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Original Research Article

Estimation of flux ratios without uptake or release data: Application to serine and methionine metabolism

Roland Nilsson[a,b,*], Irena Roci[a,b], Jeramie Watrous[c], Mohit Jain[c]

[a] Cardiovascular Medicine Unit, Department of Medicine, Karolinska Institutet, Karolinska University Hospital, SE-17176 Stockholm, Sweden
[b] Center for Molecular Medicine, Karolinska Institutet, Karolinska University Hospital, SE-17176 Stockholm, Sweden
[c] Departments of Medicine & Pharmacology, University of California, San Diego, USA

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ABSTRACT

Model-based metabolic flux analysis (MFA) using isotope-labeled substrates has provided great insight into intracellular metabolic activities across a host of organisms. One challenge with applying MFA in mammalian systems, however, is the need for absolute quantification of nutrient uptake, biomass composition, and byproduct release fluxes. Such measurements are often not feasible in complex culture systems or *in vivo*. One way to address this issue is to estimate flux ratios, the fractional contribution of a flux to a metabolite pool, which are independent of absolute measurements and yet informative for cellular metabolism. Prior work has focused on "local" estimation of a handful of flux ratios for specific metabolites and reactions. Here, we perform systematic, model-based estimation of all flux ratios in a metabolic network using isotope labeling data, in the absence of uptake/release data. In a series of examples, we investigate what flux ratios can be well estimated with reasonably tight confidence intervals, and contrast this with confidence intervals on normalized fluxes. We find that flux ratios can provide useful information on the metabolic state, and is complementary to normalized fluxes: for certain metabolic reactions, only flux ratios can be well estimated, while for others normalized fluxes can be obtained. Simulation studies of a large human metabolic network model suggest that estimation of flux ratios is technically feasible for complex networks, but additional studies on data from actual isotopomer labeling experiments are needed to validate these results. Finally, we experimentally study serine and methionine metabolism in cancer cells using flux ratios. We find that, in these cells, the methionine cycle is truncated with little remethylation from homocysteine, and polyamine synthesis in the absence of methionine salvage leads to loss of 5-methylthioadenosine, suggesting a new mode of overflow metabolism in cancer cells. This work highlights the potential for flux ratio analysis in the absence of absolute quantification, which we anticipate will be important for both *in vitro* and *in vivo* studies of cancer metabolism.

1. Introduction

Assessment of metabolic fluxes in living cells has proven critical for understanding cellular metabolism, and may potentially identify state-specific metabolic vulnerabilities in disease, including cancer (Duckwall et al., 2013; Gunther et al., 2015). Intracellular metabolic activities in living cells are often analyzed using isotope labeling experiments, in which cells are exposed to stable isotope-labeled nutrients and the resulting intracellular isotope patterns within metabolic products are measured. In model-based metabolic flux analysis (MFA), a stoichiometric, atom-level model of a metabolic network is constructed, and a number of metabolic fluxes are simultaneously estimated by fitting the model to isotope labeling data. To obtain absolute fluxes (in moles per unit time), however, requires absolute quantification of nutrient uptake rates, biomass growth, and release of metabolic products, in order to constrain the model. In microorganisms such measures are often feasible, as cells can be grown on a single carbon source whose uptake can be readily quantified, and biomass rate and composition can be measured as well (Sauer et al., 1999; Lange and Heijnen, 2001). Consequently, MFA has provided a wealth of information regarding intracellular metabolic activities in microorganisms (Tang et al., 2009). In contrast, for mammalian cells, measuring uptake and release rates has proven much more challenging (Niklas et al., 2010). While uptake rates for major nutrients (glucose, amino acids) can be quantified in cell cultures (Kell et al., 2005; Jain et al., 2012), mammalian cells require complex growth medium that also contains significant amounts of serum-derived protein and fat-containing lipoproteins which are consumed by cells (Commisso et al., 2013; Beloribi-djefa et al., 2016), and are more difficult to quantify. Moreover, the biomass generated by proliferating human cells varies by cell type, and consists not only of

* Corresponding author.

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new cellular material, but also extracellular matrix proteins deposited on culture dishes and various biosynthesized products released into the medium (Xie and Wang, 1994). Finally, for MFA of tissues or tumors *in vivo*, for example in tumor-bearing animals (Marin-Valencia et al., 2012), accurate quantification of comprehensive uptake/release measures can prove challenging. In such situations, artificial constraints must often be imposed on uptake/release fluxes to perform MFA, and may substantially influence the final results. This problem also arises when modeling a subset of the metabolic network, since estimates of fluxes crossing the "boundary" of the model are then needed for MFA, yet are rarely available.

One potential approach to overcome this limitation is to instead estimate metabolic flux ratios, defined as the relative contributions of each reaction feeding into a specific metabolite pool, since these ratios do not depend on absolute uptake/release fluxes. If data is available for isotope labeling of the metabolite of interest and for all substrates of all reactions that feed into the specific pool, then flux ratios can be estimated at this metabolite, either from steady-state (Sauer et al., 1999; Zamboni et al., 2009) or time-course data (Hörl et al., 2013). Since isotope distributions are directly related to flux ratios, this "local" approach is relatively straightforward and robust. However, usually not all required isotopomers are measurable, and although missing data can sometimes be substituted from "proxy" metabolites (Rantanen et al., 2008), still only a limited number of flux ratios have been accessible by the local method. For example, in the study of Hörl et al. (2013), flux ratios at three TCA cycle metabolites could be obtained, while a more extensive recent study using machine learning approaches estimated six flux ratios, including one novel ratio in the glyoxylate shunt (Kogadeeva and Zamboni, 2016). In human cells, and in particular in the study of cancer metabolism, flux ratios have been used to estimate the relative activity of alternative synthesis pathways. This has provided important insights into cellular metabolism: for example, the relative contributions of glucose and glutamine to lactate (DeBerardinis et al., 2007) or lipogenic acetyl-CoA (Metallo et al., 2012) have been proven to be important factors in the metabolic reprogramming of cancer cells. Yet, to our knowledge systematic estimation of flux ratios in arbitrary metabolic networks has not been performed. It is not even clear whether flux ratios are indeed a valid parametrization, that is, if flux ratios contain all information about the flux state. Also, we do not know how many flux ratios can be well-estimated in large metabolic networks, particularly in central human metabolism, or how measurement error propagates to uncertainty about flux ratios in large models.

In this paper, we propose and evaluate a systematic, model-based method for estimating flux ratios, as a means of analyzing isotope labeling experiments in the absence of uptake/release data. This approach makes use of all available isotopomer information and yields confidence intervals for all flux ratios in a metabolic network, also for metabolites that were not measured or do not carry labeled atoms, such as metabolic cofactors. We also prove that flux ratios are valid parameters of the flux state, in that they are in one-to-one correspondence with fluxes normalized to the total release flux. In simulation studies with a large human metabolic network, we find that many flux ratios are well-estimated, indicating that studies of human cellular metabolism using our approach are at least theoretically feasible. Finally, we apply this method to study serine and methionine metabolism in human cancer cells.

2. Methods

2.1. Cell culture

HeLa cells were cultured in six-well plates in RPMI-1640 medium containing either unlabeled nutrients (control), 50% U-^{13}C-methionine (Cambridge Isotopes, no. CLM- 893-H), or 50% 1-^{13}C-serine (Cambridge Isotopes no. CLM-1574-H), and supplemented with 5% fetal bovine serum (Life Technologies) dialyzed using Snakeskin 10 K MWCO dialysis tubing (Nordic Biolabs, Taby, Sweden, no. 88245-P) to remove additional metabolites. An intermediate (50%) amount of tracer was used since this tends to generate more informative MIDs for MFA (Möllney et al., 1999). After 48 h of culture in this medium (at a confluence of 85%), wells were rapidly rinsed twice with 500 μL of Hanks balanced salt solution (Sigma-Aldrich, St. Louis, no. H6648) and metabolites extracted through the addition of 540 μL of precooled 100% methanol. Cells were scraped and transferred to a 1.5 mL tube. Cell material was subjected to three freeze-thaw cycles whereby the sample tubes were partially submerged in an acetone dry ice bath for 45 s followed by partial submersion in a room temperature water bath. After complete lysis, 200 μL of ice cold methanol was added to each sample. Samples were then vortexed for 30 s, sonicated for 2 min and allowed to sit at −20 °C for 30 min to facilitate protein precipitation. Samples were then centrifuged at 14,000 rpm for 10 min at 4 °C, after which 200 μL of supernatant was transferred to new microfuge tubes, dried in vacuo using a vacuum concentrator, and the resulting pellets resuspended in 40 μL of 80:20 methanol: water for analysis.

2.2. Mass spectrometry

For liquid chromatography-mass spectrometry (LC-MS), sample volumes were transferred to glass 100 μL volume LCMS vials and kept at 4 °C in the autosampler compartment until 2 μL of sample was injected for analysis. Compounds were separated using a Thermo Vanquish UPLC coupled to a Thermo QExactive Orbitrap mass spectrometer. Separation was performed using a Millipore (Sequant) Zic-pHILIC 2.1×150 mm 5 μm column maintained at 25 °C using a flow rate of 0.3 mL/min and a 15 min linear gradient starting from 90:10 acetonitrile: 20 mM ammonium bicarbonate pH 9.6–45:55 acetonitrile: 20 mM ammonium bicarbonate pH 9.6. Detection was performed in positive and negative ion modes through sequential sample injections using a heated electrospray ionization (HESI) source operated at 2.5 kV (negative mode) and 3.5 kV (positive mode), sheath gas flow of 40, auxiliary gas flow of 20, sweep gas flow of 2, capillary temperature of 275 °C and auxiliary gas heater temperature of 350 °C. Data was collected using data-dependent tandem MS collection with MS1 parameters of 70,000 mass resolution, 100 ms maximum IT time, 3×106 AGC volume and a mass range of 67–1000 *m/z*, and MS2 parameters of 17,500 mass resolution, 50 ms maximum IT time, 1×105 AGC volume, loop count of 5, isolation window of 0.5 *m/z*, NCE of 35 and 10 s dynamic exclusion. LC-MS peaks were assigned metabolite identity by matching accurate mass and retention time against pure standards, and further confirmed by observing plausible mass isotopomer patterns in tracing experiments. Peak areas were obtained from chromatogram data by integrating total intensity within the retention time range. Mass isotopomer peaks were manually inspected in labeled samples and unlabeled controls. A small false +2 mass isotopomer of serine (ser-L) deriving from an unrelated compound was excluded by setting this MI fraction to zero. All experiments were performed in triplicate cultures with independent LC-MS analysis.

2.3. Metabolic network models

The metabolic network models used herein were assembled based on a previously described reconstruction of the human metabolite network (Duarte et al., 2007), with some modifications and simplifications. Complete model descriptions are provided as supplementary data. All reactions in each the model were able to carry flux in the forward (reference) direction. For the large network model, we also verified that the model is capable of biomass synthesis as well as terminal oxidation of all the major nutrients (glucose and amino acids) to CO_2. The large model comprises 554 reactions, of which 236 were reversible, including all transporters; 140 of these cannot carry net flux in the reverse direction, but were kept reversible since nonzero