Title

Absolute quantitation of glycolytic intermediates reveals thermodynamic shifts in *Saccharomyces cerevisiae* strains lacking *PFK1* or *ZWF1* genes

Running title: Absolute quantitation of yeast metabolites

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9

Abstract

Internal standard based absolute quantitation of glycolytic intermediates was performed to characterize the thermodynamic states of *Saccharomyces cerevisiae* metabolism. A mixture of ^{13}C -labeled glycolytic intermediates was prepared via extraction from *S. cerevisiae* cells cultivated using a synthetic medium containing $[\text{U-}^{13}\text{C}]$ glucose as the sole carbon source. The ^{13}C -labeled metabolite mixture was used as an internal standard for the analysis of *S. cerevisiae* cultivated in a medium containing natural glucose. The methodology was employed for the absolute quantitation of glycolytic intermediates of BY4742, *pfk1* Δ , and *zwf1* Δ strains of *S. cerevisiae*. Fructose-1,6-bisphosphate was the most abundant intermediate in the BY4742 strains in the log phase of growth. Estimation of the Gibbs free energy change (ΔG) from the absolute concentration revealed that several reactions, such as those catalyzed by ribose-5-phosphate keto-isomerase and phosphoglucose isomerase, were commonly at near-equilibrium in all three strains. A significant shift in thermodynamic state was also observed for the transketolase-transaldolase reaction, for which ΔG was $-6.6 \pm 0.5 \text{ kJ mol}^{-1}$ in the BY4742 strain and $5.4 \pm 0.3 \text{ kJ mol}^{-1}$ in the *zwf1* Δ strain.

1 **Introduction**

2 Budding yeast, *Saccharomyces cerevisiae*, is one of the most important
3 microorganisms for industrial production of ethanol (1, 2). The metabolic regulation of
4 glycolysis in *S. cerevisiae* has been extensively investigated by a series of physiological
5 and biochemical studies (3, 4). Although the genetic and biochemical aspects have been
6 investigated in detail, several basic aspects of *S. cerevisiae* metabolism, including
7 concentrations of intracellular glycolytic intermediates, remain to be characterized.
8 These aspects have been poorly understood because a method for exact analysis of
9 glycolytic intermediates, including sugar phosphates, has so far been lacking. Recent
10 progress in metabolomics techniques have enabled relative quantitation of the glycolytic
11 intermediates by employing capillary electrophoresis-mass spectrometry (MS) and
12 ion-pairing liquid chromatography (LC)-MS (5-8). The accumulation patterns of
13 glycolytic intermediates have been successfully compared among *S. cerevisiae* cells
14 with distinct genotypes or grown under different conditions by relative quantitation of
15 each metabolite (fold increase or decrease) (9, 10). For more detailed characterization of
16 *S. cerevisiae* metabolism, considering the thermodynamics and kinetics of metabolic
17 reactions, absolute quantitation of metabolite concentrations is required. However, the
18 internal standard based-absolute quantitation of glycolytic intermediates has been
19 hampered by the poor commercial availability of stable isotope-labeled authentic
20 standards (11, 12).

21 A recent report detailed an absolute quantitation of intracellular metabolites, by
22 preparing a mixture of isotope-labeled compounds from microbial cells cultivated in an
23 isotope-labeled medium (Fig. 1) (13-17). Metabolites uniformly labeled with ^{13}C were
24 extracted from *S. cerevisiae* cells cultivated using a synthetic medium containing

1 [U-¹³C] glucose as the sole carbon source (Fig. 1(a-d)). The mixture of ¹³C-labeled
2 metabolites could be used as an internal standard for the analysis of *S. cerevisiae*
3 cultivated in a medium containing natural glucose (Fig. 1(e, f)) (14). The methodology
4 was employed for the analysis of xylose-fermenting *S. cerevisiae* in which
5 thermodynamic analysis suggested that the activities of pentose phosphate pathway
6 enzymes and the pool of fructose-6-phosphate were potential factors limiting xylose
7 utilization (15). Furthermore, yeast single-enzyme knockout mutants grown on three
8 different carbon sources were thermodynamically analyzed (16). In addition, absolute
9 metabolite concentrations were used to quantitatively and thermodynamically
10 understand cellular metabolism in *Escherichia coli* (17).

11 The present study employed this methodology for the absolute quantitation of
12 glycolytic intermediates of BY4742, *pfk1Δ*, and *zwf1Δ* strains of *S. cerevisiae*. The
13 results confirmed that fructose-1,6-bisphosphate (FBP) was the most abundant
14 intermediate in the BY4742 strains during the log phase of growth. Estimation of the
15 Gibbs free energy change revealed that several reactions, such as those catalyzed by
16 ribose-5-phosphate ketol-isomerase (RKI) and phosphoglucose isomerase (PGI) were
17 commonly at near-equilibrium in all three strains. A significant shift in thermodynamic
18 state was also observed for the transketolase-transaldolase (TKL-TAL) reaction,
19 demonstrating that the direction of the TKL-TAL reaction changed from forward in the
20 BY4742 strain to reverse in the *zwf1Δ* strain.

Materials and methods

Yeast strains and growth conditions

S. cerevisiae strains, including S288C (MAT α SUC2), BY4742 (MAT α , his3 Δ 1, leu2 Δ 0, lys2 Δ 0, ura3 Δ 0), pfk1 Δ (BY4742 Δ pfk1::kanMX), and zwf1 Δ (BY4742 Δ zwf1::kanMX) were purchased from Thermo Scientific (Pittsburgh, PA, USA). All strains were cultured in yeast extract peptone dextrose (YPD) medium (1% bacto yeast extract, 2% bacto peptone, 2% glucose) and synthetic dextrose (SD) medium (6.7% yeast nitrogen base without amino acids and 5% non-labeled or [U-¹³C] glucose, as necessary, 0.06% leucine, 0.03% lysine hydrochloride, 0.02% histidine, and 0.02% uracil). [U-¹³C] glucose (99%) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

S. cerevisiae cells from glycerol stocks were cultured in 5 mL of YPD medium at 30°C for 24 h with shaking at 120 rpm. The cells were inoculated into 100 mL of SD medium in 500-mL Sakaguchi flasks and cultured at 30°C for 24 h with shaking at 120 rpm. The precultures were transferred to the main culture (100 mL of SD medium in 500-mL Sakaguchi flasks at 30°C for 48 h with shaking at 120 rpm). The initial OD₆₆₀ values for pre- and main cultures were set at 0.2. For the preparation of isotope-labeled standards from the S288C strain, SD medium containing [U-¹³C] glucose was used for the pre- and main cultures. The BY4742, pfk1 Δ , and zwf1 Δ strains were cultured in SD medium containing unlabeled glucose.

Analysis of extracellular metabolites

To determine the concentrations of glucose, ethanol, acetate, and glycerol in the culture medium, supernatant obtained by centrifugation at 15,000 rpm at 4°C for 10 min was applied to a high-performance liquid chromatography (HPLC) system (Shimadzu, Japan) equipped with an Aminex HPX-87H (7.8 mm, 300 mm, Bio-Rad, USA) column,

a UV/vis detector (SPD-20A, Shimadzu, Kyoto, Japan), and a refractive index detector (RID-10A, Shimadzu, Kyoto, Japan). The column temperature was set at 65°C, and 1.5 mM H₂SO₄ was used as the mobile phase with a flow rate of 0.5 mL min⁻¹. The flow cell temperature of the refractive index detector was set at 35°C.

LC-MS/MS analysis of intermediate metabolites

Culture broth was sampled rapidly and filtered through a 0.5-μm pore size filter (PTFE-type membrane, ADVANTEC, Japan). Cells on the filter were immediately immersed in 1.6 mL methanol (-80°C) and kept at -80°C until extraction (18, 19). For the preparation of isotope-labeled standards from the S288C strain, 40 mL or 45 mL culture broth was collected at 9 h or 21 h, respectively. Following addition of 1.6 mL of chloroform (-30°C) and 640 μL of Milli-Q water (4°C) and vortexing for 1 min, the mixture was centrifuged at 4500 × g for 40 min at 4°C. Two hundred and fifty microliters of the aqueous layer was transferred to a 1.5-mL tube. The extract solution was dispensed into seven Eppendorf tubes and dried using a SpeedVac SPD1010 (Thermo Scientific, Japan) at room temperature. To determine the concentrations of the ¹³C-labeled intermediates, the dried extract was dissolved in 50 μL of standard solution containing known concentrations of unlabeled metabolites. The concentration of each unlabeled metabolite in the standard solution was as follows: glucose-6-phosphate (G6P, 10.2 μM), fructose-6-phosphate (F6P, 9.8 μM), fructose-1,6-bisphosphate (FBP, 20.0 μM), dihydroxyacetonephosphate (DHAP, 22.3 μM), 2-phosphoglycerate + 3-phosphoglycerate (2PG+3PG, 20.6 μM), phosphoenolpyruvate (PEP, 20.3 μM), pyruvate (Pyr, 20.9 μM), 6-phosphogluconate (6PG, 20.0 μM), ribulose-5-phosphate (Ru5P, 18.9 μM), ribose-5-phosphate (R5P, 19.3 μM), xylulose-5-phosphate (Xu5P,

20.0 μM), and sedoheptulose-7-phosphate (S7P, 20.0 μM). For analysis of the BY4742, *pfk1 Δ* , and *zwf1 Δ* strains, cells were harvested at $\text{OD}_{660} = 1.0$ and 20 mL culture broth was collected at 9h, 9h and 18h in BY4742, *pfk1 Δ* , and *zwf1 Δ* strains. Metabolites were extracted by addition of 1.6 mL of chloroform, 540 μL of Milli-Q water, and 100 μL of isotope-labeled extract prepared from the S288C strain. The dried samples were suspended in 50 μL of Milli-Q water.

LC-MS/MS analysis (LC: Agilent 1100 series; Agilent Technologies, MS/MS: API 2000; MA, AB SCIEX) was performed under the following conditions (20) : column, ProteCol-P C18 HQ103 (2.1 $\times$ 150 mm, particle size of 3 μm); mobile phase, 10 mM tert-butyl alcohol/15 mM acetic acid in water (A) and methanol (B); flow rate: 0.2 mL min⁻¹; gradient curve, 0% B at 0 min, 0% B at 8 min, 90% B at 24 min, 0% B at 24.1 min, and 0% B at 30 min; injection volume, 3 μL ; column temperature, 35°C; mode of mass analysis, negative ion mode; nebulizer flow, 55 psi; dry gas flow rate, 10 L min⁻¹; sheath gas flow rate, 11 L min⁻¹; dry gas temperature, 300°C; sheath gas temperature, 380°C; capillary voltage, 3.5 kV. The parameters for selected reaction monitoring (SRM) of target metabolites are shown in Table S1. The peak of each target metabolite was identified by comparing its chromatographic behavior with that of an authentic standard. Peak area was determined using the software Analyst (version 1.6.2, AB SCIEX). Quantitation was performed based on the MS data using the intracellular concentration of each metabolite (mmol g⁻¹ fw⁻¹) determined using the ratio of the ¹³C peak area to the ¹²C peak area. Fresh weight (fw) was calculated as follows: (OD_{660}) $\times$ 1.50 $\times$ (sampling liquid amount).

Calculation of Gibbs free energy change (ΔG)

Gibbs free energy change (ΔG) was determined using the following equation:

$$\Delta G = \Delta G^0 + RT \ln Q$$

Here, R is the ideal gas constant in units of $\text{J mol}^{-1} \text{K}^{-1}$, T is the temperature, $\ln$ represents a logarithm to the base e , and Q is the reaction quotient at that moment in time. Data for K_{eq} and ΔG^0 were obtained from the National Institute of Standards and Technology (NIST) database of K_{eq} data compiled from the biochemical literature by Goldberg and Tewari (21), and thermodynamic studies (22), except for FBA (23), TKL-TAL (24), and enolase (ENO) (25) (Table S2). The value of E_{eq} was adapted to the value of the standard Gibbs free energy change of the reaction ($\Delta G^0 = -RT \ln K_{\text{eq}}$, where K_{eq} is the equilibrium constant).

Results

Preparation of isotope-labeled standards from the S288C strain

Figure 1 shows a schematic representation of the experimental design of this study. For preparation of the ^{13}C -labeled metabolite mixture, the S288C strain of *S. cerevisiae* was cultured in synthetic medium containing $[\text{U-}^{13}\text{C}]$ glucose as the sole carbon source (Fig. 1(a)). The S288C strain was selected for its amino acid autotrophy. A mixture of ^{13}C -labeled metabolites was extracted from the S288C cells, dispensed into seven Eppendorf tubes, and dried under reduced pressure (Fig. 1(b)). One of the extracts was dissolved in 50 μL of standard solution containing known concentrations of unlabeled metabolites (Fig. 1(c)). The concentrations of the ^{13}C -labeled metabolites in the solution were determined by LC-MS/MS analysis, using unlabeled metabolites as an internal standard (Fig. 1(d)). In this study, the concentrations of 13 metabolites in the glycolytic pathway (G6P, F6P, FBP, DHAP, 3PG, 2PG, PEP, Pyr, 6PG, Ru5P, R5P, Xu5P,

and S7P, see the Materials and Methods for abbreviations of metabolite names) were determined. Other glycolytic intermediates were not analyzed due to poor availability of standard compounds (1,3-BPG) or very low concentrations in *S. cerevisiae* cells (E4P and GAP).

The concentrations of the ^{13}C -labeled metabolites were determined based on the ratio of the peak areas of (A) non-labeled-metabolite signals from a standard solution of unlabeled metabolites of known concentrations, and (B) ^{13}C -labeled-metabolite signals from S288C (Fig. 1(d)). For example, LC-MS/MS analysis of R5P demonstrated that signals of R5P labeled with different numbers of ^{13}C atoms were observed in the distinct SRM chromatograms (Fig 2(a)). Of the metabolite signals shown in Fig. 2(a), the peaks observed at 229 (+0) > 97 and 230 (+1) > 97 were derived from natural R5P in the standard mixture. A portion of the natural R5P labeled with naturally occurring isotopes contributed to the peak at 230 (+1) > 97. The peaks at 233 (+4) > 97 and 234 (+5) > 97 show isotope-labeled R5P derived from *S. cerevisiae* S288C grown in [U- ^{13}C] glucose. In addition to the fully labeled isotopomer (234 (+5) > 97), a trace amount of partially labeled R5P was also observed (233 (+4) > 97). This is probably because the [U- ^{13}C] glucose used in this study was not completely labeled with ^{13}C .

In this study, six sets of the internal standard mixtures were prepared by different cultures of S288C. Table 1 shows the concentrations of ^{13}C -labeled glycolytic intermediates. The data shown in Table 1 indicated that the concentrations of some intermediates, such as FBP and 2PG + 3PG, varied among the extracts prepared from *S. cerevisiae* cells at the mid (9 h) and late (21 h) log phases of growth. Furthermore, the concentrations of PP pathway intermediates, such as R5P, Ru5P, and Xu5P, were shown

to be at relatively low levels, and no signal was observed for GAP or E4P under either conditions. A large variation of S7P levels (9.7 - 77.9 μM) was observed among the mixtures (Set 3, 4, 5, and 6) probably due to an unstable metabolic states in the late log phase cultures. To prepare a ^{13}C -labeled metabolite mixture containing significant amounts of GAP and E4P, suitable yeast strains and culture conditions for accumulation of the appropriate glycolytic intermediates must be determined.

Absolute quantitation of yeast central metabolites using isotope-labeled standards

As shown in the experimental design of this study (Fig. 1), the mixtures of isotope-labeled metabolites obtained from S288C (Fig. 1(b)) were used as internal standards for the absolute quantitation of intracellular metabolite concentrations of *S. cerevisiae* cells cultured with natural (non-labeled) glucose (Fig. 1(e)). Following extraction of metabolites using the chloroform-water-methanol method, the extracted metabolites were analyzed using LC-MS/MS (Fig. 1(f)). The metabolite profiles of glycolytic intermediates were determined in the BY4742, *pfk1* Δ , and *zwf1* Δ strains. Figure 3 shows the culture profiles of the BY4742, *pfk1* Δ , and *zwf1* Δ strains grown in Sakaguchi flasks containing unlabeled glucose. Similar culture profiles were observed for the BY4742 and *pfk1* Δ strains, which showed specific growth rates of $0.281 \pm 0.013 \text{ h}^{-1}$ and $0.273 \pm 0.014 \text{ h}^{-1}$, respectively. The glucose in the medium had been completely consumed at 14 h after the start of cultivation. In contrast, the *zwf1* Δ strain was still in the logarithmic growth phase ($\text{OD}_{660} = 1.0$) at 14 h after the start of cultivation, and its specific growth rate was $0.083 \pm 0.003 \text{ h}^{-1}$, one-third of that of the BY4742 and *pfk1* Δ strains. In all strains, glycerol and acetate production were approximately 2 mM, which was much lower than the observed ethanol production (32–35 mM).

S. cerevisiae cells were harvested at $OD_{660} = 1.0$, and glycolytic intermediates were extracted using a buffer containing the mixtures of isotope-labeled metabolites (Fig. 1(e)). Fig 2(b) shows representative SRM chromatograms of R5P analyzed by LC-MS/MS. Of the four peaks observed, the peaks at $229 (+0) > 97$ and $230 (+1) > 97$ were derived from R5P in the BY4742 strain grown on unlabeled glucose. The peaks at $233 (+4) > 97$ and $234 (+5) > 97$ show the signal of isotope-labeled R5P in ^{13}C -labeled standard solution. Based on the peak area ratios, the absolute metabolite concentrations of the BY4742 strain were determined. Using the same approach, the absolute concentrations of glycolytic intermediates were determined for the BY4742, *pfk1* Δ , and *zwf1* Δ strains during the logarithmic growth phase.

The quantitation method was verified using the following procedure. The isotope-labeled metabolite mixture derived from the S288C strain (Fig. 1(b)) was added to a standard solution of non-labeled metabolites of known concentrations. Here, the concentrations of the non-labeled metabolites in the mixture were determined by LC-MS/MS using the isotope-labeled metabolites as internal standards. The experimentally determined concentrations of the non-labeled metabolites were compared with theoretical values since the actual concentrations in the mixture were known. The results showed that the concentrations determined using the procedure detailed here were essentially identical to the theoretical values (Fig. 4). For comparison, the concentrations of the non-labeled metabolites in the mixture were also determined by the external standard method, using the external calibration curve. The concentrations determined by the external standard method were significantly lower than the theoretical values, due to an ion suppression effect derived from the matrix of the S288C sample (Fig. 4) (12). These results demonstrate that levels of intracellular

metabolites could be determined accurately by the internal standard based method.

Analysis of yeast glycolysis based on absolute quantitation data

The concentration of each glycolytic intermediate was compared with the absolute quantitation data. As shown in Figure 5, the levels of Embden–Meyerhof–Parnas (EMP) pathway intermediates tended to be higher than those of pentose phosphate (PP) pathway intermediates. For example, in the BY4742 strain, levels of G6P, FBP, and DHAP reached 0.18 ± 0.02 , 1.18 ± 0.16 , and 0.31 ± 0.03 $\mu\text{mol g}^{-1} \text{fw}^{-1}$, whereas the concentrations of Ru5P, R5P, and Xu5P were 0.04 ± 0.01 , 0.01 ± 0.01 , and $0.05 \pm 0.01 \mu\text{mol g}^{-1} \text{fw}^{-1}$, respectively. A similar result was observed in the absolute quantitation of intracellular metabolites in the K-12 strain of *Escherichia coli* (17). It was shown that FBP (15 mM) was the most abundant glycolytic intermediate and that concentrations of other intermediates, including Ru5P and R5P, were as much as ten times lower than those of FBP (17). In contrast, 3PG and PEP were among the most abundant metabolites in the PCC6803 strain of the cyanobacterium *Synechocystis* sp., grown under photoautotrophic conditions (1.48 and $2.51 \mu\text{mol g-drycell weight}^{-1}$) (26). The different patterns of metabolite accumulation should reflect the activity of the Calvin cycle, in which C3 intermediates, such as 3PG and PEP, are synthesized from pentose phosphates by RuBisCO.

Comparison of the accumulation profiles of *pfk1Δ* and BY4742 showed that metabolic profiles were significantly altered by the disruption of *PFK1*, which encodes phosphofructokinase (27). Because phosphofructokinase catalyzes the reaction $\text{F6P} + \text{ATP} \Rightarrow \text{FBP} + \text{ADP}$, down-regulation of the reaction caused five- to ten-fold increases in up-stream metabolites, including G6P and F6P, as well as drastic decreases in

down-stream metabolites such as FBP and DHAP (Fig. 5(a)). Furthermore, the level of S7P was drastically increased, from $0.08 \pm 0.01 \mu\text{mol g}^{-1} \text{fw}^{-1}$ in BY4742 to $0.43 \pm 0.01 \mu\text{mol g}^{-1} \text{fw}^{-1}$ in *pfk1Δ*. Similar results including the accumulation of S7P in *pfk1Δ* strain were reported in the previous study (28). On the other hand, levels of other intermediates in the PP pathway were the same in *pfk1Δ* and BY4742 strains. These observations suggest that Pfk1p plays a role in glycolysis in the BY4742 strain. However, because the culture profiles of *pfk1Δ* were similar to those of BY4742 (Fig. 3), the function of Pfk1p was likely to have been complemented by an activity of the isozyme Pfk2p (28), causing the shift in metabolite accumulation pattern observed in the *pfk1Δ* strain.

In the *zwf1Δ* strain, most metabolites in the pentose phosphate pathway were lower than in the BY4742 strain, with concentrations $< 0.006 \mu\text{mol g}^{-1} \text{fw}^{-1}$. These low concentrations resulted from disruption of the gene encoding the enzyme responsible for the entry point of the oxidative PP pathway (*ZWF1* encodes glucose-6-phosphate dehydrogenase for conversion of G6P to 6-phosphoglucono-δ-lactone) in the *zwf1Δ* strain (30). In contrast to the *pfk1Δ* strain, the levels of up-stream intermediates, including G6P and other EM pathway intermediates such as F6P, FBP, DHAP, and Pyr, were significantly decreased in the *zwf1Δ* strain, (Fig. 5(a)). The *zwf1Δ* strain exhibited slower specific growth and glucose consumption rates and had low concentrations of glycolytic intermediates (Fig. 3); together, these suggest a retardation in the glucose assimilation activity of the strain. A similar metabolic shift has been observed in the *pfk1Δ* and *zwf1Δ* strains by relative quantitation of metabolite profiles using conventional metabolome analysis (9).

1 Estimation of Gibbs free energy change of the reaction (ΔG)

2 Using absolute intracellular metabolite concentrations, the Gibbs free energy
3 changes of the reaction (ΔG) in the *pfk1* Δ , *zwf1* Δ , and BY4742 strains were determined
4 for phosphoglucose isomerase (PGI, $G6P \leftrightarrow F6P$), ribose-5-phosphate ketol-isomerase
5 (RKI, $R5P \leftrightarrow Ru5P$), and D-ribulose-5-phosphate 3-epimerase (RPE, $Ru5P \leftrightarrow Xu5P$)
6 (Fig. 5(b) and Table S2). Levels of ΔG for FBA (fructose-1,6-bisphosphate aldolase,
7 $FBP \leftrightarrow DHAP + GAP$) were also estimated, using theoretically determined GAP levels,
8 because it has been reported that the reaction catalyzed by triosephosphate isomerase
9 (TPI, $DHAP \leftrightarrow GAP$) is at near-equilibrium (31). The reactions catalyzed by
10 transketolase and transaldolase (TKL-TAL, $Ru5P + 2Xu5P \leftrightarrow 2F6P + GAP$) were
11 considered as a single reaction for determination of ΔG levels (15). Furthermore, the ΔG
12 of enolase (ENO, $2PG \leftrightarrow PEP$) was calculated using theoretically determined 2PG
13 levels, since phosphoglucomutase ($3PG \leftrightarrow 2PG$) catalyzed by phosphoglycerate
14 mutase was assumed to be at equilibrium (32).

15 Among the six reactions observed, the ΔG levels of reactions catalyzed by FBA
16 showed relatively large negative values ($-8.1 \pm 0.1 \text{ kJ mol}^{-1}$, $-10.6 \pm 0.6 \text{ kJ mol}^{-1}$, and
17 $-11.2 \pm 0.3 \text{ kJ mol}^{-1}$ in BY4742, *pfk1* Δ , and *zwf1* Δ , respectively). However, the ΔG
18 levels of the PGI, RKI, and RPE reactions were close to 0 in all three strains. The results
19 suggested that the reactions were commonly at near-equilibrium, although substrate and
20 product concentrations differed among the BY4742, *pfk1* Δ , and *zwf1* Δ strains. A
21 significant shift in thermodynamic state was observed for the TKL-TAL reaction,
22 because the ΔG levels for TKL-TAL differed among the three strains ($-6.6 \pm 0.5 \text{ kJ}$
23 mol^{-1} , $-0.6 \pm 0.9 \text{ kJ mol}^{-1}$, and $5.4 \pm 0.3 \text{ kJ mol}^{-1}$ in the BY4742, *pfk1* Δ , and *zwf1* Δ
24 strains, respectively).

Discussion

In this study, an internal standard method was employed for the absolute quantitation of glycolytic intermediates in *S. cerevisiae*. A mixture of uniformly ^{13}C -labeled metabolites was prepared by extraction of metabolites from cells of *S. cerevisiae* strain S288C cultivated in medium containing $[\text{U-}^{13}\text{C}]$ glucose as the sole carbon source (Fig. 1). The concentrations of the ^{13}C -labeled metabolites in the extracts were determined using the non-labeled authentic standards of the target compounds (Table 1). The extracts of ^{13}C -labeled metabolites were shown to be a useful mixture of internal standards for absolute quantitation of glycolytic intermediates in *S. cerevisiae* cells cultured in a natural glucose medium (Fig. 2 and Fig. 4).

Using the method developed here, levels of 13 glycolytic intermediates in the BY4742, *pfk1* Δ , and *zwf1* Δ strains were successfully determined (Fig. 5(a)). The Gibbs free energy changes (ΔG) were also estimated for the PGI, RKI, RPE, FBA, TKL-TAL, and ENO reactions (Fig. 5(b)). Among the six reactions, the ΔG for the FBA reaction showed the largest negative values in the BY4742 strain, suggesting that the reaction somehow departed from equilibrium. Indeed, the accumulation profile of the BY4742 strain showed that FBP was the most abundant of the target metabolites with concentrations greater than 1.0 mM (Fig. 5(a)). The result indicated that the high concentration of FBP pushed the FBA reaction in the forward direction. Furthermore, comparison of kinetic parameters of the enzymes indicated that FBP, a substrate for fructose-1,6-bisphosphate aldolase (FBA, $\text{FBP} \rightleftharpoons \text{DHAP} + \text{GAP}$), was saturated; the K_m for FBP is reported to be 0.055–0.45 mM (33, 34). FBA activity was not sufficient to reach equilibrium, suggesting that the FBA reaction is one of the metabolic bottlenecks

1 in glycolysis.

2 In contrast, concentrations of PP pathway intermediates, including R5P, Ru5P,
3 and Xu5P remained at low levels in the BY4742 and *pfk1Δ* strains ($0.05\text{--}0.01\ \mu\text{mol g}^{-1}$
4 fw^{-1} , Fig. 5(a)). Because the ΔG of the RKI and RPE reactions are close to zero ($\Delta G \rightleftharpoons$
5 0, Fig. 5(b)), conversion among the $\text{R5P} \rightleftharpoons \text{Ru5P} \rightleftharpoons \text{Xu5P}$ was at near-equilibrium
6 (15). Even when the concentrations of R5P, Ru5P, and Xu5P were significantly
7 decreased in the *zwf1Δ* strain (Fig. 5 (a)), the RKI and RPE reactions remained at
8 near-equilibrium (Fig. 5(b)). Similar result was also observed for the PGI reaction (Fig.
9 5). While levels of G6P and F6P differed among strains, levels of ΔG were close to zero.
10 These results suggested that PGI has sufficient activity to maintain rapid rates of the
11 forward ($\text{G6P} \rightarrow \text{F6P}$) and reverse ($\text{F6P} \rightarrow \text{G6P}$) reactions.

12 The ΔG changed from $-6.6 \pm 0.5\ \text{kJ mol}^{-1}$ in the BY4742 strain to $5.4 \pm 0.3\ \text{kJ}$
13 mol^{-1} in the *zwf1Δ* strain, indicating a significant shift in the thermodynamic state for
14 the TKL-TAL reaction (Fig. 5(b)). Thus, while the direction of the TKL-TAL reaction
15 was forward ($\text{Ru5P} + 2\ \text{Xu5P} \Rightarrow 2\ \text{F6P} + \text{GAP}$) in the BY4742 strain, the reaction
16 proceed in the reverse direction ($2\ \text{F6P} + \text{GAP} \Rightarrow \text{Ru5P} + 2\ \text{Xu5P}$) in the *zwf1Δ* strain.
17 This metabolic shift suggests that because the metabolic flux in the oxidative pentose
18 phosphate was reduced by disruption of *ZWF1*, pentose phosphates including Ru5P and
19 Xu5P were synthesized from F6P and GAP in the *zwf1Δ* strain. The ΔG of TKL-TAL
20 was close to zero in the *pfk1Δ* strain ($-0.6 \pm 0.9\ \text{kJ mol}^{-1}$), indicating that accumulation
21 of F6P in the *pfk1Δ* strain via disruption of *PFK1* resulted in TKL-TAL equilibrium.

22 The present study demonstrated that absolute quantitation of glycolytic
23 intermediates can produce basic and essential data to understand the regulation of *S.*
24 *cerevisiae* metabolism from a thermodynamic point of view. Further improvement of

the method has been attempted for more sensitive analysis of wider ranges of metabolites (35). The absolute quantitation of larger numbers of glycolytic intermediates and cofactors will enable us to investigate the metabolic regulation of *S. cerevisiae* by comparative analysis with proteomics (36) and metabolic flux data (37).

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Figure legends

Fig. 1 Experimental design. *Saccharomyces cerevisiae* S288C strain was cultured in the synthetic medium containing [U-¹³C] glucose as sole carbon source (a), from which a mixture of ¹³C-labeled metabolites was extracted (b). Following a suspension in standard solution containing unlabeled metabolites with known concentrations (c), the concentrations of ¹³C-labeled metabolites in the solution were determined by LC-MS/MS analysis using unlabeled metabolites as the internal standard (d). The mixtures of isotope-labeled metabolites obtained from S288C were used as internal standard for the absolute quantitation of intracellular metabolite of *S. cerevisiae* cells cultured in the natural (non-labeled) glucose (e). Following the extraction of metabolites using the chloroform-water-methanol method, the extracted metabolites were analyzed by using LC-MS/MS (f).

Fig. 2 Representative selected reaction monitoring (SRM) chromatograms of ribose-5-phosphate (R5P) analyzed by LC-MS/MS. The mass numbers (*m/z*) employed for each SRM transitions were shown in the figure. (a) Non-labeled- (from unlabeled R5P with known concentration in a standard solution) and ¹³C labeled-(from S288C strain grown on [U-¹³C] glucose) R5P signals. (b) Non-labeled-(from BY4742 strain grown on unlabeled glucose) and ¹³C labeled-(¹³C standard solution) R5P signals.

Fig. 3 The culture profiles of BY4742 (a), *pfk1*Δ (b) and *zwf1*Δ strains (c) by batch fermentations. The yeast cells were inoculated in 100 mL of SD medium containing 5 g/L glucose. Extracellular substances (glucose, ethanol, acetate and glycerol) measured using a high-performance liquid chromatography (HPLC) system. Black arrow

1 indicated sampling point. Each data point represents the mean (SD) values obtained
2 from 3 replicate fermentations.

3
4 **Fig. 4** Comparison of metabolite concentration determined by internal and external
5 standard methods. X axis represents theoretical (true) concentrations of each metabolite.
6 Y axis represent concentrations of each metabolite determined by the absolute
7 quantification (internal standard) method (closed circle) and by the conventional
8 (external standard) method (open circle).

9
10 **Fig. 5** The absolute quantitation of 13 glycolytic intermediates in BY4742, *pfk1Δ* and
11 *zwf1Δ* strain (a) and Gibbs free energy change of the reaction inferred from absolute
12 metabolite concentrations (b). Each data point represents the mean (SD) values obtained
13 from 3 replicate fermentations. Abbreviations: G6P, glucose-6-phosphate; F6P,
14 fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; DHAP,
15 dihydroxyacetonephosphate; 2PG + 3PG, 2-phosphoglycerate + 3-phosphoglycerate; PEP,
16 phosphoenolpyruvate; Pyr, pyruvate; 6PG, 6-phosphogluconate; Ru5P,
17 ribulose-5-phosphate; R5P, ribose-5-phosphate; Xu5P, xylulose-5-phosphate; and S7P,
18 sedoheptulose-7-phosphate. PGI, phosphoglucose isomerase; RKI, ribose-5-phosphate
19 ketol-isomerase; RPE, ribulose-5-phosphate 3-epimerase; FBP,
20 fructose-1,6-bisphosphate aldolase; TKL-TAL, transketolase and transaldolase; ENO,
21 Enolase.

Fig. 1

Fig. 2

Fig. 3

1

2

3 **Fig. 4**

4

Fig.5

Table 1 The concentration of ^{13}C -labeled glycolytic intermediates in six internal standard mixture sets prepared from *Saccharomyces cerevisiae* S288C strain cultured in the synthetic medium containing $[\text{U-}^{13}\text{C}]$ glucose as the sole carbon source

	Set 1	Set 2	Set 3	Set 4	Set 5	Set 6
Sampling time ¹	9 h	9 h	21 h	21 h	21 h	21 h
OD ₆₆₀	2.02	2.26	4.04	4.12	4.20	4.20
Volume (mL)	40	40	45	45	45	45
Metabolite (μM) ²						
G6P	29.8	14.8	37.8	50.5	41.0	49.4
F6P	14.9	10.4	26.2	28.7	24.1	22.4
FBP	74.3	69.8	6.6	14.2	9.2	10.3
DHAP	13.4	13.6	2.5	4.3	2.5	2.1
2PG+3PG	16.6	16.2	270.5	234.8	215.0	248.5
PEP	4.0	3.8	100.4	71.1	64.8	67.0
Pyr	69.3	72.0	59.6	58.8	76.1	69.7
6PG	11.2	9.5	6.6	11.1	7.0	9.6
Ru5P	2.2	1.9	0.5	0.8	0.6	0.5
R5P	5.5	5.5	3.3	3.5	1.1	0.7
Xu5P	4.3	3.8	0.6	0.6	0.6	0.4
S7P	9.0	7.3	13.5	11.0	9.7	77.9

¹ Liquid volumes of the culture and OD₆₆₀ at sampling time are described.

² Concentrations of ^{13}C -labeled metabolites in mixtures prepared from *Saccharomyces cerevisiae* S288C strain.