The relevance of transhydrogenases and heterologous phosphagen kinases for microbial cofactor metabolism

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The relevance of transhydrogenases and heterologous phosphagen kinases for microbial cofactor metabolism

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When I think back to the time point where I began my PhD, and further back to the years of the study, it is surprising how many people one recalls as have being important in a way or another. In this section, it is not possible to name them all, but to some people I owe a tribute, because without them I would not have succeeded in finishing my PhD.

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# TABLE OF CONTENTS

**SUMMARY / SOMMARIO** .......................................................................................................................... 7

**CHAPTER 1** General introduction ........................................................................................................... 11

**CHAPTER 2** Metabolic flux response to phosphoglucoisomerase knock-out in *Escherichia coli* and impact of overexpression of the soluble transhydrogenase UdhA ........................................................................................................ 55

**CHAPTER 3** Dissecting the role of soluble and membrane-bound transhydrogenases UdhA and PntAB in *Escherichia coli* ................................................................................................................................. 69

**CHAPTER 4** Functional expression of phosphagen kinase systems confers resistance to transient stresses in *Saccharomyces cerevisiae* by buffering the ATP pool ............................................................................................................. 95

**CHAPTER 5** Functional expression of arginine kinase improves recovery from pH stress of *Escherichia coli* ...................................................................................................................................................... 119

**CONCLUSIONS AND OUTLOOK** ........................................................................................................ 133

**ABBREVIATIONS** .................................................................................................................................... 135

**LIST** ..................................................................................................................................................... 139

**CURRICULUM VITAE** ............................................................................................................................ 139

5
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SUMMARY

The scientific topic of this thesis is how micro-organisms generate NADPH and how they can be equipped with ATP buffering systems that provide transient protection during energy stress. ATP and pyridine nucleotides are essential cofactors for cellular metabolism, linking catabolism to anabolism. While NADH is almost exclusively utilized for energy generation, NADPH provides reducing power for biosynthetic reactions.

In Chapter 2 and Chapter 3, we show through mutant construction and metabolic flux analysis that the two transhydrogenases of *Escherichia coli* have distinct roles in NADPH metabolism. The membrane-bound transhydrogenase PntAB is an important source of NADPH during exponential growth on glucose by catalyzing the transhydrogenation of NADH to NADPH. The soluble transhydrogenase UdhA, in contrast, catalyzes the reverse reaction from NADPH to NADH. This function is not necessary during growth on glucose but critical on substrates such as acetate, the catabolism of which generates a higher proportion of NADPH.

Vertebrates contain so-called phosphagen kinase systems, which constitute spatial and temporal energy buffering systems to overcome periods of fluctuating energy demand. In Chapter 4 we install such phosphagen kinase systems in *Saccharomyces cerevisiae* that normally does not contain such systems. We then show the functional expression of arginine kinase, one particular phosphagen kinase system. Environmental conditions of fluctuating energy demand are installed by growing *S. cerevisiae* in glucose-limited chemostat culture with interrupted feeding. In the absence of glucose feeding, the intracellular ATP level was shown to drop significantly in the control strain, but it was stabilized at a much higher level in the arginine kinase-expressing strain. Our results demonstrate that temporal energy buffering is an intrinsic property of phosphagen kinases that can be transferred to phylogenetically very distant organism.
The 5th chapter affords then a short study on the expression of arginine kinase in the bacterium *E. coli*. Specifically, we could show that arginine kinase expression significantly improves the recovery after a transient pH stress, that was anticipated to exert among other effects and ATP stress.
"Gli ostacoli sono quelle cose spaventose che si scorgono quando si perde di vista il proprio obiettivo"

Henry Ford

SOMMARIO

L'argomento scientifico di questa tesi è come l’NADPH venga generato nei microorganismi e come questi possano venire equipaggiati con sistemi tampone per l’ATP in grado di fornire una protezione transitoria durante lo stress energetico. L’ATP e i nucleotidi a piridina sono cofattori essenziali del metabolismo cellulare e collegano l’anabolismo al catabolismo. Mentre l’NADH è utilizzato quasi esclusivamente per la produzione di energia, l’NADPH fornisce potere riduttivo per le reazioni biosintetiche.

Nel Capitolo 2 e nel Capitolo 3 mostriamo tramite la costruzione di mutanti e l’analisi dei flussi metabolici che le due transidrogenasi del batterio Escherichia coli hanno ruoli distinti nel metabolismo dell’NADPH. La transidrogenasi membranare PntAB è un importante sorgente di NADPH durante la crescita esponenziale su glucosio, catalizzando la transidrogenazione dell’NADH in NADPH. Questa funzione non è necessaria durante la crescita su glucosio, ma è critica su substrati come l’acetato, il cui catabolismo genera una in proporzione più NADPH.

I vertebrati contengono il cosiddetto sistema di fosfagene chinasi, che costituisce un sistema tampone spaziale e temporale per affrontare momenti di fluttuazione energetica. Nel Capitolo 4, installiamo un tale sistema di fosfagene chinasi nel lievito Saccharomyces cerevisiae che ne è normalmente privo. In seguito mostriamo l’espressione funzionale della arginina chinasi, un particolare membro della famiglia delle fosfagene chinasi. Condizioni ambientali che causano fluttuazioni nella domanda di energia vengono generate crescendo S. cerevisiae in un medium di coltura limitato in glucosio con alimentazione discontinua. In assenza di una alimentazione di glucosio, il livello intracellulare di ATP diminuisce in maniera significativa nel ceppo di controllo, ma risulta stabilizzato e mantiene un livello più alto nel ceppo in grado di esprimere arginina chinasi. I nostri risultati dimostrano quindi come la funzione di tampone temporale sia una proprietà intrinseca delle fosfagene chinasi trasferibile ad organismi filogeneticamente molto distanti.
Il 5° capitolo affronta un breve studio sull’espressione dell’arginina chinasi nel batterio *E. coli*. Nello specifico, mostriamo come l’espressione dell’arginina chinasi migliori in maniera significativa il recupero da uno stress transiente da pH, conosciuto per generare tra gli altri effetti uno stress da ATP.
General introduction
TWO FACTORS LINK CATABOLISM TO ANABOLISM: ATP AND NADH

In principle, all living organisms have two goals. Firstly, they need to survive environmental challenges, and secondly they need to generate offspring. In every living organism, from the most primitive and simple microorganism to human being, all cellular activity relies on the availability of energy.

The universal elementary energetic quantum for biological systems is adenosine triphosphate (ATP), this being a fundamental and dogmatic issue in the universal nature of biochemistry (112). Its hydrolysis is highly exergonic and may be coupled to the numerous endergonic cellular processes so as to drive them to completion. In non-photosynthetic living organisms, ATP can be generated either by substrate-level phosphorylation or by oxidative phosphorylation.

Energy is provided by breakdown of growth substrates to smaller molecules by a series of enzymatic reactions, organized as a network of so called catabolic pathways. Here, reductive charge is produced in form of the two reduced pyridine nucleotides NADH and NADPH (Figure 1). Although structurally very similar, the two are not metabolically interchangeable. In fact, whereas the reducing power of NADH is exploited to accomplish the otherwise endergonic ATP synthesis, NADPH is involved in utilizing the free energy of metabolite oxidation for otherwise endergonic reductive biosynthesis, such as e.g. the biosynthesis of fatty acids. This ability of the metabolism to differentiate between NADH and NADPH is guaranteed by the high degree of specificity of dehydrogenases towards their respective cofactors.

![Diagram](image)

Figure 1. Link of the two pyridine nucleotides NADH and NADPH with anabolism and catabolism.

In oxidative phosphorylation (also called respiration) the free energy of metabolite oxidation is exploited to synthesize ATP. In general, organic substrates are oxidized by
enzymes exhibiting specific dehydrogenase as well as quinone reductase activities. Thus, electrons are transferred to mobile quinones, which deliver the reducing equivalent to terminal oxidoreductases. The latter enzymes are not only quinole oxidases, but also transfer the reduction potential to specific electron acceptors. The free energy of this electrons transfer is conserved by transporting $\text{H}^+$ on the outside of the membrane in which the respiratory complex is integrated. The generated electrochemical $\text{H}^+$ gradient across the membrane is then exploited by the membrane-integrated proton-translocating ATP synthase ($\text{H}^+\text{-ATPase}$), which is able to generate ATP from ADP and free phosphate.

The efficiency of oxidative phosphorylation is therefore strongly influenced by the availability of the organic substrate oxidized in the first step of respiration. During aerobic growth on glucose in *Escherichia coli*, this reduction power, essential to provide the cell with energy in form of ATP, is supplied by reduced nicotinamide adenine dinucleotide (NADH). This is oxidized to NAD$^+$ by NADH dehydrogenases I and II and the electrons are transferred by terminal oxidases to oxygen (107,163).

We shall not forget, however, that depending on the organism considered, but also on the growth condition and substrate availability, many different substrates may be utilized by dehydrogenases to oxidize quinones. In *E. coli*, other electron donor may be utilized, e.g. formate as oxygen is not present, or glycerol-3-phosphate if cells has phospholipids to breakdown (107).

The central role played by ATP and reduced pyridine nucleotides includes a series of involvements of these molecules in cellular regulation, both allosterically and non-allosterically. Because ATP synthesis and utilization involves a cyclic flow through ADP and AMP, it makes sense to find that all three adenylates play regulatory roles in fueling reactions as well as in biosynthetic pathways. Some enzymes are regulated primarily by the concentration of ATP, ADP or AMP; others respond to the ratios $[\text{ATP}]:[\text{ADP}]$ or $[\text{ATP}]:[\text{AMP}]$. A summary index of the cellular energy status, can be expressed defining the energy charge as

$$E = \frac{[\text{ATP}]+\frac{1}{2}[\text{ADP}]}{[\text{ATP}]+[\text{ADP}]+[\text{AMP}]}$$

Energy charge reflects the relative number of high-energy phosphate bonds in the adenylate pool and the aggregate of the controls by adenylates on a large number of enzymes. The compound ADP, with a single anhydride bound phosphate, is assigned one-half the charge value of ATP. The activity of adenylate kinase provides a physiological reason, in
addition to the mathematical one, for assigning to ADP one-half the charge value of ATP. This enzyme, the activity of which is quite high in E. coli, catalyzes the reaction
\[ \text{AMP} + \text{ATP} \rightleftharpoons 2 \text{ADP} \]
thereby establishing an equilibrium between ADP and ATP with a stoichiometry of 2 to 1. The energy charge is calculated from the actual intracellular concentrations of the three adenylates, but it reflects their relative proportions. The energy charge of a cell can vary mathematically from 0 (all AMP) to 1 (all ATP); but in fact, the energy charge of bacteria under normal conditions will not lie outside the range 0.87 to 0.95, and it is invariant with growth rate. The energy charge of a cell deprived of a substrate for long periods decreases slowly; when it reaches a value of approximately 0.5, the cell dies.

The necessity for the existence of two species of pyridine nucleotides in cells undergoing aerobic metabolism seems apparent as a consequence of three facts. First, dehydrogenase reactions involving pyridine nucleotides have equilibrium constants near 1. Secondly if in fueling reactions, oxidized pyridine nucleotides are the reactants, in biosynthetic reactions and energy generation, they exert their function in reduced form. Thus, for fueling reactions to proceed, the participating pyridine nucleotide pool must be largely oxidized; for biosynthetic reactions, it must be largely reduced. Indeed, within the cell, most NAD is in the oxidized form and most NADP in the reduced form. Useful parameters of the oxidoreduction state are

\[
\text{ARC (anabolic reduction charge): } \text{ARC} = \frac{[\text{NADH}]}{[\text{NADH}]+[\text{NAD}^+]} \\
\text{CRC (catabolic reduction charge): } \text{CRC} = \frac{[\text{NADPH}]}{[\text{NADPH}]+[\text{NADP}^+]} 
\]

The CRC in growing cells is maintained at a low level of 0.03 to 0.07, and the ARC is approximately 10-fold higher.

The levels of these charges undoubtedly play a vital role in coordinating fueling reactions with biosynthesis. Bacteria may set the levels in two ways. Firstly, by regulating the relative pathway usage, as depicted below. Secondly, by utilizing an enzyme called transhydrogenase, which will be extensively described in a separate section. Here a vectorial reaction (proton transport) is coupled to a scalar reaction (oxidoreduction), resulting in a change of the apparent equilibrium constant of the redox system. On energization of the mitochondrial membrane, the mass action ratio \( \Gamma \) for nicotinamide nucleotides, calculated as

\[ \Gamma = \frac{[\text{NADPH}][\text{NAD}^+]}{[\text{NADP}^+][\text{NADH}]} \]

can reach values up to 500.
The central carbon metabolism is the core of cellular metabolism

The complexity of the catabolic network is due to the different possibilities that are often available for a given conversion or synthesis of metabolites. In fact, where main pathways are branched, a significant portion of the carbon flux may be redirected to alternative pathways. The core of substrate breakdown is the central carbon metabolism (Figure 2). Here, glucose is converted to trioses either by glycolysis, pentose phosphate (PP) pathway, or Entner-Doudoroff (ED) pathway. The trioses formed either by glycolysis or by ED Pathway are then converted to AcCoA, which is either accumulated as acetate or enters the tricarboxylic acid (TCA) cycle.

The so cited pathways differ not only by their intermediates that serve also as starting point for the biosynthesis of more complex cellular building blocks, but provide also different types and amounts of pyridine nucleotides, and provides substrate-level phosphorylation of ADP to ATP. Glycolysis provides NADH but not NADPH, and PP pathway provides NADPH but not NADH. The TCA cycle provides both reduced pyridine nucleotides, but NADH to a larger extent, while the ED pathway does not generate any reductive charge. The branching points which connect two pathways are therefore of fundamental importance for control of NADPH and NADH production. Substrate-level phosphorylation of ATP is provided in glycolysis by pyruvate kinase, in TCA by succinate thiokinase, and in overflow metabolism by acetate kinase.

One of the main branching points in central carbon metabolism of E. coli is surveyed by two enzymes: phosphoglucone isomerase, the product of the pgi gene and first enzyme of the glycolytic cascade, and glucose-6-phosphate dehydrogenase, the product of the zwf gene and the first enzyme of the PP pathway. These two enzymes are well conserved throughout evolution and their cellular production rate varies very little during different growth phases (56). Phosphoglucone isomerase catalyses the reversible conversion of glucose 6-phosphate (G6P) to fructose 6-phosphate (F6P), and it is present as a dimer or tetramer (107), both in periplasm and cytoplasm. It is unclear whether these two forms have different functions, but it is known that the dimer is not the precursor of tetramer, and they are not correlated with the localization (135). The expression rate of pgi is doubled under anaerobic conditions (135). Mutants lacking phosphoglucone isomerase grow one third as fast as wild type on glucose but rapidly on fructose (57). If grown on substrates that do not provide G6P, these pgi mutants still grow, since G6P appears to be not essential (56).
Figure 2. Reaction network of E. coli central carbon metabolism. The arrows indicate physiological reaction directionality and enzymes are indicated by their 3-letter code. Abbreviations: G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; 6PG, gluconate 6-phosphate; KDPG, 2-keto-3-deoxy 6PG; P5P, pentose 5-phosphate; E4P, erythrose 4-phosphate; S7P, sedoheptulose 7-phosphate; GAP, glyceraldehyde 3-phosphate; PGA, 3-phosphoglycerate; PEP, phosphoenolpyruvate; AcCoA, acetyl coenzyme A; OGA, oxoglutarate; OAA, oxaloacetate; Eda, KDPG aldolase; Edd, 6-phosphogluconate dehydratase; UdhA, soluble transhydrogenase, PntAB, membrane-bound transhydrogenase; Zwf, G6P dehydrogenase; Pgi, phosphoglucone isomerase; PP pathway, pentose phosphate pathway; ED pathway, Entner-Doudoroff pathway; TCA, tricarboxylic acid.

G6P dehydrogenase (Zwf) catalyses the irreversible conversion of G6P to 6PG, and is normally expressed constitutively. However, an increase in the expression rate is detected in the presence of superoxide agents (56), consistently with the increased need for NADPH during oxidative stress. Mutants lacking G6P dehydrogenase do not show any significant changes in growth rates on glucose and fructose, and grow only slightly slower on gluconate (55,93).
A second relevant branching point, is situated immediately after the step catalyzed by G6P dehydrogenase. Here 6PG may either flow to subsequent steps of PP pathway, where 2 moles of NADPH are produced per mol of 6PG, or enter ED pathway, where neither NADPH nor NADH are produced. Studies on the ED pathway of E. coli began more than thirty years ago (178), but only recently this pathway found renewed interest. It is thought that the ED pathway had an essential evolutionary role in allowing E. coli and other enterobacteria to occupy the intestine of mammalian, in which sugar acids are the main substrates (147). Until now, however, it was not possible to determine which of these substrates is the most important for intestinal colonization (114).

The ED pathway consists of 6PG dehydratase (encoded by the edd gene) and the 2-keto-3-deoxy 6PG (KDPG) aldolase (encoded by the eda gene). Furthermore, two transport and phosphorylation enzymes for gluconate are present. The former one is encoded by gntU, and exhibits low affinity, whereas the other, encoded by gntT, exhibits high affinity. The latter one is inducible by gluconate and has a main role during growth with low cell density. The expression of these genes is negatively controlled by the product of the gntR gene, which is induced by gluconate. Other gluconate transporter and phosphorylation systems are known, but The question of why so many are needed is still unanswered (114). Other roles for Edd and Eda have also been proposed, including respiration recovery after SOS-response and neutralization of some toxic compounds. Eda mutants have been generated, showing that their growth rate does not change, compared to the wild type, when grown on glucose or fructose, but it is reduced to one third when grown on gluconate. Since no cofactors are needed in ED pathway, no cofactor recycling is necessary, and ED pathway should be a "fast" pathway, if compared to the PP pathway (178).

**REGULATION OF THE BALANCE BETWEEN NADH AND NADPH BY THE TRANSHYDROGENASE SYSTEM**

Pyridine nucleotide transhydrogenase catalyzes the stereospecific transfer of reduction equivalents (hydride ion equivalents) between NADH and NADPH, according to the following reaction

$$nH^+_{out} + NADP^+ + NADH \rightleftharpoons NADPH + NAD^+ + nH^+_{in}$$

Depending on the stereospecificity of the hydride transfer, two classes of transhydrogenases have been identified. The AB transhydrogenase activity is found in the
Cytosolic membrane of many heterotrophic and photosynthesizing bacteria (82,111), as well as in parasitic protozoans (160,176), in chloroplast of plants (164), and in mitochondria of animals. It couples the translocation of a proton (n=1) across the membrane to the specific transfer of both the 4A hydrogen from the nicotinamide ring of NADH to NADP⁺ and of the 4B hydrogen from NADPH to NAD⁺ (34,84), and it is therefore referred as energy-linked or proton-translocating (71). The BB transhydrogenases transfer the 4B hydrogen from both NADPH and NADH through a ping-pong bi-bi-reaction mechanism, without coupling to proton translocation (n=0) (36,164), therefore being referred as non-energy-linked. The latter activity is found only in a few heterotrophic bacteria such as E. coli (13), Pseudomonas spp. (58), and Azotobacter vinelandii (164).

Energy-linked transhydrogenases are located in the inner membrane of animal mitochondria and in the cytoplasmic membrane of many bacteria (84), and show high structural, but important differences in polypeptide organization (111). The enzyme from E. coli has an amino acid sequence similar to that of the transhydrogenase of higher animals but is split into the PntA and PntB polypeptides (α and β) having 510 and 460 amino acids residues, respectively, organized in an α₂β₂ unit (35). All proton-translocating transhydrogenases have three major structural components, designated dI, dII, and dIII, which probably assemble with a universal topology. Both dI, which bind NAD⁺ and NADH, and dIII, which binds NADP⁺ and NADPH, protrude from the matrix, on the cytosolic side of membrane. The binding-change mechanism by which the hydride transfer occurs between dI and dIII domain has recently been clarified by X-ray crystallography and separate in vitro studies of domains, as illustrated in (9,10,83,84). Briefly, with no bound substrate, the dI:dII:dIII assumes an ‘open’ conformation, which is switched to ‘occluded’ by substrate binding. So, dI and dIII come closer together and hydride transfer occurs. Structural studies on dI have suggested that transhydrogenase and alanine dehydrogenase may have a common ancestor, which was recruited by a membrane protein (the equivalent of dII/dIII) during the evolution of transhydrogenase (85). Domain dII is membrane integrated, and is split in dIIa and dIIb, part of the PntA and PntB peptides, respectively. There are twelve trans-membrane domains that form a channel through which proton translocation occurs. The structure of dII and the mechanism of proton-translocation have been studied extensively (10,71,72,83-85,111). An interesting feature has been proposed, by which the degree of protonation of residues in the dII domain regulates the activity of the energy-linked transhydrogenase (61). Besides the possible involvement of the cAMP/CRP system (40), this is the only regulatory issue known concerning transhydrogenase.
Like most proton pumps, the proton-translocating transhydrogenase can either use or generate a proton gradient, depending on the direction of the reaction catalyzed (155). The hydride transfer from NADH to NADP⁺ (forward reaction) is slow in the absence of a proton gradient, but is nevertheless coupled to proton extrusion (111). In the presence of proton gradient generated by another proton pump, the rate of the same reaction is increased by 5-10 times. The opposite (reverse) reaction is faster and its maximal velocity approaches that of the proton gradient driven forward reaction (97).

Non-energy-linked transhydrogenases are the intriguing property of only a few organisms. This class of transhydrogenases show high sequence similarity to each other but is structurally unrelated to energy-linked transhydrogenases. They are soluble flavoproteins containing flavin adenine dinucleotide (FAD) and are remarkable for the ability to form large polymers, which in E. coli are composed by seven or eight monomers (12,13). This enzymes are generally activated by NADPH and inhibited by NADP⁺ (13). The presence of a soluble transhydrogenase in E. coli escaped identification till 1999, when a sequencing error of E. coli genome was discovered, and the product of the udhA gene was found to be homologous to the soluble transhydrogenase from P. fluorescens (13). E. coli is thereby the first organism reported to possess both a soluble and a membrane-bound pyridine nucleotide transhydrogenase. Later on (25,26), a non-energy-linked transhydrogenase has been identified in potato tuber and pea leaves, whereas in the same tissue no energy-linked transhydrogenase activity was detected. So far, few investigations have been performed on the further catalytic and structural properties of soluble E. coli transhydrogenase (12,13).

Ever since its discovery, the physiological role of transhydrogenases has been a source of speculation and matter of controversy. Earlier theories hypothesized an essential function in NADPH generation or maintenance of the electrochemical potential. Since, however, transhydrogenase deletion is not lethal (179) and alternative cellular sources exist for both NADPH production and generation of the proton gradient, an essential role is excluded. On the basis of a thermodynamic and kinetic approach, Hoek and Rydström proposed a protective function in maintaining the proton gradient or under conditions of high demand for NADPH, such as during oxidative stress due to toxic agents or reoxygenation after anoxia (75). In E. coli NADPH is not only used in reductive biosynthesis, but is also of great importance in reducing glutathione, which is an essential component of the response mechanism to oxidative stress.

Besides E. coli, which at that moment is a unique and particular case, those organisms in which transhydrogenases are found, have either the energy-linked or the non-energy linked
transhydrogenase. By considering thermodynamic argumentations, Voordouw attempted to provide a rationale for the distribution of the two transhydrogenases (164). He suggested that the energy-linked transhydrogenases are responsible for the formation of NADPH where catabolism is NAD\textsuperscript{-}\textsuperscript{+}-linked, such as in *E. coli* or in animal mitochondria. The non-energy-linked transhydrogenase, in contrast, is supposed to act in reoxidizing NADPH in organism with partially or completely NADP\textsuperscript{+}-linked catabolism. This hypothesis, however, cannot explain the simultaneous presence of both transhydrogenase in *E. coli*.

The two transhydrogenase isoforms found in other micro-organisms show strong structural and catalytic similarities to the *E. coli* enzymes suggesting a similar function for these enzymes in all these organisms, although still no study has been conducted in this direction. In animals and plants, transhydrogenase is present in a mitochondrial isoform that is more similar to the *E. coli* membrane-bound one, and shows tissue-dependent level of transcription (2). Due to the reduced environmental variations to which e.g. human tissues are subjected, the presence of a soluble transhydrogenase catalyzing the NADPH to NADH reaction does not resemble to be necessary.

Organisms without a transhydrogenase are thought to have no overproduction of NADPH (164). Imbalance in the reductive charge distribution between NADPH and NADH is, however, a common problem in transhydrogenase-lacking organisms. Some of them were able to overcome the problem by using a system of two or more enzymes, which together may give a transhydrogenase-like activity (25). Special cases of NADPH metabolism are yeasts, and in particular *S. cerevisiae* where the low utilization of the PP pathway (51) causes an increased production of NADH (109). In anaerobic processes aimed at the production of ethanol, NADH cannot be exploited by the respiratory chain, and therefore it is utilized to synthesis glycerol. The yield of ethanol is strongly reduced and therefore also the rentability of the process. In *S. cerevisiae*, which do not exhibit transhydrogenase activity (23), both the membrane-bound as well as the soluble transhydrogenase from *E. coli* were expressed, based on the idea that the reductive charge would be transferred from NADH to NADPH, thereby improving the productivity. In both cases it was showed, however, that both enzymes tend to catalyze the reaction on the opposite direction (1,108). In another study, UdhA was shown to compensate for the lethal Pgi knockout in *S. cerevisiae* (51). Another biotechnological application of the same soluble transhydrogenase was found as a cofactor regeneration system in a cell-free system for the biological production of hydromorphine (12).

Further biotechnological applications of transhydrogenases rely on our knowledge of the mechanisms related to cofactor metabolism. The investigation of the role of
transhydrogenases in *E. coli*, the sole organism with both the energy-linked and the non-energy-linked transhydrogenases, may be a fundamental step in this direction. To accomplish this task it is essential to have the tools to modify cofactor metabolism and examine the effects on the whole metabolism.

**FLUX ANALYSIS IS A POWERFUL TOOL TO TRACK METABOLIC VARIATIONS**

Strategies for the dissection of the *in vivo* role of a certain gene or enzyme include the generation of deletion mutants and of strains grown under non-standard conditions. Classical approaches, such as physiological analysis, are often not able to depict the intracellular consequences of these procedures, which often occurs at a metabolic level and are reflected in metabolic fluxes. Metabolic flux analysis is aimed to the determination of metabolic fluxes, and is therefore a potentially powerful tool for the investigation of global metabolic responses (131).

Flux analysis is commonly performed by stoichiometric flux balancing of quantitative physiological data (158). This method uses linear algebra, which is used to solve a system of linear stoichiometric relationships, where the unknowns are intracellular fluxes. Since normally the system is underdetermined, it is often not possible to solve the system for a single solution.

A recent improvement in metabolic flux analysis is the use of isotope tracing. A commonly used method involves the feeding with $^{13}$C-glucose, and analysis of the $^{13}$C-labeling pattern in proteinogenic amino acids. Here the distribution of carbon fluxes at branching points of the central carbon metabolism is reflected in the labeling pattern of amino acids and this may be applied to the stoichiometric matrix to reduce the number of constraints. The practical question of the detection of the relative abundance of isotope isomers or isotopomers, reflecting the labeling pattern of amino acids, may be addressed in many ways. In a first approach, fractional $^{13}$C-enrichments at specific positions of metabolic products, resulting from growth on specifically $^{13}$C-labeled carbon substrates, are determined using 1D $^{13}$C NMR spectra of the purified compounds (41,101). Alternatively, uniformly labeled and natural abundance carbon substrates are fed. The information contained in the analyzed metabolic products is then the fraction and position of carbon bonds which have not been broken during synthesis from the labeled substrate, analyzed by 2D [$^{13}$C,$^1$H] correlation NMR spectroscopy (132,148,150). Specifically and uniformly labeled substrates have be combined in a method with allow to increase the amount of deduced information (134). Further
techniques involve the combination of NMR spectroscopy with mass spectrometry (MS), which may be complemented by the additional use of $^2$H or $^{18}$O labeled substrates, in order to further extend the collected information (149).

Considerable efforts concentrated on the development of computational methods to obtain qualitatively reliable flux analysis, which involves the laborious integration of a vast number of biochemical and physiological information in a model as close as possible to the real system (171). Recently, a new method for the determination of net metabolic fluxes from MS data and physiological data has been developed (52) and will be used in this work to analyze the rate of formation of pyridine nucleotides from central carbon metabolism pathways and through the transhydrogenase reaction.

**CELLULAR ATP BUFFERING: THE PHOSPHAGEN KINASE SYSTEM**

Despite its key role in cellular energetics, the availability of ATP is often a problem for metabolism. For example, ATP production rate depends significantly on the availability of substrates or on optimal environmental conditions. On the other hand, the energetic needs may suddenly increase because of activation of ATP consuming tasks, e.g. ion transport, or stress responses. Under optimal growth conditions, where ATP synthesis capacity possibly exceeds the cellular ATP needs, it would be very advantageous for the cell to stock ATP for moments where ATP consumption increases and metabolism cannot face such augmented energetic needs. Unfortunately, both ATP and ADP concentration, as well as the ATP to ADP ratio exert essential regulatory functions and therefore have to be kept constant under normal physiological conditions (59,167). This is an important issue, since the energy liberated by ATP hydrolysis is not fixed but strongly depends on the local nucleotide concentration, i.e. on how far the hydrolysis reaction is displaced from thermodynamical equilibrium (86).

A more or less sudden drop of ATP availability was faced by bacteria and primitive unicellular eukaryotes by optimizing the efficiency of carbon source transport systems, central carbon pathways as well as respiratory chain. However, as multicellular organisms evolved, their specialized tissues and cells like e.g. muscles, nervous cells and spermatozoa showed an increased energetic need. The tasks accomplished by these new tissues are coupled with a sudden and increased need of energy in form of ATP, which could not be entirely compensated improving the kinetic or thermodynamic parameters of energy producing pathways. Adaptations, like polyphosphate kinase in plants (157) or subcellular
compartmentation could not fully cover these energetic needs. Thus, the need for a fully new system arose, allowing the possibility to stock energy in another form.

A class of enzymes arose, able to transfer the phosphoryl group of ATP to a metabolically mostly inert molecule (a so called phosphagen) without significant loss of energy and with the possibility to constitute a large pool of such “high energy” compound if ATP production rate exceeds the need for energy. When, later, large amounts of energy are suddenly required, these enzymes will mainly work in the opposite direction, thereby reconstituting ATP from the phosphorylated phosphagen pool. We refer to this ability as the temporal energy buffering.

Eight phosphagens have been identified, all with the same basic chemical structure

\[
\text{HN} \underbrace{\text{R}}_{\text{NH}_2} \text{NH}_2
\]

(with \(R\) as in Table 1)

where the guanidino group is the target for phosphorylation (therefore phosphagen kinases are also called guanidino kinases) (43).

<table>
<thead>
<tr>
<th>R</th>
<th>Phosphagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH-(CH₂)₂-CHNH₂COOH</td>
<td>Arginine</td>
</tr>
<tr>
<td>NH-CH₂COOH</td>
<td>Glycocynamine (Guanidinoacetate)</td>
</tr>
<tr>
<td>N(CH₃)-CH₂COOH</td>
<td>Creatine</td>
</tr>
<tr>
<td>NH-(CH₂)₂-SO₂H</td>
<td>Taurocyanine</td>
</tr>
<tr>
<td>NH-(CH₂)₂-SO₃H</td>
<td>Hypotaurocyanine</td>
</tr>
<tr>
<td>NH-(CH₂)₂-HPO₄CH₃</td>
<td>Opheline</td>
</tr>
<tr>
<td>NH-(CH₂)₂-HPO₄-CH₂CH(NH₂)COOH</td>
<td>Lombricine</td>
</tr>
<tr>
<td>NH-(CH₂)₂-HPO₄-CH₂CH(N(CH₃)₂)COOH</td>
<td>Thalassemie</td>
</tr>
</tbody>
</table>

Table 1. The eight known phosphagens.

The general idea is that the system formed by arginine and arginine kinase (AK) is the common ancestor for the other guanidino kinase systems (170). This appears to be a reasonable hypothesis by considering how AK evolved as first, probably simultaneously as the appearance of metazoans (Figure 3). Moreover, the sole presence of a specific guanidino kinase is sufficient to make the system functional, whereas for the other phosphagens the simultaneous evolution of both a specific biosynthetic pathway for the phosphagen and a specific guanidino kinase was necessary. Therefore, in contrast to arginine, these phosphagens can be considered as metabolically ‘inert’ phosphagen.
Arginine is a proteinogenic amino acid and is involved in biosynthetic pathway, being thus a more “primitive” phosphagen. A second group of phosphagens (glycocyamine, lombricine, hypotaurocyamine) appeared as the result of the activity of specific amidinotransferase (Figure 4). They do not participate to other metabolic reaction and therefore metabolically inert. A decisive step towards the establishment of a highly efficient phosphagen kinase system was accomplished by the evolution of guanidino methyltransferases acting on the products of the amidinotransferase reaction. Among these new phosphagens, creatine (Cr) seems to possess unique properties which makes of the phosphocreatine / creatine kinase (PCr/CK) system the tool of choice for tissues with high and sudden energy demands (43).

Surprisingly, there is evidence for the evolution of AK from a CK ancestor, e.g. in chordates and in the sea cucumber *Stichopus japonicus* (43). The question about the driving force of such an involution are still unanswered, but some conclusions may be drawn by considering the physico-chemical properties of arginine and Cr, as well as the kinetic and thermodynamical characteristics of the corresponding guanidino kinases and the degree of complexity of the system.
Considering the mere physico-chemical properties of phosphoarginine (PArg) and PCr, it is notable that PCr is far more labile than other phosphagens, due to the presence of a methyl group vicinal to the guanidino group. This eliminates many of the resonance states, thereby destabilizing the compound (125). As a direct consequence, the apparent equilibrium constant for ATP formation under physiological conditions is around eight times higher for CK than for AK (38,42,94), thereby causing decisive differences on the ATP buffering behaviour of the two phosphagen kinase systems. A drop in pH causes a strong increase of this equilibrium constant for both CK and AK, but the effect is much stronger for CK, due to the thermodynamic properties of PCr (63,151-153).

Moreover, as mentioned before, the free energy change for ATP hydrolysis is highly dependent on the variation in ATP concentration. Under optimal physiological conditions and if ATP hydrolysis is kept far enough from equilibrium, free energy values up to -60 kJ/mol may be attained (86). The presence of a phosphagen kinase system increases and stabilizes the maximal free energy change for ATP hydrolysis up to -(75-80) kJ/mol, by keeping high and constant ATP concentrations. Because of these intrinsic thermodynamic properties, PCr can be considered as the best phosphagen for energy buffering.

In vertebrates, the amount of Cr needed is provided either by biosynthetic activities or by dietary supplement (175). In mammals, biosynthesis occurs in a two step reaction, but can
only barely cover the Cr need (Figure 5). The first step occurs in kidneys, and it is catalyzed by L-arginine:glycine amidinotransferase, which produces guanidinoacetate and L-ornithine out of L-arginine and glycine (78,175). Successively, guanidinoacetate is exported to the liver where S-adenosyl-L-methionine:N-guanidinoacetate methyltransferase catalyzes the methylation of guanidinoacetate to Cr (79,175). Cr is then exported to the blood vessels, thus reaching then the other CK-expressing tissues, such as muscles and brain. In lower vertebrates, the situation differs slightly, due to the different distribution of the biosynthetic enzymes (156). In invertebrates, no Cr biosynthesis has been detected. Those organisms express, however, CK, particularly in spermatozoa (43), thus forcing these species to accumulate Cr from the feed or from the environment (175).

![Figure 5. Biosynthesis of creatine in mammals.](image)

Transport of Cr from and to the tissues is operated by Na⁺- and Cl⁻-dependent Cr transporters (142). Intracellular Cr accumulation is one of the factors influencing the activity of these transporters (100). Cr and ornithine are strong allosteric inhibitors of AGAT, and thus of Cr biosynthesis (140,175). The intracellular Cr turnover (indirectly) and the efficiency of transport (directly) exert a strong influence on the biosynthesis of Cr (175).

Cr is degraded spontaneously to creatinine, without the need of a specific enzyme (175). This implies two important consequences for Cr-using cells. First, cells have to continuously import Cr to keep the Cr pool constant. Secondly, creatinine accumulates in the intracellular space and exerts a toxic effect because of the much lower solubility with respect to Cr. Therefore, Cr-dependent organisms have to dispose of a system to eliminate this degradation-product of the CK system.
Organisms using arginine as a substrate for AK are dealing with a much simpler system. Arginine is either synthesized in the urea cycle from ornithine or it is imported by the action of different kind of transporters (39,177). Being a proteinogenic amino acid and a basic constituent of some cellular activities, arginine has a central role in metabolism. Thus, the AK reaction can not cause a too drastic change in arginine concentration.

Besides temporal buffering, a second essential property of phosphagen kinase systems is the ability to function as an energy transport system especially in large and polar cells (165-167). For a molecule to be easily and efficiently distributed in the cell, the diffusivity is a crucial issue. Being strongly dependent from the molecular size and mass, the diffusivity of phosphagens is doubled for PCr and PArg with respect to MgATP. In addition, the diffusivity of PCr is again 10% higher than for PArg, making PCr the best suited phosphagen for fast energy transport (45).

The largest difference between the AK and the CK system concern the intracellular targeting and the tissue specificity of the enzymes. CK is known to exist as two cytosolic isoforms, M-CK and B-CK, which may form homodimers (MM-CK in skeletal muscles and heart, and BB-CK in neural tissues, brain, and other muscles) or heterodimers (in embryonic tissues). In addition, two mitochondrial isoforms (sMtCK, sarcomeric and uMtCK, ubiquitous) exist as homooctamers and homodimers. The thermodynamical, kinetic and structural properties of these isoenzymes have been extensively studied during the past years (for reviews see (33,105,128,133,166,174)).

However, mitochondrial activity has also been found for AK in some crustaceans (44,73,74), and in the tobacco hornworm Manduca sexta (30), and the presence of a mitochondrial AK in Drosophila melanogaster has been suggested (106). In the crabs Limulus polyphemus and Callinectes sapidus this activity appears to be located in the intermembrane space of mitochondria (116). However, this does not correspond to a true mitochondrial AK isoenzyme, since the AK isolated from cytosol and form mitochondria appear to be the product of the same gene (74,106). How part of the AK is targeted to the mitochondria remains unclear.

The availability of cytosolic and mitochondrial isoforms allows the introduction of a new concept: a phosphagen kinase system for spatial energy buffering, or energy transport (8,65) (Figure 7). MtCK is present in the intermembrane space of mitochondria, where the octameric form functionally interacts with the adenine nucleotide translocator (ANT) on the inner membrane and with the oligomeric porin on the outer membrane (17,127,167,173,174). The cytosolic dimeric isoenzyme, in contrast, is present either as free cytosolic enzyme or
functionally coupled to ATPases and glycolytic enzymes (167). The idea of a spatial buffering by CK is based on the observation that the ATP synthesized in mitochondria by oxidative phosphorylation will be channelled directly to MtCK which converts it to PCr. The porin complex will then export PCr into the cytosol, where it accumulates and serves as a substitute of cytosolic CK to keep a high ATP/ADP ratio, globally and locally in the optimal range for ATPases (81, 167, 173). CK is prominently present not only in tissues with high energy requirement, but more interestingly in tissues with the most abrupt fluctuations in workload. On this basis, it was hypothesized that the CK system may reduce the transient times needed to refill the ATP pool (174). In fact, the cytosolic CK reaction has a much higher turnover rate than ATP utilization or consumption rates. In addition, metabolite channeling occurs with MiCK coupled to porin and ANT (173), or cytosolic CK, e.g. coupled to sarcomeric Ca\(^{2+}\)-pumps. Besides the increase in metabolic efficiency, this reduction of transient times would also improve the transition between different work loads and metabolic states, such as occurring during transient stress (165).

Figure 7. Energy transport by CK system. Abbreviations: CK, creatine kinase; MtCK, mitochondrial creatine kinase (from (165), modified).
A further relevant feature of the CK system is pH buffering \((90, 167)\). In case of large energy utilization, intracellular acidification may occur as result of ATP hydrolysis, in particular during severe muscle exercise. Under anaerobic muscle activity, lactate production involves further liberation of protons in the cytoplasm \((90)\). This may result in loss of fibres contraction force \((32)\), as a consequence of the pH gradient disturbance as well as of the influence of low pH on proteins and membranes \((90)\). The depletion of the PCr buffer by cytosolic CK involves the utilization of one proton per generated ATP molecule. Thus, the replenishment of the ATP pool during intense energy utilization is accomplished by a reduction of local acidification \((167)\). Although AK has not yet been investigated for a possible role in proton buffering, it was shown that AK interacts with sites of high energy utilization, as CK does \((124)\). Moreover, although its kinetic parameters make of AK a less efficient system than CK, it seems theoretically possible that AK exerts a proton buffering function as well.

As mentioned above, CK interacts with some proteins for a direct channeling of substrates and products of the reaction. The interaction of CK with AMP-activated protein kinase (AMPK) may moreover exert a co-ordinated regulation of energy metabolism in skeletal muscle, where AMPK is expressed at high levels. In response to metabolic stresses as the intracellular ATP/AMP ratio falls, AMPK is activated, inhibiting enzymes involved in energy-consuming tasks, such as biosynthetic pathways \((69, 70)\). Regulation is exerted at post-transcriptional level by sequence-specific phosphorylation \((37, 172)\), and CK has three sites where such a phosphorylation may occur. It was moreover shown how AMPK is able to in vitro phosphorylate MM-CK, and that in vivo the two enzymes are co-localized in skeletal muscle \((121)\). AMPK is part of a protein cascade, being phosphorylated and activated by a distinct kinase (AMPKK), although the regulation of the latter remains to be cleared \((28, 121)\). AMPK is activated by low ATP/AMP ratio \((69, 146)\) as well as it is inhibited by increased PCr/Cr ratio in a pH-dependent manner \((121)\) (Figure 8). This is in fact a very efficient system by which the cells is able to regulate energy availability and attributes to AMPK an energy-conserving role.

By considering all these issues, CK appear to be a system which is very well suited for high-energy demanding tasks in very complex organisms. These organisms are very well protected form strong fluctuations of physiological conditions, such as temperature and intracellular pH. Under these conditions the weakness of PCr in term of thermodynamical properties is attenuated. The metabolic burden connected with the biosynthesis and the uptake
of Cr, as well as the many tissue specific isoforms of CKs, is more than compensated by the advantages of an efficient energy transport and buffering system.

**Figure 8. Regulation of CK activity by AMP-activated protein kinase.** Gray arrows indicate enzymatic reactions, black arrows indicate regulatory issues (here, + indicates activation and − indicates inhibition). Abbreviations: CK, creatine kinase; PCr, phosphocreatine; Cr, creatine; AMPK, AMP-activated protein kinase; AMPKK, AMPK-activated protein kinase.

AK, on the contrary, appears to lack the vast palette of functionalities, as exhibited by CK, although less efficient energy transport function as well as a role in pH buffering cannot be excluded. The lower dependence of PArg from physiological conditions makes of it a more powerful tool for organisms or tissues exposed to the influence of the environment, like e.g. sponges or unicellular eukaryotes. It seems however, that unicellular organisms were unable to develop a phosphagen kinase system by themselves, relying therefore on horizontal gene transfer from host-parasite relationships, as in case of arthropods and *Trypanosoma cruzi* (115). A possible cause for the missing evolution of phosphagen kinase systems in unicellular organisms may be the lack of the ancestor gene, from which AK evolved first.

These considerations may also explain the possible involution of AK from CK. An organism confronted e.g. with the appearance of sudden changes in environmental conditions or the colonization of a new habitat would take advantage from a more primitive and more resistant system, although it is less efficient as is the case of the AK/PArg system.

The debate about the possible advantage primitive unicellular organisms like yeasts or bacteria would take from the availability of an ATP buffering system appears to be intriguing as well. It was already shown how *Paramecium caudatum* could profit from the coexistence
of both adenylate cyclase and AK in his cilia (110). In the next sections, we will analyze the possible biotechnological applications of the overexpression of phosphagen kinase systems in different organisms.

**PREVIOUS STUDIES ON HETEROLOGOUS PHOSPHAGEN KINASE EXPRESSION**

The first attempt to heterologously express a phosphagen kinase was described by Koretsky and co-workers in 1989 (89). The B isoenzyme of CK was expressed in *E. coli* as fusion protein. The enzyme was found to be active *in vivo* and high levels of PCr were measured by $^{31}$P-NMR when the medium was supplemented with Cr. One year later, a study on the overexpression of the B isoenzyme of CK in the yeast *S. cerevisiae* was published. Here as well, *in vivo* activity was detected by measuring PCr levels as Cr was fed in the medium, but no corresponding change in the ATP concentration was observed (19). In both studies, however, it remained unclear whether Cr transport actually occurred and PCr was synthesized intracellularly. In fact, it is known that determination of intracellular Cr needs extensive washing of cells before disruption in order to eliminate the Cr bound to the cell wall (U. Schlattner, unpublished results). Moreover, the Cr levels measured are higher than the solubility limit for Cr (144). Thus, the measured PCr could eventually have resulted from extracellular CK activity leaking from lysed cells.

In another study, MtCK was expressed in *S. cerevisiae* and targeted to mitochondria. Despite the correct localization of the enzyme, no PCr formation could be detected after medium supplementation with Cr (E. Furter 1994, unpublished results). Similarly, cytosolic CK was expressed in the same organism, but also in this case no PCr formation was observed in correspondence to Cr feeding (27). The possible problem was reported to be the absence of intracellular Cr due to the lack of a transport system (for discussion of this topic, see Chapter 4). The overexpression of the cytosolic enzymes B-CK and M-CK was attempted in *E. coli*, but PCr was not determined and growth parameters not analyzed (M. Stolz 1995 / N. Holmberg 1998, unpublished results).

Other studies, focusing on the expression of CK in mammals and plants could show surprising effects. In perfused mouse liver overexpressing cytosolic CK, no drop of ATP and pH occurred under hypoxic conditions as is generally seen in control liver, indicating a protective effect by CK in a tissue normally lacking CK activity (103). In the same organ, overexpression of MtCK generated a PCr pool, which could be efficiently exploited (104).
The cytosolic B-CK expressed in tobacco plants resulted as well in the formation of a PCr pool, although no physiological improvement could be observed (50).

APPLICATIONS OF FUNCTIONAL PHOSPHAGEN SYSTEMS IN BIOTECHNOLOGY

In our work, we have used a new approach to investigate the effect of heterologous expression of functional phosphagen systems, concentrating our efforts in providing sufficient amounts of phosphagen to the phosphagen kinase expressed and using *E. coli* and *S. cerevisiae*. These hosts allow not only a comparison with previous studies, but also a detailed knowledge of metabolism and biochemistry. Moreover, their low pathogenicity and the frequent utilization as model strain or in industrial processes make of them an ideal system to analyze possible future applications of phosphagen kinases in biotechnology.

We have limited our investigation to the AK/PArg and CK/PCr systems, being aware of their structural and functional differences, as outlined before. As already described, the CK/PCr system has been investigated quite extensively. The vast palette of functionalities, providing a large free energy change for ATP, and the kinetic characteristics make of CK the most efficient phosphagen kinase to work with. Moreover, Cr is inert, only interacting with CK and not being involved in other biosynthetic pathways. However, the relative instability of PCr at low pH makes the CK/PCr system only suitable in organisms able to keep a strict control over intracellular pH. In addition, the need to install a Cr-uptake mechanism or Cr-biosynthesis together with co-expression of different CK isoenzymes contribute to a considerable burden for the cells. Therefore, it was of particular importance to evaluate possible negative effect connected to system complexity. Finally, the inhibitory effects of creatinine have to be considered and, if necessary, a system to avoid accumulation should be implemented.

Since the AK/PArg system is not relevant for humans, this system in only poorly characterized. The overexpression study presented here is the first attempt of a functional heterologous expression of AK. Therefore, it is difficult to concretely estimate the impact on the chosen hosts. About the functions other than the temporal ATP buffering one can only speculate at the moment. AK is also a simpler and monomeric enzyme, which makes of it a less efficient actor in comparison to CK. Nevertheless, this lack of complexity probably confers to the AK/PArg system an improved compatibility with unicellular hosts. Arg can be provided directly as medium component or we can rely on endogeneous synthesis.
By considering expression studies for biotechnological purposes, it is of fundamental importance to consider possible drawbacks. In their natural cellular environment, phosphagen kinases have been reported to directly interact with many proteins. We shall consider possible interactions in our expression system as well. AMPK is analogous to the yeast protein \textit{SNF1}, which appears to also exert the same function (68-70). It is reasonable to consider, both AMPK and \textit{SNF1} utilizes the same recognition sequence to phosphorylate target proteins. We analyzed the protein sequence of AK and we found two putative sites of AMPK-dependent phosphorylation (Ser130 and Thr32). Due to the lack of structural data, it is however difficult to estimate whether these sites are effectively accessible for AMPK. Similarly, we cannot estimate the impact of phosphorylation on activity, and therefore of a possible regulation of AK and CK by \textit{SNF1} in \textit{S. cerevisiae}. In \textit{E. coli} one can neglect such a possible interaction, since no protein homologous to the AMPK has been identified in this organism. As second possible drawback, we should consider a possible inhibition of the \textit{Cr} biosynthesis pathway by \textit{Cr}, as described above. The relevant point is the expression of the whole system in a single cell and not in different tissues as it is in mammals, therefore possibly resulting in a stronger inhibitory effect.

A functional phosphagen kinase system is expected to provide cells with an energy buffer be used in case of sudden and intense energy consumption. In normal batch cultures, however, this is a condition which rarely occurs. In order to find a possible application for the heterogeneously expressed phosphagen kinase systems it is thus important, to apply conditions which cause serious depletion of the ATP pool or negatively affect the capability of ATP formation. Environmental stress is known to generate such situations, as will be described below.

\textbf{STRESS MECHANISMS IN \textit{E. coli} AND \textit{S. cerevisiae}}

The intriguing ability of certain micro-organisms to overcome the stress situations found in hostile and quickly changing environments has been extensively studied in the last decades. This great interest originates mostly form the close relationship between stress response and many activities of fundamental importance for mankind. Many biotechnological applications, such as wine production (80,122), cheese (20) and bread making, brewing (4,123), and industrial processes involving the generation of stressful conditions (46,136) require robust, stress-resistant cells. But stress resistance not always is intended as a positive property of micro-organisms. Bacterial pathogenicity is often strongly related to the efficiency
of stress resistance mechanisms, e.g. as in the case of gastro-intestinal tract colonization by enteric pathogenic bacteria (99,102). Moreover, food preservation relies as well on the generation of combined stresses to prevent food spoilage (22).

*E. coli* can respond to a vast palette of stresses, by activating both a general stress response and more specific mechanisms. Some of these involve a more or less intense depletion of energy or a disturbance in the formation or utilization of energy cofactors. Among these energetic stresses, the presence of weak organic acids or of strong inorganic acids is one of the most relevant. Acid stress is also the most frequent encountered hostile condition in enteric bacteria (7), where it may affect cellular activity at different levels. Strong inorganic acids lower drastically the extracellular pH, and this variation is reflected more or less strongly in the cytoplasm, depending on the organism. Since proteins show maximal activity at given pH values, the shift from this optimum may cause inhibition or denaturation of surface or intracellular protein (15), depending on the severity of pH change. But weak organic acids are considered to have stronger deleterious effect on microbial growth and their mechanism of action is slightly different (53,143). Organic acids enter the cell in the protonated form, which is mostly lipophilic, and deprotonates in the cytoplasm. So, internal acidification will occur with the same deleterious effects as for inorganic acids, but eventually with reduced changes in extracellular pH (11). Moreover, the efficacy of weak acid stress, relies on the possible toxicity of the so liberated anion, as it is the case e.g. for acetate, propionate and benzoate (129). Focussing attention on weak organic acids is of sure interest since presence of weak acids in culture media often occurs as the results of by-products accumulation (14).

As in all situations where survival is menaced, micro-organisms try to face the problem as efficiently as possible. Acid stress may result on one side from the stable change in conditions, especially in the case of organism colonizing a new environment. Here, instead of fighting the adverse conditions, micro-organisms adapted their structure or their metabolism to the external conditions, evolving into acidophilic or alkalophilic organism, which exhibit their optimum at reduced or increased pH (14). On the other side, acid stress may occur as a consequence of sudden and momentary environmental variations. In those cases, the development of resistance mechanism is preferred, which will act only if needed. It is surprising, how different are the approaches developed by bacteria and unicellular eukaryotes to actively or passively overcome acid stress. Convincing evidence had been provided that acid stress resistance arises as the combination of several systems of actors and regulators, which often overlap (15).
To prevent entry of extracellular protons and intracellular acidification as a consequence, micro-organisms take advantage from a series of passive defence mechanisms (Figure 9). In *E. coli*, the first significant barrier to the environment is the cell wall itself. It seems that the efficacy of cell wall against proton influx is due to its structure and to porins (15, 95), but their exact contribution to pH stress resistance is still unclear (15). However, it was hypothesized that, in the case of *E. coli*, porins are responsible to partially reflect external pH changes in the periplasmic space (22). The buffering capacity of periplasm, guaranteed by the large amount of peptidoglycans (14) is able to compensate for small proton influxes. As the periplasm becomes acidified, the cytoplasmic membrane is the second strong barrier and the real defence against intracellular acidification. The low proton permeability of cytoplasmic barrier is of fundamental importance for energy transduction as well. The high protection level of cytoplasmic membrane from acidification of cytoplasm relies on its composition, which provides a net positive surface charge, thereby reducing the protons flux (22, 66), as well as on the control of ion channels (96). In *E. coli*, adaptation to slightly acidic pH will cause a change in the cytoplasmic membrane composition to increase the protective effect of cell membrane (21, 31).

However, all these passive barriers can compensate only small changes of extracellular pH (1-2 pH units) and are even of little help against the cytoplasm acidification by weak organic acids (22). Moreover, buffering capacity of cytoplasm is much reduced if compared to periplasm (14), so proton flux through the cell membrane influences strongly the cytoplasmic pH, and acidification of cell interior occurs. Active counteraction of cytoplasm acidification is accomplished in first place by proton pumping. One of the actors is ATPase, which extrudes one proton in the periplasm by consuming one molecule of ATP. Kobayashi and co-workers observed how in enteric bacteria ATPase synthesis was increased up to 3-fold under mild acid stress (pH 5) (88). It has moreover always been recognized, how acid stress response strongly depends from the availability of potassium in the medium (92). In fact, there are two types of potassium transporter which affects the intracellular proton concentration. One is an H⁺/K⁺-antiporter, extrudes protons, imports protons and represents the main way in which *E. coli* cell regulates the potassium availability (18, 47). However, during recovery from pH stress, no net increase of intracellular potassium was observed (22). In a recent study, Zakharyan and Trchounian suggested the presence of the Kup potassium transporter, acting as an H⁺/K⁺-antiporter (180). It was however not clear whether this mechanism only works for proton import and maintenance the proton gradient or if it can also efficiently extrude both protons
and potassium ions to the periplasm. Moreover, the NhA H⁺/Na⁺-antiporter has also been related to proton extrusion from acidified cytoplasm (14,15).

Figure 9. Stress resistance mechanism in E. coli. Abbreviations used: Glu, glutamate; Arg, arginine; GABA, γ-aminobutyric acid; Kup, H⁺/K⁺ symporter; TrkA, H⁺/K⁺ antiporter; NhaA, Na⁺/K⁺ antiporter; GadC, glutamate/GABA antiporter, GadAB, glutamate decarboxylase; AdiA, arginine decarboxylase; RpoS, stationary-phase dependent sigma factor σ²; HdeAB, periplasmic chaperones. Color code: grey, proteins present at neutral as well as at acidic pH; white, proteins expressed only at acidic pH by RpoS-dependent transcription. Dashed arrows represent activities with unclear mechanism.

The effectiveness of proton extrusion in the periplasm for strong or long-term acid stress has often been matter of debate. In fact, it seems that, although periplasmic acidification is as deleterious as for cytoplasm, protons cannot be accumulated beyond a certain limit in the periplasm. Porins appear to be the major if not the only way for the cell to extrude protons to the extracellular space, thereby being a probable limitation in the process. So, low intracellular pH also induces a series of acid resistance systems (ARS) which are true defensive systems that reduce the negative effects of acidification or eliminate protons without need for a transporter. These ARS are all induced by RpoS and are therefore present.
in the stationary phase. A first ARS is the glucose-repressed and oxidative system, which may involve many proteins. The identity and the role of those proteins are not clear at the time, although it seems that they reduce the effect of protons on DNA and proteins (98). Recently, a part of this system was identified as the periplasmic chaperones HdeAB. These proteins are synthesized in the cytoplasm and exported to the periplasm, where they are active as heterodimers, binding unfolded proteins and preventing their aggregation (60). The analysis of protein expression under weak and strong acid stress by two-dimensional gel electrophoresis showed the specific overexpression of many proteins involved in sugar metabolism. It is however not clear whether these proteins are induced by RpoS, or the exact role they may have in stress resistance (11). Another system, involving the cytoplasmic regulatory protein Fur, has been found to play a role in stress resistance, but principally in sensing pH changes. The exact role of this resistance system have however still to be explained (54,67). The glucose-repressed ARS is present during growth in LB medium only, and the complex components responsible for the induction have not yet been identified, but glutamate could be one of those (5). As the name tells this system is not present in minimal medium complemented with glucose (7).

The second ARS is a glutamate-dependent one and provides the highest acid resistance. It allows cells to survive several hours at an external pH of 2, and it is most effective if the pH is lower than 3 (5). The system relies on the availability of glutamate in the medium (endogenous glutamate seems not to suffice) which is imported and converted to γ-aminobutyric acid (GABA), by the glutamate decarboxylase GadAB upon consumption of a proton (29). The generated GABA molecule is then exported to the periplasm in exchange of another glutamate molecule by the glutamate/GABA antiporter GadC. In the more acidic periplasm, the GABA molecule is protonated, contributing to the alkalization of periplasm. The contribution to the reduction of periplasm acidification may be of extreme importance since porins limit the extrusion of periplasmic protons. So, the efficacy of these systems relies in the reduction of acidity by elimination of one proton in both cytoplasm and periplasm (15). RpoS induces gadBC at every pH, but in LB medium, acid also induces the gadA regardless from growth phase (5,29). Another important function exerted by the glutamate-dependent ARS, seems to be the possibility to enhance energy production under anaerobic conditions, by coupling electrogenic antiport and amino acid decarboxylation. In fact, by consuming cytoplasmatic protons, a proton gradient across the cytoplasmatic membrane can be created and exploited to generate ATP. The uniqueness of the glutamate decarboxylase with this regard and in comparison to other decarboxylases is its ability to work at lower cytoplasmatic
pH (130,141). Since in *E. coli* the ATP production is seriously limited during anaerobiosis, this system could be an efficient way to overcome the problem.

A third ARS, offering a more modest level of protection is an arginine-dependent one. Analogously to the glutamate-dependent ARS, here arginine is imported and converted to agmatine, by the arginine decarboxylase AdiA upon consumption of a proton. The thus generated agmatine molecule is then exported to the periplasm in exchange of another glutamate molecule by an unknown glutamate/agmatine antiporter (29). The system protects the cells for short-term stresses at pH 2.5, appearing to be active at its maximum under pH 3, and is not only induced by RpoS, but also activated by the CysB regulatory protein (5). The arginine-dependent ARS requires anaerobiosis to be active at full strength, and this may speak for a possible role in ATP generation under lack of oxygen, as for the glutamate-dependent ARS (7).

Strengthening the ability of *E. coli* to fight acid stress, this organism and in general many enteric bacteria have the ability to show acid habituation (or adaptation) (AH). The AH response involves a pre-incubation of the cell at a mild acidic pH. These cells show then higher resistance against stress at lower pH. The mechanism of resistance in this case seems to involve principally an improved repair of DNA damage caused by low pH. The AH system seems also to allow the cell to reduce the deleterious effect of the anion released by some weak organic acids, such as benzoic acid (7).

The presence of all these overlapping and complex system to overcome acid stress confers the ability to exert a strong control over cytoplasmic pH to *E. coli*, which varies only of 0.1 pH unit per every unit of extracellular pH change (22), and is kept at a constant value of 7.6 for external pH values between 4.5 and 9.0 (11). However, in order to fight strong stresses, the environmental conditions have to match some minimal requirements, such as the availability of amino acids or other complex components (see above). Moreover, weak acids strongly affect growth, even at low concentrations, and this appears to be due more to the released anion in the cytoplasm than because of acidification. In a recent study, it is reported *E. coli* grow with at least halved maximum growth rate if 8 mM acetate, 2 mM benzoate, or 5 mM propionate are present in growth medium (126).

It is generally recognized, that recovery from acid stress requires the investment of a large amount of resources which would else be utilized for metabolic purposes. Among these, the dissipation of proton motive-force as well as the increased ATP-demanding proton extrusion are considered to play a relevant role during recover from stress (6,14).
S. cerevisiae is both a biotechnologically useful organism and a frequent food spoilage agent. Industrial applications involving S. cerevisiae include not only brewing, bakery and wine production, but also the production of the yeast itself, as starting material for the successive applications (4,123). During these processes, cells are subjected to a large numbers of stress factors, including heat, change in pH, toxic chemicals, ions, oxidizing compounds, and momentary lack of nutrients. If grown under optimal conditions, S. cerevisiae shows little sign of stress response, but immediately switches on stress resistance mechanisms if conditions change (62). S. cerevisiae has the amazing capacity to easily overcome stress conditions which results deleterious for many other organisms. This ability to survive is conferred by a multifaceted stress response (62). In general, the onset of stress conditions that are strong or long enough to be sensed, causes the cell to stop growth and enter a stationary phase where emergency defences are activated, and a process is started where the cell try to adapt to the new conditions. This last step may last from minutes to several hours in yeast cultures, after which the normal exponential growth is recovered. These cells are now adapted to this new stress factor (118). Although yeast is able to activate a general stress response as well, the key role is played by specialized mechanism in response to specific stresses. As it is the case for E. coli, also in S. cerevisiae some of these stress responses affect the formation or the utilization of energy cofactors, thereby impairing growth. Among those, acid stress and carbon starvation are for sure among the stresses encountered more frequently during industrial processes (4).

The distinction between the stress caused by strong acids and weak acids is necessary (Figure 10). The low pK of the former causes intracellular acidification as a consequence of the lowered extracellular pH, while the latter have a higher pK, and only acidify cytoplasm by entering in the undissociated form, liberating the proton only in the intracellular space (120). In both cases, however, the lowered cytoplasmic pH would have a strong negative impact on the cellular mechanisms if the cell would not fight it by alkalizing the intracellular space (24). S. cerevisiae alkalize the cytoplasm by proton transport, both through the combined action of Na⁺/H⁺- and Cl⁻/HCO₃⁻-antiporter, and by the work of the plasma membrane ATPase Pma1, which is the most abundant membrane protein, constituting over 20% of the total membrane protein (76,137). Besides exploiting the proton gradient to generate energy in form of ATP, the ATPase is also responsible maintain the correct ion balance across the membrane. The fulfilment of this activity is achieved by extruding protons to the cell exterior upon consumption of ATP (137). In case of extensive intracellular acidification, the ATPase was shown to be activated (76,119,161,162). The maintenance of the intracellular pH by this
mechanism can be energetically expensive (48,137) (138), consuming up to 60% of the total cellular ATP (139). The resulting significant ATP depletion may restrict growth (120,162,168). Therefore, the activity of Pma1 has to be tightly regulated, and this occurs post-transcriptionally by the action of the stress-induced protein Hsp30. This regulator protein is expressed during late heat or acid stress response and inhibits the activity of Pma1, thereby having a probable energy conservation role. The knockout of Hsp30 produces a quicker alkalization of cytoplasm, but also a reduced growth during the recovery phase (119).

![Diagram showing acid stress response of S. cerevisiae.](image)

Figure 10. Acid stress response of *S. cerevisiae.*

If the stressful condition is caused by weak organic acids, intracellular acidification may be a less serious matter (16,120). In this case, the anionic acid form is liberated in the cytoplasm, inhibiting growth in different ways, depending on the acid nature. Benzoate is known to inhibit glycolytic enzymes generating thereby a reduced ATP production (76,91). Sorbate in presence of oxygen cause mitochondrial disorganization and resulting formation of high levels of superoxide free radicals (117). Other acids, such as acetate or propionate simply inhibit metabolic reactions or cause an increased turgor pressure (120). Other than in other yeasts, many of which can efficiently metabolize weak acids, *S. cerevisiae* responds to stress caused by weak acids, by transporting the anionic form to the extracellular space (3). During weak organic acids, the ABC transporter Pdr12 is activated both on a transcriptional and a post-transcriptional level, approaching the abundance of Pma1 in the plasma membrane. The role of this transporter is to catalyze the ATP-dependent extrusion of weak organic acid
anions (113,118). Hence, the response to weak acid stress causes severe ATP depletion (2 ATP molecules per molecule weak acid entering cytoplasm), needing a regulation system to avoid an excessive energy consumption and consequent growth impairment (119,145,159,162,168). Repression of Pdr12 is achieved through post-transcriptional modification by the action of the Ca$^{2+}$/calmodulin-dependent protein kinase Cmk1p (77). The adaptive response to weak organic acids involves the inhibition of Cmk1, and therefore an increased activity of Pdr12 (120). Pdr12 is regulated also on a transcriptional level, although the exact mechanism is not known. The weak acid anion extruded by Pdr12 becomes protonated as a consequence of the lower extracellular pH, so the cell has to avoid the futile cycle of anion expulsion and successive re-enter as undissociated acid (118,169). This is probably achieved by a reduction of the porosity of the cell wall, by a mechanism which is currently still unknown (120).

Besides increased ATP consumption, energetic stress may be achieved also by reducing ATP formation. The availability of energy in form of ATP strictly depends on the availability of the carbon source. Glucose is the preferred carbon source for S. cerevisiae, and it is therefore not surprising that pathways involved in glucose sensing and signalling are crucial regulators of cellular physiology (64) (Figure 11). If enough glucose is available, ATP availability is high and AMP levels are low. As a consequence, the Ras/cAMP system and the AMP-dependent protein kinase (PKA) are activated, thereby allowing to progress over the nutrients decision point in the G1-phase of the cell cycle. The same ATP and AMP levels also inhibit the AMP-activated protein kinase SNF1 and the signalling factor cascade leading to the elicitation of the general stress response. As the glucose concentration drops, the ATP concentration increases and the AMP concentration increases. Therefore, the activation of SNF1 occurs, which inhibits all the ATP consuming tasks. At the same time the signalling cascade relative to the activation of the general stress response is activated. Moreover, growth is blocked by the reduced presence of PKA and of the RAS/cAMP system (49,87,154).

![Figure 11. Stress response to carbon starvation in Saccharomyces cerevisiae. Black arrows indicate interactions or regulatory issues (here, \(\oplus\) indicates activation and \(\ominus\) indicates inhibition), grey arrows indicate metabolic reactions, and dotted arrows indicate transport. Abbreviations used: AMPK, AMP-activated protein kinase; PKA, AMP-dependent protein kinase; STREs, stress-response elements; cAMP, cyclic AMP.](image-url)
As seen before, SNFL could also interact with phosphagen kinase, as observed in humans (87,121). That would lead to the inhibition of the phosphagen kinase if stress is too severe, and ATP levels drop under a certain level. Thus a short-term transient glucose limitation would be the ideal condition to study the possible impact of a phosphagen kinase under carbon starvation stress.

In Chapter 2 and Chapter 3, we have analyzed defined deletion mutants of enzymes of the central carbon metabolisms of E. coli in combination with the transhydrogenases. Through physiological observation and metabolic flux analysis, we have investigated the role for both the energy-linked and the non-energy-linked transhydrogenase, and depict the natural relevance of the presence of two transhydrogenases in E. coli. A possible regulation of these two enzymes within the framework of a central role in central carbon metabolism is discussed.

In Chapter 4 and Chapter 5, we show the effect of heterologous expression of both the AK/PArg and the CK/PCr ATP buffering system in S. cerevisiae and E. coli under conditions of reduced ATP availability, such as transient acidic and starvation stress.
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49


CHAPTER 2

Metabolic flux response to phosphoglucose isomerase knock-out in Escherichia coli and impact of overexpression of the soluble transhydrogenase UdhA

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Abstract

Blocking glycolytic breakdown of glucose by inactivation of phosphoglucone isomerase in Escherichia coli led to a greatly reduced maximum specific growth rate. Examination of the operational catabolic pathways and their flux ratios using [U-\(^{13}\)C\(_6\)]-glucose labeling experiments and METAFoR analysis provide evidence for the pentose phosphate (PP) pathway as the primary route of glucose catabolism in the knock-out mutant. The resulting extensive flux through the PP pathway disturbs apparently the reducing power balance, since overexpression of the recently identified soluble transhydrogenase UdhA improves significantly the growth rate of the Pgi mutant. The presented results provide first evidence that UdhA restores the cellular redox balance by catalyzing electron transfer from NADPH to NADH.
INTRODUCTION

By providing energy, building blocks, and co-factors, central carbon metabolism constitutes the biochemical backbone of all cells. Nevertheless, our understanding of systemic properties of the central metabolic network lacks behind the accumulated wealth of detailed biochemical and genetic knowledge on its individual components. One reason is the redundancy of this complex network, in the sense that more than one reaction or pathway catalyzes a given conversion of intermediates. Thus, firm conclusions on metabolic consequences of genetic manipulations can often not be drawn on the basis of physiological characterization only (7). When based on isotopic tracer data, however, methods of metabolic flux analysis can potentially distinguish between alternative pathways (15,17,19).

One approach is based on tracing intact carbon fragments in cells grown on mixtures of uniformly [U-13C6] and unlabeled glucose (16,18). The resulting 13C labeling patterns of metabolic intermediates are analyzed by nuclear magnetic resonance (NMR) spectroscopy of amino acids that are synthesized from these intermediates. Because alternative pathways that lead to common intermediates or products yield different intact fragments that originate from a single glucose source molecule, specific multiplet patterns in the 13C fine structures that reflect the in vivo usage of reactions are generated. Probabilistic equations relate the determined intensities of the multiplet components to the relative abundance of intact carbon fragments (16). Thus, this method, also referred to as metabolic flux ratio (METAFoR) analysis, identifies the active metabolic pathways and the ratios of their fluxes during a labeling experiment (11,13,14,16,18).

Here we use METAFoR analysis by NMR to investigate the metabolic consequences of phosphoglucone isomerase (Pgi) inactivation in Escherichia coli. Since Pgi is located at the first juncture of different pathways for glucose catabolism (Figure 1), its inactivation is particularly useful for studying general metabolic network behavior because glucose catabolism must then proceed via the pentose phosphate (PP) and/or the Entner-Doudoroff (ED) pathway. This flux rerouting has a profound influence on the balance of the reducing power budget because NADPH is formed in large excess when catabolism proceeds exclusively via these pathways. In contrast to NADH, NADPH is solely used in anabolic reactions and not in respiration (9). If and to which extent the energy-coupled, membrane-bound transhydrogenase PntAB and/or the soluble transhydrogenases UdhA are involved in maintaining the balance between NADH and NADPH in E. coli is currently an exciting matter of debate.
Figure 1. Reaction network of E. coli central carbon metabolism. The arrows indicate physiological reaction directionality and key enzymes are indicated by their 3-letter code in the grey ellipses. Abbreviations: G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; 6PG, 6-phosphogluconate; KDPG, 2-keto-3-deoxy 6PG; P5P, pentose-5-phosphate; E4P, erythrose-4-phosphate; 7PG, seduheptulose-7-phosphate; GAP, glyceraldehyde-3-phosphate; DAP, dihydroxy acetone-phosphate; PGA, 3-phosphoglycerate; SER, serine; GLY, glycine; PEP, phosphoenolpyruvate; PYR, pyruvate; ACoA, acetyl coenzyme A; CIT, citrate; ICT, isocitrate; OGA, oxoglutarate; SUC, succinate; FUM, fumarate; MAL, malate; OAA, oxaloacetate; GOX, glyoxylate; Eda, KDPG aldolase; Edd, 6-phosphogluconate dehydratase; Ppc, PEP carboxylase; PckA, PEP carboxykinase; Mdh, malate dehydrogenase; MaeA, NAD-linked malic enzyme; MaeB, NADP-linked malic enzyme; transhydrogenase, pyridine nucleotide transhydrogenase; Zwf, G6P dehydrogenase.
MATERIALS AND METHODS

Strains and growth conditions. Here we used the two E. coli strains wild-type MG1655 (λ, F, rph-l) and a Pgi mutant (λ, F, IN(rrnD-rrnE), rph-l, pgi::Tn10) (a gift of G. Sprenger, Biotechnology, Forschungszentrum Jülich, Germany) that was constructed by P1 phage transduction of the pgi-Tn10 marker (2) into W3110. Luria-Bertani (LB) and M9 minimal medium were prepared as described previously (13). M9 medium was supplemented with either glucose or fructose at a final concentration of 0.5% (w/v). Aerobic cultivation was performed in 1-l baffled shake flasks with maximally 150 ml medium at 30°C on a gyratory shaker at 200 rpm.

For 13C labeling experiments, cultures were grown in M9 medium with 0.45% (w/v) unlabeled glucose and 0.05% (w/v) uniformly labeled [U-13C6]-glucose (13C >98%, Isotech). In these cases, the inoculum volume was well below 1% of the culture volume, so that the presence of unlabeled biomass could be neglected for the analysis of the 13C-labeling patterns. These cultures were always harvested in the mid-exponential growth phase at an optical density at 600 nm (OD600) of about 1.

Analytical procedures. Cell growth was monitored by determination of OD600 and cellular dry weight (cdw) was calculated from previously determined OD600-to-cdw correlations. Glucose, acetate, and protein concentrations were determined with commercial kits (Beckman) or by HPLC. Physiological parameters were calculated as described previously (13).

To prepare crude cell extracts, LB-grown cultures were washed and resuspended in one volume 0.9% (w/v) NaCl, 10 mM MgSO4, and disrupted by two sonication steps at 100 W for 1 min each. After centrifugation at 10,000 g for 30 min, the supernatant was transferred to a new tube and used directly for determination of protein concentration and enzyme activities. Specific activities of Pgi (6) and transhydrogenase (5,12) were determined as described previously. Specific ED pathway activity was determined from the combined Edd and Eda reactions in a 750 µl mixture containing 5 mM 5PG, 10 mM MgSO4, and 200 mM Tris-HCl (pH 7.2). The reaction was started by adding 50 µl crude cell extract and was incubated at 30°C for 30 min. After addition of 750 µl 0.2% (w/v) 2,4-dinitrophenylhydrazine in 500 mM HCl, incubation at RT for 10 min, and stopping the reaction by adding 500 µl 4 M NaOH, the reaction product pyruvate was detected by recording the extinction at 450 nm (1).
**NMR spectroscopy and data analysis.** Preparation of protein hydrolyzates and recording of 2D $^{13}$C-$^1$H correlation NMR spectra for aliphatic and aromatic amino acid resonances were performed as described previously (13,16). The program FCAL (8,18) was used for integration of $^{13}$C-$^{13}$C scalar coupling fine structures and the calculation of relative abundances, $f$, of intact carbon fragments originating from a single source molecule of glucose (16). Briefly, these $f$ values were calculated with probabilistic equations from the relative intensities, $I$, of the superimposed multiplets in the $^{13}$C-$^{13}$C scalar coupling fine structures of resonances in 48 carbon atoms of the amino acids. The $f$ values in amino acids carbon atoms provide then information on the metabolic origin of their precursors molecules in central metabolism, i.e. P5P, E4P, PGA, PEP, PYR, OGA, and OAA (16).

**Genetic manipulations.** Inducible overexpression of the soluble transhydrogenase UdhA (3) was achieved by PCR amplification of *udhA* from chromosomal DNA of *E. coli* MG1655 with the primers 5'-CGGGATCCGA TGCCATAGTA ATAGG-3' and 5'-CCCAAGCTT TTTAAAACAG GCGGTT-3' (chromosomal sequences are underlined). The resulting PCR product was digested with *BamHI* and *HindIII* and cloned under the control of the IPTG-inducible trc promoter of pTrc99A (Pharmacia).
RESULTS AND DISCUSSION

Phenotypic characterization. Pgi enzyme activities of 966±5 and 5±5 U/mg protein in crude cell extracts from control and mutant batch cultures, respectively, proved complete absence of Pgi activity in the mutant. Under aerobic conditions, mutant cultures grew well on fructose as the sole carbon source with a maximum specific growth rate ($\mu_{\text{max}}$) of 0.40 h$^{-1}$, as was reported previously (6). This indicates that G6P is not an essential component of E. coli biomass because it cannot be synthesized from fructose in pgi mutants. Providing glucose as the sole carbon source, the Pgi mutant grew significantly slower and consumed less glucose (Table 1). The biomass yield of the Pgi mutant on glucose, however, was slightly improved when compared with the wild-type, which is consistent with previous observations (6). This indicates a kinetic limitation of glucose catabolism, which is supported by the complete absence of the metabolic overflow product acetate (data not shown).

Metabolic flux response to pgi knockout. To investigate metabolic consequences of blocking the first step of glycolysis, E. coli wild-type and the Pgi mutant were subjected to a [U-13C$\beta$]-glucose labeling experiment in batch culture. Visual inspection of the resulting 13C-13C scalar coupling fine structures in the amino acids immediately revealed several striking differences between wild-type and mutant (Figure 2). Using probabilistic equations (METAFoR analysis), these fine structures were then quantitatively analyzed and then recruited to derive metabolic flux ratios (16,18).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-type</th>
<th>Pgi mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\mu$ (h$^{-1}$)</td>
<td>0.74 ± 0.02$^a$</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>$q_{\text{glyc}}$ (g g$^{-1}$ h$^{-1}$)$^b$</td>
<td>2.4 ± 0.1</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>$Y_{X/S}$ (g g$^{-1}$)$^c$</td>
<td>0.46 ± 0.03</td>
<td>0.54 ± 0.02</td>
</tr>
</tbody>
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Table 1. Exponential growth parameters of aerobic E. coli cultures with glucose as the sole carbon source.

$^a$ Standard deviation from triplicate experiments

$^b$ Specific glucose consumption rate

$^c$ Yield of biomass on glucose during the exponential growth phase
Figure 2. $^{13}$C scalar coupling fine structures observed for fractionally $^{13}$C-labeled amino acids obtained from wild-type E.coli MG1655 (left) and the Pgi mutant (right): (A) phenylalanine $^{13}$Ca, (B) alanine $^{13}$C$, (C)$ aspartate $^{13}$Ca, (D) glutamate $^{13}$Ca, (E) histidine $^{13}$C$, and (F) histidine $^{13}$C$. The carbon atoms are derived from the metabolic intermediates given in the panels. In each panel, the relative abundances of the different carbon fragments shown in (A) are indicated, where carbon-carbon bonds that remained intact during amino acid biosynthesis from glucose are shown in bold. In (E), the doublet of doublets arising from $^{13}$C$-^{13}$C$-^{13}$C is further split by the two-bond scalar coupling $^{2}$JCC, which reveals the presence of intact $^{13}$C-fragments originating from a single source molecule of glucose in ribose, which arise from the action of the oxidative branch of the PP pathway (14,16). The relative abundancies were calculated as described (16,17) and served to determine the biosynthetic origin of metabolic intermediates (Figure 3).
The ratios obtained for MG1655 (Figure 3) were very similar to those reported for *E. coli* JM101 when grown under the identical conditions (13). In accordance with textbook knowledge (9), the glyoxylate shunt (Figure 3H) and the gluconeogenic reactions catalyzed by PckA and MaeA/B (Figure 3F and G) were inactive during aerobic exponential growth on glucose. The only significant difference between MG1655 and JM101 was the relative flux through the anaplerotic Ppc reaction, 72% (Figure 3E) and 45% (13), respectively. The comparably high value of MG1655 demonstrates that it uses the TCA cycle predominantly for generating the building blocks OAA and OGA, and to a lesser extent for generating ATP via oxidative phosphorylation. *E. coli* JM101, on the other hand, exhibited a more balanced TCA cycle use for building block synthesis and energy generation (13).

![Figure 3. Origins of metabolic intermediates (A to K) during aerobic exponential growth of *E. coli* MG1655 (white bars) and the Pgi mutant (grey bars). In certain cases, the NMR data permit the determination only of upper bounds (ub) or lower bounds (lb) on the origin of metabolites. The experimental error was estimated from the analysis of redundant $^{13}$C scalar coupling fine structures and the signal-to-noise ratio of the $^{13}$C,$^{1}$H COSY spectra employing the Gaussian law of error propagation. The fraction of the total pool for a particular metabolite quantifies the ratio of this metabolite that is derived from a specified substrate to the sum of all other substrates that contribute to the pool of this metabolite. In cases where only two reactions contribute to one metabolite, e.g., OAA from PEP and PEP from OAA, the remaining fraction of the total pool can be attributed to the competing reaction. Abbreviations are explained in Figure 1.](image)

The METAFor data of the Pgi mutant differed significantly from those of the wild-type, most pronounced for the flux ratios that are related to the PP pathway (Figure 3A-D). Most telling is the upper bound of the fraction of PEP molecules that were derived through at least one transketolase reaction (Figure 3B), which is at around 5-10% for the wild-type but almost 100% for the mutant. This strongly suggests that glucose catabolism proceeds predominantly via the PP pathway in the mutant. Consistently, the fraction of P5P molecules that were not affected by fast exchange via transketolase is increased in the mutant (Figure 3A). Furthermore, the fraction of OAA originating from PEP (Figure 3E) was reduced more
than 2-fold in the mutant, when compared to the wild-type. This shows that the TCA cycle operates predominantly for the generation of ATP in the mutant.

Consistent with previous results (6), the primary flux response to Pgi inactivation appears to be the flux rerouting via the PP pathway. METAFor analysis by NMR, however, provides an upper bound for the fraction of PEP molecules that were derived via transketolase, and we thus cannot exclude residual glucose catabolism via the ED pathway based on the labeling data (16). In fact, ED pathway in vitro activity (conversion of 6PG to PYR) was identically low in glucose-grown cultures of both strains (1.2 ± 0.1 U/mg (protein)). Hence, we conclude that the ED pathway likely catalyzes a minor fraction of glucose catabolism in both wild-type and mutant strain. Consequently, the physiological consequences of PP pathway flux rerouting due to a Pgi knock-out is apparently manifested in significantly reduced specific growth and glucose uptake rates, and thus a kinetic limitation of glucose catabolism.

UdhA overexpression increases \( \mu_{\text{max}} \) of the Pgi mutant. Kinetic limitations of catabolism might be due to (i) the difficulty of E. coli metabolism to reoxidize NADPH, (ii) the inability of the PP pathway to support higher fluxes, or (iii) the inhibition of one or more essential reactions by accumulation of metabolites. Since have no evidence for the latter possibility (iii) from HPLC analysis of culture supernatants, the first two possibilities appear more likely. Reoxidation of NADPH can potentially be achieved by three reactions in E. coli, (i) the NADPH-dependent malic enzyme (either via backflux through the NADH-dependent malic enzyme or via a futile cycle from PYR via MAL, OAA, and PEP to PYR), (ii) the membrane-bound transhydrogenase PntAB (4), and (iii) the soluble transhydrogenase UdhA (3) (Figure 1). Significant involvement of the malic enzyme can be excluded because virtually no PYR originates from MAL (Figure 3G), and only a small fraction of PEP originates from OAA (Figure 3F). Similarly, PntAB is unlikely to catalyze sufficient reoxidation of NADPH because pgi\(^-\) pntAB\(^-\) double mutants grow as slowly as the pgi\(^-\) mutant (10), i.e., the additional knock-out of PntAB does not further reduce the rate of glucose catabolism, which would be expected if reoxidation through PntAB would play an important role. The primary metabolic function of PntAB appears to be in the generation of NADPH because zwf\(^-\) pnt\(^-\) mutants grow slower than a zwf\(^-\) mutant (10), as is expected when PntAB produces NADPH in the absence of PP pathway fluxes (in the zwf mutant).

To verify that insufficient reoxidation of NADPH is relevant for the kinetic limitation of glucose catabolism in Pgi mutants, we thus overexpressed the soluble transhydrogenase UdhA in both wild-type and Pgi mutant. The IPTG-induced expression level was chosen such that
two- to three-fold increased transhydrogenase activity was achieved, when compared to controls of the same strain that were transformed with the empty pTrc99a and subjected to the same IPTG concentration. While this moderate overexpression had no effect on \( \mu_{\text{max}} \) of the wild-type, \( \mu_{\text{max}} \) of the Pgi mutant was increased by about 25\% (from 0.22 ±0.00 to 0.27 ±0.01 h\(^{-1}\)). This observation provides the first evidence for a physiological role of the soluble transhydrogenase UdhA in the reoxidation of NADPH. A further increase in UdhA overexpression to a transhydrogenase activity about ten-fold the control activity did not lead to a further increase in \( \mu_{\text{max}} \) (data not shown). Thus, growth of the Pgi mutant cannot be restored fully to wild-type rates by further improving the capacity for NADPH reoxidation. This suggests that reduced growth of the Pgi mutant is caused by both the increased demand for NADPH reoxidation and the limited capacity of the PP pathway.

ACKNOWLEDGMENTS

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CHAPTER 3

Dissecting the role of soluble and membrane-bound transhydrogenases UdhA and PntAB in *Escherichia coli*

FABRIZIO CANONACO, SYLVIA HERI, ELIANE FISCHER, AND UWE SAUER

69
ABSTRACT

To elucidate the metabolic role for the soluble and the membrane-bound transhydrogenases UdhA and PntAB in Escherichia coli, we constructed mutants lacking one or both enzymes under conditions where NADPH formation was artificially modified. Specifically, we eliminated phosphoglucone isomerase (Pgi) or glucose 6-phosphate dehydrogenase (Zwf), thereby forcing carbon flux exclusively through the NADPH-generating pentose phosphate pathway in the former mutant, or avoiding this pathway in the latter mutant. The obtained mutants were analyzed on glucose, acetate, or glycerol as the sole carbon source. The main function of the soluble transhydrogenase appears to be the conversion of NADPH to NADH, as shown by the lethality of the combined deletion of pgi and udhA on glucose and by the beneficial effect of UdhA overexpression in the pgi knockout mutant. A role of UdhA in NADPH formation is excluded, as shown by the unchanged transhydrogenase flux if UdhA is knocked out. The membrane-bound transhydrogenase, in contrast, converts NADH to NADPH, as shown by the increase use of PP pathway in PntAB mutants and by the reduced growth if Zwf is knocked out additionally. The normal function of UdhA appears to be NADPH reoxidation on substrates that lead to higher formation of NADPH than glucose, e.g. acetate. Quantification of transhydrogenase transcript levels provides first evidence that expression of UdhA is upregulated in conditions that require reoxidation of NADPH, and PntAB is downregulated. In conditions that require reoxidation of NADH, UdhA is downregulated.
INTRODUCTION

A large number of anabolic reactions are required to produce the macromolecular components that make up functional cells, but the core of this intricate anabolic reaction network are eleven intermediates of central carbon metabolism and the cofactors ATP, NADH, and NADPH (23). These intermediates and cofactors must be supplied by carbon catabolism at appropriate stoichiometries for balanced growth; hence anabolism and catabolism are delicately balanced to enable growth on a wide variety of substrates. The reduction aequivalents NADH and NADPH serve distinct biochemical functions. While NADH is the main electron donor for the respiratory chain, NADPH is the usual reductant in anabolic processes. After decades of detailed biochemical studies, the pathways of carbon and energy metabolism (ATP and NADH) are known and relatively well understood (15, 23, 27). Despite its important role in linking catabolism and anabolism, however, such quantitative understanding of NADPH metabolism is still missing for most microbes.

The primary NADPH-generating reactions are typically considered to be those of the oxidative pentose phosphate (PP) pathway and the NADPH-dependent isocitrate dehydrogenase in the tricarboxylic acid (TCA) cycle (15) (REF. Fraenkel, E. coli book) (Figure 1). Additionally, nicotinamide nucleotide transhydrogenases may be involved, but ever since their discovery, the physiological role of transhydrogenases has been a source of speculation and often a matter of controversy (16, 17, 19). In principle, the reversible transhydrogenase reaction

\[ \text{NADPH} + \text{NAD}^+ + \text{H}^+ \text{in} \leftrightarrow \text{NADP}^+ + \text{NADH} + \text{H}^+ \text{out} \]

that may be catalyzed by either a membrane-bound, proton-translocating or a soluble, energy-independent isoform (1, 17) could function as a valve to reoxidize a surplus of NADPH from catabolism or as a NADPH-generating reaction. In eukaryotes, the proton-translocating transhydrogenase appears to have a flexible function as a buffer against dissipation of either the mitochondrial redox potential or the energy supply (17). In microbial systems, however, the physiological role of the two types of transhydrogenases remains an exciting matter of debate.

Organisms without functional transhydrogenases, such as the yeast Saccharomyces cerevisiae, cannot tolerate significant imbalances between catabolic NADPH production and anabolic NADPH consumption, hence must delicately balance both processes (2, 10). In the bacterium Escherichia coli, however, metabolic flux analyses revealed that catabolic NADPH formation exceeds the anabolic requirement under certain growth conditions (9), as was also shown for other bacteria (8, 22, 33). In sharp contrast to most microbes, E. coli contains both
a soluble and a membrane-bound transhydrogenase isoform, encoded by the \textit{udhA} (4) and \textit{pntAB} (6) genes, respectively. In particular the physiological role of the soluble isoform remains obscure, but heterologous overexpression of Udha or its analogs in yeast was shown to catalyze the conversion of NADPH to NADH (10, 25), since the [NADPH]-to-[NADP+] ratio is generally in a more reduced state than is the [NADH]-to-[NAD+] ratio (16).

Here we quantitatively elucidate the physiological role of the two transhydrogenases Udha and PntAB in \textit{E. coli} NADPH metabolism, more generally, and the extent to which catabolism is coupled to anabolism via NADPH. The experimental strategy is based on perturbing NADPH metabolism by defined mutations. Metabolic consequences of these perturbations are monitored by metabolic flux analysis based on $^{13}$C-labeling experiments, which is at present the exclusive methodology for quantification of NADPH formation (8, 9, 22, 32, 33).
Figure 1. Reaction network of *E. coli* central carbon metabolism. The arrows indicate physiological reaction directionality and enzymes are indicated by their 3-letter code. Abbreviations: G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; 6PG, gluconate 6-phosphate; KDPG, 2-keto-3-deoxy 6PG; GAP, glyceraldehyde 3-phosphate; PEP, phosphoenolpyruvate; ACoA, acetyl coenzyme A; OGA, oxoglutarate; OAA, oxaloacetate; Eda, KDPG aldolase; Edd, 6-phosphogluconate dehydratase; UdhA, soluble transhydrogenase, PntAB, membrane-bound transhydrogenase; Zwf, G6P dehydrogenase; Pgi, phosphogluucose isomerase; PP pathway, pentose phosphate pathway; ED pathway, Entner-Doudoroff pathway; TCA, tricarboxylic acid.
EXPERIMENTAL PROCEDURES

Strains, plasmids and growth conditions. All genetic manipulation were performed in \textit{E. coli} strain DH5\textalpha{} and physiological experiments were done with wild-type \textit{E. coli} MG1655 (\textit{E. coli} Genetic Stock Center, Yale University, New Haven, MT) and mutants derived thereof (Table 1). The knockout construction plasmids pKD13, pKD46, and pCP20 were obtained from B.L. Wanner (Purdue University, Lafayette, IN, USA) (7). pKD13 contains a kanamycin cassette (\textit{kan}) flanked by FLP recombinase recognition targets, as well as the ampicillin resistance gene (\textit{bla}). pKD46 carries the \textit{bla} gene, a temperature sensitive origin of replication with a non-permissive temperature of 37°C and the Red recombinase system, which enhances the recombination rate under the control of the arabinose-inducible promoter \textit{P}_{araB}. pCP20 contains the gene for chloramphenicol resistance (\textit{cat}) and the \textit{bla} gene. Moreover, it has a temperature sensitive origin of replication and expresses the yeast FLP recombinase. pUdhA contains the \textit{E. coli} \textit{udhA} gene under the control of the IPTG-inducible \textit{trc} promoter (5). The expression was induced with 100 \textmu{}M IPTG.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Known genetic markers</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5\textalpha{}</td>
<td>$F' / \text{endA1 hsdRI7 (}\text{r}\text{m}_{\text{K}^+} \text{) glnV55 thi-1 recA1 gyrA (Nal}^+ \text{)}$ $\text{relA1 (lacIZYA-argF)U169 deoR (}\phi80\text{dlacA(lacZ)M15})$</td>
<td>(34)</td>
</tr>
<tr>
<td>MG1655</td>
<td>$F' / \lambda^+ \text{rph-1}$</td>
<td>(20)</td>
</tr>
<tr>
<td>Pgi</td>
<td>MG1655 \textit{pgi}</td>
<td>this work</td>
</tr>
<tr>
<td>Zwf-EDP</td>
<td>MG1655 \textit{zwf edd eda}</td>
<td>this work</td>
</tr>
<tr>
<td>UdhA</td>
<td>MG1655 \textit{udhA}</td>
<td>this work</td>
</tr>
<tr>
<td>PntAB</td>
<td>MG1655 \textit{pntAB}</td>
<td>this work</td>
</tr>
<tr>
<td>UdhA-PntAB</td>
<td>MG1655 \textit{udhA pntAB}</td>
<td>this work</td>
</tr>
<tr>
<td>Pgi-UdhA</td>
<td>MG1655 \textit{pgi udhA}</td>
<td>this work</td>
</tr>
<tr>
<td>Zwf-EDP-UdhA</td>
<td>MG1655 \textit{zwf edd eda udhA}</td>
<td>this work</td>
</tr>
<tr>
<td>Zwf-EDP-PntAB</td>
<td>MG1655 \textit{zwf edd eda pntAB}</td>
<td>this work</td>
</tr>
</tbody>
</table>

Table 1. Used \textit{E. coli} strains. For proteins encoded by the deleted genes see also the legend to Figure 1.
Luria-Bertani (LB) complex medium and M9 minimal medium were prepared as described previously (29) and all physiological experiments were done in M9 medium. The M9 medium was supplemented with 5.0 g/l of either glucose, acetate, glycerol, or gluconate as the sole carbon source. Unless indicated otherwise, aerobic batch cultures were grown in 500 ml baffled shake flasks with maximally 50 ml medium at 37°C on a gyratory shaker at 200 rpm. When necessary, 50 mg/l ampicillin, 50 mg/l kanamycin, or 25 mg/l chloramphenicol were added.

For $^{13}$C-labeling experiments, cultures were grown in 30 ml M9 medium, supplemented with 3 g/l [1-$^{13}$C]-glucose or with a mixture of 0.6 g/l [U-$^{13}$C]-glucose and 2.4 g/l unlabeled glucose. After inoculation with maximally 1% (v/v) of an M9 culture and growth up to an optical density at 600 nm (OD$_{600}$) of 1.0-1.5, culture aliquotes were harvested, centrifuged at 2,500 g, and 4°C for 8 min, washed with 10 mM MgSO$_4$ and 9 g/l NaCl, and stored at -20°C.

**Genetic modifications / Strains construction.** Plasmid preparation and purification, and DNA extraction from agarose gels were performed using commercial kits (Promega, Madison WI, USA). All other recombinant DNA techniques were done according to standard protocols (28). Colony polymerase chain reaction (PCR) was performed by dissolving part of a colony from a LB plate into 50 µl of ddH$_2$O and incubation at 99°C for 10 min. 0.5-2.0 µl of the lysate were then used as the template for PCR.

Defined knockout mutants were generated by deleting genes from start to stop codon (7). Briefly, PCR-fragments of the FRT-flanked kanamycin gene from plasmid pKD13 were amplified with primers that contained sequence extension homologous to flanking regions of target genes or operons. The following primers were used:

for the *pgi* gene 5'-aatcgtgctatatgttattagccagatactgacatatatttccggggatccggtgacc-3' and 5'-aacatagctgaaggcactaaacactatcatatatttttgtaggctggagctgcttc-3';

for the *zwf edd eda* operon 5'-caagtgagaaagctatgcgtatgtgattttttactagttggcgggatccggtgacc-3' and 5'-gacttttacagctgccctttctacagctgcttcagccgatccggtgacc-3';

for the *udhA* gene 5'-aagctctatgaggtttctgctgaggttgtattttgcgtctaggtgagctgcttc-3' and 5'-aaaaactcaataataacctatttcgactctgtgctgggatccggtgacc-3';

for the *pntAB* operon 5'-ctcagcagaggcttattttgctgcgtccaggtttcatctgcttgagctgcttc-3' and 5'-acacagccaaaccatcatataaaccagtagggaaggtgtaggctggagctgcttc-3' (chromosomal sequences underlined).

The *DpnI*-digested PCR-fragments were purified and transformed into the target strain, containing the temperature-sensitive Red recombinase plasmid pKD46. Correct insertion of
PCR fragments in kanamycin-resistant transformants was verified by colony PCR using primers homologous to regions adjacent the knocked-out gene or operon. The pKD46 Red-helper plasmid was cured by growing clones with correctly inserted PCR fragments at 37°C on LB plates with kanamycin. Plasmid loss was verified by ampicillin sensitivity at 30°C. To excise the chromosomally-integrated kanamycin cassette, FLP-recombinase plasmid pCP20 was transformed to cured clones at 30°C. Correct excision in chloramphenicol-resistant and kanamycin-sensitive clones was verified by colony PCR using the same primers as before. Finally, pCP20 was cured by growing clones at 37°C on LB plates without antibiotics, and chloramphenicol sensitivity was verified at 30°C.

**GC-MS analysis.** The biomass pellets were resuspended and hydrolyzed with 1.5 ml 6 M HCl at 105°C for 24 h in sealed glass tubes. Hydrolysates were dried in a vacuum centrifuge at room temperature and derivatized at 85°C in 50 μl tetra-hydrofuran (Fluka, Switzerland) and 50 μl of N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide (MTBSTFA) (Fluka Switzerland) for 60 min. The mass isotope distribution in the amino acids was then analyzed by GC-MS of the derivatized samples as described (12).

The GC-MS-derived mass distributions of proteinogenic amino acids were corrected for naturally occurring isotopes, and the corrected mass distributions were used for METAFoR (metabolic flux ratio) analysis (12). Calculation of the metabolic flux ratios was done with the MATLAB-based (Mathworks, Natick, MA, USA) software Fiat Flux (N. Zamboni, unpublished) using the equations described in (12). The relative abundances of intact carbon fragments originating from a single source molecule of glucose were calculated from the corrected mass distributions of the amino acids.

Following the principles outlined by Sauer et al. (30), metabolic net fluxes were determined using Fiat Flux by considering a stoichiometric matrix containing 25 fluxes and 22 metabolite balances (including balances for the measured substrates and products glucose, acetate and CO₂ and the cofactors NADH and NADPH) derived from the bioreaction network considered in Figure 1. Since this system of linear equations is underdetermined, the previously accumulated flux ratios were used as additional constraints (N. Zamboni, unpublished). Briefly, the sum of the weighed square residuals of the constraints from both metabolite balances and flux ratios was minimized using the MATLAB function *fmincon*. The residuals were weighed by division with the experimental error. For flux ratios implemented as upper and lower bounds only error residuals from positive or negative deviations from the experimental value, respectively, were considered.
Determination of physiological parameters and errors. Cell growth was monitored by determination of optical density at 600 nm (OD\textsubscript{600}), glucose, acetate, and protein concentrations were determined enzymatically using commercial kits (Beckman-Coulter, Zürich, Switzerland or Dispolab, Dielsdorf, Switzerland) as described previously (9). Physiological parameters in batch cultures were calculated as described previously (29). Briefly, in batch cultures, the exponential growth phase was identified by log-linear regression of OD\textsubscript{600} versus time, with maximal growth rate ($\mu_{\text{max}}$) as the regression coefficient. To obtain specific production rates, OD\textsubscript{600} values were converted to cellular dry weight (cdw) using a predetermined correlation factor of 0.51 g/l cdw per OD\textsubscript{600} unit (5). The specific substrate uptake rate ($q_s$) was calculated by log-linear regression of substrate versus biomass concentration, dividing the regression coefficient by the maximal growth rate. Errors of metabolic net fluxes and metabolic flux ratios were calculated as described in (12). All other errors are calculated either as standard deviations or as average error of the mean. For a general function $f(x,y)$ the latter is calculated as $\Delta f(x,y) = \sqrt{(\partial f/\partial x)^2 + (\partial f/\partial y)^2}$.

In vitro enzyme activities. Crude cell extracts were prepared from M9 medium batch cultures that were harvested at around OD\textsubscript{600} of 1. Cell pellets were washed once with 9 g/l NaCl and 10 mM MgSO\textsubscript{4}, and disrupted by sonication at 100 W on ice for 2 min. Enzyme activities and protein concentrations were determined either directly in crude cell extracts or in supernatants after centrifugation at 10,000 g for 30 min. Specific activities of phosphoglucone isomerase were determined as described previously (14). The transhydrogenase protocol was slightly modified (26). Briefly, the change in absorbance at 375 nm was monitored at 25°C in a mixture containing 50 mM Tris-HCl (pH 7.6), 2 mM MgCl\textsubscript{2}, 500 $\mu$M NADPH (Fluka, Switzerland), 1 mM 3-acetylpyridine adenine dinucleotide (Sigma, Switzerland), and 10-100 $\mu$l crude cell extract. Since PntAB is membrane-bound, this activity was determined in cell extracts. The specific activity was then obtained by dividing the measured slope by the protein concentration.

Quantitative RT-PCR. Total RNA was purified from 1 ml of a batch culture with an OD\textsubscript{600} of about unity with the RNeasy Mini Kit (Qiagen). The obtained RNA solution was frozen in liquid nitrogen and stored at -70°C for further use. Aliquots were analyzed on agarose gels containing formaldehyde for quantification and determination of purity (28). RT-PCR was then performed using the OneStep RT-PCR Kit (Qiagen), according to the suppliers
instructions. Reaction mixtures were setup with 100 ng of total RNA and 0.6 μM of gene-specific primers. Reverse transcription was done for 30 min at 50°C and the cDNA was amplified by amplification cycles of 30 s at 94°C, 30 s at 55°C, and 1 min/kb at 72°C, using the following primers:

for *udhA* 5'-AAAATGTTGGCCGCGGTGC-3'
and 5'-CATCGTCGGG TAGTTAAGGTGTT-3';
for *pntA* 5'-CCAAGAGAACGGT TAAACCAATGAAA-3'
and 5'-ATAAGCACC CGGATAAAACTAAGGAA-3';
for *rpoD* 5'-GATCAACGACATGGCGATTAGGTGTT-3'
and 5'-CTTCCTCCAGCGTGTAGTCGGTGTTCATA-3'.

To stop amplification of the obtained cDNA during the exponential phase, we tested 10, 15, 20, 25, and 30 amplification cycles. Since 20 cycles gave the best trade-off between amplification yield and easiness of quantification (data not shown), all other RT-PCR experiments were done with 20 cycles. Total RNA samples were routinely tested for the absence of chromosomal DNA contaminations by using control primers hybridizing outside the open reading frame. The quality of the RNA preparations was also confirmed by spectrophotometric and electrophoretic assays (28). A 2 μl aliquot was analyzed on an agarose gel and quantified by visual inspection. Results were normalized according to the amplification product with *rpoD*-specific primers.
RESULTS

Construction and physiological characterization of knockout mutants. To elucidate the function of the two transhydrogenases in *E. coli*, we constructed single and double mutants of both transhydrogenases and two families of knockout mutants with increased or reduced formation of NADPH during glucose catabolism. For this purpose, a marker-free deletion method was chosen (7), thus minimizing potential side effects. Increased NADPH formation in the first mutant family was achieved by deleting the *pgi* gene, which encodes the phosphoglucone isomerase, thereby forcing carbon flux from glucose to proceed exclusively through the PP or ED pathways (Figure 1). The Pgi mutant grew with a significantly reduced maximal specific growth rate of 0.22 h\(^{-1}\) on glucose, as was described previously (14). As a consequence of the *pgi* deletion, the *in vitro* activity of phosphoglucone isomerase in the Pgi strain was reduced to 10.6 ± 0.1 IU/mg during growth on glucose, while the wild-type exhibited an activity of 966.3 ± 0.5 IU/mg.

In the wild-type background, the deletion of transhydrogenases affects the maximal specific growth rate only in the case of the membrane-bound transhydrogenase. Surprisingly, double mutant grew as fast as the wild-type strain (Table 2). In the Pgi mutant background, however, knockout of UdhA precludes growth on glucose.

The second mutant family was constructed in the background of Zwf-EDP mutant that lacks the first reaction of the PP pathway, glucose 6-phosphate dehydrogenase, and the entire ED pathway (Figure 1). As a consequence, NADPH formation is reduced when compared to the wild-type situation. The Zwf-EDP strain grew slightly slower on glucose than wild-type (Table 2), as was described before (13). Differently from the Pgi mutant family, knockout of the soluble transhydrogenase in strain Zwf-EDP-UdhA had no negative effects. In sharp contrast, deletion of the membrane-bound transhydrogenase in strain Zwf-EDP-PntAB, was detrimental to growth, resulting in very poor growth rates (Table 2).

It should be noted that all transhydrogenase mutants showed significantly lower maximum biomass concentration and yield of biomass on glucose than their control strain (Table 2).
<table>
<thead>
<tr>
<th>Strain</th>
<th>Maximum specific growth rate (h(^{-1}))</th>
<th>Maximum biomass concentration (g·l(^{-1}))</th>
<th>Yield of biomass on substrate during exponential phase (g/g)</th>
<th>Specific glucose uptake rate (mmol·g(^{-1})·h(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG1655</td>
<td>0.67 ± 0.01</td>
<td>2.24 ± 0.02</td>
<td>0.47 ± 0.03</td>
<td>7.9 ± 0.5</td>
</tr>
<tr>
<td>UdhA</td>
<td>0.67 ± 0.01</td>
<td>1.62 ± 0.03</td>
<td>0.78 ± 0.24</td>
<td>4.8 ± 1.5</td>
</tr>
<tr>
<td>PntAB</td>
<td>0.61 ± 0.03</td>
<td>1.89 ± 0.02</td>
<td>0.71 ± 0.25</td>
<td>4.8 ± 1.7</td>
</tr>
<tr>
<td>UdhA-PntAB</td>
<td>0.69 ± 0.02</td>
<td>1.77 ± 0.03</td>
<td>0.40 ± 0.06</td>
<td>9.7 ± 1.5</td>
</tr>
<tr>
<td>Pgi</td>
<td>0.22 ± 0.02</td>
<td>not determined</td>
<td>not applicable</td>
<td>not determined</td>
</tr>
<tr>
<td>Pgi-UdhA</td>
<td>no growth</td>
<td>not applicable</td>
<td>not applicable</td>
<td>not applicable</td>
</tr>
<tr>
<td>Zwf-EDP</td>
<td>0.41 ± 0.01</td>
<td>2.12</td>
<td>0.24 ± 0.03</td>
<td>9.6 ± 1.3</td>
</tr>
<tr>
<td>Zwf-EDP-UdhA</td>
<td>0.48 ± 0.01</td>
<td>1.71</td>
<td>0.74 ± 0.27</td>
<td>3.6 ± 1.3</td>
</tr>
<tr>
<td>Zwf-EDP-PntAB</td>
<td>0.05 ± 0.03</td>
<td>not determined</td>
<td>not applicable</td>
<td>not determined</td>
</tr>
</tbody>
</table>

Table 2. Physiological parameters of knockout mutants during exponential batch growth on glucose.

To get insights on the relevance of transhydrogenase activity, we measured it in glucose cultures (Figure 2). Transhydrogenase activity does not vary in the Pgi and Zwf-EDP strains during growth on glucose. In the UdhA strain the transhydrogenase activity is reduced by 43% and in the PntAB strain by 71%. In the UdhA-PntAB strain, transhydrogenase activity was zero.

Surprisingly, transhydrogenase remains at a high level when UdhA is deleted in the Zwf-EDP strain, indicating that either UdhA is not active in this background, or that the loss in activity is compensated by upregulation of PntAB. In the Zwf-EDP-PntAB strain, in contrast, an increase in transhydrogenase activity is observed, indicating that upregulation of UdhA is required to partially compensate for the missing membrane-bound transhydrogenase.
Figure 2. Specific transhydrogenase activity in knockout mutants. The activity was determined in crude cell extract, harvested from exponentially growing batch cultures in M9 medium with 5 g/l glucose. Values are the means of independent duplicate experiments. Maximal deviations were always smaller than 3%.

Physiological behaviour of strains overexpressing the soluble transhydrogenase. Since the Pgi-UdhA mutant is not viable on glucose, we tested here whether overexpression of UdhA, improves growth of the Pgi mutant. Plasmid-based overexpression of the soluble transhydrogenase UdhA led to a 2-fold increased transhydrogenase activity (data not shown). In the absence of phosphoglucose isomerase on glucose, overexpression of the soluble transhydrogenase increased the growth rate by 13% and the substrate uptake rate by 40% as was shown before for a similar strain (5). The growth rate of the Zwf-EDP mutant, however, was not affected by overexpression of UdhA (Table 3). Growth on gluconate causes a reduction of the net formation rate of both NADH and NADPH, leaving the NADPH:NADH ratio unchanged (see Appendix A). Under these conditions, the overexpression of the soluble transhydrogenase on wild type background causes a 10% reduction of growth rate and a 5% reduction of the maximal biomass concentration (Table 3).
Table 3. Physiological parameters of strains overexpressing the soluble transhydrogenase UdhA. Cultures were grown in M9 medium with 100 μM IPTG (final concentration) and 5 g/l glucose or 5 g/l gluconate (n.d.: non determined).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Maximum specific growth rate (h⁻¹)</th>
<th>Maximum biomass concentration (g/l)</th>
<th>Specific substrate uptake rate (mmol·g⁻¹·h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Gluconate</td>
<td>Glucose</td>
</tr>
<tr>
<td>MG1655 [pTrec99A]</td>
<td>0.70 ± 0.02</td>
<td>0.64 ± 0.00</td>
<td>2.13 ± 0.02</td>
</tr>
<tr>
<td>Pgi [pTrec99A]</td>
<td>0.23 ± 0.01</td>
<td>n.d.</td>
<td>1.70 ± 0.02</td>
</tr>
<tr>
<td>Zwf-EDP [pTrec99A]</td>
<td>0.44 ± 0.02</td>
<td>n.d.</td>
<td>1.75 ± 0.03</td>
</tr>
<tr>
<td>MG1655 [pUdhA]</td>
<td>0.65 ± 0.02</td>
<td>0.58 ± 0.00</td>
<td>2.11 ± 0.01</td>
</tr>
<tr>
<td>Pgi [pUdhA]</td>
<td>0.26 ± 0.01</td>
<td>n.d.</td>
<td>1.65 ± 0.02</td>
</tr>
<tr>
<td>Zwf-EDP [pUdhA]</td>
<td>0.44 ± 0.02</td>
<td>n.d.</td>
<td>1.80 ± 0.02</td>
</tr>
</tbody>
</table>

**Determination of metabolic net fluxes and flux ratios.** To obtain insights into the relative usage of pathways and the formation rates of reducing equivalents, we determined metabolic flux ratios (Table 4) and metabolic net fluxes (Table 5) in the constructed mutants (for complete flux data see Appendix B and C). In the UdhA strain, the usage of the ED pathway is doubled and the PP pathway is reduced, while in the PntAB strain the flux to PP pathway is increased by 22%. Similarly to the PntAB strain, the double transhydrogenase mutant UdhA-PntAB showed a 34% increase in PP pathway and a 17% decrease in glycolysis usage. This modification in the usage of the fueling pathways causes changes in the net formation rates for NADPH and NADH, as estimated by net flux analysis (Table 5).

In the wild-type, transhydrogenases contributes significantly to NADPH formation (Table 5). In the UdhA mutant we observe the same flux as in wild-type, but in PntAB and UdhA-PntAB strains the transhydrogenase flux is zero. This indicates that PntAB is a significant NADPH producing pathway in wild-type *E. coli* during batch growth on glucose.

Metabolic fluxes in the Zwf-EDP mutant reveal that reducing equivalent metabolism is significantly perturbed, with a very high formation rate of NADH and, as a consequence, reduced flux through the transhydrogenase to NADH. Surprisingly, however, there is still a small but significant flux through the PP pathway. This may be catalyzed by the periplasmic
glucose oxidase. Deletion of phosphoglucone isomerase leads to an even more radical metabolic perturbation it is the only case where the transhydrogenase flux is reversed, now actually requiring NADPH to NADH flux. Moreover, these flux results demonstrate also the biochemically expected flux responses of blocking flux into glycolysis or the PP pathway.

### Table 4. Metabolic flux ratios (METAFoR) obtained by GC-MS analysis from [U-13C]-and [l-,3C]-glucose labeled (♀) cultures. For abbreviations refer to Figure 1

<table>
<thead>
<tr>
<th>Metabolic flux ratios</th>
<th>MG1655</th>
<th>UdhA</th>
<th>PntAB</th>
<th>UdhA</th>
<th>MG1655</th>
<th>Pgi</th>
<th>Zwf-EDP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pUdhA</td>
<td></td>
<td></td>
<td>pUdhA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serine through glycolysis ‡</td>
<td>0.76 ± 0.01</td>
<td>0.80 ± 0.01</td>
<td>0.67 ± 0.01</td>
<td>0.63 ± 0.01</td>
<td>0.75 ± 0.01</td>
<td>0.00 ± 0.00</td>
<td>0.98 ± 0.01</td>
</tr>
<tr>
<td>Pyruvate through ED Pathway ‡</td>
<td>0.00 ± 0.07</td>
<td>0.18 ± 0.07</td>
<td>0.12 ± 0.05</td>
<td>0.08 ± 0.05</td>
<td>0.12 ± 0.06</td>
<td>0.28 ± 0.05</td>
<td>0.00 ± 0.01</td>
</tr>
<tr>
<td>OAA from PEP</td>
<td>0.76 ± 0.05</td>
<td>0.67 ± 0.04</td>
<td>0.54 ± 0.04</td>
<td>0.91 ± 0.07</td>
<td>0.69 ± 0.07</td>
<td>0.35 ± 0.06</td>
<td>0.37 ± 0.06</td>
</tr>
<tr>
<td>PEP from OAA</td>
<td>-0.04 ± 0.01</td>
<td>0.00 ± 0.05</td>
<td>0.00 ± 0.01</td>
<td>0.00 ± 0.18</td>
<td>0.02 ± 0.02</td>
<td>0.01 ± 0.01</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>Pyruvate from malate (lb)</td>
<td>0.02 ± 0.02</td>
<td>0.04 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.06 ± 0.19</td>
<td>0.01 ± 0.02</td>
<td>0.02 ± 0.02</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>Pyruvate from malate (ub)</td>
<td>0.07 ± 0.06</td>
<td>0.13 ± 0.05</td>
<td>0.16 ± 0.03</td>
<td>0.66 ± 0.61</td>
<td>0.02 ± 0.07</td>
<td>0.07 ± 0.05</td>
<td>0.06 ± 0.05</td>
</tr>
<tr>
<td>PEP through PP pathway (ub)</td>
<td>0.10 ± 0.17</td>
<td>0.09 ± 0.07</td>
<td>0.25 ± 0.05</td>
<td>0.08 ± 0.08</td>
<td>0.10 ± 0.17</td>
<td>0.85 ± 0.07</td>
<td>0.00 ± 0.07</td>
</tr>
</tbody>
</table>

Table 5. Selected net fluxes obtained by flux balancing of physiological data (Table 2 and 3) and using the METAFoR values (Table 4). Net NADH and NADPH formation rates are calculated as the sum of the net fluxes of reactions by which cofactors are formed and their errors are calculated as average error of the mean. For abbreviations refer to Figure 1.

<table>
<thead>
<tr>
<th>Net flux</th>
<th>MG1655</th>
<th>UdhA</th>
<th>PntAB</th>
<th>UdhA</th>
<th>MG1655</th>
<th>Pgi</th>
<th>Zwf-EDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>(mmol·g (cdw)⁻¹·h⁻¹)</td>
<td>pUdhA</td>
<td></td>
<td></td>
<td>pUdhA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose uptake rate</td>
<td>7.50 ± 0.01</td>
<td>6.90 ± 0.61</td>
<td>8.11 ± 0.10</td>
<td>7.38 ± 0.11</td>
<td>7.75 ± 0.04</td>
<td>2.50 ± 0.01</td>
<td>8.80 ± 0.05</td>
</tr>
<tr>
<td>G6P → F6P</td>
<td>4.95 ± 0.03</td>
<td>4.60 ± 0.43</td>
<td>4.76 ± 0.06</td>
<td>4.04 ± 0.06</td>
<td>5.03 ± 0.66</td>
<td>0.01 ± 0.00</td>
<td>7.82 ± 0.05</td>
</tr>
<tr>
<td>6PG → P5P</td>
<td>2.12 ± 0.04</td>
<td>1.55 ± 0.01</td>
<td>2.70 ± 0.05</td>
<td>2.98 ± 0.06</td>
<td>2.11 ± 0.19</td>
<td>2.06 ± 0.04</td>
<td>0.94 ± 0.01</td>
</tr>
<tr>
<td>6PG → KDPG</td>
<td>0.39 ± 0.07</td>
<td>0.71 ± 0.25</td>
<td>0.62 ± 0.05</td>
<td>0.33 ± 0.05</td>
<td>0.58 ± 0.11</td>
<td>0.42 ± 0.04</td>
<td>0.00 ± 0.01</td>
</tr>
<tr>
<td>NADPH formation rate</td>
<td>6.95 ± 0.46</td>
<td>6.15 ± 0.65</td>
<td>9.89 ± 0.24</td>
<td>10.91 ± 0.09</td>
<td>6.77 ± 0.48</td>
<td>6.49 ± 0.17</td>
<td>8.45 ± 0.21</td>
</tr>
<tr>
<td>NADH formation rate</td>
<td>23.55 ± 0.99</td>
<td>21.27 ± 2.12</td>
<td>30.86 ± 0.65</td>
<td>29.20 ± 0.25</td>
<td>23.71 ± 2.54</td>
<td>10.98 ± 0.39</td>
<td>42.51 ± 0.05</td>
</tr>
<tr>
<td>transhydrogenase</td>
<td>-3.63 ± 0.47</td>
<td>-4.42 ± 0.86</td>
<td>0.01 ± 0.23</td>
<td>0.08 ± 0.09</td>
<td>-3.52 ± 0.19</td>
<td>3.54 ± 0.03</td>
<td>-2.26 ± 0.05</td>
</tr>
</tbody>
</table>
Growth of knockout mutants on acetate and gluconate. To elucidate the normal function of the two transhydrogenases, we grew the generated knockout mutants on acetate and gluconate, substrates which are expected to generate more and less NADPH, respectively. Growth on acetate strongly increases the formation of NADPH, while the NADH formation is only slightly increased, resulting in an increase of the NADPH:NADH ratio. Gluconate decreases formation of both NADPH and NADH, but leaves the NADPH:NADH ratio unchanged (see Appendix A).

Knockout of the soluble transhydrogenase appears to be lethal if cells are grown on acetate (Table 6), the substrate that generates a surplus of NADPH. While PntAB grows undisturbed on acetate, knockout of pntAB in the Zwf-EDP strain is lethal, probably because this mutant cannot generate sufficient NADPH. On gluconate, all strains lacking one or both transhydrogenases showed the same growth behaviour as the respective control strain (Table 6).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Maximum specific growth rate (h⁻¹)</th>
<th>Maximal biomass concentration (g cdw.l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acetate</td>
<td>Gluconate</td>
</tr>
<tr>
<td>wild type</td>
<td>0.24 ± 0.02</td>
<td>0.65 ± 0.03</td>
</tr>
<tr>
<td>UdhA</td>
<td>no growth</td>
<td>0.65 ± 0.04</td>
</tr>
<tr>
<td>PntAB</td>
<td>0.20 ± 0.02</td>
<td>0.62 ± 0.03</td>
</tr>
<tr>
<td>UdhA-PntAB</td>
<td>0.03 ± 0.00</td>
<td>0.61 ± 0.02</td>
</tr>
<tr>
<td>Zwf-EDP-UdhA</td>
<td>no growth</td>
<td>not determined</td>
</tr>
<tr>
<td>Zwf-EDP-PntAB</td>
<td>no growth</td>
<td>0.67 ± 0.02</td>
</tr>
</tbody>
</table>

Table 6. Physiological parameters of knockout mutants on acetate and gluconate. Maximal specific growth rate and maximal biomass concentration were determined in strains as described in Table 1. Cultures were grown in M9 supplemented with 4.9 g/l acetate (Ac) or 5.1 g/l gluconate (Glu).

Determination of the udhA and pntA transcript levels. To address the question of how transhydrogenases are regulated, the levels of udhA and pntA mRNA were determined for all transhydrogenase mutants and for wild-type by quantitative RT-PCR on different carbon sources. The rpoD gene, encoding the RNA polymerase subunit σ⁷⁰, was used as an internal
standard (24). Aliquots of the RT-PCR reactions were separated on agarose gels and quantified by visual inspection (Figure 3). At this point the results obtained for pntA and udhA were divided by the intensity found for rpoD. The normalized values were expressed as fold-changes of the transcript level in wild type grown on glucose (Figure 4).

Figure 3. Quantification of RT-PCRs on agarose gels. Quantitative RT-PCR was performed with total RNA isolated from mutants grown on glucose, acetate, or glycerol as the sole carbon source. Reaction aliquots were then loaded onto a 1% agarose gel. M indicates the marker lane, 1 the amplification of rpoD, 2 the amplification of udhA, and 3 the amplification of pntA. Conditions analyzed: A, MG1655 on glucose; B, MG1655 on acetate; C, MG1655 on glycerol; D, UdhA on glucose; E, UdhA on glycerol; F, UdhA-PntAB on glucose; G, MG1655 on glucose; H, PntAB on glucose; I, PntAB on acetate; J, PntAB on glycerol; K, Pgi on glucose; L, MG1655:pUdhA on glucose; M, Zwf-EDP on glucose; N, Zwf-EDP-UdhA on glucose; O, Zwf-EDP-PntAB on glucose.

Figure 4. Relative transcript levels of udhA (A) and pntA (B) determined by quantitative RT-PCR. Cultures were grown in batch culture with either 5.0 g/l glucose (grey bars), 0.49 g/l acetate (white bars), or 5.1 g/l glycerol (black bars) as the sole carbon source. Values normalized to the transcript level on glucose in MG1655.

Only changes exceeding 0.5- to 1.5-fold of the wild type level were considered as significant (Figure 4). Transcription of pntA was upregulated under conditions with increased
NADPH formation, i.e. growth on acetate (Table 1) or with high PP pathway fluxes in the Pgi strain. The Pgi mutant on glucose and wild-type on acetate showed a 3.3-fold decrease in \( pntA \) mRNA levels. Under conditions, where the NADPH formation was decreased (growth on glycerol (Table 1) or with high glycolysis fluxes in the Zwf-EDP strain), we detected either a moderate upregulation of \( pntA \) or a significant downregulation of \( udhA \) transcription. The Zwf-EDP mutant showed on glucose a 1.7-fold increase in \( pntA \) mRNA level and a 3.3-fold decrease in \( udhA \) mRNA level, whereas the wild type grown on glycerol showed a 5.0-fold decrease of \( udhA \) transcription.

If \( udhA \) was deleted, \( pntA \) transcription was increased by 1.7-fold during growth on glycerol, whereas it showed a 2.0-fold decrease during growth on glucose. This would speak in favor of a compensation for the missing transhydrogenase by transcriptional regulation of the intact one. However, an analogous compensation in \( udhA \) transcription level was not observed in the PntAB mutant grown on glucose and acetate as sole carbon source. Finally, in both Zwf-EDP-UdhA and Zwf-EDP-PntAB we observed an increase in the transcription level of the intact transhydrogenase during growth on glucose.
**DISCUSSION**

*Escherichia coli* is at present the sole organism known to contain both a soluble and a membrane-bound transhydrogenase (3). We show here that both isoforms have distinct physiological functions. The main role of the membrane-bound transhydrogenase is the formation of NADPH by NADH reoxidation. In the wild-type, the knockout of the membrane-bound transhydrogenase causes a significant increase in the use of NADPH-generating PP pathway. Moreover, the Zwf-EDP-PntAB mutant is almost incapable of growth, which can be rationalized by the fact that the two primary NADPH generating reactions are missing in this strain. In wild-type the PP pathway and PntAB contribute to about 75% of the NADPH production. The transhydrogenase activity in the Zwf-EDP-PntAB mutant was 2.5 fold higher than in the Zwf-EDP mutant, most likely due to increased expression of UdhA (Figure 4). Thus, the activation of UdhA in the absence of PntAB may be an attempt to compensate for the missing NADH reoxidation activity. However, considering the extremely low growth rate of 0.03 h\(^{-1}\) of the Zwf-EDP-PntAB mutant argues against a NADPH formation activity of UdhA.

The primary role of the soluble transhydrogenase UdhA appears to be NADPH reoxidation, because the knockout of UdhA in the Pgi mutant, which is the only strain with a surplus of NADPH, is lethal on glucose. Overexpression of UdhA in the Pgi mutant, in contrast, is advantageous and increases the maximal specific growth rate significantly. The catalytic properties of UdhA are also more appropriate for NADPH reoxidation because UdhA is repressed by NADP\(^+\) and activated by NADPH (3). These results are consistent with the observed recovery of the lethal Pgi mutation in *S. cerevisiae* by expression of *E. coli* UdhA (11). The *in vitro* rate of NADPH reoxidation by PntAB is much slower as the rate of NADPH formation (21,31). We expect therefore no important role of PntAB in NADPH reoxidation.

Since UdhA appears to have no function during batch growth on glucose, what then is its normal role? The results suggest that UdhA is important for growth on substrates which generate increased NADPH formation, such as for example acetate, since the UdhA knockout is lethal on this substrate. The ability to optimally behave during acetate consumption is of utmost importance for *E. coli*, since this by-product is exploited after consumption of glucose. Acetate is only an example, and the ability to reoxidize NADPH by soluble transhydrogenase is applicable in all other situations where increased NADPH formation occurs. This result is consistent with the overexpression of an UdhA-like transhydrogenase in *S. cerevisiae*, which likewise catalyzed the conversion of NADPH into NADH (25).
Determination of transcriptional levels of transhydrogenases grown in presence of acetate and glycerol provide first insights into regulation of these enzymes. Concomitant with increased NADPH availability in the Pgi mutant on glucose or in wild-type on acetate, we observe a downregulation of pntAB. Analogously, downregulation of udhA occurs if NADPH formation is reduced in the Zwf-EDP mutant on glucose or in wild-type grown on glycerol. This indicates the presence of a regulatory mechanism at transcriptional level.

Acknowledgements. We thank Barry Wanner for the gift of pKD13, pKD46, and pCP20.
APPENDIX A

Cells grown on glucose, acetate, glycerol, or gluconate as sole carbon source show different net NADPH and NADH formation rates. To estimate the extent of such changes, we calculated the expected NADPH:NADH ratio for growth on these carbon sources (Table A1), on the basis of previously calculated fluxes (18). The net formation of both NADPH and NADH is increased on acetate, but of NADPH on a greater extent. On glycerol, the net formation of NADH is strongly increased, since one molecule of NADH is synthesized per metabolized molecule of glycerol (24). Moreover, since glycerol is directly converted to GAP (Figure 1), less NADPH is available.

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>Reaction</th>
<th>Specific formation rates (mmol · (g cdw)^{-1} · h^{-1}) during growth on</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td>NADPH</td>
<td>G6P → 6PG</td>
<td>1.86</td>
</tr>
<tr>
<td></td>
<td>6PG → P5P</td>
<td>1.80^1</td>
</tr>
<tr>
<td></td>
<td>Isocitrate → OGA</td>
<td>3.36</td>
</tr>
<tr>
<td></td>
<td>Sum</td>
<td>7.02</td>
</tr>
<tr>
<td>NADH</td>
<td>GAP → PGA</td>
<td>17.29</td>
</tr>
<tr>
<td></td>
<td>OGA → Succinate</td>
<td>2.37</td>
</tr>
<tr>
<td></td>
<td>Succinate → Fumarate</td>
<td>2.37^2</td>
</tr>
<tr>
<td></td>
<td>Malate → OAA</td>
<td>2.37^2</td>
</tr>
<tr>
<td></td>
<td>Pyruvate → AcCoA</td>
<td>10.73</td>
</tr>
<tr>
<td></td>
<td>PGA → Serine</td>
<td>1.28</td>
</tr>
<tr>
<td></td>
<td>Glycerol → Glyc-3-P</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Malate → Pyruvate</td>
<td>0.00</td>
</tr>
<tr>
<td>Sum</td>
<td></td>
<td>36.41</td>
</tr>
<tr>
<td>NADPH:NADH ratio</td>
<td></td>
<td>0.19</td>
</tr>
</tbody>
</table>

Table A1. Fluxes through NADPH and NADH producing reactions. For abbreviations refer to Figure 1. Malic enzyme is considered as NADH-dependent.

1 On glycerol and acetate, we consider the same flux through ED pathway as on glucose (3% of the flux from G6P to 6PG). On gluconate this flux corresponds to 10.62 mmol · (g cdw)^{-1} · h^{-1} (93% of G6P to 6PG) (18).

2 This values are considered to be the same as for OGA→Succinate, if glyoxylate shunt is not active. A flux through ED pathway corresponding to 3% of the flux from G6P to 6PG is considered on glucose, glycerol and acetate, 93% on gluconate.
### APPENDIX B

**In vivo reaction rates (mmol \cdot g (cdw)^{-1} \cdot h^{-1})**

<table>
<thead>
<tr>
<th>Reaction</th>
<th>MG1655</th>
<th>UdhA</th>
<th>PntAB</th>
<th>UdhA</th>
<th>PntAB</th>
<th>MG1655</th>
<th>Pgi</th>
<th>Zwf-EDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose uptake rate</td>
<td>7.50±0.01</td>
<td>6.90±0.61</td>
<td>8.11±0.10</td>
<td>7.38±0.11</td>
<td>7.75±0.94</td>
<td>2.50±0.01</td>
<td>8.80±0.05</td>
<td></td>
</tr>
<tr>
<td>G6P → 6PG</td>
<td>2.51±0.03</td>
<td>2.26±0.21</td>
<td>3.32±0.04</td>
<td>3.31±0.05</td>
<td>2.69±0.28</td>
<td>2.48±0.01</td>
<td>0.94±0.01</td>
<td></td>
</tr>
<tr>
<td>6PG → P5P + CO2</td>
<td>2.12±0.04</td>
<td>1.55±0.10</td>
<td>2.70±0.05</td>
<td>2.98±0.06</td>
<td>2.11±0.19</td>
<td>2.06±0.04</td>
<td>0.94±0.01</td>
<td></td>
</tr>
<tr>
<td>G6P → F6P</td>
<td>4.95±0.03</td>
<td>4.60±0.43</td>
<td>4.76±0.06</td>
<td>4.04±0.06</td>
<td>5.03±0.66</td>
<td>0.01±0.00</td>
<td>7.82±0.05</td>
<td></td>
</tr>
<tr>
<td>6PG → T3P + Pyruvate</td>
<td>0.39±0.07</td>
<td>0.71±0.25</td>
<td>0.62±0.05</td>
<td>0.33±0.05</td>
<td>0.58±0.11</td>
<td>0.42±0.04</td>
<td>0.00±0.01</td>
<td></td>
</tr>
<tr>
<td>F6P + ATP → 2T3P</td>
<td>5.80±0.05</td>
<td>5.07±0.45</td>
<td>6.05±0.08</td>
<td>5.44±0.09</td>
<td>5.90±0.78</td>
<td>1.28±0.03</td>
<td>7.88±0.05</td>
<td></td>
</tr>
<tr>
<td>2 P5P → S7P + T3P</td>
<td>0.57±0.01</td>
<td>0.38±0.03</td>
<td>0.79±0.01</td>
<td>0.85±0.02</td>
<td>0.58±0.06</td>
<td>0.68±0.01</td>
<td>0.18±0.00</td>
<td></td>
</tr>
<tr>
<td>P5P + E4P → F6P + T3P</td>
<td>0.33±0.01</td>
<td>0.14±0.03</td>
<td>0.56±0.01</td>
<td>0.60±0.02</td>
<td>0.34±0.06</td>
<td>0.60±0.01</td>
<td>-0.07±0.00</td>
<td></td>
</tr>
<tr>
<td>P5P + E4P → E4P + F6P</td>
<td>0.57±0.01</td>
<td>0.38±0.03</td>
<td>0.79±0.01</td>
<td>0.85±0.02</td>
<td>0.58±0.06</td>
<td>0.68±0.01</td>
<td>0.18±0.00</td>
<td></td>
</tr>
<tr>
<td>T3P → PGA</td>
<td>12.25±0.06</td>
<td>10.91±1.06</td>
<td>13.21±0.27</td>
<td>11.73±0.18</td>
<td>12.64±1.70</td>
<td>3.57±0.03</td>
<td>15.60±0.11</td>
<td></td>
</tr>
<tr>
<td>PGA → PEP</td>
<td>11.04±0.07</td>
<td>9.71±1.07</td>
<td>12.10±0.26</td>
<td>10.48±0.16</td>
<td>11.48±1.69</td>
<td>3.29±0.04</td>
<td>14.38±0.11</td>
<td></td>
</tr>
<tr>
<td>PEP → Pyr</td>
<td>7.93±0.37</td>
<td>6.88±0.77</td>
<td>8.97±0.26</td>
<td>4.69±0.16</td>
<td>8.86±1.88</td>
<td>2.37±0.09</td>
<td>11.13±0.13</td>
<td></td>
</tr>
<tr>
<td>Pyruvate → AcCoA + CO2</td>
<td>6.95±0.12</td>
<td>5.93±1.28</td>
<td>8.50±0.25</td>
<td>6.23±0.12</td>
<td>7.67±1.74</td>
<td>2.36±0.10</td>
<td>9.84±0.15</td>
<td></td>
</tr>
<tr>
<td>OAA + AcCoA → ICT</td>
<td>2.32±0.46</td>
<td>2.34±0.61</td>
<td>3.87±0.23</td>
<td>4.63±0.05</td>
<td>1.98±0.34</td>
<td>1.95±0.17</td>
<td>6.56±0.21</td>
<td></td>
</tr>
<tr>
<td>ICT + OGA → CO2</td>
<td>2.32±0.46</td>
<td>2.34±0.61</td>
<td>3.87±0.23</td>
<td>4.63±0.05</td>
<td>1.98±0.34</td>
<td>1.95±0.17</td>
<td>6.56±0.21</td>
<td></td>
</tr>
<tr>
<td>OGA + Fumarate + CO2</td>
<td>1.45±0.46</td>
<td>1.48±0.62</td>
<td>3.05±0.04</td>
<td>3.75±0.04</td>
<td>1.13±0.35</td>
<td>1.68±0.17</td>
<td>5.69±0.21</td>
<td></td>
</tr>
<tr>
<td>Fumarate → Malate</td>
<td>1.45±0.46</td>
<td>1.48±0.62</td>
<td>3.05±0.04</td>
<td>3.75±0.04</td>
<td>1.13±0.35</td>
<td>1.68±0.17</td>
<td>5.69±0.21</td>
<td></td>
</tr>
<tr>
<td>Malate → OAA</td>
<td>0.84±0.12</td>
<td>1.15±0.22</td>
<td>2.27±0.23</td>
<td>0.51±0.08</td>
<td>0.96±0.12</td>
<td>1.50±0.12</td>
<td>4.97±0.18</td>
<td></td>
</tr>
<tr>
<td>Malate → Pyruvate + CO2</td>
<td>0.61±0.35</td>
<td>0.33±0.40</td>
<td>0.78±0.13</td>
<td>3.24±0.07</td>
<td>0.18±0.26</td>
<td>0.19±0.09</td>
<td>0.72±0.05</td>
<td></td>
</tr>
<tr>
<td>OAA + ATP + PEP + CO2</td>
<td>0.00±0.01</td>
<td>0.00±0.05</td>
<td>0.00±0.01</td>
<td>0.00±0.02</td>
<td>0.02±0.03</td>
<td>0.05±0.01</td>
<td>0.20±0.01</td>
<td></td>
</tr>
<tr>
<td>PEP + CO2 → OAA</td>
<td>2.59±0.36</td>
<td>2.31±0.40</td>
<td>2.64±0.13</td>
<td>5.26±0.08</td>
<td>2.13±0.24</td>
<td>0.81±0.08</td>
<td>2.92±0.06</td>
<td></td>
</tr>
<tr>
<td>AcCoA → Acetate</td>
<td>3.08±0.49</td>
<td>2.04±0.82</td>
<td>3.19±0.37</td>
<td>0.00±0.10</td>
<td>4.18±2.03</td>
<td>0.00±0.16</td>
<td>1.71±0.24</td>
<td></td>
</tr>
<tr>
<td>NADPH → NADH</td>
<td>-3.63±0.47</td>
<td>-4.42±0.86</td>
<td>0.01±0.23</td>
<td>0.08±0.09</td>
<td>-3.52±0.19</td>
<td>3.54±0.26</td>
<td>-2.26±0.25</td>
<td></td>
</tr>
<tr>
<td>Net CO2 formation rate</td>
<td>12.23</td>
<td>10.69</td>
<td>17.58</td>
<td>16.95</td>
<td>12.31</td>
<td>7.96</td>
<td>22.42</td>
<td></td>
</tr>
</tbody>
</table>

Table B. In vivo reaction rates in transhydrogenase mutants obtained by GC-MS analysis of hydrolysates from $^{13}$C-glucose labeling experiments. Reactions are indicated without cofactors. For abbreviations refer to Figure 1. Errors are calculated as described in (12).
### APPENDIX C

**Metabolic net fluxes normalized to the glucose uptake rate**

<table>
<thead>
<tr>
<th>Reaction</th>
<th>MG1655</th>
<th>UdhA</th>
<th>PntAB</th>
<th>UdhA</th>
<th>MG1655</th>
<th>Pgi</th>
<th>Zwf-EDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6P → 6PG</td>
<td>0.335 ± 0.004</td>
<td>0.328 ± 0.042</td>
<td>0.409 ± 0.007</td>
<td>0.448 ± 0.009</td>
<td>0.346 ± 0.055</td>
<td>0.992 ± 0.006</td>
<td>0.107 ± 0.002</td>
</tr>
<tr>
<td>6PG → P5P + CO₂</td>
<td>0.283 ± 0.006</td>
<td>0.225 ± 0.024</td>
<td>0.333 ± 0.007</td>
<td>0.403 ± 0.010</td>
<td>0.272 ± 0.041</td>
<td>0.825 ± 0.016</td>
<td>0.107 ± 0.002</td>
</tr>
<tr>
<td>G6P → F6P</td>
<td>0.660 ± 0.004</td>
<td>0.667 ± 0.086</td>
<td>0.586 ± 0.010</td>
<td>0.547 ± 0.011</td>
<td>0.649 ± 0.116</td>
<td>0.005 ± 0.000</td>
<td>0.889 ± 0.008</td>
</tr>
<tr>
<td>6PG → T3P + Pyruvate</td>
<td>0.052 ± 0.009</td>
<td>0.103 ± 0.037</td>
<td>0.077 ± 0.006</td>
<td>0.045 ± 0.007</td>
<td>0.074 ± 0.017</td>
<td>0.166 ± 0.016</td>
<td>0.000 ± 0.001</td>
</tr>
<tr>
<td>F6P + ATP → 2T3P</td>
<td>0.774 ± 0.007</td>
<td>0.736 ± 0.093</td>
<td>0.746 ± 0.013</td>
<td>0.737 ± 0.016</td>
<td>0.761 ± 0.136</td>
<td>0.513 ± 0.011</td>
<td>0.895 ± 0.008</td>
</tr>
<tr>
<td>2P5P → S7P + T3P</td>
<td>0.076 ± 0.002</td>
<td>0.055 ± 0.007</td>
<td>0.097 ± 0.002</td>
<td>0.115 ± 0.003</td>
<td>0.074 ± 0.012</td>
<td>0.272 ± 0.005</td>
<td>0.020 ± 0.000</td>
</tr>
<tr>
<td>P5P + E4P → F6P + T3P</td>
<td>0.044 ± 0.002</td>
<td>0.020 ± 0.005</td>
<td>0.068 ± 0.002</td>
<td>0.082 ± 0.002</td>
<td>0.044 ± 0.009</td>
<td>0.241 ± 0.005</td>
<td>0.008 ± 0.000</td>
</tr>
<tr>
<td>P5P + E4P → E4P + F6P</td>
<td>0.076 ± 0.002</td>
<td>0.055 ± 0.007</td>
<td>0.097 ± 0.002</td>
<td>0.115 ± 0.003</td>
<td>0.074 ± 0.012</td>
<td>0.272 ± 0.005</td>
<td>0.020 ± 0.000</td>
</tr>
<tr>
<td>T3P → PGA</td>
<td>1.633 ± 0.009</td>
<td>1.584 ± 0.209</td>
<td>1.628 ± 0.038</td>
<td>1.589 ± 0.033</td>
<td>1.650 ± 0.294</td>
<td>1.427 ± 0.014</td>
<td>1.773 ± 0.016</td>
</tr>
<tr>
<td>PEP → Pyr</td>
<td>1.472 ± 0.010</td>
<td>1.409 ± 0.200</td>
<td>1.492 ± 0.036</td>
<td>1.420 ± 0.030</td>
<td>1.480 ± 0.281</td>
<td>1.316 ± 0.016</td>
<td>1.634 ± 0.016</td>
</tr>
<tr>
<td>Pyruvate → AcCoA + CO₂</td>
<td>0.926 ± 0.016</td>
<td>0.861 ± 0.201</td>
<td>1.048 ± 0.034</td>
<td>0.843 ± 0.020</td>
<td>0.989 ± 0.254</td>
<td>0.946 ± 0.041</td>
<td>1.118 ± 0.019</td>
</tr>
<tr>
<td>OAA + AcCoA → ICT</td>
<td>0.309 ± 0.061</td>
<td>0.339 ± 0.094</td>
<td>0.477 ± 0.029</td>
<td>0.627 ± 0.011</td>
<td>0.755 ± 0.054</td>
<td>0.781 ± 0.066</td>
<td>0.746 ± 0.024</td>
</tr>
<tr>
<td>ICT → OGA + CO₂</td>
<td>0.309 ± 0.061</td>
<td>0.339 ± 0.094</td>
<td>0.477 ± 0.029</td>
<td>0.627 ± 0.011</td>
<td>0.755 ± 0.054</td>
<td>0.781 ± 0.066</td>
<td>0.746 ± 0.024</td>
</tr>
<tr>
<td>OGA → Fumarate + CO₂</td>
<td>0.193 ± 0.061</td>
<td>0.214 ± 0.091</td>
<td>0.376 ± 0.029</td>
<td>0.508 ± 0.009</td>
<td>0.146 ± 0.048</td>
<td>0.674 ± 0.068</td>
<td>0.647 ± 0.024</td>
</tr>
<tr>
<td>Fumarate → Malate</td>
<td>0.193 ± 0.061</td>
<td>0.214 ± 0.091</td>
<td>0.376 ± 0.029</td>
<td>0.508 ± 0.009</td>
<td>0.146 ± 0.048</td>
<td>0.674 ± 0.068</td>
<td>0.647 ± 0.024</td>
</tr>
<tr>
<td>Malate → OAA</td>
<td>0.112 ± 0.015</td>
<td>0.166 ± 0.035</td>
<td>0.279 ± 0.028</td>
<td>0.069 ± 0.011</td>
<td>0.123 ± 0.022</td>
<td>0.598 ± 0.048</td>
<td>0.565 ± 0.021</td>
</tr>
<tr>
<td>Malate → Pyruvate + CO₂</td>
<td>0.082 ± 0.047</td>
<td>0.048 ± 0.059</td>
<td>0.096 ± 0.016</td>
<td>0.439 ± 0.011</td>
<td>0.023 ± 0.033</td>
<td>0.073 ± 0.036</td>
<td>0.081 ± 0.006</td>
</tr>
<tr>
<td>OAA + ATP → PEP + CO₂</td>
<td>0.000 ± 0.002</td>
<td>0.000 ± 0.008</td>
<td>0.000 ± 0.002</td>
<td>0.000 ± 0.002</td>
<td>0.003 ± 0.004</td>
<td>0.018 ± 0.005</td>
<td>0.023 ± 0.001</td>
</tr>
<tr>
<td>PEP + CO₂ → OAA</td>
<td>0.345 ± 0.047</td>
<td>0.335 ± 0.065</td>
<td>0.325 ± 0.016</td>
<td>0.713 ± 0.015</td>
<td>0.275 ± 0.045</td>
<td>0.322 ± 0.032</td>
<td>0.332 ± 0.007</td>
</tr>
<tr>
<td>AcCoA → Acetate</td>
<td>0.411 ± 0.065</td>
<td>0.297 ± 0.121</td>
<td>0.394 ± 0.045</td>
<td>0.000 ± 0.014</td>
<td>0.540 ± 0.269</td>
<td>0.000 ± 0.065</td>
<td>0.194 ± 0.027</td>
</tr>
<tr>
<td>NADPH → NADH</td>
<td>-0.484 ± 0.063</td>
<td>-0.642 ± 0.138</td>
<td>0.001 ± 0.029</td>
<td>0.011 ± 0.012</td>
<td>-0.454 ± 0.060</td>
<td>1.416 ± 0.104</td>
<td>-0.257 ± 0.029</td>
</tr>
</tbody>
</table>

**Table C.** Metabolic net fluxes (%) in transhydrogenase mutants obtained by normalization of data from Appendix B to the glucose uptake rate. Reactions are indicated without cofactors. For abbreviations refer to Figure 1. Errors are calculated as average error of the mean.
REFERENCES


Functional expression of phosphagen kinase systems confers resistance to transient stresses in *Saccharomyces cerevisiae* by buffering the ATP pool

FABRIZIO CANONACO, UWE SCHLATTNER, PAMELA S. PRUETT, THEO WALLIMANN, AND UWE SAUER

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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 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. While 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.
INTRODUCTION

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 (21). Hence, metabolic ATP generation in a cell must be tightly balanced with ATP consuming processes. Small derivations 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 rapidly replenish ATP during phases of high energy demand, according to the following reaction (42):

\[ \text{MgADP}^- + \text{PCr}^{2-} + H^+ \rightleftharpoons \text{MgATP}^{2-} + \text{Cr} \]

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 a phosphagen kinase system (6). 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 (6), with arginine kinase (AK; EC 2.7.3.3) as a prominent example, occurring in insects (13), crustaceans (45) and in certain unicellular organisms (30). In analogy to CK, AK catalyzes the reaction:

\[ \text{MgADP}^- + \text{PArg}^{2-} + H^+ \rightleftharpoons \text{MgATP}^{2-} + \text{Arg}. \]

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 (6,42). In addition to the above general functions, the CK/PCr system has the unique capability to establish a spatial energy buffering as well (11). 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 (34). 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 (15,23). 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 (28,30). 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 environmental stresses, including exposure to weak acids
and copper (10,17,41) but also as a secondary effect of stress challenges like desiccation and
heat (1,29). This 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 (17). The energetic expense for this proton pumping activity may require up to
60% of the total ATP production (35), thus constituting an ATP drain similar to muscle
contraction. Likewise starvation and several other stress challenges are known to exert high
ATP demands (14).

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 towards 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 which can be easily
genetically manipulated.
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 E. coli DH5α (F' endA1 hsdR17(rK-mK) glnV44 thi-1 recA1 gyrA(Nalr) relA1 Δ(lacZΔM15)U169 deoR (φ80dλlacΔ(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 (16,26).

All physiological experiments were done in yeast minimal medium (YMM), containing 0.85 g/l KH₂PO₄, 0.15 g/l K₂HPO₄, 0.5 g/l MgSO₄, 0.1 g/l NaCl, 0.1 g/l CaCl₂, 500 μg/l H₂BO₃, 63 μg/l CuSO₄·5H₂O, 100 μg/l KI, 200 μg/l FeCl₃, 450 μg/l MnSO₄·H₂O, 235 μg/l Na₂MoO₄·2H₂O, 712 μg/l ZnSO₄·7H₂O, 20 μg/l biotin, 2 mg/l calcium pantothenate, 2 μg/l folic acid, 10 mg/l inositol, 0.4 mg/l nicotinic acid, 0.2 mg/l p-aminobenzoic acid, 0.4 mg/l pyridoxine hydrochloride, 0.2 mg/l riboflavin, and 0.4 mg/l thiamin hydrochloride. If not specified otherwise, the medium was also supplemented with 5 g/l (NH₄)₂SO₄ and 5 g/l glucose as nitrogen and carbon sources respectively. Uracil (20 mg/l), tryptophane (50 mg/l), or leucine (240 mg/l) were added, if necessary.

Growth conditions. Aerobic batch cultivations were performed in 500 ml baffled shake flasks with maximally 50 ml 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 either 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 zero or 0.5% (w/v) glucose as well as zero 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 either 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 medium at 30°C on a gyratory shaker at 300 rpm. Cultures were grown to an optical density at 600 nm (OD$_{600}$) of about 0.5, before the pH was set to 2 with 10% (v/v) H$_3$PO$_4$. 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 l using a weight-controlled pump and the pH was controlled at 5.0 by the addition of 2M KOH. The airflow was kept constant at 1.0 L/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, UK). 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 (M. Stolz and T. Wallimann, unpublished) with the primers 5'-CGCACTAGTATGCCTCCTCAAA-3' and 5'-CGCGAATTCTTATTTCTGAGCTGG-3'. The resulting SpeI / EcoRI 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 (R. Furter and T. Wallimann, unpublished) with the primers 5'-GACTAGTATGTTTTCAAATCTATCTAA-3' and 5'-CCGCTCGAGTCACTTCCTGCGCAAAC-3'. The resulting SpeI / XhoI fragment was then cloned into p426HXT7. pAGAT was constructed by PCR amplification of the L-arginine-glycine amidinotransferase gene from pig (18) from plasmid pBS/SK⁺:RGAT-7 with the primers 5'-CGCGGATCCATGCTGCGGGTGC-3' and 5'-CGCGAATTCTCAGTCCAAGTAGGAC-3'. The resulting BamHI / EcoRI fragment was then cloned into p425HXT7. pGAMT was constructed by PCR amplification of the human guanidino acetate methyltransferase gene (20) from pGEM-T-4-hGAMT-7 with the primers 5'-CGCAGATCCATGCTGCGGGTGC-3' and 5'-CGCGAATTCTCAGCTTCTGAGCTGG-3'.
The resulting SpeI/EcoRI fragment was cloned into p426HXT7. pAK was constructed by PCR amplification of the Limulus polyphemus AK gene (38) from plasmid pET-22b(+):AK with the primers 5'-GGAATTCATGGTGGACCAGGCAACATTTG-3' and 5'-TGCGGTCGACTTAGGCAGCAGCCTTTTCCATC-3'. The resulting EcoRI/SaiI fragment was cloned into p424HXT7. pCreaT was constructed by PCR amplification of the Rattus norvegicus creatine transporter gene (22) from plasmid pRC/CMV-HCRT. The resulting EcoRI/EcoRI fragment was cloned into p424HXT7 and correct insertion was checked by digestion analysis. 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 pcv of cold water and resuspending in 2 pcv 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 M (NH₄)₂SO₄, and 1 mM phenylmethanesulfonylfluorid (PMSF) at 0°C. 4 pcv ice-cold, acid-washed 0.45-0.55 mm glass beads were added, 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, the supernatant 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 (24,43). 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-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 MgCl₂, 83 μM EGTA, 1 mM β-mercaptoethanol, 4 mM ADP, and 0.1 U/ml rabbit B-CK. Calibration was done with Cr concentrations between 1 to 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 U/ml glutamate dehydrogenase, 10 U/ml urease, and 6 U/ml arginase to crude cell extracts and monitoring the change in absorbance at 340 nm (25).

PArg concentrations were determined as described previously (28). 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 since incubation at lower temperatures did not result in complete disruption of the yeast cells (data not shown). Stability of PArg during this treatment was experimentally verified. While acid incubation at 95°C for 2 min lead to significant hydrolysis of PArg, PArg concentrations after 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 Na₂SO₄ and centrifuged for 5 min at 13'000 rpm to remove residual barium ions. Trichloroacetic acid was added to 2.3% (v/v) 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/l polyvinyl alcohol and 185 mg/l malachite green.

Intracellular ATP and ADP concentrations were determined as described previously (7,40). 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 dimethylsulfoxide and 750 μl 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 Biochemicals). ADP was converted to ATP by incubating another aliquot for 30 min at 37°C with 1 mM phosphoenolpyruvate and 1’250 U/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 1.162·10⁻¹¹ ml and a concentration of 10⁸ cells/ml at an OD₆₀₀ of 1.5 (44).
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. In order to calculate specific production rates, OD₆₀₀ values were converted to cellular dry weight (cdw) using a predetermined correlation factor (0.52 g/l 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 (q₀₂) was calculated dividing the CER by the biomass concentration (X) and the culture volume (V), on the basis of the relationship q₀₂ = CER / (X · 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* (Figure 1), we first over-expressed the cytosolic brain-type isoenzyme (B-CK) and/or the ubiquitous mitochondrial isoenzyme (uMtCK). Transformants exhibited CK activity of 0.5 – 0.9 IU/mg protein (Figure 2), corresponding to the CK activity found in many chicken tissues (32). Based on a previous report (3), we expected Cr uptake from the medium, although yeast genome data suggested the absence of a specific Cr transporter gene (27,36). During growth in YMM supplemented with 10 mM Cr, however, neither CK-expressing nor control yeast accumulated any appreciable amounts of intracellular Cr (Table 1).

![Diagram of cellular uptake and biogenesis of the phosphagens analyzed](image)

Figure 1. *S. cerevisiae* engineered with either arginine kinase or creatine kinase systems. Scheme showing cellular uptake and biogenesis of the phosphagens analyzed. Black boxes indicate heterologous enzymes that are not naturally present in *S. cerevisiae*. Abbr.: AGAT, L-arginine:glycine amidinotransferase; AK, arginine kinase; CK, creatine kinase; GAMT, guanidinoacetate methyltransferase; SAM, S-adenosyl methionine; SAH, S-adenosyl homocysteine.
Table 1. 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).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Intracellular Cr conc. (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>pBCK</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>pAGAT</td>
<td>0.12 ± 0.2</td>
</tr>
<tr>
<td>pGAMT</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>pAGAT pGAMT</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>pAGAT pGAMT pBCK</td>
<td>3.3 ± 0.2</td>
</tr>
</tbody>
</table>

To activate potential unspecific Cr transport systems or silent genes (33) 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), nor any growth in cultures with Cr as the unique carbon or nitrogen source.
To provide intracellular Cr to CK-expressing *S. cerevisiae*, we tried two strategies. As first, we expressed the creatine specific transporter from *R. norvegicus* (22). Unfortunately, the expression from the plasmid pCreaT resulted deleterious for growth even at reduced level, not allowing cultured cells to attain OD_{600} higher than 0.3 (data not shown). Anyway, we could not detect any increased amount of creatine in the cell extract of CreaT expressing cells (data not shown), and therefore the CreaT expression experiments were abandoned.

As a second strategy, we installed the biosynthetic pathway for Cr that naturally does not occur in yeast (27,36). Expression of human L-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 due to 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 (37). Thus, functional establishment of an AK system should depend solely on heterologous expression of AK, which is not naturally present in yeast (45). Independent of the amount of exogenously supplied Arg, AK activities in crude extracts of cells harboring pAK were 3.7 IU/mg, while 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 (Figure 3). In this respect, an essentially identical behavior was observed for control and AK expressing strains. While 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, whereas intracellular Arg concentrations in these AK expressing strains were similar to those seen in controls.
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 which provoke a drop in ATP and therefore possibly confer an advantage to cells expressing a phosphagen kinase system (17,29).

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 $\mu_{\text{max}}$ value, as well as the time needed to recover growth (defined as ‘recovery time’), were investigated. Cultures harbouring the complete CK-system as above did not show any improvement with respect to the controls (Figure 4b). In contrast, AK conferred the ability to reduce the recovery time from 3.3 h to 2.3 h (Figure 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.
Figure 4. Influence of intracellular acidification on growth of engineered yeast strains. Growth of *S. cerevisiae* CEN.PK 113-6B either harbouring (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 show at the bottom. The figure shows one representative out of 3 independent experiments performed.

*Expression of AK stabilizes intracellular ATP levels during short-term starvation.* Since 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 (5). In order to expose *S. cerevisiae* for defined periods to energetic stress, glucose-limited chemostat experiments were used, which place
cultures in a metabolic state where they efficiently generate energy and biomass from glucose as the limiting nutrient (4,31). 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 5 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 seconds): 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 5 volume changes. The only exceptions were 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 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 2). Hence, this profile was chosen for further analysis.

<table>
<thead>
<tr>
<th>Feed type</th>
<th>Plasmid</th>
<th>OD₆₀₀₀</th>
<th>q_CO₂ (mmol·g⁻¹·h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Continuous</td>
<td>control</td>
<td>4.44</td>
<td>0.69 ± 0.07</td>
</tr>
<tr>
<td>Continuous</td>
<td>pAK</td>
<td>4.92</td>
<td>0.52 ± 0.05</td>
</tr>
<tr>
<td>Discontinuous</td>
<td>control</td>
<td>3.25</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td>Discontinuous</td>
<td>control</td>
<td>3.28</td>
<td>n.a.</td>
</tr>
<tr>
<td>Discontinuous</td>
<td>pAK</td>
<td>4.28</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>Discontinuous</td>
<td>pAK</td>
<td>4.34</td>
<td>0.07 ± 0.01</td>
</tr>
</tbody>
</table>

Table 2. Growth and specific CO₂ production rate. OD₆₀₀₀ and specific CO₂ production rate (q_CO₂) were determined in steady-state before and in pseudo steady-state in continuous cultures of control and AK-expressing *S. cerevisiae*. 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.

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 2). 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 CO₂ production rate in the AK-expressing culture (Table 2). Upon imposing the above chosen discontinuous feeding profile, a new pseudo steady-state was attained after about 5 medium changes with a significantly decreased biomass concentration (Table 2). However, the AK-expressing culture was apparently less stressed by the harsh conditions, since 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 mM to 1.5 mM in wild-type S. cerevisiae and remained at this level until the onset of feeding (Figure 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 level (Figure 6). A similar intracellular ATP concentration was observed during the 30 s feeding interval in pseudo steady-state (Figure 5). In contrast, ADP levels were found to be relatively constant under all conditions in both strains (Figure 6).

![Figure 5. Time course of CO₂ production and intracellular ATP during starvation stress in AK-engineered yeast.](image)

The specific CO₂ 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). The bold line represents the discontinuous feeding profile (30 s on, 150 s off). Values are given as mean ± SD of three independent measurements over a period of two days.
Figure 6. Intracellular ATP and ADP in control and AK-engineered yeast. Concentrations of ATP (grey bars) and ADP (white bars) in steady-state before stress begins (label 'not stressed') and in pseudo steady-stress (label '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 3 independent measurements over a period of two days in pseudo steady-state. The samples were withdrawn at least 30 seconds after the feed offset and before the feed onset.
DISCUSSION

The present study shows for the first time a successful functional expression of phosphagen kinase systems in *S. cerevisiae*. The AK- and the 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 about 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.

Since 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 (33). Thus, not only homologues to known Cr transporters are absent in the yeast genome (27,36), but also unspecific Cr transport via general amino acid permeases (12) 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 (3). In these cultures, when maintained at 100 mM Cr, Brindle et al. (3) reported the accumulation of 50-100 mM intracellular Cr, determined in a biochemical assay. However, this cannot be taken as a 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 (Schlattner et al., unpublished results). In fact, the very low concentration of PCr observed in these yeast cells rather suggests that the Cr is separated from the cytosolic CK and therefore mainly (if not entirely) extracellular. Remnant PCr may have been produced by CK 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 \( \text{H}^+ \)-ATPase (17). An extra boost of energy from the accumulated PArg pool may help maintain AK-expressing cells to resume growth upon relieve 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 \( S. \text{cerevisiae} \) 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. Since the intracellular level of ADP was similar in both cultures, it appears that most of the hydrolyzed ATP accumulated as AMP. This is due to the presence of three isoenzymes and a rather high enzymatic activity of yeast adenylate kinase (2). These enzymes catalyze the formation of ATP and AMP from two molecules of ADP, thus buffering the cellular energy charge (21). The intracellular concentration of ATP and ADP determined here compare favourably with those of another \( S. \text{cerevisiae} \) strain that was grown under almost identical culture conditions (39). 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. (39), who investigated metabolic responses to a glucose pulse in glucose-limited chemostat culture.

Given the protective effects of AK in \( S. \text{cerevisiae} \), 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 (9). 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 (42). In fact, with a \( K_m \) (PCr) for chicken B-CK of 1.4 mM, these PCr concentrations do not favour fast conversions. Furthermore, the intracellular pH is much lower in yeast (pH 5.5-6.2) (17,19) as compared to the natural environment of CK, e.g. in the resting muscle (pH 7.0-7.2) (42). The acidic intracellular pH of yeast reduces B-CK activity by about 50% and leads to partial hydrolysis of PCr, which is less stable than PArg under acidic conditions (6). 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 towards 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 (34). 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, due to imperfect mixing (8).

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for the pRC/CMV-CHOT15 plasmid. and Ross Ellington, Florida State University, for the
AKcDNA.
REFERENCES


pombe as well as the extent of duration of the heat shock response. *J Gen Microbiol* 136, 1763-1770.


CHAPTER 5

Functional expression of arginine kinase improves recovery from pH stress of *Escherichia coli*

FABRIZIO CANONACO, UWE SCHLATTNER, THEO WALLIMANN, AND UWE SAUER
ABSTRACT

Acid stress in Escherichia coli involves a complex resource- and energy-consuming response mechanism. By overexpression of arginine kinase from Limulus polyphemus in E. coli, we improved the recovery from a transient pH stress. While wild type E. coli resumed growth after a transient pH reduction to pH 3 for 1 h with a rate that was 25% lower than before the stress, the arginine kinase expressing strain continued to grow as rapidly as before. This effect is presumably caused by the physiological function of arginine kinase as a short term energy buffer in the form of phosphoarginine, but a pH-buffering effect cannot be excluded.
INTRODUCTION

Cells of higher organisms often contain so-called phosphagen kinases that serve as transient ATP buffering systems during high or fluctuating energy requirements. Perhaps the most well-known of these is creatine kinase (CK) which is found in all vertebrates, as well as in sponges and in some worms (8). CK isoenzymes exert spatial and temporal energy buffering functions and are involved in intracellular pH homeostasis (24). Arginine kinase (AK; EC 2.7.3.3) is a conceptually simpler phosphagen kinase, which occurs in insects (13), crustaceans (25) and in certain unicellular organisms (17). Although transient ATP buffering is the main function of AK (8), an involvement in pH buffering cannot be excluded. AK uses arginine (Arg) to create a metabolically inert pool of phosphoarginine (PArg), according to the following reaction (25):

\[
\text{MgADP}^+ + \text{PArg}^2+ + \text{H}^+ \rightleftharpoons \text{MgATP}^2+ + \text{Arg}.
\]

Although microorganisms are often exposed to rapidly changing environmental conditions and fluctuating availability of energy, phosphagen kinases seem to have evolved only with metazoans (8). Exceptions are the unicellular eukaryotes Trypanosoma spp. and Paramecium caudatum, probably as the result of horizontal gene transfer (1,16-18). Since the high motility of these latter organisms requires large bursts of energy, the availability of an ATP buffering system obviously conferred a significant evolutionary advantage.

Bacteria frequently encountered environmental stresses that generate a severe demand for ATP (14), as for example exposure to low pH in the case of Escherichia coli (3). The most prominent response to pH stress is activation of an ATPase, which compensates variations in cytoplasmic pH by extruding protons to the periplasm, with concomitant consumption of ATP (14). Moreover, severe intracellular acidification impairs ATP production from fueling pathways as a consequence of enzyme inhibition (4). Further protective mechanisms include proton extruding ion antiporters and symporters (4,11,26) and proton-consuming amino acid decarboxylases (7).

Recently, we showed that heterologous expression of AK in the unicellular yeast Saccharomyces cerevisiae provided a transient ATP buffer, which also helped to overcome a transient, energy-demanding stress of short term starvation (6). Functional expression of creatine kinase was demonstrated in E. coli (15), but data from our own lab showed that creatine kinase had no apparent beneficial effect on cellular physiology (5). Since AK expression was successful in yeast, we extend here our studies on transient ATP-buffering systems to prokaryotes by overexpressing AK from Limulus polyphemus in E. coli.
**Experimental Procedures**

*Strains and plasmids construction.* E. coli wild-type strain MG1655 (λ-, F', rph-1) was used for all physiological experiments, and E. coli DH5α (F' / endA1 hsdR17(tk-mk−) glnV44 thi-1 recA1 gyrA(Nalr) relA1 Δ(lacZYA-argF)U169 deoR (φ80dlacA(lacZ)M15)) was used for genetic constructions. The expression vector pTrc99A (Pharmacia Biotech) was used for heterologous gene expression. Expression was driven from the strong, IPTG-inducible trc promoter (2). The AK-encoding gene of *L. polyphemus* was PCR-amplified from plasmid pET-22b(+):AK (22) with the primers 5'-GGAATTCATGGTGGACCAGGCAACATTG-3' and 5'-TGCGGTCGACTTAGGCAGCAGCCTTTTCCATC-3'. The resulting EcoRl I Sall fragment was cloned into pTrc99A to yield pAK, which was transformed in E. coli by electroporation using a GenePulser (Biorad).

*Media and growth conditions.* Luria-Bertani (LB) and M9 minimal medium were prepared as described previously (21). M9 medium was supplemented with glucose at a final concentration of 5 g/l and with 50 mg/l ampicillin for plasmid maintenance. For induction of AK expression, IPTG was added prior to inoculation at the desired concentration. Cultivation was performed aerobically in 500 ml baffled shake flasks with maximally 50 ml medium at 37°C on a gyratory shaker at 200 rpm.

Transient pH stress experiments were performed by growing cultures to an optical density at 600 nm (OD₆₀₀) of about 0.5 in M9 medium with a pH of 6.5, before the pH was reduced to 3 with 10% (v/v) H₃PO₄. After 1 h, pH 6.5 was re-established using 100 mM KOH.

Benzoate and acetate stress experiments were performed by growing cultures to an OD₆₀₀ of about 0.5 in M9 medium, before sodium benzoate or sodium acetate were added at final concentrations of 2 mM and 8 mM, respectively.

*Analytical procedures.* Cell growth was monitored by the increase in OD₆₀₀. Crude cell extracts for determination of AK activity and ATP concentration were prepared from stationary phase cells by 10 to 50-fold concentrated culture aliquots in 0.9% NaCl and 10 mM MgSO₄. Cells were then disrupted by sonication at 100 W for 1 min on ice.

For determination of AK activity, samples were frozen at -70°C and analyzed within a week. AK activity using pH-stat analysis, arginine concentration, and intracellular ATP and ADP concentrations were determined as described previously (6,9,23). Intracellular metabolite concentrations were calculated from the experimentally determined concentration using a cellular volume of 3.2 μl / mg of protein.
Determination of physiological parameters. In batch cultures, the exponential growth phase was identified by log-linear regression of OD$_{600}$ versus time, with maximum growth rate ($\mu_{\text{max}}$) as the regression coefficient. To calculate specific production rates, OD$_{600}$ values were converted to cellular dry weight (cdw) using a predetermined correlation factor of 0.51 g/l (cdw) per OD$_{600}$ unit. The biomass concentration was calculated as cdw per volume unit.
RESULTS

Establishing a functional arginine kinase system. Upon transformation of pAK into E. coli MG1655, the maximum in vitro activity of AK was attained at an IPTG concentration of 100 μM (Figure 1). This value corresponds to about the same level of specific AK activity as found in muscles of L. polyphemus (18).

Figure 1. Specific arginine kinase activity at different levels of IPTG induction. Enzymatic activity was determined in crude cell extracts harvested from stationary phase, pAK carrying E. coli, the control is E. coli with pTrc99A. The hatched bar indicates cells grown in the presence of 10 mM Arg. Error bars indicate the standard deviation from triplicate determinations.

To determine the impact of Arg feeding on intracellular Arg levels, we grew AK-expressing and control strains at different extracellular Arg concentrations (Figure 2). While the AK-expressing strain always had slightly lower intracellular Arg concentrations, the difference with respect to the control strain became smaller when 10 mM or more Arg were added to the medium. The control strain already reached the maximum intracellular Arg concentration of about 20 mM at 5 mM Arg supplementation, while the AK strain attained the maximum intracellular concentration at 10 mM Arg supplementation.
Figure 2. Intracellular arginine levels at different extracellular arginine concentrations. Intracellular Arg concentrations were determined at different levels of Arg supplementations during growth of *E. coli* MG1655 harboring the control plasmid (■) or pAK (□).

**Expression of AK improves recovery after transient pH stress.** AK expression had no appreciable influence on the maximum specific growth rate or the biomass yield on glucose during batch growth, when compared to the control (Table 1). In fact, both values were slightly reduced. The presence of 10 mM Arg in the medium increased both $\mu_{\text{max}}$ and $Y_{\text{X/S}}$ in control cultures but had little effect on AK expressing cells (Table 2).

<table>
<thead>
<tr>
<th>IPTG concentration (mM)</th>
<th>Maximum specific growth rate (h$^{-1}$)</th>
<th>Biomass yield on glucose (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>0.67 ± 0.01</td>
<td>0.36 ± 0.01</td>
</tr>
<tr>
<td>0</td>
<td>0.62 ± 0.01</td>
<td>0.35 ± 0.01</td>
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<td>50</td>
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<td>0.32 ± 0.00</td>
</tr>
<tr>
<td>400</td>
<td>0.54 ± 0.02</td>
<td>0.33 ± 0.01</td>
</tr>
</tbody>
</table>

Table 1. AK induction level on growth rate and yield. The standard deviations on the indicated values are from duplicate experiments.
Next, we analyzed the potential impact of AK expression during transient pH stress, that was expected to exert an ATP drain among other effects (3,14). Specifically, the pH of exponentially growing cultures (OD600 of 0.5) was shifted for 1 h from pH 6.5 to pH 3, and afterwards followed by re-establishing pH 6.5 (Figure 3 and Table 2).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Maximum specific growth rate (h(^{-1}))</th>
<th>Biomass yield on glucose (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>before pH stress</strong></td>
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<td></td>
</tr>
<tr>
<td>control, 0 mM arginine</td>
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<td>pAK, 0 mM arginine</td>
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<td>control, 10 mM arginine</td>
<td>0.67 ± 0.00</td>
<td>0.36 ± 0.01</td>
</tr>
<tr>
<td>pAK, 10 mM arginine</td>
<td>0.56 ± 0.01</td>
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<tr>
<td><strong>after pH stress</strong></td>
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<tr>
<td>control, 10 mM arginine</td>
<td>0.51 ± 0.00</td>
<td>0.32 ± 0.00</td>
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<tr>
<td>pAK, 10 mM arginine</td>
<td>0.67 ± 0.01</td>
<td>0.31 ± 0.00</td>
</tr>
</tbody>
</table>

Table 2. Growth rate and yield before and after a transient pH reduction for 1 h from 6.5 to 3. In all cases, the M9 medium was supplemented with 100 μM IPTG. The standard deviations on the indicated values are from duplicate experiments.

Figure 3. Impact of a transient medium acidification to pH 3 during growth of AK-expressing (◇) and control (○) strains. The induction level was 100 μM IPTG and the M9 medium was supplemented with 10 mM arginine. Cultures were either kept at normal pH (pH 6.5; dashed lines) or subjected to transient pH stress (1 h at pH 3; continuous lines). The figure shows one representative of 3 independent experiments performed.
A pH reduction to 3 is well below the value for which the induction of the acid tolerance response is reported (12). Indeed, the intracellular ATP level dropped from 5.5 ± 0.2 mM in unstressed cells to 4.2 ± 0.2 mM during low-pH stress in both the control and the AK-expressing strain (Figure 4). While lag-phases were similar in both strains after the stress, the post-stress growth rates differed significantly. The AK-expressing strain resumed growth at about the same rate as observed before the stress, but the control grew significantly slower.

![Graph showing intracellular ATP levels during transient pH stress](image)

**Figure 4. Intracellular ATP levels during the transient pH stress.** Intracellular ATP concentrations were determined in unstressed cultures (white bars) and during transient stress (grey bars) of E. coli MG1655 harboring the control plasmid or pAK. The M9 medium was supplemented with 10 mM Arg. The pH of one of the cultures was reduced to 3 and after 1 h a sample was harvested both from stressed and unstressed cultures. Intracellular ATP concentrations were determined as the mean from three independent measurements in two parallel cultures.

**Expression of AK during acetate and benzoate stress.** By effect of the anion liberated in the cytoplasm, weak organic acids are known to generate a larger stress effect than strong acids (12,20). Acetate and benzoate stresses were applied here to control and AK-expressing strains by incubating mid-exponential phase cultures with sub-lethal quantities of acetate and benzoate (Figure 5). The anionic form of these acids becomes protonated at neutral pH, and is thus able to diffuse into the cytoplasm, where dissociation occurs.

No significant differences were observed between the control and AK strain concerning the maximum cell density attained. The concentrations of added acetate and benzoate were
chosen to reduce the maximal specific growth rate by about 20-30% (19). We observed a reduction of the maximal growth rate by 11% and 21% in the strain transformed with the control plasmid after addition of acetate or benzoate, respectively (Table 3). However, if pAK is transformed and AK-expression is induced in presence of 10 mM arginine, the negative effect was even stronger. Since the maximal growth rate was reduced by 27% and 32% after addition of acetate or benzoate, respectively.

![Graph](image)

**Figure 5.** Effect of sub-lethal acetate and benzoate concentrations on growth of AK-expressing strain. Growth of *E. coli* MG1655 expressing AK (dashed lines) or just harboring the control plasmid p7c99A (continuous lines) is given as the increase in cell density. Growth was performed in M9 medium supplemented with 0.5% glucose (w/v), 50 mg/l ampicillin, 100 µM IPTG, and 10 mM arginine. Cultures were either allowed to grow undisturbed (control cultures; ) or supplemented with 2 mM sodium benzoate (a; ) or 8 mM sodium acetate (b; ) after reaching OD~600~ 0.5. The figure shows one representative out of 2 independent experiments performed.
Table 3. Growth rates of AK cultures stressed with sub-lethal concentrations of acetate and benzoate. Maximal growth rates ($\mu_{\text{max}}$) of *E. coli* MG1655 expressing AK (dashed lines) or just harboring the control plasmid pTrc99A (continuous lines) during incubation with acetate or benzoate are given. Growth was performed in M9 medium supplemented with 0.5% glucose (w/v), 50 mg/l ampicillin, 100 µM IPTG, and 10 mM arginine. Cultures were either allowed to grow undisturbed (non-stressed cultures) or supplemented with 8 mM sodium acetate (Ac-stressed cultures) or 2 mM sodium benzoate (Bz-stressed cultures) after reaching OD$_{600}$ 0.5. The figure shows the mean values from the two independent experiments performed.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>maximum specific growth rate (h$^{-1}$) non-stressed cultures</th>
<th>maximum specific growth rate (h$^{-1}$) Ac-stressed cultures</th>
<th>maximum specific growth rate (h$^{-1}$) Bz-stressed cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>0.69 ± 0.02</td>
<td>0.61 ± 0.01</td>
<td>0.54 ± 0.01</td>
</tr>
<tr>
<td>pAK</td>
<td>0.61 ± 0.02</td>
<td>0.41 ± 0.04</td>
<td>0.44 ± 0.03</td>
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</tbody>
</table>
DISCUSSION

Previously, we demonstrated an improved resistance to transient pH and starvation stress by metabolic engineering of a functional phosphagen kinase system into the yeast S. cerevisiae (6). Although another phosphagen kinase was functionally expressed in a prokaryote before (15), we show here for the first time a beneficial effect of phosphagen kinase overexpression in a prokaryote; namely improved recovery from a transient pH stress in E. coli. Apparently, AK expression has little if any protective effect during the stress, since global intracellular ATP levels were similarly reduced in control and the AK strain. Although both strains resumed growth immediately upon reestablishment of the original pH, the AK-expressing strain attained reproducibly a much higher growth rate than its control. Thus, it appears that AK expression improves recovery from a transient stress, which reduced intracellular ATP concentration. The lack of a beneficial effect of AK in presence of organic acids may be due to their inhibitory role on biosynthetic pathways not strictly involved in ATP-generation (19).

Our study provides indirect evidence for the formation of the storage phosphagen PArg, since the intracellular Arg level was always lower in strains overexpressing AK, when compared to the control. Moreover, more than 10 mM extracellular Arg was necessary to achieve maximum intracellular Arg concentrations in the AK expressing strain, while 5 mM was sufficient for the control, presumably because a significant portion of Arg was converted to PArg by the AK.

The beneficial effect of arginine kinase may be related to overcome an energy-related or a low-pH-related buffering. While pH buffering by arginine kinase was so far not shown explicitly in organisms that express arginine kinase naturally, our results would also be consistent with proton scavenging by dephosphorylation of phosphoarginine (see equation 1).

The results presented here and in a previous study (6) show that even lower eukaryotes and prokaryotes can be metabolically engineered with transient ATP-buffering systems working in the range of seconds and perhaps minutes. This may be of biotechnological relevance for industrial conditions that are characterized by fluctuating energy demands and availability, as is found for example in large-scale bioreactors with imperfect mixing and thus local zones with oxygen or carbon source limitation (10).

Acknowledgements. We would like to thank Ross Ellington and Pamela S. Pruett of Florida State University for providing the AK cDNA.
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Conclusions and outlook
Our investigations provided new insights in microbial cofactor metabolism. **Chapter 2** and **Chapter 3** show that transhydrogenases are important enzymes for NADPH metabolism. For the particular example of *Escherichia coli*, we could show that they provide efficient means of decoupling catabolic NADPH production from the anabolic NADPH demand. To our knowledge, this is the first study that quantitatively shows that the membrane-bound transhydrogenase contributes to 34% of the anabolic NADPH requirement in exponentially growing *E. coli* on glucose. These enzymes are thus important for radical redirection of carbon flow in chemicals overproducing micro-organisms by metabolic engineering. An important question that needs to be addressed is how the cell regulates the activity of the soluble transhydrogenase. It cannot be active during all conditions because it would then just equilibrate the more reduced NADPH / NADP+ pool with the more oxidized NADH / NAD+ pool, which would be detrimental to growth. Hence, although we have evidence for *in vitro* activity of the soluble transhydrogenase on glucose – a conditions where it should be in active – there is likely a mechanism that restricts the *in vivo* activity. The complementation of the lethal transhydrogenase deletion phenotype under certain conditions, could be exploited to detect enzymes with transhydrogenase-like activity, for example in *Bacillus subtilis*, about whose NADPH metabolism little is known.

The use of heterologous phosphagen kinase systems in metabolic engineering of microorganisms, confirmed how energy buffering systems may be advantageous under specific conditions of reduced ATP availability, such as during stress. In **Chapter 4** and **Chapter 5** of this thesis, only a restricted palette of conditions has been applied, but since reduced ATP availability is involved in a large number of microbial stress responses, more conditions should be investigated, for example hypoxic stress. Such conditions are present in large scale processes in which cells are often exposed to fluctuating availability of nutrients such as carbon source and oxygen because of imperfect mixing.

Some difficulties were encountered in the successful expression of a functional creatine kinase system, while the establishment of the arginine kinase system was much simpler. This may be a consequence of the evolutionary distance to the host cells used. Creatine kinase may thus be more appropriate to achieve similar goals in higher organisms. Such an example would be immobilized hepatocytes cultures, because of the reduced oxygen availability. Lastly, in the case of arginine kinase overexpression in *E. coli* we have not fully proved that indeed phosphoarginine was used as a transient energy buffer. Thus, this question should be also addressed.
LIST OF ABBREVIATIONS

6PG  gluconate 6-phosphate
AcCoA acetyl coenzyme A
AdiA arginine decarboxylase
ADP adenosine di-phosphate
AGAT L-arginine:glycine amidinotransferase
AK arginine kinase
AMP adenosine mono-phosphate
cAMP cyclic AMP
AMPK AMP-activated protein kinase
AMPKK AMPK-activated protein kinase
ANT adenine nucleotide translocator
Arg arginine
ARS acid resistance systems
ATP adenosine tri-phosphate
cdw cell dry weight
CER CO₂ evolution rate
CK creatine kinase (B-CK, cytosolic brain isoform, M-CK; cytosolic muscle isoform; sMtCK, sarcomeric mitochondrial creatine kinase; uMtCK, ubiquitous mitochondrial creatine kinase)
Cr creatine
DTT dithiothreitol
E. coli Escherichia coli
E4P erythrose 4-phosphate
ED pathway Entner-Doudoroff pathway
Eda KDPG aldolase
Edd 6-phosphogluconate dehydratase
EDTA ethylenediamine tetraacetic acid
EGTA ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid
F6P fructose 6-phosphate
G6P glucose 6-phosphate
GABA γ-aminobutyric acid
GadAB glutamate decarboxylase
GadC glutamate/GABA antiporter
GAMT guanidinoacetate methyltransferase
GAP glyceraldehyde 3-phosphate
GK glycocyamine kinase
Glu glutamate
HdeAB periplasmic chaperones
HTK hypotaurocyamine kinase
IPTG isopropyl-β-D-1-thiogalactopyranoside
KDPG 2-keto-3-deoxy 6PG
Kup H⁺/K⁺ symporter
LB medium Luria-Bertani medium
Leu leucine
LK lombricine kinase
MAL malate
μmax maximum specific growth rate
METAFoR metabolic flux ratios
MTBSTFA N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide
NAD(H) oxidized (reduced) nicotinamide adenine dinucleotide
NADP(H) oxidized (reduced) nicotinamide adenine dinucleotide phosphate
NhaA Na⁺/K⁺ antiporter
NMR nuclear magnetic resonance
OAA oxaloacetate
OD₆₀₀ optical density at 600 nm
OGA oxoglutarate
OK ophaline kinase
P₅P pentose 5-phosphate
PArg phosphoarginine
PCr phosphocreatine
PCR polymerase chain reaction
pcv packed cell volume
PEP phosphoenolpyruvate
PGA 3-phosphoglycerate
Pgi phosphoglucone isomerase
PKA AMP-dependent protein kinase
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tbody>
<tr>
<td>PMSF</td>
<td>phenylmethanesulfonylfluorid</td>
</tr>
<tr>
<td>PntAB</td>
<td>membrane-bound transhydrogenase</td>
</tr>
<tr>
<td>PP pathway</td>
<td>Pentose phosphate pathway</td>
</tr>
<tr>
<td>PYR</td>
<td>pyruvate</td>
</tr>
<tr>
<td>q&lt;sub&gt;CO2&lt;/sub&gt; / q&lt;sub&gt;O2&lt;/sub&gt;</td>
<td>specific CO&lt;sub&gt;2&lt;/sub&gt; evolution rate / O&lt;sub&gt;2&lt;/sub&gt; consumption rate</td>
</tr>
<tr>
<td>q&lt;sub&gt;s&lt;/sub&gt;</td>
<td>specific substrate-uptake rate</td>
</tr>
<tr>
<td>RpoS</td>
<td>stationary-phase dependent sigma factor σ&lt;sup&gt;s&lt;/sup&gt;</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>Saccharomyces cerevisiae</td>
</tr>
<tr>
<td>S7P</td>
<td>sedoheptulose 7-phosphate</td>
</tr>
<tr>
<td>SAH</td>
<td>S-adenosyl homocysteine</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosyl methionine</td>
</tr>
<tr>
<td>TCA cycle</td>
<td>tricarboxylic acid cycle</td>
</tr>
<tr>
<td>ThK</td>
<td>thalassesmine kinase</td>
</tr>
<tr>
<td>TK</td>
<td>taurocyamine kinase</td>
</tr>
<tr>
<td>TrkA</td>
<td>H&lt;sup&gt;+&lt;/sup&gt;/K&lt;sup&gt;+&lt;/sup&gt; antiporter</td>
</tr>
<tr>
<td>Trp</td>
<td>tryptophane</td>
</tr>
<tr>
<td>UdhA</td>
<td>soluble transhydrogenase</td>
</tr>
<tr>
<td>Ura</td>
<td>uracile</td>
</tr>
<tr>
<td>YMM</td>
<td>yeast minimal medium</td>
</tr>
<tr>
<td>Y&lt;sub&gt;X/s&lt;/sub&gt;</td>
<td>yield of biomass on substrate</td>
</tr>
<tr>
<td>Zwf</td>
<td>G6P dehydrogenase</td>
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139