Dissecting the Role of 5′-AMP for Allosteric Stimulation, Activation, and Deactivation of AMP-activated Protein Kinase*

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AMP-activated protein kinase (AMPK) is a heterotrimeric protein kinase that is crucial for cellular energy homeostasis of eukaryotic cells and organisms. Here we report on the activation of AMPK α1β1γ1 and α2β2γ1 by their upstream kinases (Ca2+/calmodulin-dependent protein kinase β and LKB1-MO25α-STRADα), the deactivation by protein phosphatase 2Ca, and on the extent of stimulation of AMPK by its allosteric activator AMP, using purified recombinant enzyme preparations. An accurate high pressure liquid chromatography-based method for AMPK activity measurements was established, which allowed for direct quantitation of the unphosphorylated and phosphorylated artificial peptide substrate, as well as the adenine nucleotides. Our results show a 1000-fold activation of AMPK by the combined effects of upstream kinase and saturating concentrations of AMP. The two AMPK isoforms exhibit similar specific activities (6 μmol/min/mg) and do not differ significantly by their responsiveness to AMP. Due to the inherent instability of ATP and ADP, it proved impossible to assay AMPK activity in the absolute absence of AMP. However, the half-maximal stimulatory effect of AMP is reached below 2 μM. AMP does not appear to augment phosphorylation by upstream kinases in the purified in vitro system, but deactivation by dephosphorylation of AMPK α-subunits at Thr-172 by protein phosphatase 2Ca is attenuated by AMP. Furthermore, it is shown that neither purified NAD+ nor NADH alters the activity of AMPK in a concentration range of 0–300 μM, respectively. Finally, evidence is provided that ZMP, a compound formed in 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside-treated cells to activate AMPK in vivo, allosterically activates purified AMPK in vitro, but compared with AMP, maximal activity is not reached. These data shed new light on physiologically important aspects of AMPK regulation.

AMP-activated protein kinase (AMPK)2 and its homologues in insects, plants, and yeast are fuel sensors of the eukaryotic cell and master regulators of energy metabolism (1–3). AMPK is a heterotrimeric serine/threonine protein kinase consisting of α-, β-, and γ-subunits. In mammals, each subunit exists in different isoforms (α1, α2, β1, β2, γ1, γ2, and γ3), which may give rise to 12 different heterotrimeric isoform-subunit combinations. A prerequisite for significant protein kinase activity of AMPK is phosphorylation of the catalytic α-subunit at Thr-172 (4), but additional phosphorylation sites in α- and β-subunits of AMPK have been reported (5). Upstream kinases capable of activating AMPK have been identified recently as LKB1-MO25-STRAD (6–8) and CaMKKβ (9, 10), collectively called AMPK kinases (AMPKKs). AMP allosterically stimulates AMPK activity by binding to the γ-subunit, which carries four CBS domains organized in two pairs called Bateman domains (11). A mutant truncated form of the catalytic α-subunit, α1-(1–312), still requiring phosphorylation of Thr-172 for enzyme activity, is independent of allosteric activation by AMP (12). The β-subunit carries a glycogen-binding domain (13, 14) and tethers the α- and γ-subunits together (15). Besides directly stimulating AMPK activity, AMP inhibits dephosphorylation of AMPK and in addition was reported to promote phosphorylation of AMPK by upstream kinase(s) (16). However, neither CaMKKβ nor LKB1 themselves are directly activated by AMP, and the question of whether binding of AMP to the allosteric site of AMPK renders the enzyme a better substrate for its upstream kinases remains controversial (6, 8–10). The degree of stimulation of AMPK by AMP in vitro was of rather moderate extent (1.5–4-fold) (6, 8–10), a fact that would seem difficult to reconcile with a system demanding immediate and high responsiveness in vivo to the rapidly changing energy requirement of many cell types. In living cells and organisms, AMPK is activated by metabolic stresses, but many of the downstream effects of AMPK were originally demonstrated using the compound 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) (2). AICAR is a nucleoside that is taken up by cells and converted to AICAR-monophosphate (ZMP), an AMP

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2 The abbreviations used are: AMPK, 5′-AMP-activated protein kinase; AMPKK, AMPK kinase; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; CaMKKβ, Ca2+/calmodulin-dependent protein kinase kinase β; Cr, creatine; GST, glutathione S-transferase; LKB1, serine/threonine kinase 11 (STK11); MO25, mouse protein 25; PCr, phospho-creatine; PP2Cα, protein phosphatase-2C α isoform; SAMS, synthetic peptide HMRSAMS-GLHLVKKRR; STRAD, STE20-related adaptor protein; ZMP, AICAR-monophosphate; HPLC, high pressure liquid chromatography.

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an analogue that mimics the effects of AMP on the AMPK system (17).

Despite recent progress in understanding the roles of AMPK in cells, tissues, and in the whole organism, the enzyme characteristics of the AMPK heterotrimer have not been described in much detail. A major reason for this was the lack of sufficient amounts of highly purified AMPK protein available. AMPK has been mainly purified from tissue or cell lysates, but recently we have established an expression system for mammalian AMPK in bacteria, yielding milligram quantities of highly purified AMPK (18), and several groups, including ourselves, have successfully used such recombinant material for a number of different studies (5, 8, 10, 19–23). Importantly, wild type AMPK produced in bacteria is almost entirely inactive but can be highly activated by upstream kinases that are capable of phosphorylating AMPK α-subunits at Thr-172 (18).

AMPK activity is commonly determined by radioactive labeling of artificial peptide substrates, e.g. the so-called SAMS peptide, and spotting of the phosphorylated peptide onto charged membranes, followed by scintillation counting (24). The SAMS peptide is based on the AMPK recognition sequence of acetyl-CoA carboxylase and has been used most frequently for AMPK activity determination, although alternatives like the AMARA peptide (where the AMARA synthetic peptide is AMARAASAAALARRR) have been described (25). GST fusion products of a larger domain of acetyl-CoA carboxylase, comprising the AMPK recognition sequence, have been used to determine the consensus sequence of phosphorylation by AMPK (26), and recently, a pull-down assay using GST-SAMS was developed for AMPK activity determination in crude lysates (27). In this study, we developed a dedicated analytical method and scrutinized the roles of AMP in AMPK regulation.

EXPERIMENTAL PROCEDURES

Recombinant AMPK—Wild type AMPK α1β1γ1, α2β2γ1, and constitutively active mutant AMPK α1T172Dβ1γ1 were bacterially expressed as published previously (18). All AMPK preparations were pre-purified on nickel-nitrilotriacetic acid superflow columns (Qiagen) and further processed to highest purity. Specific kinase activity and purity, as determined from Coomassie Blue-stained gels after separation by SDS-PAGE, served as criteria for enzyme homogeneity (see “Results”). Stock solutions of the enzyme were kept in 50% glycerol at −20 °C.

Activation of AMPK by Upstream Kinases and Activity Assay—AMPK activity was assessed with SAMS-peptide as substrate target and nonradioactive ATP, which was HPLC-purified directly before use or stored frozen until usage (see below). The assay buffer consisted of 40 mM HEPES, pH 7.1, 75 mM NaCl, 2 mM dithiothreitol, 1–10 mM MgCl2. AMP, ATP, SAMS, as well as AMPK (and CaMKKβ or LKB1-MO25α-STRADα for wild type AMPK) were added as indicated in the figure legends. A plasmid encoding the GST fusion construct of CaMKKβ cDNA for bacterial expression was a kind gift from H. Tokumitsu (Kagawa Medical University, Kagawa, Japan), and GST-CaMKKβ was prepared as described previously (28). The tricistronic construct, expression, and purification of the heterotrimeric complex LKB1-MO25α-STRADα was prepared in our laboratory (23). AMPK assays were performed in Eppendorf tubes on a thermal shaker at 30 °C and 300 rpm. Samples were taken at the indicated time points, and the reaction was stopped by freezing the samples in liquid nitrogen, followed by storage at −20 °C until further analysis.

Re-purification of Activated Wild Type AMPK—After activation by either CaMKKβ, the pH of the sample was adjusted to 8.0 by addition of the appropriate amount of phosphate buffer (500 mM sodium phosphate buffer, 250 mM NaCl, pH 9) and loaded onto a 1-ml nickel-Sepharose HiTrap column (GE Healthcare). The column was washed with a minimum of 20 ml of washing buffer (20 mM sodium phosphate buffer, 150 mM NaCl, pH 8.0) containing a low concentration of imidazole (20 mM) and eluted with high imidazole (250 mM imidazole in washing buffer). Samples were analyzed by SDS-PAGE, and selected fractions were stored at −20 °C after addition of glycerol to a final concentration of 50% (w/v). All protein concentrations were determined using protein assay reagent (Bio-Rad) with bovine serum albumin as a standard.

HPLC Analysis—AMP, ADP, ATP, NAD+, and NADH were separated and quantified on an anion exchange column (Nucleosil 4000-7 PEI, 50/4 from Macherey-Nagel, Oelhensingen, Switzerland) with a linear gradient (0–1.5 mM NaCl in 10 mM Tris-Cl, pH 8.0) using an HPLC system equipped with two independent UV-visible spectrometers (Shimadzu, Reinach, Switzerland). Elution of samples was monitored at 259 and 220 nm. The latter wavelength was used to trace possible contaminants. Commercially available ATP (519979; Roche Diagnostics) always contained traces of AMP (and ADP), even immediately after dissolving from newly opened fresh containers. Therefore, ATP used throughout this study was HPLC-purified by the above method and routinely checked for its AMP content, which was always below 0.1% after HPLC processing. SAMS and SAMS-phosphate were separated and quantified on a cation exchange column (Source 15S, 1 ml, from GE Healthcare) with a linear gradient (0–1.5 mM NaCl in 20 mM sodium phosphate, pH 7.5) at 220 nm.

SDS-PAGE and Western Blotting—Protein samples were diluted in Laemmli buffer, snap-frozen in liquid nitrogen at indicated time points, and subjected to SDS-PAGE (12% polyacrylamide). After transfer to nitrocellulose membranes (Millipore, Merlyn, Switzerland), proteins were immunoblotted using either anti-AMPKα, phospho-specific anti-AMPKα Thr-172 (Cell Signaling Technology, BioConcept, Allschwil, Switzerland, catalogue numbers 2532, 2535, respectively), or anti-AMPKβ primary antibodies (a kind gift of Dr. David Carling, MRC, Hammersmith Hospital, London, UK) and goat-anti rabbit peroxidase conjugated secondary antibodies (catalogue number DC03L; Calbiochem). Signals were detected with enhanced chemiluminescence (Applichem, Axxon Lab, Baden-Dättwil, Switzerland) and Kodak x-ray-sensitive films (GE Healthcare). Western blots were quantified by densitometry on a Kodak Imagestation 440CF using Kodak Digital Science Software version 2.0.4 (PerkinElmer Life Sciences).

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RESULTS

The standard method for AMPK activity determination depends on an artificial peptide substrate, called SAMS that is incubated with AMPK in presence of \([\gamma-^{32}P]\text{ATP (ATP + SAMS }\rightarrow\text{ ADP + SAMS-P)}\). The amount of radioactively labeled \([^{32}P]\text{SAMS-phosphate is determined by scintillation counting, as the only accessible parameter at the end of the reaction. AMPK, however, interacts with all adenine nucleotides. AMP is an allosteric stimulator; ADP is a product of the kinase reaction, and ATP is a substrate. Additionally, high concentrations of ADP and AMP likely inhibit binding of ATP to the kinase domain by a competitive mechanism. High ATP is also believed to inhibit stimulation of AMPK by AMP by competitive binding to the allosteric site (17).}

We therefore considered accurate determination of the concentrations of all reaction partners, namely adenine nucleotides, SAMS, and SAMS-phosphate.

_HPLC Detection Enhances Reliability of the AMPK Activity Assay_—Separation of adenine nucleotides is commonly performed with anion exchange chromatography. The SAMS-peptide with a theoretical pI of 12.3 was found to interact strongly with cation exchange material at pH 7.5, and the introduction of negative charges through phosphorylation reduces its binding to the negatively charged column matrix. Thus, a salt gradient allowed for separation of SAMS from SAMS-phosphate. Samples were taken from an AMPK activity assay, and two sequential runs on the different HPLC columns were analyzed. Fig. 1A shows a representative overlay of two HPLC runs showing separation of adenine nucleotides, as well as SAMS and SAMS-phosphate. The applicability of the analytical procedure for activity determination of AMPK is demonstrated in Fig. 1B. Samples of an activity assay were taken at different time points, divided, snap-frozen, and stored for later injection. The data show the reciprocal decrease or increase of the ATP/ADP and SAMS-phosphate/SAMS ratios, respectively. AMP was added at constant concentrations to stimulate AMPK activity. A comparatively greater decrease in [ATP] compared with [SAMS] was observed consistently with different samples of AMPK after a longer incubation of samples. This might be due to “energy waste” by an inherent ATPase activity of AMPK rather than to unspecified decay of ATP or ADP over time, as it appears to be associated with substrate turnover. AMPK in the absence of SAMS did not degrade ATP (data not shown). As shown in Fig. 1B, the formation of ADP and SAMS-phosphate perfectly correlates within the first 10 min of the assay. As AMPK assays were generally stopped and analyzed after an incubation time of 5–10 min, [SAMS-phosphate] and [ADP] equally reflect AMPK activity. Furthermore, the concentrations of all educts and products in a single sample are interrelated and can be determined individually, thereby increasing the accuracy of the method. We generally used SAMS ([SAMS] + [SAMS-phosphate] = constant) and adenine nucleotides ([ADP] + [ATP] = constant) to evaluate the data, but in most cases determination of [ADP] or [SAMS] was found satisfactory. Thus, a single data point is sufficiently exact and can be used for calculation of specific activity.

_specific activity of fully activated recombinant AMPK_—Highly purified heterotrimeric wild type AMPK α1β1γ1 and α2β2γ1 was activated with recombinant preparations of either of the two known upstream kinases of AMPK, CaMKKβ (GST-tagged) or the LKB1-MO25-α-STRADα heterotrimer was used at saturating concentrations. To reach full activation with CaMKKβ, a 1:1 ratio (w/w) of upstream kinase to AMPK had to be used, whereas with the heterotrimeric LKB1-MO25α-STRADα complex a ratio of 1:50 was sufficient to reach full activity. The specific activity of AMPK after activation and full stimulation by AMP (Fig. 2A) was in the range of 6 μmol of SAMS-phosphate/mg of wild type AMPK/min at 30 °C. This value was similar for both isoforms of AMPK and much higher than reported previously with recombinant AMPK, _e.g._ 0.3 μmol/min using recombinant AMPK activated by partially purified upstream kinase preparation from liver containing LKB1 (5) or 0.2 μmol/min activated by recombinant CaMKKβ (10). Native AMPK α1 complexes have been purified from tissue to near homogeneity and exhibited similar high specific activities of 8.2 μmol/min using radioactive detection method and SAMS as a substrate (29). To compare

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**FIGURE 1. HPLC analysis employed for in vitro AMPK activity assays.** A separation of adenine nucleotides (solid line; 259 nm) and SAMS/SAMS-phosphate (dashed line; 214 nm) was achieved by anion and cation exchange chromatography, respectively (for conditions see “Experimental Procedures”). Single HPLC runs were completed within 10 min. The signals for ADP and SAMS-phosphate (SAMS-P) correspond to ~10 nmol. B, time course of AMPK activity assay and quantitation of all reaction partners. Samples from an AMPK activity assay were taken at the indicated time points, split into halves, and injected into the HPLC system equipped with either an anion or cation exchange column. Each compound was quantified from the resulting chromatogram using appropriate standards. Results are means values (n = 3, ± S.D.).
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FIGURE 2. Activation and re-purification of recombinant wild type AMPK. Wild type α1β1γ1 and α2β2γ1 AMPK (25 μg/ml) were activated in the presence of 2 mM ATP and 1 mM AMP at 30 °C for 1 h with CaMKKβ (25 μg/ml) or LKB1-MO25α-STRADβ (0.5 μg/ml). Activated AMPK was then diluted in assay buffer to 5 μg/ml (200 μM ATP, 100 μM AMP), followed by the addition of 500 μM SAMS. Immobilized metal affinity chromatography was exploited for re-purification of AMPK activated by upstream kinases (see “Experimental Procedures” for details). A, specific activities of different AMPK samples before and after the activation or re-purification step, as determined by HPLC quantification of SAMS phosphate. B, Coomassie Blue-stained gel of the same samples separated by SDS-PAGE (500 ng/lane). C, Western blot of the same samples utilizing AMPK-β-specific antibodies for detection.

FIGURE 3. Time course of activation of wild type α2β2γ1 by CaMKKβ. A, specific activities of AMPK taken at different time points of incubation with CaMKKβ (as described in the legend of Fig. 2). B, analysis of the samples by Western blotting utilizing AMPK-β-specific antibodies. C, ratio between specific activities of samples taken at different time points of incubation with CaMKKβ with or without addition of AMP to the SAMS assay. AMPK (25 μg/ml) was incubated with HPLC-purified ATP (1 mM) and CaMKKβ (25 μg/ml). At indicated time points, a sample of the activation mixture was assayed for 5 min (200 μM ATP, 500 μM SAMS) with or without addition of 20 μM AMP. Activities were determined by the HPLC method.

The HPLC-based method with the most widely used radioactive detection method of AMPK activity determination, we purchased a partially purified preparation of rat liver AMPK (Upstate, Lucerna-Chem, Lucerne, Switzerland) with a declared specific activity (0.8 μmol/mg/min at 30 °C, as determined by SAMS assay, using the radioactive method), and determined its activity by our HPLC method. Under conditions identical to the company's kinase assay protocol (100 μM ATP, 100 μM AMP, and 100 μM SAMS), we measured 0.2 μmol/mg/min or using optimized assay conditions (200 μM ATP, 50 μM AMP, and 500 μM SAMS) reached a value of 0.4 μmol/mg/min, suggesting that the HPLC-based analytical method is, if anything, underestimating the specific activities by a factor of 2–4 in comparison to the commonly used radioactive assay procedure. This would indicate that the specific activity of highly purified recombinant AMPK is even higher, although no attempts were undertaken to determine activity using traditional approaches. Furthermore, the re-purification step of fully activated AMPK resulted in no significant loss of specific activity (Fig. 2A), suggesting that once AMPK has been activated by upstream kinases these high specific activities can be preserved in vitro in the absence of AMPKKs.

Autophosphorylation MayReduce AMP Sensitivity of AMPK—The same AMPK samples as shown in Fig. 2A were also analyzed by SDS-PAGE (Fig. 2B). Upon activation by AMPKK, shifts of electrophoretic mobility were observed in α-subunits as a consequence of phosphorylation of AMPK at Thr-172 by AMPKKs, as has been observed previously (10). The immunostaining of the β-subunit in Western blots also revealed changes after activation by AMPKK (Fig. 2C), suggesting that β-subunits partly shift to higher apparent molecular weight in SDS-PAGE. In contrast, the β-subunits of the enzymatically inactive α1D157Aβ1γ1 and α2D157Aβ2γ1 mutants did not shift toward higher molecular weight in SDS-PAGE and are not incorporating radioactivity from [γ-32P]ATP upon activation by CaMKKβ or LKB1-MO25α-STRADβ (data not shown), strongly suggesting that the altered electrophoretic mobility of the β-subunit is caused by autophosphorylation. However, a certain proportion of the β-subunit-specific signal did not shift toward higher molecular weight (Fig. 2C). At present, we do not have an explicable interpretation for this phenomenon. The appearance of multiple AMPK β-subunit immunoreactive signals, which may represent different phospho-species of the β-subunits, prompted us to investigate a time course of AMPK activation. The kinetics of the activation process with AMPK α2β2γ1 is shown in Fig. 3A, reaching saturation after 30 min. Western blotting of samples taken at different time points of
incubation with upstream kinases (up to 4 h) demonstrates that the β2-subunit phosphorylation still increased after full activity was reached (Fig. 3B). Interestingly, the time course of increasing autophosphorylation, as reflected by the lowered electrophoretic mobility of the β2-subunit in SDS-PAGE, is concomitant with a decrease of the stimulatory effect by AMP (Fig. 3C).

**Activation of AMPK with Upstream Kinases Is Not Stimulated by AMP**—AMP does not directly regulate the activities of the upstream kinases LKB1-MO25α-STRADα or CaMKKB (6, 8–10). However, the question of whether binding of AMP to AMPK renders the enzyme a better substrate for upstream kinases is still a matter of discussion. We therefore used bacterially expressed truncated α1-(1–312) comprising only the kinase domain of AMPK and thus lacking allosteric effects by AMP, in comparison with the two heterotrimeric isoforms of AMPK carrying their functional γ-subunits and allowing for allosteric regulation. AMPK was incubated in the presence and absence of AMP with the two upstream kinases (LKB1-MO25α-STRADα or CaMKKB) and analyzed by Western blotting. Identical signal intensities derived from the phospho-specific anti-AMPKα Thr-172 antibody were obtained with the two wild type AMPK α1β1γ1 and α2β2γ1 isoforms as shown in Fig. 4 (upper panels) after incubation of the different AMPK species with CaMKKB or LKB1-MO25α-STRADα, regardless of whether AMP was added or not, suggesting that binding of AMP to the allosteric site does not augment phosphorylation at the α-Thr-172 site of AMPK by upstream kinases. Yet [AMP] at the end of the activation assay was 0.3–0.5 μM in samples where no AMP was externally added, which could contribute to some stimulation of phosphorylation. However, such an effect, if present in our in vitro system, could hardly be observable by other available techniques. Despite equal loading of AMPK α-subunit (Fig. 4, lower panels), the phospho-Thr-172 signals of α1-(1–312), however, remained very faint in comparison with the heterotrimeric AMPK (Fig. 4, upper panels, lanes 8, 9 and 18, 19). As also true for the two wild type heterotrimers, prolonged exposure to the film did not reveal any differences between Thr-172 phosphorylation in the truncated α1-(1–312) mutant in the presence or absence of AMP (data not shown). If α1 and α2 phospho-Thr-172 signals are compared (Fig. 4, upper panels, lanes 2, 3 and 5, 6), a lower signal was consistently observed in all incubations with AMPK α1, which raises the possibility that AMPK α2 is a better substrate for both upstream kinases, CaMKKB and LKB1-MO25α-STRADα, if compared with AMPK α1. A possible effect of the β-subunit isoform that is different in the α1 and α2 complexes cannot be excluded, and further studies are required to address this question more specifically.

**Stimulation of Fully Activated AMPK by AMP**—The degree of AMPK stimulation by AMP and the concentration range where AMP exerts its stimulatory effect are highly relevant for in vivo functioning of the kinase. Both were investigated using the HPLC method described herein.

ATP solution freshly prepared from powder and analyzed by HPLC revealed a significant amount of AMP as contaminant (>0.5%, depending on handling and storage conditions). To obtain an AMP-free ATP stock solution, highly concentrated ATP was purified by HPLC, but this HPLC-purified ATP still contained trace amounts of AMP (<0.1% relative to ATP, with a detection limit at 0.03–0.05 nmol). Using this HPLC-purified ATP at 200 μM and without externally added AMP, at the end of an AMPK activity assay, 0.76 and 0.51 μM of AMP (0.25–0.38% relative to ATP) were measured in the assay mixture with activated and repurified AMPK α1β1γ1 and α2β2γ1, respectively. In the absence of its substrate, the SAMS peptide, AMPK did not produce significant amounts of AMP or ADP (data not shown), which excludes the possibility that contaminating enzyme activities, e.g. adenylate kinase or such, could have caused the increased content of AMP during the time course of the assays. More likely, the non-enzymatic decay of the generally very labile reaction product ADP to AMP can explain these elevated AMP levels, as the concentration of AMP did indeed increase concomitantly with the turnover rate of ATP by AMPK. The AMP content of each sample was therefore calculated from the HPLC data, thus reflecting the true AMP con-
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FIGURE 5. AMP dependence of activated re-purified wild type AMPK. AMPK α1β1γ1 (diamonds, broken line) and α2β2γ1 (triangles, solid line) (5 μg/ml) were assayed at 200 μM ATP (HPLC-purified) and 500 μM SAMS. The AMP concentration in the assay mixture was determined by HPLC for each sample at the end of the reaction (AMP measured). Even if no AMP was added, significant concentrations of AMP were detected in the assay mixture (0.76 or 0.51 μM for α1β1γ1 or α2β2γ1 AMPK, respectively). The arrow indicates the AMP concentration necessary for half-maximal stimulation. Inset, reciprocal plotting of the curves and calculation of trend lines using least square fitting.

AMP concentration at the end point of each assay at the various times indicated (Fig. 5).

Despite using HPLC-purified ATP and the pure recombinant enzymes, determination of AMPK activity in absence of AMP remains an enigma, since, due to the reasons specified above, it was not possible to obtain assay conditions with truly “zero” AMP. Moreover, other experimental approaches to completely remove AMP from the assay mixture, e.g. using AMP-deaminase and/or 5’-nucleotidase, were not successful, i.e. the concentration of AMP could still not be reduced below 0.5 μM (data not shown).

Although determination of AMPK activity in the absence of AMP has proven practically impossible, it is evident that the curve in Fig. 5, if extended toward lower AMP content, is sharply decreasing toward much lower specific activities of AMPK (Fig. 5, dotted lines). Reciprocal plotting of the activity data using the simplest model available that is a hyperbolic curve (Hill coefficient = 1) results in a linear relationship with high correlation coefficients (Fig. 5; R²α1β1γ1 and R²α2β2γ1 are 0.97 and 0.95, respectively). However, we acknowledge that the assumption of a hyperbolic relationship between AMPK activity and AMP content might not be entirely valid for AMPK, where a Hill coefficient of 2 was proposed based on experimental evidence (11). If using a Hill coefficient of 2 and appropriate reciprocal plotting (1/[AMP]²), however, the linear correlation is comparably low (R²α1β1γ1 and R²α2β2γ1 are 0.83 and 0.90, respectively).

Half-maximal stimulation of AMPK by AMP occurs below 2 μM (Fig. 5, arrow). As discussed above, AMPK activity assays corresponding to the lowest accessible AMP content still contained significant amounts of AMP. Although we can only speculate about curve progression at low [AMP], it is clear that graphic extrapolation of the curve toward low [AMP] (Fig. 5, dotted lines) also results in lowered values for half-maximal stimulation, suggesting that AMPK might be sensitive to sub-micromolar concentrations of AMP. Hence, 2 μM AMP constitutes the upper limit for half-maximal stimulation, and likely values are significantly below.

The stimulatory effect of AMP on AMPK activity is often calculated relative to conditions where no AMP was added. According to our data, AMP stimulates AMPK activity 1.5–3-fold (Figs. 3C and 5), and the extent of this stimulation may depend on autophosphorylation following activation of AMPK by upstream kinases (Fig. 3B). However, even without addition of AMP to the assay, [AMP] is above 0.5 μM as determined by HPLC (Fig. 5). Thus, the stimulatory effect of AMP must be higher than 1.5-fold (Fig. 5), if considering residual AMP and the sharply decreasing specific activities at low [AMP]. In other words, a consequence of graphic extrapolation of the curve (Fig. 5, dotted lines) is an increased relative stimulatory effect by AMP. Although we cannot quantify the stimulation, as we do not know the exact curve progression, AMP may well increase AMPK activity 10-fold or more. Thus, even careful interpretation of the data suggests a rather high stimulatory potency of AMP with half-maximal effect at low micromolar or even sub-micromolar [AMP]. Moreover, full stimulation of activated AMPK by AMP occurs at around 10 μM, although AMPK activity is close to maximal levels already at 5 μM AMP with both AMPK isoforms (Fig. 5).

Nonactivated AMPK Is Almost Totally Inactive in the Presence and Absence of AMP—Although AMPK activity cannot be determined in the total absence of AMP, due the trace amounts of AMP likely generated from ADP during the assay, we can measure AMPK activity in the absence of upstream kinases. Activity of recombinant AMPK prior to activation by AMPKKs is very low but clearly detectable. The specific activities in the absence of externally added AMP were 0.005–0.007 μmol/mg/min for both AMPK heterotrimers. These values increased only slightly to 0.01 μmol/mg/min upon addition of AMP at saturating concentrations (data not shown).

Contaminating AMP in NAD⁺ Is Responsible for Stimulation of AMPK—The cellular redox potential, which is reflected by NAD⁺/NADH, was recently proposed to regulate AMPK activity in addition to AMP/ATP ratios (30). Accordingly, constitutively active AMPK (T172D) and wild type AMPK were purported to be activated in a dose-dependent manner by NAD⁺, whereas NADH inhibited AMPK activities. Indeed, a dose-dependent stimulatory effect of NAD⁺ was also observed initially in our hands, using NAD⁺ from a commercial supplier (Fig. 6B). Analysis of NAD⁺ by HPLC, however, revealed a significant impurity (Fig. 6A), which we suspected to represent AMP, as this is a likely degradation product of NAD⁺. Therefore, AMP alone and NAD⁺ spiked with AMP were subjected to HPLC analysis using identical running conditions. Overlay of the spectra showed that AMP exactly co-eluted with the major contaminant that is present in NAD⁺ preparations (Fig. 6A, inset). At NAD⁺ concentrations of 30 and 300 μM, the final AMP content in the AMPK assay mixture was determined to be at 1.3 and 10.9 μM, respectively. On the other hand, HPLC-purified NAD⁺ was unable to stimulate AMPK activity (Fig. 6C). In the absence of NAD⁺, AMP at concentrations approximating the contamination stimulated AMPK comparably (Fig. 6D). Furthermore, NAD⁺ purchased from a second manufacturer showed in a freshly prepared solution 0.1% AMP directly.
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Stimulation of Fully Activated AMPK by ZMP—The compound 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) has frequently been used to activate AMPK in cell cultures and in whole animal studies (33). AICAR is taken up by cells and converted to AICAR-phosphate (ZMP), itself representing a metabolic intermediate of adenine nucleotide synthesis. In vivo, ZMP might mediate AMPK activation via a direct allosteric mechanism (17). Here we determined the allosteric effect of ZMP directly on recombinant AMPK in vitro. According to our results, ZMP did not stimulate AMPK to full activity. In comparison with 10 μM AMP (sufficient for full stimulation of wild type AMPK), the potency of ZMP was significantly lower at 25 μM, and even at high concentrations (1 mM) less than 60% of full stimulation by AMP was reached. However, it should be emphasized that AMPK activity assays always contain trace amounts of AMP. Thus, it is not possible to quantitate the stimulatory effect of ZMP alone.

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DISCUSSION

AMPK is an important energy-sensing kinase playing a key role for the maintenance of the cellular energy charge represented by a high cellular ATP to AMP ratio (34). In order to perform this task, AMPK is required to adjust its activity rapidly to changes of cellular energy demand, suggesting that regulation of AMPK must be tightly controlled, highly sensitive, and very dynamic (35).

Here we present new data concerning the activation of AMPK by AMP and the action of the latter allosteric effector directly on AMPK and/or the corresponding upstream kinase and phosphatase system. To address these questions, we used highly purified recombinant AMPK, obtained from bacterial lysates after co-expression of AMPK subunits in Escherichia coli (18). A general concern that may be raised against using recombinant AMPK and other recombinant material originating from bacteria for studies of enzyme function may be a potentially altered functionality. Nevertheless, ~80% of the proteins used to solve three-dimensional structures submitted to the Protein Data Bank in 2003 were prepared in an E. coli expression system (36), and E. coli remains the most important host for protein production in industrial scale (37). We have used bacterially expressed recombinant AMPK since its biochemical characteristics compare very favorably with AMPK that has been purified from tissue or cell cultures (see “Results”). Recombinant wild type AMPK alone is basically inactive, suggesting that bacteria are not capable of phosphorylating Thr-172 of the α-subunit. Particularly, phosphorylation at Thr-172 is below the detection limit if probed with the corresponding phospho-specific anti-AMPK Thr-172 antibodies. Moreover, we presume that bacterially expressed AMPK is fully dephosphorylated, also evidenced by facilitated identification of new phosphorylation sites using the very same material (5). In contrast, native AMPK is post-translationally modified at multiple sites (4, 5, 38); thus, the absence of these modifications in recombinant AMPK provides a clear advantage if one intends to study the effect of a particular modification. This “maiden-like” property of recombinant wild type AMPK allows for determination of activities prior to activation by upstream kinases, revealing low but clearly detectable kinase activities. Furthermore, the recombinant enzyme is highly activated by upstream kinases and, in addition, is stimulated by AMP. Moreover, we observe very high specific activities that are comparable to highly purified enzyme preparations from cells or tissues (29). As opposed to eukaryotic cells, bacteria do not express endogenous AMPK; thus, it is possible to obtain homogeneous preparations of AMPK isoenzymes, depending solely on the cDNA used for transformation, thereby eliminating the problem of co-purifying endogenously synthesized AMPK isoforms as always present in cells and tissues.

FIGURE 7. Allosteric effect of NADH on activated re-purified wild type AMPK. A, HPLC of 10 nmol of NADH (Roche Diagnostics) revealing no significant contaminations. B, NADH was added at the indicated concentrations to activated re-purified αβγy1 (5 μg/ml) in the presence of 200 μM ATP, 50 μM AMP, 500 μM SAMS. Samples were analyzed by HPLC, and specific activities were calculated from the chromatograms.

FIGURE 8. Inhibition of PP2Ca by AMP. Activated and re-purified αβγy1 (5 μg/ml), αβγy1 (5 μg/ml), and α1(1–312) AMPK (25 μg/ml) were treated with PP2Ca (10 μg/ml for heterotrimers and 0.3 μg/ml for the monomer) for 20 min at 30 °C. A, samples were analyzed by Western blotting after SDS-PAGE using phospho-specific anti-AMPK Thr-172 antibodies (three independent experiments). B, densitometric quantitation of the phospho-specific anti-AMPK Thr-172 signals (n = 3; ± S.E., * p < 0.05). C, AMPK activity was determined after PP2Ca-treatment at different AMP concentrations by incubation with 200 μM ATP, 50 μM AMP, and 500 μM SAMS for 10 min using the HPLC-based procedure. Remaining activities are shown by percentage relative to the non-PP2Ca-treated AMPK.
Here we show that bacterially expressed AMPK is activated 1000-fold by the combined effect of activation by its upstream kinases, CaMKKβ or LKB1-MO25α-STRADα, together with its allosteric stimulator AMP. Both upstream kinases activate AMPK to a similar extent, and the two wild type isoforms of AMPK α1B1–y1 and α2B2–y1 studied exhibit similar specific activities, as well as equal responses to AMP. We further suggest high sensitivities of AMPK toward AMP at low micromolar concentrations. Half-maximal stimulation of AMPK was reached at 2 μM AMP, and, if residual AMP content is considered, half-maximal stimulation would occur already at significantly lower [AMP]. Consistent with previous reports, we observe 1.5–3-fold stimulation by AMP (6, 8–10), but a much higher stimulatory potential of AMP is expected if the residual AMP is taken into account. These findings support the notion that AMPK is a highly sensitive energy-sensing kinase.

Neither LKB1-MO25-STRAD nor CaMKKβ is directly stimulated by AMP (6, 8–10). However, phosphorylation of immunoprecipitated AMPK by a partly purified LKB1-MO25-STRAD complex was stimulated by AMP 1.5–3.5-fold (6), and in another study AMP did not affect activation of AMPK by the LKB1 complex (8). According to our results AMP exhibits no direct effect on AMPKks, nor does binding of AMP to the allosteric site of the AMPK γ-subunit appear to augment phosphorylation at Thr-172 of the α-subunit by the upstream kinases CaMKKβ and LKB1-MO25α-STRADα (Fig. 4). In contrast, we reported previously that phosphorylation of recombinant AMPK by a partially purified preparation of heart upstream kinases is augmented by AMP (19). We cannot currently explain this discrepancy, but it is possible that another unknown AMPKK is operational in heart (39). Thus, AMP-augmented AMPK phosphorylation in vivo or in partially purified extracts should not be ruled out, but the mechanism of such regulation so far remains elusive.

Deactivation of AMPK α2 by its phosphatase PP2Cα is significantly attenuated by elevated concentrations of AMP, and the same tendency, although not statistically significant, is seen with AMPK α1 (Fig. 8). Similar results were reported previously (16), supporting the notion that the AMPK-AMP complex serves as a less favorable substrate for PP2Cα. Such deceleration of the dephosphorylation rate by increased AMP concentrations couples phosphorylation of the α-subunit of AMPK at Thr-172, a prerequisite for significant AMPK activity, to the cellular AMP levels. Thus, AMP plays two direct roles for regulation of AMPK activity simultaneously: (i) allosteric stimulation of activity and (ii) repression of deactivation by PP2Cα.

Ponticos et al. (40) proposed regulation of AMPK activity by phosphocreatine to creatine (Pcr/Cr) ratios via a direct allosteric mechanism. Although representing an attractive model, the purported inhibitory effect of Pcr has been recently challenged by Taylor et al. (21). Similarly, we have been unable to detect a direct allosteric effect on AMPK activity or on its upstream kinases by Pcr or Cr in the concentration range of 0.3–20 mM.4 A very strong direct interaction of AMPK with creatine kinase, also reported by the same authors (40), seems unlikely as well, because if this were true, all AMPK present in a cell would be completely complexed by creatine kinase, a very abundant enzyme in tissues of high and fluctuating energy requirements (41, 42). However, a phosphorylation of creatine kinase by AMPK (40) should not be ruled out.

In this study, we also investigated the potential of NAD+ and NADH to allosterically regulate AMPK activity as proposed by Rafaeloff-Phail et al. (30). We employed the HPLC system to analyze solutions of NAD+ and identified contaminating AMP, a likely degradation product, causing significant allosteric activation of AMPK. However, neither HPLC-purified NAD+ nor NADH exhibits any direct effect on AMPK activity at concentrations up to 300 μM. Therefore, the originally attractive hypothesis that AMPK could act as a sensor for the cellular redox potential should be carefully re-evaluated.

AICAR has been used extensively to activate AMPK in living cells and organisms (33). AICAR is taken up by cells and converted to ZMP, which is thought to activate AMPK via direct allosteric activation. Here we also investigated potential direct allosteric effects of ZMP on AMPK in vitro. Our results demonstrate that, in comparison to AMP, the stimulatory potential of ZMP is lower, and also high concentrations fail to fully stimulate AMPK, suggesting that mimicking of AMP by ZMP in vivo is incomplete. It is entirely conceivable that activation of AMPK, although not reaching maximal levels, could be sufficient for a full cellular response considering the time factor during which the compound acts within cells. Alternatively, other additional mechanisms might exist that lead to activation of AMPK by AICAR or ZMP in vivo.

In conclusion, the study presented here, using a defined in vitro system with purified AMPK, upstream kinases, and phosphatase, contributes to a better comprehension of physiologically important aspects of AMPK function and regulation. As AMP does not augment phosphorylation of AMPK in vitro, the question arises whether inhibition of PP2Cα through elevated AMP in response to stress can explain the observed changes of Thr-172 phosphorylation of AMPK in vivo or in cultured cells. Alternatively, additional previously unknown mechanisms may exist. Further studies are required to investigate these issues.

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