Letter to the Editor

Extreme Pathways and Kirchhoff's Second Law

Constraints-based models

As an alternative to kinetic models for the analysis of genome-scale networks, data-driven constraints-based models have emerged. Constraints-based modeling is centered on the successive application of fundamental physicochemical constraints and experimental data on the function of reconstructed genome-scale networks to narrow the range of their possible functions (Palsson, 2000). This approach has proven successful for modeling cellular metabolism (Edwards et al., 2001). Analytical tools such as flux balance analysis (FBA), and extreme pathway analysis (ExPA) utilize mass, redox, and energy balance constraints to model steady-state behavior of reconstructed networks. FBA uses linear optimization to find a flux distribution that maximizes a stated objective function (Varma and Palsson 1994; Bonarius et al., 1997), and ExPA describes all the possible steady-state flux distributions of the network (Schilling et al., 2000). The application of mass balance constraints in FBA and ExPA is analogous to Kirchhoff's first law for electrical circuits: namely the production and utilization rate of a metabolite must balance in a steady state. Further utilized constraints are based on the thermodynamic irreversibility of reactions and the maximum flux through any reaction or transporter.

Systemic thermodynamic constraints

A recent paper (Beard et al., 2002) provides a framework for incorporating systemic thermodynamic constraints analogous to Kirchhoff's second law into constraints-based models. Just as the voltage drop around a closed loop in an electrical circuit must be zero, so too must the free energy change around a biochemical loop be equal to zero, because free energy is a state variable. When multispecies reactions are included in the network, closed reaction loops can be identified through an algebraic transformation of the stoichiometric matrix (Beard et al., 2002). Going around a loop returns the system to the same state from which it originated (see Fig. 1). For example, any series of reaction steps that together has an overall reaction of $A + 2B + C \rightarrow A + 2B + C$ must have a free energy change of zero. From the second law of thermodynamics, each reaction in the network must have a negative free energy change in the direction of the net flux. No net flux is possible through a balanced biochemical loop in the steady state. Thus the application of the loop constraint will not change the optimal value of an objective function, such as production of biomass, that is composed of throughput fluxes. However, it does restrict the allowable intracellular distribution of fluxes.

Extreme pathways and the loop law

The method of Beard et al. (2002) involves a bilinear constraint on fluxes and free energies, resulting from implementation of the second law of thermodynamics. However, if only fluxes are to be computed, the loop constraint can be applied using the extreme pathways without the need for a bilinear optimization. Extreme pathways are a set of convex basis vectors that describe all the steady-state functions of a biochemical network (Schilling et al., 2000). Extreme pathways are flux maps through a biochemical network that have the following properties: 1) they are a unique set of convex basis vectors that circumscribe all possible steady-state flux distributions through the network; 2) they characterize time invariant properties of biochemical networks; 3) they are contiguous sets of fluxes (a flux map) that each satisfy the mass balance of the system and reaction irreversibility constraints; and 4) they can have multiple inputs or outputs (Price et al., 2002). Extreme pathways can be divided into three basic categories based upon their exchange fluxes (Schilling et al., 2000). Each of these three categories can be understood relative to energy usage.

Type I extreme pathways are those that have exchange fluxes across the system boundaries that correspond to noncurrency metabolites (Fig. 2, *left*). These extreme pathways can be energetically interpreted analogously to charging a battery. These extreme pathways drive the cycling of

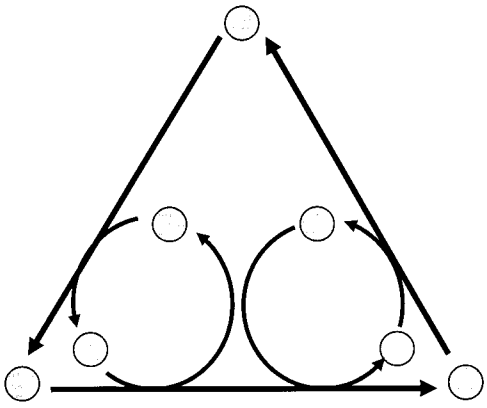

FIGURE 1 An example of a "complex" loop.

Submitted May 21, 2002 and accepted for publication June 28, 2002.

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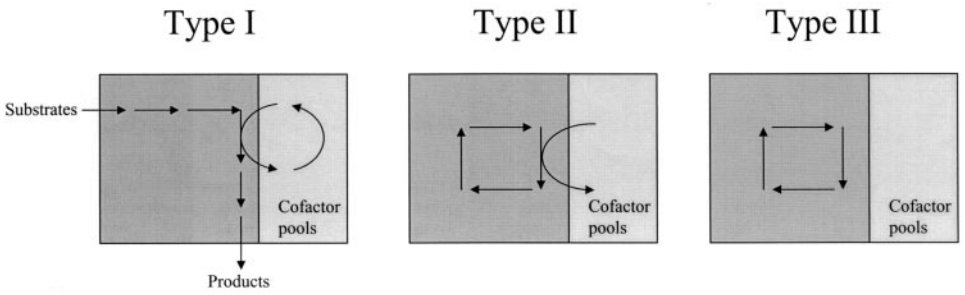

FIGURE 2 Types of Extreme Pathways. Type I extreme pathways are those that have exchange fluxes across the system boundaries that correspond to noncurrency metabolites. Type II extreme pathways have only currency metabolites that cross system boundaries. These currency metabolites can thus be thought of as being accumulated or depleted in the cell. Type III extreme pathways do not contain any exchange fluxes, and thus correspond to internal loops.

metabolic currencies, such as ATP, which then drive other cellular processes.

Type II extreme pathways are those that have "exchange fluxes" corresponding only to currency metabolites (e.g., ATP, NADH), with the rest of the pathway being an internal cycle (Fig. 2, *center*). These pathways represent futile cycles and are analogous to draining a battery. They are unidirectional in the absence of a driving force on the cofactor pool.

Type III extreme pathways are those that have no exchange fluxes (Fig. 2, *right*). Thus, these represent internal cycles. The fluxes through interior cycles must necessarily be zero to satisfy the loop constraints.

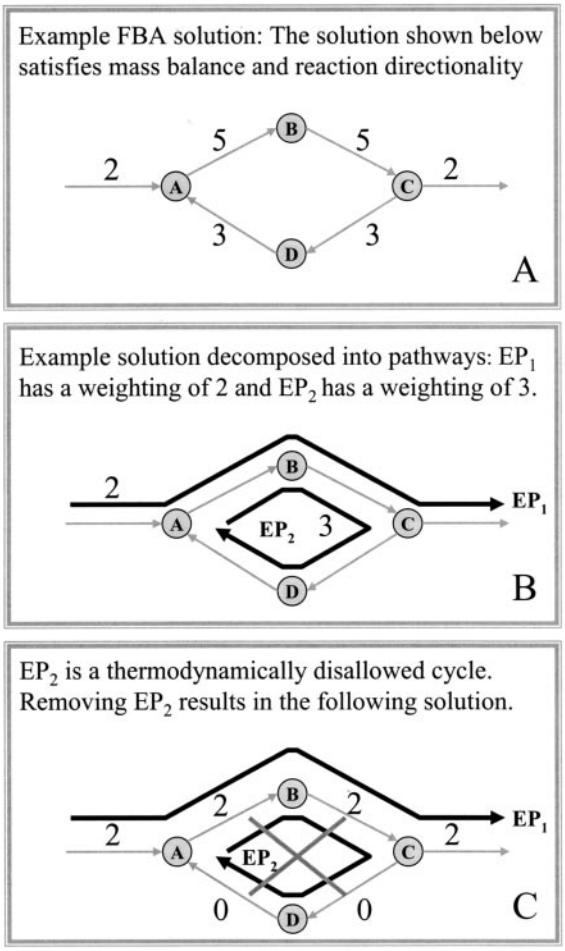

FIGURE 3 Simple illustration of loop constraint.

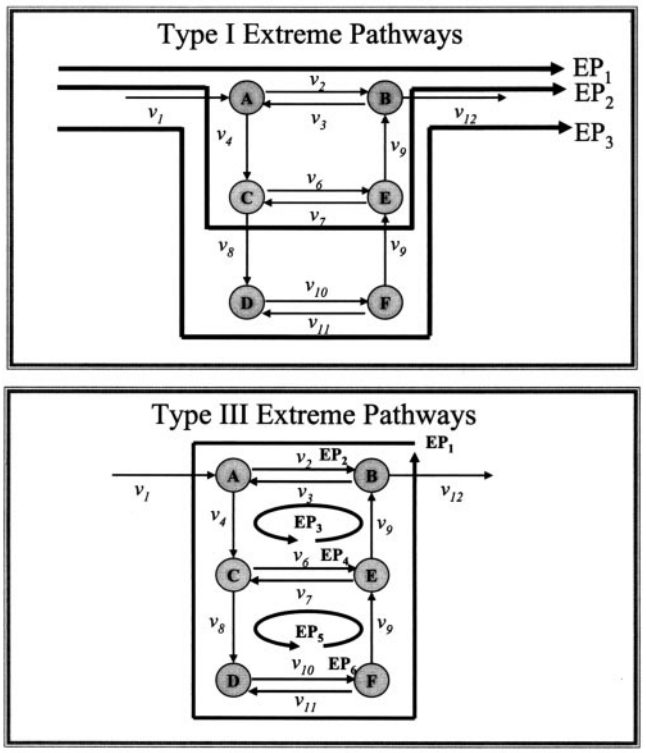

FIGURE 4 Extreme pathways of an example system. There are three type I extreme pathways, and six type III extreme pathways. Three of the type III extreme pathways correspond simply to the reversible reactions. Solutions that are non-negative linear combinations of only the type I extreme pathways account for all possible flux distributions in the network that do not violate the loop law.

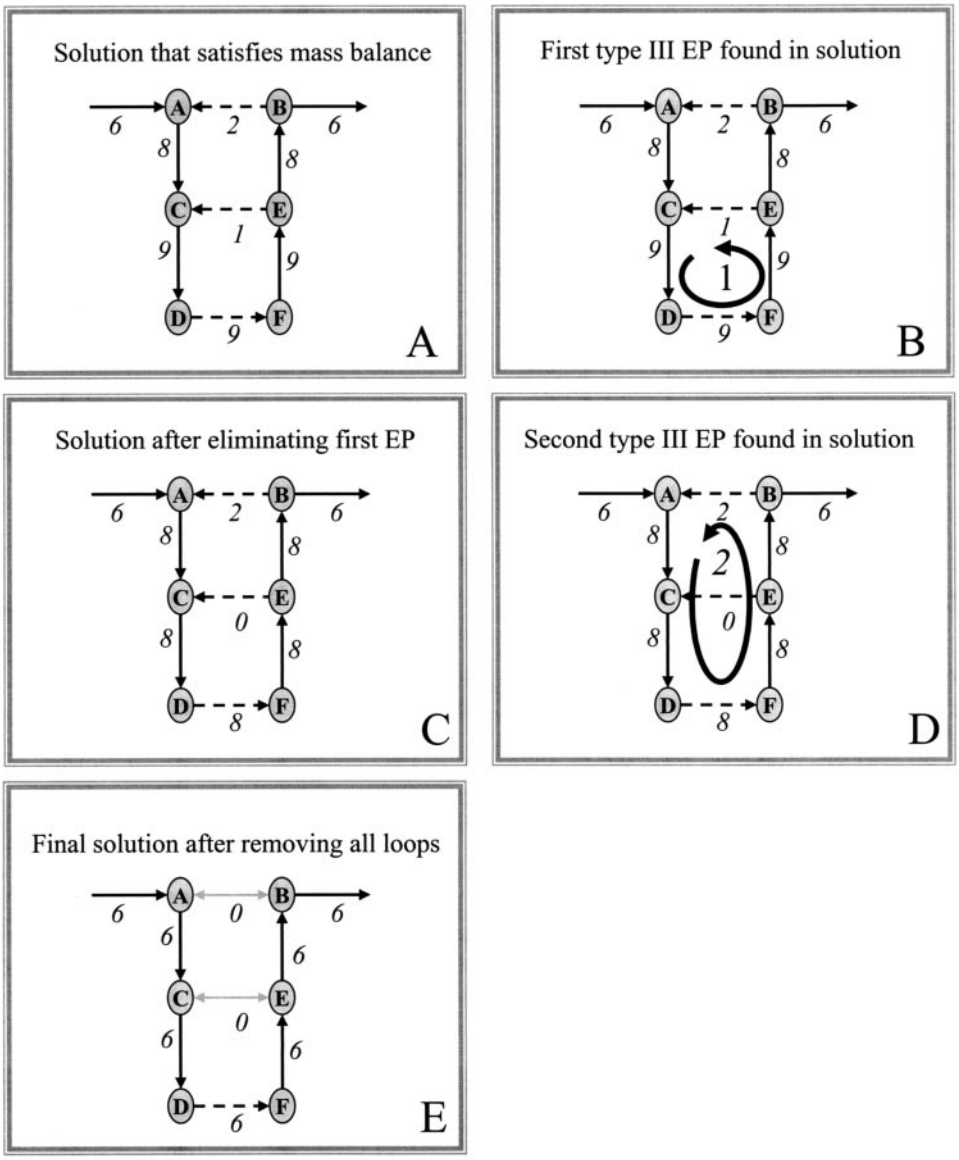

FIGURE 5 Application of the loop law to FBA solutions. (*A*) A flux distribution that satisfies mass balance. However, this solution violates the loop law. Type III extreme pathways can be used to eliminate infeasible loops. (*B*) The first type III extreme pathway has a value of one. (*C*) The resulting flux distribution with the loop shown in (*B*) eliminated. (*D*) A second type III extreme pathway is left in the solution. (*E*) The final solution with the extreme pathway shown in (*D*), with a weighting of two, eliminated from the solution. The dotted lines indicate that a reaction is reversible.

The imposition of the loop law on the reaction fluxes is equivalent to setting the flux through type III extreme pathways to zero. Type III extreme pathways can be calculated for any defined biochemical network by not allowing any fluxes to cross system boundaries. This generated set of type III extreme pathways then forms a convex basis for all the complex internal loops existing in the network. The use of a type III pathway can be readily eliminated in the FBA solution, thus removing thermodynamically disallowed cycles. This constraint is equivalent to that proposed in Beard et al. (2002), and can be applied without needing to do a bilinear optimization.

Simple examples

A simple example system can demonstrate how the loop constraints work (Fig. 3). This simple example is chosen for conceptual clarity and does not represent biochemically realistic systems. However, the principles outlined in the example are the same as for realistic biochemical loops. Figure 3 *A* shows an example FBA solution that balances the fluxes through the system and does not violate the directionality of any of the reactions. All FBA solutions can be decomposed into weightings on the extreme pathways. For the example system, two extreme pathways describe the system. The weightings of the two extreme pathways are shown in Fig. 3 *B*. However, the loop law disallows the internal cycle represented by extreme pathway *2* (EP_2). The disallowed cycle can be readily eliminated from the solution as a post-processing step, leaving a solution that does not violate the loop law (Fig. 3 *C*).

A slightly more complicated example system is shown in Fig. 4, with all of the reversible reactions decoupled into two separate reactions. This example system contains nine

extreme pathways: three type I extreme pathways and six type III extreme pathways. These extreme pathways form a set of biochemically realistic basis vectors for the flux solution space. All possible steady-state flux distributions must be non-negative linear combinations of the extreme pathways. Application of the loop constraint requires that the flux through all of the type III extreme pathways be set to zero. Thus, the type III extreme pathways are effectively eliminated from the convex basis, leaving only the three type I extreme pathways to span the flux solution space. Non-negative combinations of these three extreme pathways yield all of the steady-state flux distributions that do not violate the loop law.

An example FBA solution for the example network in Fig. 4 is shown in Fig. 5 *A*. It is not necessary to completely decouple a solution into weightings on extreme pathways to apply the loop law. Once the type III extreme pathways have been calculated, the network simply needs to be searched for these cycles, and each cycle that exists in the solution needs to be eliminated until no more are present. The minimum weighting on the type III extreme pathway needed to eliminate the loop is used. In the simple example system of Fig. 5, the FBA solution contains two loops that need to be deleted. As shown in Fig. 5 *B*, the bottom type III needs to be deleted. The minimum flux value in the loop relative to the stoichiometry of the extreme pathways is used to determine the magnitude of the type III extreme pathway being used, which, in this case, is *1*. Once this flux is deleted (Fig. 5 *C*), only one loop remains. This loop is the combination of the two type III extreme pathways determined earlier (Fig. 5 *D*). The minimum flux value is 2, and so the loop is scaled accordingly and deleted from the solution. With the loops removed, a valid solution remains that does not violate the loop law.

A priori knowledge of irreversibility

Appropriately defining irreversible reactions can capture to a significant degree the constraints imposed by the loop law. Upon examination of the sample system, it can be seen that the three reactions that are shown to be reversible are actually constrained to be unidirectional based solely on the loop law. In the example system of Fig. 4, setting the three initially reversible reactions to be irreversible (left to right) would constrain the solution space exactly as it was constrained by the application of the loop law: only the type I extreme pathways would have been calculated. Thus, in many cases, the inclusion of irreversibility constraints on many reactions, as has commonly been done in FBA, can encapsulate a good portion of the thermodynamic information. Equivalently, application of the loop law can recover irreversibility constraints that have not been implemented a priori.

Recapitulation

The application of the loop law to eliminate thermodynamically infeasible solutions gives us an additional set of useful physicochemical constraints to determine allowable behavior of biochemical reaction networks. By utilizing the type III extreme pathways, the loop law can be implemented in flux balance without needing to perform a bilinear optimization. The importance of adding these constraints into genome-scale models now needs to be evaluated. In addition, the energy balance theory presented by Beard et al. (2002) provides a foundation for constraints-based analysis of reaction-free energies in large-scale biochemical systems and thus expands the scope of information available from constraints-based modeling of biochemical networks.

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