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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[1]; 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): $MgADP^- + PCr^{2-} + H^+ \rightleftarrows MgATP^{2-} + Cr$.

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: $MgADP^- + PArg^{2-} + H^+ \rightleftarrows MgATP^{2-} + Arg$.

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 isoforms, 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, CO_2 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 could potentially improve resistance to those stress challenges that constitute a significant energetic burden. 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 *ura*3–52 *leu*2–3,112 *trp*1–289). Construction of expression plasmids was done in *Escherichia coli* DH5α (F^-/*endA1 hsdR17*(r_k-m_k^+) *glnV44 thi-1 recA1 gyrA*(Nal^r) *relA1* Δ*(lacZYA-argF)U169 deoR* (ϕ80*dlac*Δ*(lacZ)M15*)). The plasmids p424HXT7 (*TRP1*), 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 KH_2PO_4, 0.15 g/liter K_2HPO_4, 0.5 g/liter $MgSO_4$, 0.1 g/liter NaCl, 0.1 g/liter $CaCl_2$, 500 μg/liter H_3BO_3, 63 μg/liter $CuSO_4 \cdot 5H_2O$, 100 μg/liter KI, 200 μg/liter $FeCl_3$, 450 μg/liter $MnSO_4 \cdot H_2O$, 235 μg/liter $Na_2MoO_4 \cdot 2H_2O$, 712 μg/liter $ZnSO_4 \cdot 7H_2O$, 20 μg/liter biotin, 2 mg/liter calcium pantothenate, 2 μg/liter folic acid, 10 mg/liter 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 5 g/liter $(NH_4)_2SO_4$ 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. Cr adaptation experiments were performed by growing *S. cerevisiae* CEN.PK 113–7D for up to 100 generations in consecutive 3-ml batch cultures at 30 °C and 300 rpm. Two types of carbon and nitrogen source combinations were used, with 0.1% (w/v) glucose, 33 mM Cr, and 0.5% (w/v) ammonium sulfate or 0.5% (w/v) glucose, 0.1% (w/v) ammonium sulfate, and 22.5 mM Cr, respectively. As positive and negative control, cultures with combinations of 0 or 0.5% (w/v) glucose, as well as 0 or 0.5% (w/v) ammonium sulfate, were also used. After 24 h, the next batch culture was inoculated with 1.5% (v/v) in fresh medium, and the intracellular Cr concentration was measured. To detect metabolism of Cr, 3-ml yeast minimal medium cultures were grown for up to 6 days at 30 °C and 300 rpm, with 40 mM Cr (as the sole carbon source) and 0.5% (w/v) ammonium sulfate or 0.5% glucose (w/v) and 28 mM Cr (as the sole ammonium source).

Transient pH stress experiments were performed in 10-ml culture tubes with 3 ml of medium at 30 °C on a gyratory shaker at 300 rpm. Cultures were grown to an OD_{600} of about 0.5, before the pH was set to 2 with 10% (v/v) H_3PO_4. After 1 h, pH 5 was re-established using 100 mM 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.

Starvation stress experiments were performed in glucose-limited chemostat cultures at a dilution rate of 0.1 h^{-1}. The culture volume was kept constant at 1 liter using a weight-controlled pump, and the pH was controlled at 5.0 by the addition of 2 M KOH. The airflow was kept constant at 1.0 liter/min, and the agitation speed was adjusted to 1′000 rpm. The temperature was kept constant at 30 °C. Oxygen and carbon dioxide concentration in the culture effluent gas were determined with a quadrupole mass spectrometer (Fisons Prima 600; Fisons, Oxbridge, United Kingdom). After cultures reached a stable steady state, defined as at least 5 volume changes with constant O_2, CO_2, and OD_{600} readings, the medium feed pump was programmed so that the feed was interrupted for 150 s and initiated for 30 s. These 180-s cycles were maintained until a new steady state was achieved, usually within 5 volume changes. For determination of intracellular metabolite concentrations, aliquots were withdrawn at least 30 s after the feeding was interrupted and before it was started again.

Constructions of Plasmids and Molecular Biology Procedures—pBCK was constructed by PCR amplification of the chicken B-CK gene from plasmid pT23–1[2] with the primers 5′-CGCACTAGTATGCCCTCCTCAAA-3′ and 5′-CGCGAATTCTTATTTCTGAGCTGG-3′. The resulting *Spe*I/*Eco*RI fragment was then cloned into p424HXT7. pMtCK was constructed by PCR amplification of the chicken sarcomeric MtCK gene with a cytochrome c_1 pre-sequence added as in-frame fusion for mitochondrial targeting from plasmid pFG8[3] with the primers 5′-GACTAGTATGTTTTCAAATCTATCTAA-3′ and 5′-CCGCTCGAGTCACTTCCTGCCAAACT-3′. The resulting *Spe*I/*Xho*I fragment was then cloned into p426HXT7. pAGAT was constructed by PCR amplification of the L-arginine-glycine amidinotransferase gene from pig (21) from plasmid pBS/SK−⁻:RGAT-7 with the primers 5′CGCGGATCCATGCTGCGGGTGC-3′ and 5′-CGCGAATTCTCAGTCCAAGTAGGAC-3′. The resulting *Bam*HI/*Eco*RI fragment was then cloned into p425HXT7. pGAMT was constructed by PCR amplification of the human guanidino acetate methyltransferase gene (22) from pGEM-T-4-hGAMT-7 with the primers 5′-CGCACTAGTATGAGCGCCCCCA-3′ and 5′-CGCGAATTCTCAGCCTTTGGTCAC-3′. The resulting *Spe*I/*Eco*RI fragment was cloned into p426HXT7. pAK was constructed by PCR amplification of the *Limulus polyphemus* AK gene (23) from plasmid pET-22b(+):AK with the primers 5′-GGAATTCATGGTGGACCAGGCAACATTG-3′ and 5′-TGCGGTCGACTTAGGCAGCAGCCTTTTCCATC-3′. The resulting *Eco*RI/*Sal*I fragment was cloned into p424HXT7. Plasmids were transformed in *S. cerevisiae* using the S.c. EasyComp kit (Invitrogen).

Analytical Procedures—Cell growth was monitored by the increase in OD_{600}. 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 $MgCl_2$, 1 mM EDTA, 5% (v/v) glycerol, 4 mM dithiothreitol, 0.3 M $(NH_4)_2SO_4$, and 1 mM phenylmethanesulfonyl 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 12′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—CK 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 $MgCl_2$, 83 μM EGTA, 1 mM β-mercaptoethanol, 4 mM ADP, and 10 mM of either PCr (CK) or PArg (AK), and the rate of stoichiometric proton consumption by conversion of PCr and ADP to Cr and ATP was monitored by titration.

Intracellular Metabolite Concentrations—Cr concentrations in supernatants and crude cell extracts were determined with 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 $MgCl_2$, 83 μM EGTA, 1 mM β-mercaptoethanol, 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 triethanolamine, 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-maleate, pH 7.0, and disrupted by boiling for 1 min at 95 °C 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-

[2] M. Stolz and T. Wallimann, unpublished data.
[3] R. Furter (deceased) and T. Wallimann, unpublished data.

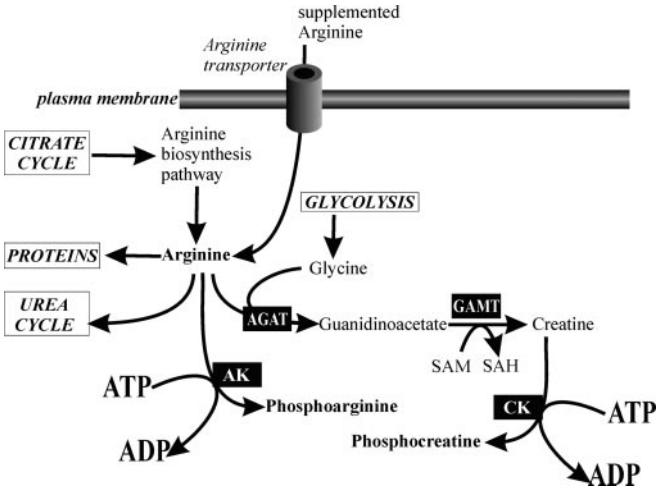

FIG. 1. ***S. cerevisiae* engineered with either arginine kinase or creatine kinase systems.** The scheme shows cellular uptake and biogenesis of the phosphagens analyzed. *Black boxes* indicate heterologous enzymes that are not naturally present in *S. cerevisiae*. *SAM*, *S*-adenosyl methionine; *SAH*, *S*-adenosyl homocysteine.

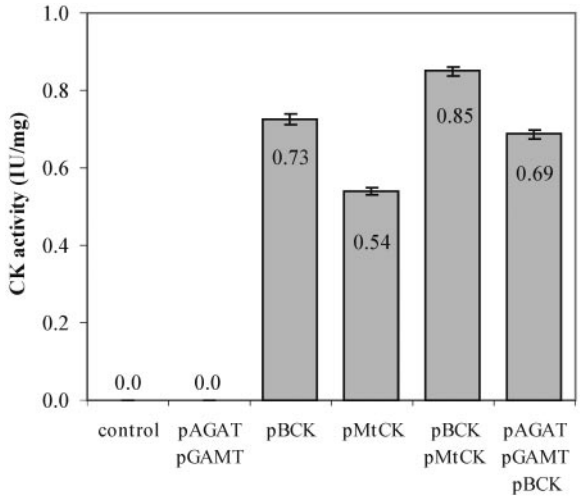

FIG. 2. **Specific creatine kinase activity in different CK-engineered yeast strains.** Enzymatic activity was determined in crude cell extracts harvested from stationary phase of *S. cerevisiae* CEN.PK 113–6B (*control*) and transformed yeast strains grown on yeast minimal medium with 0.5% glucose. The cytosolic brain-type isoform (as pBCK), as well as the mitochondrial sarcomeric isoform (as pMtCK), of CK are expressed alone, in combination, or together with the Cr synthesis genes pAGAT and pGAMT. Control with the Cr synthesis genes alone is also shown.

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 Na_2SO_4 and centrifuged for 5 min at 13′000 rpm to remove residual barium ions. Trichloroacetic acid was added to 2.3% (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) H_2SO_4, 10 mM Na_2MoO_4, and 10% (v/v) of a color reagent containing 10 g/liter polyvinyl alcohol and 185 mg/liter malachite 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 HS II (Roche Molecular Biochemicals). ADP was converted to ATP by incubating another aliquot for 30 min at 37 °C with 1 mM phosphoenolpyruvate and 1,250 units/ml pyruvate kinase, and the total ATP level was then determined. ADP concentration 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 $1.162 \cdot 10^{-11}$ ml and a concentration of 10^8 cells/ml at an OD_{600} of 1.5 (29).

Determination of Physiological Parameters—In batch cultures, the exponential growth phase was identified by log-linear regression of OD_{600} *versus* time, with maximal growth rate (μ_{max}) as the regression coefficient. To calculate specific production rates, OD_{600} values were converted to cellular dry weight (cdw) using a predetermined correlation factor (0.52 g/liter cdw per OD_{600} unit). The biomass concentration was calculated as cdw per volume unit. The CO_2 evolution rate (CER) was defined as the relative CO_2 production (ΔCO_2) multiplied by the effluent gas flow rate (F), on the basis of the relationship $CER = \Delta CO_2 \cdot F$. The specific production rate for CO_2 (q_{CO2}) was calculated by dividing the CER by the biomass concentration (X) and the culture volume (V), on the basis of the relationship $q_{CO2} = CER/(X \cdot V)$.

RESULTS

Establishing a Functional Creatine Kinase System by Co-expression 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 L-arginine:glycine amidinotransferase (AGAT) and rat guanidinoacetate methyltransferase (GAMT) yielded an intracellular concentration of about 3.2 mM Cr (Table I), 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

TABLE I
Intracellular creatine concentrations

Cr was determined in *S. cerevisiae* strains expressing creatine synthesis genes (pAGAT and pGAMT) and/or the cytosolic brain-type creatine kinase (pBCK).

Strain	Intracellular Cr concentration
	mM
Control	0.0 ± 0.0
pBCK	0.0 ± 0.0
pAGAT	0.12 ± 0.2
pGAMT	0.0 ± 0.0
pAGAT pGAMT	3.2 ± 0.2
pAGAT pGAMT pBCK	3.3 ± 0.2

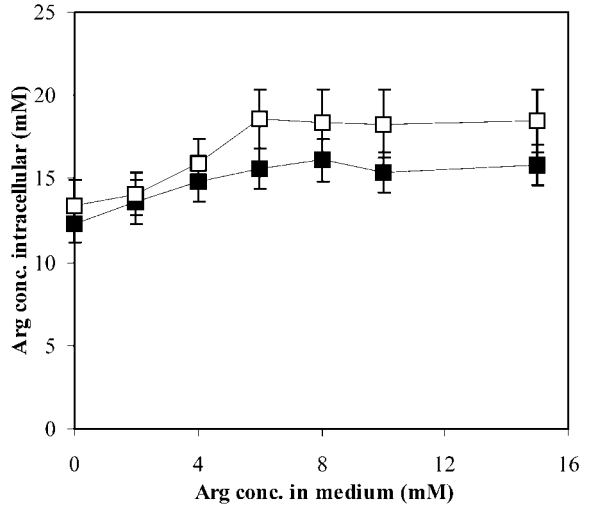

FIG. 3. **Dependence of intracellular arginine levels on medium arginine concentrations.** Arginine was determined in control (CEN.PK 113–6B p424HXT7; ■) and AK-expressing strain (CEN.PK 113–6B pAK; □) at different arginine concentrations in medium.

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 observed any improvement in maximal cdw and μ_{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_{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 μ_{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. 4*b*) In contrast, AK conferred the ability to reduce the recovery time from 3.3 to 2.3 h (Fig. 4*a*). 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 μ_{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

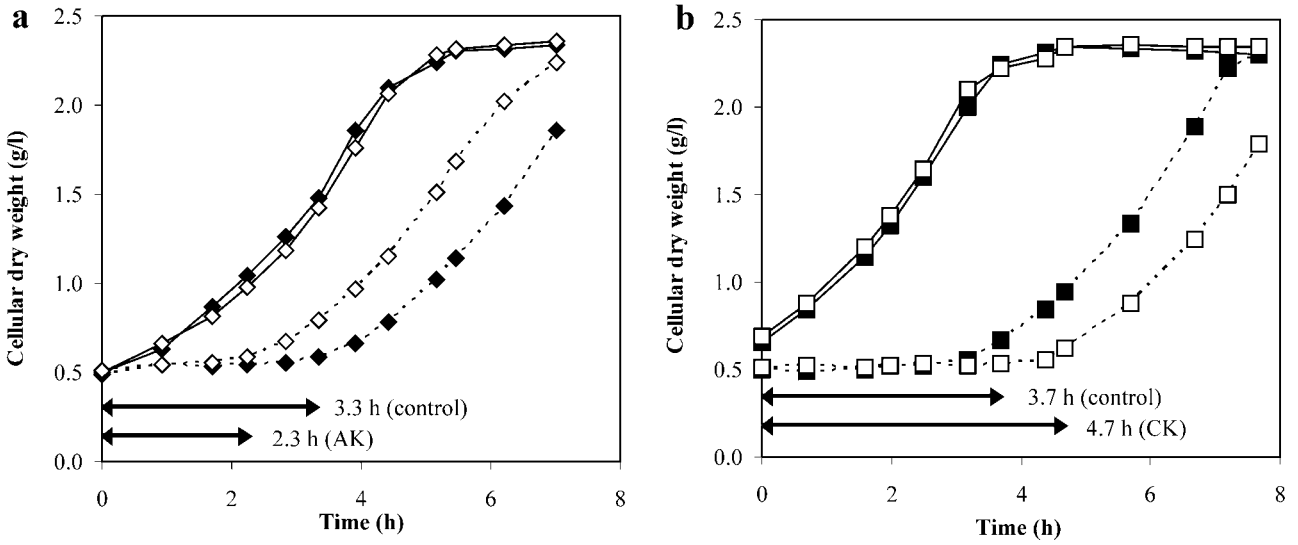

FIG. 4. **Influence of intracellular acidification on growth of engineered yeast strains.** Growth of *S. cerevisiae* CEN.PK 113–6B harboring either (*a*) the AK (◇) or (*b*) the CK (□) system is given as the increase in biomass concentration, together with the relative controls (◆ or ■). Yeast cultures were grown in shake flasks on yeast minimal medium, either kept at normal pH (pH 5; *continuous lines*) or subjected to transient pH stress (1 h at pH 2; *dashed lines*). The stress experiment was initiated by addition of acid at $t = 0$; the time needed to recover full growth rate after return to normal pH is shown at the *bottom*. The figure shows one representative of three independent experiments performed.

TABLE II
Growth and specific CO_2 production rate

OD_{600} and specific CO_2 production rate (q_{CO2}) in steady state before and in pseudo steady state in continuous cultures of control and AK-expressing *S. cerevisiae* are shown. Continuous feeding was done at a D of 0.1 h^{-1}, and discontinuous feeding was obtained with a D of 0 h^{-1} for 150 s and 0.1 h^{-1} for 30 s. For discontinuous feeding, the q_{CO2} values during the 150-s starvation period are given. The data of two independent experiments are given for pseudo steady states.

Feed type	Plasmid	OD_{600}	q_{CO2}
			mmol·g⁻¹·h⁻¹
Continuous	Control	4.44	0.69 ± 0.07
Continuous	pAK	4.92	0.52 ± 0.05
Discontinuous	Control	3.25	0.15 ± 0.02
Discontinuous	Control	3.28	NA
Discontinuous	pAK	4.28	0.07 ± 0.01
Discontinuous	pAK	4.34	0.07 ± 0.01

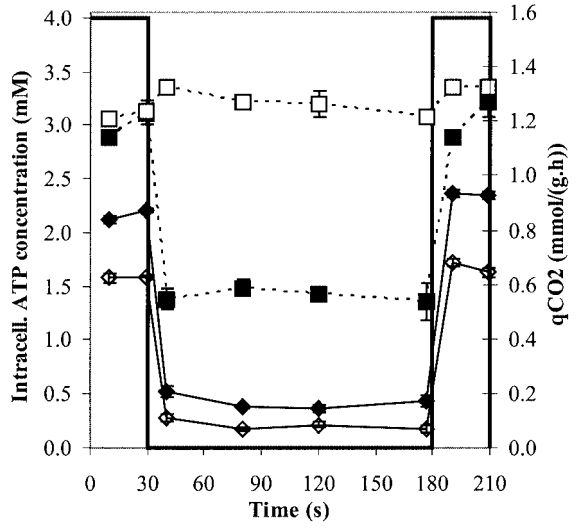

FIG. 5. **Time course of CO_2 production and intracellular ATP during starvation stress in AK-engineered yeast.** The specific CO_2 production rate (q_{CO2}) (◇ and ◆; *continuous lines*) and intracellular ATP concentration (□ and ■; *dashed lines*) during starvation in chemostats of *S. cerevisiae* CEN.PK 113–6B pAK (*open symbols*) and control (*filled symbols*) are shown. The *bold line* represents the discontinuous feeding profile (30 s on, 150 s off). Values are given as mean ± S.D. of three independent measurements over a period of 2 days.

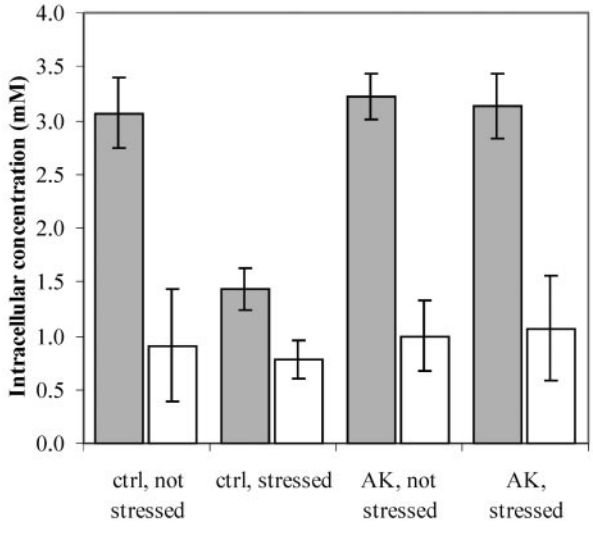

FIG. 6. **Intracellular ATP and ADP in control and AK-engineered yeast.** Shown are concentrations of ATP (*gray bars*) and ADP (*white bars*) in steady state before stress begins (*not stressed*) and in pseudo steady state (*stressed*) in continuous chemostats of control (*ctrl*) and AK-expressing *S. cerevisiae*. Concentrations during pseudo steady state were determined in the middle of the 150-s feed interruption. The data represent the mean of three independent measurements over a period of 2 days in pseudo steady state. The samples were withdrawn at least 30 s after the feed offset and before the feed onset.

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 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 *S. cerevisiae* strains to analyze intracellular ADP concentrations (31). In these cultures, when maintained at 100 mM Cr, Brindle *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.[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 *S. cerevisiae* and that transformation with AGAT and GAMT 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 *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^+-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 then 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 *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 isoenzymes 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 *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 *et al.* (41), who investigated metabolic responses to a glucose pulse in glucose-limited chemostat culture.

Given the protective effects of AK in *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, GAMT) 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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