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Determination of Multiple Steady States in Oxidation of Monophenols by Tyrosinase with Enzymatic-Enzymatic-Chemical Model

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Abstract−A family of an enzymatically catalyzed reaction network is studied, which involves the oxidation of monophenols by tyrosinase with enzymatic-enzymatic-chemical model in an isothermal continuous flow stirred tank reactor (CFSTR). This system consists of 11 coupled non-linear equations and is determined to have the capacity to exhibit computational multiple steady states. A set of rate constants and two corresponding steady states are computed. The phenomena of bistability, hysteresis and bifurcation are discussed. Moreover, the capacity of steady state multiplicity is extended to its family of reaction networks.

Key words: Multiple Steady States, Tyrosinase, Enzymatic-Enzymatic-Chemical, Hysteresis, Bifurcation

INTRODUCTION

Exotic dynamic phenomena, such as unstable steady states, undamped oscillations, multiple steady states and chaos, can be predicted in non-isothermal chemical reaction systems involving simple reactions [Chang et al., 1989; Kim and Rhee, 1989; Kim et al., 1989]. In isothermal chemical systems some nonlinear dynamics have been shown experimentally [Epelboin et al., 1972; Geiseler and Bar-Eli, 1981; Orbán and Epstein, 1985; Dutt and Müller, 1996]. This indicates that instabilities derive from the intricacy of chemistry itself, instead of from thermal effects for non-isothermal systems. It is important for the chemical engineer to be able to identify chemical systems that have capacity to exhibit multiple steady states, since such an identification helps design safer and more efficient reactors.

Biochemical systems, which usually consist of many species and reactions, can also give rise to those complex reaction behaviors [Hatzimanikatis and Bailey, 1997; Bailey, 1998; Li, 1998; Hynne et al., 2001]. Chemical reaction network theory studies connections between reaction network structure and capacity of unstable behavior generated by the corresponding isothermal differential equations. Reaction networks are classified by a non-negative integer index called the deficiency, which is determined from the reaction network structure [Feinberg, 1987] (The calculation of the deficiency of a network is briefly mentioned in Appendix). The Deficiency Zero Theorem, developed by Horn [1972], Horn and Jackson [1972], and Feinberg [1972, 1977], indicates that all reaction networks of deficiency zero, no matter how complex and no matter what values the rate constants take, admit at most one steady state, that steady state is stable, and there does not exist any cyclic solution. However, networks of deficiency greater than zero have different pictures. For example, deficiency one networks contain members that have capacity to generate multiple steady states and still others that do not. The Deficiency One Theorem [Feinberg, 1987] and the Deficiency One Algorithm [Feinberg, 1988] provide means to distinguish between those two types of mechanisms.

To determine the possibility of multiple steady states in a complex reaction network of high deficiency, one efficient method is to study its subnetworks. A subnetwork of the reaction network under study is just a network that consists of reactions belonging to the original reaction network. High deficiency networks admitting multiple steady states often contain deficiency one subnetworks that also exhibit multiple steady states [Li, 1992]. A modified Subnetwork Analysis [Li, 1998] was proposed, which can be applied to both forest-like and circular reaction networks for the determination of the capacity of multiple steady states, if one of its subnetworks has steady state multiplicity.

Numerous reports on the tyrosinase (EC 1.14.18.1) action mechanism have appeared to explain the characteristics of enzyme activity, and in particular to clarify the presence of the lag period [Mason, 1956; Wilcox et al., 1985; Cabanes et al., 1987; Rodriguez-López et al., 1992; Naish-Byfield et al., 1992]. However, the steady state multiplicity of this system, consisting of 11 coupled non-linear equations, has not been studied so far. In the present work we determine the capacity of computational multiple steady states in a family of oxidation of monophenols by tyrosinase with enzymatic-enzymatic-chemical model in an isothermal CFSTR by using the Deficiency One Algorithm and the Subnetwork Analysis.

THEORETICAL BACKGROUND

1. Reaction Networks and Mass Action Differential Equations

It is well known that the copper-containing enzyme tyrosinase catalyzes two different reactions: the hydroxylation of monophenols to *o*-diphenols (cycle I in Fig. 1) and the oxidation of *o*-diphenols to *o*-quinones (cycle II in Fig. 1). Cabanes et al. [1987] proposed a mechanism (as shown in Fig. 1) involving the combination of these two cycles and a chemical reaction step to explain the appearance of a lag. This model was named an enzymatic-enzymatic-chemical model with substrate regeneration (E_ZE_ZC-S.R.), by analogy with the nomenclature used for electrochemical-chemical reactions. These binuclear copper sites can be prepared in three enzymatic forms: met, oxy and deoxy. In Fig. 1, the symbols E_{met}, E_{oxy}, E_{deoxy}, M, D,

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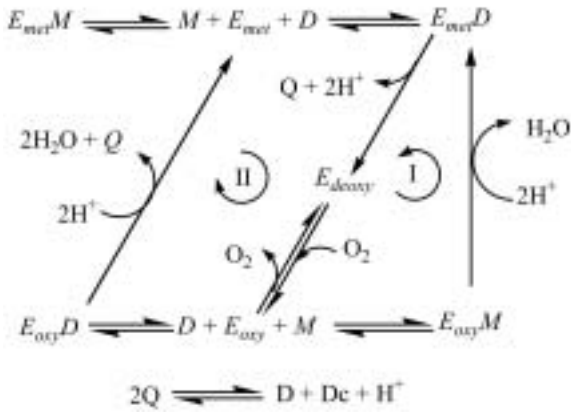

Fig. 1. Kinetic mechanisms for oxidation of monophenols by tyrosinase.

Q and Dc denote the metal form of the enzyme, the oxygen form of the enzyme, the de-oxygen form of the enzyme, the monophenols, the diphenols, the quinones and aminechrome, while $E_{met}M$, $E_{oxy}M$, $E_{met}D$ and $E_{oxy}D$ denote catalyst occupied by species M and D, respectively. In brief, during cycle II, *o*-diphenols bind both to E_{met} and E_{oxy}, rendering $E_{met}D$ and $E_{oxy}D$ intermediates, which give rise to two *o*-quinones. During cycle I, the binding of monophenols to oxy form renders $E_{met}D$. However, the binding of monophenols to E_{met} (with no catalytic activity on monophenols) scavenges a portion of tyrosinase from the catalytic turnover as a dead-end complex.

The chemical recycling step ($2Q \rightleftarrows D + Dc + H^+$) follows these two cycles to regenerate *o*-diphenols and aminechrome, which could be back to the pools of these two cycles. According to Fig. 1, the overall enzymatic-enzymatic-chemical reaction contains the following elementary steps:

$$\begin{aligned}
&E_{met}+M \rightleftarrows E_{met}M\\
&E_{oxy}+M \rightleftarrows E_{oxy}M\\
&E_{met}+D \rightleftarrows E_{met}D \rightleftarrows E_{deoxy}+Q\\
&E_{oxy}+D \rightleftarrows E_{oxy}D \rightleftarrows E_{met}+Q\\
&E_{oxy} \rightleftarrows E_{deoxy}\\
&E_{oxy}M \rightleftarrows E_{met}D\\
&2D \rightleftarrows Q+Dc
\end{aligned} \tag{1}$$

The assumptions used in this work are: 1. The reactions occur in an isothermal CFSTR, 2. The reactor volume is constant, 3. The copper-containing enzyme tyrosinase, both occupied and unoccupied, are all retained in the reactor by using, for example, porous membranes or screens to prevent escape of immobilized enzyme pellets, 4. The concentrations of H_2O, O_2 and in Fig. 1 do not change apparently with time and are viewed as a constant, 5. The elementary steps in Eq. (1) are all chemically reversible and the mass action kinetics are followed. Thus, the oxidation of monophenols by tyrosinase in an isothermal CFSTR is represented by the reaction network (2) and its corresponding dynamical ordinary differential equations are listed in Eq. (3).

$$\begin{aligned}
&A_8 \rightleftarrows 0 \rightleftarrows A_9\\
&A_{10} \rightarrow 0 \leftarrow A_{11}\\
&A_1+A_8 \rightleftarrows A_4\\
&A_2+A_8 \rightleftarrows A_5\\
&A_1+A_9 \rightleftarrows A_6 \rightleftarrows A_3+A_{10}\\
&A_2+A_9 \rightleftarrows A_7 \rightleftarrows A_1+A_{10}\\
&A_2 \rightleftarrows A_3\\
&A_5 \rightleftarrows A_6\\
&2A_{10} \rightleftarrows A_9+A_{11}
\end{aligned} \tag{2}$$

(A_1: E_{met}, A_2: E_{oxy}, A_3: E_{deoxy}, A_4: $E_{met}M$, A_5: $E_{oxy}M$, A_6: $E_{met}D$, A_7: $E_{oxy}D$, A_8: M, A_9: D, A_{10}: Q, A_{11}: Dc, and 0: zero complex)

$$\begin{aligned}
\frac{dc_1}{dt} &= -k_{A_1+A_8\rightarrow A_4}c_1c_8 + k_{A_4\rightarrow A_1+A_8}c_4\\
&\quad - k_{A_1+A_9\rightarrow A_6}c_1c_9 + k_{A_6\rightarrow A_1+A_9}c_6 + k_{A_7\rightarrow A_1+A_{10}}c_7 - k_{A_1+A_{10}\rightarrow A_7}c_1c_{10}\\
\frac{dc_2}{dt} &= -k_{A_2+A_8\rightarrow A_5}c_2c_8 + k_{A_5\rightarrow A_2+A_8}c_5\\
&\quad - k_{A_2\rightarrow A_3}c_2 + k_{A_3\rightarrow A_2}c_3 - k_{A_2+A_9\rightarrow A_7}c_2c_9 + k_{A_7\rightarrow A_2+A_9}c_7\\
\frac{dc_3}{dt} &= k_{A_2\rightarrow A_3}c_2 - k_{A_3\rightarrow A_2}c_3 + k_{A_6\rightarrow A_3+A_{10}}c_6 - k_{A_3+A_{10}\rightarrow A_6}c_3c_{10}\\
\frac{dc_4}{dt} &= k_{A_1+A_8\rightarrow A_4}c_1c_8 - k_{A_4\rightarrow A_1+A_8}c_4\\
\frac{dc_5}{dt} &= k_{A_2+A_8\rightarrow A_5}c_2c_8 - k_{A_5\rightarrow A_2+A_8}c_5 - k_{A_5\rightarrow A_6}c_5 + k_{A_6\rightarrow A_5}c_6\\
\frac{dc_6}{dt} &= k_{A_5\rightarrow A_6}c_5 - k_{A_6\rightarrow A_5}c_6 - k_{A_6\rightarrow A_3+A_{10}}c_6\\
&\quad + k_{A_3+A_{10}\rightarrow A_6}c_3c_{10} + k_{A_1+A_9\rightarrow A_6}c_1c_9 - k_{A_6\rightarrow A_1+A_9}c_6\\
\frac{dc_7}{dt} &= k_{A_2+A_9\rightarrow A_7}c_2c_9 - k_{A_7\rightarrow A_2+A_9}c_7 - k_{A_7\rightarrow A_1+A_{10}}c_7 + k_{A_1+A_{10}\rightarrow A_7}c_1c_{10}\\
\frac{dc_8}{dt} &= -k_{A_1+A_8\rightarrow A_4}c_1c_8 + k_{A_4\rightarrow A_1+A_8}c_4 - k_{A_2+A_8\rightarrow A_5}c_2c_8\\
&\quad + k_{A_5\rightarrow A_2+A_8}c_5 - k_{A_8\rightarrow 0}c_8 + k_{0\rightarrow A_8}\\
\frac{dc_9}{dt} &= -k_{A_2+A_9\rightarrow A_7}c_2c_9 + k_{A_7\rightarrow A_2+A_9}c_7 - k_{A_1+A_9\rightarrow A_6}c_1c_9\\
&\quad + k_{A_6\rightarrow A_1+A_9}c_6 - k_{A_9\rightarrow 0}c_9 + k_{0\rightarrow A_9} + k_{2A_{10}\rightarrow A_9+A_{11}}c_{10}^2 - k_{A_9+A_{11}\rightarrow 2A_{10}}c_9c_{11}\\
\frac{dc_{10}}{dt} &= k_{A_6\rightarrow A_3+A_{10}}c_6 - k_{A_3+A_{10}\rightarrow A_6}c_3c_{10} + k_{A_7\rightarrow A_1+A_{10}}c_7\\
&\quad - k_{A_1+A_{10}\rightarrow A_7}c_1c_{10} - k_{A_{10}\rightarrow 0}c_{10} - 2k_{2A_{10}\rightarrow A_9+A_{11}}c_{10}^2 + 2k_{A_9+A_{11}\rightarrow A_{10}}c_9c_{11}\\
\frac{dc_{11}}{dt} &= k_{2A_{10}\rightarrow A_9+A_{11}}c_{10}^2 - k_{A_9+A_{11}\rightarrow 2A_{10}}c_9c_{11} - k_{A_{11}\rightarrow 0}c_{11}
\end{aligned} \tag{3}$$

where c_i, i=1, 2, …, 11 denote the concentrations of species A_1, A_2, …, A_{11} within the reactor and $k_{i\rightarrow j}$ is a rate constant for reaction i⟶j in network (2).

The last seven lines in Eq. (2) are the elementary steps in mechanism Eq. (1). The first two lines in Eq. (2) display the inflow of reactants (M and D) and the outflow of remaining reactants (M and D) and the product (Q and Dc). In reaction network terms [Feinberg, 1987], to account for the inflow of A_8 and A_9 in the feed stream, the pseudo-reactions $0 \rightarrow A_8$ and $0 \rightarrow A_9$ are added to true chemistry Eq. (1) (The physical meaning of "0" (zero complex) represents the surroundings). Compared with the dynamical equations in Eq. (3), the rate constants $k_{0\rightarrow A_8}$ and $k_{0\rightarrow A_9}$ are assigned, respectively, to be equal to c_8^f/θ and c_9^f/θ. (c_i^f denotes the feed concentration of species i (=8, 9) and θ denotes the residence time). Also to account for the outflow of A_8, A_9, A_{10} and A_{11} in the effluent stream, pseudo-reactions $A_8 \rightarrow 0$, $A_9 \rightarrow 0$, $A_{10} \rightarrow 0$ and $A_{11} \rightarrow 0$ are added to true chemistry Eq. (1). The flow rates $k_{A_8\rightarrow 0}$, $k_{A_9\rightarrow 0}$, $k_{A_{10}\rightarrow 0}$ and $k_{A_{11}\rightarrow 0}$ are all assigned to be equal to the reciprocal of residence time $1/\theta$. Thus, in reaction network terms, we consider the reactions given in Eq. (1) operating in an open system to be modeled by reaction

network (2), instead of Eq. (1).

From Eq. (3), we find that a mass conservation condition must be satisfied, i.e. the summation of concentrations of species A_1, A_2, A_3, A_4, A_5, A_6 and A_7 remains a constant. This means that the total number of enzymes, both occupied and unoccupied, is a constant and it must be satisfied by all the steady states. It is

$$\frac{d(c_1+c_2+c_3+c_4+c_5+c_6+c_7)}{dt}=0. \quad (4)$$

2. Deficiency One Algorithm and Subnetwork Analysis

At steady states, the compositions c_i do not change with time. Therefore, from Eq. (3) we have that $dc_i/dt=0$, for $i=A_1, A_2, \ldots, A_{11}$. The question we are asking is this: Can there exist for network (2) an assignment of residence time θ, rate constants $k_{i\to j}$ and feed concentrations c_i^f such that its corresponding mass action differential Eq. (3) admit more than one steady state? When we say that a reaction network has the capacity to admit multiple steady states, we mean that there is at least one assignment of residence time θ, rate constants $k_{i\to j}$ and feed concentrations c_i^f such that the corresponding (mass action) equations admit at least two steady states. Obviously, it would be difficult to answer this question if one does not have a systematic way to analyze the reaction system (2).

In this paper the Deficiency One Algorithm [Feinberg, 1988] and the Subnetwork Analysis [Li, 1998] are applied to determine the multiplicity of steady states in network (2) and their parent networks. In reaction network terms, each network has a deficiency, which is an integer equal to or greater than zero and can be calculated easily by the structure of a reaction network. The Deficiency One Algorithm provides a necessary and sufficient condition for a deficiency one network to admit multiple steady states. By the analysis of this algorithm, the "signatures" of steady state multiplicity for a deficiency one reaction network are represented by many sets of linear inequality systems in terms of a vector μ. This vector μ correlates two steady states, say c' and c", corresponding to a set of rate constants in the following way:

$$\mu=[\mu_1, \ldots, \mu_N] = [\ln(c_1'/c_1''), \ldots, \ln(c_N'/c_N'')], \quad N=\text{number of species} \quad (5)$$

If there exists such a nonzero μ with the specified properties generated by the Deficiency One Algorithm, the deficiency one network under study has the capacity to admit multiple steady states. Otherwise, the network can admit at most one steady state.

The Deficiency One Algorithm is a powerful method to determine steady state multiplicity of a deficiency one reaction network. However, there are some complex reaction networks lying outside of the algorithm's applicable range. Take reaction network (2) for an example: it has a deficiency of three. Therefore, the Deficiency One Algorithm cannot be applied. The Subnetwork Analysis extends the applicable range of the algorithm by studying the subnetworks of a network with higher deficiency than one. Li [1992] realized that networks of deficiency greater than one that admit multiple steady states often (not always) contain subnetworks that also have the capacity of exhibiting steady state multiplicity. An example has been shown [Li, 1992] that a parent network cannot admit multiple steady states, no matter what values the rate constants might have, although one of its deficiency one subnetworks has the capacity to exhibit steady state multiplicity. Thus, it is not trivial to ask the question: If a network contains a subnetwork that admits multiple steady states, under what conditions will the network also admit multiple steady states? The Subnetwork Analysis provides sufficient conditions for the capacity of multiple steady states in a network of deficiency great than one if one of its subnetworks is admitting steady state multiplicity. The terminology of the Subnetwork Analysis and its implementation can be found in Li [1998].

RESULTS AND DISCUSSION

1. Multiple Steady States in a Deficiency One Subnetwork

The complex reaction network (2) has deficiency three. According to the deficiency oriented theory, a "minimal" network admitting multiple steady states should have a deficiency of at least one. According to the Subnetwork Analysis, the subnetworks with the same rank as the parent network (2), which is 10 (see Appendix), would meet one of the criteria for extending directly the steady-state multiplicity to their family networks. Thus, we are interested to know if there exists a deficiency one subnetwork of network (2) with rank 10, which generates multiple steady states. By using the Deficiency One Algorithm, such a deficiency one subnetwork exhibiting steady state multiplicity is determined. It is obtained by deleting $A_{10}\to 0\leftarrow A_{11}$ from network (2) and displayed below in Eq. (6). Actually, we have found it is the only deficiency one subnetwork with rank 10 admitting multiplicity.

$$\begin{aligned}
&A_8 \rightleftarrows 0 \rightleftarrows A_9\\
&A_1+A_8 \rightleftarrows A_4\\
&A_2+A_8 \rightleftarrows A_5\\
&A_1+A_9 \rightleftarrows A_6 \rightleftarrows A_3+A_{10}\\
&A_2+A_9 \rightleftarrows A_7 \rightleftarrows A_1+A_{10}\\
&A_2 \rightleftarrows A_3\\
&A_5 \rightleftarrows A_6\\
&2A_{10} \rightleftarrows A_9+A_{11}
\end{aligned} \quad (6)$$

According to the algorithm, it has the capacity to admit multiple steady states if the μ defined in Eq. (5) satisfies the set of linear inequalities Eq. (7a) generated by the algorithm. It is easy to see that Eq. (7b) is a set of nonzero solutions to Eq. (7a). Eq. (7a) indicates the relationships of the two steady states, which can be used to identify mechanism as the steady states of some species are measured [Ellison and Feinberg, 2000]. The more the numbers of the inequalities and the equations of Eq. (7a) are satisfied by the experimental data, the more likely that mechanism (6) is followed. (The reverse of all the inequalities in Eq. (7a) is also a signature of multiple steady states, which is obtained by replacing c' and c" in Eq. (5) by each other).

$$\begin{aligned}
&\mu_1+\mu_9>\mu_6\\
&\mu_6>\mu_5\\
&\mu_2+\mu_8>\mu_9\\
&\mu_9>0\\
&0>\mu_8\\
&\mu_5>\mu_2+\mu_8\\
&\mu_2+\mu_9>\mu_7\\
&\mu_2>\mu_3\\
&\mu_7>\mu_1+\mu_{10}\\
&\mu_3>\mu_9
\end{aligned}$$

$\mu_1+\mu_{10}>\mu_9$
$\mu_3+\mu_{10}>\mu_6$
$\mu_1+\mu_8=\mu_4$
$\mu_9+\mu_{11}=2\mu_{10}$ (7a)
$\mu=[\mu_1, \ldots, \mu_{11}]=[4, 7, 6, -1, 3, 4, 7, -5, 1, 0.5, 0]$ (7b)

By the μ given in Eq. (7b), a set of rate constants, displayed in Eq. (8a), and its two corresponding steady states, c' and c" in Eq. (8b), are computed (The formulas for the computation of two steady states and a set of rate constants can be found in Feinberg [1988]).

$$A_8 \underset{9.01061}{\overset{13.9267}{\rightleftharpoons}} 0 \underset{13.9267}{\overset{2.05252}{\rightleftharpoons}} A_9$$
$$A_1+A_8 \underset{0.21071}{\overset{212.948}{\rightleftharpoons}} A_4$$
$$A_2+A_8 \underset{17.4816}{\overset{10699.6}{\rightleftharpoons}} A_5$$
$$A_1+A_9 \underset{164.461}{\overset{196.794}{\rightleftharpoons}} A_6 \underset{12.2454}{\overset{115.996}{\rightleftharpoons}} A_3+A_{10}$$
$$A_2+A_9 \underset{3461.32}{\overset{4364.62}{\rightleftharpoons}} A_7 \underset{21.5570}{\overset{186.199}{\rightleftharpoons}} A_1+A_{10}$$
$$A_2 \underset{1268.06}{\overset{1261.10}{\rightleftharpoons}} A_3$$
$$A_5 \underset{96.9312}{\overset{110.858}{\rightleftharpoons}} A_6$$
$$2A_{10} \underset{3.43656}{\overset{0.03435}{\rightleftharpoons}} A_9+A_{11} \quad (8a)$$

$c_1' \approx 0.509329$	$c_1'' \approx 0.009329$
$c_2' \approx 0.500456$	$c_2'' \approx 0.000456$
$c_3' \approx 0.501242$	$c_3'' \approx 0.001242$
$c_4' \approx 1.745930$	$c_4'' \approx 4.745930$
$c_5' \approx 0.526198$	$c_5'' \approx 0.026198$
$c_6' \approx 0.509329$	$c_6'' \approx 0.009329$
$c_7' \approx 0.500456$	$c_7'' \approx 0.000456$
$c_8' \approx 0.003392$	$c_8'' \approx 0.503392$
$c_9' \approx 0.790988$	$c_9'' \approx 0.290988$
$c_{10}' \approx 8.895229$	$c_{10}'' \approx 5.395229$
$c_{11}' \approx 1.000000$	$c_{11}'' \approx 1.000000$ (8b)

Note that the mass balance condition in Eq. (4) is satisfied by

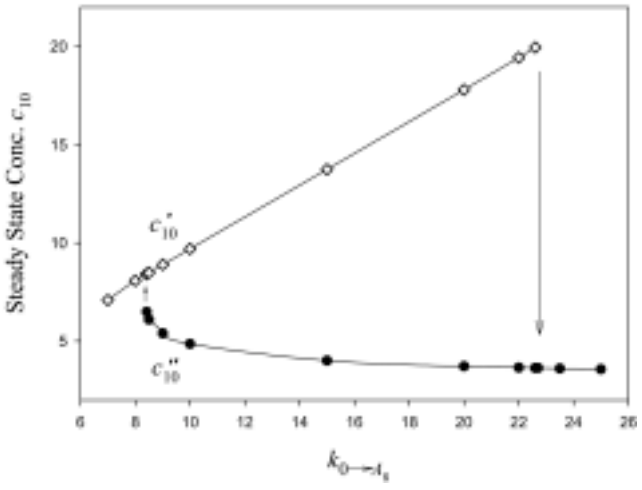

Fig. 2. The change of the steady state concentration c_{10} with the flow rate $k_{0\to A_8}$ for network (8).

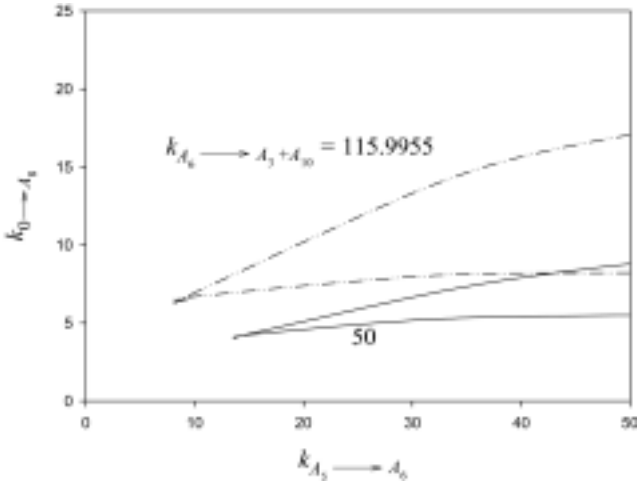

Fig. 3. The locus of the multiple steady state bifurcation for network (8) in the ($k_{A_5\to A_6}$, $k_{0\to A_8}$) plane for different values of the rate constant $k_{A_6\to A_3+A_{10}}$.

the Eq. (8b):

$$c_1'+c_2'+c_3'+c_4'+c_5'+c_6'+c_7'=c_1''+c_2''+c_3''+c_4''+c_5''+c_6''+c_7'' \approx 4.792940. \quad (9)$$

In Fig. 2, the steady states and bistability occurring in network (8) are illustrated as hysteresis with variation of $k_{0\to A_8}$. The steady states c' (the upper points in Fig. 2) and c" (the lower points in Fig. 2) in Eq. (8b) are stable and an unstable steady state (not shown in Fig. 2) lies somewhere between c' and c". The steady state c" in Eq. (8b) established at a higher $k_{0\to A_8}$ (>22.61) is associated with a lower concentrations c_{10}. As $k_{0\to A_8}$ is lower than 8.41, the steady state c' associated with a higher concentration c_{10} is obtained. As $k_{0\to A_8}$ is in between, a hysteresis loop containing three steady states, two stable ones and an unstable one, occurs and the steady state depends on the initial concentrations.

Fig. 3 shows a two-parameter ($k_{A_5\to A_6}$, $k_{0\to A_8}$) plane for different values of the rate constant $k_{A_6\to A_3+A_{10}}$ for network (8) (The dotted line corresponds to 115.9955 and the solid line to 50, respectively). Inside the cusp regions, there are three steady states, two stable ones and an unstable one. They display the inside regions of a hysteresis loop similar to Fig. 2. Right on the curves of the cusp, there are two steady states, one stable and the other unstable. They represent the two end points of a hysteresis loop. Only a single steady state exists outside the cusp region in Fig. 3, which displays the outside region of a hysteresis loop.

2. Multiple Steady States in Parent Networks

To extend the capacity of multiple steady states of a subnetwork to its parent network, the Subnetwork Analysis provides some sufficient conditions. Unfortunately, the μ vector in Eq. (7a) contains an equality (the last line) such that the sufficient condition of the analysis is not satisfied. By the addition of any one of the two deleted irreversible reactions, $A_{10}\to 0$ and $A_{11}\to 0$, to the deficiency one subnetwork (6), a deficiency two subnetwork is obtained. With this deficiency two subnetwork, we are allowed to adjust more parameters than those for the deficiency one subnetwork (6) in the necessary and sufficient condition [Li, 1999], which was used to prove the Subnetwork Analysis and should be satisfied for the determination of multiple steady states in a reaction network of any defi-

ciency. By the addition of the deleted reactions to the deficiency one subnetwork (6) one by one and by adjusting the parameters and the μ vector, the deficiency two subnetworks of the network (2) and the deficiency three network (2) itself all have the capacity to admit multiple steady states. A modified μ vector such that the network (2) exhibits multiple steady states is shown in Eq. (10). It meets the inequalities and equalities of Eq. (7a), except for the last equality. The rate constants and corresponding two steady states are indicated in Eqs. (11), computing according to the vector μ given in Eq. (10) (The formulas for the computation of two steady states and a set of rate constants can be found in the appendix of Li [1999]).

$$\mu=[\mu_1, \ldots, \mu_{11}]=[4, 7, 6, -1, 3, 4, 7, -5, 1, 0.49, 0] \tag{10}$$

$$A_8 \underset{48.6897}{\overset{0.18066}{\longleftarrow}} 0 \underset{0.18066}{\overset{9.26262}{\longleftarrow}} A_9$$

$$A_{10} \xrightarrow{0.18066} 0$$

$$A_{11} \xrightarrow{0.18066} 0$$

$$A_1+A_8 \underset{0.21071}{\overset{0.54263}{\longleftarrow}} A_4$$

$$A_2+A_8 \underset{143.132}{\overset{185.792}{\longleftarrow}} A_5$$

$$A_1+A_9 \underset{822.306}{\overset{2.55287}{\longleftarrow}} A_6 \underset{98.8949}{\overset{930.477}{\longleftarrow}} A_3+A_{10}$$

$$A_2+A_9 \underset{17306.6}{\overset{56.6191}{\longleftarrow}} A_7 \underset{104.966}{\overset{920.427}{\longleftarrow}} A_1+A_{10}$$

$$A_2 \underset{10155.9}{\overset{10119.8}{\longleftarrow}} A_3$$

$$A_5 \underset{660.531}{\overset{731.430}{\longleftarrow}} A_6$$

$$2A_{10} \underset{0.04981}{\overset{1.04193}{\longleftarrow}} A_9+A_{11} \tag{11a}$$

$c_1' \approx 0.509329$	$c_1'' \approx 0.009329$
$c_2' \approx 0.500456$	$c_2'' \approx 0.000456$
$c_3' \approx 0.501242$	$c_3'' \approx 0.001242$
$c_4' \approx 1.745930$	$c_4'' \approx 4.745930$
$c_5' \approx 0.526198$	$c_5'' \approx 0.026198$
$c_6' \approx 0.509329$	$c_6'' \approx 0.009329$
$c_7' \approx 0.500456$	$c_7'' \approx 0.000456$
$c_8' \approx 1.331079$	$c_8'' \approx 197.5497$
$c_9' \approx 304.8764$	$c_9'' \approx 112.1578$
$c_{10}' \approx 9.035205$	$c_{10}'' \approx 5.535205$
$c_{11}' \approx 5.535205$	$c_{11}'' \approx 5.535205$

(11b)

Fig. 4 shows a two-parameter ($k_{A_3+A_{10}\to A_6}$, $k_{A_5\to A_6}$) plane for different values of the rate constant $k_{A_{10}\to 0}$ (=0.180662, 0.145 and 0.140) of network (11). Inside the cusp regions, there are three steady states. Right on the curves of the cusp, there are two steady states. Only a single steady state exists outside the cusp region in Fig. 4. To maintain the existence of the steady state multiplicity under a fixed rate constant $k_{A_{10}\to 0}$, the smaller the rate constant $k_{A_5\to A_6}$ is, the smaller the rate constant $k_{A_3+A_{10}\to A_6}$ is required and the narrower its range. To maintain the existence of the steady state multiplicity under a lower rate constant $k_{A_{10}\to 0}$, it is required to increase the rate constant $k_{A_3+A_{10}\to A_6}$.

The application of the Subnetwork Analysis can be used to extend the steady-state multiplicity of network (2) to its parent networks with higher deficiency based on the vector μ given in Eq. (10). Consider a family member of network (2). The addition of a pair of the reversible reaction $A_5 \rightleftarrows A_3+A_{10}$ ($E_{oxy}M \rightleftarrows E_{deoxy}+Q$) to network (2) leads to the network (12a) of deficiency three with a circle ($A_5 \rightleftarrows A_6 \rightleftarrows A_3+A_{10} \rightleftarrows A_5$). Following the Subnetwork Analysis, the augmented network (12a) also has the capacity to admit multiple steady states. The rate constants and corresponding two steady states are indicated in Eq. (12), computing by using the vector μ given in Eq. (10).

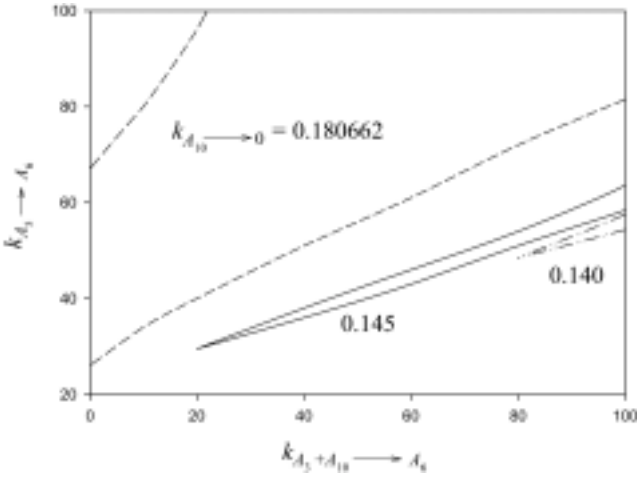

Fig. 4. The locus of the multiple steady state bifurcation for network (11) in the ($k_{A_3+A_{10}\to A_6}$, $k_{A_5\to A_6}$) plane for different values of rate constant $k_{A_{10}\to 0}$.

$$A_8 \underset{48.6897}{\overset{0.18066}{\longleftarrow}} 0 \underset{0.18066}{\overset{9.26262}{\longleftarrow}} A_9$$

$$A_{10} \xrightarrow{0.18066} 0$$

$$A_{11} \xrightarrow{0.18066} 0$$

$$A_1+A_8 \underset{0.21071}{\overset{0.54263}{\longleftarrow}} A_4$$

$$A_2+A_8 \underset{143.132}{\overset{185.792}{\longleftarrow}} A_5$$

$$A_1+A_9 \underset{822.306}{\overset{2.55287}{\longleftarrow}} A_6 \underset{88.6825}{\overset{815.752}{\longleftarrow}} A_3+A_{10}$$

$$A_2+A_9 \underset{17306.6}{\overset{56.6191}{\longleftarrow}} A_7 \underset{104.966}{\overset{920.427}{\longleftarrow}} A_1+A_{10}$$

$$A_2 \underset{10155.9}{\overset{10119.8}{\longleftarrow}} A_3$$

$$A_5 \underset{635.984}{\overset{684.518}{\longleftarrow}} A_6$$

$$2A_{10} \underset{0.04981}{\overset{1.04193}{\longleftarrow}} A_9+A_{11}$$

$$A_5 \underset{1.79995}{\overset{38.6436}{\longleftarrow}} A_3+A_{10} \tag{12a}$$

$c_1' \approx 0.509329$	$c_1'' \approx 0.009329$
$c_2' \approx 0.500456$	$c_2'' \approx 0.000456$
$c_3' \approx 0.501242$	$c_3'' \approx 0.001242$
$c_4' \approx 1.745930$	$c_4'' \approx 4.745930$
$c_5' \approx 0.526198$	$c_5'' \approx 0.026198$
$c_6' \approx 0.509329$	$c_6'' \approx 0.009329$
$c_7' \approx 0.500456$	$c_7'' \approx 0.000456$
$c_8' \approx 1.331079$	$c_8'' \approx 197.5497$

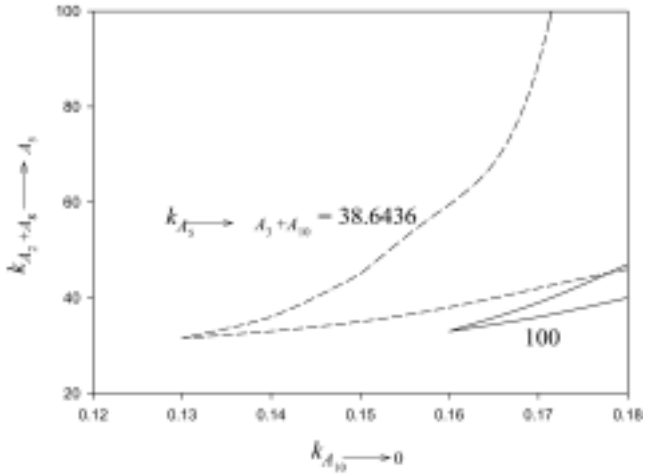

Fig. 5. The locus of the multiple steady state bifurcation for network (12) in the ($k_{A_{10}\to 0}$, $k_{A_2+A_8\to A_5}$) plane for different values of rate constant $k_{A_5\to A_3+A_{10}}$.

$c_9' \approx 304.8764$ $c_9'' \approx 112.1578$
$c_{10}' \approx 9.035205$ $c_{10}'' \approx 5.535205$
$c_{11}' \approx 5.535205$ $c_{11}'' \approx 5.535205$ (12b)

Fig. 5 shows a two-parameter ($k_{A_{10}\to 0}$, $k_{A_2+A_8\to A_5}$) plane for different values of the rate constant $k_{A_5\to A_3+A_{10}}$ (=38.6436 and 100) for network (12a). The number of steady states is 3, 2 and 1 for the region inside the cusp, right on the cusp and outside of it, respectively. Fig. 5 shows that, to maintain the existence of the steady state multiplicity under a fixed rate constant $k_{A_5\to A_3+A_{10}}$, the smaller the rate constant $k_{A_2+A_8\to A_5}$ is, the smaller the rate constant $k_{A_{10}\to 0}$ is required and the narrower its range. To maintain the existence of the steady state multiplicity under a higher rate constant $k_{A_5\to A_3+A_{10}}$, it is required to increase the rate $k_{A_{10}\to 0}$ and to decrease the rate constant $k_{A_2+A_8\to A_5}$.

Following the Subnetwork Analysis, the family members of network (2) displayed in network (13) have the capacity to exhibit multiple steady states for the vector μ in Eq. (10). The first nine lines of network (13) are just the deficiency three network (2). The last line of reaction network (13) describes any of the reactions which can be represented by a linear combination of those reactions on the right-hand side of the equation. The parameters a, b, c, …, j in the last line of network (13) are any real numbers. A negative parameter means the reverse of the reaction arrow. The network (13) might have a high deficiency, such as circular network (12) of deficiency three, and even other networks of high deficiency.

$$A_8 \rightleftarrows 0 \rightleftarrows A_9$$
$$A_{10} \to 0 \leftarrow A_{11}$$
$$A_1+A_8 \rightleftarrows A_4$$
$$A_2+A_8 \rightleftarrows A_5$$
$$A_1+A_9 \rightleftarrows A_6 \rightleftarrows A_3+A_{10}$$
$$A_2+A_9 \rightleftarrows A_7 \rightleftarrows A_1+A_{10}$$
$$A_2 \rightleftarrows A_3$$
$$A_5 \rightleftarrows A_6$$
$$2A_{10} \rightleftarrows A_9+A_{11}$$
$$y_i \to y_j = a(A_8 \to 0)+b(A_9 \to 0)+c(A_2+A_8 \to A_5)+d(A_1+A_9 \to A_6)$$
$$+e(A_2+A_9 \to A_7)+f(A_2 \to A_3)+g(A_7 \to A_1+A_{10})$$
$$+h(A_6 \to A_3+A_{10})+i(A_5 \to A_6)+j(2A_{10} \to A_9+A_{11}) \quad (13)$$

CONCLUSION

Subnetwork Analysis is used to determine the capacity of computational multiple steady states in a family of enzymatically catalyzed reaction network (13). A minimal subnetwork of network (13), which generates multiple steady states, is determined in network (6). The bistability, hysteresis and bifurcation phenomena are discussed. The inequalities and equations listed in Eqs. (7a) might provide some information of reaction mechanism identification if steady states of some species are measured. The results of this work might help us to study the complex reaction networks in other enzymatically catalyzed systems, such as the enzymatic production of L-DOPA (dihydroxyphenylalanine). From a biochemical engineering point of view, it can be also applied to biosensors for phenols, waste-water treatment containing phenolic pollutants and depigmentation of melanin in plants and animals, even if they have different stoichiometry.

APPENDIX THE CALCULATION OF DEFICIENCY

The calculation of the deficiency for a reaction network is introduced briefly. The details can be found in Feinberg [1987]. The deficiency δ for a network is computed by

$$\delta = n - l - s \quad (A.1)$$

where n, l and s denote, respectively, the number of complexes, the number of linkage classes, and the rank of the network. Take network (2) for an example. Its deficiency is three since $\delta = n - l - s = 19 - 6 - 10 = 3$. For network (6), $\delta = n - l - s = 17 - 6 - 10 = 1$. For network (12a), $\delta = n - l - s = 19 - 6 - 10 = 3$.

NOMENCLATURE

A_1, A_2, … : chemical species
c_1, c_2, … : molar concentrations for species A_1, A_2, … [mol/l]
c_1^f, c_2^f, … : molar concentrations in the feed stream for species A_1, A_2, … [mol/l]
c', c" : composition vectors at steady states [mol/l]
$k_{i\to j}$: rate constant for reaction i⟶j [(mol/l)$^{1\text{-reaction order}}\cdot s^{-1}$]
$k_{i\to 0}$: flow rate [s^{-1}]
l : number of linkage classes
n : number of complexes
N : number of species in a reaction network
s : the rank of a network
t : time [s]

Greek Letters

δ : deficiency of a network
μ : variables defined in Eq. (5)
θ : reactor residence time [s]

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