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# Heading in the right direction: thermodynamics-based network analysis and pathway engineering

Meric Ataman[1,2] and Vassily Hatzimanikatis[1,2]

Thermodynamics-based network analysis through the introduction of thermodynamic constraints in metabolic models allows a deeper analysis of metabolism and guides pathway engineering. The number and the areas of applications of thermodynamics-based network analysis methods have been increasing in the last ten years. We review recent applications of these methods and we identify the areas that such analysis can contribute significantly, and the needs for future developments. We find that organisms with multiple compartments and extremophiles present challenges for modeling and thermodynamics-based flux analysis. The evolution of current and new methods must also address the issues of the multiple alternatives in flux directionalities and the uncertainties and partial information from analytical methods.

**Addresses**
[1] Laboratory of Computational Systems Biotechnology (LCSB), Swiss Federal Institute of Technology (EPFL), CH-1015 Lausanne, Switzerland
[2] Swiss Institute of Bioinformatics (SIB), CH-1015 Lausanne, Switzerland

Corresponding author: Hatzimanikatis, Vassily (vassily.hatzimanikatis@epfl.ch)

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## Introduction

In the last 15 years, the number of annotated genome sequences has grown tremendously, and this has led to reconstruction of genome scale metabolic models (GEM) for many organisms, from unicellular prokaryotes to higher organisms such as mouse and human [1]. These metabolic models are *in silico* representations of all biochemical reactions that take place in the cell. Through various methods, such as Flux Balance Analysis (FBA), different phenotypes of organisms can be simulated and analyzed [2]. Directionalities and allowable flux ranges for metabolic reactions are the main constraints that delineate the boundaries for GEMs. The two most important uses of thermodynamics-based analysis of metabolic networks are the determination of reaction directionality and the estimation of how far from, or close to, equilibrium the reactions in the network operate.

In most of the metabolic flux balance studies that discuss and analyze reaction thermodynamics and energetics, the authors consider the reactions as irreversible (unidirectional) based on the standard Gibbs free energy of reaction. Soh and Hatzimanikatis [3,4] suggested differentiating between 'reaction directionality' and the commonly used term 'reaction reversibility'. Reaction reversibility is a *kinetic* property of the enzyme and it is used to denote that the enzymes are able to catalyze the reactions in both directions, that is, the *forward* and *backward* reactions. If an enzyme is catalytically reversible, then the directionality of the reaction depends on the displacement of the reaction from thermodynamic equilibrium. In the context of a metabolic network, with or without thermodynamic constraints, a reversible reaction can be either *bidirectional*, that is, it is able to operate in both the forward and reverse directions, or *unidirectional*, that is, it can operate only in one of the directions. The catalytic reversibility is an enzyme property that depends on the enzyme amino acid sequence, and therefore it can be different between organisms. The information about catalytic reversibility is available for a relatively small number of the enzymes in the biological databases and for a very small number of organisms. Therefore, by determining the reaction directionality, thermodynamic constraints provide important information that substitute for the lack of information about reaction reversibility.

Three main approaches have been used for the introduction of thermodynamic constraints (network thermodynamics): (i) the energy balance analysis (EBA) [5], (ii) the network-embedded thermodynamic analysis (NET analysis) [6,7], and (iii) the thermodynamics-based flux analysis (TFA), which has been also called thermodynamics-based metabolic flux analysis (TMFA) [8] or thermodynamics-based flux balance analysis (TFBA) [4]. All three methods introduce a new set of constraints that enforce the reactions fluxes to operate within the feasible bounds of energy constraints. The general EBA problems constrain the directionality and bounds of the fluxes using the value of the Gibbs free energy, either as a constant or as continuous variable within defined ranges. NET analysis and TFA constrain also the fluxes using the value of the Gibbs free energy but as a linear function of the logarithms of the metabolite concentrations (or activities). However, NET analysis requires a predetermination of the directionality of the fluxes, and the thermodynamic constraints determine if the flux is feasible in the defined direction and what are the

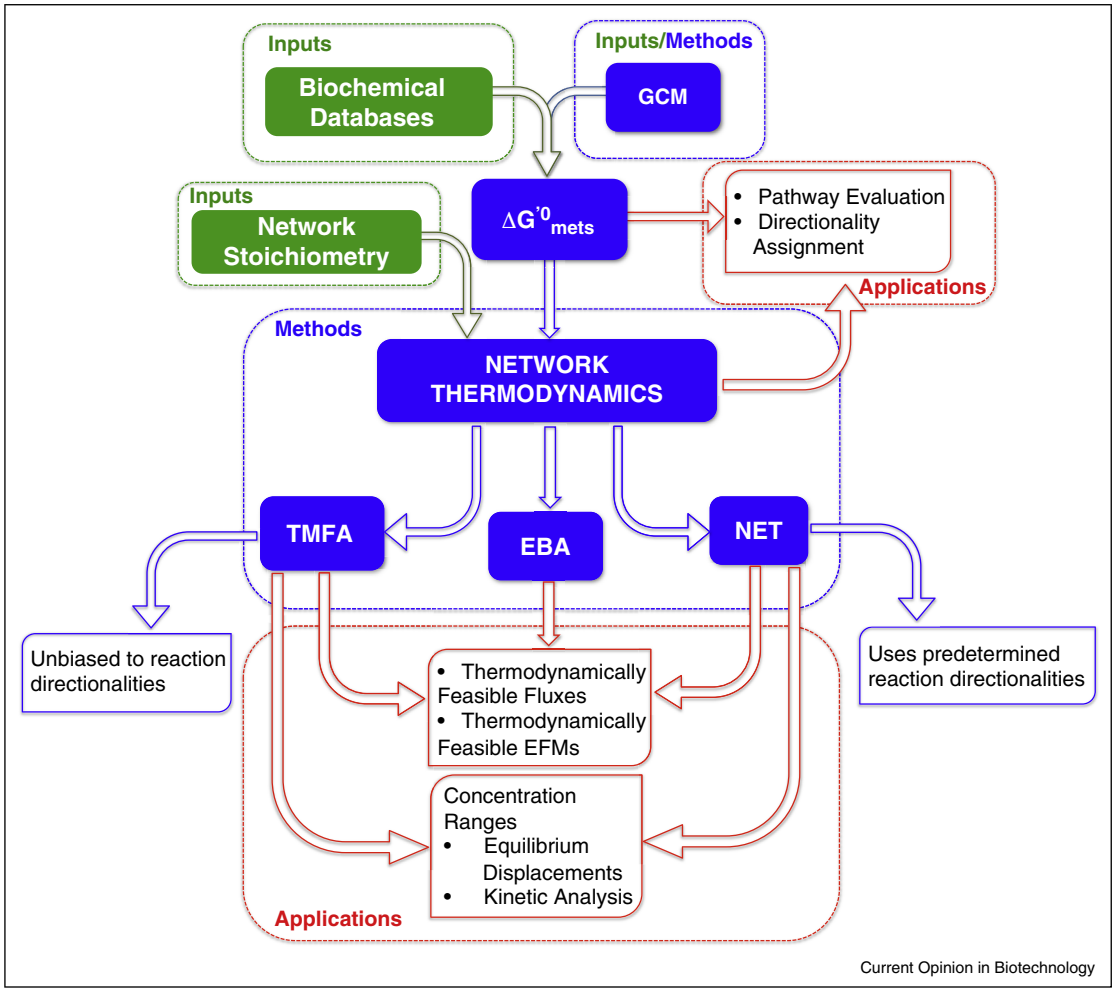
thermodynamically feasible concentration ranges. The TFA considers initially all catalytically reversible fluxes as thermodynamically bidirectional and employs a mixed-integer linear programming formulation that accounts for concentration ranges and it computes the flux directionality based on the thermodynamically feasible concentration profiles. Therefore TFA introduces the minimum bias about reaction directionality and it simultaneously computes thermodynamically feasible flux *and* concentration ranges. Moreover, the EBA and NET analysis formulations represent special cases of the TFA formulation. Hence, we believe that any analysis that uses thermodynamic constraints should apply TFA, or a similar formulation in order to avoid incomplete or false predictions about the properties of the network.

We review here the recent publications that have applied thermodynamic constraints in metabolic flux balance analysis. The major applications of thermodynamics have benefited the study of metabolism through three main uses: (i) the application of thermodynamic constraints to assign directionalities and thus constraint the allowable flux space and improve the predictions of metabolic modeling; (ii) the evaluation of the feasibility of synthetic and metabolically engineered pathways; and (iii) the integration of metabolomics data into metabolic models and their analysis and interpretation in the context of metabolic networks (Figure 1).

## Assigning directionality based on Gibbs free energy of reactions in GEMs

Directionalities of metabolic fluxes in GEMs have significant impact on network properties, such as yield on biomass, gene/reaction essentiality. Directionality specifications in GEMs are based on literature, databases, biochemical textbooks and information from similar

**Figure 1**




Applications of thermodynamics in metabolic networks. Standard Gibbs free energy values can be used to evaluate pathways, and to assign directionalities in GEMs. Integration of network-thermodynamics allows computation of thermodynamically feasible flux profiles and EFMs and feasible concentration ranges. These ranges can be further used for estimating the equilibrium displacements and building kinetic models of metabolic networks.