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Redirecting metabolic flux in *Saccharomyces cerevisiae* through regulation of cofactors in UMP production

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Abstract Although it is generally known that cofactors play a major role in the production of different fermentation products, their role has not been thoroughly and systematically studied. To understand the impact of cofactors on physiological functions, a systematic approach was applied, which involved redox state analysis, energy charge analysis, and metabolite analysis. Using uridine 5′-monophosphate metabolism in *Saccharomyces cerevisiae* as a model, we demonstrated that regulation of intracellular the ratio of NADPH to $NADP^+$ not only redistributed the carbon flux between the glycolytic and pentose phosphate pathways, but also regulated the redox state of NAD(H), resulting in a significant change of ATP, and a significantly altered spectrum of metabolic products.

Keywords Cofactor · Uridine 5′-monophosphate · Redox state analysis energy · Charge analysis · Metabolite analysis

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Introduction

Metabolic engineering has the potential to considerably improve process productivity, through the manipulation of flux through specific pathways [1, 19]. Most current metabolic engineering studies have focused on manipulating enzyme levels through the amplification, addition, or deletion of a particular pathway. However, recent studies demonstrated that cofactors play a major role in biochemical reactions, and the flux of cofactor-dependent pathways is controlled by the levels and availability of the required form of the cofactor [3–5, 12, 21]. Cofactor manipulations have the potential to be a powerful tool for enhancing the concentration, yield, and production of target metabolites [16, 18].

The most important cofactors are ATP, NAD(H), and NADP(H). ATP, which widely serves as a substrate, a product, an activator, or/and an inhibitor, plays an important role in many metabolic pathways and in the production of almost all of the metabolites by industrial strains [25]. Although the structures of NAD(H) and NADP(H) are similar, they have fundamentally distinct functions in most biochemical pathways [2]. Altering the ratio of NADH to NAD^+ significantly affects the uptake of glucose, cell growth, and the metabolic flux distribution in glycolysis [10, 17, 23], while NADP(H) plays a role in anabolic reactions, and flux in the pentose phosphate pathway [9, 16].

Considering the importance of cofactors in cell physiology, their manipulation appears to be an efficient way to regulate metabolic flux. The fluxes of ATP-, NAD(H)-, and NADP(H)-dependent pathways are determined by the availabilities of these cofactors, which could be improved by either weakening the metabolic branches competing for ATP, NAD(H), or NADP(H), or introducing regeneration systems such as acetatekinase, NAD-dependent

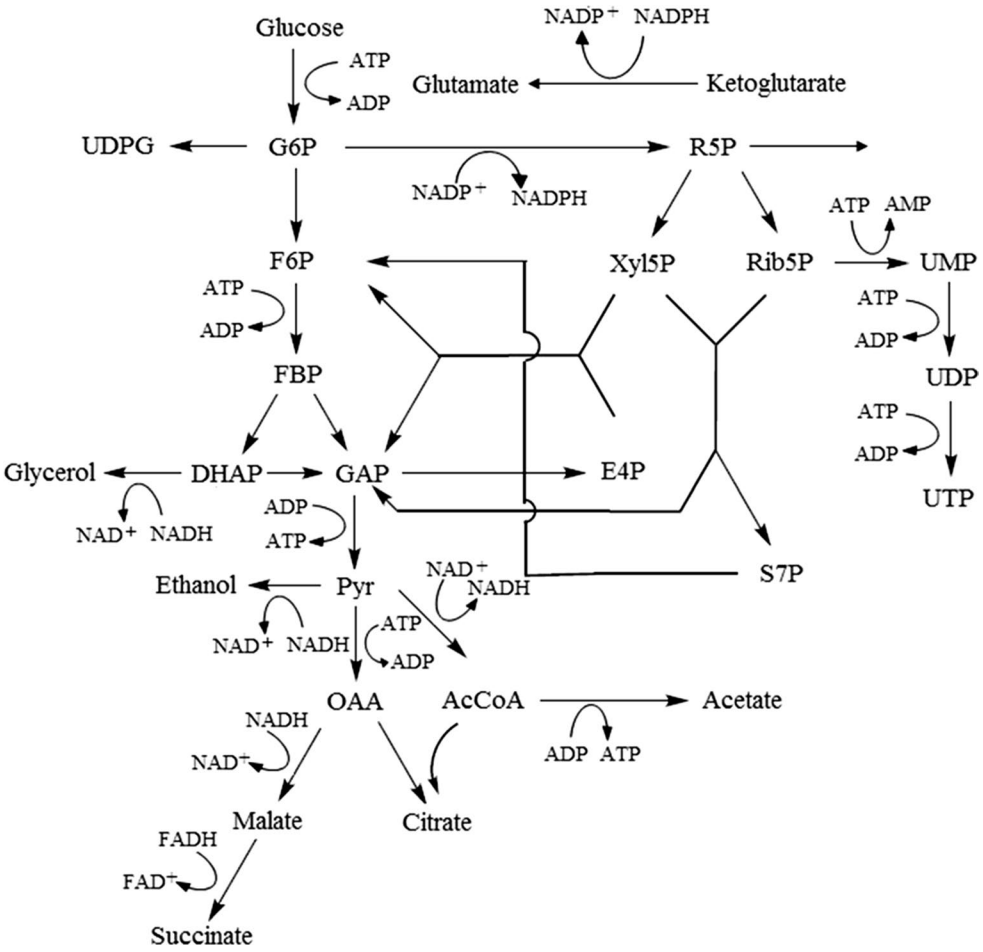

Fig. 1 Schematic pathway of the biosynthesis of UMP from orotic acid under microaerobic conditions. *G6P* glucose-6-phosphate, *F6P* fructose-6-phosphate, *FBP* fructose 1,6- bisphosphate, *GAP* glyceraldalde hyde-3-phosphate, *DHAP* dihydroxyacetone phosphate, *Pyr* pyruvate, *OAA* oxalosuccinate, *AcCoA* acetyl coenzyme A, *R5P* ribulose-5-phosphate, *Xyl5P* xylulose-5-phosphate, *Rib5P* ribose-5-phosphate, *S7P* sedoheptulose-7-phosphate, *E4P* erythrose-4-phosphate, *UMP* uridine 5′-monophosphate, *UDP* uridine 5′-diphosphate, *UTP* uridine 5′-triphosphate and *UDPG* uridine 5′-diphosphoglucose

formate dehydrogenase, NADH-dependent NADHase, NADP-dependent glucose 6-phosphate dehydrogenase, or NADPH-dependent glutamate dehydrogenase [15]. Cofactor-oriented bioprocess optimization has developed expeditiously, and has successfully extended over the boundaries of metabolic engineering. However, more researchers are beginning to realize that the introduction of cofactor-related pathway does not always achieve targets, which suggests that we have not fully understood the cofactor-related mechanisms of the cells. To our knowledge, scarce data have been published about effects on the whole system, including other metabolic pathways, energy transfer, and other cofactors. Additional analysis of cofactor-oriented regulation will lead to a better understanding of cell physiology regulation, and allow for further bioprocess optimization.

We used uridine-5′-monophosphate (UMP) metabolism in *Saccharomyces cerevisiae* as a model system to demonstrate that regulation of NADP(H) metabolism not only redistributed the carbon flux between the glycolytic and pentose phosphate pathways, but also affected intracellular redox state and ATP level, and altered the spectrum of metabolic products (Fig. 1). This work provides a new perspective for understanding the systems relationship between metabolic flux and cofactor-oriented regulation.

Materials and methods

Strains, media, and cultivation

UMP production was analyzed in *S. cerevisiae* strain As 2.398, derived from *S. cerevisiae* 1002 (China Center of Industrial Culture Collection).

The seed culture medium contained, in grams per liter: yeast extract 10, peptone 20, and glucose 20. Medium pH was adjusted to 5.8 with 1.0 M NaOH and 0.1 M HCl. Seed cultures were added at 5 % (v/v) into a 5-L fermenter (NBS Bioflo-110) containing 3 L of fermentation medium, which was composed of in grams per liter: glucose 50, peptone 5, yeast extract 2, $(NH4)_2HPO_4$ 2, $MgSO_4{\cdot}7H_2O$ 1, and KH_2PO_4 2. Medium pH was adjusted to 7.4 as above. Cultivation was at 30 °C for 48 h.

Cells were collected by centrifugation (8000×*g*, 10 min at 4 °C) and washed twice with distilled water, before lyophilization and storage at −20 °C.

Biocatalytic reactions

Reaction mixtures contained orotic acid 60 mM, glucose 300 mM, NaH_2PO_4 80 mM, $MgCl_2$ 5 mM, NH_4Cl 10 mM, dimethylbenzene 0.5 % (v/v), and cells (dry cell weight, DCW) 40 g. Reactions were at 30 °C in a 5-L fermenter containing 3 L medium. The pH was adjusted to 8.0 with 1.0 M NaOH. Glucose solution (800 mM) (maintained at 30 °C) was continuously fed into the reactor after 6 h, at 17.5 mL/h.

Metabolite analysis

Concentrations of UMP, UTP, and UDPG were measured by high-performance liquid chromatography (HPLC) using a SepaxHP-C18 column (250 mm × 4.6 mm × 5 μm) and a UV detector at 260 nm. The column was eluted with 6 % (v/v) phosphoric acid (adjusted to pH 6.6 with triethylamine) at a flow rate of 1 mL/min at room temperature.

Concentrations of glucose-6-phosphate (G6P), fructose-1, 6-biphosphate (FBP), and 6-phosphogluconate (6PG) were determined by enzyme analysis according to Vaseghi [20]. Acetate and ketoglutarate were measured by HPLC using an Aminex HPX-87H column and a UV detector at 210 nm. The column was eluted with 5 mM sulfuric acid at a flow rate of 0.5 mL/min and 60 °C. Ethanol and glycerol were measured in yeast culture supernatants using gas chromatography (Perkin-Elmer GC 8310) with a Poropaq Q-packed column. The injector column and flame ionization detectors were maintained at 210 °C, and N_2 was used as a carrier gas at a flow rate of 45 mL/min. Isopropanol was used as an internal standard. Glucose concentration was determined using an automated enzyme analysis system (model SBA-40C).

NAD^+ and NADH concentrations were determined as described previously [8], and $NADP^+$ and NADPH concentrations were determined according to Zhang [24]. Spectrophotometric enzyme assays were performed for $NADP^+$-GDH and NAD^+-GDH according to Alexander [7]. ATP was analyzed by the luciferin–luciferase reaction using the Entiten ATP assay system FF2000, and was detected using a Modulus microplate multimode reader. ADP and AMP were measured as the difference after enzymatic conversion to ATP.

Results

Effects of α-ketoglutarate on glucose consumption and UMP production

Sufficient supplies of phosphoribosyl pyrophosphate (PRPP) and ATP are required for high-level UMP production, with PRPP synthesis as the rate-limiting step. PRPP synthesis is related to the supply of ribose-5-phosphate, which is derived from the pentose phosphate pathway, so α-ketoglutarate was added to cultures to regulate the ratio of NADPH to $NADP^+$, which is considered the most important cofactor for pentose phosphate regulation. The rate of glucose consumption was faster than that of control, and UMP production increased by 36.3 %, to 34.9 mM in the presence of α-ketoglutarate (Fig. 2).

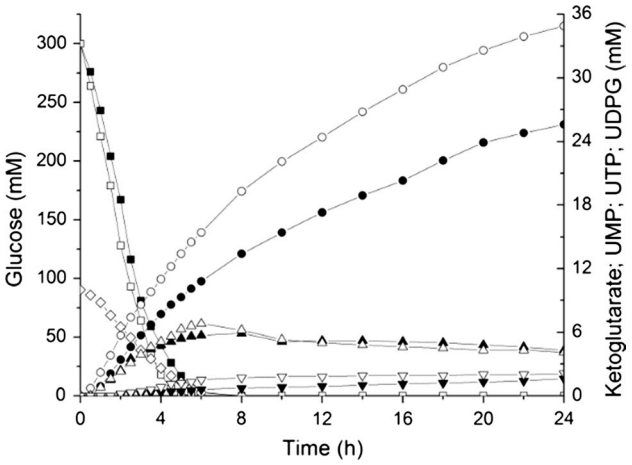

Fig. 2 Effects of α-ketoglutarate on glucose consumption and UMP production. Control (*filled square* glucose, *filled circle* UMP, *filled triangle* UTP, *filled inverted triangle* UDPG), with 10 mM α-ketoglutarate addition (*unfilled square* glucose, *unfilled circle* UMP, *unfilled triangle* UTP, *unfilled inverted triangle* UDPG). The data are presented as means from three independent experiments (variation was always <5 %)

Effects of α-ketoglutarate on $NADP^+$, NADPH, and the ratio of NADPH to $NADP^+$

The concentration of $NADP^+$ decreased immediately followed by a transient increase to the former steady-state level when glucose was added into the medium (Fig. 3). And significant changes of $NADP^+$, NADPH and the ratio of NADPH to $NADP^+$ were observed in the presence of α-ketoglutarate. NADPH was immediately oxidized to $NADP^+$ by NADPH-dependent GDH (Table 1). This conclusion was confirmed by decreased α-ketoglutarate (Fig. 2). The improved availability of $NADP^+$ reduced the NADPH-level, establishing a new redox balance of the ratio of NADPH to $NADP^+$. This ratio gradually recovered with the depletion of α-ketoglutarate.

Effects of α-ketoglutarate on NAD^+, NADH, the ratio of NADH to NAD^+, and the NAD^+-related metabolites

NADH/NAD^+ plays a major role in catabolism. Continued glycolysis requires that NADH be produced and oxidized

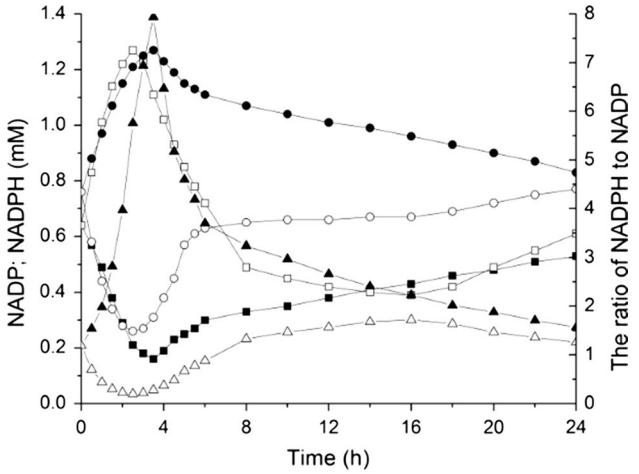

Fig. 3 Effects of α-ketoglutarate on $NADP^+$, NADPH and the ratio of NADPH to NADP. Control (*filled square* $NADP^+$, *filled circle* NADPH, *filled triangle* the ratio of NADPH to $NADP^+$), with 10 mM α-ketoglutarate addition (*unfilled square* $NADP^+$, *unfilled circle* NADPH, *unfilled triangle* the ratio of NADPH to $NADP^+$). The data are presented as means from three independent experiments (variation was always <5 %)

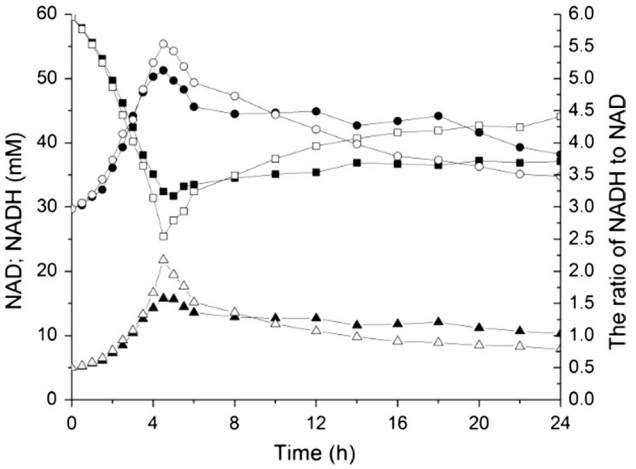

Fig. 4 Effects of α-ketoglutarate on NAD^+, NADH and the ratio of NADH to NAD^+. Control (*filled square* NAD^+, *filled circle* NADH, *filled triangle* the ratio of NADH to NAD^+), with 10 mM α-ketoglutarate addition (*unfilled square* NAD^+, *unfilled circle* NADH, *unfilled triangle* the ratio of NADH to NAD^+). The data are presented as means from three independent experiments (variation was always <5 %)

Table 1 Specific activities of $NADP^+$-GDH and NAD^+-GDH in *Saccharomyces cerevisiae*

	0 h	4 h	8 h	16 h	24 h
$NADP^+$-GDH	0.83	0.78	0.73	0.67	0.64
NAD^+-GDH	0.063	0.059	0.052	0.047	0.042

The data are presented as means from three independent experiments (variation was always <5 %)

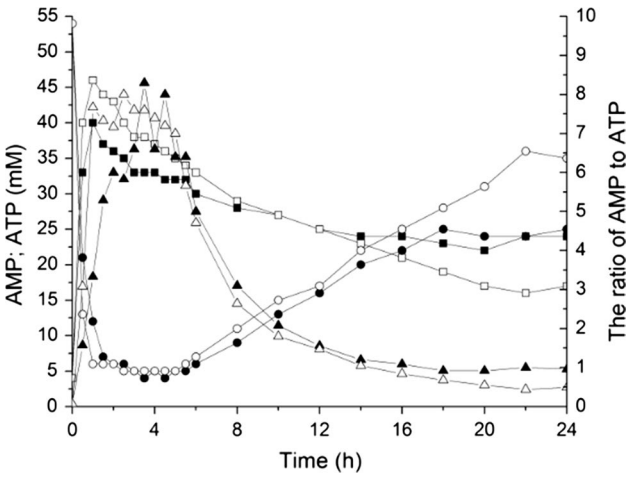

Fig. 5 Effects of α-ketoglutarate on AMP, ATP, and the ratio of AMP to ATP. Control (*filled square* AMP, *filled circle* ATP, *filled triangle* the ratio of AMP to ATP), with 10 mM α-ketoglutarate addition (*unfilled square* AMP, *unfilled circle* ATP, *unfilled triangle* the ratio of AMP to ATP). The data are presented as means from three independent experiments (variation was always <5 %)

to NAD^+. For *S. cerevisiae*, NAD^+ regeneration can occur by alcohol dehydrogenase and glycerol-3-phosphate dehydrogenase. A rapid and temporary decline in the ratio of NADH to NAD^+ was observed when glucose was added into the medium, because the rate-limiting step of glycolytic pathway inhibited the regeneration of NAD^+ through alcohol dehydrogenase (Fig. 4). Cells then stimulated the other NADH-dependent dehydrogenase, leading to a recovery of the ratio of NADH to NAD^+. In the presence of α-ketoglutarate, the trends of decline in prior stage and the recovery in later stage seem more obvious.

Effects of α-ketoglutarate on ATP, AMP, and the ratio of AMP to ATP

Before the glucose addition, the ATP level, AMP level, and the ratio of ATP to AMP were 54, 5 μM, and 0.1, respectively. In the first 2 h after the glucose was added, a significant fall of ATP level was shown in this experiment, which induced an increase in the ratio of AMP to ATP (Fig. 5). The partial recovery was followed during the remaining time. When α-ketoglutarate was added into the medium, a faster decline of ATP level in the first few hours and a relatively high level of ATP at later hours were observed. Combined with the decreased glycolytic flux, this result suggested that there is a negative correlation between ATP and glycolytic flux. This is in agreement with that of Lasson et al. [14].

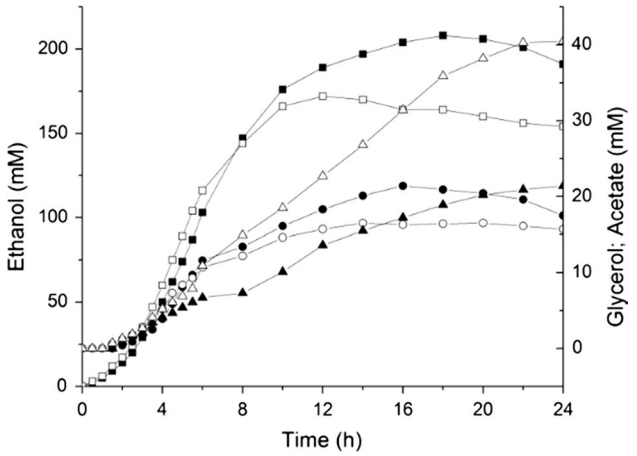

Fig. 6 Effects of α-ketoglutarate on NAD(H)-related metabolites. Control (*filled square* ethanol, *filled circle* glycerol, *filled triangle* acetate), with 10 mM α-ketoglutarate addition (*unfilled square* ethanol, *unfilled circle* glycerol, *unfilled triangle* acetate). The data are presented as means from three independent experiments (variation was always <5 %)

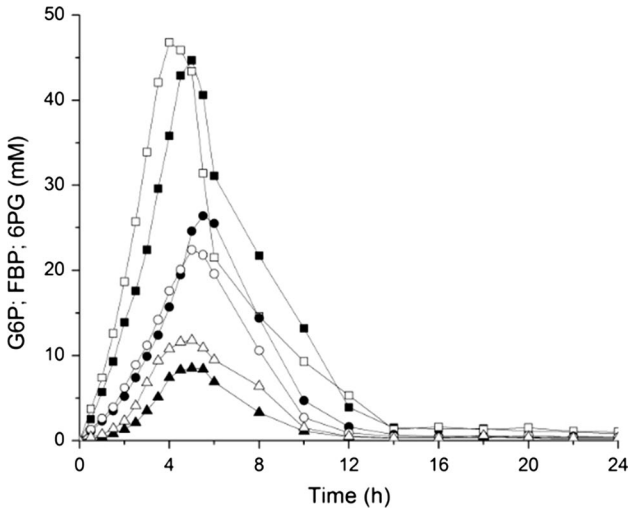

Fig. 7 Effects of α-ketoglutarate on metabolites around G6P node. Control (*filled square* G6P, *filled circle* FBP, *filled triangle* 6PG), with 10 mM α-ketoglutarate addition (*unfilled square* G6P, *unfilled circle* FBP, *unfilled triangle* 6PG). The data are presented as means from three independent experiments (variation was always <5 %)

Effects of α-ketoglutarate on metabolic intermediates in glycolytic pathway and pentose phosphate pathway

To investigate the effect of α-ketoglutarate on the metabolic redistribution, metabolic intermediates in glycolytic pathway and pentose phosphate were assayed in this experiment. Accumulation of G6P, FBP, and 6PG occurred within the first 6 h, and was followed by that of ethanol, glycerol, and acetate (Figs. 6, 7). However, accumulation rates were different when α-ketoglutarate was added into the medium. Faster G6P and 6PG accumulation rates were observed, while there was no considerable correlation between the accumulation rates of FBP and α-ketoglutarate. Figure 7 showed that a significantly altered spectrum of metabolic products was observed in this experiment. Concentrations of ethanol and glycerol decreased to 147 and 15.7 mM, respectively, while acetate increased nearly twofold to 40.5 mM.

Discussion

Previous studies showed that the flux of cofactor-dependent pathways is controlled not only by enzyme availability, but also by cofactor levels and redox state. Cofactor manipulation has the potential to be a powerful tool in regulating the distribution of metabolic flux. This strategy has been applied to *Escherchia coli* [3–5] and *S. cerevisiae* [11, 20]. In this study, we generated more efficient and feasible routes for *S. cerevisiae* UMP metabolism, and demonstrated that physical changes of cells respond to cofactor regulation.

In addition to orotate phosphoribosyltransferase and orotidine-5′-monophosphate decarboxylase, a sufficient PRPP supply was important for improving nucleotide yield. Ribose-5-phosphate and ATP are both precursors for PRPP synthesis [6, 22], and ribose-5-phosphate is synthesized by the oxidative route of the pentose phosphate pathway [13], while ATP is regenerated by substrate-level phosphorylation in glycolysis. Rational distribution between the glycolytic and pentose phosphate pathways is key for improving UMP yield. However, in the absence of an NADPH-consuming pathway, it is accumulated as the carbon source was directed into the pentose phosphate pathway. NADPH is a competitive inhibitor of glucose-6-phosphate dehydrogenase, which is also under substrate $NADP^+$ feedback control, resulting in a low flux through the pentose phosphate pathway.

To redirect metabolic flux, the strategy of supplementing the culture medium with specific NADPH-dependent enzyme substrate was adopted to manipulate $NADP^+$ availability. The α-ketoglutarate was chosen to be added into the medium to optimize the cofactor-oriented bioprocesses. In *S. cerevisiae*, two NADPH-dependent and a NADH-dependent glutamate dehydrogenases, encoded by *GDH1, GDH3,* and *GDH2*, respectively, catalyze the synthesis of glutamate from ammonium and α- ketoglutarate [7]. The coordinated regulation of these enzymes results in glutamate biosynthesis and the balanced utilization of α-ketoglutarate under catalytic conditions. Due to the low activity of NAD^+-dependent enzyme, there was no change in the ratio of NADH to NAD^+. However, a sharp decline occurred in NADPH, and a consequent rise of $NADP^+$

was observed in this experiment, which was attributed to the increased NADPH-dependent GDH and was proved by decreased α-ketoglutarate (Fig. 2). The low ratio of NADPH to $NADP^+$ mimicked NADPH deficiency, relieving 6-phosphate dehydrogenase suppression induced by the competitive inhibitor NADPH, and directing carbon flux toward the pentose phosphate pathway.

However, not all physical changes of cells responded to cofactor regulation. In fact, microbes adapt to these changes by many mechanisms such as altering spectrum of metabolic products, energy changes, and so on. As we know, ATP is also a proper candidate for flux coordination between glycolysis and pentose phosphate pathway. First of all, it acts as substrate in first steps of glycolytic pathway that is supposed to be important for controlling the rate of glycolysis, i.e., hexokinase, phosphofructokinase. Addition of glucose would lead initially to a net ATP consumption, and result in the decline of ATP level. Furthermore, ATP is also known to be an allosteric regulator inhibiting key enzymes of glycolytic pathway and pentose phosphate pathway. The decreased pool of ATP relieved the suppression of hexokinase, phosphofructokinase, as well as glucose-6-phosphate dehydrogenase (G6PDH), leading to the increased fluxes of glycolytic pathway and pentose phosphate pathway. Further derepression of G6PDH could be achieved by addition of α-ketoglutarate for the lower ratio of NADPH to $NADP^+$. Larger amounts of carbon source flowed into PPP, and stimulated the synthesis of PRPP, which was considered the major ATP-consuming process in the UMP production. In fact, the demand for ATP that resides outside the glycolytic pathway was considered as the major reason for the control of glycolytic flux. The strong ATP demand further accelerated the decline of ATP, and gave the cells a feint to ATP deficiency, thus stimulating ATP regeneration and leading to a faster glucose consumption rate and increased glycolytic flux. Although both glycolytic flux and PPP flux were active, the extents of changing were different. The high ratio of pentose phosphate pathway to glycolytic pathway flux facilitated during UMP synthesis.

For the fixed total quantity of glucose, the redistribution between glycolytic pathway and PPP indicated that low amount of carbon source would flow into glycolytic pathway after 8 h, which could be confirmed by the decreased quantities in the end products of glycosis (ethanol + glycerol + acetate). Lower ATP supply could not meet the demand of UMP synthesis, so the cells had to redirect metabolic flux from the non-ATP production pathway to ATP production pathway. Under aerobic condition, majority of intracellular ATP was synthesized when NADH oxidized to NAD^+ through the electron transfer chain. However, under microaerobic condition, energy production is mainly from substrate-level phosphorylation. We analyzed the regenerative capacity of ATP by measuring the moles of ATP available per mole of metabolite synthesized. Ethanol, glycerol, and acetate were the primary metabolites with 1 mol ATP produced per mole ethanol formed for a net generation; 1 mol ATP consumed per mole glycerol formed for a net consumption; and two ATP formations with acetate. In this experiment, the most notable observation was the shift of acetate concentration, which indicated that there was a great increase in ATP availability.

Intracellular NADH and NAD^+ metabolism were closely related to ATP regeneration. Cells could manipulate ATP availability through manipulating NADH-dependent metabolic flux shift, i.e., if ethanol production is inhibited, the yeast would have to increase its glycerol production to maintain their redox balance, thus leading to lower the ATP production. In this case, further oxidized glucose to acetate was chosen to achieve higher ATP availability. An analysis of the metabolic fluxes showed a significant increase in the flux to acetate, accompanied by a decrease in the flux to ethanol and a marked decrease in the flux to glycerol. The increase in the acetate flux (NAD^+) in combination with the decrease in the flux to ethanol and glycerol (NADH) indicated that there is a great change of redox state in cells. However, no remarkable difference on the ratio of NADH to NAD^+ was observed. These findings supported the viewpoint as suggested by Berrios-Rivera et al. [5] that the ratio of NADH to NAD^+ is not always a good indicator of the oxidation state of the cell because the turnover can be fast in an effort to achieve a redox balance. In general, cell metabolism is always in a steady state, where NAD^+ consumption and production are equal. When one of them is more strongly stimulated, the cells would make physiological response quickly to activate the other in an effort to establish a new redox balance. The corporate effects of improved intracellular NADH regeneration and efficient mechanisms for consumption of NADH resulted in a nearly unaltered redox balance. Instead, a change in the ratio of (ethanol + glycerol) to acetate can be considered as a good indirect indicator of a change in redox state.

In conclusion, in assessing the effects of α-ketoglutarate on the production of UMP and the key cofactors, a systems approach was applied that involved metabolite analysis, cofactor analysis, and energy analysis. UMP metabolism in *S. cerevisiae* as a model was used to demonstrate that the disturbance to the ratio of NADPH to $NADP^+$ induced by α-ketoglutarate not only redistributed the carbon flux between the glycolytic and pentose phosphate pathways, but also had influence on redox state and ATP availability, resulting in a significantly altered spectrum of metabolic products. Our work provides a new insight into understanding of the impact of cofactors on physiological function.

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