Determination of non-steady-state fluxes and kinetic information using stable isotope labelling and metabolite pool size data: theory and application

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Introduction

Incubation of plant material in labelled isotopes often reaches neither isotopic nor metabolic steady state within the constrained time span of experimental measurements. This can be problematic since at a non-steady state, flux balance cannot be established. For this reason here we develop a mathematical approach to calculate fluxes within systems that do not approximate steady state. The approach is based on mass balance of all forms of molecules and employs trapezoid Euler's numerical method to deal with differential equations. The general method for deriving non-steady-state fluxes is established, and construction of overdermined systems for non-steady states is proposed. Determination of fluxes based on GC-MS analysis is analysed in detail. We show that non-steady-state fluxes can be derived based on time-dependent metabolite pool size and specific labelling data. Moreover, when a number of data points are available, the approach is able to predict the dependence of flux on pool size of substrate and as such to reveal which enzymes do not follow conventional Michaelis-Menten type kinetics. Therefore, regulatory enzymes can be identified for further study. In addition, kinetic parameters can be estimated on the basis of this approach for Michaelis-Menten type kinetics. As a first example of the application of this approach we apply it to the analysis of primary metabolism in tuber discs isolated from wild type potato plants. We demonstrate that this approach is able to identify irrational flux combinations that violate mass balance, and as such allows a structural analysis of the metabolic network under evaluation. Despite the challenges presented by these findings certain fluxes including the interchange between glucose-6-P and fructose-6-P could be readily calculated. We discuss the potential of the approach both in analysing fluxes of metabolic networks and more generally within kinetic modeling strategies.

Theory for determination of non-steady-state fluxes

Although fluxes of an intracellular pool do not balance in situations under which steady state is not approximated, mass balance is universally held for all forms of molecules and is defined as follows. Change in the concentration of a specific form of molecules in a metabolite pool= total influx of the molecules in the specific form into the pool – total efflux of the molecules in the form out of the pool. Here a specific form of molecules is referred to as any identifiable form that is either unlabelled or specifically positional labelled form of the molecules. For example, when using GC-MS analysis it is very difficult to get positional information. However, it is able to quantify two forms of molecules: unlabelled and unilabelled molecules. For any metabolite pool, the number of mass balance equations depends on how many forms of a molecule are experimentally identifiable within the pool. For example, for a 2-carbon molecule, if all positions are different and identifiable, four mass-balance equations can be established. For a 3-carbon molecule there can be up to 8 mass-balance equations.

Taking the assumption that labelled and unlabelled molecules with the same concentration lead to the same flux, irrespective of presence or absence of label and of position of labelling within the molecule, mass balance equations can be described as follows.

$$\frac{dX}{dt} = EF^* \quad (1)$$

Here X is an m-dimensional vector of concentration for the specific form (e.g. unilabelled glucose), and F^* is an n-dimensional vector which contains influx and efflux for the specific form of molecules. E is the m x n stoichiometric matrix.

Alternatively, equation (1) can be written in the form of equation (2).

$$\frac{d(SP)}{dt} = ES^*F \quad (2)$$

Here P is an m-dimensional vector that contains pool sizes (all forms of molecules), S is an m-dimensional vector that contains the percentages of the specific form of molecules of given metabolite pools. SP is the m-dimensional concentration vector of the specific form of molecules, X. F is an n-dimensional vector that contains the total flux of all forms of molecules, and S^* is an n-dimensional vector that contains the percentage of the specific form of molecules corresponding to all influx and efflux in metabolite pools. The superscript * is to distinguish S^* from S. S^*F is F^* in equation (1).

Following application of trapezoid Euler's method to equation (2), it becomes

$$\frac{(SP)^{k+1} - (SP)^k}{\Delta t} = \frac{(ES^*F)^{k+1} + (ES^*F)^k}{2} = E\frac{(S^*F)^{k+1} + (S^*F)^k}{2} \quad (3)$$

where the time interval between k and k+1 is Δt, and the stoichiometric matrix, E, is time-independent.

When the time interval between k and k+1 is such that the vector F does not change significantly, we may assume that F is independent of time during this period, i.e. $(F)^{k+1} = (F)^k$ For this case, equation (3) becomes

$$\frac{(SP)^{k+1} - (SP)^k}{\Delta t} = E\frac{(S^*)^{k+1} + (S^*)^k}{2}(F)^k = E\overline{S^*}(F)^k \quad (4)$$

Here the number of columns in $\overline{S^*}$ is the same as that in S^*. Each element in vector $\overline{S^*}$ is the average of the two elements of at k and k+1 time. The mass-balance equation (4) directly links the measurable quantities (pool size and percentage of molecules in a specific form) with time-dependent fluxes, F. Therefore, it is the basis for calculating fluxes based on experimental data.

Determination of fluxes and kinetic information based purely on GC-MS analysis

Flux determination of example networks

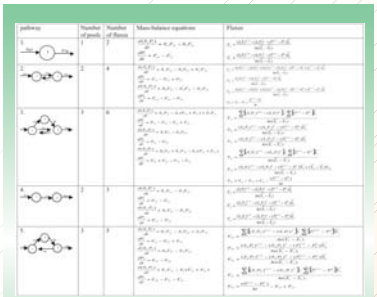

Table 1. Calculation of fluxes based on pool size and specific labelling data. Fluxes can be determined based on mass balance equations for unbranched (pathway 1), reversible (pathways 2 and 4), cyclic (pathway 5) and reversible and cyclic (pathway 3) pathway

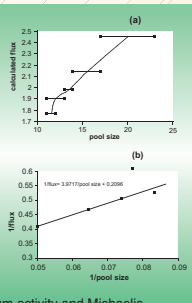

Figure 1. Determination of enzymatic kinetics and parameters based on pool size and specific labelling data. For pathway 1 in Table 1 (a): dependence of calculated flux on measured pool size shows that the enzyme follows conventional Michaelis-Meneten kinetics; (b): a Lineweaver-Burk plot is able to determine the maximum activity and Michaelis constant of the enzyme

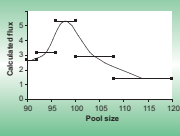

Figure 2. Enzymatic kinetics based on the specific labelling and pool size data reveals that the enzyme is inhibited by its substrate.

Case study: estimation of some non-steady state fluxes in pathways of primary metabolism of potato tubers

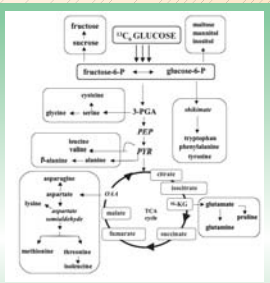

Figure 3. Schematic diagram constructed based on biochemical knowledge, for the purpose of flux analysis following incubation in ^{13}C labelled isotopes

(Roessner-Tunali, U., Liu, J., Leisse, A., Balbo, I., Perez-Melis A., Willmitzer, L., Fernie, A. R., 2004. Kinetics of labelling of organic and amino acids in potato tubers by gas chromatography-mass spectrometry following incubation in ^{13}C labelled isotopes. Plant Journal, 39, 668-679.)

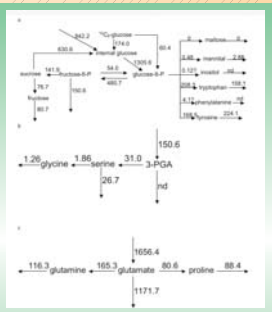

Figure 4. Refined pathway structure and calculated fluxes for primary metabolism of discs isolated from wild-type potato tubers, based on Figure 3

Discussion

This work develops a mathematical approach to calculate fluxes within systems that do not approximate steady state. The approach is based on mass balance of all forms of molecules and employs trapezoid Euler's numerical method to deal with differential equations. A general method for deriving non-steady-state fluxes is established, and construction of overdetermined systems for non-steady states is proposed. This method is particularly important for plant heterotrophic systems, as incubation of plant material in labelled isotopes often does not reach both isotopic and metabolic steady state within the constrained time span of experimental measurements. As an example, determination of fluxes based on GC-MS analysis is analysed in detail. We show that non-steady-state fluxes can be derived based on time-dependent metabolite pool size and specific labelling data. Moreover, when a number of data points are available, the approach is able to predict the dependence of flux on pool size of substrate and as such to reveal which enzymes within a system do not follow conventional Michaelis-Menten type kinetics suggesting a novel mechanism for the identification of regulatory enzymes, although it should be noted that these clearly need to be confirmed through experimentation. Finally, as a first example, some non-steady-state fluxes in metabolic pathways of primary metabolism of discs isolated from wild-type potato tubers are estimated based on experimental measured data following GC-MS analysis.

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