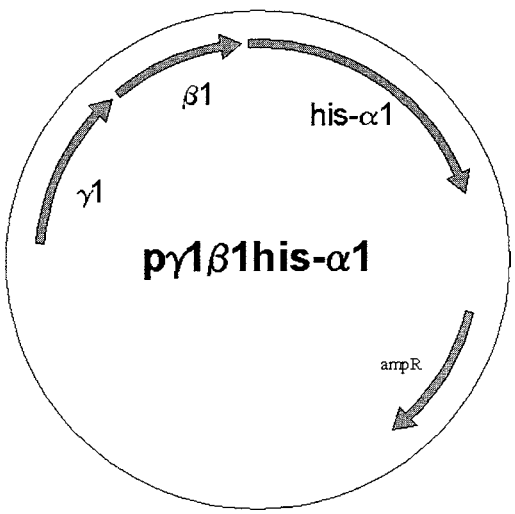
available so far, the enzyme has proved to be notoriously difficult to produce in significant amounts. Our new bacterial expression system, however, is able to produce milligram amounts of functional trimeric AMPK. This may allow us to solve a variety of major questions concerning the molecular structure and function of AMPK.

The polycistronic format of the vector ensures the availability of all subunits in a single cell. The co-expression of individual subunits is obviously sufficient for the formation of heterotrimeric native AMPK complexes. In addition, the heterotrimer also seems to assemble without co-expression of any specialized proteins that would aid in folding or assembly, as well as without post-translational modifications. The upstream kinase (AMPK kinase) plus AMP can activate the protein complex. Thus, N-terminal myristoylation of the β-subunit or N-acetylation of the γ-subunit [54,55] are not essential for activity or stability of the complex *per se*. Moreover, recombinant AMPK from bacteria is entirely unphosphorylated at Thr-172 in the α1 subunit. With a Thr-172 $\rightarrow$ Asp mutant mimicking a phosphorylated residue, we could confirm that such a mutant protein is constitutively enzymically active and provide evidence for additional phosphorylation sites in AMPK still to be identified.

Taking into account that recombinant wild-type AMPK was expressed in bacteria and as such was completely inactive, it seems very likely that all other putative phosphorylation sites are not phosphorylated either. Thus the recombinant protein represents an invaluable tool for the elucidation of both the regulation of AMPK by phosphorylation of specific sites and the autophosphorylation events leading to activation. Further, the characterization of AMPK kinase, which so far has evaded identification and characterization, may be greatly facilitated by the availability of ample and highly purified AMPK protein. Another advantage of the expression system is the availability of AMPK with defined subunit composition. As mentioned above, purification of AMPK from tissue is bound to result in a complex mixture containing various AMPK isoforms [56]. Purification from transfected cell culture with plasmids encoding α, β and γ subunits of AMPK also led to limited purity, depending on

Figure 3 | Expression vector for expression of heterotrimeric AMPK in *E. coli*

The T7 RNA polymerase-driven vector encodes γ, β and hexahistidine-tagged α subunits of AMPK. The transcription results in a single polycistronic messenger ensuring stoichiometric production of all subunits.



the transfection rate and the presence of endogenous AMPK subunits (D. Carling, personal communication). Hence, the production of large quantities of pure complexes consisting only of a single combination of subunit isoforms will eliminate all those uncertainties inherent to AMPK produced from natural sources. Such recombinant AMPK will allow a detailed biochemical and biophysical characterization of AMPK and a thorough comparative study of the different isoforms, including isoenzyme stability, allosteric regulation and kinetic properties. The bacterial expression of proteins in virtually unlimited quantity and superior quality has often also been a prerequisite for structural characterization by X-ray crystallography [57–59]. Our high-level expression system is therefore expected to facilitate crystallization of the proteins and solution of a high resolution X-ray structure of AMPK, which will definitely be needed for rational drug design.

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