Functional Expression of Phosphagen Kinase Systems Confers Resistance to Transient Stresses in *Saccharomyces cerevisiae* by Buffering the ATP Pool*

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Phosphagen kinase systems provide different advantages to tissues with high and fluctuating energy demands, in particular an efficient energy buffering system. In this study we show for the first time functional expression of two phosphagen kinase systems in *Saccharomyces cerevisiae*, which does not normally contain such systems. First, to establish the creatine kinase system, in addition to overexpressing creatine kinase isoenzymes, we had to install the biosynthesis pathway of creatine by co-overexpression of l-arginine:glycine amidinotransferase and guanidinoacetate methyltransferase. Although we could achieve considerable creatine kinase activity, together with more than 3 mM intracellular creatine, this was not sufficient to confer an obvious advantage to the yeast under the specific stress conditions examined here. Second, using arginine kinase, we successfully installed an intracellular phosphagen pool of about 5 mM phosphoarginine. Such arginine kinase-expressing yeast showed improved resistance under two stress challenges that drain cellular energy, which were transient pH reduction and starvation. Although transient starvation led to 50% reduced intracellular ATP concentrations in wild-type yeast, arginine kinase overexpression stabilized the ATP pool at the pre-stress level. Thus, our results demonstrate that temporal energy buffering is an intrinsic property of phosphagen kinases that can be transferred to phylogenetically very distant organisms.

The availability of biochemical energy, with ATP as the primary energy currency, is fundamental to most cellular processes. Although ATP and its congeners are involved in literally hundreds of biochemical reactions, the intracellular concentration of ATP is generally kept very constant at about 2–5 mM, depending on organisms and tissues, with a turnover rate of the ATP pool that is in the range of a few seconds (1). Hence, metabolic ATP generation in a cell must be balanced tightly with ATP-consuming processes. Small deviations from the standard cellular concentrations of free ATP, ADP, and AMP serve important regulatory roles in fine tuning this delicate balance.

This balance between energy-consuming and -producing processes is particularly challenged in tissues that experience periods of high and fluctuating energy demand, such as brain, heart, or skeletal muscle. To maintain constant ATP levels, these tissues express creatine kinase (CK; EC 2.7.3.2) that uses creatine (Cr) to create a metabolically inert pool of phosphocreatine (PCr). Among other functions, this PCr pool serves as a temporal energy buffer that can replenish ATP rapidly during phases of high energy demand, according to the following reaction (2): MgADP + PCr2— + H+ ⇄ MgATP2— + Cr.

The CK system is found in many vertebrate tissues and is probably the best known example of what is more generally referred to as the phosphagen kinase system (3). The common feature of these kinases is their capability to synthesize a metabolically inert pool of phosphorylated compounds (phosphagens) during normal metabolic conditions and to replenish the ATP from this pool during periods of high energetic demand. Eight phosphagen kinases are found in the animal kingdom (3), with arginine kinase (AK; EC 2.7.3.3) as a prominent example, occurring in insects (4), crustaceans (5), and in certain unicellular organisms (6). In analogy to CK, AK catalyzes the following reaction: MgADP + PArg2— + H+ ⇄ MgATP2— + Arg.

In addition to their supposedly primary role as temporal energy buffer, phosphagen kinase systems serve a number of other functions, which include buffering of the intracellular pH and preventing a rise in intracellular ADP levels that would trigger multiple metabolic responses (2, 3). In addition to the above general functions, the CK/PCr system has the unique capability to establish a spatial energy buffering, as well (2, 3, 7). This complex functionality is also reflected by oligomeric composition and compartmentalization of the CK isozymes, with cytosolic isoenzymes (B-CK, M-CK) forming dimers and mitochondrial isoenzymes (sMtCK, uMtCK) forming dimers and octamers (8). In mice, expression of CK in the naturally CK-deficient liver led to multiple, beneficial effects for this organ, including tolerance to hypoxia and endotoxins (9, 10). Although microorganisms are often exposed to rapidly changing environmental conditions and fluctuating availability of energy, phosphagen kinase systems occur only in few unicellular organisms, e.g. *Paramecium caudatum* and *Trypanosoma cruzi* (6, 11). Thus, we hypothesized that lower unicellular eukaryotes, such as the yeast *Saccharomyces cerevisiae*, would potentially benefit if artificially equipped with such phosphagen kinase systems. In particular for *S. cerevisiae*, intracellular acidification is a well-known consequence of various environ-

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1 The abbreviations used are: CK, creatine kinase; AK, arginine kinase; cdw, cell dry weight; Cr, creatine; D, dilution rate; Mt, mitochondrial; PArg, phosphoarginine; PCr, phosphocreatine; CER, CO2 evolution rate; AGAT, l-arginine:glycine amidinotransferase; GAMT, guanidinoacetate methyltransferase.
mental stresses, including exposure to weak acids and copper (12–14) but also as a secondary effect of stress challenges like desiccation and heat (15, 16). Acidification is counteracted by a greatly increased activity of the plasma membrane H^+ -ATPase, which expels protons from the cytoplasm at the expense of ATP hydrolysis (14). The energetic expense for this proton pumping activity may require up to 60% of the total ATP production (17), thus constituting an ATP drain similar to muscle contraction. Likewise starvation and several other stress challenges are known to exert high ATP demands (18).

In this study, we attempted to install phosphagen kinase systems in lower eukaryotes, thereby addressing the hypothesis that functional expression of such phosphagen kinases in a biological background that is free of endogenous phosphagen kinases and that can be genetically manipulated easily. This work is also a first step toward a more detailed investigation on molecular functions and mechanisms of the phosphagen kinase systems in a biological background that is free of endogenous phosphagen kinases and that can be genetically manipulated easily.

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, and Media—** S. cerevisiae CEN.PK 113–7D (MATa) was used for Cr adaptation experiments. All other experiments were performed with strain CEN.PK 113–6B (MATa ura3–52 leu2–3,112 trp1–289). Construction of expression plasmids was done in Escherichia coli DH5α (F ‐r174Δpir) strain by using the Gateway system (Invitrogen, Carlsbad, CA). The plasmids p424HXT7 (TRPI), p425HXT7 (LEU2), and p426HXT7 (URA3) were used for heterologous gene expression. Constitutive expression is driven from the truncated promoter of the high affinity glucose transporter gene HXT7 and is terminated by the CYC1 terminator (19, 20).

All physiological experiments were done in yeast minimal medium containing 0.85 g/liter KH₂PO₄, 0.15 g/liter K₂HPO₄, 0.5 g/liter MgSO₄ 7H₂O, 0.1 g/liter NaCl, 0.1 g/liter CaCl₂, 50 μg/liter HEPES, 83 μg/liter CaSO₄·H₂O, 100 μg/liter KI, 200 μg/liter FeCl₃, 450 μg/liter MnSO₄·H₂O, 235 μg/liter Na₂MoO₄·2H₂O, 712 μg/liter ZnSO₄·7H₂O, 20 μg/liter biotin, 2 mg/liter calcium pantothenate, 2 μg/liter folic acid, 10 μg/ml inositol, 0.4 mg/liter nicotinic acid, 0.2 mg/liter p-aminobenzoic acid, 0.4 mg/liter pyridoxine hydrochloride, 0.2 mg/liter riboflavin, and 0.4 mg/liter thiamin hydrochloride. If not specified otherwise, the medium was also supplemented with 0.5% glucose, and 5 g/liter glucose as nitrogen and carbon sources, respectively. Uracil (20 mg/liter), tryptophane (50 mg/liter), or leucine (240 mg/liter) were added, if necessary.

**Growth Conditions—** Aerobic batch cultivations were performed in 500-ml baffled shake flasks with maximally 50 ml of medium at 30 °C on a gyratory shaker at 300 rpm. Cultures were grown to an OD₆₀₀ of about 0.5, before the pH was set to 5. After 1 h, pH 5 was re-established using 100 μM KOH. The recovery time was determined as the period required to reach a growth rate of at least 0.1 h⁻¹ after re-establishing pH 5.

**Analytical Procedures**—Cell growth was monitored by the increase in OD₆₀₀. Crude cell extracts for determination of intracellular metabolite concentrations were prepared by washing cells in 4 packed cell volumes of cold water and resuspending in 2 packed cell volumes of buffer containing 20 mM Tris–HCl, pH 7.9, 10 mM MgCl₂, 1 mM EDTA, 5% (v/v) glycerol, 4 mM dithiothreitol, 0.3 mM NADH, and 1 mM phenylmethylsulfonil fluoride at 0 °C. Four packed cell volumes of ice-cold, acid-washed 0.45–0.55-mm glass beads were added, and the mixture was vortexed for 1 min and incubated for 2 min on ice. This procedure was repeated up to 10 times until complete disruption, as was verified by microscopic control. The liquid phase was centrifuged for 60 min at 13,000 g and 4 °C, and the supernatant was frozen in liquid nitrogen and stored at −70 °C for further analysis. For determination of protein activity, samples were analyzed within a week.

**Phosphagen Kinase Activities—** Cr and AK activity were determined using pH-stat analysis (24, 25). Briefly, crude cell extracts were incubated at pH 7.0 and 25 °C with 6.3 mM KCl, 8.3 mM MgCl₂, 83 μM EGTA, 1 mM β-nerecaethanol, 4 mM ADP, and 10 mM of either PCK (Cr) or AK (AK), and the rate of stoichiometric proton consumption by conversion of PCK and ADP to Cr and ATP was monitored by titration.

**Intracellular Metabolite Concentrations—** Cr concentrations in supernatants and crude cell extracts were determined by the above pH-stat analysis by incubating cell extracts at pH 8.0 and 25 °C in a nitrogen atmosphere in a buffer containing 63 mM KCl, 5 mM MgCl₂, 83 μM EGTA, 1 mM β-nerecaethanol, 4 mM ADP, and 0.1 units/ml rabbit B-CK. Calibration was done with Cr concentrations between 1 and 20 mM. For Cr measurement, cells were washed three to five times with water prior to crude cell extract preparation.

**Arg concentrations** were determined enzymatically by adding 200 mM triehanolamine, 12 mM α-ketoglutarate, 130 μM NADH, 2 mM ADP, 1.3 units/ml glutamate dehydrogenase, 10 units/ml urease, and 6 units/ml arginase to crude cell extracts and monitoring the change in absorbance at 340 nm (26).

**PArg concentrations** were determined as described previously (11). Briefly, cultures aliquots were frozen in liquid nitrogen and stored at 70 °C. Cells were resuspended in 50 mM KCl, 2 mM EDTA, and 10 mM Tris–HCl (pH 7.0), and incubated by boiling with 0.6% (v/v) trichloroacetic acid. This boiling step was necessary, because incubation at lower temperatures did not result in complete disruption of the yeast cells (data not shown). Stability of PArg during this treat-
ment was experimentally verified. Although acid incubation at 95 °C for 2 min lead to significant hydrolysis of PArg, PArg concentrations after a 1-min incubation at 95 °C were within 10% of those found after incubation at 0 °C. The supernatant was then transferred to a new tube, and the pH was set to 8.2. To precipitate adenyl nucleotides, 1.5% (w/v) barium acetate was added, and the mixture was incubated overnight on ice. The supernatant was supplemented with 100 μM Na2MoO4 and centrifuged for 5 min at 13,000 rpm to remove residual barium ions. Trichloroacetic acid was added to 2.5% (v/v) to the clarified supernatant, and samples were hydrolyzed for 1 min at 100 °C. The liberated organic phosphate was determined colorimetrically at 623 nm after addition of 1.5% (v/v) H2SO4, 10 mM Na2MoO4, and 10% (v/v) of a color reagent containing 10 g/liter polyvinyl alcohol and 185 mg/liter mala- chite green.

Intracellular ATP and ADP concentrations were determined as described previously (27, 28). Briefly, 1-ml culture samples were quickly withdrawn (within 1 s) on acid-washed glass beads that were pre-cooled at −20 °C. 50-μl aliquots of culture broth were supplemented with 200 μl of dimethyl sulfoxide and 750 μl of 25 mM Hepes, pH 7.75. Aliquots were then frozen at −70 °C for further analysis. ATP concentrations were determined using an ATP bioluminescence kit II HS (Roche Molecular Biochemicals). ADP was converted to ATP by incubating another aliquot for 30 min at 37 °C with 1 μM phosphoenolpyruvate and 1.250 units/ml pyruvate kinase, and the total ATP level was then determined. ADP concentrations were calculated as the difference between total ATP and the ATP level in the other aliquot. Calibration was done with ATP and ATP/ADP mixtures, in the concentration range of 1 and 1,000 nM. Intracellular metabolite concentrations were calculated from the experimentally determined concentration using a cellular volume of 1.162·10−13 ml and a concentration of 108 cells/ml at an OD600 of 1.5 (29).

Determination of Physiological Parameters—In batch cultures, the exponential growth phase was identified by log-linear regression of OD600 versus time, with maximal growth rate (μmax) as the regression coefficient. To calculate specific production rates, OD600 values were converted to cellular dry weight (cdw) using a predetermined correlation factor (0.52 g/liter cdw per OD600 unit). The biomass concentration was calculated as cdw per volume unit. The CO2 evolution rate (CER) was defined as the relative CO2 production (ΔCO2) multiplied by the effluent gas flow rate (F), on the basis of the relationship CER = ΔCO2 · F. The specific production rate for CO2 (qCO2) was calculated by dividing the CER by the biomass concentration (X) and the culture volume (V), on the basis of the relationship qCO2 = CER/X · V.

RESULTS

Establishing a Functional Creatine Kinase System by Coexpression of Creatine Biosynthetic Enzymes Plus Creatine Kinase—To establish a functional CK system in S. cerevisiae (Fig. 1), we first overexpressed the cytosolic brain-type isoenzyme (B-CK) and/or the ubiquitous mitochondrial isoenzyme (uMtCK). Transformants exhibited CK activity of 0.5–0.9 international units/mg protein (Fig. 2), corresponding to the CK activity found in many chicken tissues (30). Based on a previous report (31), we expected Cr uptake from the medium, although yeast genome data suggested the absence of a specific Cr transporter gene (Ref. 33 and the National Center for Biotechnology Information website at www.ncbi.nlm.nih.gov). During growth in yeast minimal medium supplemented with 10 mM Cr, however, neither CK-expressing nor control yeast accumulated any appreciable amounts of intracellular Cr (data not shown).

To activate potential unspecific Cr transport systems or silent genes (34) that may facilitate some Cr uptake, we attempted to adapt S. cerevisiae to Cr consumption. Specifically, we grew S. cerevisiae CEN.PK 113–7D in successive batches with Cr as additional carbon or nitrogen source, in combination with glucose or ammonium, respectively. In no case, however, did we detect intracellular Cr (data not shown) or any growth in cultures with Cr as the unique carbon or nitrogen source.

To provide intracellular Cr to CK-expressing S. cerevisiae, we installed the biosynthetic pathway for Cr that naturally does not occur in yeast (Ref. 33 and the National Center for Biotechnology Information website at www.ncbi.nlm.nih.gov). Expression of human t-arginine:glycine amidinotransferase (AGAT) and rat guanidinoacetate methyltransferase (GAMT) yielded an intracellular concentration of about 3.2 mM Cr (Table 1), presumably resulting from utilization of endogenous glycine and Arg. The minor concentration of Cr detected in cells expressing only AGAT is probably because of the accumulation of guanidinoacetate, which, at high concentrations, is also detected by the Cr assay (data not shown).

Establishing a Functional Arginine Kinase System in S. cerevisiae—S. cerevisiae displays the endogenous capability for Arg biosynthesis and transport (35). Thus, functional establishment of an AK system should depend solely on heterologous expression of AK, which is not naturally present in yeast (5). Independent of the amount of exogenously supplied Arg, AK activities in crude extracts of cells harboring pAK were 3.7 international units/mg, whereas control cultures did not exhibit any AK activity (data not shown). To exclude that Arg
became limiting in our system, we determined intracellular Arg concentration in cultures grown at different extracellular Arg concentrations. Increasing extracellular Arg supplementation up to 6 mM was reflected by increasing intracellular Arg concentrations. At concentration exceeding 6 mM, however, no further increase of intracellular Arg was observed (Fig. 3). In this respect, an essentially identical behavior was observed for control and AK-expressing strains. Although intracellular PArg concentrations were below detection level in the control strain, AK-expressing strains exhibited intracellular PArg concentrations of about 5 mM at all investigated extracellular Arg concentrations (data not shown), whereas intracellular Arg concentrations in these AK-expressing strains were similar to those seen in controls (Fig. 3).

Expression of AK But Not CK Shortens the Lag Phase after pH Stress—We grew S. cerevisiae CEN.PK 113–6B expressing either AK (pAK and 10 mM Arg supplementation in the medium) or the complete CK system (pBCK, pAGAT, and pGAMT) on yeast minimal medium in shake flasks. Under standard growth conditions, we did not observe any improvement in maximal cdw and $\mu_{\text{max}}$ with respect to the controls (data not shown). We then examined specific stress conditions that provoke a drop in ATP and therefore possibly confer an advantage to cells expressing a phosphagen kinase system (14, 16). A transient acidic stress was applied by shifting the pH of mid-log phase cultures (at an $OD_{\text{600}}$ of 0.8–1.0) for 1 h from pH 5 to pH 2 and subsequently returning to pH 5. Under these conditions, the ability of the cultures in recovering to the original $\mu_{\text{max}}$ value, as well as the time needed to recover growth (defined as recovery time), were investigated. Cultures harboring the complete CK system as above did not show any improvement with respect to the controls (Fig. 4b). In contrast, AK conferred the ability to reduce the recovery time from 3.3 to 2.3 h (Fig. 4a). As the experiment was repeated two times (data not shown), the recovery time of the control varied between 3 and 5 h, whereas the recovery time was always at least 1 h shorter with AK-expressing cells. The $\mu_{\text{max}}$ of controls and cells expressing AK remained unchanged. Thus it became obvious that the AK system facilitated a shortening of the time needed to recover full growth after pH stress.

Expression of AK Stabilizes Intracellular ATP Levels during Short Term Starvation—Because heterologous AK expression improved resistance to transient pH stress, we wanted to investigate whether this improvement could also be seen under starvation stress and directly related to the ATP buffering capacity of AK (36). To expose S. cerevisiae for defined periods to energetic stress, glucose-limited chemostat experiments, which place cultures in a metabolic state where they efficiently generate energy and biomass from glucose as the limiting nutrient (37, 38), were used. The S. cerevisiae wild-type culture was grown at a D of 0.1 h$^{-1}$ until a stable steady state was attained after about five reactor volume changes. Subsequently, the feed pump was programmed to cycles of activity and pause for defined periods so that the cultures experienced alternating periods of starvation and slow growth during the intervals of the discontinuous feed. Specifically, we tested the following on/off cycle periods (in s): 60/60, 60/120, 30/120, 30/180, 20/150, and 30/150. For each discontinuous feeding profile, we allowed cultures to attain a new stable biomass concentration that was usually attained after five volume changes. The only exception was the 30/180 profile, which led to a washout of the culture, and the 60/60 profile, which did not allow for attaining a steady state. The stable biomass concentrations did not reflect a true physiological steady state; hence it is referred to as a pseudo steady state. Generally, biomass concentrations in these pseudo steady states during discontinuous feeding were lower than during continuous feeding (data not shown), which is consistent with the notion that the periods of starvation constitute an energetic burden. The strongest decrease in biomass concentration was observed with a discontinuous feeding profile of 30 s at a D of 0.1 h$^{-1}$ and 150 s at a D of 0 h$^{-1}$ (Table II). Hence, this profile was chosen for further analysis.

As with the wild-type control, AK-expressing cells were then grown in glucose-limited chemostat at a D of 0.1 h$^{-1}$. The steady-state biomass concentration was about 10% higher in the AK-expressing culture (Table II). Consistent with this higher biomass yield from the available glucose in the medium, less substrate carbon was used for respiration, as is illustrated by the reduced specific CO$_2$ production rate in the AK-expressing culture (Table II). Upon imposing the above chosen discontinuous feeding profile, a new pseudo steady state was attained after about five medium changes with a significantly decreased biomass concentration (Table II). However, the AK-expressing culture was apparently less stressed by the harsh conditions, because its pseudo steady-state biomass concentration was about 30% higher than the concentration of the control strain under the same conditions.

To verify whether the improved biomass yield of the AK-expressing culture was indeed related to the temporal energy-buffering function of AK, we determined intracellular ATP and ADP concentrations in these cultures. Within seconds after feed interruption, the intracellular ATP pool dropped from 3.1 to 1.5 mM in wild-type S. cerevisiae and remained at this level until the onset of feeding (Fig. 5). The AK-expressing culture, however, did not display such a drop in ATP levels. During the continuous feeding steady state, control and AK-expressing cultures exhibited rather similar intracellular ATP and ADP
levels (Fig. 6). A similar intracellular ATP concentration was observed during the 30-s feeding interval in pseudo steady state (Fig. 5). In contrast, ADP levels were found to be relatively constant under all conditions in both strains (Fig. 6).

**DISCUSSION**

The present study shows for the first time a successful functional expression of phosphagen kinase systems in *S. cerevisiae*. The AK and CK systems could be engineered in *S. cerevisiae*. Both transgenic strains showed enzymatic activities that (i) are able to generate a phosphagen pool (5 mM PArg and 2 mM PCr as calculated from the Cr) and (ii) are high enough to allow equilibrium conditions, i.e. a rather fast flux from the phosphagen to ATP that theoretically could recover the cellular energy charge under energy stress.
Because yeast does not naturally synthesize Cr, means to generate intracellular Cr are a major prerequisite for installing a functional CK system. Our results obtained with the yeast model strain \textit{CEN.PK} clearly demonstrate the absence of measurable intracellular Cr concentrations during growth or incubation with 15 mM Cr, independent of CK expression. Long term selection experiments were designed to possibly enable Cr uptake and utilization as an additional carbon or nitrogen source. They did not show any improvement of culture physiology over a period of about 100 generations in liquid media, which is usually sufficient to identify such improved metabolic phenotypes (34). Thus, not only are homologues to known Cr transporters absent in the yeast genome (Ref. 33 and the National Center for Biotechnology Information website at www.ncbi.nlm.nih.gov), but also unspecific Cr transport via general amino acid permeases (39) does not occur. These results contradict an earlier NMR study that expressed rabbit muscle CK in different \textit{Saccharomyces} strains to analyze intracellular ADP concentrations (31). In these cultures, when maintained at 100 mM Cr, Brindle \textit{et al.} (31) reported the accumulation of 50–100 mM intracellular Cr, determined in a biochemical assay. However, this cannot be taken as final proof for cellular Cr uptake. At these extremely high concentrations, Cr is at the solubility limit and may be trapped in the extracellular matrix and at the whiskered cell wall of yeast cells, thus escaping washing procedures.\textsuperscript{4} In fact, the very low concentration of PCr observed in these yeast cells rather suggests that Cr is separated from the cytosolic CK and is therefore mainly (if not entirely) extracellular. Remnant PCr may have been produced by CK that is possibly liberated from a few damaged cells. Although we can presently not exclude a faint, unspecific Cr uptake at extremely high extracellular Cr concentrations, we nevertheless conclude that generally Cr uptake does not occur in \textit{S. cerevisiae} and that transformation with AGAT and G4MT is necessary to generate an intracellular Cr pool.

Of the two phosphagen kinase systems that we installed in yeast, only AK appears to confer an appreciable advantage to \textit{S. cerevisiae} under conditions that impose an energetic stress. AK yeast showed a clearly reduced lag phase in growth after transient pH reduction. Artificial decrease of the extracellular pH forces cells to counteract acidification of the cytoplasm, probably by increased activity of the plasma membrane H\textsuperscript{+}-ATPase (14). An extra boost of energy from the accumulated PArg pool may help maintain AK-expressing cells to resume growth upon relief of the transient pH stress faster than the wild-type control. This phenotype is likely not a truly increased resistance to lower pH, because the conditions were chosen such that little if any cell death occurred.

The hypothesis of PArg as a temporal energy buffer in AK-expressing yeast was addressed more specifically in chemostat cultures that were exposed to defined periods of growth and starvation. These transient stress challenges reduced the biomass yield of wild-type \textit{S. cerevisiae} significantly but had only very little negative influence on AK-expressing cells. Consistent with the above hypothesis, we found stable intracellular ATP levels during the starvation phase of the recombinant yeast. Because the intracellular level of ADP was similar in both cultures, it appears that most of the hydrolyzed ATP accumulated as AMP. This is because of the presence of three isozymes and a rather high enzymatic activity of yeast adenylate kinase (40). These enzymes catalyze the formation of ATP and AMP from two molecules of ADP, thus buffering the cellular energy charge (1). The intracellular concentration of ATP and ADP determined here compare favorably with those of another \textit{S. cerevisiae} strain that was grown under almost identical culture conditions (41). Moreover, the time scale of the rapid ATP decrease at the onset of starvation is in qualitative agreement with the data of Theobald \textit{et al.} (41), who investigated metabolic responses to a glucose pulse in glucose-limited chemostat culture.

Given the protective effects of AK in \textit{S. cerevisiae}, the question remains why the CK system did not confer similar advantages under the examined stress conditions, a phenomenon already seen with CK-transgenic tobacco (42). Several reasons may account for this discrepancy. First, the synthesized pool of 3 mM intracellular Cr may give rise to not more than 2 mM PCr, which is much lower than the 5–30 mM PCr occurring in native animal tissues (2). In fact, with a $K_m$(PCr) for chicken B-CK of 1.4 mM, these PCr concentrations do not favor fast conversions. Furthermore, the intracellular pH is much lower in yeast (pH 5.5–6.2) (14, 43), as compared with the natural environment of CK, e.g. in the resting muscle (pH 7.0–7.2) (2). The acidic intracellular pH of yeast reduces B-CK activity by about 50–70\% and leads to partial hydrolysis of PCr, which is less stable than PArg under acidic conditions (3). Finally, the heterologous expression of three proteins (CK, AGAT, G4MT) on different plasmids may impose a metabolic burden onto the yeast cultures that counteracts beneficial effects. In contrast to CK, the AK system seems to combine several advantages for expression in unicellular organisms that can undergo intracellular pH fluctuations; it uses an intrinsic substrate (Arg) and is able to accumulate a more acid-stable phosphagen (PArg) to higher concentrations.

Our results with AK demonstrate that energy buffering is an intrinsic property of phosphagen kinases that can be transferred to phylogenetically very distant organisms. This is a first step toward the analysis of phosphagen kinases in a background that is naturally free of those kinases. Yeast, with its ease of genetic manipulation and the availability of specific mutants, is ideally suited to study in more detail the molecular physiology of phosphagen kinases, e.g. Cr stimulation of oxidative phosphorylation (8). Moreover, installing a functional phosphagen kinase system such as shown here for AK appears to be a pertinent metabolic engineering strategy to improve the biotechnological potential of microbes that are exposed to energetically stressful conditions. One such condition may be large scale processes in which cells are often exposed to fluctuating availability of nutrients such as carbon source and oxygen because of imperfect mixing (44).

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Expression of Phosphagen Kinase Systems in Yeast