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

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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 [U-^{13}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, pfk1Δ, and zwf1Δ 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 ketol-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 ± 0.5 kJ mol^{-1} in the BY4742 strain and 5.4 ± 0.3 kJ mol^{-1} in the zwf1Δ strain.

[**Key words:** *Saccharomyces cerevisiae*; Absolute quantitation; Central carbon metabolism; Liquid chromatography–mass spectrometry; Gibbs free energy change]

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

A recent report detailed an absolute quantitation of intracellular metabolites, by preparing a mixture of isotope-labeled compounds from microbial cells cultivated in an isotope-labeled medium (Fig. 1) (13–17). Metabolites uniformly labeled with ^{13}C were extracted from *S. cerevisiae* cells cultivated using a synthetic medium containing [U-^{13}C] glucose as the sole carbon source (Fig. 1A–D). The mixture of ^{13}C-labeled metabolites could be used as an internal standard for the analysis of *S. cerevisiae* cultivated in a medium containing natural glucose (Fig. 1E, F) (14). The methodology was employed for the analysis of xylose-fermenting *S. cerevisiae* in which thermodynamic analysis suggested that the activities of pentose phosphate pathway enzymes and the pool of fructose-6-phosphate were potential factors limiting xylose utilization (15). Furthermore, yeast single-enzyme knockout mutants grown on three different carbon sources were thermodynamically analyzed (16). In addition, absolute metabolite concentrations were used to quantitatively and thermodynamically understand cellular metabolism in *Escherichia coli* (17).

The present study employed this methodology for the absolute quantitation of glycolytic intermediates of BY4742, pfk1Δ, and zwf1Δ strains of *S. cerevisiae*. The results confirmed that fructose-1,6-bisphosphate (FBP) was the most abundant intermediate in the BY4742 strains during the log phase of growth. Estimation of the Gibbs free energy change revealed that several reactions, such as those catalyzed by ribose-5-phosphate ketol-isomerase (RKI) and phosphoglucose isomerase (PGI) were commonly at near-

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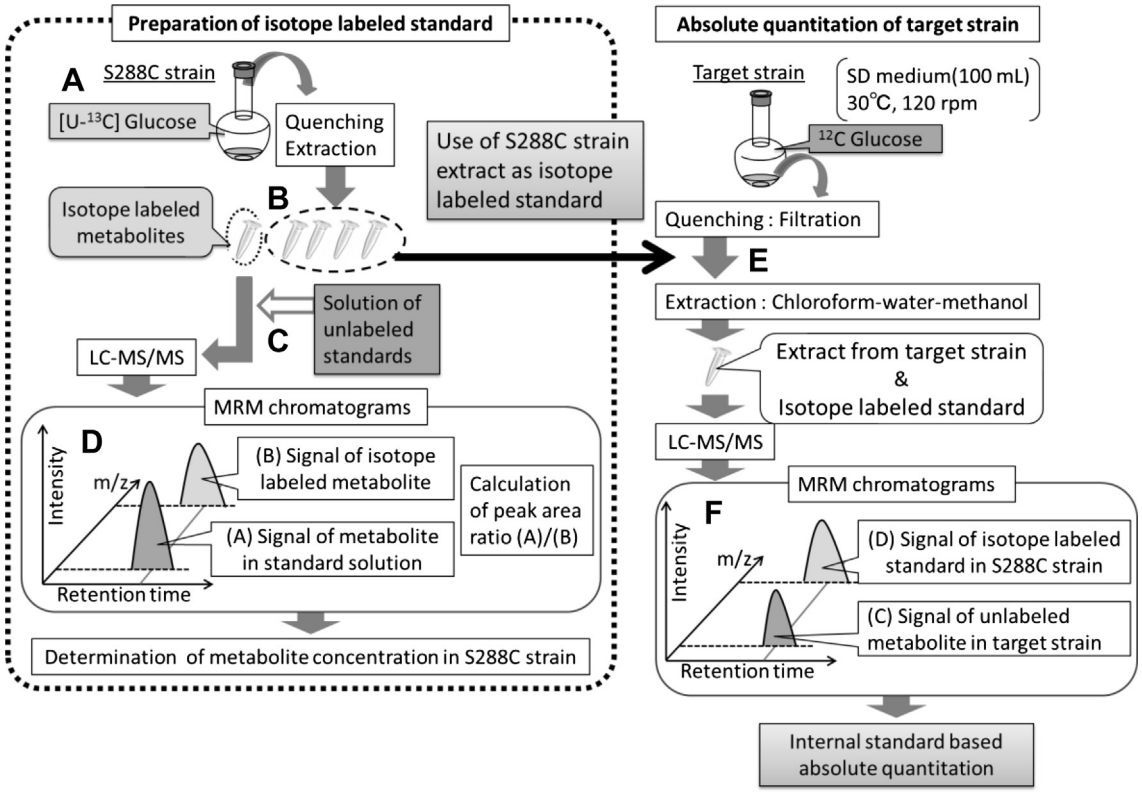

FIG. 1. Experimental design. *Saccharomyces cerevisiae* S288C strain was cultured in the synthetic medium containing [U-^{13}C] glucose as sole carbon source (A) from which a mixture of ^{13}C-labeled metabolites was extracted (B). Following a suspension in standard solution containing unlabeled metabolites with known concentrations (C), the concentrations of ^{13}C-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).

equilibrium in all three strains. A significant shift in thermodynamic state was also observed for the transketolase–transaldolase (TKL–TAL) reaction, demonstrating that the direction of the TKL-TAL reaction changed from forward in the BY4742 strain to reverse in the zwf1Δ strain.

MATERIALS AND METHODS

Yeast strains and growth conditions *S. cerevisiae* strains, including S288C (MATα SUC2), BY4742 (MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0), pfk1Δ (BY4742 Δpfk1::*kanMX*), and zwf1Δ (BY4742 Δzwf1::*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-^{13}C] glucose, as necessary, 0.06% leucine, 0.03% lysine hydrochloride, 0.02% histidine, and 0.02% uracil). [U-^{13}C] 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 OD_{660} 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-^{13}C] glucose was used for the pre- and main cultures. The BY4742, pfk1Δ, and zwf1Δ 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, Kyoto, Japan) equipped with an Aminex HPX-87H (7.8 mm, 300 mm, Bio-Rad, Hercules, CA, USA) column, a UV/vis detector (SPD-20A, Shimadzu), and a refractive index detector (RID-10A, Shimadzu). The column temperature was set at 65°C, and 1.5 mM H_2SO_4 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 metabolties Culture broth was sampled rapidly and filtered through a 0.5-μm pore size filter (PTFE-type membrane, Advantec, Tokyo, 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. The aqueous layer was dispensed to a 1.5-mL tube by 250 μL. The extract solution was dispensed into seven Eppendorf tubes and dried using a SpeedVac SPD1010 (Thermo Fisher Scientific, Waltham, MA, USA) at room temperature. To determine the concentrations of the ^{13}C-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), dihydroxyacetone phosphate (DHAP, 22.3 μM), 2-phoshoglycerate + 3-phoshoglycerate (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 sedohepturose-7-phosphate (S7P, 20.0 μM). For analysis of the BY4742, pfk1Δ, and zwf1Δ strains, cells were harvested at OD_{660} = 1.0 and 20 mL culture broth was collected at 9 h, 9 h and 18 h in BY4742, pfk1Δ, and zwf1Δ 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, Santa Clara, CA, USA; MS/MS: API 2000; AB SCIEX, MA, USA) was performed under the following conditions (20): column, ProteCol-P C18 HQ103 (2.1 × 150 mm, particle size of 3 μm); mobile phase, 10 mM tributylamine/15 mM acetic acid in water (A) and methanol (B); flow rate: 0.2 mL min^{-1}; gradient curve, 100% A: 0% B at 0 min, 100% A: 0% B at 8 min, 10% A: 90% B at 24 min, 100% A: 0% B at 24.1 min, and 100% A: 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