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Theoretical aspects of ^{13}C metabolic flux analysis with sole quantification of carbon dioxide labeling

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Abstract

The potential of using sole respirometric CO_2 labeling measurement for ^{13}C metabolic flux analysis was investigated by metabolic simulations. For this purpose a model was created, considering all CO_2 forming and consuming reactions in the central catabolic and anabolic pathways. To facilitate the interpretation of the simulation results, the underlying metabolic network was parameterized by physiologically meaningful flux parameters such as flux partitioning ratios at metabolic branch points and reaction reversibilities. For real case flux scenarios of the industrial amino acid producer *Corynebacterium glutamicum* and different commercially available ^{13}C-labeled tracer substrates, observability and output sensitivity towards key flux parameters was investigated. Metabolic net fluxes in the central metabolism, involving, e.g. glycolysis, pentose phosphate pathway, tricarboxylic acid cycle, anaplerotic carboxylation, and glyoxylate pathway were found to be determinable by the respirometric approach using a combination of [1-^{13}C] and [6-^{13}C] glucose in two parallel studies. The reversibilities of bidirectional reactions influence the isotopic labeling of CO_2 only to a negligible degree. On one hand, they therefore cannot be determined. On the other hand, their precise values are not required for the quantification of net fluxes. Computer-aided optimal experimental design was carried out to predict the quality of the information from the respirometric tracer experiments and identify suitable tracer substrates. A combination of [1-^{13}C] and [6-^{13}C] glucose in two parallel studies was found to yield a similar quality of information as compared to an approach with mass spectrometric labeling analysis of secreted products. The quality of information can be further increased by additional studies with [1,2-$^{13}C_2$] or [1,6-$^{13}C_2$] glucose. Respirometric tracer studies with sole labeling analysis of CO_2 are therefore promising for ^{13}C metabolic flux analysis.

Keywords: Sensitivity; Optimal experimental design; Mass spectrometry; Respirometric tracer; [^{13}C] CO_2

1. Introduction

In recent years, the use of isotopic labeling techniques for understanding of metabolism expands continuously along with increasing interest in various fields of bioscience (Fell, 1997; Nielsen, 2001; Stephanopoulos, 1999). The approaches used are mainly focused to certain parts of metabolic network and often linked to substantial approximations. To gain more detailed insights into the studied biological system, extended mathematical and computer models can be implemented (Baranyai and Blum, 1989; Mulquiney et al., 1999; Mulquiney and Kuchel, 2003; Schmidt et al., 1997; Wiechert et al., 1997; Wittmann and Heinzle, 1999; Wright and Reimers, 1988; Yang et al., 2004a; Zupke and Stephanopoulos, 1994). Metabolic flux analysis, i.e. the quantification of in vivo activities of biochemical reactions and pathways, is one of the major applications of isotopic labeling techniques. It has proven very useful for the understanding of functional and regulatory activities of cells in its entirety (Stephanopoulos et al., 1998). The developed approaches typically utilize labeling analysis of cellular constituents formed during growth of the examined cells. These are amino acids from the cell protein (Dauner and Sauer, 2000; Christensen and Nielsen, 1999), nucleotides from DNA (Macallan et al., 1998), or monomers from glycogen (Shulman and Rothman,

Abbreviations: AMM, atom mapping matrix; IDV, isotopomer distribution vector; IMMs, isotopomer mapping matrices; MDV, mass isotopomer distribution vector; MS, mass spectrometry; PPP, pentose phosphate pathway; TCA, tricarboxylic acid

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2001). However, there are different situations where these approaches cannot be applied. This involves non-growing cells, often observed, e.g. in production processes of primary or secondary metabolites (Okamoto et al., 1997; Hashimoto and Katsumata, 1999; Tamehiro et al., 2003; Bizani and Brandelli, 2004). These processes do not provide ^{13}C-labeled biomass that could be used for labeling analysis. Further cases are given, when the separation of the ^{13}C-labeled cellular material, required prior to labeling analysis, is complicated or even impossible, e.g. when the cells are immobilized (Bayraktar and Mehmetoglu, 2000) or when complex insoluble nutrients are present. These limitations can be partly overcome by metabolic flux methods, which are based on labeling analysis of secreted products (Wittmann and Heinzle, 2002; Kiefer et al., 2004). However, this requires sufficient amounts of the products to be analyzed, no interference with medium components and case-specific development of analytical protocols for the analytes of interest. An attractive candidate to be used for labeling measurement in flux analysis under these conditions is CO_2. CO_2 is released by cells under aerobic as well as anaerobic conditions, both during growth and non-growth phases. Labeling analysis of CO_2 can be easily performed by measurement of dissolved CO_2 in the culture broth using membrane inlet mass spectrometry or by measurement of CO_2 in the exhaust gas using capillary inlet mass spectrometry (Heinzle, 1987). A thorough theoretical revision on the use of CO_2 in labeling studies, i.e. on the observability of fluxes, optimal experimental approaches, expected precision of estimated fluxes has, however, not been carried out. Selected studies have been carried out for quantification of pentose phosphate pathway activity by measuring [^{14}C] CO_2 (Katz and Wood, 1963; Larrabee, 1989), [^{13}C] CO_2 (Bonarius et al., 2001) or [^{18}O] CO_2 (Model and Rittenberg, 1967; Yang et al., 2004b). Though these studies are restricted to rather simple biochemical networks, they give an illustration that CO_2 might be a promising candidate for labeling analysis in metabolic flux analysis. On the other hand, it has been presumed that CO_2 may provide little or no information for the quantification of metabolic fluxes by use of its isotopic labeling data (de Graaf, 2000).

The present work is a detailed theoretical investigation on the potential of using sole CO_2 labeling measurement for metabolic flux analysis. Based on a metabolic model that takes all CO_2 forming and consuming reactions in central catabolic and anabolic pathways into account, questions on the observability and output sensitivities towards key flux parameters are addressed. Simulations are further applied to identify optimal experimental strategies for respirometric flux analysis. In this way, it is elaborated how far the required information, usually collected in single tracer studies with labeling analysis of different metabolites can be replaced by a combination of tracer experiments with differently labeled substrates and labeling analysis of only CO_2. In the simulations, the metabolic network of the industrial amino acid producer *Corynebacterium glutamicum* is considered as a real case scenario (Wittmann and Heinzle, 2002).

2. Modeling of respirometric ^{13}C metabolic flux analysis

Intracellular metabolic fluxes are not directly measurable. Therefore, mathematical models are required for metabolic flux analysis (Stephanopoulos, 1999). In the case of ^{13}C tracer studies, the models usually comprise a combination of stoichiometric and isotopomer balancing. For the respirometric ^{13}C tracer experiments, which are based on labeling analysis of CO_2, all metabolic reactions involving CO_2 as a substrate or product have to be considered in the model.

2.1. Cellular reactions related to CO_2

CO_2 is involved in different carboxylation and decarboxylation reactions in catabolism and anabolism as given in Table 1. It is released in different catabolic reactions of dehydrogenases and enzymes converting C_4 metabolites of the TCA cycle into C_3 metabolites of the glycolysis. The latter enzymes, comprising malic enzyme, phosphoenolpyruvate carboxykinase, and oxaloacetate decarboxylase are involved in gluconeogenesis, e.g. during growth on acetate (Wendisch et al., 2000), or in regulation of the energy level of the cell during growth on carbohydrates such as glucose or fructose (Kiefer et al., 2004; Petersen et al., 2000; Wittmann and Heinzle, 2002). In addition to catabolism, CO_2 is involved in anabolic pathways towards different amino acids (Table 1). CO_2 also plays a role for the synthesis of pyrimidine nucleotides involving a carboxylation by carbamoyl-P-synthase and a decarboxylation catalyzed by orotidine monophosphate decarboxylase (Michal, 1999). Due to the fact that the consumed and the released CO_2 are not identical with respect to the origin of the carbon atom, both reactions have to be included. For *C. glutamicum*, it has been previously stated, that the biosynthesis of lipids, lipopolysaccharides and peptidoglycan is linked to net formation of CO_2 (Marx et al., 1996; de Graaf, 2000). A close inspection of the underlying biochemical reactions, however, shows that this is not the case. The peptidoglycan synthesis is not resulting in the formation of CO_2 (Michal, 1999). During fatty acid biosynthesis, CO_2 is incorporated by acetyl-CoA carboxylase (HCO_3^- + acetyl-CoA → malonyl-CoA) (Michal, 1999). This incorporation, however, is only transient, because the same carbon atom is again released as CO_2 in the subsequent metabolic reaction by acyl-malonyl acyl-carrier protein condensing enzyme. Hence, there is no net production of CO_2 during the biosynthesis of either lipids or lipopolysaccharides.

2.2. Stoichiometric network

A mathematical model combining stoichiometric balancing with isotopomer balancing was applied in the present work. Hereby, a few characteristics were included in the set-up of the model in order to achieve its straightforward