Kinetic Constraints for Formation of Steady States in Biochemical Networks

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ABSTRACT The constraint-based analysis has emerged as a useful tool for analysis of biochemical networks. This work introduces the concept of kinetic constraints. It is shown that maximal reaction rates are appropriate constraints only for isolated enzymatic reactions. For biochemical networks, it is revealed that constraints for formation of a steady state require specific relationships between maximal reaction rates of all enzymes. The constraints for a branched network are significantly different from those for a cyclic network. Moreover, the constraints do not require Michaelis-Menten constants for most enzymes, and they only require the constants for the enzymes at the branching or cyclic point. Reversibility of reactions at system boundary or branching point may significantly impact on kinetic constraints. When enzymes are regulated, regulations may impose severe kinetic constraints for the formation of steady states. As the complexity of a network increases, kinetic constraints become more severe. In addition, it is demonstrated that kinetic constraints for networks with co-regulation can be analyzed using the approach. In general, co-regulation enhances the constraints and therefore larger fluctuations in fluxes can be accommodated in the networks with co-regulation. As a first example of the application, we derive the kinetic constraints for an actual network that describes sucrose accumulation in the sugar cane culm, and confirm their validity using numerical simulations.

INTRODUCTION

Following advances in the acquisition of biological data at the genomic, transcriptomic, proteomic, and metabolomic levels, metabolic networks can be reconstructed in many organisms (Allen et al., 2003; Famili et al., 2003; Ma and Zeng, 2003; Duarte et al., 2004). For reconstructed metabolic networks, the constraint-based analysis has emerged as a useful tool for analysis of integrated functions (Edwards et al., 2001; Famili and Palsson, 2003; Klamt and Stelling, 2003; Palsson, 2000; Price et al., 2003; Schilling and Palsson, 2000; Schuster et al., 1999, 2000; Stelling et al., 2002). The core of constraint-based approaches is twofold: one is that the network is at a steady state; and the other is that physicochemical constraints, such as the constraints of stoichiometry, thermodynamics, and maximal reaction rates, confine the possible phenotypic outcome, which is the feasible solution of the network. Although those constraints are based on fundamental properties of enzymatic reactions, they are not related to the formation of a steady state per se. Specifically, stoichiometric constraints restrict the molar relation of reactants; thermodynamic constraints confine the direction of reactions (Beard et al., 2002, 2004; Price et al., 2002); and maximal reaction rates define the capacity of isolated enzymatic reactions. Clearly, none of the above constraints guarantees the formation of a steady state. Biologically, one of the prerequisites for forming a phenotype is that the metabolic network forms a state that is with positive and finite metabolite concentrations. Under this condition, the network may form stable states, one possibility of which is a steady state. At a steady state, steady-state mass balance (i.e., flux balance) is maintained. Such a state can be described in matrix form

$$S \cdot J = 0, \tag{1}$$

where S is the stoichiometric matrix and J is the vector of reaction rates. The validity of Eq. 1 is essential for constraint-based analysis (Klamt and Stelling, 2003). However, what are the conditions to maintain Eq. 1?

As discussed by Segre (2004), a metabolic network can be described by its hardware and software. The hardware is represented by a static reaction network, and the software is the underlying regulatory strategies. In the context of constraint-based analysis, the hardware can define matrix S and vector J. If Eq. 1 is assumed to be valid, constraint-based analysis may use it to analyze integrated functions of the network. No kinetic information is further required (Klamt and Stelling, 2003; Price et al., 2003). However, the software of the network has some noticeable features. Enzymatic reactions are highly nonlinear in character as a consequence of the dependence of reaction rates on substrate concentrations and regulations. Moreover, enzymes can be saturated by their substrates, and consequently, the reaction rates are limited by maximal reaction rates (Palsson, 2000; Famili et al., 2003). In relatively simple networks with those features, it has been shown that establishment of a stable state requires specific constraints on kinetic parameters, particularly maximal reaction rates (Liu, 1999a,b, 2001). To obtain a stable steady state based on the parameters in literature, many of the parameters need to be adjusted (Aon and Cortassa, 2002; Rohwer and Botha, 2001; Teusink et al.,

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2000). Although many of the kinetic parameters may not be readily obtained, some of them can be derived from measurable quantities. For example, maximal reaction rates are directly related to concentrations of enzymes that can be measurable at proteomic level. In constraint-based analysis, maximal reaction rates were referred to as the capacity constraints (Palsson, 2000; Famili et al., 2003). Moreover, investigation of non-isolated Michaelis-Menten-type reaction suggests that the conventional kinetic forms are valid under a range of in vivo conditions. For example, if the concentration ratio of enzyme versus substrate is not very high, Michaelis-Menten formalism is applicable for non-isolated reactions (Stoleriu et al., 2004a,b). Therefore, in addition to maximal reaction rates, kinetic description of a metabolic network may be reliably obtained.

In this article, we introduce the concept of kinetic constraints for metabolic networks based on kinetic description of enzymatic reactions and show that maximal reaction rates are the constraints only for isolated reactions. In a biochemical network, we show that constraints for formation of a steady state require specific relationships between maximal reaction rates of all enzymes. Reversibility of reactions at system boundary or branching point may significantly impact on kinetic constraints. Moreover, regulations can impose severe constraints on the formation of steady states, and co-regulated networks may significantly enhance these constraints. Furthermore, the theory developed is applied to analyze the kinetic constraints for an actual network that describes sucrose accumulation in the sugar cane culm.

RESULTS

Concepts of kinetic constraints

One of the main characteristics of enzyme-catalyzed reactions is that enzymes can be saturated by their substrates. At saturation of an enzyme, the reaction rate reaches a maximum and further increase of substrate concentration cannot further increase the reaction rate. This feature is captured by Michaelis-Menten formalism, which laid the foundation of enzymatic kinetics. The conventional Michaelis-Menten kinetics, described by Eq. 2, can be derived based on the basic mass-action law and quasi-steady-state assumptions (Segel and Selmrod, 1989; Stoleriu et al., 2004a),

$$J = \frac{J^{\max} S}{k + S}, \tag{2}$$

where $J^{\max}$ and k are the maximal reaction rate and Michaelis-Menten constant of the enzyme, respectively. For an isolated enzymatic reaction, the reaction capacity is limited by the maximal enzymatic rate. For example, the flux through an isolated enzyme E_1, J_1, is limited by the maximal reaction rate of E_1, $J_1^{\max}$; namely $J_1 < J_1^{\max}$. In a biochemical network, many enzymatic reactions with various limited reaction capacities interplay. How do the interacting enzymatic reactions form a steady state? In this work, we refer to kinetic constraints as the constraints of forming steady states due to the interplay of enzymatic kinetics. In the Appendix, we summarize the principle and relevant topics of kinetic constraints in detail. Here, for introducing the concept of kinetic constraints, we analyze a sequential reaction network with two enzymatic reactions: $S_1 \overset{E_1}{\rightarrow} S_2 \overset{E_2}{\rightarrow}$. If we assume that the flux catalyzed by E_1, J_1, is the input of the network, a steady state of the network is

$$J_1 - \frac{J_2^{\max} S_2}{k_2 + S_2} = 0. \tag{3}$$

Here $J_2^{\max}$ and k_2 are the maximal reaction rate and Michaelis-Menten constant of E_2, respectively. The value S_2 is the metabolite concentration. Equation 4 can be readily derived from Eq. 3, as

$$S_2 = \frac{J_1 k_2}{J_2^{\max} - J_1}. \tag{4}$$

As S_2 has to be positive and finite, a steady state requires that $J_1 < J_2^{\max}$. Therefore, J_1 must be smaller than the smaller one of $J_1^{\max}$ and $J_2^{\max}$. Clearly, when the reaction catalyzed by E_1 is isolated, the constraint for J_1 is described only by $J_1 < J_1^{\max}$. However, when the two reactions interact, J_1 is restricted by kinetic properties of both enzymes: $J_1 < J_1^{\max}$ and $J_1 < J_2^{\max}$. For a sequential network with n enzymes, kinetic constraints, $J_1 < J_i^{\max}$ and $J_i < J_i^{\max}$ ($i = 2 \ldots n$), can be readily derived following the derivation of Eq. A6. Clearly, these constraints only need the maximal reaction rates and they do not need Michaelis-Menten constants for any enzyme. Analysis of the simple example shows that the concept of kinetic constraints is important for understanding the formation of steady states in a biochemical network.

In general, biochemical networks can be very complicated (Stryer, 1997). They can be unidirectional, reversible, branched, or cyclic, and regulations of enzymes exist in all networks. To understand how steady states are formed in a complex network with regulations, it is essential to understand how kinetic constraints for formation of steady states are affected by network structures and regulations. We therefore analyze how reaction interactions and regulations affect kinetic constraints using the examples in Table 1. In the following, we will assume that for all networks J_1 is the input flux, and it is at the system boundary.

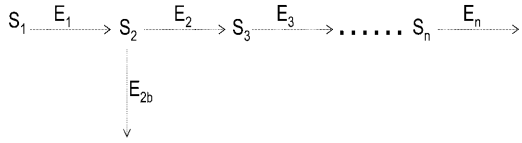
Kinetic constraints for branched networks with Michaelis-Menten kinetics

To proceed, we examine a branched network, *Network 1*, in Table 1. When a network is branched, there are numerous possibilities for its network construction. However, the principle for deducing the steady-state conditions is the same.

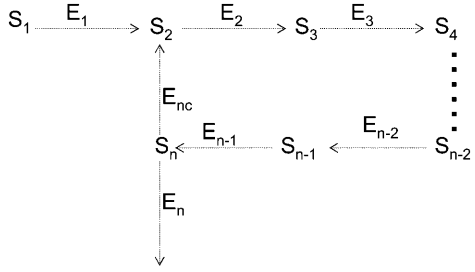
In the Appendix, Network 1 is analyzed in detail. It is shown that, if all reactions follow irreversible Michaelis-Menten kinetics (see Principle for Deriving Kinetic Constraints:

TABLE 1 Summary of biochemical networks under study

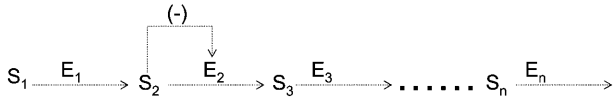
Network 1. A branched network.



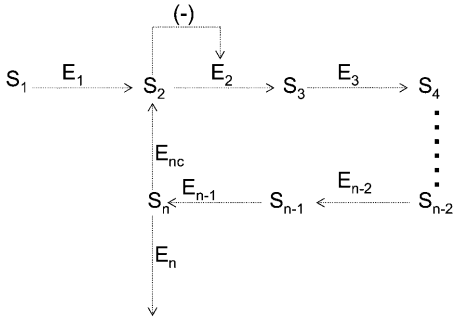
Network 2. A cyclic network.



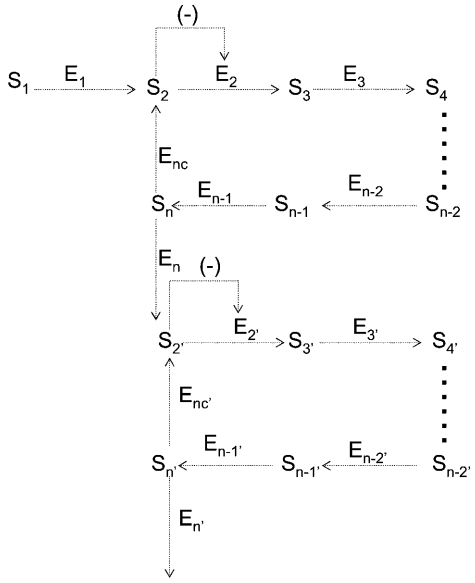
Network 3. A sequential network with substrate inhibition.



Network 4. A cyclic network with substrate inhibition.



Network 5. An example network for demonstrating effects of network complexity on kinetic constraints.



Irreversible Michaelis-Menten Kinetics for All Reactions as an Example, in the Appendix), the kinetic constraints for formation of a steady state are independent of all Michaelis-Menten constants for enzymes E_1, E_3 to E_n, although they are dependent on k_2 and k_{2b}. If reversible reactions do not occur at system boundary or branching point (see Effects of Reversible Reactions on Kinetic Constraints, in the Appendix), the above conclusion remains. However, if the reactions at system boundary or branching point become reversible, the reversibility of these reactions significantly impacts on kinetic constraints. Again, the significances of the results are discussed in detail in the Appendix. In the following, we further discuss a special case in which $k_2 = k_{2b}$, to show how the constraints are related to maximal reaction rates.

For $k_2 = k_{2b}$, the constraints for formation of a steady state at which all metabolite concentrations are non-negative and finite (Eq. A12) become

$$\begin{aligned} &J_1 < J_1^{\max} \\ &J_1 < J_2^{\max} + J_{2b}^{\max} \\ &J_1 < \frac{J_i^{\max}(J_2^{\max} + J_{2b}^{\max})}{J_2^{\max}}. \\ &J_i < J_i^{\max} \\ &\quad i = 2, \ldots n. \end{aligned} \tag{5}$$

It is clear that the constraints require specific relationship of maximal rates of all enzymes.

If the constraints do not satisfy Eq. 5, Network 1 cannot establish a steady state in the sense that the concentrations of at least one metabolite are not non-negative and finite.

Kinetic constraints for cyclic networks with Michaelis-Menten kinetics

As an example, we analyze *Network 2*. At a steady state, the following mass-balance equation must be valid:

$$\begin{aligned} &J_1 - \frac{J_2^{\max} S_2}{k_2 + S_2} + \frac{J_{nc}^{\max} S_n}{k_{nc} + S_n} = 0 \\ &\frac{J_2^{\max} S_2}{k_2 + S_2} - \frac{J_3^{\max} S_3}{k_3 + S_3} = 0 \\ &\cdots\cdots \\ &\frac{J_{n-2}^{\max} S_{n-2}}{k_{n-2} + S_{n-2}} - \frac{J_{n-1}^{\max} S_{n-1}}{k_{n-1} + S_{n-1}} = 0 \\ &\frac{J_{n-1}^{\max} S_{n-1}}{k_{n-1} + S_{n-1}} - \frac{J_n^{\max} S_n}{k_n + S_n} - \frac{J_{nc}^{\max} S_n}{k_{nc} + S_n} = 0. \end{aligned} \tag{6}$$

Analysis of Eq. 6 reveals that the constraints for formation of a steady state are independent of all Michaelis-Menten constants for enzymes E_1 to E_{n-1}, although they are dependent on k_n and k_{nc}. Here we discuss the special case of $k_n = k_{nc}$ while the effects of k_n and k_{nc} on the constraints can be quantitatively analyzed by varying their values.

For $k_n = k_{nc}$, the constraints for formation of a steady state at which all metabolite concentrations are non-negative and finite are

$$
\begin{aligned}
&J_1 < J_1^{\max} \\
&J_1 < J_n^{\max} \\
&J_1 < \frac{J_i^{\max} J_n^{\max}}{J_n^{\max} + J_{nc}^{\max}}. \\
&J_i < J_i^{\max} \\
&\quad i = 2, \ldots n-1. \qquad (7)
\end{aligned}
$$

To clearly demonstrate how kinetic constraints restrict the formation of steady states, we assume that there are five enzymes in Network 2. For the five-enzyme network, all possibilities for forming steady states are restricted by Eq. 7. As an example, here we use the following parameter setting: $J_1 = 3, J_1^{\max} = 10$, $J_5^{\max} = 8$, and $J_{5c}^{\max} = 4$. Michaelis-Menten constants are not required for theoretically analyzing the constraints, but they are set to be unity for numerical simulation. Following Eq. 7, a steady state requires that the smallest of $J_2^{\max}$, $J_3^{\max}$ and $J_4^{\max}$ must be larger than 4.5. Fig. 1 shows the dependence of the evolution of the network on $J_2^{\max}$, $J_3^{\max}$, and $J_4^{\max}$. In Fig. 1, *a* and *b*, we fix $J_2^{\max} = 6$, and $J_3^{\max} = 5$. In Fig. 1 *a*, $J_4^{\max} = 4.6 > 4.5$, the network settles onto a steady state for all metabolites. However, in Fig. 1 *b*, $J_4^{\max} = 4.4 < 4.5$; and although S_2 and S_3 are still able to reach a steady state, S_4 accumulates infinitely. Therefore, the network as a whole cannot reach a steady state. In a similar manner, if $J_2^{\max} < 4.5$, then S_2 cannot reach a steady state; and if $J_3^{\max} < 4.5$, then S_3 cannot reach a steady state. If $J_2^{\max}$, $J_3^{\max}$, and $J_4^{\max}$ are smaller than 4.5 simultaneously, none of S_1, S_2, and S_3 is able to reach a steady state (Fig. 1 *c*). In Fig. 1, *a*–*c*, S_5 always establishes a steady state (data not shown).

For isolated enzymatic reactions, it is known that each reaction is limited by its maximal reaction rate. In contrast, in a biochemical network, the constraints due to maximal reaction rates of all enzymes cannot simply lead to the formation of a steady state. It is revealed that constraints for formation of a steady state require specific relationships between maximal reaction rates of all enzymes. The constraints for a branched network are significantly different from those for a cyclic network, implying that network structure is of importance for formation of steady states. Moreover, the constraints do not require Michaelis-Menten constants for most enzymes, and they only require these constants for the enzymes at the branching or cyclic point. If the constants are unknown, the effects of changing the values of the constants on the constraints can be quantitatively analyzed.

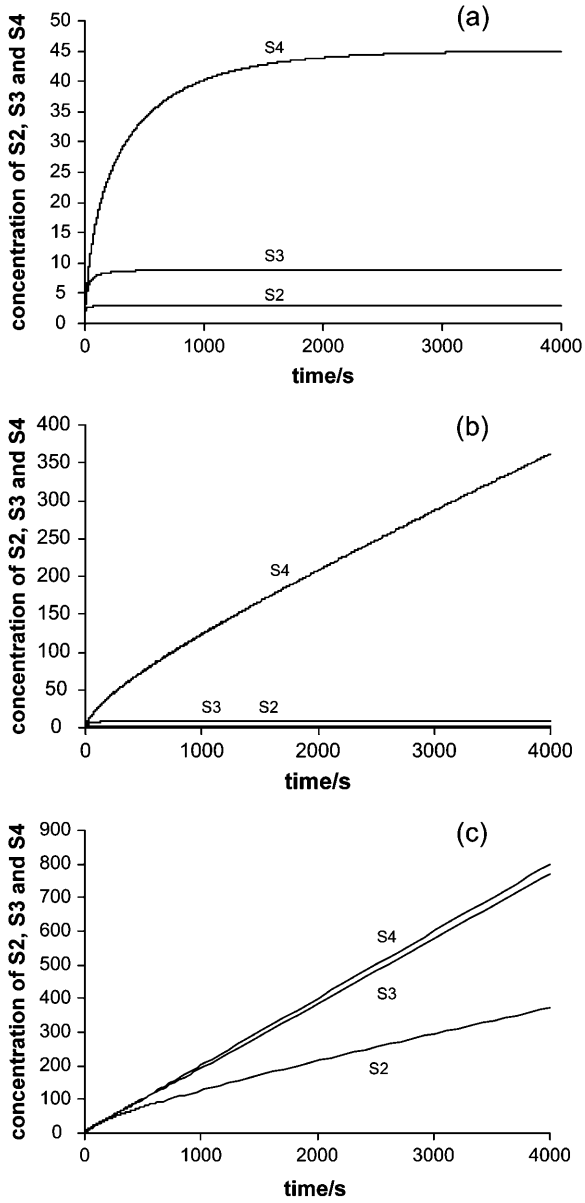


FIGURE 1 Effects of kinetic constraints on formation of a steady state for Network 2 in Table 1. $J_1^{\max} = 10$, $J_5^{\max} = 8$, and $J_{5c}^{\max} = 4$. (*a*) Eq. 7 is valid ($J_2^{\max} = 6$, $J_3^{\max} = 5$, $J_4^{\max} = 4.6 > 4.5$). All metabolites reach a steady state after a transient period. (*b*) Eq. 7 is invalid due to $J_4^{\max}$ being too small ($J_2^{\max} = 6$, $J_3^{\max} = 5$, $J_4^{\max} = 4.4 < 4.5$). Although metabolites S_2 and S_3 establish a steady state, S_4 cannot reach a steady state. (*c*) Eq. 7 is invalid due to all of $J_2^{\max}$, $J_3^{\max}$, and $J_4^{\max}$ being too small ($J_2^{\max} = 4.2 < 4.5$; $J_3^{\max} = 4.0 < 4.5$, $J_3^{\max} = 3.8 < 4.5$), none of S_2, S_3, and S_4 can establish a steady state.

Regulation and kinetic constraints

When an enzyme is regulated, its kinetic properties change. How do regulations affect kinetic constraints for formation of steady states in a network? Following the above analysis, we know that kinetic constraints depend on the network structure. It is expected that different regulations such as activation and inhibition may also affect the constraints in different ways, since kinetic constraints depend on description of kinetics. In the following, we examine effects of substrate inhibition. However, any network with known regulation rules can be analyzed using the same approach.

A classic regulation is Michaelis-Menten kinetics with substrate inhibition (Degn, 1968; Shen and Larter, 1994). *Network 3* shows a sequential network with E_2 being inhibited by its substrate S_2. The kinetic equation for Michaelis-Menten kinetics with substrate inhibition can be

readily derived (Degn, 1968; Shen and Larter, 1994). A steady state for Network 3 is described by

$$\begin{aligned} J_1 - \frac{J_2^{\max} S_2}{k_2 + S_2 + k_2^{\mathrm{eq}} S_2^2} &= 0 \\ \frac{J_2^{\max} S_2}{k_2 + S_2 + k_2^{\mathrm{eq}} S_2^2} - \frac{J_3^{\max} S_3}{k_3 + S_3} &= 0 \\ \cdots\cdots & \\ \frac{J_{n-1}^{\max} S_{n-1}}{k_{n-1} + S_{n-1}} - \frac{J_n^{\max} S_n}{k_n + S_n} &= 0. \end{aligned} \tag{8}$$

Here k_2^{eq} is the equilibrium constant for substrate inhibition (Degn,1968; Shen and Larter, 1994).

Based on Eq. 8, we obtain that, to establish a steady state at which all metabolite concentrations, S_i ($i = 2, \ldots n$), are non-negative and finite, the following constraints must be satisfied:

$$\begin{aligned} J_1 &< J_1^{\max} \\ J_1 &< J_i^{\max} \\ J_1 &< \frac{J_2^{\max}}{1 + 2\sqrt{k_2 k_2^{\mathrm{eq}}}} \\ J_i &< J_i^{\max}. \end{aligned} \tag{9}$$

Equation 9 shows that substrate inhibition imposes a further constraint $J_1 < J_2^{\max}/(1+2\sqrt{k_2 k_2^{\mathrm{eq}}})$ in this sequential network.

Network 4 is a cyclic pathway based on Network 2 with E_2 being inhibited by its substrate S_2. For the cyclic pathway, the constraints for formation of steady states are described as

$$\begin{aligned} J_1 &< J_1^{\max} \\ J_1 &< J_n^{\max} \\ J_1 &< \frac{J_n^{\max}}{J_n^{\max} + J_{nc}^{\max}} \frac{J_2^{\max}}{1 + 2\sqrt{k_2 k_2^{\mathrm{eq}}}}. \\ J_1 &< \frac{J_i^{\max} J_n^{\max}}{J_n^{\max} + J_{nc}^{\max}} \\ J_i &< J_i^{\max} \\ i &= 3, \ldots n-1. \end{aligned} \tag{10}$$

Comparing Eq. 7 with Eq. 10 reveals that substrate inhibition to enzyme E_2 imposes a further constraint $J_1 < (J_n^{\max}/(J_n^{\max} + J_{nc}^{\max}))(J_2^{\max}/(1+2\sqrt{k_2 k_2^{\mathrm{eq}}}))$ in this cyclic network. Following Eq. 10, we know that if $J_2^{\max} < (1+2\sqrt{k_2 k_2^{\mathrm{eq}}})J_i^{\max}$ for any enzyme E_i ($i = 3, \ldots n-1$), substrate inhibition of E_2 becomes an important constraint for formation of a steady state. Only if $J_2^{\max}$ is larger than all of $(1+2\sqrt{k_2 k_2^{\mathrm{eq}}})J_i^{\max}$ for $i = 3, \ldots n-1$, the inhibition becomes unimportant for formation of steady states.

The analysis can be extended to include a network with any complexity. For example, if J_n in Network 4 is an input to another pathway that is assumed to be the same as Network 4, we have *Network 5*. In addition to Eq. 10, the kinetic constraints for Network 5 also require Eq. 11:

$$\begin{aligned} J_n &< J_{n'}^{\max} \\ J_n &< \frac{J_{n'}^{\max}}{J_{n'}^{\max} + J_{nc'}^{\max}} \frac{J_{2'}^{\max}}{1 + 2\sqrt{k_{2'} k_{2'}^{\mathrm{eq}}}} \\ J_n &< \frac{J_{i'}^{\max} J_{n'}^{\max}}{J_{n'}^{\max} + J_{nc'}^{\max}}. \\ J_{i'} &< J_{i'}^{\max} \\ i' &= 3, \ldots n'-1. \end{aligned} \tag{11}$$

It is plausible that as the complexity of a network increases, kinetic constraints will become more severe.

To clearly demonstrate how kinetic constraints due to regulation and complexity of network restrict the formation of steady states, we analyze Network 4 with five enzymes in detail. The following values of parameters are used: $J_1^{\max} = 10$, $J_2^{\max} = J_3^{\max} = J_4^{\max} = 6.0$, $J_5^{\max} = 8$, $J_{5c}^{\max} = 4$, $k_2 = 1$, and $k_2^{\mathrm{eq}} = 1$. The Michaelis-Menten constants for E_3, E_4, and E_5 are not required for analyzing the kinetic constraints. Fig. 2 summarizes the constraints of fluxes J_1 and J_5 for various network constructions. Initially, if both J_1 and J_5 are isolated, they are restricted by the maximal reaction rates of E_1 and E_5, which are 10 and 8, respectively (*column A*). If there is no regulation, the kinetic constraints are calculated using Eq. 7 (for simplicity, we set $J_2^{\max} = J_3^{\max} = J_4^{\max} = 6.0$, therefore, the constraints due to $J_2^{\max}$, $J_3^{\max}$, and $J_4^{\max}$ are the same; see Fig. 1 for detailed analysis for the case of varying $J_2^{\max}$, $J_3^{\max}$, and $J_4^{\max}$). Therefore, $J_1 < (J_2^{\max} J_n^{\max})/(J_n^{\max} + J_{nc}^{\max}) = 4.0$ (*column B*). However, when E_2 is inhibited by its substrate S_2, kinetic constraint becomes $J_1 < (J_n^{\max}/(J_n^{\max} + J_{nc}^{\max}))(J_2^{\max}/(1+2\sqrt{k_2 k_2^{\mathrm{eq}}})) = (4.0/3.0) = 1.33$ based on Eq. 10. Fig. 2 clearly demonstrates

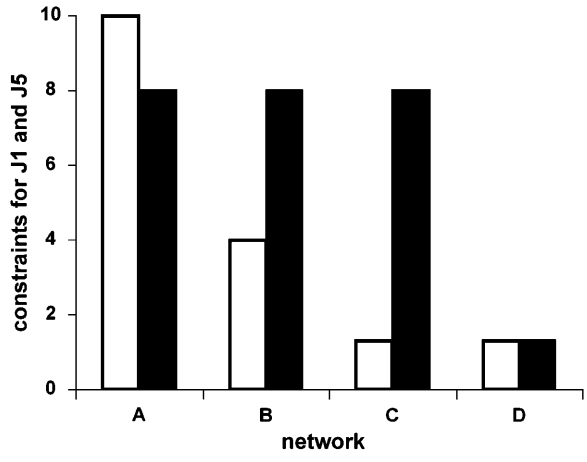


FIGURE 2 Effects of regulation and network complexity on kinetic constraints. The constraints are depicted for fluxes J_1 and J_5 for Networks 4 and 5 in Table 1. J_1, blank column; J_5, filled column. (*A*) Both J_1 and J_5 are the fluxes for isolated reactions. (*B*) All reactions in Network 4 have no regulations and they follow irreversible Michaelis-Menten kinetics (Network 2). (*C*) The reaction catalyzed by E_2 is inhibited by its substrate, S_2 (Network 4). (*D*) The reaction catalyzed by E_2 is inhibited by its substrate, S_2, and flux J_5 is an input to another network (Network 5).

that regulation may impose severe constraints for formation of steady states (*column C*). As an example, Fig. 3 shows the development of S_2 for two neighboring values of J_1. In Fig. 3, all Michaelis-Menten constants are set to be unity for numerical simulation. If $J_1 = 1.32 < 1.33$ (Fig. 3 *a*), a steady state is established. However, if $J_1 = 1.34 > 1.33$ (Fig. 3 *b*), no steady state is established for S_2. In a similar manner, all possibilities for kinetic constraints in Fig. 2 can be numerically analyzed, and they confirm that Eq. 10 displays the constraints for formation of steady states in Network 4 (data not shown). If Network 4 is extended to Network 5, J_5 is an input to the lower part. If it is assumed that the lower part in Network 5 is exactly same as the upper part, a further constraint $J_5 < 1.33$ must be satisfied to maintain a steady state for the whole network. The kinetic constraints for Network 5 are also included in Fig. 2 for comparison (*column D*). It is clear that, as the complexity of network increases, kinetic constraints become more severe.

For Networks 4 and 5, the kinetic constraints only require the Michaelis-Menten and equilibrium constants for E_2. If they are not known, the effects of changing their values can be assessed. For example, if we only know $1 \le k_2 \le 10$, and$1 \le k_2^{eq} \le 10$, following Eq. 10 we have the following constraints: J_1 must be <1.33, otherwise Network 4 cannot establish a steady state. Moreover, if $J_1 < 0.36$, a steady state is guaranteed.

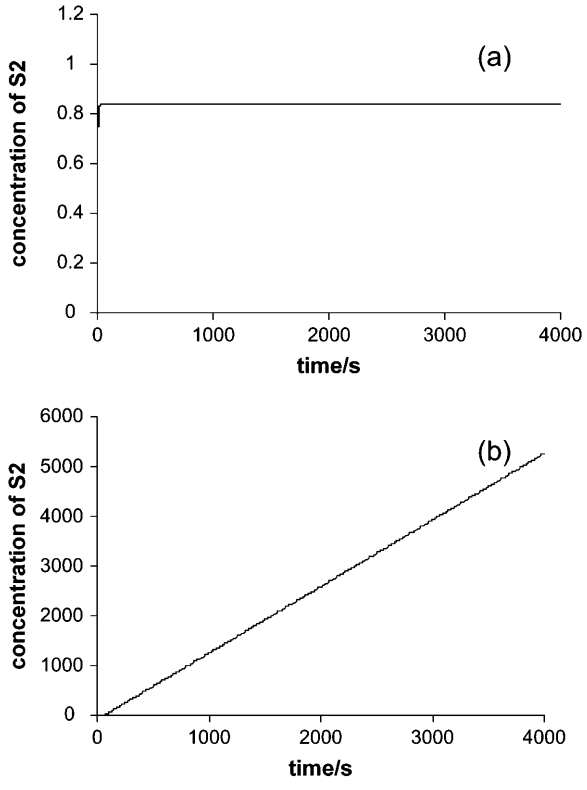


FIGURE 3 For the case in which the reaction catalyzed by E_2 is inhibited by its substrate, S_2 (column *C* in Fig. 2), effects of kinetic constraints on development of metabolite S_2 for two neighboring J_1. (*a*) Kinetic constraint in Fig. 2 is valid ($J_1 = 1.32 < 1.33$). A steady state is established for S_2. (*b*) Kinetic constraint in Fig. 2 is invalid ($J_1 = 1.34 > 1.33$). S_2 will never establish a steady state.

Co-regulation and kinetic constraints

It has recently been shown (Ihmels et al., 2004) that, for some pathways, only a subset of genes displays significant co-expression, and these sets of genes consist mainly of linear arrangements of enzymes. Moreover, it has been shown (Ihmels et al., 2004) that, at a branching point, co-regulation of incoming reaction and one of the two outgoing reactions is prevalent. Segre (2004) analyzed a simple kinetic model, showing that co-regulation might lead to indefinite increase in fluxes.

The approach introduced above is able to assess how kinetic constraints restrict formation of steady states in co-regulated networks. For simplicity, in the first instance, we assume that when enzymes are co-regulated, their maximal reaction rates are increased by the same percentage. Analysis of the kinetic constraints for various networks reveals that for most networks, co-regulation linearly enhances the constraints. For example, for Network 1, if the maximal reaction rates of all enzymes except for E_{2b} are upregulated by a factor α, the kinetic constraints become

$$\begin{aligned} J_1 &< \alpha J_1^{max} \\ J_1 &< \alpha J_2^{max} + J_{2b}^{max} \\ J_1 &< \frac{J_i^{max}(\alpha J_2^{max} + J_{2b}^{max})}{J_2^{max}} \\ J_i &< \alpha J_i^{max}. \end{aligned} \tag{12}$$

It is clear that the restriction to fluxes is linearly enhanced for all cases, but the rate of enhancement (i.e., the slope of J_1 versus α and J_i versus α ($i = 2,\ldots n$)) is different, depending on the values of maximal reaction rates.

Interestingly, for cyclic networks, co-regulation may nonlinearly enhance the kinetic constraints. As an example, we analyze Network 4 and revisit analysis for Fig. 2. When the network is co-regulated for all enzymes apart from the enzyme at cyclic point (E_{5c}), the kinetic constraint is $J_1 < (\alpha^2 J_5^{max}/(\alpha J_5^{max} + J_{5c}^{max}))(J_2^{max}/(1+2\sqrt{k_2 k_2^{eq}}))$. Using the same values of parameters as those for Fig. 2, we know $J_1 < (4\alpha^2/2\alpha + 1)$. As α increases, the constraint is enhanced nonlinearly. If E_{5c} is also enhanced by a factor of α, the constraint becomes $J_1 < 4\alpha/3$. Since $(4\alpha^2/2\alpha+1)$ is always larger than $4\alpha/3$ for $\alpha > 1$, the network is able to absorb larger fluctuations in fluxes if E_{5c} is not co-regulated.

Moreover, if the enzymes in a network are co-regulated with different strengths and the strengths can be quantified, the kinetic constraints of co-regulated networks can also be analyzed using the approach introduced. In terms of the analysis of co-regulation with the same strengths, co-regulated networks are able to establish a steady state in

less restricted conditions and therefore the networks more possibly absorb large fluctuations in fluxes.

Kinetic constraints for the network that accumulates sucrose in the sugar cane culm

The approach introduced in this work clearly demonstrates that kinetic constraints are of vital importance to the formation of steady states in biochemical networks. Regulations and co-regulations may have important implications in kinetic constraints. As a first example of the application of this approach, we apply it to the analysis of an actual network that describes sucrose accumulation in the sugar cane culm (Rohwer and Botha, 2001). The network was analyzed in detail in terms of its kinetic properties (Rohwer and Botha, 2001). We employ the model as an example, and derive the kinetic constraints for the network. We further employ numerical analysis to confirm the validity of those constraints.

As described in literature (Rohwer and Botha, 2001), the network includes eight metabolites, two input fluxes, and two output fluxes. The enzymes in the network are regulated in complicated ways, and enzyme kinetics are highly nonlinear. For simplicity, we use the original notations. However, for the consistency with this work, we use symbol J rather than V to represent reaction rates and maximal reaction rates. The two input fluxes are as follows. The value J_1 is the flux from external fructose to internal fructose; and J_2 is the flux from external glucose to internal glucose. Following the approach developed above and in the Appendix, the kinetic constraints are derived.

The kinetic constraints for glucose and fructose to establish a steady state are

$$J_1 < \frac{J_3^{\max}[ATP]}{(k_{\mathrm{mATP}} + [ATP])} - \frac{J_{\mathrm{f8}}^{\max}[UDPGlc]}{k_{\mathrm{eq8}}\left(\frac{[SUC][UDP]}{k_{\mathrm{iFru}}} + \frac{J_{\mathrm{f8}}^{\max}}{J_{\mathrm{r8}}^{\max}k_{\mathrm{eq8}}}\left(k_{\mathrm{mUDPGlc}}\left(1 + \frac{[UDP]}{k_{\mathrm{iUDP}}}\right) + 1 + \frac{[SUC]}{k_{\mathrm{iSUC}}}\right)\right)}$$

$$J_2 < \frac{J_3^{\max}[ATP]}{(k_{\mathrm{mATP}} + [ATP])}. \tag{13}$$

Equation 13 shows that, although enzymatic kinetics in the network involves many parameters (43 parameters in total), the kinetic constraints only require a few of them. In particular, the kinetic constraints for glucose uptake only require two parameters of the reaction catalyzed by hexokinase: the maximal reaction rate and the Michaelis-Menten constant of ATP. Using the values of the two parameters and steady-state ATP concentration (Rohwer and Botha, 2001), the kinetic constraints are readily obtained, and they are

$$J_2 < 0.1576. \tag{14}$$

The exact determination of the kinetic constraints for fructose uptake also requires the values of some kinetic parameters for sucrose synthase and the steady-state concentrations of sucrose, UDP, and UDP-glucose. Without making the exact calculation, Eq. 13 also shows that the kinetic constraints for fructose uptake are more restricted than those for glucose uptake. Therefore, for fructose to possibly form a steady state, J_1 has to be smaller than 0.1576. Clearly, this constraint is not exact. However, we know that if J_1 is larger than 0.1576, it is not possible for fructose to establish a steady state.

Numerical calculations confirm that Eq. 13 displays the kinetic constraints for glucose and fructose to form a steady state. Fig. 4 *a* shows that as long as $J_2 < 0.1576$, a steady state is established for glucose. However, if $J_2 > 0.1576$, no steady state is possible for glucose. Fig. 4 *b* confirms that the kinetic constraints for fructose to establish a steady state are more restricted. As long as $J_1 > 0.125$, fructose cannot settle onto a steady state. The difference of the kinetic constraints for glucose and fructose to establish a steady state is due to the contribution of the second term of the first equation in Eq. 13. In a similar manner, the kinetic constraints for sucrose and hexose phosphate to establish a steady state can be derived, and they are described by Eq. 15, as

$$J_1 + J_2 < 2J_{11}^{\max} + \frac{J_{10}^{\max}[Fru6P]}{k_{\mathrm{mFruc6P}} + [Fru6P]}. \tag{15}$$

The factor 2 in Eq. 15 is due to stoichiometric relation between sucrose 6-phosphate and hexose phosphate. It is clear that Eq. 15 only requires a few kinetic parameters and the steady-state concentration of *Fru6P*. Equation 15 can be readily examined. For example, if $J_{10}^{\max} = 0$, numerical calculation confirms that as long as $J_1 + J_2 < 2J_{11}^{\max}$, a steady state can be established for sucrose and hexose phosphate. Otherwise, no steady state is possible for sucrose and hexose phosphate. Finally, the kinetic constraints for sucrose 6-phosphate to establish a steady state can be derived (data not shown); it depends on the steady-state concentrations of many metabolites.

It is clear that kinetic constraints for the network that accumulates sucrose in the sugar cane culm can be derived based on the approach developed in this work. Numerical analysis confirms that those kinetic constraints are valid.

CONCLUDING REMARKS

Although it is difficult to acquire kinetic parameters such as Michaelis-Menten constants, particularly for in vivo

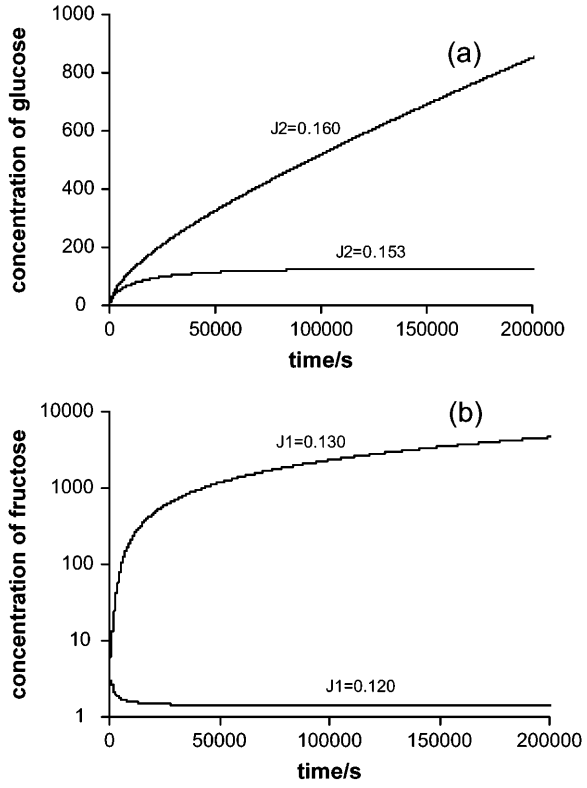


FIGURE 4 The kinetic constraints for glucose and fructose to establish a steady state in the network accumulating sucrose in the sugar cane culm (Rohwer and Botha, 2001). The values of all parameters are the same as those in Rohwer and Botha (2001). (*a*) $J_1 = 0.12$. Effects of kinetic constraints Eqs. 13 and 14 on formation of a steady state for glucose. (*b*) $J_2 = 0.153$. Effects of kinetic constraints Eq. 13 on formation of a steady state for fructose. In *b*, the *y* axis is in logarithmic scale.

conditions, kinetic descriptions such as Michaelis-Menten kinetics can be reliably derived, even for some in vivo conditions (Stoleriu et al., 2004a,b). Based on kinetic description, kinetic constraints for formation of steady states can be derived and they imply that enzymes in a network work together to lead to a steady state. These constraints can be quantified, since they mainly require the relationship between maximal reaction rates of all enzymes. In literature (Palsson, 2000; Famili et al., 2003), individual maximal reaction rates for each enzyme are usually stated as constraints of reaction capacity. However, in a network, the interplay of enzymes, rather than maximal reaction rates of each enzyme, are of importance for formation of steady states. Moreover, it is shown that regulations may impose severe kinetic constraints for the formation of steady states. As the complexity of a network increases, kinetic constraints become more severe. It is demonstrated that kinetic constraints for networks with co-regulation can be analyzed using the approach. In general, co-regulation enhances the constraints and therefore larger fluctuations in fluxes can be accommodated in the networks. Based on the analysis, it is plausible that, for a very complex metabolic network, network structure and regulations may impose severe kinetic constraints, resulting in fluxes in the network being restricted to certain (and small) ranges. To accommodate fluctuations of fluxes, co-regulation becomes essential. This may be one of the postulations for explaining the observed co-regulation patterns (Ihmels et al., 2004; Segre, 2004). Clearly, regulations in a metabolic network occur at different levels. The analysis in this work concentrates on the regulations at enzymatic reaction level. Efforts have been made to include transcriptional regulatory networks and gene expression data in constraint-based analysis (Akesson et al., 2004; Covert and Palsson, 2002; Covert et al., 2001).

In general, biochemical networks comprise a large number of enzymatic reactions, and their structure is complicated. Networks can be unidirectional, reversible, branched, or cyclic. Moreover, enzyme regulations generally exist (Stryer, 1997). Our analysis shows that for the reactions occurring at system boundary or branching point, their reversibility significantly impacts on kinetic constraints for formation of steady states. Therefore, In addition to their roles in flux control (Koch, 1967), reversible reactions may play an important role in maintaining steady states in biochemical networks. Moreover, analysis of the kinetic constraints for an actual network that accumulates sucrose in the sugar cane culm clearly shows that naturally occurring networks are indeed restricted by kinetic constraints.

Enzyme catalysis can be regulated by compounds which are themselves reaction substrates and products, and the consequent regulatory networks are the basis of biological functioning in cells. In a pathway, such a process settles onto a stable, though possibly time-dependent, state. Although metabolic networks can be described in terms of flux control (Fell, 1997), spatiotemporal behavior (Goldbeter, 1996), and energy utility (Ross and Schell, 1987), ultimately, their behavior arises from the formation of stable states (Liu et al., 1997). One of the stable states is a steady state. Based on mass-action kinetics, general theories about reaction networks have been developed, and the properties of reaction networks have been extensively studied (Clarke, 1981, 1988; Feinberg, 1989; Horn and Jackson, 1972; Heinrich and Schuster, 1996). Recently, it has been shown that, for receptor-ligand interactions, existence, uniqueness and global stability of positive steady states can be guaranteed under mass-action kinetics (Chaves et al., 2004). Enzymatic kinetics are usually derived from traditional mass-action kinetics together with simplifying assumptions such as the existence of a quasi-steady state (Segel and Selmrod, 1989; Stoleriu et al., 2004a). At the level of enzymatic reactions, the kinetic rate laws exhibit some special features such as saturation and regulation (Heinrich and Schuster, 1996). Those features are due predominantly to the catalyzing functions of enzymes, and they are captured by Michaelis-Menten-type kinetics. At the level of mass-action kinetics of an enzyme-catalyzed reaction, although the rate laws for all reactions follow mass-action kinetics, the concentration of any form of an enzyme

cannot arbitrarily change as it is limited by the total concentration of the enzyme. Therefore, although the saturation and regulation features of enzymatic rate laws cannot be immediately described by mass-action kinetics (Heinrich and Schuster, 1996), they are the consequences of the mass-action kinetics in which the concentrations of all forms of an enzyme are limited. To copy with those features, generalized mass-action kinetics were suggested (Schauer and Heinrich, 1983). This work shows that the features of enzymatic reactions may have implications for the formation of steady states in biochemical networks. Once a steady state is established in a network, various approaches can be applied to study the properties of the networks. For example, flux balance analysis may analyze the integrated functions. Steady-state perturbation experiments may reveal the underlying regulatory mechanisms (Andrec et al., 2005; Torralba et al., 2003; Vance et al., 2002).

When enzymes are regulated, a network of enzymatic reactions is capable of generating various steady- and time-dependent states. Our analysis shows that substrate inhibition, a typical type of regulation, may impose severe constraints for formation of steady states. Therefore, in addition to the capabilities in generating spatiotemporal behavior (Shen and Larter, 1994), regulations may have significance in restricting formation of possible stable states. When time-dependent states emerge, formation of stable states can be analyzed in terms of the balance of average fluxes (Liu, 1999a,b, 2001; Liu and Crawford, 2000). Moreover, the relationship between a stable state and the nature of environmental fluctuations can be established (Liu and Crawford, 2000).

It is clearly demonstrated that co-regulations can dramatically enhance capability for a network to form a stable state. Co-regulation patterns based on the evidence of experimental observations can be readily analyzed in terms of their effects on kinetic constraints. It is expected that the analysis can be extended to include how co-regulated networks are able to accommodate various environmental fluctuations, following the methods previously introduced for an extracted small model system with product activation (Liu and Crawford, 2000).

Clearly, an essential assumption for constraint-based analysis is that a steady state is established. Searching for possible functions beyond the constraints for which a steady state is established is unrealistic for constraint-based analysis. In this sense, kinetic constraints are of vital importance when constraint-based analysis is applied. Combination of stoichiometric, thermodynamic, and kinetic constraints will appropriately restrict the search for biological phenotypes.

APPENDIX: PRINCIPLE FOR DERIVING KINETIC CONSTRAINTS AND RELEVANT TOPICS—A BRANCHED NETWORK AS AN EXAMPLE

Using Network 1 as an example, this Appendix shows the principle for deriving kinetic constraints in detail, examines the effects of reversible reactions on kinetic constraints and the assumptions for applying those constraints, and discusses the incorporation of some experimentally measurable data into the determination of kinetic constraints.

Flux balance at a steady state

The mass balance of Network 1 is described by

$$\begin{aligned}\frac{dS_2}{dt} &= J_1 - J_2 - J_{2b}\\ \frac{dS_3}{dt} &= J_2 - J_3\\ &\cdots\cdots\\ \frac{dS_n}{dt} &= J_{n-1} - J_n.\end{aligned} \tag{A1}$$

At a steady state, all expressions in Eq. A1 are equal to zero, and they describe the flux balance.

Principle for deriving kinetic constraints: irreversible Michaelis-Menten kinetics for all reactions as an example

For this case, the steady state of Eq. A1 becomes Eq. A2:

$$\begin{aligned}J_1 - \frac{J_2^{max}S_2}{k_2 + S_2} - \frac{J_{2b}^{max}S_2}{k_{2b} + S_2} &= 0\\ \frac{J_2^{max}S_2}{k_2 + S_2} - \frac{J_3^{max}S_3}{k_3 + S_3} &= 0\\ &\cdots\cdots\\ \frac{J_{n-1}^{max}S_{n-1}}{k_{n-1} + S_{n-1}} - \frac{J_n^{max}S_n}{k_n + S_n} &= 0,\end{aligned} \tag{A2}$$

where J_i^{max} and k_i are the maximal reaction rate and Michaelis-Menten constant of E_i ($i = 2,..n$), respectively. The values J_{2b}^{max} and k_{2b} are the maximal reaction rate and Michaelis-Menten constant for enzyme E_{2b}, respectively. The value S_i ($i = 1,...n$) is the metabolite concentration.

The kinetic constraints can be derived as follows. The first expression in Eq. A2 contains only one variable, S_2. Therefore, it can be directly solved as

$$S_2 = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}, \tag{A3}$$

with

$$\begin{aligned}a &= J_1 - J_2^{max} - J_{2b}^{max}\\ b &= J_1(k_2 + k_{2b}) - J_2^{max}k_{2b} - J_{2b}^{max}k_2\\ &= a(k_2 + k_{2b}) + J_2^{max}k_2 + J_{2b}^{max}k_{2b}\\ c &= k_2k_{2b}J_1.\end{aligned} \tag{A4}$$

Based on Eqs. A3 and A4, if $a > 0$, we have $b > 0$ and $\sqrt{b^2 - 4ac} < b$. Therefore, S_2 has no positive solution. Therefore, the kinetic constraints for S_2 to reach a steady state is $a < 0$, which leads to

$$J_1 < J_2^{max} + J_{2b}^{max}. \tag{A5}$$

This constraint can be alternatively deduced and validated by analyzing the boundness of S_2, as previously suggested for small models (Liu and Crawford, 2000) . Following the first expression in Eq. A1, it is known that, as long as Eq. A5 is valid, $(dS_2/dt) < 0$ when $S_2 \rightarrow \infty$. Therefore, Eq. A5 implies that S_2 is able to establish a steady state that is finite. For a sequential network, $J_{2b}^{max} = 0$. Therefore, Eq. A5 can be readily generalized to

$$J_1 < J_i^{max}. \quad \text{(A6)}$$

For the branched network, when S_2 is at a steady state, the flux distribution between J_2 and J_{2b} can be determined following

$$J_1 - J_2 - J_{2b} = 0$$
$$\frac{J_2}{J_{2b}} = \frac{J_2^{max}(k_{2b} + S_2)}{J_{2b}^{max}(k_2 + S_2)}. \quad \text{(A7)}$$

Equation A8 can be readily deduced from Eq. A7 as

$$J_2 = \frac{J_2^{max}(k_{2b} + S_2)}{J_2^{max}(k_{2b} + S_2) + J_{2b}^{max}(k_2 + S_2)} J_1. \quad \text{(A8)}$$

Moreover, following Eq. A2, we derive that, if S_i ($i = 3, \ldots n$) is at a steady state, Eq. A9 must be valid,

$$S_i = \frac{J_2 k_i}{J_i^{max} - J_2}.$$
$$i = 3, \ldots n. \quad \text{(A9)}$$

Combination of Eqs. A8 and A9 gives the kinetic constraints for formation of a steady state for metabolite S_i ($i = 3, \ldots n$), which are in the form of

$$J_1 < \frac{J_i^{max}(J_2^{max}(k_{2b} + S_2) + J_{2b}^{max}(k_2 + S_2))}{J_2^{max}(k_{2b} + S_2)}.$$
$$i = 3, \ldots n. \quad \text{(A10)}$$

The reaction rate catalyzed by each enzyme is also limited by its maximal reaction rate. Therefore, Eq. A11 also displays the constraints for Eq. A2,

$$J_i < J_i^{max}$$
$$i = 1, \ldots n. \quad \text{(A11)}$$

In summary, the complete set of kinetic constraints for all metabolites in Network 1 to form a steady state is described by Eq. A12, if all the reactions in Network 1 follow irreversible Michaelis-Menten kinetics:

$$J_1 < J_1^{max}$$
$$J_1 < J_2^{max} + J_{2b}^{max}$$
$$J_1 < \frac{J_i^{max}(J_2^{max}(k_{2b} + S_2) + J_{2b}^{max}(k_2 + S_2))}{J_2^{max}(k_{2b} + S_2)}.$$
$$J_i < J_i^{max}$$
$$i = 2, \ldots n. \quad \text{(A12)}$$

S_2 in Eq. A12 is in the form of Eqs. A4 and A5. It is more convenient to use Eq. A12 to discuss kinetic constraints, in particular if S_2 is measurable (see Incorporation of Experimentally Measurable Data into Kinetic Constraints, below, for details). Equation A12 does not require Michaelis-Menten constants for enzymes E_i ($i = 3, \ldots n$), although it requires these constants for enzymes E_2 and E_{2b}.

Effects of reversible reactions on kinetic constraints

If an enzymatic reaction is reversible, we assume that its rate law follows the uni-uni mechanism of Michaelis-Menten kinetics (Heinrich and Schuster, 1996), as

$$J = \frac{J^{max}\frac{S}{k_S} - J_r^{max}\frac{P}{k_P}}{1 + \frac{S}{k_S} + \frac{P}{k_P}}. \quad \text{(A13)}$$

Here J^{max} and J_r^{max} denote the maximal reaction rates of the forward and reverse reactions, respectively. The values k_S and k_P are the Michaelis-Menten constants of substrate, S, and product, P, respectively. For Network 1, we will show that, for the reactions that do not occur at the boundary of the network or branching point, reversibility of reactions do not affect kinetic constraints. However, for the reactions at boundary of the network and branching point, introduction of reversibility of reactions may have significant effects on kinetic constraints.

When the reaction catalyzed by E_3 in Network 1 is reversible, the steady state of Network 1 is described by

$$J_1 - \frac{J_2^{max}S_2}{k_2 + S_2} - \frac{J_{2b}^{max}S_2}{k_{2b} + S_2} = 0$$
$$\frac{J_2^{max}S_2}{k_2 + S_2} - \frac{J_3^{max}\frac{S_3}{k_{S3}} - J_{3r}^{max}\frac{S_4}{k_{S4}}}{1 + \frac{S_3}{k_{S3}} + \frac{S_4}{k_{S4}}} = 0$$
$$\frac{J_3^{max}\frac{S_3}{k_{S3}} - J_{3r}^{max}\frac{S_4}{k_{S4}}}{1 + \frac{S_3}{k_{S3}} + \frac{S_4}{k_{S4}}} - \frac{J_4^{max}S_4}{k_4 + S_4} = 0$$
$$\ldots\ldots$$
$$\frac{J_{n-1}^{max}S_{n-1}}{k_{n-1} + S_{n-1}} - \frac{J_n^{max}S_n}{k_n + S_n} = 0. \quad \text{(A14)}$$

The principle for deriving kinetic constraints for Eq. A14 is the same as that for Eq. A2. For Eq. A14, analysis of kinetic constraints exactly follows Eqs. A3–A8. However, Eq. A9 becomes

$$S_3 = \frac{k_{S3}\left(J_2 + J_2\frac{S_4}{k_{S4}} + \frac{J_{3r}^{max}S_4}{k_{S4}}\right)}{J_3^{max} - J_2}$$
$$S_i = \frac{J_2 k_i}{J_i^{max} - J_2}.$$
$$i = 4, \ldots n. \quad \text{(A15)}$$

Although Eq. A15 is significantly different from Eq. A9, it also leads to Eq. A10. Therefore, the kinetic constraints for formation of a steady state for Eq. A14 are the same as those for Eq. A2. The kinetic constraints are also described by Eq. A12. In a similar manner, when the reactions catalyzed by E_i ($i = 4, \ldots n-1$) become reversible, kinetic constraints can be derived, and they are described by Eq. A12. However, if the reactions at the boundary or branching point (the rates of which are J_1, J_2, and J_{2b} in Network 1) become reversible, kinetic constraints for formation of a steady-state change.

When the reaction catalyzed by E_2 is reversible, the steady state of Network 1 is described by

$$J_1 - \frac{J_2^{max}\frac{S_2}{k_{S2}} - J_{2r}^{max}\frac{S_3}{k_{S3}}}{1 + \frac{S_2}{k_{S2}} + \frac{S_3}{k_{S3}}} - \frac{J_{2b}^{max}S_2}{k_{2b} + S_2} = 0$$
$$\frac{J_2^{max}\frac{S_2}{k_{S2}} - J_{2r}^{max}\frac{S_3}{k_{S3}}}{1 + \frac{S_2}{k_{S2}} + \frac{S_3}{k_{S3}}} - \frac{J_3^{max}S_3}{k_3 + S_3} = 0$$
$$\ldots\ldots$$
$$\frac{J_{n-1}^{max}S_{n-1}}{k_{n-1} + S_{n-1}} - \frac{J_n^{max}S_n}{k_n + S_n} = 0. \quad \text{(A16)}$$

For Eq. A16, Eq. A5 remains the same. However, Eq. A7 becomes

$$J_1 - J_2 - J_{2b} = 0$$

$$\frac{J_2}{J_{2b}} = \frac{J_2^{max}\left(1 - \frac{J_{2r}^{max}}{J_2^{max}}\frac{k_{S2}}{k_{S3}}\frac{S_3}{S_2}\right)(k_{2b} + S_2)}{J_{2b}^{max}\left(k_{S2} + S_2 + \frac{k_{S2}}{k_{S3}}S_3\right)}. \quad \text{(A17)}$$

Equation A17 leads to

$$J_2 = \frac{J_2^{max}\left(1 - \frac{J_{2r}^{max}}{J_2^{max}}\frac{k_{S2}}{k_{S3}}\frac{S_3}{S_2}\right)(k_{2b} + S_2)}{J_{2b}^{max}\left(k_{S2} + S_2 + \frac{k_{S2}}{k_{S3}}S_3\right) + J_2^{max}\left(1 - \frac{J_{2r}^{max}}{J_2^{max}}\frac{k_{S2}}{k_{S3}}\frac{S_3}{S_2}\right)(k_{2b} + S_2)}J_1. \quad \text{(A18)}$$

Moreover, a steady state for S_i ($i = 3, \ldots n$) in Eq. A16 also requires Eq. A9. Combination of Eqs. A18 and A9 leads to the kinetic constraints for formation of a steady state for metabolites S_i ($i = 3, \ldots n$) in Eq. A16, and they are described by

$$J_1 < \left(1 + \frac{J_{2b}^{max}\left(k_{S2} + S_2 + \frac{k_{S2}}{k_{S3}}S_3\right)}{J_2^{max}\left(1 - \frac{J_{2r}^{max}}{J_2^{max}}\frac{k_{S2}}{k_{S3}}\frac{S_3}{S_2}\right)(k_{2b} + S_2)}\right)J_i^{max}.$$

$$i = 3 \ldots n. \quad \text{(A19)}$$

Equation A19 clearly shows that the kinetic constraints for S_i ($i = 3, \ldots n$) to reach a steady state depend also on S_3, due to the reaction catalyzed by E_2 being reversible. If S_3 is sufficiently small ($S_3 \rightarrow 0$), the reaction catalyzed by E_2 becomes irreversible and Eq. A19 becomes Eq. A10. As S_3 increases, Eq. A19 becomes less restricted. The effects of reversibility of the reaction catalyzed by E_2 on kinetic constraints can be understood as follows. With the increase of S_3, the reverse reaction is enhanced. Therefore, the flux entering the branch catalyzed by E_2 becomes smaller for a specific J_1. In other words, for specific $J_i^{max}(i = 3, \ldots n)$, S_i ($i = 3, \ldots n$) may reach a steady state for a wider range of J_1 as far as the branch catalyzed by E_2 is concerned. If S_3 reaches such a value that the reaction catalyzed by E_2 is at its equilibrium (i.e., $(J_{2r}^{max}/J_2^{max})(k_{S2}/k_{S3})(S_3/S_2) = 1$), Eq. A19 becomes $J_1 < \infty$. Once this happens, the flux entering the branch catalyzed by E_2 is zero. As far as this branch is concerned, there is no constraint for J_1. Clearly, for this case, Network 1 becomes a sequential network to the branch catalyzed by E_{2b} whose constraints are analyzed in Principle for Deriving Kinetic Constraints: Irreversible Michaelis-Menten Kinetics for All Reactions as an Example, above. In summary, when the reaction catalyzed by E_2 is reversible, the kinetic constraints for formation of a steady state are in the form of

$$J_1 < J_1^{max}$$

$$J_1 < J_2^{max} + J_{2b}^{max}$$

$$J_1 < \left(1 + \frac{J_{2b}^{max}\left(k_{S2} + S_2 + \frac{k_{S2}}{k_{S3}}S_3\right)}{J_2^{max}\left(1 - \frac{J_{2r}^{max}}{J_2^{max}}\frac{k_{S2}}{k_{S3}}\frac{S_3}{S_2}\right)(k_{2b} + S_2)}\right)J_i^{max}.$$

$$J_i < J_i^{max}$$

$$i = 3, \ldots n. \quad \text{(A20)}$$

Equation A20 does not require Michaelis-Menten constants for enzymes E_i ($i = 3, \ldots n$). In a similar manner, if any of the reactions catalyzed by E_1 and E_{2b} becomes reversible, the kinetic constraints will change accordingly.

Incorporation of experimentally measurable data into kinetic constraints

In principle, kinetic constraints can always be derived based on the approaches introduced. However, when a network becomes complicated, analysis of kinetic constraints may become a difficult task. Introduction of some experimentally measurable data may significantly simplify the analysis of kinetic constraints. For example, S_2 in Eq. A12 should be in the form of Eqs. A3 and A4. However, for experimentally measurable S_2, kinetic constraints can be directly calculated using Eq. A12. In a similar manner, if both S_2 and S_3 are known, the kinetic constraints for the case for which the reaction catalyzed by E_2 is reversible, can be calculated using Eq. A20. No further efforts are required to link S_2 and S_3 with kinetic parameters.

More generally, the approach introduced can be extended to include the formation of any finite stable state that can be either a steady state or a time-dependent state (Liu and Crawford, 2000). For a metabolite concentration, S, we know that, as long as $(dS/dt) < 0$ when $S \rightarrow \infty$, S cannot increase infinitely. For this case, S is bounded and emergence of finite stable states becomes possible. Therefore, we may derive kinetic constraints for formation of a finite stable state by analyzing the conditions under which $(dS/dt) < 0$ when $S \rightarrow \infty$. If a network is subject to environmental fluctuations, appropriate temporal average should be introduced to describe the effects of the fluctuations on flux distribution (Liu and Crawford, 2000).

Kinetic constraints and level of networks

Following the conventional practice, when the kinetics of an enzymatic reaction are described, concentrations of metabolites are considered to be variables, and concentrations of enzymes are assumed to be parameters that do not change with time. This implies that the derived kinetic constraints are directly applicable to the level of enzymatic reactions. If the synthesis and degradation of enzymes are considered, the concentrations of enzymes become variables. For this case, kinetic constraints for formation of steady states should be examined for the conditions in which concentrations of enzymes are also variables.

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