BIOMATH
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Biomath Forum
ORIGINAL ARTICLE

Multistationarity in triple-site mixed mechanism phosphorylation network

Suha Jayyousi Dajani[1], Maya Mincheva[2]

[1]Department of Mathematics and Computer Science,
Lake Forest College, Illinois 60045, USA
sdajani@lakeforest.edu

[2]Department of Mathematical Sciences,
Northern Illinois University, Illinois 60115, USA
mmincheva@niu.edu

Received: 8 October 2024, Published: 28 March 2025

Abstract: **In this work, we investigate the existence of multistationarity for a triple-site mixed phosphorylation network, where the phosphorylation part contains distributive and processive components, while the dephosphorylation part is purely distributive. We obtain a simple inequality which defines a region in parameter space such that the parametric ordinary differential equations (ODE) system modeling the mixed network is multistationary, i.e., it has multiple positive steady states. We obtain a sufficient condition for uniqueness of the steady state in the form of parametric inequalities. Lastly, we show that the emergence of multistationarity is enabled by the catalytic constants regardless of the position of the processive part in the triple-site mixed mechanism phosphorylation network.**

Keywords: **mixed phosphorylation, parametric ODE system, convex parameters, multistationarity, bistability**

I. INTRODUCTION

Phosphorylation is a chemical mechanism in which a phosphate group is attached to a protein; whereas dephosphorylation is the detachment of a phosphate group from a phosphorylated protein. A single-site phosphorylation consists of one phosphorylation event and one dephosphorylation event, while multi-site phosphorylation is formed by multiple events. Multi-site phosphorylation balances protein function and plays prominent part in intracellular processes [1]. Processive phosphorylation occurs when a kinase binds once to all substrate sites before disassociating; in distributive phosphorylation at most one site of the protein is modified by the kinase at each binding. If a phosphorylation mechanism contains both processive and distributive parts it is of mixed nature.

Research on the existence of multistationarity in biochemical networks has been published extensively. One of the influential papers related to this research is “Mixed Mechanism of Multi-Site Phosphorylation” by Suwanmajo and Krishnan [2]. They start their paper by presenting purely processive and purely distributive mechanism in single-site and multi-site phosphorylation models, which are followed by various mixed mechanisms in double-site and triple-site phosphorylation models, encompassing up to three phosphorylation events. While studying these models in detail requires significant effort as the number of phosphorylation sites increases, they use simulation and obtain purely numerical results related to identifying multistationarity, stability, and oscillations in multi-site phosphorylation models.

Conradi and Mincheva published a paper on the existence of multistationarity in a purely distributive double-site phosphorylation model [3]. As a result of

Citation: Suha Jayyousi Dajani, Maya Mincheva, Multistationarity in triple-site mixed mechanism phosphorylation network, Biomath 14 (2025), 2503285, https://doi.org/10.55630/j.biomath.2025.03.285

their studies, they obtain regions in parameter space where either multiple positive steady states exist or a unique steady state exists. Moreover, a general condition on multistationarity is obtained for reaction networks with bounded concentrations [4].

We analyze an ODE system model of a triple-site mixed mechanism phosphorylation that consists of a combination of processive and distributive components adopted from [2]. Degree theory is essential to determining the number of positive steady states, which is always odd and is either single or multiple [5, 6].

First, we determine the parameter region where multiple positive steady states exist for the triple-site mixed mechanism phosphorylation ODE system model (Theorem 1). Based on Theorem 1, we select parameter values such that the triple-site mixed mechanism model has three positive steady states.

Then, we show that the ODE system is bistable by confirming that two of the positive steady states are asymptotically stable and one is unstable. Lastly, we show that if the processive part of the network is moved in the reaction network, multistationarity exists and depends on specific rate constants.

In Section II, we introduce some preliminaries on reaction networks, construct their ODE system model using mass-action kinetics; multistationarity, and convex parameters are introduced. In Section III, we describe the phosphorylation and dephosphorylation processes in a triple-site mixed mechanism phosphorylation network. In Section IV, the ODE system of the triple-site mixed mechanism phosphorylation network is presented. Finally, in Section V, we present the main results on multistationarity and bistability.

The Appendices contain some theoretical results on degree theory and a list of matrices and long expressions.

Notation. The following notation is used in this paper:

1) $\mathbb{R}^n_{\geq 0}$ is the nonnegative orthant of $\mathbb{R}^n$.
2) $\mathbb{R}^n_{>0}$ is the positive orthant of $\mathbb{R}^n$.
3) The matrix $\mathrm{diag}(x) \in \mathbb{R}^{n\times n}$ is a diagonal matrix with $\mathrm{diag}(x)[i,i] = x_i$ for $i = 1, 2, \dots, n$.
4) For $x \in \mathbb{R}^n_{>0}$, we introduce the vector notation
$$\frac{1}{x} = \left(\frac{1}{x_1}, \dots, \frac{1}{x_n}\right).$$
5) For $x, v \in \mathbb{R}^n$ we use the notation
$$x^v = (x_1^{v_1}, \dots, x_n^{v_n}).$$

II. PRELIMINARIES

A. Reaction networks and their ODE System

A chemical reaction that consists of m species A_i and n elementary reactions can be written as

$$\sum_{i=1}^{m} \alpha_{is} A_i \xrightarrow{k_s} \sum_{i=1}^{m} \beta_{is} A_i, \quad s = 1, 2, \dots n, \tag{1}$$

where the coefficients $\alpha_{is} \geq 0$ and $\beta_{is} \geq 0$ are small integers called *stoichiometric coefficients* and $k_s > 0$ are the *rate constants*.

The concentrations of A_i at time $t \geq 0$ will be denoted as $x_i(t)$. We assume that mass action kinetics is used for the rate functions, where the reaction rate is proportional to the product of the concentrations of the reacting species raised to the power of their respective molecularities. The reaction rate function $r_s(k, x)$ of the s-th elementary reaction is

$$r_s(k, x) = k_s \prod_{i=1}^{m} x_i^{\alpha_{is}}. \tag{2}$$

Suppose that the column vectors y_s contain the stoichiometric coefficients α_{is} of the i-th reactant species A_i in the s-th reaction. These vectors form the $(m \times n)$ *kinetic order matrix* Y.

We use the columns y_i of the kinetic order matrix Y to define the monomial vector of rate functions $r_i(k, x)$ in the following way:

$$\phi(x) = \begin{pmatrix} x^{y_1} \\ \vdots \\ x^{y_n} \end{pmatrix}. \tag{3}$$

Thus, the rate functions vector $r(k, x)$ can be written as the product

$$r(k, x) = \mathrm{diag}(k)\phi(x),$$

where $k = (k_1, \dots, k_n)$ is the vector of rate constants.

Next, we define the entries of the $m \times n$ *stoichiometric matrix* Γ as

$$\Gamma_{ij} = \beta_{ij} - \alpha_{ij}.$$

Now, we can introduce the ODE system of reaction network (1) in vector form

$$\dot{x} = \Gamma\, r(k, x), \tag{4}$$

where Γ is the $m \times n$ stoichiometric matrix and $r(k, x) = (r_1, \dots, r_n)^T$ is the vector of reaction rates with r_s defined as in (2).

If $\operatorname{rank}(\Gamma) = s \le m$, then there exist $(m - s)$ conservation relations. A full-rank matrix W^T where W is of order $m \times (m-s)$ such that $W^T \Gamma = 0$ exists.

The conservation relations are defined as $W^T x = c$, where $c = W^T x(0) \in \mathbb{R}^{m-s}_{>0}$ and $x(0) \ge 0$ is the initial condition of (4).

The solution of the ODE system (4) is contained in the set

$$\omega_c = \{x \in \mathbb{R}^m_{\ge 0} \,|\, W^T x = c\}, \tag{5}$$

which is convex, closed, and forward invariant by definition.

B. Multistationarity and stability of steady states

A *positive steady state* x^* satisfies the ODE system (4) and the conservation relations $W^T x = c$. Thus x^* is contained in the set ω_c defined in (5).

Definition 1 (Multistationarity)**.** A chemical reaction network is called multistationary if there exist at least two positive steady states x^* and x^{**} in ω_c for a fixed c such that $\Gamma\, r(k, x^*) = \Gamma\, r(k, x^{**}) = 0$.

Since the rank of the Jacobian matrix $J(k, x)$ of the right-hand site of (4) is at most s, which is the rank of Γ, the characteristic polynomial $\det(zI - J)$ has $(m-s)$ identical zero roots, and can be written as

$$q(z) = z^{m-s}(z^s - a_1 z^{s-1} + ... + (-1)^s a_s). \tag{6}$$

Thus, we will work with the polynomial

$$p(z) = z^s - a_1 z^{s-1} + ... + (-1)^s a_s, \tag{7}$$

in place of (6), which is the restriction of (6) to the subspace $x + \operatorname{image}(\Gamma)$ [3].

A steady state x^* is *linearly stable* if all eigenvalues of the Jacobian $J(k, x)$ evaluated at x^* have negative real parts. In our case, this means that the polynomial $p(z)$ of the Jacobian $J(k, x)$ evaluated at the steady state $x = x^*$ has s roots with negative real parts. In fact, we will study the sign of the last non-zero coefficient $(-1)^s a_s$ of $p(z)$ in place of $(-1)^m a_m$, where $s \le m$. This is one way to simplify the computations for finding parameter regions. Another method is by reducing the number of reactions, which will be explained next.

Remark 1. An important theorem proved by Banaji states that: "If a chemical reaction network contains irreversible reactions only and its mathematical model has multiple positive non-degenerate steady states, then if a reverse reaction [with appropriately chosen rate constants] is added to the network, the new chemical reaction network also has multiple positive (non-degenerate) steady states" [7]. By working with the corresponding reaction network with irreversible reactions we can reduce the number of parameters and the computations.

C. Convex parameters

In this section, we introduce the convex parameters [8]. They will be used to parameterize the Jacobian matrix of the ODE system (4).

At a nonnegative steady state for the dynamical system $\dot{x} = \Gamma\, r$,

$$\Gamma\, r = 0, \quad \text{where} \quad r = [r_1,\, r_2, ..., r_n]^T, \quad r \ge 0.$$

Since $r \ge 0$ satisfies a finite number of inequalities, their intersection forms a *polyhedral cone*

$$C = \{r \,|\, \Gamma\, r = 0, r \ge 0\}.$$

referred to as a *flux cone* and r as a *flux vector*. Any flux vector can be represented as a non-negative linear combination of the extreme vectors $\{E_1, \dots, E_l\}$ of C.

Let E be the matrix with columns $E_1, \dots E_l$.

Definition 2 (Convex parameters)**.** A vector of convex parameters is a vector of the form

$$(j, h) = (j_1, j_2, ..., j_l, h_1, h_2, ..., h_l) \in \mathbb{R}^l_{>0} \times \mathbb{R}^m_{\ge 0},$$

where $h_i = 1/x_i^*$, $i = 1, 2, ..., m$, such that the rate function vector $Ej \in \mathbb{R}^n_{>0}$ [9] at a positive steady state x^*.

Alternatively, the Jacobian in convex parameters can be computed as [8, 10–15]

$$J(k, x^*) = J(j, h) = \Gamma\, \operatorname{diag}(Ej)\, Y^T\, \operatorname{diag}(h)\,. \tag{8}$$

We will work with the Jacobian matrix $J(j, h)$ when symbolic calculations are involved. For the numerical calculation on finding positive steady states where we use Maple, we will need the corresponding rate constants k and total concentrations c values. We explain next how to obtain the rate constants and total concentrations values by the convex parameters values.

Remark 2 (The rate constants k and the total concentrations c in terms of the convex parameters (j, h) [8])**.** Using (3), the rate constants vector can be written in terms of the convex parameters (j, h) as

$$k = \operatorname{diag}(Ej)\, \phi(h).$$

For the total concentrations c, we have

$$W^T \frac{1}{h} = c, \quad \text{where} \ \frac{1}{h} = \left(\frac{1}{h_1}, \frac{1}{h_2}, ..., \frac{1}{h_m}\right).$$

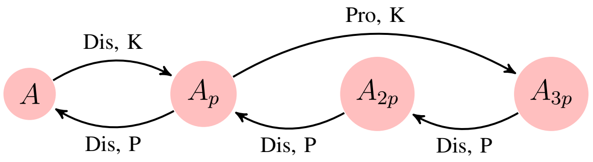

Fig. 1: Triple-site mixed mechanism phosphorylation model.

III. PHOSPHORYLATION NETWORKS

We introduce a biological process called phosphorylation, where a protein catalyst enzyme, called kinase, transforms protein into a phosphorylated protein by attaching a phosphate group. The reverse process, dephosphorylation, occurs when the phosphatase enzyme detaches a phosphate group from a phosphorylated protein [1].

A. Phosphorylation mechanisms

Phosphate groups attach to a protein and detach from a phosphorylated protein in two ways: processive and distributive. If the attachment of kinase and substrate occurs once, after all phosphate groups are added, then this mechanism of phosphorylation is referred to as *processive*.

On the other hand, if kinase is attached to a substrate, each time a phosphate group is added, then this mechanism is *distributive*. Similarly, a phosphate group can be detached from a substrate processively or distributively. If a reaction network contains only processive phosphorylation and dephosphorylation, then this network is *purely processive*. Meanwhile, if a reaction network contains only distributive phosphorylation and dephosphorylation, then this network is *distributive*.

While networks can be purely processive or distributive, if a reaction network contains a mix of processive and distributive phosphorylation and dephosphorylation, then it is referred to as *a mixed mechanism phosphorylation network.*

When the binding sites of the phosphorylation and dephosphorylation mechanism occur in order it is referred to as sequential. In a sequential mechanism the last site to be phosphorylated is dephosphorylated first [16].

B. Triple-site mixed mechanism network

Figure 1 shows the phosphorylation of A, A_p, and A_{2p} by kinase K, where A represents the protein, A_p represents a mono-phosphorylated protein, A_{2p} represents a double-phosphorylated protein, and A_{3p} represents a triple-phosphorylated protein. Phosphorylation is followed by dephosphorylation of A_{3p}, A_{2p}, and A_p by phosphatase P. The abbreviations *Dis* and *Pro* represent distributive and processive, respectively. The last two parts of the phosphorylation in Figure 1 are processive, while the first part of the phosphorylation and all parts of the dephosphorylation are distributive. Therefore, this triple-site phosphorylation model is of mixed mechanism nature. By definition, the mixed mechanism we consider is sequential.

The reaction network that corresponds to the triple-site mixed mechanism phosphorylation model in Figure 1 is shown in equation (9). (The bold parts are processive, while the remaining parts are distributive.)

$$\begin{aligned} &\mathrm{A}+\mathrm{K} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{AK} \xrightarrow{k_3} \mathrm{A_p}+\mathrm{K} \underset{k_5}{\overset{k_4}{\rightleftharpoons}} \\ &\mathbf{A_pK} \xrightarrow{\boldsymbol{k_6}} \mathbf{A_{2p}K} \xrightarrow{\boldsymbol{k_7}} \mathbf{A_{3p}} + \mathbf{K}, \\ &\mathrm{A_{3p}}+\mathrm{P} \underset{k_9}{\overset{k_8}{\rightleftharpoons}} \mathrm{A_{3p}P} \xrightarrow{k_{10}} \mathrm{A_{2p}}+\mathrm{P} \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} \\ &\mathrm{A_{2p}P} \xrightarrow{k_{13}} \mathrm{A_p}+\mathrm{P} \underset{k_{15}}{\overset{k_{14}}{\rightleftharpoons}} \mathrm{A_pP} \xrightarrow{k_{16}} \mathrm{A}+\mathrm{P}. \end{aligned} \tag{9}$$

IV. ODE SYSTEM OF THE TRIPLE-SITE MIXED MECHANISM NETWORK

We denote the concentration of species A by $[A]$ as it is customary in chemistry. The concentrations of the kinase K, phosphatase P, and all of the substrates are denoted by $x_1, x_2, \ldots, x_{12}$ as follows:

$$\begin{aligned} x_1 &= [A], & x_2 &= [K], & x_3 &= [AK], \\ x_4 &= [A_p], & x_5 &= [A_pK], & x_6 &= [A_{2p}], \\ x_7 &= [A_{2p}K], & x_8 &= [A_{3p}], & x_9 &= [P], \\ x_{10} &= [A_{3p}P], & x_{11} &= [A_{2p}P], & x_{12} &= [A_pP]. \end{aligned} \tag{10}$$

Banaji's result (see Remark 1) allows us to remove the reverse reactions from the phosphorylation network (9) and consider the following simplified phosphorylation network where the reactions have been renumbered:

$$\begin{aligned} &\mathrm{A}+\mathrm{K} \xrightarrow{k_1} \mathrm{AK} \xrightarrow{k_2} \mathrm{A_p}+\mathrm{K} \xrightarrow{k_3} \\ &\mathbf{A_pK} \xrightarrow{\boldsymbol{k_4}} \mathbf{A_{2p}K} \xrightarrow{\boldsymbol{k_5}} \mathbf{A_{3p}} + \mathbf{K}, \\ &\mathrm{A_{3p}}+\mathrm{P} \xrightarrow{k_6} \mathrm{A_{3p}P} \xrightarrow{k_7} \mathrm{A_{2p}}+\mathrm{P} \xrightarrow{k_8} \\ &\mathrm{A_{2p}P} \xrightarrow{k_9} \mathrm{A_p}+\mathrm{P} \xrightarrow{k_{10}} \mathrm{A_pP} \xrightarrow{k_{11}} \mathrm{A}+\mathrm{P}. \end{aligned} \tag{11}$$

Thus, we obtain the rate functions of (11), where the law of mass action kinetics is implemented as follows:

$$\begin{aligned} r_1 &= k_1x_1x_2, & r_2 &= k_2x_3, & r_3 &= k_3x_2x_4, \\ r_4 &= k_4x_5, & r_5 &= k_5x_7, & r_6 &= k_6x_8x_9, \\ r_7 &= k_7x_{10}, & r_8 &= k_8x_6x_9, & r_9 &= k_9x_{11}, \\ r_{10} &= k_{10}x_4x_9, & r_{11} &= k_{11}x_{12}. \end{aligned} \tag{12}$$

The irreversible reaction network (11) is transformed to the following ODE system:

$$\begin{aligned}
\dot{x}_1 &= -k_1x_1x_2 + k_{11}x_{12},\\
\dot{x}_2 &= -x_2(k_1x_1 + k_3x_4) + k_2x_3 + k_5x_7,\\
\dot{x}_3 &= -k_2x_3 + k_1x_1x_2,\\
\dot{x}_4 &= -x_4(k_3x_2 + k_{10}x_9) + k_2x_3 + k_9x_{11},\\
\dot{x}_5 &= -k_4x_5 + k_3x_2x_4,\\
\dot{x}_6 &= -k_8x_6x_9 + k_7x_{10},\\
\dot{x}_7 &= -k_5x_7 + k_4x_5,\\
\dot{x}_8 &= -k_6x_8x_9 + k_5x_7,\\
\dot{x}_9 &= -x_9(k_6x_8 + k_8x_6 + k_{10}x_4) + k_7x_{10}\\
&\quad + k_{11}x_{12} + k_9x_{11},\\
\dot{x}_{10} &= -k_7x_{10} + k_6x_8x_9,\\
\dot{x}_{11} &= -k_9x_{11} + k_8x_6x_9,\\
\dot{x}_{12} &= -k_{11}x_{12} + k_{10}x_4x_9.
\end{aligned} \tag{13}$$

The system (13) can be written in a vector form as

$$\dot{x} = \Gamma\, r(k,x), \tag{14}$$

where the stoichiometric matrix is given in (25) in Appendix VII-E and $r(k,x) = (r_1,\dots,r_{11})^T$ is the vector of rate functions in equation (12).

The system (13) has the following three mass conservation relations:

$$\begin{aligned}
x_9(t) + x_{10}(t) + x_{11}(t) + x_{12}(t) &= c_1,\\
x_2(t) + x_3(t) + x_5(t) + x_7(t) &= c_2,\\
x_1(t) + x_3(t) + x_4(t) + x_5(t) + x_6(t) + x_7(t)&\\
+x_8(t) + x_{10}(t) + x_{11}(t) + x_{12}(t) &= c_3,
\end{aligned}$$

which can be written in a matrix form as $W^Tx(t) = c$, where

$$W^T = \begin{pmatrix} 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 1 & 1 & 1\\ 0 & 1 & 1 & 0 & 1 & 0 & 1 & 0 & 0 & 0 & 0 & 0\\ 1 & 0 & 1 & 1 & 1 & 1 & 1 & 1 & 0 & 1 & 1 & 1 \end{pmatrix}$$

and $c = (c_1, c_2, c_3)^T > 0$ with $c = W^Tx(0)$.

The solutions of (13) are contained in the set

$$\omega_c = \{x \in \mathbb{R}^{12}_{\geq 0} \mid W^Tx = c\}. \tag{15}$$

It is easy to show that ω_c is compact, forward invariant, and convex. Moreover, the boundary, $\partial\omega_c$, does not contain any steady states of the dynamical system (13). We can also conclude that the solution $x(t)$ of the ODE system (13) is bounded for all $t \geq 0$ since ω_c is compact.

V. MAIN RESULTS

Our main result is Theorem 1 which will be applied to the coefficient $(-1)^9a_9(j,h)$ of (7) for $s = 9$ to determine whether the ODE system (13) is multistationary, and then identify the parameter region where multiple steady states exist [3,9].

First, we compute the characteristic polynomial of the Jacobian matrix $J(j,h)$ by using Maple [17], and identify the coefficient $(-1)^9a_9(j,h)$. Since j_1^3 and j_2^6 can be factored out, their product does not affect the sign of $(-1)^9a_9(j,h)$.

To simplify the analysis, we let $h_2 = h_9 = s$ and

$$\bar{h} = (h_1, h_3, \dots, h_8, h_{10}, h_{11}, h_{12}).$$

We obtain a quadratic polynomial in s where

$$(-1)^9a_9(s) = g(s) = \alpha(\bar{h})s^2 + \beta(\bar{h})s + \gamma(\bar{h}).$$

The coefficients of $g(s)$ are given in Appendix VII-C, and there are several options for their signs as follows:

1) $\alpha > 0$, $\beta > 0$, and $\gamma > 0$.
2) $\alpha < 0$, $\beta > 0$, and $\gamma > 0$.
3) $\alpha < 0$, $\beta < 0$, and $\gamma > 0$.
4) $\alpha > 0$, $\beta < 0$, and $\gamma > 0$ (For the purposes of this study, we will not be focusing on this case).

Remark 3. Suppose that case (1) above is satisfied, then $g(s) > 0$ for all $s > 0$ and some $\bar{h} > 0$. On the other hand, if case (2) or case (3) is satisfied, then there exist $s > 0$ and $\bar{h} > 0$ such that $g(s) < 0$.

The first three cases are crucial for determining whether the dynamical system (13) has a unique positive steady state or multiple positive steady states. Suppose that case (1) is satisfied for some parameter values of h; then, we will show in Theorem 2 that the ODE system (13) has a unique steady state. On the other hand, if cases (2) or (3) are satisfied, then we will show in Theorem 1 that the ODE system (13) has multiple positive steady states for some values of h. If (4) is satisfied, multistationarity is still possible if $g(h,s) < 0$. However, the proof will be more challenging.

Next, we introduce the following proposition to help us prove Theorems 1 and 2.

Proposition 1. Consider the coefficients α, β, and γ in (23). Then,

1) $\gamma > 0$, for all $h > 0$.
2) The sign of the coefficient α in (23) is determined by

$$\begin{aligned}\operatorname{sign}\alpha = \operatorname{sign}\big(&h_3h_{10}h_{11}(h_5 + h_7)\\ &-h_5h_7h_{12}(h_{10} + h_{11})\big).\end{aligned}$$

3) Suppose that $\bar{h} > 0$ satisfies

$$\begin{aligned} h_3h_{10}h_{11}(h_5+h_7) - h_5h_7h_{12}(h_{10}+h_{11}) > 0, \\ h_5h_8h_{12} - h_1(h_{12}-2h_3)(h_6+h_8) > 0, \\ h_1h_3 - (2h_1+h_3)h_8 > 0. \end{aligned} \tag{16}$$

Then, the coefficients α, β, and γ are all positive and $g(s) > 0$ for all $s > 0$.

4) Suppose that $\bar{h} > 0$ satisfies

$$h_3h_{10}h_{11}(h_5+h_7) - h_5h_7h_{12}(h_{10}+h_{11}) < 0.$$

Then, for a fixed value of $\bar{h}$, we have fixed values for $\alpha = \bar{\alpha}$,$\beta = \bar{\beta}$, and $\gamma = \bar{\gamma}$. We obtain a quadratic polynomial $\bar{g}(s) = -\bar{\alpha}s^2 + \bar{\beta}s + \bar{\gamma}$. The signs of the leading coefficient $\bar{\alpha}$ and the free coefficient $\bar{\gamma}$ are negative and positive, respectively, while the sign of $\bar{\beta}$ is either positive or negative. Moreover, $\bar{g}(0) = \bar{\gamma} > 0$, and when $s \to \infty$, $\bar{g}(s) \to -\infty$.

5) Suppose that

$$h_3h_{10}h_{11}(h_5+h_7) - h_5h_7h_{12}(h_{10}+h_{11}) < 0$$

is satisfied. Then, by part 4 there exists $s > 0$ such that $g(s) < 0$.

6) Following part 3, if the inequalities in (16) are satisfied, then for all $s > 0$, we have $g(s) > 0$.

A. Multistationarity in the triple-site mixed mechanism network

The next theorem presents the condition under which the ODE system (13) exhibits multiple positive steady states in some ω_c defined in (15) for some values of c.

Theorem 1 (Existence of multiple positive steady states)**.** *Suppose that the inequality*

$$h_3h_{10}h_{11}(h_5+h_7) - h_5h_7h_{12}(h_{10}+h_{11}) < 0 \tag{17}$$

is satisfied. Then, for some values of the total concentrations c where $W^Tx = c$, the ODE system (13) has multiple positive steady states contained in the set ω_c as defined in (15).

Proof: Suppose that the convex parameters

$$\bar{h} = (h_1, h_3, h_4, h_5, h_6, h_7, h_8, h_{10}, h_{11}, h_{12})^T > 0$$

are fixed and satisfy the inequality in (17) while $h_2 = h_9 = s$ are kept as variables. It follows by Proposition 1 part (5) that $(-1)^9a_9(s) = g(s) < 0$ for some value of $s > 0$. We fix any such steady state $x^* = \frac{1}{h}$ and let $\tilde{c} = W^Tx^*$.

Thus, we fix $\tilde{c} = W^Tx^*$ such that $\omega_{\tilde{c}}$ will contain the multiple positive steady states of the ODE system (13). Based on the degree theory introduced in Appendix VII-A, in particular equation (21), Corollary 3, and Corollary 2, for every steady state $x^* = \frac{1}{h}$ such that $(-1)^9a_9(x^*) < 0$, two other positive steady states, x^{**} and x^{***} in $\omega_{\tilde{c}}$ must exist such that $(-1)^9a_9(x^{**}) > 0$ and $(-1)^9a_9(x^{***}) > 0$, respectively.

In summary, x^*, x^{**}, and x^{***} satisfy $\Gamma\, r(k^*, x) = 0$ and $W^Tx = \tilde{c}$. Therefore, multistationarity exists for the ODE (13) if the inequality in (17) is satisfied for some values of c. ■

Remark 4. By Theorem 1, every $x^* = \frac{1}{h}$ such that $(-1)^9a_9(h) < 0$ defines $\tilde{c} = W^Tx^* \in \mathbb{R}^3_{>0}$, such that the set $\omega_{\tilde{c}}$ contains multiple positive steady states of the ODE system (13).

The next corollary follows by Lemma 1 in Appendix VII-B. The condition for multistationarity is given in terms of the rate constants k. This condition is useful when trying to solve the ODE system (13) together with $W^Tx = c$ numerically for given values of k and c.

Corollary 1. *Suppose that $k \in \mathbb{R}^{11}_{>0}$ satisfy*

$$k_2\left(\frac{1}{k_5} + \frac{1}{k_4}\right) - \left(\frac{1}{k_9} + \frac{1}{k_7}\right)k_{11} < 0. \tag{18}$$

Then, for some values of the total concentration c, the ODE system (13) has multiple positive steady states contained in the set ω_c as defined in (15).

Remark 5. We note that the number of positive steady states, x, that satisfy $\Gamma\, r(k^*, x) = 0$ and $W^Tx = \tilde{c}$ when the inequality in (18) is satisfied, is at least three and always an odd number by Corollary 3. For some values of the rate constants k, we have obtained three such positive steady states (see Section V-B). For some other values of k that satisfy the inequality in (18), there could be $5, 7$, or more steady states but always an odd number that is greater than 1.

B. Bistability in the triple-site mixed network

Recall that a reaction network is multistationary if its ODE system has at least two positive steady states. In the case of the triple-site mixed network, the steady states have to belong to the same ω_c for some fixed $c > 0$. We say that a multistationary system is *bistable* if its ODE system has three positive steady states where two are linearly stable and one is unstable.

Krishnan and his colleagues used *bifurcation* techniques to study the multistationarity, and consequently bistability of steady states in several models of mixed mechanism phosphorylation networks [2].

They found that for some values of the rate constants, multistationarity only exists in either purely distributive or mixed mechanism models where a distributive part is in both phosphorylation and dephosphorylation. Hence, for the case of the triple-site mixed phosphorylation network (11), bistability cannot be ruled out.

Next, we find parameter values such that the ODE system (13) is multistationary and moreover, bistable. We will select values for h such that the inequality (17) is satisfied which guarantees multistationarity by Theorem 1. The values of $j_1 > 0$ and $j_2 > 0$ are not restricted in any way and we will set them to $j_1 = 200$ and $j_2 = 400$.

We compute the rate constants values for k and total concentrations' values for c by Remark 2 such that the ODE system (13) has multiple positive steady states in the set $\omega_{\tilde{c}}$ where $W^T x^* = \tilde{c}$ and $x^* = \frac{1}{h}$. With the help of Maple, we will solve the ODE system (13) with the selected k's where the first second and ninth equations have been replaced by the three equations of $W^T x^* = \tilde{c}$ and obtain three positive steady states.

The following example shows how we can use *Maple* to find multistationarity and bistability for the ODE system (13).

1) Select values for $h_3 = \frac{1}{400}$, $h_5 = \frac{1}{200}$, $h_7 = \frac{3}{400}$, $h_{10} = \frac{1}{100}$, $h_{11} = \frac{1}{80}$, $h_{12} = \frac{3}{100}$, so that the inequality (17) is satisfied.
2) Select values for $h_1 = h_4 = h_6 = h_8 = 1$ and let $h_2 = h_9 = s$.
3) Substitute the values of h in $(-1)^9 a_9 = g(s)$. The result is
$$g(s) = -8.5625s^2 - 4.24796875s + 0.033421875.$$
4) Solve $g(s) < 0$ for $s > 0$. The solution after rounding is $(-\infty, -0.504) \cup (0.008, \infty)$.
5) Pick $s = 0.01$.
6) The total concentrations for the set $\omega_{\tilde{c}}$ which contains multiple steady states are $\tilde{c}_1 = 313.\bar{3}, \tilde{c}_2 = 833.\bar{3}$, and $\tilde{c}_3 = 950.\bar{6}$.
7) The corresponding rate constants values are $k_1 = 2$, $k_2 = 0.5$, $k_3 = 4$, $k_4 = 2$, $k_5 = 3$, $k_6 = 4$, $k_7 = 4$, $k_8 = 4$, $k_9 = 5$, $k_{10} = 2$, and $k_{11} = 6$.
8) The three positive steady states are
    (i) $(46.02, 4.43, 816.25, 0.86, 7.59, 0.02, 5.1, 0.02, 238.48, 3.8, 3.03, 68.02)$.
    (ii) $(0.02, 250.53, 15.52, 0.68, 340.36, 29.77, 226.9, 29.8, 5.7, 170.2, 136.1, 1.3)$.
    (iii) $(1, 99.\bar{9}, 400, 1, 199.\bar{9}, 0.\bar{9}, 133.\bar{3}, 0.\bar{9}, 100, 99.\bar{9}, 79.\bar{9}, 33.\bar{3})$.

The last steady state is the one that we selected as $x^* = \frac{1}{h}$ to fix the total concentrations values, $W^T x^* = \tilde{c}$.

To show the stability of the first two steady states and its relationship to bistability we work with the Hurwitz command in *Maple*.

For the first two steady states the Hurwitz command returns the value of true. By the necessary condition for stability, we have that the third steady states is unstable since $(-1)^9 a_9(h) < 0$, [18]. As a result, the ODE system (13) of the triple-site mixed mechanism phosphorylation model in Figure 1 admits three positive steady states where two are stable and one is unstable for the given values of the rate constants k in 7) and the total concentrations $\tilde{c}$ in 6) showing that the ODE system (13) is bistable for this particular set of parameter values.

C. Uniqueness of the steady state in the triple-site mixed network

In this section, we state the conditions for the uniqueness of a steady state of the dynamical system 14.

Theorem 2 (Uniqueness of the steady state)**.** *Consider the ODE system (13), and suppose that the steady states reciprocals $h \in \mathbb{R}^{12}_{>0}$ satisfy the inequalities*

$$\begin{aligned} h_3h_{10}h_{11}(h_5 + h_7) - h_5h_7h_{12}(h_{10} + h_{11}) > 0, \\ h_5h_8h_{12} - h_1(h_{12} - 2h_3)(h_6 + h_8) > 0, \\ h_1h_3 - (2h_1 + h_3)h_8 > 0. \end{aligned} \quad (19)$$

Then, for any value of the total concentrations of $c \in \mathbb{R}^3_{>0}$, the ODE system (13) has a unique positive steady state x^* such that $x_2^* = x_9^*$ in ω_c.

Proof: Suppose that h satisfy the inequalities in (19). Then, it follows from part (6) of Proposition 1 that for all $s > 0$, $(-1)^9 a_9(s) > 0$. If this is the case, then by Corollary 2 and equation (21) for all $x^* = \frac{1}{h}$, where $Z^T x^* = Z^T x_0 > 0$, there is a unique steady state. For each such fixed x^* we have $c^* = W^T x^* > 0$ such that $x^* \in \omega_c^*$ and $\Gamma r(k^*, x^*) = 0$. The latter means, that every set, ω_{c^*} where $c^* = W^T x^* > 0$, contains only the steady state $x^* > 0$. ■

Remark 6.

(a) If we select h-values such that (19) is satisfied and any values for $j_1 > 0, j_2 > 0$, then by Remark 2, we can find the corresponding rate constant values k such that the ODE system (13) has a unique steady state x^* in ω_c where $c = W^T \frac{1}{h}$.

(b) Other conditions for uniqueness of a steady state for any $c > 0$ in terms of the rate constants $k > 0$ are

$$k_2\left(\frac{1}{k_5}+\frac{1}{k_4}\right)-\left(\frac{1}{k_9}+\frac{1}{k_7}\right)k_{11}>0,$$
$$\big((k_1k_5-2k_2k_6)k_4-2k_6k_2k_5\big)k_9+k_2k_4k_5k_6>0.$$

These conditions can be obtained by using the coefficient $(-1)^9 a_9(k, x)$ of the characteristic polynomial for the Jacobian $J(k, x)$ parameterized in k and $x > 0$.

The presence of a distributive part in both the phosphorylation and dephosphorylation part is essential for the existence of multistationarity in the triple-site mixed phosphorylation network. We have tested the triple-site mixed phosphorylation network where the phosphorylation is processive and the dephosphorylation is distributive (computations not included). Since the corresponding last non-zero coefficient $(-1)^9 a_9(s)$ is positive for all values of s, the existence of multiple positive steady states is excluded.

In [2, 19] a double-site mixed network is studied and it is shown that its ODE system also has a unique positive steady state for any $c > 0$. In [20] it is shown for the n-site processive network, that there is a unique steady state which is globally asymptotically stable.

In the next section, we answer the question if multistationarity still exists and if the inequality in (17) that guarantees it, is the same provided the position of the processive part changes in the triple-site mixed phosphorylation network in (9).

D. Symmetry in triple-site mixed mechanism networks

In this section, we attempt to answer the question - if multistationarity will persist when the processive part in (9) is moved to the front of the phosphorylation; also if the processive part is moved to the dephosphorylation part, and is positioned first at the front and then at the end of the reaction network. Our initial guess, that multistationarity persists for suitably chosen values of the reciprocal steady states $h > 0$ (and correspondingly k) and for some values c is confirmed.

We label the reaction network in (11) as $\mathcal{N}_1$. Next, we list the other triple-site mixed reaction networks where the processive part is in bold:

$$\begin{aligned}
\mathcal{N}_2:\ &\mathbf{A}+\mathbf{K}\xrightarrow{\boldsymbol{k_1}}\mathbf{AK}\xrightarrow{\boldsymbol{k_2}}\mathbf{A_pK}\xrightarrow{k_3}\\
&\mathrm{A_{2p}}+\mathrm{K}\xrightarrow{k_4}\mathrm{A_{2p}K}\xrightarrow{k_5}\mathrm{A_{3p}}+\mathrm{K},\\
&\mathrm{A_{3p}}+\mathrm{P}\xrightarrow{k_6}\mathrm{A_{3p}P}\xrightarrow{k_7}\mathrm{A_{2p}}+\mathrm{P}\xrightarrow{k_8}\\
&\mathrm{A_{2p}P}\xrightarrow{k_9}\mathrm{A_p}+\mathrm{P}\xrightarrow{k_{10}}\mathrm{A_pP}\xrightarrow{k_{11}}\mathrm{A}+\mathrm{P},
\end{aligned}$$

$$\begin{aligned}
\mathcal{N}_3:\ &\mathrm{A}+\mathrm{K}\xrightarrow{k_1}\mathrm{AK}\xrightarrow{k_2}\mathrm{A_p}+\mathrm{K}\xrightarrow{k_3}\\
&\mathrm{A_pK}\xrightarrow{k_4}\mathrm{A_{2p}}+\mathrm{K}\xrightarrow{k_5}\mathrm{A_{2p}K}\xrightarrow{k_6}\mathrm{A_{3p}}+\mathrm{K},\\
&\mathrm{A_{3p}}+\mathrm{P}\xrightarrow{k_7}\mathrm{A_{3p}P}\xrightarrow{k_8}\mathrm{A_{2p}}+\mathrm{P}\xrightarrow{k_9}\\
&\mathbf{A_{2p}P}\xrightarrow{\boldsymbol{k_{10}}}\mathbf{A_pP}\xrightarrow{\boldsymbol{k_{11}}}\mathbf{A}+\mathbf{P},\\
\mathcal{N}_4:\ &\mathrm{A}+\mathrm{K}\xrightarrow{k_1}\mathrm{AK}\xrightarrow{k_2}\mathrm{A_p}+\mathrm{K}\xrightarrow{k_3}\\
&\mathrm{A_pK}\xrightarrow{k_4}\mathrm{A_{2p}}+\mathrm{K}\xrightarrow{k_5}\mathrm{A_{2p}K}\xrightarrow{k_6}\mathrm{A_{3p}}+\mathrm{K},\\
&\mathbf{A_{3p}}+\mathbf{P}\xrightarrow{\boldsymbol{k_7}}\mathbf{A_{3p}P}\xrightarrow{\boldsymbol{k_8}}\mathbf{A_{2p}P}\xrightarrow{k_9}\\
&\mathrm{A_p}+\mathrm{P}\xrightarrow{k_{10}}\mathrm{A_pP}\xrightarrow{k_{11}}\mathrm{A}+\mathrm{P}.
\end{aligned}$$

Remark 7. We kept the same notation for the concentrations as in equation (10) and obtained the same inequality (17) as a condition for multistationarity for networks $\mathcal{N}_1$ and $\mathcal{N}_4$ for some values of c. On the other hand, for networks $\mathcal{N}_2$ and $\mathcal{N}_3$, the inequality which guarantees multistationarity for some values of c is

$$h_3h_5h_{10}(h_{11}+h_{12})-h_7h_{11}h_{12}(h_3+h_5)<0. \quad (20)$$

Remark 8. If we state the corresponding inequalities to (17) and (20) in terms of the rate constants, then it becomes clear that the catalytic constants ensure the emergence of multistationarity in such triple mixed mechanism networks. Below we give the corresponding inequalities for networks $\mathcal{N}_i$, $i = 2, 3, 4$; for network $\mathcal{N}_1$, the inequality is (18):

$$\begin{aligned}
\mathcal{N}_2:\ &k_7\left(\frac{1}{k_9}+\frac{1}{k_{11}}\right)-\left(\frac{1}{k_2}+\frac{1}{k_3}\right)k_5<0,\\
\mathcal{N}_3:\ &k_8\left(\frac{1}{k_{10}}+\frac{1}{k_{11}}\right)-\left(\frac{1}{k_2}+\frac{1}{k_4}\right)k_6<0,\\
\mathcal{N}_4:\ &k_2\left(\frac{1}{k_6}+\frac{1}{k_4}\right)-\left(\frac{1}{k_9}+\frac{1}{k_8}\right)k_{11}<0.
\end{aligned}$$

VI. CONCLUSION

We have presented a sufficient condition for multistationarity (Theorem 1 and Corollary 1) in the triple-site mixed mechanism phosphorylation network ODE system (13). For a set of parameter values, we show that the ODE model (13) is bistable (Section V-B). In Theorem 2, we have presented a sufficient condition for a unique steady state of the ODE system (13) for all values of the total concentrations c. In Section V-D, we show that if the position of the processive part changes in the triple-site mixed mechanism phosphorylation network (11), then the inequality which guarantees the existence of multistationarity always involves the catalytic constants.

An intriguing question for future study is whether the ODE system of the triple-site mixed mechanism phosphorylation network (11) exhibits bistability across all parameter values that satisfy the multistationarity condition (17).

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VII. APPENDICES

A. Degree theory

Assume that the columns of a full-rank matrix S form an orthonormal basis of Im(Γ). Then, it suffices to study the matrix S^TJS in place of J [3]. In Lemma 4.1 of [3], it is shown that $\det(S^TJ(k,x)S) = (-1)^{m-3}a_{m-3}(k,x)$. (The number 3 could be replaced by the number of conservation relations.) Similarly, the set ω_c can be projected onto a lower-dimensional space by considering an orthonormal matrix Z whose columns are an orthonormal basis for Im$(\Gamma)^\perp$. The set Ω_p corresponding to ω_c in $\mathbb{R}^{m-3}$ is defined as

$$\Omega_p = \left\{ y \in \mathbb{R}^9_{\geq 0} \,|\, S\,y + Z\,p \geq 0 \right\}.$$

We have to introduce the corresponding dynamical system:

$$S^T\,\dot{x} = S^T\,\Gamma\,r(k,x(y,z)), \quad S^T\,\dot{x} = g(k,y),$$

or

$$\dot{y} = g(k,y), \quad y = S^T\,x, \quad g(k,y) = S^T\Gamma\,r(k,x).$$

Moreover, for $t = 0$, we have

$$\begin{aligned} y(0) &= y_0 = S^Tx_0, \\ p(0) &= p_0 = Z^Tx_0. \end{aligned}$$

The set Ω_p is compact, convex, forward invariant, and does not contain any boundary steady states.

Therefore, the degree of the function $g(k,y)$ on the set Ω_p is defined as:

$$\deg(g,\Omega_p,0) = \sum_{\left\{h\,\in\mathbb{R}^m_{>0} | Z^T\frac{1}{h} = Z^Tx_0\right\}} \text{sign}((-1)^{m-3}a_{m-3}(j,h)). \quad (21)$$

Remark 9.

1) Note that $g(k,y)$ is parameterized with respect to k, while the degree is defined in terms of h and j. If (j,h) are given, we can compute k and c by Remark 2.
2) We will work with $(-1)^9a_9(j,h)$ to obtain parameter values and parameter region for h, and thus for k and c such that $\dot{y} = g(k,y)$ has multiple steady states in Ω_p. However, this means that $\dot{x} = \Gamma\,r(k,x)$ has the same number of steady states in ω_c, since $x = Sy$.

The next corollary follows directly from Theorem 5.2 from [3].

Corollary 2. *The function $g(k,y)$, where $y \in \Omega_p$, has* $\deg(g,\Omega_p,0) = 1$.

The following corollary shows that at least one steady state always exists and the number of steady states is always an odd number. For the proof of this corollary, see Corollary 5.3 in the supplementary file of [3].

Corollary 3. *Suppose that the set D is bounded, open, convex, and forward invariant such that ∂D does not contain any steady states of $\dot{x} = \mathbb{F}(x)$, where $\mathbb{F}$ is a smooth function on $\bar{D}$. If the steady states in D are regular, then the number of steady states in D is odd. Furthermore, if $(-1)^{m-3}a_{m-3}$ alternates its sign between positive and negative (when it is evaluated at different steady states), then D contains more than one regular steady state.*

B. Inequality (17) in terms of the rate constants k

Lemma 1. *Suppose that the convex parameters h satisfy the following inequality*

$$h_3(h_5 + h_7)h_{10}h_{11} - h_5h_7(h_{10} + h_{11})h_{12} > 0. \quad (22)$$

Then, the rate constants k satisfy

$$k_2\left(\frac{1}{k_5} + \frac{1}{k_4}\right) - \left(\frac{1}{k_9} + \frac{1}{k_7}\right)k_{11} > 0.$$

Proof: Suppose that h_i satisfy (22). Then, we need to show that we can write this inequality in terms of the reaction rate constants k.

Consider the reaction rate function $r_2 = k_2x_3$ in equation (12). Solving for k_2 gives us

$$k_2 = \frac{r_2}{x_3}.$$

We substitute the convex parameters $r_2 = j_1$ from equation (24) and $h_3 = \frac{1}{x_3^*}$ into $k_2 = \frac{r_2}{x_3}$ to obtain

$$k_2 = \frac{r_2}{x_3} = j_1h_3, \quad h_3 = \frac{k_2}{j_1}.$$

In a similar manner, we calculate

$$h_5 = \frac{k_4}{j_2}, \qquad h_7 = \frac{k_5}{j_2}, \qquad h_{10} = \frac{k_7}{j_2},$$
$$h_{11} = \frac{k_9}{j_2}, \qquad h_{12} = \frac{k_{11}}{j_1}.$$

We substitute the value of h into equation (22) and we obtain

$$\frac{k_2}{j_1}\left(\frac{k_4}{j_2} + \frac{k_5}{j_2}\right)\frac{k_7}{j_2}\frac{k_{13}}{j_2} - \frac{k_4}{j_2}\frac{k_5}{j_2}\left(\frac{k_7}{j_2} + \frac{k_9}{j_2}\right)\frac{k_{11}}{j_1} > 0.$$

Finally, we multiply both sides of the inequality by $j_1j_2^3$ and divide by $k_4k_5k_7k_9$ to obtain

$$k_2\left(\frac{1}{k_5} + \frac{1}{k_4}\right) - k_{11}\left(\frac{1}{k_9} + \frac{1}{k_7}\right) > 0.$$

■

C. Coefficients of $g(s)$

$$\begin{aligned}
\alpha &= -((-h_3(h_5 + h_7)h_{11} + h_{12}h_5h_7)h_{10} \\
&\quad + h_{12}h_5h_7h_{11})(((h_6 + h_8)h_4 + h_6h_8)h_1 + h_4h_6h_8), \\
\beta &= (((((h_{12}h_8h_5 - h_1(h_{12} - 2h_3)(h_6 + h_8))h_{11} \\
&\quad + h_{12}(((-2h_1 - h_3)h_8 + h_1h_3)h_6 + h_1h_3h_8))h_{10} \\
&\quad + h_{12}(((-2h_1 - h_3)h_8 + h_1h_3)h_6 + h_1h_3h_8)h_{11})h_7 \\
&\quad + 2h_3h_6h_8h_{10}h_{11}(h_1 + h_{12}))h_5 \\
&\quad + 2h_3h_6h_7h_8h_{10}h_{11}(h_1 + h_{12}))h_4 \\
&\quad + h_1((h_{12} + h_7)h_5 + h_{12}h_7)h_6h_{10}h_3h_{11}h_8, \\
\gamma &= ((((((((h_7 + h_8)h_6 + h_7h_8)h_5 + h_6h_7h_8)h_4 \\
&\quad + h_5h_6h_7h_8)h_3 + h_4h_5h_6h_7h_8)h_1 \\
&\quad + h_3h_4h_5h_6h_7h_8)h_{12} \\
&\quad + h_1h_3h_4h_5h_6h_7h_8)h_{11} + h_1h_{12}h_3h_4h_5h_6h_7h_8)h_{10} \\
&\quad + h_1h_{11}h_{12}h_3h_4h_5h_6h_7h_8.
\end{aligned} \quad (23)$$

D. Jacobian matrix

Solving $\Gamma r = 0$ for the system (13); we obtain

$$\begin{aligned}
&j_1 = r_1 = r_2 = r_{10} = r_{11}, \\
&j_2 = r_3 = r_4 = r_5 = r_6 = r_7 = r_8 = r_9,
\end{aligned} \quad (24)$$

where $j_1, j_2 \geq 0$. Consequently, we can write r as

$$r = \sum_{i=1}^{2} j_i\, E_i, \quad j_i \geq 0,$$
$$E_1 = [1, 1, 0, 0, 0, 0, 0, 0, 0, 1, 1]^T,$$
$$E_2 = [0, 0, 1, 1, 1, 1, 1, 1, 1, 0, 0]^T,$$

are the extreme vectors of the flux cone [8].

The Jacobian matrix of the ODE system (13) in terms of k and x is:

$$\begin{pmatrix}
-\frac{r_1}{x_1} & -\frac{r_1}{x_2} & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & \frac{r_{11}}{x_{12}} \\
-\frac{r_1}{x_1} & -\frac{r_1+r_3}{x_2} & \frac{r_2}{x_3} & -\frac{r_3}{x_4} & 0 & 0 & \frac{r_5}{x_7} & 0 & 0 & 0 & 0 & 0 \\
\frac{r_1}{x_1} & \frac{r_1}{x_2} & -\frac{r_2}{x_3} & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & -\frac{r_3}{x_2} & \frac{r_2}{x_3} & -\frac{r_3+r_{10}}{x_4} & 0 & 0 & 0 & 0 & -\frac{r_{10}}{x_9} & 0 & \frac{r_9}{x_{11}} & 0 \\
0 & \frac{r_3}{x_2} & 0 & \frac{r_3}{x_4} & -\frac{r_4}{x_5} & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & -\frac{r_8}{x_6} & 0 & 0 & -\frac{r_8}{x_9} & \frac{r_7}{x_{10}} & 0 & 0 \\
0 & 0 & 0 & 0 & \frac{r_4}{x_5} & 0 & -\frac{r_5}{x_7} & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & \frac{r_5}{x_7} & -\frac{r_6}{x_8} & -\frac{r_6}{x_9} & 0 & 0 & 0 \\
0 & 0 & 0 & -\frac{r_{10}}{x_4} & 0 & -\frac{r_8}{x_6} & 0 & -\frac{r_6}{x_8} & -\frac{r_6+r_8+r_{10}}{x_9} & \frac{r_7}{x_{10}} & \frac{r_9}{x_{11}} & \frac{r_{11}}{x_{12}} \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & \frac{r_6}{x_8} & \frac{r_6}{x_9} & -\frac{r_7}{x_{10}} & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & \frac{r_8}{x_6} & 0 & 0 & \frac{r_8}{x_9} & 0 & -\frac{r_9}{x_{11}} & 0 \\
0 & 0 & 0 & \frac{r_{10}}{x_4} & 0 & 0 & 0 & 0 & \frac{r_{10}}{x_9} & 0 & 0 & -\frac{r_{11}}{x_{12}}
\end{pmatrix}.$$

The Jacobian matrix of the ODE system (13) in convex parameters is:

$$\begin{pmatrix}
-j_1h_1 & -j_1h_2 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & j_1h_{12} \\
-j_1h_1 & -(j_1+j_2)h_2 & j_1h_3 & -j_2h_4 & 0 & 0 & j_2h_7 & 0 & 0 & 0 & 0 & 0 \\
j_1h_1 & j_1h_2 & -j_1h_3 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & -j_2h_2 & j_1h_3 & -(j_1+j_2)h_4 & 0 & 0 & 0 & 0 & -j_1h_9 & 0 & j_2h_{11} & 0 \\
0 & j_2h_2 & 0 & j_2h_4 & -j_2h_5 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & -j_2h_6 & 0 & 0 & -j_2h_9 & j_2h_{10} & 0 & 0 \\
0 & 0 & 0 & 0 & j_2h_5 & 0 & -j_2h_7 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & j_2h_7 & -j_2h_8 & -j_2h_9 & 0 & 0 & 0 \\
0 & 0 & 0 & -j_1h_4 & 0 & -j_2h_6 & 0 & -j_2h_8 & -(2j_2+j_1)h_9 & j_2h_{10} & j_2h_{11} & j_1h_{12} \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & j_2h_8 & j_2h_9 & -j_2h_{10} & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & j_2h_6 & 0 & 0 & j_2h_9 & 0 & -j_2h_{11} & 0 \\
0 & 0 & 0 & j_1h_4 & 0 & 0 & 0 & 0 & j_1h_9 & 0 & 0 & -j_1h_{12}
\end{pmatrix}$$

E. Stoichiometric and kinetic matrices

The stoichiometric matrix Γ and the kinetic matrix Y for the triple-site mixed mechanism phosphorylation network are:

$$\Gamma = \begin{pmatrix}
-1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 \\
-1 & 1 & -1 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 \\
1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 1 & -1 & 0 & 0 & 0 & 0 & 0 & 1 & -1 & 0 \\
0 & 0 & 1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 1 & -1 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & -1 & 0 & 1 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 1 & -1 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & -1 & 1 & -1 & 1 & 1 & 1 \\
0 & 0 & 0 & 0 & & 1 & -1 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & -1 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & -1
\end{pmatrix}, \ Y = \begin{pmatrix}
1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
1 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 \\
0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 1 & 0 & 1 & 0 & 1 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1
\end{pmatrix}. \tag{25}$$