Journal of Bioscience and Bioengineering
VOL. 118 No. 3, 350–355, 2014

www.elsevier.com/locate/jbiosc

Metabolic distance estimation based on principle component analysis of metabolic turnover

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Received 20 May 2013; accepted 12 February 2014
Available online 26 March 2014

Visualization of metabolic dynamism is important for various types of metabolic studies including studies on optimization of bio-production processes and studies of metabolism-related diseases. Many methodologies have been developed for metabolic studies. Among these, metabolic turnover analysis (MTA) is often used to analyze metabolic dynamics. MTA involves observation of changes in the isotopomer ratio of metabolites over time following introduction of isotope-labeled substrates. MTA has several advantages compared with ^{13}C-metabolic flux analysis, including the diversity of applicable samples, the variety of isotope tracers, and the wide range of target pathways. However, MTA produces highly complex data from which mining useful information becomes difficult. For easy understanding of MTA data, a new approach was developed using principal component analysis (PCA). The resulting PCA score plot visualizes the metabolic distance, which is defined as distance between metabolites on the real metabolic map. And the score plot gives us some hints of interesting metabolism for further study. We used this method to analyze the central metabolism of *Saccharomyces cerevisiae* under moderated aerobic conditions, and time course data for 77 isotopomers of 14 metabolites were obtained. The PCA score plot for this dataset represented a metabolic map and indicated interesting phenomena such as activity of fumarate reductase under aerated condition. These findings show the importance of a multivariate analysis to MTA. In addition, because the approach is not biased, this method has potential application for analysis of less-studied pathways and organisms.

[**Key words:** Dynamic analysis of metabolic pathway; Metabolomics; TCA cycle; *Saccharomyces cerevisiae*; Yeast; Stable isotope; Fumarate respiration; ^{13}C-glucose; Principal component analysis]

Recent advances in the comprehensive analysis of metabolites, i.e., metabolomics, have enabled differentiation of sample types and elucidation of contributing metabolites (1,2). These techniques have been successfully used in research areas such as phenotyping (1) and in quality control processes (2). However, snapshots of the metabolome only provide static information that is sometimes difficult to apply to dynamic aspects of metabolism such as the direction of a reaction or distribution of flux, whereas some research areas such as metabolic engineering (3) and studies of metabolism-related diseases (4) sometimes require dynamic information.

At present, analysis of metabolic dynamics is commonly performed by ^{13}C-metabolic flux analysis (^{13}C-MFA). ^{13}C-MFA is based on simultaneous partial differential equations (5,6). Recently, turnover analysis of metabolites by using isotope tracers has also been used for dynamic analyses of several metabolic pathways simultaneously (7,8). For convenience, in this article, we have termed this turnover analysis of many metabolites as metabolic turnover analysis (MTA). Although both methods share the same purpose, i.e., revealing metabolic dynamism, they are based on different approaches. Therefore, these 2 methods have different applications. ^{13}C-MFA is designed to generate intensive and precise results under controlled conditions. In particular, ^{13}C-MFA utilizes steady-state labeling patterns and ratios of downstream metabolites. The substrate is usually partially labeled by an isotope. For example, in the study of central metabolism, 1-$^{13}C_1$-glucose is commonly used as a label, and the labeling patterns and ratios of amino acids are analyzed. ^{13}C-MFA is used to calculate the fluxes from these steady-state labeling patterns and a preliminarily defined metabolic map. Therefore, ^{13}C-MFA is a strong tool for studies conducted using highly controlled conditions, such as optimization of metabolic engineering (9). Recently isotopic non-stationary MFA (INST-MFA) is also developing. INST-MFA calculates flux of steady state cell from the time resolving data of isotopomer ratio and absolute quantity of metabolites (10). Compared with ^{13}C-MFA, INST-MFA can use variety labeling source.

On the other hand, MTA has fewer constraints than ^{13}C-MFA. MTA can be used to analyze cells that are typically not in steady state conditions, such as those of the plant body (8). It can be performed using fully labeled tracers such as $^{13}CO_2$ (8,11) and $^{15}NH_3$ (7). Further, it can also be used to observe various pathways such as nitrogen assimilation (7). MTA is used for analyzing the changes in the isotopomer ratio over time for each metabolite after addition of a labeled substrate (Fig. 1). In turnover analysis, only the pathways which have detectable metabolites can be analyzed. Therefore, the target pathways had been limited by technology of analytical chemistry. Recent advances in metabolomics technology (12) had enabled expansion of the target pathway of MTA. However, it also created a significant challenge in data analysis. Because each isotopomer of several metabolites are measured at time course, the

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2014.02.014

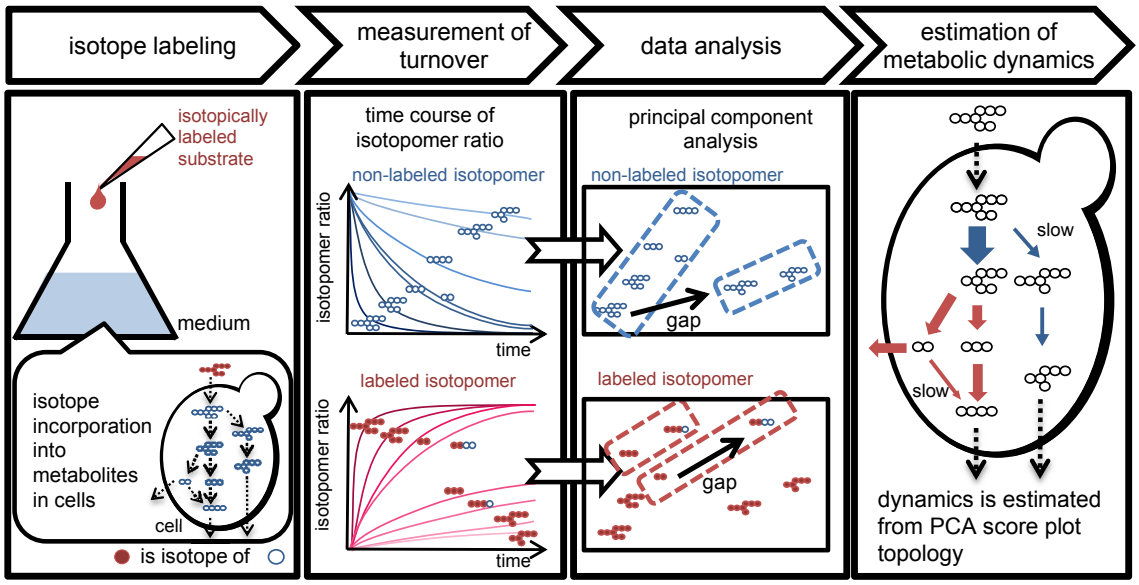

FIG. 1. Concept of data mining with principal component analysis. In our concept, the similarity among metabolic turnover data represents the metabolic dynamics. Principal component analysis (PCA) effectively shows the similarity in a two-dimensional plane. Moreover, plots of non-labeled isotopomers show a rough overview of the metabolic pathway (upper part) and plots of labeled isotopomers show relatively active alternative metabolic pathways.

size of MTA data increases enormously after combination with a metabolomics approach.

In this article, we demonstrate new approach to mine informative ideas from the huge dataset of MTA. For convenience, we defined metabolic distance as the number of reaction steps between 2 metabolites. For instance, a few reaction steps such as those between glucose and glucose-6-phosphate are described as a short metabolic distance, and many reaction steps such as those between glucose and glutamate are described as a long metabolic distance. Although estimation of the reaction route of metabolites is starting point for study of metabolism, the estimation is sometimes wrong because of unknown pathways or just neglect of alternative pathways. For example, pyruvate is converted to oxaloacetate in 9 reaction steps through the TCA cycle. Meanwhile, this conversion could also occur in one step using pyruvate carboxylase.

We hypothesized that real metabolite distance can be estimated from metabolic turnover. Because the metabolites in a cell are gradually replaced with the labeled isotopomers starting from the upstream of the metabolic pathways, the metabolite at a shorter metabolic distance from the label source should be labeled faster than the metabolites at longer metabolic distances in the same pathway. Therefore, the similarity among turnover data of each isotopomer are closely related with metabolic distance. Furthermore, the MTA data of metabolites that have the same metabolic distance from one precursor may reflect the flux of each pathway. Although the pool size, sub-localization and bypass of intermediate have to be considered, visualization of similarity among each turnover data is helpful to analyze metabolic distance. In this report, we suggest a new data mining approach to elucidate metabolic distance by combining multivariate analysis and MTA to mine interesting relation among turnover data (Fig. 1).

In previous report, hierarchical cluster analysis (HCA) was performed to classify the pattern of metabolic turnover (11). The authors found anomalistically classified metabolites against references. This approach was advanced from the view point of making connections among metabolites by performing multivariate analysis to metabolic turnover. However, the study did not analyzed labeled isotopomers. Because the isotope number of an isotopomer sometimes defined by pathway which they are produced, turnover of labeled isotopomers is also informative.

In this point of view, we applied the principal component analysis (PCA) to whole MTA data. The resulted score plot represents the metabolic pathway used to generate the isotopomers. In particular, the score plot of non-labeled mono-isotopomers, which simply decrease after addition of the labeled substrate, shows a rough illustration of the metabolic map based on metabolic distance. In contrast, the score plot of labeled isotopomers, whose number is affected by branch points, shows a more detailed illustration of the metabolic pathway. The score plot can be used to visualize metabolic distance and enable us to figure out characteristics of the metabolism and contrary parts against our knowledge or estimation of metabolism (Fig. 1). We employed our method for metabolic dynamic analysis of central metabolism in *Saccharomyces cerevisiae*, which is one of the most studied model organisms. We selected central metabolism for the analysis because it has several branches, confluences, cycles, and reverse reactions that are difficult to visualize solely by quantitative analysis of metabolites.

MATERIALS AND METHODS

Simulation of isotopomer ratio Simulation data for change in the isotopomer ratio over time were calculated using Excel 2007 (Microsoft, WA, USA). The equation and initial parameters are described in Table S1. The sampling time were t = 0, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, and 10,000 with 10% of relative standard deviation (RSD). Then the data was added 10% of RSD and applied to PCA (Table S2).

Reagents Citric acid, glyceraldehyde-3-phosphate, and malic acid were purchased from Nacalai Tesque (Kyoto, Japan). Ribose-5-phosphate, ribulose-5-phosphate, ribulose-1,5-bisphosphate, erythrose-4-phosphate, fructose-1,6-bisphosphate, 3-phosphoglyceric acid, 2-phosphoglyceric acid, glucose-6-phosphate, fructose-6-phosphate, and dihydroxyacetone phosphate were purchased from Sigma (MO, USA). Phosphoenolpyruvate was purchased from Wako (Osaka, Japan). 1,4-Piperazinediethanesulfonic acid (PIPES) was purchased from Dojindo (Kumamoto, Japan). Sedoheptulose-7-phosphate was a gift from Dr. Shigeoka and Dr. Tamoi (Kinki University, Nara, Japan). U-$^{13}C_6$-D-Glucose was purchased from Cambridge Isotope Laboratory (MA, USA). For extraction and analysis, we obtained HPLC-grade distilled water from Wako, HPLC-grade chloroform from Merck (Darmstadt, Germany), HPLC-grade methanol from Kishida (Osaka, Japan), and HPLC-grade ammonium formate from Sigma.

Yeast cultivation The strain used in this study was *S. cerevisiae* BY4742 (*MATα*, *leu2Δ0*, *his3Δ1*, *lys2Δ0*, *ura3Δ0*). To obtain a single-colony isolate, glycerol-stocked cells were streaked onto a yeast extract peptone dextrose (10 g/L yeast