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Multi-equilibrium property of metabolic networks: Exclusion of multi-stability for SSN metabolic modules

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SUMMARY

It is a fundamental and important problem whether or not a metabolic network can admit multiple equilibria in a living organism. Due to the complexity of the metabolic network, it is generally a difficult task to study the problem as a whole from both analytical and numerical viewpoints. In this paper, a structure-oriented modularization research framework is proposed to analyze the multi-stability of metabolic networks. We first decompose a metabolic network into four types of basic building blocks (called metabolic modules) according to the particularity of its structure, and then focus on one type of these basic building blocks—the single substrate and single product with no inhibition (SSN) module, by deriving a nonlinear ordinary differential equation (ODE) model based on the Hill kinetics. We show that the injectivity of the vector field of the ODE model is equivalent to the nonsingularity of its Jacobian matrix, which enables us equivalently to convert an unverifiable sufficient condition for the absence of multiple equilibria of an SSN module into a verifiable one. Moreover, we prove that this sufficient condition holds for the SSN module in a living organism. Such a theoretical result not only provides a general framework for modeling metabolic networks, but also shows that the SSN module in a living organism cannot be multi-stable.

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KEY WORDS: metabolic network; multiple equilibria; multi-stability; SSN module

1. INTRODUCTION

Metabolic networks are one type of the most important molecular networks in living organisms. As nonlinear dynamical systems, they can generally admit three dynamic features: mono-stability (only one stable steady state), bi-stability or multi-stability (more than one stable steady states), and oscillation (periodic or non-periodic orbits) [1–4]. Mono-stability is very common in biological systems. With the recent development of biotechnology, bi-stability [5, 6] and oscillation [7, 8] were also observed at molecular level in various living organisms.

A question naturally arises on whether or not a given metabolic network is multi-stable in all its feasible regions. For its great biological significance, this question attracts much attention from both biologists and mathematicians. Take the photosynthetic carbon metabolic network in the mesophyll cells of plants as an example. If it has two or more steady states, one of them will

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correspond to the higher or highest photosynthesis rate, which clearly can be used to increase the grain yields by driving the system into this steady state; otherwise, the only thing one can do is to improve the photosynthesis at the existing steady state [9–11]. Thus, it is crucial to accurately judge whether or not this molecular network is able to admit multiple equilibria so that a correct strategy can be adopted.

It is not only expensive but also difficult, if not impossible, to answer such a question via biological experiments. Hence, a system modeling approach to tackle this problem is strongly demanded. However, in the traditional theoretical analysis, necessary information about model parameters is always required. Due to the limitation of measurement tools and measurement errors, most of the model parameters are either unavailable or uncertain. This makes it difficult to analyze the model. Moreover, the biological variability, which makes the model parameters vary drastically from different individuals, limits the applications of the theoretical results based on a model with fixed parameter values. Thus, to detect multi-stability, another good way is to analyze the network from a structure perspective, which needs only the topological structure information but has a wide range suitability to various individuals or systems.

There are some pioneering works in the area of detecting multiple equilibria of some specific systems. In 1981, a conjecture of Thomas asserts that a necessary condition for multiple equilibria is the presence of a positive circuit in the interaction graph, i.e. a circuit in which the product of the signs of the edges is positive [12]. Since then, it took more than two decades for people to prove the Thomas conjecture from a particular case to a general case [13–17]. Based on the Thomas conjecture and other three open conjectures [18–20], Kaufman *et al.* gave another necessary condition for multiple equilibria of a dynamical system described by a set of ordinary differential equations (ODEs) [21]. Craciun and Feinberg [22–24] analyzed the chemical network constituted by the reactions in an isothermal homogeneous continuous flow stirred tank reactor (CFSTR [25]). They described the rate of each chemical reaction in the CFSTR by the law of mass-action and gave sufficient conditions for the absence and presence of multiple positive equilibria, respectively [22]. Moreover, by regarding a cell as a CFSTR, they extended the work in [22, 23] to an enzyme-driven reaction network [24]. Chesi proposed a recursive algorithm to compute equilibrium point of genetic regulatory network [26] and analyzed their global asymptotic stability [27].

Generally, a metabolic network in a living organism is a large-scale system. Hence, it is difficult to directly study it as a whole, especially when the only available information is the network structure. To overcome such a difficulty, we resort to the modularization idea, which is widely used in the area of systems control [28–30], and view a metabolic network as an assembly of several basic building blocks. Specifically, by exploiting the topological structures, we first decompose a general metabolic network into four types of basic building blocks, which are called metabolic modules, and then investigate the multi-stability of the original network through studying the dynamical characteristics of the basic modules and their interactions. Such a strategy can not only reduce the difficulty in studying a complex metabolic network, but also make full use of its structural information. After getting a profound insight in the basic building blocks, we can use them to reconstruct a metabolic network.

To practice such a modularization idea, we model a general metabolic network in a mathematical manner. Generally, an enzyme-catalyzed metabolic reaction contains several intermediate reactions, such as the binding of substrate and enzyme and the degrading of the intermediate enzyme–substrate complex. If the details of all those intermediate reactions are known, one can model the network based on the law of mass action and use the theory of Craciun *et al.* [22–24] to investigate its multi-stability. Unfortunately, the detailed intermediate process of a metabolic reaction is unknown for many cases. Compared with the law of mass action, less information about the intermediate reactions is required when the Michaelis–Menten kinetics [31] or Hill kinetics [32] is chosen to describe the rate of a metabolic reaction. Moreover, the parameters in the Hill or Michaelis–Menten kinetics have clear biological meaning and can be regulated by experimental techniques. Thus, we model the enzyme-driven metabolic network based on the Hill kinetics in this work, and investigate whether or not it can admit multiple equilibria from a structure perspective.

In this paper, we show that a metabolic network can be constructed by four basic modules, and specifically focus on one key type of the basic modules, i.e. the single substrate and single

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product without inhibition (SSN) module, and prove that it cannot be multi-stable. By our results, although an SSN module satisfies the necessary condition for multiple equilibria given in the Thomas conjecture [12], there is still no more than one equilibrium. This implies that the necessary condition is not sufficient.

This paper is organized as follows. Section 2 introduces the main idea of the modularization decomposition of a metabolic network, defines the SSN metabolic module and provides a general framework to model it. Section 3 proves the SSN metabolic modules in living organisms cannot be multi-stable. Section 4 gives a biological example to illustrate the application of the theoretical results. Section 5 provides some concluding remarks and some topics worth investigating.

2. MODELING SSN MODULE

A metabolic network is generally composed of a large number of enzyme-catalyzed reactions. Some of them contain only one substrate and one product, and the others do not. Furthermore, there may exist inhibitors for some metabolic reaction, which acts to decrease the reaction rate. Due to the special feature of enzyme-catalyzed reactions, we can classify metabolic reactions into four classes according to the number of substrates and products and the existence of inhibitors, as follows.

Definition 2.1
A metabolic reaction is called a single substrate and single product (SS) reaction, if it contains only one substrate and one product; otherwise, called a multiple substrates or multiple products (MM) reaction. An SS (or MM) metabolic reaction is called an SS (or MM) reaction with inhibition, SSI (or MMI) for short, if there exist some inhibitors of the reaction; otherwise, called an SS (or MM) reaction with no inhibition, SSN (or MMN) for short.

Due to the particularity of activation, we will discuss it in detail as future work and do not consider it in this article. And in this sense, the four classes of reactions defined in Definition 2.1, i.e. SSI, MMI, SSN, and MMN reactions, can cover all metabolic reactions. Then we can decompose a metabolic network into four types of basic building blocks, and call them SSN, SSI, MMN, and MMI metabolic modules, respectively. In fact, initially taking each reaction as a module and then extending it to the maximum subnetwork through adding its neighbor nodes is a nice and efficient way to do the modularization decomposition. We aim to study a metabolic network with the modularization idea, regard it as a system consisting of these basic modules, and then investigate the original network through studying the characteristics of the basic modules and their interactions. There are several steps for such a process, from the optimal modularization decomposition to the characteristics of each module, to the interactions among these modules, and to the integration of the four types of basic modules. As a first step in this line, we will discuss the SSN module in this paper.

2.1. Definitions

In this section, we will give the definition for SSN module.

Definition 2.2 (*Reaction graph*)
For a group of SSN metabolic reactions, take each metabolite as a node. If two nodes appear in a same reaction, link them with a directed edge from the substrate to the product. Then we get a graph, called reaction graph of the group of SSN reactions.

Remark 2.1
We regard a reversible reaction as two reactions. For example, take $A \overset{E}{\rightleftharpoons} B$ as the forward reaction $A \overset{E}{\rightarrow} B$ and the reverse reaction $B \overset{E}{\rightarrow} A$.

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Remark 2.2
Any node in a reaction graph must be a substrate or a product of some reaction. Hence, it will have at least one connecting edge.

Definition 2.3
In the reaction graph of a group of SSN metabolic reactions, a node is called an input node, if the direction of each edge connecting it points to other node; a node is called an output node, if the direction of each edge connecting it points to itself. The other nodes are called state nodes. A state node that directly connects with an input (or output) node is called a head (or an end) node.

Definition 2.4
A reaction is said to be relevant to a metabolite S, if S is the reactant, product or inhibitor of this reaction.

Definition 2.5 (*SSN module*)
For a given metabolic network, denote $\widetilde{\mathscr{M}}$ the set of all the metabolites, and $\widetilde{\mathscr{R}}$ the set of all the reactions. The pair $\mathscr{S}\times\mathscr{R}$ is called an SSN module within the metabolic network, and $\mathscr{S}$ and $\mathscr{R}$ are called the state node set and the reaction set of the SSN module, respectively, if the following conditions are satisfied:

(i) $\mathscr{S}$ is a nonempty subset of $\widetilde{\mathscr{M}}$.
(ii) $\mathscr{R}$, a nonempty subset of $\widetilde{\mathscr{R}}$, is constituted of all the reactions which are relevant to the metabolites in $\mathscr{S}$.
(iii) The reactions in $\mathscr{R}$ are all SSN reactions.
(iv) If there exist both input and output nodes, then for any $S\in\mathscr{S}$, there exists one directed path[‡] from some input node to some output node passing S in the reaction graph of $\mathscr{R}$.
(v) The undirected graph constructed as follows is a connected graph: remove all the input and output nodes (if there exist) and the edges connected with them in the reaction graph of $\mathscr{R}$, and replace each directed edge by an undirected one.

Remark 2.3
The first three conditions of Definition 2.5 are the elementary requirements. The condition (iv) is from biological systems. In a living organism, any metabolite must be synthesized from other metabolites and be converted into an output. The condition (v) is essential for the modularization decomposition of a metabolic network.

Remark 2.4
Although each reaction in an SSN module is with single substrate and single product, an SSN module could be with multiple inputs and multiple outputs, i.e. multiple input and output nodes.

Similarly, we can define the SSI, MMI, and MMN metabolic modules, which will be studied in detail in our future works. We now give an example to elucidate the concepts in the above definitions.

Suppose that there is a group of metabolic reactions, $H\xrightarrow{E_1}S_1$, $H\xrightarrow{E_2}S_3$, $S_1\xrightarrow{E_3}S_2$, $S_1\xrightarrow{E_4}S_3$, $S_2\xrightarrow{E_5}S_3$, $S_2\xrightarrow{E_6}S_4$, $S_2\xrightarrow{E_7}S_5$, $S_3\xrightarrow{E_8}S_4$, $S_3\xrightarrow{E_9}P_2$, $S_4\xrightarrow{E_{10}}P_1$, $S_4\xrightarrow{E_{11}}P_2$, $S_5\xrightarrow{E_{12}}S_1$, $S_5\xrightarrow{E_{13}}P_1$, where H, S_i, and P_k are metabolites (i.e. substrates and products) and E_j are enzymes. They are all SSN reactions, and their reaction graph is shown in Figure 1. By Definition 2.3, H is an input node, P_1 and P_2 are output nodes, S_1 is a head node, S_3 is both a head and an end node, and S_4 and S_5 are end nodes. Denote $\mathscr{S}=\{S_1,S_2,S_3,S_4,S_5\}$ and $\mathscr{R}$ the set of those reactions. Then $\mathscr{S}\times\mathscr{R}$ is an SSN module.

A metabolic network in a living organism is usually very complex and is generally composed of various types of metabolic reactions, including SSN, SSI, MMN, and MMI reactions. Thus, the

[‡]Here, a path in a directed graph is a sequence of nodes such that from each of its nodes there is a directed edge to the next node in the sequence.

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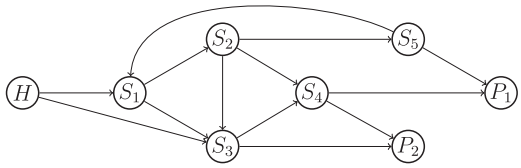

Figure 1. The reaction graph of the reaction set $\mathscr{R}$.

one with only SSN modules is a special case, but SSN metabolic module exists in many metabolic networks. Take the reversible reaction $R5P \overset{Rpi}{\longleftrightarrow} Ru5P$ in the photosynthetic carbon metabolism as an example, where $R5P$, $Ru5P$, and Rpi represent, respectively, the Ribose 5-phosphate, Ribulose 5-phosphate and Ribose 5-phosphate isomerase. Let $\mathscr{S}=\{R5P, Ru5P\}$, and $\mathscr{R}$ be the set of $R5P \xrightarrow{Rpi} Ru5P$, $Ru5P \xrightarrow{Rpi} R5P$ and the other reactions relevant to $R5P$ and $Ru5P$. Then $\mathscr{S}\times\mathscr{R}$ is an SSN module of the photosynthetic carbon metabolism.

2.2. Modeling SSN module

Let $\mathscr{S}\times\mathscr{R}$ be an SSN metabolic module containing n state nodes and m reactions, and denote

$$\mathscr{S}=\{S_1,\ldots,S_n\},$$
$$\mathscr{R}=\{A_1\to B_1,\ldots,A_m\to B_m\}.$$

Assume that all the reactions in $\mathscr{R}$ obey the Hill kinetics. Then, the Hill function

$$v_j=\frac{V_{maxj}(C_{A_j})^{n_j}}{(K_{Mj})^{n_j}+(C_{A_j})^{n_j}}\triangleq\frac{V_{maxj}(C_{A_j})^{n_j}}{K_j+(C_{A_j})^{n_j}} \tag{1}$$

can be used to describe the rate of the enzyme-catalyzed reaction $A_j \xrightarrow{E_j} B_j$, where C_{A_j} is the concentration of the metabolite A_j, V_{maxj} represents the maximum rate of the reaction, K_{Mj} is the Michaelis–Menten constant of the substrate A_j, n_j is the Hill coefficient, $K_j=(K_{Mj})^{n_j}$. If $n_j=1$, (1) is also called Michaelis–Menten equation. The Hill kinetics or the Michaelis–Menten kinetics is a good choice to describe the rate of a biochemical reaction, which has been widely accepted by both biologists and mathematicians. Moreover, an enzyme-catalyzed metabolic reaction can be formulated by these kinetics without the detailed information on its intermediate process.

Let $C_i\triangleq C_{S_i}$ represent the concentration of the metabolite S_i, and $C=(C_1,\ldots,C_n)^{\mathrm{T}}$, where T means the transpose of a matrix. Note that the rate of change of the concentration of S_i is given by the difference between the rate(s) of the reaction(s) generating S_i and the rate(s) of the reaction(s) consuming S_i. Then

$$\frac{\mathrm{d}C_i}{\mathrm{d}t}=\sum_{A_j\to B_j\in\mathscr{R},\quad B_j=S_i} v_j-\sum_{A_j\to B_j\in\mathscr{R},\quad A_j=S_i} v_j, \tag{2}$$

where v_j is given in (1). Then, we can get a model of the SSN module $\mathscr{S}\times\mathscr{R}$,

$$\frac{\mathrm{d}C}{\mathrm{d}t}=\begin{pmatrix}\dfrac{\mathrm{d}C_1}{\mathrm{d}t}\\ \vdots\\ \dfrac{\mathrm{d}C_n}{\mathrm{d}t}\end{pmatrix}=\begin{pmatrix}R_1(C;P)\\ \vdots\\ R_n(C;P)\end{pmatrix}\triangleq R(C;P), \tag{3}$$

where $R_i(C;P)$ is given by the right-hand side of (2), P is vector-valued model parameter. $R(C;P)$ is called the rate function of the model.

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Remark 2.5
If node A_j is an input node, then its concentration C_{A_j} in (1) is not a variable of the model (3) but a parameter.

Definition 2.6
For a fixed parameter P_0, an equilibrium of (3) is a state C that satisfies $\mathrm{d}C/\mathrm{d}t=0$, i.e. a solution of the algebraic equations $R(C; P_0)=0$. System (3) or the SSN module $\mathscr{S}\times\mathscr{R}$ is said to have the capability of multiple equilibria, if there exists a parameter P_0 such that the algebraic equations $R(C; P_0)=0$ have more than one positive solutions.

3. THEORETICAL ANALYSIS

Since a metabolic process in biological systems must have input and output, we only investigate the SSN module that has both input and output nodes. If the metabolites of the input nodes cannot get any supplement from external sources, then all the metabolites of both the input nodes and the state nodes will be entirely exhausted and converted into the metabolites of the output nodes after a period of time, and all the reactions will come to an end. If the metabolites of input nodes are supplied inconstantly from external sources, then the concentrations of the state node metabolites will fluctuate accordingly. We now focus on the case where the metabolites of input nodes have constant supply, which means that the concentration of a input node metabolite is constant.

Equation (3) is a basic model for SSN modules by using ODEs. Our objective is to show $R(C; P)$ in (3) is injective for any P, which implies the absence of multiple equilibria for SSN modules. To achieve this objective, we first show that the injectivity of $R(C; P)$ for all P is equivalent to the nonsingularity of the Jacobian matrix $\partial R(C; P)/\partial C$ of $R(C; P)$ with respect to C, and next show $\det(\partial R(C; P)/\partial C)\neq 0$ for all C and P. Although model (3) can be represented by using stoichiometric matrix, great difficulty will lie in computing $\det(\partial R(C; P)/\partial C)$ and proving $\det(\partial R(C; P)/\partial C)\neq 0$. So we need to equivalently convert model (3) into another effective form.

Define

$$\mathbb{R}^{\mathscr{S}}=\left\{\sum_{i=1}^{n} z_i S_i : z_i\in\mathbb{R}=(-\infty,\infty)\right\}.$$

It is obvious that $\mathbb{R}^{\mathscr{S}}$ is a vector space spanned by $\mathscr{S}=\{S_1,\ldots,S_n\}$. For any reaction $A\to B\in\mathscr{R}$, if A (or B) is a state node in $\mathscr{S}$, we can view it as a vector in $\mathbb{R}^{\mathscr{S}}$; if A (or B) is an input (or output) node, we make a convention viewing it as the zero vector in $\mathbb{R}^{\mathscr{S}}$. Denote

$$\varepsilon_i=(0,\ldots,1,\ldots,0)^{\mathrm{T}},$$

whose entries are all zero except the ith position. Then

$$C=(C_1,\ldots,C_n)^{\mathrm{T}}=\sum_{i=1}^{n} C_i\varepsilon_i,$$

$$\begin{aligned}\frac{\mathrm{d}C}{\mathrm{d}t}&=\left(\frac{\mathrm{d}C_1}{\mathrm{d}t},\ldots,\frac{\mathrm{d}C_n}{\mathrm{d}t}\right)^{\mathrm{T}}=\sum_{i=1}^{n}\frac{\mathrm{d}C_i}{\mathrm{d}t}\varepsilon_i\\&=\sum_{i=1}^{n}\left\{\sum_{A_j\to B_j\in\mathscr{R},B_j=S_i} v_j-\sum_{A_j\to B_j\in\mathscr{R},A_j=S_i} v_j\right\}\varepsilon_i\\&=\sum_{i=1}^{n}\left\{\sum_{j=1}^{m}\delta_{B_jS_i}v_j-\sum_{j=1}^{m}\delta_{A_jS_i}v_j\right\}\varepsilon_i\end{aligned}$$

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$$= \sum_{j=1}^{m} \left\{ \sum_{i=1}^{n} \delta_{B_j S_i} \varepsilon_i - \sum_{i=1}^{n} \delta_{A_j S_i} \varepsilon_i \right\} v_j$$

$$\triangleq \sum_{j=1}^{m} (\beta_j - \alpha_j) v_j,$$

where

$$\delta_{MS_i} = \begin{cases} 1 & \text{if } M = S_i, \\ 0 & \text{otherwise,} \end{cases}$$

and the column vectors $\beta_j = \sum_{i=1}^{n} \delta_{B_j S_i} \varepsilon_i$ and $\alpha_j = \sum_{i=1}^{n} \delta_{A_j S_i} \varepsilon_i$ in $\mathbb{R}^n$ are the coordinates of the vectors B_j and A_j in $\mathbb{R}^{\mathscr{S}}$ with respect to the basis $\mathscr{S}$, respectively. Denote

$$\mathscr{R}_1 = \{A_j \to B_j : A_j \text{ is an input node}\},$$

$$\mathscr{R}_2 = \{A_j \to B_j : A_j \text{ is not an input node}\}.$$

Then, we can equivalently convert the model (3) into the following form:

$$\frac{\mathrm{d}C}{\mathrm{d}t} = R(C; P) = \sum_{j=1}^{m} (\beta_j - \alpha_j) v_j$$

$$= \sum_{j, A_j \to B_j \in \mathscr{R}_1} (\beta_j - \alpha_j) v_j - \sum_{j, A_j \to B_j \in \mathscr{R}_2} (\beta_j - \alpha_j) v_j. \tag{4}$$

Now, we can go on the model analysis based on the new equivalent model (4).

Proposition 3.1
Let $F : \mathbb{R}^n \to \mathbb{R}^n$ be a map, and D be a subset of $\mathbb{R}^n$. For a system of ODEs

$$\frac{\mathrm{d}x}{\mathrm{d}t} = F(x), \tag{5}$$

if F (also called the vector field of the system) is injective in D, then the system cannot admit multiple equilibria in D, i.e. the equations $F(x) = 0$ have no more than one root in D.

Proof
Assume, on the contrast, that the system could admit multiple equilibria in D. Then, there would exist $x_1, x_2 \in D$ $(x_1 \neq x_2)$ such that $F(x_1) = 0 = F(x_2)$, which implies that $F(\cdot)$ is not injective. This contradicts the assumption. Thus, the proposition is true. □

Proposition 3.1 provides us a sufficient condition to rule out multiple equilibria, but it is difficult to verify. Thus, we would like to equivalently transform this condition into a verifiable one.

Lemma 3.1
Suppose n and m be some fixed positive integers. Let $D \subset \mathbb{R}^n$ be an open set, and $\mathscr{R} = \{(\alpha_j, \beta_j) : \alpha_j, \beta_j \in \mathbb{R}^n, j = 1, \ldots, m\}$, $\{N_1, N_2\}$ be a partition of $N = \{1, \ldots, m\}$, i.e. $N_1 \cap N_2 = \emptyset$ and $N_1 \cup N_2 = N$, and $\{r_k : k \in N_2, r_k \in \{1, \ldots, n\}\}$ be a sequence. Let $F(\cdot, p) : \mathbb{R}^n \to \mathbb{R}^n$ be a map of the following form:

$$F(x, p) = \sum_{k \in N_1} (\beta_k - \alpha_k) a_k f_k(u_k, p_k) + \sum_{k \in N_2} (\beta_k - \alpha_k) a_k f_k(x_{r_k}, p_k), \tag{6}$$

where $x \in \mathbb{R}^n$, $u_k \in \mathbb{R}$, $a_k \in \mathbb{R}_+ = (0, \infty)$, p_k can be either real scalar or vector, $p = (\ldots, u_k, a_k, p_k, \ldots)$. Assume that for any fixed p_k, the function $f_k(\cdot, p_k) : \mathbb{R}_+ \to \mathbb{R}_+$ is increasing and continuously differentiable. Then the Jacobian matrix of F with respect to x is nonsingular everywhere on D for any p equivalent to F that is injective on D for any p.

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Proof of this lemma is given in Appendix A.

Remark 3.1
The result in Lemma 3.1 is not true for a general function F. In fact, there is some similarity between Lemma 3.1 and the Jacobian conjecture. The Jacobian conjecture was first proposed by Keller in 1939: letting $F:\mathbb{R}^n\to\mathbb{R}^n$ be a polynomial function, if the determinant of the Jacobian matrix of $F(x)$ is nonzero everywhere, then $F(\cdot)$ has a polynomial inverse [33]. If the Jacobian conjecture is true, then the nonsingularity of the Jacobian matrix of F implies the injectivity of F. After more than 50 years, Pinchuk gave a counterexample by constructing a bivariate polynomial $F(x,y)=(p(x,y),q(x,y))^{\mathrm{T}}$ and showed that the Jacobian matrix of F is nonsingular, but F is not injective [34, 35]. However, for the particular class of functions in Lemma 3.1, we can prove that its injectivity and the nonsingularity of its Jacobian matrix are equivalent.

To derive the main result, we need the following lemma, whose proof is given in Appendix B.

Lemma 3.2
For an SSN module $\mathscr{S}\times\mathscr{R}$, assume $\mathscr{S}=\{S_1,\ldots,S_n\}$ and $\mathscr{R}=\{A_j\to B_j : j=1,\ldots,m\}$, where n and m are some fixed positive integers. Let $\mathbb{R}^{\mathscr{S}}=\{\sum_{i=1}^n z_i S_i : z_i\in\mathbb{R}\}$, and the column vectors β_j and α_j in $\mathbb{R}^n$ be the coordinates of the vectors B_j and A_j in $\mathbb{R}^{\mathscr{S}}$ with respect to the basis $\mathscr{S}$, respectively. Then

(i) for any group of reactions $\{A_{j_1}\to B_{j_1},\ldots,A_{j_n}\to B_{j_n}\}\subset\mathscr{R}_2$, if $A_{j_k}=S_k$ for all $k=1,\ldots,n$, then
$$\det(\beta_{j_1}-\alpha_{j_1},\ldots,\beta_{j_n}-\alpha_{j_n})=0 \quad \text{or } (-1)^n;$$
(ii) if the module has both input and output nodes, then there exists a group of reactions $\{A_{j_1}\to B_{j_1},\ldots,A_{j_n}\to B_{j_n}\}\subset\mathscr{R}_2$ such that $A_{j_k}=S_k$ and
$$\det(\beta_{j_1}-\alpha_{j_1},\ldots,\beta_{j_n}-\alpha_{j_n})=(-1)^n.$$

Remark 3.2
Actually, $\alpha_{j_k}=\varepsilon_k$ when $A_{j_k}=S_k$.

Now, we can give the main result.

Theorem 3.1
Suppose that an SSN metabolic module $\mathscr{S}\times\mathscr{R}$ has both input and output nodes, and is modeled as (3). Then it cannot admit multiple equilibria, i.e. $R(C;P)=0$ has no more than one set of positive solution for any fixed $P\in\mathbb{R}^l_+$, where l is the corresponding dimension of the parameter P.

Proof
It suffices to prove the theorem for the equivalent system (4). By Proposition 3.1 and Lemma 3.1, it suffices to show that the Jacobian matrix $\partial R(C;P)/\partial C$ of the rate function $R(C;P)$ in (4) is nonsingular for any $P\in\mathbb{R}^l_+$ and $C\in\mathbb{R}^n_+$, i.e.
$$\det\left(\frac{\partial R(C;P)}{\partial C}\right)\neq 0 \quad \forall P\in\mathbb{R}^l_+, \quad C\in\mathbb{R}^n_+. \tag{7}$$

Assume that $\mathscr{S}=\{S_1,\ldots,S_n\}$ and $\mathscr{R}=\{A_j\to B_j : j=1,\ldots,m\}$, where n and m are some appropriate positive integers. Then
$$\det\left(\frac{\partial R}{\partial C}\right)=\det\left(\frac{\partial R}{\partial C_1},\ldots,\frac{\partial R}{\partial C_n}\right)=\det\left(\sum_{j_1\in\mathscr{N}_1}(\beta_{j_1}-\alpha_{j_1})v'_{j_1},\ldots,\sum_{j_n\in\mathscr{N}_n}(\beta_{j_n}-\alpha_{j_n})v'_{j_n}\right)$$
$$=\sum_{j_1\in\mathscr{N}_1}\cdots\sum_{j_n\in\mathscr{N}_n}\prod_{k=1}^n v'_{j_k}\det(\beta_{j_1}-\alpha_{j_1},\ldots,\beta_{j_n}-\alpha_{j_n}), \tag{8}$$

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where

$$v'_{j_k} = \frac{\mathrm{d}v_{j_k}}{\mathrm{d}C_k} = \frac{\mathrm{d}v_{j_k}}{\mathrm{d}C_{A_{j_k}}},$$

$$\mathscr{N}_k = \{j_k : j_k \in \{1, \ldots, m\}, A_{j_k} \to B_{j_k} \in \mathscr{R}_2, A_{j_k} = S_k\}, \quad k = 1, \ldots, n.$$

When $V_{\max j_k}$, K_{j_k}, $C_k \triangleq C_{A_{j_k}}$ and n_{j_k} are all positive, so is the term $\prod_{i=1}^{n} v'_{j_k}$. (8) and Lemma 3.2 lead to

$$\begin{aligned} &\det\left(\frac{\partial R(C;P)}{\partial C}\right) > 0, \quad n \text{ is even} \\ &\det\left(\frac{\partial R(C;P)}{\partial C}\right) < 0, \quad n \text{ is odd} \end{aligned} \qquad \forall P \in \mathbb{R}^l_+, \ C \in \mathbb{R}^n_+,$$

which implies that (7) is true. Thus, the theorem is true. □

For an SSN metabolic module with both input and output nodes, Theorem 3.1 tells us that the system (3) cannot admit multiple equilibria when the metabolites of the input nodes can have a constant supplement from external sources. Together with the discussion at the beginning of this section, we can conclude that the SSN metabolic module with both input and output nodes cannot admit multiple equilibria, regardless of the existence and the external sources for the metabolites of input nodes.

Since metabolic networks in living organisms have both input and output, the SSN modules in living organisms cannot admit multiple equilibria, and thus, is not multi-stable.

Remark 3.3

The result in Theorem 3.1 does not require that the reaction rate must be described by the Hill kinetics. Actually, the expression of the reaction rate could be relaxed to a product of a parameter and a monotone increasing function, i.e. satisfying the condition in Lemma 3.1.

4. APPLICATION

One application of the above result is to rule out the multi-stability of any SSN module in a metabolic network. Moreover, when some subnetwork can be viewed as an SSN module approximately, the result is still valid. We now take the central carbon metabolism [36–38] in *E. coli* as an example.

Figure 2 shows a subnetwork in the central carbon metabolism of *E. coli*, which includes 17 reactions. Each node represents one metabolite. Metabolite nomenclatures read GA3P: glyceraldehyde-3-phosphate; DPG: 1,3-diphosphoglycerate; NAD: nicotinamide adenine dinucleotide; NADH: dihydronicotinamide adenine dinucleotide; ADP: adenosine-5′-diphosphate; ATP: adenosine-5′-triphosphate; 3PG: 3-phosphoglycerate; 2PG: 2-phosphoglycerate; PEP: phosphoenolpyruvate; PYR: pyruvate; OAA: oxaloacetate; NADP: nicotinamide adenine dinucleotide phosphate; NADPH: dihydronicotinamide adenine dinucleotide phosphate; Fum: fumarate; Q: quinine; QH_2: quinol; Mal: malate; Cit: citrate; Suc: succinate; CoA: coenzyme A; SucCoA: succinyl CoA; iCit: isocitrate; KG: a-ketoglutarate; Ac: cis-aconitate.

Pi, HCO_2^-, Q, QH_2, NAD^+, $NADP^+$, NADH, NADPH, ADP, and ATP on the edges are actually small molecules or energy storage molecules in the cell, whose concentrations can be generally taken as constants. This means that their concentrations can be viewed as parameters in the model. Hence, all of the reactions can be viewed as SSN reactions, except the reaction $OAA + AcCoA \to Cit + CoA$. We approximately take $OAA + AcCoA$ as a complex, denoted by $OAAa$. Then, the metabolic network in Figure 2 can be reduced to an SSN module shown in Figure 3. Therefore, based on Theorem 3.1, it cannot admit multiple equilibria, regardless of the parameter values. In other words, the network is not a multi-stable system.

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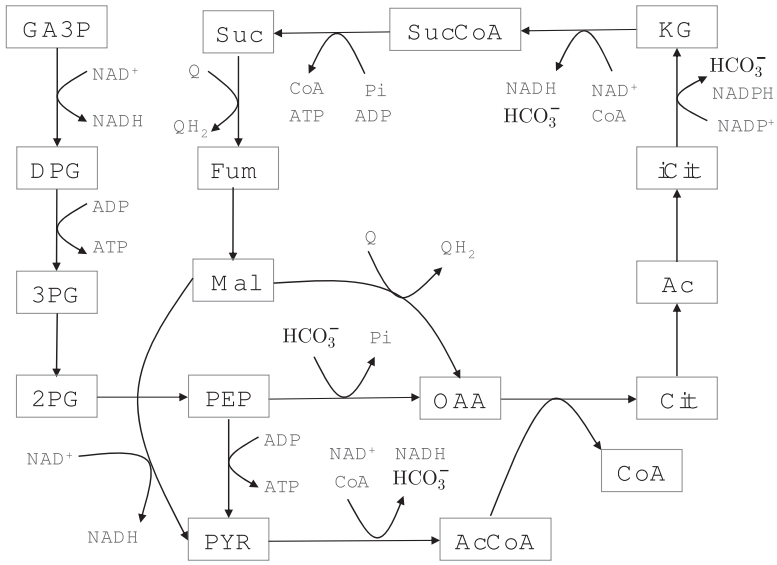

Figure 2. A subnetwork of *E. coli* central carbon metabolism. An arrow represents a reaction. The symbols at the beginning of an arrow are substrates, and those at the head of an arrow are products. The enzymes of these reactions are omitted to simplify the representation.

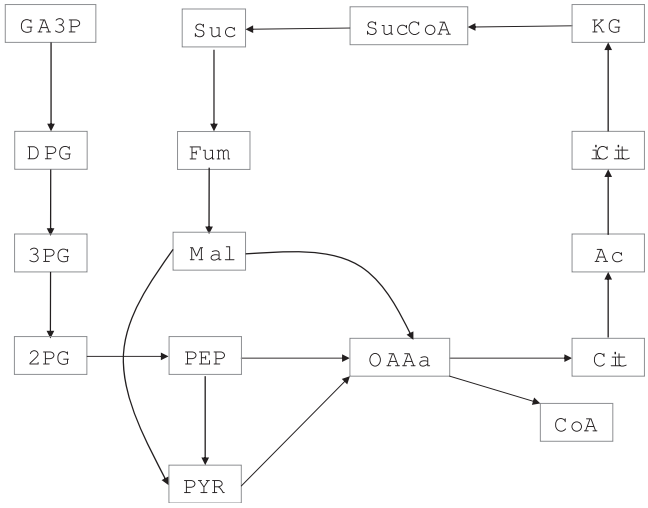

Figure 3. The SSN metabolic module in *E. coli* central carbon metabolism, which is reduced from Figure 2.

5. CONCLUDING REMARKS

To reduce the complexity and make a full use of the structure information in studying whether or not a metabolic network can admit multiple equilibria, we proposed a structure-oriented modularization framework and decomposed a general metabolic network into four types of basic building blocks (i.e. SSN, SSI, MMN, and MMI modules). With this idea, we intended to study the original network through investigating the characteristics of the basic modules and their interactions.

In this paper, we modeled and investigated one kind of the basic building blocks—SSN modules. The Hill kinetics, which is a widely accepted expression in biological systems, has been adopted to describe the rate of an enzyme-catalyzed metabolic reaction. In particular, we modeled a general

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SSN metabolic module by a set of nonlinear ODEs without requiring detailed information about intermediate processes of the metabolic reactions.

We first gave a sufficient condition to rule out multiple equilibria of a general dynamical system described by a set of ODEs, i.e. the vector field of the system is injective. For an SSN module, by using the particularity of its topological structure we proved that the injectivity of the vector field of the model and the nonsingularity of its Jacobian matrix are equivalent, and then converted the unverifiable sufficient condition into a verifiable one. We also proved that if an SSN module has both input and output nodes, then the corresponding model satisfies this condition, and thereby cannot admit multiple equilibria. Since the metabolism in living organisms must have input and output, this result implies that it cannot be multi-stable. In addition to applying the result accurately, we also gave a biological example to show how to apply it approximately in an metabolic network. Note that the theoretical result in this paper is effective even for the case without the detailed information about the stoichiometric matrix.

As the first step to elucidate the essential mechanism of a general metabolic network in living organisms, this paper mainly focused on the analysis of one of the four types of basic building blocks, i.e. SSN module. To reveal the basic design principle and dynamical properties of metabolic networks, we will further study the other three types of basic building blocks (SSI, MMN, and MMI modules) and their interactions.

APPENDIX A: PROOF OF LEMMA 3.1

Proof

Denote

$$f_k'(x_{r_k}, p_k) = \frac{\partial f_k(x_{r_k}, p_k)}{\partial x_{r_k}}.$$

Then

$$\frac{\partial F(x, p)}{\partial x_i} = \sum_{k \in N_2} (\beta_k - \alpha_k) a_k \delta_{r_k i} f_k'(x_{r_k}, p_k),$$

where

$$\delta_{r_k i} = \begin{cases} 1 & \text{if } r_k = i, \\ 0 & \text{otherwise.} \end{cases}$$

Let $y = (y_1, \ldots, y_n)^{\mathrm{T}} \in \mathbb{R}^n$ be a column vector. Note that the Jacobian matrix $\partial F/\partial x = (\partial F/\partial x_1, \ldots, \partial F/\partial x_n)$. Then

$$\begin{aligned} \frac{\partial F(x, p)}{\partial x} y &= \sum_{i=1}^{n} y_i \frac{\partial F(x, p)}{\partial x_i} \\ &= \sum_{k \in N_2} (\beta_k - \alpha_k) y_{r_k} a_k f_k'(x_{r_k}, p_k). \end{aligned} \tag{A1}$$

With the fact that $\det(\partial F(x, p)/\partial x) = 0$ if and only if there exists a non-zero vector $y \in \mathbb{R}^n$ such that $(\partial F(x, p)/\partial x) y = 0$ in mind, we will first show the necessity. Now, we have that $F(x, p)$ is injective on D for any p. Assume that Lemma 3.1 was not true. Then there would exist $\bar{x}$, $\bar{p}$ and a non-zero vector $\bar{y}$ such that

$$\frac{\partial F(\bar{x}, \bar{p})}{\partial x} \bar{y} = 0. \tag{A2}$$

Take $\tilde{n}_k = \bar{n}_k$ and $\tilde{u}_k = \bar{u}_k$. Define $\tilde{x}$ and $\hat{x}$ as follows:

$$\tilde{x}_i = \begin{cases} 2\bar{x}_i, & \bar{y}_i > 0, \\ \bar{x}_i, & \bar{y}_i = 0, \\ \frac{1}{2}\bar{x}_i, & \bar{y}_i < 0, \end{cases} \qquad \hat{x}_i = \begin{cases} \frac{1}{2}\bar{x}_i, & \bar{y}_i > 0, \\ \bar{x}_i, & \bar{y}_i = 0, \\ 2\bar{x}_i, & \bar{y}_i < 0. \end{cases}$$

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Then $\bar{y}$ is a non-zero vector implies $\tilde{x} \neq \hat{x}$. Denote

$$Z_k = f_k(\tilde{x}_{r_k}, \tilde{p}_k) - f_k(\hat{x}_{r_k}, \tilde{p}_k). \tag{A3}$$

Since for any fixed p_k, if the function $f_k(\cdot, p_k)$ is increasing and continuously differentiable, then Z_k has the same sign as $\bar{y}_{r_k}$, and $f_k'(\bar{x}_{r_k}, \bar{p}_k) > 0$. Thus, for any $\tilde{p}_k$, there exists $\tilde{a}_k > 0$ such that

$$\tilde{a}_k Z_k = \bar{y}_{r_k} \bar{a}_k f_k'(\bar{x}_{r_k}, \bar{p}_k). \tag{A4}$$

Combining with (A1), (A2), and (A3), we can get

$$\sum_{k \in N_2} (\beta_k - \alpha_k) \tilde{a}_k f_k(\tilde{x}_{r_k}, \tilde{p}_k) = \sum_{k \in N_2} (\beta_k - \alpha_k) \tilde{a}_k f_k(\hat{x}_{r_k}, \tilde{p}_k), \tag{A5}$$

or equivalently,

$$\begin{aligned} &\sum_{k \in N_1} (\beta_k - \alpha_k) \tilde{a}_k f_k(\tilde{u}_k, \tilde{p}_k) + \sum_{k \in N_2} (\beta_k - \alpha_k) \tilde{a}_k f_k(\tilde{x}_{r_k}, \tilde{p}_k) \\ &\quad = \sum_{k \in N_1} (\beta_k - \alpha_k) \tilde{a}_k f_k(\tilde{u}_k, \tilde{p}_k) + \sum_{k \in N_2} (\beta_k - \alpha_k) \tilde{a}_k f_k(\hat{x}_{r_k}, \tilde{p}_k). \end{aligned}$$

That is $F(\tilde{x}, \tilde{p}) = F(\hat{x}, \tilde{p})$. Thus, $F(x, \tilde{p})$ is not injective. It contradicts the condition. Hence, the necessity is true.

Next, we will show the sufficiency. Now, we have $\det(\partial F(x, p)/\partial x) \neq 0$ for all $x \in D$ and any p. Assume that $F(x, \tilde{p})$ was not injective for some $\tilde{p}$. Then, there would exist $\tilde{x}$ and $\hat{x}$ in D ($\tilde{x} \neq \hat{x}$) such that

$$F(\tilde{x}, \tilde{p}) = F(\hat{x}, \tilde{p}),$$

or equivalently, (A5) holds.

Take $\bar{n}_k = \tilde{n}_k$ and $\bar{u}_k = \tilde{u}_k$. Define $\bar{x}$ and $\bar{y}$ as follows:

$$\bar{x}_i = \tfrac{1}{2}(\tilde{x}_i + \hat{x}_i),$$

$$\bar{y}_i = \begin{cases} 1, & \tilde{x}_i > \hat{x}_i, \\ 0, & \tilde{x}_i = \hat{x}_i, \\ -1, & \tilde{x}_i < \hat{x}_i. \end{cases}$$

Then $\tilde{x} \neq \hat{x}$ implies that $\bar{y}$ is a non-zero vector. Similarly, we can show that Z_k and $\bar{y}_{r_k}$ have the same sign. Thus, there exists $\bar{a}_k > 0$ such that (A4) holds. Combining with (A1), (A3), and (A5), we have

$$\det\left(\frac{\partial F(\bar{x}, \bar{p})}{\partial x}\right) = 0,$$

which contradicts the condition. Thus, the sufficiency is also true. □

APPENDIX B: PROOF OF LEMMA 3.2

Proof

We first show the item (i) of the lemma. Note that $\alpha_{j_k} = \varepsilon_k$ when $A_{j_k} = S_k$. Then, the matrix $(\beta_{j_1} - \alpha_{j_1}, \ldots, \beta_{j_n} - \alpha_{j_n})$ has the following form:

$$A = (a_{ij})_{n \times n} = \begin{pmatrix} -1 & * & \ldots & * & * \\ * & -1 & \ldots & * & * \\ \vdots & \vdots & & \vdots & \vdots \\ * & * & \ldots & -1 & * \\ * & * & \ldots & * & -1 \end{pmatrix}. \tag{B1}$$

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In the rest part of this proof, we will replace A_{j_k} by S_k and write α_{j_k} as ε_k. Since B_{j_k} is the product of reaction $S_k \to B_{j_k}$, it cannot be an input node. For a fixed k, if $B_{j_k} = S_i$, then $a_{ik} = 1$ and $a_{pk} = 0$ $(p \neq i, k)$ in matrix A; otherwise, if B_{j_k} is an output node, then $\beta_{j_k} = (0, \ldots, 0)^{\mathrm{T}}$, consequently $\beta_{j_k} - \alpha_{j_k} = \alpha_{j_k} = -\varepsilon_k$, which implies $a_{pk} = 0$ $(p \neq k)$.

Our objective is to show $\det A$ is either $(-1)^n$ or 0. The main idea of the proof is to transform the matrix A into a block matrix $\begin{pmatrix} Q & \\ & -I_{n-q} \end{pmatrix}$ by some elementary operations, where $\det Q = 0$ if $q \neq 0$ and $-I_{n-q}$ is the $(n-q) \times (n-q)$ identity matrix. The procedure of the transformation is as follows.

Step 1: Let $B = (b_{ij})_{n \times n} = A$.

Step 2: For matrix B, if 1 there exists a $k \in \{1, \ldots, n\}$ such that $b_{kk} = -1$ and $b_{ik} = 0 (i \neq k)$, and 2 there exists $j \in \{1, \ldots, n\}$ and $j \neq k$ such that $b_{kj} \neq 0$, then, add a scalar multiple of column k to the other columns to make the entries in row k except that the (k, k) position to be 0, and we have

$$\begin{pmatrix} -1 & * & \ldots & 0 & \ldots & * & * \\ * & -1 & \ldots & 0 & \ldots & * & * \\ \vdots & \vdots & & \vdots & & \vdots & \vdots \\ 0 & 0 & \ldots & -1 & \ldots & 0 & 0 \\ \vdots & \vdots & & \vdots & & \vdots & \vdots \\ * & * & \ldots & 0 & \ldots & -1 & * \\ * & * & \ldots & 0 & \ldots & * & -1 \end{pmatrix}.$$

Step 3: If the obtained matrix satisfies the two conditions in Step 2, denote it by B again and return to Step 2; otherwise, go to Step 4.

Step 4: If the obtained matrix is not $-I_n$ (I_n is the $n \times n$ identity matrix), then by switching the positions of two rows in the matrix, we can transform it into a block matrix of the form,

$$\begin{pmatrix} -1 & * & \ldots & * & 0 & \ldots & 0 \\ * & -1 & \ldots & * & 0 & \ldots & 0 \\ \vdots & \vdots & & \vdots & \vdots & & \vdots \\ * & * & \ldots & -1 & 0 & \ldots & 0 \\ 0 & 0 & \ldots & 0 & -1 & \ldots & 0 \\ \vdots & \vdots & & \vdots & \vdots & & \vdots \\ 0 & 0 & \ldots & 0 & 0 & \ldots & -1 \end{pmatrix} \triangleq \begin{pmatrix} Q & 0_{q \times (n-q)} \\ 0_{(n-q) \times q} & -I_{n-q} \end{pmatrix}, \qquad \text{(B2)}$$

where $1 \leqslant q \leqslant n$, $0_{(n-q) \times q}$ and $0_{q \times (n-q)}$ are $(n-q) \times q$ and $q \times (n-q)$ zero matrices, respectively. I_{n-q} is the $(n-q) \times (n-q)$ identity matrix.

From the definition of β_{j_k} and α_{j_k}, one can see that matrix $Q = (q_{ij})_{q \times q}$ in (B2) has the following features:

(i) $q_{ii} = -1$, $i = 1, \ldots, q$,
(ii) for any fixed j, there exists one and only one number i such that $q_{ij} = 1$ and $q_{kj} = 0$ $(k \neq i, j)$.

Thus, the sum of each column in Q is 0. This leads to

$$\det(Q) = 0.$$

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Note that the column operations in Step 2 do not change the determinant of the original matrix A. Thus, if the matrix got from Step 3 is $-I_n$, then

$$\det(\beta_{j_1}-\alpha_{j_1},\ldots,\beta_{j_n}-\alpha_{j_n})=\det A=\det(-I_n)=(-1)^n;$$

otherwise, we need to continue the row operations in Step 4 and transform it into the block form (B2). Because the row operations in Step 4 change only the sign of the determinant of the original matrix A, we have

$$|\det(\beta_{j_1}-\alpha_{j_1},\ldots,\beta_{j_n}-\alpha_{j_n})|=|\det A|=\left|\det\begin{pmatrix} Q & 0_{q\times(n-q)} \\ 0_{(n-q)\times q} & -I_{n-q}\end{pmatrix}\right|=|\det(Q)\det(-I_{n-q})|=0.$$

That is

$$\det(\beta_{j_1}-\alpha_{j_1},\ldots,\beta_{j_n}-\alpha_{j_n})=0.$$

Next, we will show the item (ii) of the lemma. Denote $A/B=A\cap B^c$ the set excluding the elements of set B from A. A node S is called an upper state node of node M, if S is a state node, and there exists a reaction $S\to M$ from S to M. All the upper state nodes of M constitute the upper state node set of M, denoted by

$$\mathscr{U}(M)=\{S: S\in\mathscr{S}, S\to M\in\mathscr{R}\}. \tag{B3}$$

If M is an output node, then all the elements of $\mathscr{U}(M)$ are end nodes. If M is a head node or an input node, then $\mathscr{U}(M)$ is an empty set. Assume that there are l output nodes of the SSN module $\mathscr{S}\times\mathscr{R}$, denoted by $\{P_1,\ldots,P_l\}$.

Now, we will find a group of n reactions satisfying the item (ii) of the lemma. The main idea is to construct a reaction path backwards from an output node to an input node. If the path does not contain all state nodes, then repeat the construction procedure. Then we can show that such an group of reactions is what we need.

Step 1: Take $r=1$, $i=0$.

Step 2: Let $M_0\triangleq P_1$ and $N_1\triangleq M_0$. Take one of the upper state nodes of M_0 and denote it by M_1. Set $i=1$.

Step 3: If $i\leqslant n-1$ and $\mathscr{U}(M_i)/\{M_i,\ldots,M_1\}\neq\emptyset$, then take one element of the set $\mathscr{U}(M_i)/\{M_i,\ldots,M_1\}$ and denote it by M_{i+1}; set $N_{i+1}\triangleq M_i$ and replace i by $i+1$, go to Step 4. Otherwise, if $i\leqslant n-1$ and $\mathscr{U}(M_i)/\{M_i,\ldots,M_1\}=\emptyset$, then set $j=i$ and go to Step 5.

Step 4: If $i=n$, terminate the procedure; otherwise, return to Step 3.

Step 5: If there exists $k\leqslant j$ such that $\mathscr{U}(M_j)/\{M_j,\ldots,M_1\}=\emptyset,\ldots,\mathscr{U}(M_{j-k+1})/\{M_j,\ldots,M_1\}=\emptyset,\mathscr{U}(M_{j-k})/\{M_j,\ldots,M_1\}\neq\emptyset$, then take one element of the set $\mathscr{U}(M_{j-k})/\{M_j,\ldots,M_1\}$ and denote it by M_{j+1}; set $N_{j+1}\triangleq M_j$ and $i\triangleq j+1$; if $i=n$, terminate the procedure; if $i\leqslant n-1$, return to Step 3. Otherwise, if $\mathscr{U}(M_{j-k+1})/\{M_j,\ldots,M_1\}=\emptyset$ for all $k\leqslant j$, replace i and r by j and $r+1$, respectively, go to Step 6.

Step 6: If $r>l$, then terminate the procedure. If $r\leqslant l$, let $M_0\triangleq P_r$. If $\mathscr{U}(M_0)/\{M_i,\ldots,M_1\}\neq\emptyset$, then take one element of the set $\mathscr{U}(M_0)/\{M_i,\ldots,M_1\}$ and denote it by M_{i+1}, and set $N_{i+1}\triangleq M_i$ and replace i by $i+1$, return to Step 3; otherwise, replace r by $r+1$, and repeat this step.

Through the above procedure, we select n_0 state nodes $\{M_1,\ldots,M_{n_0}\}\triangleq\mathscr{M}$. We now show $\mathscr{M}=\mathscr{S}$, i.e. $n_0=n$. Note that the procedure can be terminated only at Step 4, Step 5, or Step 6. If it is terminated at Step 4 or Step 5, then it is obvious that $n_0=n$. If it is terminated at Step 6, by the conditions of the procedure in Step 5 and Step 6, we have $\mathscr{U}(P_r)/\mathscr{M}=\emptyset(\forall r\in\{1,\ldots,l\})$ and $\mathscr{U}(M_k)/\mathscr{M}=\emptyset(\forall k\in\{1,\ldots,n_0\})$. Consequently

$$\left(\bigcup_{r=1}^{l}\mathscr{U}(P_r)\right)\cup\left(\bigcup_{k=1}^{n_0}\mathscr{U}(M_k)\right)\subset\mathscr{M}. \tag{B4}$$

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Assume, on the contrast, that $n_0<n$. Then there would exist $S_i\in\mathscr{S}$ but $S_i\notin\mathscr{M}$. By the item (iv) of Definition 2.5, there is a directed path from S to some output node P, which implies

$$S_i\in\left(\bigcup_{r=1}^{l}\mathscr{U}(P_r)\right)\cup\left(\bigcup_{k=1}^{n_0}\mathscr{U}(M_k)\right).$$

This together with (B4) implies $S_i\in\mathscr{M}$. It is a contradiction. Hence, $n_0=n$.

Therefore, we can find a group of n reactions $\{M_1\to N_1,\ldots,M_n\to N_n\}$ satisfying $N_i\in\{M_1,\ldots,M_{i-1}\}\cup\{P_1,\ldots,P_l\}$ $(i=1,\ldots,n)$. In fact, $\{M_1,\ldots,M_n\}$ is a permutation of $\{S_1,\ldots,S_n\}$, so $\{M_1,\ldots,M_n\}$ is also a basis of vector space $\mathbb{R}^{\mathscr{S}}$. Let the column vectors $\tilde{\beta}_k$ and $\tilde{\alpha}_k$ in $\mathbb{R}^n$ be the coordinates of the vectors N_k and M_k in $\mathbb{R}^{\mathscr{S}}$ with respect to the basis $\{M_1,\ldots,M_n\}$. Then

$$(\tilde{\beta}_1-\tilde{\alpha}_1,\ldots,\tilde{\beta}_n-\tilde{\alpha}_n)=\begin{pmatrix}-1 & * & \ldots & * & *\\ 0 & -1 & \ldots & * & *\\ \vdots & \vdots & & \vdots & \vdots\\ 0 & 0 & \ldots & -1 & *\\ 0 & 0 & \ldots & 0 & -1\end{pmatrix},$$

which gives

$$\det(\tilde{\beta}_1-\tilde{\alpha}_1,\ldots,\tilde{\beta}_n-\tilde{\alpha}_n)=(-1)^n.$$

Denote by σ the permutation from $\{S_1,\ldots,S_n\}$ to $\{M_1,\ldots,M_n\}$, i.e. $M_k=\sigma(S_k)=S_{\sigma(k)}$, rewrite $\{M_1\to N_1,\ldots,M_n\to N_n\}$ as

$$\{S_{\sigma(1)}\to N_1,\ldots,S_{\sigma(n)}\to N_n\}.$$

Adjusting the order of the above reactions appropriately gives

$$\{S_1\to B_{j_1},\ldots,S_n\to B_{j_n}\},$$

where B_{j_k} represents some N_i. The only difference between the values of the determinants $\det(\beta_{j_1}-\varepsilon_1,\ldots,\beta_{j_n}-\varepsilon_n)$ and $\det(\tilde{\beta}_1-\tilde{\alpha}_1,\ldots,\tilde{\beta}_n-\tilde{\alpha}_n)=(-1)^n$ lies in their signs. This together with the item (i) of the lemma renders

$$\det(\beta_{j_1}-\varepsilon_1,\ldots,\beta_{j_n}-\varepsilon_n)=(-1)^n.$$

□

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