Stability as a Criterion in Metabolic Design

Metabolic engineering has advanced from minor alterations of existing pathways to significant re-routing of the metabolic path for better utilization of substrates or formation of non-native products. Most metabolic engineering efforts aim to achieve a steady state or quasi-steady state. Non-steady states in metabolic engineering typically result in accumulation or disappearance of intermediate metabolites. Non-steady states may occur as the expression levels of the pathway genes drift outside the working range due to physiological conditions or stochastic variation. This can result in gradual deterioration of performance, or system failure which would be catastrophic for the cell. Thus, pathway design should focus on avoiding system failure.

Through evolution, native metabolic pathways apparently have solved the robustness problem by selecting a robust network structure such that the feasible range of each parameter is sufficiently large. In addition, various regulatory mechanisms are in place to dynamically control the kinetic parameters under various physiological conditions. In contrast, non-native or metabolically engineered native pathways are potentially more prone to system failure when a kinetic parameter moves away from the initially designed level. This may cause accumulation or depletion of metabolites and the disappearance of a stable steady state. Since a stable steady state disappears after a bifurcation occurs, the bifurcational robustness should therefore be an important criterion for designing non-native pathways. Artificial dynamic controllers are potentially useful, but it is desirable to choose underlying network configurations or parameter ranges that are inherently robust to bifurcation if possible.

The robustness problem calls for a modeling approach that integrates kinetic parameters with systems performance. Kinetic parameters are perturbed in such models to examine the consequences of drifting. Unfortunately, key kinetic parameters (e.g., Vmax's) are system-dependent and usually unknown. Previous efforts have addressed the uncertainty of metabolic parameters through the random sampling of parameters to form an ensemble of models. Various approaches are then used to extract useful information from the ensemble upon large parameter changes, or infinitesimal perturbations that define control coefficients. Since non-native pathway design normally starts with little knowledge of kinetic parameters, it is sensible to investigate bifurcational robustness for an ensemble of models and to quantify it using the probability of system failure.

We defined as the bifurcational robustness as the distance away from an unstable region in parameter space when the system is constrained to a steady state. This bifurcational robustness is readily measured using parameter continuation integration until the Jacobian becomes singular. This measures the ability of a dynamical metabolic system to return to a fixed point upon perturbation. Building a theoretical foundation of robustness, measuring it, and in particular defining a simple way to quantify it, represent unique challenges in systems biology. For small perturbations, local stability criteria are well defined using linear stability analysis. For large perturbations, one must explore global properties of the system. It is important to make the distinction between bifurcational robustness, which quantifies the tendency to avoid sudden change in dynamic regime due to parameter changes, and local sensitivity, which quantifies the changes in performance (flux, period of oscillation) as a function of changes in parameters within the same dynamic regime. Although measures of performance may decrease, this does not necessarily indicate bifurcation.

Natural metabolic pathways may be presumed to be at least bifurcationally robust against stochastic changes in protein expression levels. Thus, the models of natural metabolic pathways should be similarly

robust. We have provided a quantitative way to characterize bifurcational robustness in the presence of random parameter changes. Without a quantitative index, optimization of models for bifurcational robustness becomes difficult, if not impossible. Therefore, our goal here is to develop a quantitative index for bifurcational robustness, and show that such an index enables the optimization of the bifurcational robustness of metabolic models. The developed index is easy to compute and applies to metabolic systems of various scale and complexity. Interestingly, the mathematical form of our robustness index resembles the definition of entropy in thermodynamics and information theory. We have previously shown that this entropy-like index, denoted as S, negatively correlates with empirically-measured bifurcational robustness. Metabolic systems with a small S are highly robust against bifurcation, and are more likely to retain a steady state under random perturbations affecting every enzyme than systems with a large S.

Using the presumption that natural pathways should be stable and thus relatively low entropy, we can identify kinetic models and even pathways within models which are likely to have. We have used this approach and shown that stability does indeed identify potential problems during model construction. We demonstrated these capabilities by using an ensemble model of *E. coli* consisting of 193 reactions which encompass glycolysis, the TCA cycle, the synthesis of all 20 canonical amino acids as well as nucleotides. Starting from a computer generated model using stoichiometric and regulatory data from the EcoCyc database, the model was analyzed using entropy as a measure of bifurcational robustness. Using this index and on the premise that adequate models of cellular metabolism should be sufficiently stable & robust, pathways with high numbers of high entropy enzymes were inspected for correct incorporation of regulatory information from the EcoCyc database. Three changes were made to the kinetic rate laws, while leaving the network stoichiometry unchanged. After these changes were made, entropy of the system significantly decreased. These changes were verified with reference to literature reports of individual enzyme characterization, which had apparently been wrongly incorporated in the EcoCyc database.

The usefulness of robustness as a criterion extends beyond simply building models of natural metabolism but also includes the design of non-native pathways for production in novel systems. We applied robustness analysis to several *in vitro* systems which have been characterized in the literature. In these literature reports, the performance of the system was found to be reduced by the increase of a certain enzyme or feed rate. We found that the method proved versatile enough to successfully predict these features in three different pathways investigated in different laboratories and powerful enough to do so without *a priori* knowledge of specific enzyme parameter values. While the characterized pathways were optimized based on intuition during their experimental characterization, it's possible that longer pathways with more enzymes would be much more difficult to optimize without rational balancing methods like those presented here. Importantly, although some of the phenomena were experimentally determined, it was not necessarily known that instability of the system—causing a step change in the nature of the steady state, rather than a smooth change predictable by sensitivity analysis—could be an underlying reason. Additionally, it was significant that *increase* of enzyme activity was found to cause instability, since many typical metabolic engineering strategies involve simply overexpressing all enzymes as much as possible. Overall these applications make robustness and stability critical considerations for model and pathway design.