N-site Phosphorylation Systems with 2N-1 Steady States

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Abstract Multisite protein phosphorylation plays a prominent role in intracellular processes like signal transduction, cell-cycle control and nuclear signal integration. Many proteins are phosphorylated in a sequential and distributive way at more than one phosphorylation site. Mathematical models of \( n \)-site sequential distributive phosphorylation are therefore studied frequently. In particular, in Wang and Sontag (J Math Biol 57:29–52, 2008), it is shown that models of \( n \)-site sequential distributive phosphorylation admit at most \( 2n - 1 \) steady states. Wang and Sontag furthermore conjecture that for odd \( n \), there are at most \( n \) and that, for even \( n \), there are at most \( n + 1 \) steady states. This, however, is not true: building on earlier work in Holstein et al. (Bull Math Biol 75(11):2028–2058, 2013), we present a scalar determining equation for multistationarity which will lead to parameter values where a 3-site system has 5 steady states and parameter values where a 4-site system has 7 steady states. Our results therefore are counterexamples to the conjecture of Wang and Sontag. We furthermore study the inherent geometric properties of multistationarity in \( n \)-site sequential distributive phosphorylation: the complete vector of steady state ratios is determined by the steady state ratios of free enzymes and unphosphorylated protein and there exists a linear relationship between steady state ratios of phosphorylated protein.
Keywords  Sequential-distributed phosphorylation · Mass-action kinetics · Multistationarity · Determining equation

1 Introduction

Protein phosphorylation and dephosphorylation are important intracellular processes and many proteins are phosphorylated at more than one phosphorylation site. Phosphorylation can either be processive or distributive and sequential or random (see, for example, Gunawardena (2005), Ryerson and Enciso (2013), Salazar and Höfer (2007, 2009), Thomson and Gunawardena (2009)). Here we focus on sequential distributive phosphorylation of a generic protein $A$ at $n$ sites by a kinase $E_1$ and its sequential distributive dephosphorylation by a phosphatase $E_2$ (cf. Fig. 1). This process plays an important role in signal transduction, cell-cycle control or nuclear signal integration Salazar and Höfer (2007, 2009). A common interpretation of different (stable) steady states is that of an intracellular mechanism for information storage Thomas and Kaufman (2001a, b); Thomson and Gunawardena (2007). From this point of view, the maximal possible number of steady states is an important quantity to assess the information storage capacity of the system.

Under the assumption of mass-action kinetics one obtains a polynomial dynamical system in a straightforward way Conradi and Flockerzi (2012). This dynamical system consists of $3n + 3$ ordinary differential equations with polynomial right-hand side involving $6n$ parameters. Its variables represent the concentrations of the chemical species: kinase $E_1$ and phosphatase $E_2$, unphosphorylated protein $A$ and the phosphoforms $A_{iP}$, the kinase–substrate complexes $A_{iP}E_1$ and the phosphatase–substrate complexes $A_{iP}E_2$. Of these $3n + 3$ variables only $n + 3$ can be measured with reasonable effort: the concentration of $E_1$, $E_2$, $A$ and the $A_{iP}$. Hence parameter values are subject to high uncertainty and one is either led to apply reductionist modeling approaches tailored to the system and question at hand (as suggested, for example, in Enciso et al. (2014)) or to studying the whole parametrized family of polynomial ODEs (as, for example, in Holstein et al. (2013), Pérez Millán et al. (2012), Wang and Sontag (2008) and the present publication).

![Fig. 1 Sequential distributive phosphorylation and dephosphorylation of $A$ at $n$-sites by kinase $E_1$ and phosphatase $E_2$. Subscript $iP$ with $0 \leq i \leq n$ denotes the phosphorylated forms of $A$ (‘phosphoforms’) and the number of phosphorylated sites (with $A = A_0P$ and $A_p = A_1P$). Each encounter of $A_{iP}$ and $E_1$ ($A_{iP}$ and $E_2$) results in one phosphorylation (dephosphorylation). Hence $n$ encounters of $A_{iP}$ and $E_1$ ($A_{iP}$ and $E_2$) are required for phosphorylation (dephosphorylation) of $n$-sites. For biochemical details see, for example, Gunawardena (2005), Salazar and Höfer (2007, 2009), Thomson and Gunawardena (2009).](10.1007/978-3-030-78837-9_1)
The steady states of this parametrized family have been studied in a variety of publications: Reference Gunawardena (2005) establishes a functional relationship between the steady state ratio of kinase and phosphatase on the one hand and the steady state value of the fully phosphorylated protein on the other hand. The authors furthermore study the effect of the number \( n \) of phosphorylation sites on the graph of that function. For fixed parameter values, the steady state values of the phosphoforms \( A_1 P \) satisfy the algebraic relationships described in Gunawardena (2007), Kumar and Gunawardena (2008). In particular, measurements of the \( A_1 P \) taken from a given system (protein–kinase–phosphatase) have to satisfy these algebraic relations, provided the system is distributive. These algebraic relations are therefore called invariants in Gunawardena (2007), Kumar and Gunawardena (2008), and it is suggested to exploit these invariants to discriminate different phosphorylation mechanisms. In Karp et al. (2012), it is explained how such invariants can be obtained for arbitrary biochemical reaction networks. The steady states of post-translational modification systems, like the one depicted in Fig. 1, admit a rational parameterization Thomson and Gunawardena (2009). In Pérez Millán et al. (2012), this has been specialized to the system studied here: it belongs to the class of chemical reaction systems with toric steady states (defined in Pérez Millán et al. (2012)) and a particular rational parameterization is described. It is also shown that, for such systems with toric steady states, necessary and sufficient conditions for multistationarity (i.e. the existence of multiple steady states) take the form of linear inequality systems.

The number of steady states has been studied in a variety of publications as well. We start with results concerning \( n = 2 \): here bistability has been reported numerically for the first time in Markevich et al. (2004). An implicit description of the region in parameter space where multistationarity occurs is given in Conradi et al. (2008) and explicit parameter conditions guaranteeing existence of three positive steady states have been presented in Conradi and Mincheva (2014). For arbitrary \( n \), bistability has been established numerically in Salazar and Höfer (2007, 2009) and both, multistationarity and multistability have been reported in Thomson and Gunawardena (2009). The obvious fact that all phosphorylation sites compete for the same kinase (phosphatase) has been described as a possible explanation for the occurrence of multistationarity, especially as the system depicted in Fig. 1 lacks explicit feedback loops; see Feliu and Wiuf (2012) where this phenomenon is called enzyme-sharing. Finally, in Wang and Sontag (2008) it has been shown that this system has at most \( 2n - 1 \) positive steady states. There the authors also show the existence of parameter values where the system has \( n \) \((n + 1)\) steady states for \( n \) even (odd) and conjecture that \( n \) \((n + 1)\) is an upper bound for the number of steady states. If, as described above, steady states are considered as an intracellular means to store information, then this conjecture asserts that the achievable capacity of the system \((n \) or \( n + 1 \) steady states resp.) is far from the theoretical upper bound \((2n - 1)\). Later on, in Sect. 5, we will provide counterexamples for \( n = 3 \) and \( n = 4 \). Hence the conjecture is not true in general, however, we do not provide any information as to whether the theoretical maximum can be achieved for biochemically meaningful parameter values.

In the previous publication Holstein et al. (2013), we have analyzed multistationarity for arbitrary \( n \geq 2 \): there we present a collection of feasible linear inequality systems and show that solutions of these systems define parameter values where multistationary
arity occurs (together with two steady states as witness). In the present contribution, we shed some new light on the results of Holstein et al. (2013) by introducing a crucial reparametrization (cf. Eq. (15)). We then advance in two directions.

First, we obtain in Eq. (29a) a univariate polynomial \( P \) of degree \( 2n + 1 \) whose admissible positive zeros are in one-to-one correspondence with positive steady states. Here, a positive zero \( \xi_0 \) of \( P \) is called admissible if a certain polynomial \( G \) of degree \( n \) is positive at \( \xi_0 \) (cf. Proposition 4.1). Multistationarity then requires \( \geq 2 \) admissible positive roots of \( P \). By applying an argument already used in Wang and Sontag (2008) we can show that \( P \) has at most \( 2n - 1 \) positive roots (cf. Remark 4.3).

Incorporating the admissibility condition, we pass from \( P = 0 \) to a scalar determining equation \( \theta = 0 \) in Proposition 4.2 so that positive zeros of \( \theta \) are automatically admissible and thus in one-to-one correspondence with positive steady states. For \( n = 3 \) and \( n = 4 \) we furthermore exploit the structure of \( \theta \) to explicitly construct parameter values where \( \theta \) has 5 and 7 positive roots, respectively (cf. Figs. 2, 3; Table 1). We also explain how the same construction can be applied to obtain parameter values for at least \( n + 1 \) steady states for \( n > 4 \).

Second, we investigate the geometry of multistationarity: if parameters are such that \( \theta \) admits \( \geq 2 \) positive roots, then measurement of two different steady state values of kinase, phosphatase and protein alone suffices to reconstruct the complete vector of ratios of both steady states (Proposition 6.1). We use this fact to devise a graphical test based on measurement data to discard the possibility that the measured data give rise to multistationarity (Proposition 6.2 and Remark 6.3). In the spirit of Gunawardena (2007), Kumar and Gunawardena (2008) our Propositions 6.1 and 6.2 can be interpreted as invariants characterizing steady states when parameter values are in the multistationarity regime (as opposed to the invariants described in Gunawardena (2007), Kumar and Gunawardena (2008) that hold regardless of whether or not parameters are in the multistationarity regime). To the best of our knowledge these invariants have not been described before.

This paper is organized as follows: Sects. 2 and 3 introduce the necessary notations and the basic facts from Holstein et al. (2013), leading to the crucial reparametrization (15). In the spirit of Wang and Sontag (2008), Sect. 4 presents a scalar determining equation for multistationarity which will be studied, in Sect. 5, for an explicit triple phosphorylation network possessing \( 5 = 2 \cdot 3 - 1 \) positive steady states. We also present a 4-site phosphorylation network with \( 7 = 2 \cdot 4 - 1 \) positive steady states. The concluding Sect. 6 discusses the geometry of multistationarity, addresses the constraints on corresponding steady state ratios and comments on measurement and reconstruction issues. In Appendix, we present explicit formulae for the network matrices associated to a triple phosphorylation in Fig. 1.

2 Notation

We use the symbol \( \mathbb{R}^m \) to denote Euclidean \( m \)-space, the symbol \( \mathbb{R}_{\geq 0}^m \) to denote the nonnegative orthant and \( \mathbb{R}_{> 0}^m \) to denote the interior of the nonnegative orthant. Vectors are considered as column vectors and, for convenience, usually displayed as row vectors using \( ^T \) to denote the transpose. For example, \( x \in \mathbb{R}^m \) will usually be
displayed as \((x_1, \ldots, x_m)^T\). The vector \(x \in \mathbb{R}^m\) with \(x_i = 1\) for \(i = 1, \ldots, m\) will be denoted by \(1\).

We will use the symbol \(e_j\) to denote elements of the standard basis of Euclidian vector spaces and use the superscript \((i)\) to distinguish basis vectors of vector spaces of a different dimension \(3i + 3\):

\[ e_{j}^{(i)} \ldots \text{ denotes elements of the standard basis of } \mathbb{R}^{3i+3}. \]

For positive vectors \(x \in \mathbb{R}^m_{>0}\) we use the shorthand notation \(\ln x\) to denote

\[ \ln x := (\ln x_1, \ldots, \ln x_m)^T \in \mathbb{R}^m. \]

Similarly, for \(x \in \mathbb{R}^m\), we use \(e^x\) to denote

\[ e^x := (e^{x_1}, \ldots, e^{x_m})^T \in \mathbb{R}^m_{>0}, \]

and, for \(x \in \mathbb{R}^m\) with \(x_i \neq 0\), \(i = 1, \ldots, m\),

\[ x^{-1} := \left( \frac{1}{x_1}, \ldots, \frac{1}{x_m} \right)^T \in \mathbb{R}^m. \]

Finally, \(x^{y^T}\) with \(x \in \mathbb{R}^m_{>0}\) and \(y \in \mathbb{R}^m\) will be defined by

\[ x^{y^T} := e^{y^T \ln x} = \prod_{i=1}^{m} x_i^{y_i} \in \mathbb{R}^m_{>0}, \]

3 Steady States of a Dynamical System Derived from Fig. 1

By describing every reaction at the mass action level, we derive a dynamical system from Fig. 1. For this purpose we use the notation introduced in Holstein et al. (2013). We also summarize those results of Holstein et al. (2013) that are relevant for this contribution. We would like to emphasize that the dynamical system determined here and the one considered in Wang and Sontag (2008) are identical (up to a change of variables).

The mass action network derived from Fig. 1 (with \(n\) an arbitrary but fixed positive number) consists of the following \(3 + 3n\) chemical species: the protein (substrate) \(A\) together with \(n\) phosphoforms \(A_p, \ldots, A_{n_p}\); the kinase \(E_1\) together with \(n\) kinase–substrate complexes \(A E_1, \ldots, A_{n-1} E_1\) and the phosphatase \(E_2\) together with \(n\) phosphatase–substrate complexes \(A_p E_2, \ldots, A_{n_p} E_2\). To each species, a variable \(x_i\) denoting its concentration is assigned:

\[ x_1 = E_1, \ x_2 = A, \ x_3 = E_2, \ x_{1+3i} = A_{(i-1)p} E_1, \ x_{2+3i} = A_{i p}, \ x_{3+3i} = A_{i p} E_2 \]

(1)
with $A_{0P} = A$ and $A_{1P} = A_P$ ($i = 1, \ldots, n$). We collect all variables in a $(3 + 3n)$-dimensional vector $x := (x_1, \ldots, x_{3+3n})^T$. As it will turn out, the chosen labeling entails a simple block structure for the matrices associated to the dynamical system (5) of the network in Fig. 1, cf., for example, the block structure (9) for the generators of the nonnegative cone in the kernel of the stoichiometric matrix.

Assuming a distributive mechanism, a single phosphorylation occurs with each encounter of substrate and kinase, and $n$ phosphorylations therefore require $n$ encounters of substrate and kinase. Similarly, $n$ dephosphorylations following a distributive mechanism require $n$ encounters of substrate and phosphatase. Each phosphorylation and each dephosphorylation therefore consist of 3 reactions and consequently the network consists of $6n$ reactions. To each reaction we associate a rate constant. We use $k_i$ for phosphorylation and $l_i$ for dephosphorylation reactions and obtain the following reaction network:

$$
E_1 + A_{i-1}P \xrightarrow{k_{3i-2}} A_i P \xrightarrow{k_{3i}} E_1 + A_{i}P, \quad i = 1, \ldots, n
$$

$$
E_2 + A_{i}P \xrightarrow{l_{3i-2}} A_i P \xrightarrow{l_{3i}} E_2 + A_{i-1}P, \quad i = 1, \ldots, n.
$$

Using this notation, $k_{3i-2}$ ($l_{3i-2}$) denotes the association constant, $k_{3i-1}$ ($l_{3i-1}$) the dissociation constant and $k_{3i}$ ($l_{3i}$) the catalytic constant of the $i$th phosphorylation (dephosphorylation) step. We collect all rate constants in a column vector

$$
\kappa := \text{col} (\kappa(1), \ldots, \kappa(n)) \in IR_{>0}^{6n}
$$

where the sub-vectors $\kappa(i) := (k_{3i-2}, k_{3i-1}, k_{3i}, l_{3i-2}, l_{3i-1}, l_{3i})^T$ are stacked over each other.

For every $n$, one can derive the stoichiometric matrix $S \in IR^{(3+3n) \times 6n}$ and the rate exponent matrix $\mathcal{Y} \in IR^{(3+3n) \times 6n}$ from (2), cf. Holstein et al. (2013) for example. These define two monomial functions and a dynamical system in the following way where we denote the columns of $\mathcal{Y}$ with $y_i$:

- Monomial functions $\Phi : IR_{>0}^{3+3n} \rightarrow IR_{>0}^{6n}$ and $r(\kappa, \cdot) : IR_{>0}^{3+3n} \rightarrow IR_{>0}^{6n}$:

$$
\Phi (x) := x^{\mathcal{Y}^T} \equiv e^{\mathcal{Y}^T \ln x} \quad \text{and} \quad r(\kappa, x) := \text{diag} (\kappa) \Phi (x).
$$

The $6n$-dimensional vector $r(\kappa, x)$ is called the reaction rate vector.

- Dynamical system:

$$
\dot{x} = S r(\kappa, x) = S \text{ diag} (\kappa) x^{\mathcal{Y}^T}.
$$

If the $q$ rows of a matrix $Z \in IR^{q \times (3+3n)}$ form a basis for the left kernel of $S$ then the level sets

$$
\{ x \in IR^{3+3n} : Z x = \text{const.} \}
$$

are invariant under the flow of (5) as one has $Z x(t) = Z x(0)$ along solutions $x(t)$ of (5). This observation motivates the classical definition of multistationarity.
**Definition 3.1 (Multistationarity)** The system \( \dot{x} = Sr(\kappa, x) \) from (5) is said to exhibit multistationarity if and only if there exist a positive vector \( \kappa \in \mathbb{R}_{>0}^6 \) and at least two distinct positive vectors \( a, b \in \mathbb{R}_{>0}^{3+3n} \) with

\[
S r(\kappa, a) = 0, \\
S r(\kappa, b) = 0, \\
Z a = Z b.
\]

The Eqs. (6a) and (6b) describe the steady state property of \( a \) and \( b \) whereas the Eq. (6c) asks for these steady states to belong to the same coset of the stoichiometric matrix \( S \).

For the purpose of this contribution, the monomial function \( \Phi \) and the matrix \( Z \) are of particular interest. We refer to Appendix for expressions defining the matrix \( S \) and for the explicit model of network (1) for \( n = 3 \) (cf. Holstein et al. (2013)). Using the ordering of species and reactions introduced above in Eq. (1) one obtains the matrix \( Z \in \mathbb{R}^{3\times(3+3n)} \) of conservation laws and the rate exponent matrix \( \gamma \in \mathbb{R}^{(3+3n)\times6n} \) in the following way:

(I) With

\[
Y_0 (i) := \left[ e^{(i)}_1 + e^{(i)}_{3i-1} \ e^{(i)}_{3i} \ e^{(i)}_{3i+1} \ e^{(i)}_{3i+2} \ e^{(i)}_{3i+3} \ e^{(i)}_{3i+4} \right] \in \mathbb{R}^{(3+3i)\times6},
\]

the rate exponent matrix \( \gamma \in \mathbb{R}^{(3+3n)\times6n} \) is given by

\[
\gamma^T := \left[ \begin{array}{c|c|c|c}
Y_0 (1)^T & 0_{6\times3} & 0_{6\times3} & 0_{6\times3} \\
Y_0 (2)^T & 0_{6\times3} & 0_{6\times3} & 0_{6\times3} \\
Y_0 (3)^T & 0_{6\times3} & 0_{6\times3} & 0_{6\times3} \\
\vdots & \ddots & \ddots & \ddots \\
Y_0 (n-1)^T & 0_{6\times3} & 0_{6\times3} & 0_{6\times3} \\
Y_0 (n)^T & 0_{6\times3} & 0_{6\times3} & 0_{6\times3} \\
\end{array} \right].
\]

(II) The matrix \( Z \in \mathbb{R}^{3\times(3+3n)} \) of conservation laws is given by

\[
Z = \left[ \begin{array}{c|c|c|c}
1 & 0 & 0 & 1 & 0 & 0 & \cdots & 1 & 0 & 0 \\
-1 & 1 & -1 & 0 & 1 & 0 & \cdots & 0 & 1 & 0 \\
0 & 0 & 1 & 0 & 0 & 1 & \cdots & 0 & 0 & 1 \\
\end{array} \right] .
\]

We note that the three rows of the present \( Z \) form a basis for the left kernel of \( S \) as the three rows of the matrix \( Z^{(n)} \) defined in formula (9) of Holstein et al. (2013). The first row of \( Z \), for example, refers to the conservation of the total \( E_1 \)-concentration.

We now recall the discussion of the pointed polyhedral cone \( \text{ker}(S) \cap \mathbb{R}_{\geq 0}^{6n} \) (cf. Lemma 3.5 of Holstein et al. (2013)) and the computation of steady states (cf. Theorem...
4.2 and Remark 4.3 of Holstein et al. (2013)). First, we define the matrix

\[ E := \begin{bmatrix} E_0 & \cdots & E_0 \end{bmatrix} \in \mathbb{R}^{6^n \times 3n} \] with \[ E_0 := \begin{bmatrix} 1 & 0 & 1 \\ 1 & 0 & 0 \\ 0 & 0 & 1 \\ 0 & 1 & 1 \\ 0 & 1 & 0 \\ 0 & 0 & 1 \end{bmatrix} \tag{9} \]

so that the columns of \( E \) form a basis of \( \ker(S) \). In addition, the columns of \( E \) are generators of \( \ker(S) \cap \mathbb{R}_{\geq 0}^{6^n} \). Secondly, we define the matrix

\[ L := \begin{bmatrix} L(0) \\ L(1) \\ \vdots \\ L(n) \end{bmatrix} \in \mathbb{Z}^{(3+3n) \times 3} \] for \[ L(0) := \begin{bmatrix} 1 & n - 1 & -1 \\ -1 & -n & 0 \\ 1 & n - 2 & -1 \end{bmatrix}, \]

\[ L(i) := \begin{bmatrix} 0 & i - 2 & -1 \\ -1 & i - n & 0 \\ 0 & i - 2 & -1 \end{bmatrix} \] \tag{10} \]

and observe that the matrix \( L \) has the same range as the matrix \( M \) defined in (Holstein et al., 2013, Eqs. (17a)–(17c)) because of

\[ M = LR \] with \[ R = \begin{bmatrix} -1 & 0 & 0 \\ 0 & -1 & 1 \\ 0 & 0 & -1 \end{bmatrix}. \]

Now we can summarize those points of Holstein et al. (2013) that are relevant for the following discussion:

**Proposition 3.2** (Multistationarity) **Recalling the dynamical system** \( \dot{x} = Sr(\kappa, x) \) **and the matrices** \( Z, E \) **and** \( L \) **from** (8), (9) **and** (10) **one has the following equivalences:**

1. A given \( a \in \mathbb{R}^{3+3n}_{\geq 0} \) is a positive steady state of \( \dot{x} = Sr(\kappa, x) \) if and only if there exists a \( \lambda \in \mathbb{R}^{3n}_{\geq 0} \) with

\[ \kappa = \kappa(a, \lambda) := \text{diag} \left( a^{-\lambda^T} \right) E \lambda \]. \tag{11} \]

2. A given \( b \in \mathbb{R}^{3+3n}_{\geq 0} \) is a positive steady state of \( \dot{x} = Sr(\kappa(a, \lambda), x) \) if and only if

\[ \ln (b) - \ln (a) \in \text{im} (L) \] \tag{12} \]

holds true, i.e., if and only if there exists a \( g \in \mathbb{R}^{3}_{\geq 0} \) with

\[ b = \text{diag} \left( g^L \right) a \]. \tag{13} \]
(3) Two positive steady states $a$ and $b = \text{diag}(g^L)a$, $g \in \mathbb{R}^3_{>0}$, of $\dot{x} = Sr(\kappa(a, \lambda), x)$ satisfy $Za = Zb$ from (6c) if and only if $g \in \mathbb{R}^3_{>0}$ is a solution of the 3-dimensional coset condition

$$\Theta(g, a) := Z\left(\text{diag}(g^L) - I\right)a = Z\text{diag}(a)(g^L - 1) = 0, \quad g \in \mathbb{R}^3_{>0}. \quad (14)$$

For $g \neq 1$, the steady states $a$ and $b := \text{diag}(g^L)a$ are distinct positive steady states for the network $\dot{x} = Sr(\kappa(a, \lambda), x)$ within the same coset of the stoichiometric matrix $S$.

The proof follows directly from Holstein et al. (2013). For part (2), we recall $\text{im}(M) = \text{im}(L)$ and note that $\mu = \ln(b) - \ln(a) \in \text{im}(L)$, satisfying $b = \text{diag}(g^L)a$, can be written as

$$\mu = L\ln(g) \quad \text{for} \quad g = (g_1, g_2, g_3)^T \in \mathbb{R}^3_{>0} \quad (15)$$

because of $g^L = e^L\ln(g) = e^\mu$ and $b = \text{diag}(g^L)a = \text{diag}(e^\mu)a$ (cf. (13)). This parametrization via $g \in \mathbb{R}^3_{>0}$ will be crucial in what follows. The above choice of $L$ will turn out to be advantageous since all entries of the first and third column of $L$ come from $\{-1, 0, 1\}$. We note that the matrix $L$ in (10) constrains the components of $g^L$ and thus imposes a special geometry on the steady states $a$ and $b$. For a biological interpretation, we refer to the discussion in Sect. 6.

In the subsequent Sects. 4 and 5, we will take advantage of part (3) of Proposition 3.2. We will fix an $a \in \mathbb{R}^{3n+3}_{>0}$ and a $\lambda \in \mathbb{R}^{3n}_{>0}$ with $E\lambda > 0$ and define the parameter $\kappa$ as $\kappa(a, \lambda)$ according to (11). Then any solution $g \in \mathbb{R}^3_{>0}$ of (14), different from 1, will provide a steady state $b = \text{diag}(g^L)a$, different from $a$, within the same coset of the stoichiometric matrix $S$.

### 4 A Scalar Determining Equation for Multistationarity

The previous section shows that multistationarity for the system (5), derived from network (2), can be characterized by the 3-dimensional coset condition (14). In the spirit of Wang and Sontag (2008), we will prove that the simple form (8) of the matrix $Z$, representing the conservation laws, allows a reduction to a scalar equation

$$P(\xi, a) = 0$$

where $P(\xi, a)$ is a polynomial in $\xi := g_2$, the second component of $g$ (cf. the representations (27) and (29) below). A zero $\xi_0 = \xi_0(a)$ of $P$ will be called an admissible zero (for (14)) if and only if the corresponding $g = g(\xi_0(a), a)$ belongs to $\mathbb{R}^3_{>0}$, i.e., if and only if the zero $\xi_0 = \xi_0(a)$ is positive and a certain scalar polynomial inequality $G(\xi_0(a), a) > 0$ holds true (see Proposition 4.1 and (28) below).

We first turn to the matrix $L$ of Eq. (10), denote the second column of $L(i)$ by $\ell(i)$ and define

$$\ell = (\ell_1, \ldots, \ell_{3+3n})^T = (\ell_{T(0)}, \ldots, \ell_{T(n)})^T \in \mathbb{Z}^{3+3n}$$
with $\ell^{T}_{(0)} = (n-1,-n,n-2)$ and $\ell^{T}_{(i)} = (i-2,i-n,i-2)$ for $i = 1, \ldots, n$. Moreover we fix an arbitrary $a \in \mathbb{R}^{3n+3}$ and introduce $\omega = \omega(a)$ via

$$\omega = (\omega_1, \omega_2, \omega_3)^T := Z a$$

with the total enzyme concentrations $\omega_1 = \sum_{k=0}^{n} a_{1+3k}$ and $\omega_3 = \sum_{k=0}^{n} a_{3+3k}$. For a more compact notation, we suppress the dependence on $a \in \mathbb{R}^{3n+3}$ for the moment.

The 3-dimensional system (14) for the unknown $g \in \mathbb{R}^3_{>0}$ and the positive parameter $a \in \mathbb{R}^{3n+3}$ can thus be written as

$$\omega_1 = g_3^{-1} \left[ a_1 g_1 \xi^{\ell_1} + a_4 \xi^{\ell_4} + \cdots + a_{1+3n} \xi^{\ell_{1+3n}} \right], \quad (17a)$$

$$\omega_3 = g_3^{-1} \left[ a_3 g_1 \xi^{\ell_3} + a_6 \xi^{\ell_6} + \cdots + a_{3+3n} \xi^{\ell_{3+3n}} \right], \quad (17b)$$

together with

$$\omega_2 = -a_1 g_1 g_3^{-1} \xi^{\ell_1} - a_3 g_1 g_3^{-1} \xi^{\ell_3} + g_1^{-1} \left[ a_2 \xi^{\ell_2} + \cdots + a_{2+3n} \xi^{\ell_{2+3n}} \right]. \quad (18)$$

Because of $\ell_1 = 1 + \ell_3$, the system (17) can be written as

$$a_1 \xi \cdot g_1 \xi^{\ell_3} - \omega_1 g_3 = -\left[ a_4 \xi^{\ell_4} + \cdots + a_{1+3n} \xi^{\ell_{1+3n}} \right], \quad (19a)$$

$$a_3 \xi \cdot g_1 \xi^{\ell_3} - \omega_3 g_3 = -\left[ a_6 \xi^{\ell_6} + \cdots + a_{3+3n} \xi^{\ell_{3+3n}} \right]. \quad (19b)$$

We exploit the structure of the subvectors $\ell_{(i)}$ to represent the system (19) as a $\xi$-dependent linear system for $g_1, g_3$. For this purpose we introduce the polynomials

$$\Omega_4(\xi) = a_4 + a_7 \xi + \cdots + a_{1+3n} \xi^{n-1}, \quad \Omega_6(\xi) = a_6 + a_9 \xi + \cdots + a_{3+3n} \xi^{n-1} \quad (20a)$$

and note the relations to

$$\omega_1 = a_1 + \Omega_4(1), \quad \omega_3 = a_3 + \Omega_6(1). \quad (20b)$$

For later purposes, we also introduce the $n$th order polynomial

$$\Omega_2(\xi) := a_2 + a_5 \xi + \cdots + a_{2+3n} \xi^n \quad \text{with} \quad \omega_2 = -a_1 - a_3 + \Omega_2(1), \quad (20c)$$

where $\omega_2$ is not necessarily positive (cf. (16)). With the help of $\Omega_4(\xi)$ and $\Omega_6(\xi)$ the system (19) reads

$$\begin{bmatrix} a_1 \xi^{n-1} - \omega_1 \\ a_3 \xi^{n-2} - \omega_3 \end{bmatrix} \begin{bmatrix} g_1 \\ g_3 \end{bmatrix} = -\frac{1}{\xi} \begin{bmatrix} \Omega_4(\xi) \\ \Omega_6(\xi) \end{bmatrix}. \quad (21)$$

If

$$\Delta(\xi) := \frac{a_1 \xi}{\omega_1} - \frac{a_3 \xi}{\omega_3} = \frac{a_1}{\omega_1} (\xi - \xi^*) \quad (21)$$
is nonzero, that is, if
\[ \xi \neq \xi^* := \frac{\omega_1/a_1}{\omega_3/a_3} > 0, \tag{22} \]
then system (19) possesses the unique solution
\[
\begin{align*}
g_1 &= g_1(\xi) := \xi^{1-n} F_1(\xi)/\Delta(\xi), \\
g_3 &= g_3(\xi) := \xi^{-1} F_3(\xi)/\Delta(\xi)
\end{align*} \tag{23a/b}
\]
for the following polynomials \( F_1 \) and \( F_3 \) in \( \xi \) of degree \( n-1 \) and \( n \), respectively:
\[
\begin{align*}
F_1(\xi) &:= \frac{\Omega_6(\xi)}{\omega_3} - \frac{\Omega_4(\xi)}{\omega_1} \tag{24a} \\
F_3(\xi) &:= \frac{a_1 \xi}{\omega_1} \frac{\Omega_6(\xi)}{\omega_3} - \frac{a_3}{\omega_3} \frac{\Omega_4(\xi)}{\omega_1} \tag{24b}
\end{align*}
\]
Concerning the polynomials \( F_1 \) and \( F_3 \) that depend on \( a \) in a nonlinear way, we observe the following identities:
\[
F_3(\xi) = \frac{\Omega_4(\xi)}{\omega_1} \Delta(\xi) + \frac{a_1 \xi}{\omega_1} F_1(\xi) = \frac{\Omega_6(\xi)}{\omega_3} \Delta(\xi) + \frac{a_3}{\omega_3} F_1(\xi). \tag{25}
\]
We note that, in case of (22), \( g_1 \) is positive for positive \( \xi \) if and only if \( F_1(\xi) \) and \( \Delta(\xi) \) are of the same sign. By (23b) and (25), \( g_3 \) is positive for such positive \( g_1 \) so that we have arrived at the following fact:

**Proposition 4.1 (Positivity of \((g_1, g_3)\))** Given a positive \( \xi \) with \( \xi \neq \xi^* \), the (19)-solution \((g_1(\xi), g_3(\xi))\), given by (23), is positive if and only if
\[
G(\xi) := F_1(\xi) \Delta(\xi) > 0 \tag{26}
\]
holds true.

For the case \( \xi = \xi^* \), we refer to Remark 4.3(b) below. In what follows, we assume (22) to be true.

If these rational solutions (23) of the linear system (19) are inserted into (18) one arrives—with the notations (20)—at the equivalent \((2n+1)\)—order polynomial equation.
\[ Q(\xi) := \Delta(\xi) F_3(\xi) \frac{\Omega_2(\xi)}{\omega_2} - \left[ \frac{a_1 \xi + a_3}{\omega_2} \xi \left[ F_1(\xi) \right]^2 + \xi F_1(\xi) F_3(\xi) \right] = 0 \]

with \( Q(1) = 0 \) by \( g = (g_1(1), 1, g_3(1))^T = (1, 1, 1)^T \) being the trivial solution of equation (14). By the \( F_1 \)-representations of \( F_3 \) in (25), \( Q \) can be written as

\[
Q_1(\xi) = \Delta^2(\xi) \frac{\Omega_4(\xi)}{\omega_1} \frac{\Omega_2(\xi)}{\omega_2} - \xi \left[ \frac{a_1 \xi + a_3}{\omega_2} + \frac{a_1 \xi}{\omega_1} \right] F_1^2(\xi) - \Delta(\xi) F_1(\xi) \left[ \xi \frac{\Omega_4(\xi)}{\omega_1} - \frac{\Omega_2(\xi)}{\omega_2} \frac{a_1 \xi}{\omega_1} \right]
\]
and as

\[
Q_3(\xi) = \Delta^2(\xi) \frac{\Omega_6(\xi)}{\omega_3} \frac{\Omega_2(\xi)}{\omega_2} - \xi \left[ \frac{a_1 \xi + a_3}{\omega_2} + \frac{a_3}{\omega_3} \right] F_1^2(\xi) - \Delta(\xi) F_1(\xi) \left[ \xi \frac{\Omega_6(\xi)}{\omega_3} - \frac{\Omega_2(\xi)}{\omega_2} \frac{a_3}{\omega_3} \right].
\]

We now take a linear combination of these expressions with nonnegative scalars \( h_1 \) and \( h_3, h := (h_1, h_3) \neq (0, 0) \), and define

\[
P_h(\xi) := \omega_2 h_1 Q_1(\xi) + \omega_2 h_3 Q_3(\xi) = A_h(\xi) \Delta^2(\xi) + B_h(\xi) \Delta(\xi) F_1(\xi) - C_h(\xi) F_1^2(\xi)
\]  

(27a)

for

\[
A_h(\xi) = \left( h_1 \frac{\Omega_4(\xi)}{\omega_1} + h_3 \frac{\Omega_6(\xi)}{\omega_3} \right) \Omega_2(\xi),
\]
(27b)

\[
B_h(\xi) = \left( h_1 \frac{a_1 \xi + a_3}{\omega_1} + h_3 \frac{a_3}{\omega_3} \right) \Omega_2(\xi) - \left( h_1 \frac{\Omega_4(\xi)}{\omega_1} + h_3 \frac{\Omega_6(\xi)}{\omega_3} \right) \omega_2 \xi,
\]
(27c)

\[
C_h(\xi) = \xi \left[ (h_1 + h_3)(a_1 \xi + a_3) + \left( h_1 \frac{a_1 \xi}{\omega_1} + h_3 \frac{a_3}{\omega_3} \right) \omega_2 \right].
\]
(27d)

Since \( L \) is a matrix with integer entries, (17) and (18) make sense for all \( g \) with \( g_j \neq 0 \), \( j = 1, 2, 3 \). Hence \( P_h \) can be considered as a function of \( \xi \in \mathbb{R} \). By Proposition 4.1, a zero \( \xi_0 \) of \( P_h \) with \( \xi_0 \neq \xi^* \) will be called an \textit{admissible} zero (for (14)) if

\[
\xi_0 > 0 \quad \text{and} \quad G(\xi_0) = F_1(\xi_0) \Delta(\xi_0) > 0
\]

(28)

hold true. Obviously, \( \xi_0 = 1 \) is an admissible zero of \( P_h \) with \( g_1(1) = 1 = g_3(1) \) in case of \( \xi^* \neq 1 \).

In the special case with \( h_1 = \omega_1 \) and \( h_3 = \omega_3 \) in (27), one has

\[
P(\xi) := \omega_1 \omega_2 Q_1(\xi) + \omega_2 \omega_3 Q_3(\xi) = A(\xi) \Delta^2(\xi) + B(\xi) \Delta(\xi) F_1(\xi) - C(\xi) F_1^2(\xi)
\]

(29a)
dependence on a of admissible zeros of (29a), we refer to Remark 4.3 where we reprove the upper bound found in Wang and Sontag (2008).

Determining equation of the coset condition (14). Positive solutions $g$ of (14) are characterized by the scalar determining equation $\theta(\xi, a) = 0$ in (30) whereby we explicitly mention the nonlinear dependence on $a \in \mathbb{R}^{3+3n}_>$. 

\begin{align*}
\text{Proposition 4.2} \quad & \text{(Determining equation for } \xi > 0, \xi \neq \xi^*) \text{ The determining equation for admissible solutions } g \in \mathbb{R}^{3}_> \text{ of the coset condition (14) is given by} \\
& \quad \theta(\xi, a) := 2C(\xi, a)F_1(\xi, a) - \Delta(\xi, a)[B(\xi, a) + (B^2(\xi, a) + 4A(\xi, a)C(\xi, a))]^{1/2} = 0 \\
& \text{for the polynomials } A, B \text{ and } C \text{ from (29). Any positive zero } \xi = \xi(a) \text{ of } \theta(\xi, a), \text{ different from } \xi^*(a), \text{ defines a positive steady state} \\
& \quad b = \text{diag } \left( g^L \right) a \neq a \\
& \text{of the network } \dot{x} = Sr(\kappa(a, \lambda), x) \text{ (cf. (5), (11)) for } g = (g_1(\xi(a), a), \xi(a), g_3(\xi(a), a))^T \text{ from (23).} \\
\text{Remark 4.3} \quad & \text{(At most } 2n - 1 \text{ admissible zeros (cf. Wang and Sontag (2008)))} \\
& \text{(a) We first assume (22), i.e., } \xi \neq \frac{\omega_1/\omega_3}{\omega_1/\omega_3}. \text{ Since the leading coefficient of } P(\xi) \text{ is positive and } P(0) \text{ is positive, there exists at least one negative zero } \xi_{-1} \text{ of } P. \text{ Obviously, } P(\xi^*) \text{ is negative. We suppose that } P(\xi) \text{ has } 2n \text{ distinct positive zeros and that } P \text{ is negative on an interval } (\xi', \xi'') \ni \xi^* \text{ with } P(\xi') = 0 = P(\xi''), \xi' > 0. \text{ In case } F_1(\xi) \text{ has a zero } \xi^# \in (\xi', \xi''), \text{ the value } P(\xi^#) = \Delta^2(\xi^#)\Omega_6(\xi^#)\Omega_2(\xi^#)/\omega_3 \text{ would be positive. Hence } F_1 \text{ cannot change its sign on } (\xi', \xi''). \text{ The } g_1 \text{-expression (23a) thus implies that only one of the values } g_1(\xi') \text{ and } g_1(\xi'') \text{ is positive. Summarizing, } P(\xi) \text{ has at most } 2n - 1 \text{ positive zeros under (22) (cf. Wang and Sontag (2008) where this kind of argument has been introduced).} \)
(b) We now turn to the case \( \xi = \frac{\omega_1}{a_1} \) with \( \Delta(\xi) = 0 \) (cf. (21)). System (17) is solvable if and only if
\[
\frac{\omega_1}{\omega_3} = \frac{\Omega_4(\xi)}{\Omega_6(\xi)} .
\] (31a)

Under (31a), \( F_1 \) and \( F_3 \) vanish at \( \xi \) (cf. (24)) and the positive solution of (17) is of the form
\[
g_1 = g_1(\eta) = \frac{1}{\xi} \left( \frac{\Omega_4(\xi)}{\omega_1} + \frac{a_1 \xi}{\omega_1} \eta \right) = \frac{1}{\xi} \left( \frac{\Omega_6(\xi)}{\omega_3} + \frac{a_3}{\omega_3} \eta \right)
\] (31b)
with \( \eta > 0 \). Eq. (18) is thus equivalent to
\[
A(\xi) + B(\xi) \eta - C(\xi) \eta^2 = 0
\] (31c)
with positive \( C(\xi) \) and positive \( A(\xi) \), cf. (29). Hence there exists a unique positive zero \( \eta_0 \) of (31c). Consequently, this value of \( \xi \) can yield at most one positive solution \( g \) of (14). We note that, under (31a), this \( \xi \) is a zero of \( P \) of order at least 2.

Hence we conclude that \( P(\xi) \) has at most \( 2n - 1 \) admissible zeros. We might add, as a side remark, that \( a = \alpha \cdot \frac{1}{2}, \alpha > 0 \), is the unique positive steady state of (5) since (29a) is equivalent to \( (\xi^n - 1)(\xi^{n+1} - 1) = 0 \) possessing just \( \xi = 1 = \xi^* \) as positive (double) zero.

Summarizing, by an argument similar to the one of Wang and Sontag (2008) we have shown, that the \( a \)-dependent polynomial \( P(\xi) \) in (29a) possesses at most \( 2n - 1 \) distinct admissible zeros so that there are at most \( 2n - 1 \) distinct steady states of (5) within one coset of the stoichiometric matrix \( S \). Moreover we have established that the distinct zeros of \( \theta(\xi, a) \) in (30) give rise to distinct steady states of (5) within one coset.

Finally, we note that the choices \( h_1 = 0 \) and \( h_3 = \omega_3 \) in (27a) lead to an analogous result in case of
\[
\omega_2(a) + \omega_3(a) = \sum_{k=0}^{n} a_{2+3k} + \sum_{k=1}^{n} a_{3+3k} - a_1 > 0 .
\] (32)

For
\[
A_0(\xi) := \Omega_6(\xi) \Omega_2(\xi) ,
\] (33a)
\[
B_0(\xi) := a_3 \Omega_2(\xi) - \omega_2 \Omega_6(\xi) ,
\] (33b)
\[
C_0(\xi) := \xi \left[ (a_1 \xi + a_3) \omega_3 + a_3 \omega_2 \right] .
\] (33c)
the distinct zeros of
\[
\begin{align*}
\theta_0(\xi, a) &:= 2C_0(\xi, a) F_1(\xi, a) - \Delta(\xi, a) \left[ B_0(\xi, a) + (B_0^2(\xi, a) + 4A_0(\xi, a)C_0(\xi, a))^{1/2} \right] = 0 
\end{align*}
\] (33d)
give rise to distinct steady states of (5) within one coset. To this end, we just observe that $A_0(\xi)$ is positive for $\xi > 0$ and that the condition (32) guarantees the positivity of $C_0(\xi)$ for $\xi > 0$. In the following section we apply the determining equation (33d) to construct a triple phosphorylation network with more than 3 steady states. Obviously, the choices $h_1 = \omega_1$ and $h_3 = 0$ in (27a) entail an analogous result.

5 Phosphorylation Systems with the Maximal Number of Steady States

We consider phosphorylation systems with $n$ sites for $n = 2, 3$ and 4 and give examples of multistationarity with the maximal number $2n - 1$ of steady states. For $n = 2$ we refer to the Example 4.8 in Holstein et al. (2013). We continue with the case $n = 3$.

Suppressing the $a$-dependence, $\theta_0(\xi, a) = 0$ from (33d) can be written as

$$2C_0(\xi)\left[\omega_1 \Omega_6(\xi) - \omega_3 \Omega_4(\xi)\right] + a_3 \omega_1 \left[B_0(\xi) + (B^2_0(\xi) + 4A_0(\xi)C_0(\xi))^{1/2}\right]$$

$$= a_1 \omega_3 \xi \left[B_0(\xi) + (B^2_0(\xi) + 4A_0(\xi)C_0(\xi))^{1/2}\right]$$

where the $n$ parameters $a_{3j+1}$, $j = 1, 2, ..., n$, appear just on the left-hand side and in a linear way. So they might be tuned to fulfill some prescribed constraints. This fact is the main motivation for passing from the determining equation $\theta_0 = 0$ in (30) to the determining equation $\theta_0 = 0$ in (33d).

For the triple phosphorylation, we choose a positive $a \in \mathbb{R}^{3+3}$ and fix the rate constant vector

$$\kappa = \kappa(a) = \text{diag} \left(a^{-3T} \right) E_1$$

so that $a$ is a positive steady state of the network (5). Obviously, one has $\theta_0(1, a) = 0$.

In particular, we choose $a$ in the partitioned form

$$a^* = (1, 1, 1|a_4, 1, 1|a_7, 1, 0.1|a_{10}, 0.32, 60)^T \in \mathbb{R}^{12}_{>0}$$

(34)

and compute analytically the remaining $n = 3$ parameters $a_4$, $a_7$ and $a_{10}$ so that $\theta_0(\xi, a^*)$ has the triple zero $\xi = 1$ and a further (simple) zero $\xi = \frac{1}{2}$, i.e., so that $n = 3$ constraints are met. That is, we solve the equations

$$\frac{\partial}{\partial \xi} \theta_0(\xi, a^*) \bigg|_{\xi = 1} = 0, \quad \frac{\partial^2}{\partial \xi^2} \theta_0(\xi, a^*) \bigg|_{\xi = 1} = 0, \quad \theta_0 \left(\frac{1}{2}, a^*\right) = 0$$

and obtain the analytical solution:
\[ a_4 = \frac{7787061638}{39861237827} - \frac{10658368327}{79722475654} \left( 112932093987944456 - 14276293028087\sqrt{5505644539} \right), \]

\[ a_7 = -\frac{112932093987944456 - 14276293028087\sqrt{5505644539}}{20 \left( -340903663256564611 + 4572282020317\sqrt{5505644539} \right)}, \]

\[ a_{10} = -\frac{476228483659}{39861237827} - \frac{221291854961 \left( 112932093987944456 - 14276293028087\sqrt{5505644539} \right)}{39861237827 \left( -340903663256564611 + 4572282020317\sqrt{5505644539} \right)}. \]

The resulting numerical values (up to 4 decimals) are given by

\[ a_4 := a^*_4 = 5.9026(84)\ldots, \quad a_7 := a^*_7 = 2.1344(85)\ldots, \quad a_{10} := a^*_{10} = 248.9413(34)\ldots. \] (35)

Thus, \( a_4, a_7 \) and \( a_{10} \) are positive as required, the inequality (32) is obviously satisfied. The numerical value of \( \xi^* \) at \( a^* \) is 4.1542\ldots and thus different from the zeros of \( \theta_0 \). These were the constraints that motivated the choice of the remaining components of \( a^* \) in (34). Finally, the numerical value of the rate constant vector \( \kappa = \kappa(a^*) \) is

\[
\begin{align*}
(2, 0.1694\ldots, 0.1694\ldots, 2, 1, 1|2, 0.4684\ldots, 0.4684\ldots, 2, 10, 10|2, 0.0040\ldots, 0.0040\ldots, 6.25, 0.0166\ldots, 0.0166\ldots)^T.
\end{align*}
\]

A one-parameter continuation

\[ a = a^* + \delta e_{10}, \quad -.05 < \delta < .05, \]

in (33d) is leading to the bifurcation diagram in Fig. 2 in the \((\delta, \xi)\)-plane. For \( \delta = -.03 \), the numerical values for the five admissible zeros \( \xi^{(j)} \) of (33d) and the five admissible steady states \( b^{(j)} \) of (5) can be found in Table 1.

Numerical computations lead to the conclusion that \( b^{(1)}, b^{(3)} \) and \( b^{(5)} \) are exponentially stable steady states of (5) whereas the Jacobian at \( b^{(2)} \) as well as the Jacobian at \( b^{(4)} \) possesses one positive eigenvalue.

For \( n \geq 3 \), the above argument can be applied to an \( n \)-site phosphorylation to create networks with \( n + 1 \) steady states for (5) by tuning the \( n \) parameters \( a_{3j+1}, j = 1, 2, \ldots, n \). For odd \( n \), one is then, generically, expecting \( n + 2 \) such steady states. Using this rationale for even \( n = 4 \), we have constructed a phosphorylation network with a determining Eq. (33d) with five prescribed zeros at 0.5, 1, 1.03, 1.05 and 1.07 by choosing \( a^* \in \mathbb{R}_{>0}^{15} \) as

\[
\begin{align*}
a^*_i &= 1, \quad a^*_2 = 1, \quad a^*_3 = 1, \quad a^*_4 = 1.983448, \quad a^*_5 = 1, \quad a^*_6 = 1, \\
a^*_7 &= 469.6162955, \quad a^*_8 = 1, \quad a^*_9 = 400, \quad a^*_{10} = 73.8036, \quad a^*_{11} = .32, \quad a^*_{12} = 60, \quad (36) \\
a^*_{13} &= .5807998, \quad a^*_{14} = 7, \quad a^*_{15} = 1.8.
\end{align*}
\]
Fig. 2 Numerical continuation of $\theta_0(\xi, a) = 0$ from (33d) with the data from (34) and (35). Pitchfork bifurcation at $(\delta_0, \xi_0) = (0, 1)$ (BP) and two saddle-node bifurcations (LP) at $(\delta_-, \xi_-) = (-.04488, .66691)$ and $(\delta_+, \xi_+) = (.03352, .41262)$. For $\delta = 0$ one encounters the prescribed triple zero $\xi = 1$, the zero $\xi = 1/2$ and an additional zero near $0.36222$. For $\delta = -0.03$, one has 5 distinct $\xi$-values $\xi^{(j)}$ leading to 5 distinct steady states $b^{(j)}$ of (5) ($j = 1, ..., 5$, cf. Table 1). Solid lines correspond to $\xi$’s yielding exponentially stable steady states, dashed lines to $\xi$’s yielding unstable steady states (Color figure online)

Table 1 The five admissible steady states $b^{(j)} \in \mathbb{R}^{12}_{>0}$ of (5) for $\delta = -0.03$ and the corresponding zeros $\xi^{(j)}$ of (33d) up to 4 decimals; the numerical values of the rate constant vectors $\kappa = \kappa(a)$ and $\kappa(a^*)$ coincide up to the first 4 decimals, but the components $\kappa_{14}(a) = \kappa_{15}(a) = 0.00401749, ...$ and $\kappa_{14}(a^*) = \kappa_{15}(a^*) = 0.00401701, ...$ differ

| Phos. # | $b^{(1)}$ | $b^{(2)}$ | $b^{(3)}$ | $b^{(4)} \equiv a$ | $b^{(5)}$ |
|---------|----------|----------|----------|----------------|----------|
| $i = 0$ | 1.4730   | 2.1298   | 1.0793   | 1              | 0.9618   |
|         | 4.7498   | 2.4000   | 1.4726   | 1              | 0.7700   |
|         | 4.2424   | 2.1440   | 1.3722   | 1              | 0.8246   |
| $i = 1$ | 41.3012  | 17.2813  | 9.3826   | 5.9026         | 4.3718   |
|         | 1.6493   | 1.3655   | 1.1583   | 1              | 0.8980   |
|         | 6.9970   | 2.9277   | 1.5895   | 1              | 0.7406   |
| $i = 2$ | 5.1859   | 3.5554   | 2.6688   | 2.1344         | 1.8438   |
|         | 0.5726   | 0.7768   | 0.9112   | 1              | 1.0474   |
|         | 0.2429   | 0.1665   | 0.1250   | 1.32           | 0.0863   |
| $i = 3$ | 209.9882 | 235.8919 | 244.8175 | 248.9113       | 250.7710 |
|         | 0.0636   | 0.1414   | 0.2293   | 0.32           | 0.3909   |
|         | 50.6175  | 56.8616  | 59.0132  | 60             | 60.4482  |
| $\xi$   | 0.3472   | 0.5689   | 0.7866   | 1              | 1.1662   |
As it turns out, this determining equation has two additional positive zeros, one near .59 and one near 51.07. See Fig. 3 for \( a = a^* + \delta e_1, -10^{-3} < \delta < 10^{-3} \).

6 The Geometry of Multistationarity

Here we discuss multistationarity and the constraints imposed on steady states within one coset of the stoichiometric subspace.

6.1 Relation to Sign Patterns \( s_1, \ldots, s_7 \) from Holstein et al. (2013)

As a consequence of Holstein et al. (2013), any two distinct steady states \( a \) and \( b \) of (5) (for \( n \) arbitrary) within one coset of the stoichiometric subspace satisfy the following: the sign pattern \( \text{sign}(\ln b/a) \) obeys one of the formulae \( s_1 \cdots s_7 \) from Holstein et al. (2013). For the steady states of the 3-site phosphorylation system we observe that the sign vector for \( \ln (b^{(j+1)}/b^{(j)}) \) is given by \( s_2 := (-, -|-, -|-, -|+, +|+, +|+, +) \) for \( j = 1, 2, 3, 4 \) so that these steady states are ordered with respect to \( s_2 \).

For the example with \( n = 4 \) with steady states \( b^{(j)} \) belonging to increasing \( \xi_j \) \((j = 1, \ldots, 7)\) with values 0.5, 0.59..., 1, 1.03, 1.05, 1.07, 51, ... and \( b^{(3)} = a \): the \( \ln (b^{(j)}/a) \), \( j = 1, 2 \), belong to \( s_4 \), the \( \ln (b^{(j)}/a) \), \( j = 4, 5, 6 \), belong to \( s_1 := (+, +|+, +|+, +|+, +|+, +) \). Finally, \( \ln (b^{(7)}/a) \) belongs to \( s_5 := (+, +|+, +|+, +|+, +|+, +) \).

Moreover, the \( \ln (b^{(j+1)}/b^{(j)}) \) belong to the sign patterns \( s_7 := (+, -|+, +|+, -|+, +|+, +|+, +) \) for \( j = 1, 5 \), to \( s_1 \) for \( j = 2, 3, 4 \) and to \( s_5 \) for \( j = 6 \).

6.2 Geometric Constraints on Multistationarity

According to the ordering of variables in (1), we introduce the following notation for \( g^L = \frac{b}{a} \) with the matrix \( L \) from (10):

\[
g^L = \begin{pmatrix} \Gamma_{E_1}, \Gamma_A, \Gamma_{E_2} | \Gamma_{AE_1}, \Gamma_{AP}, \Gamma_{AP} \Gamma_{E_2} | \Gamma_{AP} \Gamma_{E_1}, \Gamma_{A_2 P}, \Gamma_{A_2 P} \Gamma_{E_2} | \ldots \\
\end{pmatrix}^T
\]

with

\[
\Gamma_{E_1} = (g^L)_1 = \frac{g_1 g_2^{n-1}}{g_3}, \quad \Gamma_{E_2} = (g^L)_3 = \frac{g_1 g_2^{n-2}}{g_3}, \\
\Gamma_{A_{i-1}P} E_1 = (g^L)_{1+3i} = \frac{g_2^i}{g_2^2 g_3}, \quad \Gamma_{A_{i}P} = (g^L)_{2+3i} = \frac{g_2^i}{g_1 g_2^n}.
\]
(a) Continuation for $-10^{-3} \leq \delta \leq 10^{-3}$ in the $(\delta, \xi)$-plane

(b) Zoom to $-4 \cdot 10^{-6} \leq \delta \leq +4 \cdot 10^{-6}$; cyan diamonds indicate six zeros for $\delta = 0$

Fig. 3 Numerical continuation of $\theta_0(\xi, a) = 0$ from (33d) with the data from (36) showing 6 zeros 0.5, 0.5910929..., 1, 1.03, 1.05 and 1.07—there is a 7th zero 51.07286... near $\xi = 51$. Solid lines correspond to $\xi$’s yielding exponentially stable steady states, dashed lines to $\xi$’s yielding unstable steady states. The label LP denotes saddle-node bifurcation points, the label BP transcritical bifurcation points (Color figure online)
\[ \Gamma_{A_i P E_2} = (g^L)^{3+3i} = (g^L)_{1+3i} \]

for \( i = 1, \ldots, n \). We recall the form

\[ g_1 = \xi^{1-n} F_1(\xi) / \Delta(\xi), \quad g_2 = \xi, \quad g_3 = \xi^{-1} F_3(\xi) / \Delta(\xi) \]

of the (19)-solutions where \( \xi \) is to be a positive zero of (30) or (33d) (cf. (23)). So we obtain for the partitioning

\[ g^L = \left( \begin{array}{ccc} \Gamma^T_{(0)} & \Gamma^T_{(1)} & \cdots & \Gamma^T_{(n)} \end{array} \right)^T \in \mathbb{R}_{>0}^{3+3n} \]

the following identities:

\[ \Gamma^T_{(0)} := \left( \Gamma_{E_1}, \Gamma_A, \Gamma_{E_2} \right) = \left( \xi, \frac{F_1(\xi)}{F_3(\xi)}, \frac{\Delta(\xi)}{\Delta(\xi)} \right), \]

\[ \xi = \frac{\Gamma_{E_1}}{\Gamma_{E_2}}, \quad (38) \]

\[ \Gamma^T_{(1)} := \left( \Gamma_{A E_1}, \Gamma_{A P}, \Gamma_{A P E_2} \right) = \left( \frac{\Delta(\xi)}{F_3(\xi)}, \frac{\Delta(\xi)}{F_1(\xi)}, \frac{\Delta(\xi)}{F_3(\xi)} \right) = \left( \Gamma_A \Gamma_{E_1}, \xi \Gamma_A, \Gamma_{A P} \Gamma_{E_2} \right). \]

\[ \Gamma^T_{(i)} := \left( \Gamma_{A (i-1) P E_1}, \Gamma_{A_i P}, \Gamma_{A_i P E_2} \right) = \xi^{i-1} \left( \Gamma_{A E_1}, \Gamma_{A P}, \Gamma_{A P E_2} \right) \]

\[ = \xi^{i-1} \Gamma^T_{(1)} \]

In particular one has for \( i = 1, \ldots, n \):

\[ \Gamma_{A_{i-1} P E_1} = \xi^{i-1} \Gamma_A \Gamma_{E_1}, \quad (41a) \]

\[ \Gamma_{A P} = \xi^{i-1} \Gamma_A \Gamma_{E_1}, \quad (41b) \]

\[ \Gamma_{A_i P E_2} = \xi^{i-1} \Gamma_A \Gamma_{E_2} = \xi^{i-1} \Gamma_A \Gamma_{E_2}. \]

We summarize these geometric properties in the following fact:

**Proposition 6.1** Let \( \kappa \in \mathbb{R}_{>0}^{6n} \) be given and assume network (2) admits multistationarity, that is, there exist two distinct positive vectors \( a \) and \( b \) such that

\[ Sr(\kappa, a) = Sr(\kappa, b) = 0, \quad Z(b - a) = 0. \]

Then the steady state concentrations \( a_1 \) and \( b_1 \) of the kinase together with the steady state concentrations \( a_3 \) and \( b_3 \) of the phosphatase and \( a_2 \) and \( b_2 \) of the unphosphorylated protein allow the reconstruction of the ratios

\[ (g^L)^i = \frac{b_i}{a_i}, \quad i = 4, \ldots, 3 + 3n, \]
with
\[
\Gamma^T_{(1)} = (\Gamma_A \Gamma_{E_1}, \xi \Gamma_A, \xi \Gamma_A \Gamma_{E_2}) = \left( \frac{b_4}{a_4}, \frac{b_5}{a_5}, \frac{b_6}{a_6} \right)
\]
and
\[
\Gamma^T_{(i)} = (\Gamma_{A_{(i-1)p} E_1}, \Gamma_{A_i p} \Gamma_{A_i p E_2}) = \xi^{i-1} \left( \frac{b_4}{a_4}, \frac{b_5}{a_5}, \frac{b_6}{a_6} \right) \quad \text{for } i = 1, \ldots, n.
\]
In particular one has for \( i = 1, \ldots, n-1 \)
\[
\xi = \frac{\Gamma_{E_1}}{\Gamma_{E_2}} = \frac{\Gamma_{A_{(i-1)p} E_1}}{\Gamma_{A_{i-p}}} = \frac{\Gamma_{A_i p E_1}}{\Gamma_{A_{(i-1)p} E_1}} = \frac{\Gamma_{A_{(i+1)p} E_2}}{\Gamma_{A_{i-p} E_2}}.
\]

6.3 Reconstruction of Steady State Ratios from Measured Kinase \( E_1 \), Phosphatase \( E_2 \) and Substrate \( A \)

Consider the experimental investigation of a specific multisite phosphorylation system (2) whereby the rate constants \( \kappa \) and the total concentrations are fixed, but might not (all) be known. Suppose we know a priori that the system exhibits multistationarity for the given rate constants and total concentrations. Then steady state data of the concentration of kinase, phosphatase and protein in two different steady states \( a \) and \( b \) (for these total concentrations) are sufficient to reconstruct all fractions \( b_i/a_i \) of the two steady states. That is, it suffices to measure \( a_1, a_2, a_3 \) and \( b_1, b_2, b_3 \) to reconstruct all the ratios \( b_i/a_i, i = 1, \ldots, 3 + 3n \).

6.4 A Graphical Test for the Coset Condition

Next we elaborate on (42). For the steady state concentrations of the phosphoforms \( a_{3i+2} \) and \( b_{3i+2} \), it implies
\[
\frac{b_{3i+2}}{b_{3i-1}} = \xi \frac{a_{3i+2}}{a_{3i-1}} \quad \text{for } i = 1, \ldots, n.
\]
Hence the fractions \( b_{3i+2}/b_{3i-1} \) and \( a_{3i+2}/a_{3i-1} \) are collinear. Likewise we find for the fractions of kinase–substrate and of phosphatase–substrate complexes
\[
\frac{b_{3i+1}}{b_{3i-2}} = \xi \frac{a_{3i+1}}{a_{3i-2}} \quad \text{and} \quad \frac{b_{3i+3}}{b_{3i}} = \xi \frac{a_{3i+3}}{a_{3i}} \quad \text{for } i = 1, \ldots, n.
\]
We summarize this in the following fact:
Proposition 6.2 (Collinearity of relative steady states) Given $\kappa \in \mathbb{R}_{>0}^{6n}$ and steady states $a, b \in \mathbb{R}_{>0}^{3+3n}$ of (5), we define

$$\alpha_i := \frac{a_{i+3}}{a_i}, \quad \beta_i := \frac{b_{i+3}}{b_i}, \quad i = 1, \ldots, 3n.$$ 

If $a$ and $b$ belong to the same coset (i.e., $Z(b - a) = 0$), then the pairs $(\alpha_i, \beta_i)$ are collinear, i.e., the pairs $(\alpha_i, \beta_i)$ are on the line $\beta = \xi \alpha$ with slope $\xi = \frac{b_1/a_1}{b_3/a_3}$.

Remark 6.3 (Graphical test for steady states to satisfy the coset condition) Suppose for the phosphoforms $A, AP, \ldots, AnP$ two different sets of steady state values have been measured (i.e., there exists data for $a_2, a_5, \ldots, a_{2+3n}$ and $b_2, b_5, \ldots, b_{2+3n}$). If these belong to two steady states within one and the same coset (i.e., are components of two steady states $a, b \in \mathbb{R}_{>0}^{3+3n}$ with $Z(b - a) = 0$), then the points

$$\alpha_i := \frac{a_{3i+2}}{a_{3i-1}}, \quad \beta_i := \frac{b_{3i+2}}{b_{3i-1}}, \quad i = 1, \ldots, n,$$

are collinear. Hence, when one measures two steady state values of $A, \ldots, AnP$ so that the points $(\alpha_i, \beta_i)$ are not collinear then these two steady states do not give rise to multistationarity.

Appendix: The Network Matrices for $n \geq 2$

The matrices $\mathcal{Y}, Z, E$ and $L$ can be obtained from Eqs. (7), (8), (9) and (10) of this manuscript. We recall the definition of the stoichiometric matrix $S$ from Sect. 3 of Holstein et al. (2013). With the following sub-matrices

$$n_{11} = \begin{bmatrix} -1 & 1 & 1 & 0 & 0 & 0 \\ -1 & 1 & 0 & 0 & 0 & 1 \\ 0 & 0 & 0 & -1 & 1 & 1 \end{bmatrix}, \quad n_{12} = \begin{bmatrix} -1 & 1 & 1 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & -1 & 1 & 1 \end{bmatrix},$$

$$n_{21} = \begin{bmatrix} 1 & -1 & -1 & 0 & 0 & 0 \\ 0 & 0 & 1 & -1 & 1 & 0 \\ 0 & 0 & 0 & 1 & -1 & -1 \end{bmatrix}, \quad n_{22} = \begin{bmatrix} -1 & 1 & 0 & 0 & 0 & 1 \\ 0 & 0 & 0 & 0 & 0 & 0 \end{bmatrix}.$$ 

of dimension $3 \times 6$, one has
$S := \begin{bmatrix}
  n_{11} & n_{12} & n_{12} & n_{12} & n_{12} & n_{12} \\
  n_{21} & n_{22} & 0_{3 \times 6} & 0_{3 \times 6} & \cdots & 0_{(n-2) \times 3 \times 6} \\
  0_{3 \times 6} & n_{21} & n_{22} & 0_{3 \times 6} & \cdots & 0_{3 \times 6} \\
  0_{3 \times 6} & n_{21} & n_{22} & 0_{3 \times 6} & \cdots & 0_{3 \times 6} \\
  \vdots & \vdots & \vdots & \vdots & \ddots & \vdots \\
  0_{3 \times 6} & n_{21} & n_{22} & 0_{3 \times 6} & \cdots & n_{21}
\end{bmatrix} \in \mathbb{R}^{(3+3n) \times 6n}.
$

For the convenience of the reader, we close this appendix with the data for $n = 3$:

$S = \begin{bmatrix}
  -1 & 1 & 1 & 0 & 0 & 0 & -1 & 1 & 1 & 0 & 0 & 0 \\
  -1 & 1 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 \\
  0 & 0 & 0 & -1 & 1 & 1 & 0 & 0 & 0 & -1 & 1 & 1 \\
  1 & -1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
  0 & 0 & 1 & -1 & 1 & 0 & -1 & 1 & 0 & 0 & 0 & 0 \\
  0 & 0 & 0 & 1 & -1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 \\
  0 & 0 & 0 & 0 & 0 & 0 & 1 & -1 & -1 & 0 & 0 & 0 \\
  0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & -1 & 1 & 1 & 0 \\
  0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & -1 & 1 \\
  0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & -1 \\
  0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 \\
\end{bmatrix},
$

$Y^T = \begin{bmatrix}
  1 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
  0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
  0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
  0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
  0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
  1 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 \\
  0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 \\
  0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 \\
  1 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 \\
  0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 \\
  0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 \\
  0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 \\
  0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 \\
\end{bmatrix}.$
$E = \begin{bmatrix} 1 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 1 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 \end{bmatrix}$ with $SE = 0$,

$L = (L_1, L_2, L_3) = \begin{bmatrix} 1 & 2 & -1 \\ -1 & -3 & 0 \\ 1 & 1 & -1 \end{bmatrix}$ with $y^T L_1 = 0$.

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