The $2n-1$ steady-state bound is sharp for sequential distributive phosphorylation

Abstract

The sequential distributive n-site phosphorylation cycle, in which one kinase and one phosphatase act on a substrate with n ordered sites, is the standard mass-action model of multisite protein modification. Wang and Sontag proved that it has at most $2n-1$ positive steady states for any rate constants and any conserved totals. Whether this bound is attained has been known only for $n \le 4$. We prove that it is attained for every n. More precisely, for any $2n-1$ distinct positive numbers we construct, by explicit algebraic formulas, positive rate constants and totals for which the system has exactly $2n-1$ positive steady states whose free-kinase to free-phosphatase ratios are the prescribed numbers. All of these steady states are nondegenerate, so the maximal count persists on a nonempty open subset of the full $(6n+3)$-dimensional parameter space, and rational data suffice. The construction rests on a square-root change of variable that turns the steady-state equation on a special parameter locus into an interpolation problem, on a positive two-term polynomial recurrence, and on an interlacing argument that gives an explicit admissible range of enzyme totals; for instance, equally spaced auxiliary roots work for every kinase-to-phosphatase ratio above $(4n^2-1)/8$. We also prove a determinant formula which shows that $n-1$ of the constructed steady states are unstable for every n, and we certify in exact rational arithmetic that for $n \le 10$ the remaining n are asymptotically stable. The existence of $2n-1$ positive steady states for every n is formally verified in Lean 4 with Mathlib.

1 Introduction

1.1 Background

Reversible phosphorylation at multiple sites is one of the most common ways in which cells regulate protein function [2, 25]. In the simplest mechanistic description, a single kinase E adds phosphate groups to a substrate one site at a time in a fixed order, a single phosphatase F removes them in the reverse order, and each enzyme releases the substrate after every catalytic event. This is the *sequential distributive* mechanism, also called the multiple futile cycle. Under mass-action kinetics it is the reaction network

$$\begin{aligned} S_{i-1} + E &\underset{b_i}{\overset{a_i}{\rightleftharpoons}} C_i \xrightarrow{c_i} S_i + E, \\ S_i + F &\underset{\beta_i}{\overset{\alpha_i}{\rightleftharpoons}} Y_i \xrightarrow{\gamma_i} S_{i-1} + F, \qquad i = 1, \dots, n, \end{aligned} \tag{1.1}$$

where S_i is the substrate with i phosphorylated sites, C_i and Y_i are the kinase–substrate and phosphatase–substrate complexes, and all $6n$ rate constants are positive. There are $3n+3$ species and three conserved totals,

$$E_{\mathrm{T}} = E + \sum_{i=1}^{n} C_i, \qquad F_{\mathrm{T}} = F + \sum_{i=1}^{n} Y_i, \qquad S_{\mathrm{T}} = \sum_{i=0}^{n} S_i + \sum_{i=1}^{n} (C_i + Y_i). \tag{1.2}$$

Markevich, Hoek, and Kholodenko observed that already the two-site cycle can be bistable, with no feedback other than competition for the shared enzymes [23]. This started a long line of work on the steady states of (1.1); see [20, 5, 27, 16, 22, 6, 21, 4, 1, 13, 28] and the surveys [9, 11, 12]. The network has become a standard test case for algebraic and analytic methods in reaction network theory, because it is large, biologically meaningful, and has a steady-state variety with a monomial parametrization [24].

The basic counting result is due to Wang and Sontag.

Theorem 1.1 (Wang and Sontag [29])**.** *For every $n \geq 1$, all positive rate constants, and all positive totals $E_{\mathrm{T}}, F_{\mathrm{T}}, S_{\mathrm{T}}$, the system (1.1) has at most $2n-1$ positive steady states with those totals.*

This is Theorem 3 of [29]. In the same paper they showed that $n+1$ (for n even) or n (for n odd) positive steady states do occur, and that, under a genericity condition, there are at most $n+1$ when the enzymes are sufficiently scarce relative to the substrate. On this basis they conjectured that $n+1$ is never exceeded. For $n=2$ the numbers $n+1$ and $2n-1$ agree. Flockerzi, Holstein, and Conradi [17] refuted the conjecture: they produced rate constants with five steady states for $n=3$ and seven for $n=4$, so that the bound $2n-1$ is attained for $n \leq 4$. Their method prescribes zeros of a determining equation and requires the solution of a system of polynomial inequalities that grows with n. Since then the bound $2n-1$ has been conjectured to be sharp for all n [15, 14], and the question is recorded as open beyond $n=4$ in [14]. Thomson and Gunawardena had given numerical evidence that the number of steady states grows with n [27]. Feliu, Rendall, and Wiuf [15] proved that for every n there are parameters with $2\lfloor n/2 \rfloor + 1$ steady states of which $\lfloor n/2 \rfloor + 1$ are asymptotically stable, and explained why their reduction to a Michaelis–Menten limit cannot give $2n-1$ for $n \geq 3$. Giaroli, Rischter, Pérez Millán, and Dickenstein [19] described explicit open parameter regions with $2\lfloor n/2 \rfloor + 1$ positive steady states, and remarked that degeneration techniques are unlikely to reach $2n-1$. Thus for every $n \geq 5$ there has been a gap between the largest number known to occur, $2\lfloor n/2 \rfloor + 1$, and the upper bound $2n-1$, and the gap grows linearly in n. The dynamics of the cycle beyond steady states is also of current interest [8, 3, 7].

1.2 Results

This paper closes the gap.

Theorem 1.2. *Let $n \geq 1$ and let $u_0, \ldots, u_{2n-2}$ be distinct positive real numbers. There exist positive rate constants $a_i, b_i, c_i, \alpha_i, \beta_i, \gamma_i$ and positive totals $E_{\mathrm{T}}, F_{\mathrm{T}}, S_{\mathrm{T}}$ such that the system (1.1) has exactly $2n-1$ positive steady states with these totals, and the values of the ratio E/F of free kinase to free phosphatase at these steady states are exactly $u_0, \ldots, u_{2n-2}$. Every one of these steady states is nondegenerate. If the numbers $\sqrt{1+8u_j}$ are all rational, then the rate constants, the totals, and all steady-state concentrations can be chosen rational.*

Here a positive steady state is *nondegenerate* if the Jacobian of the mass-action vector field, restricted to the stoichiometric subspace, is invertible there. Nondegeneracy makes the count stable under perturbation, and the upper bound holds for all parameters, so we obtain a full-dimensional region of maximal multistationarity.

Corollary 1.3. *For every $n \geq 1$ there is a nonempty open subset of $\mathbb{R}^{6n+3}_{>0}$, the space of all rate constants and totals, on which (1.1) has exactly $2n-1$ positive steady states, all nondegenerate.*

In particular the maximum number of positive steady states of the n-site sequential distributive cycle is $2n-1$ for every n, and the set of parameters attaining it has positive Lebesgue measure.

The construction is explicit. Write $x_j = \sqrt{1+8u_j}$. A two-term recurrence produces two polynomials N, J of degree $n-1$ with positive coefficients from the x_j (Section 3); an exact polynomial division depending on one more number r converts them into the polynomials B, D whose coefficients are, up to the normalization (2.8), the rate constants (Section 4); and the totals are $(E_{\mathrm{T}}, F_{\mathrm{T}}, S_{\mathrm{T}}) = (2r, 2, 2r+2)$. The only condition is that the coefficients come out positive. We determine an explicit range of r for which they do.

Theorem 1.4. *In the setting of Theorem 1.2, put $x_j = \sqrt{1+8u_j}$. The construction of Sections 3–5 yields positive rate constants, and hence the conclusions of Theorem 1.2 with $E_{\mathrm{T}}/F_{\mathrm{T}} = r$, for every real number r such that*

$$r > \max_j u_j \qquad \text{and} \qquad 4r + \sqrt{1+8r} \;\geq\; \sum_{j=0}^{2n-2} x_j. \tag{1.3}$$

For $x_j = j+2$ the second condition follows from the first, so every $r > (4n^2-1)/8$ is admissible.

Finally we obtain information on stability. Order the prescribed ratios increasingly, $u_0 < u_1 < \cdots < u_{2n-2}$.

Theorem 1.5. *In the construction of Theorem 1.4, the $n-1$ steady states with odd index j are unstable: the Jacobian on the stoichiometric subspace has an odd number of positive real eigenvalues there. At the n steady states with even index this number is even.*

This follows from a determinant formula, Proposition 6.2, which is valid for all rate constants and relates the determinant of the restricted Jacobian at any positive steady state to the slope of a scalar function. For the rational family $x_j = j+2$ with the smallest admissible integer r we have computed the characteristic polynomials of the restricted Jacobians exactly and applied the Routh criterion in rational arithmetic (Proposition 8.2): for every $n \leq 10$ the n even-indexed steady states are asymptotically stable and the $n-1$ odd-indexed ones are hyperbolic saddles with a one-dimensional unstable manifold. This is n-fold multistability, the largest number compatible with $2n-1$ alternating steady states; for $n \geq 3$ it exceeds the number $\lfloor n/2 \rfloor + 1$ established for all n in [15]. We conjecture that it holds for every n (Conjecture 8.3).

1.3 Idea of the proof

At a positive steady state all concentrations are monomials in three quantities: the ratio $u = E/F$, the free unphosphorylated substrate $s = S_0$, and $f = F$. The three totals then become $E_{\mathrm{T}} = uf(1+sB(u))$, $F_{\mathrm{T}} = f(1+suD(u))$, $S_{\mathrm{T}} = sA(u) + usf(B(u)+D(u))$ for polynomials A, B, D with positive coefficients determined by the rate constants, and conversely every such triple of polynomials comes from rate constants (Proposition 2.1). The two enzyme totals determine s and f as rational functions of u, and the steady states are the solutions of one scalar equation $H(u) = S_{\mathrm{T}}$. The equation is rational of high degree, and the difficulty is that its $3n$ effective coefficients must all be positive while $2n-1$ roots are placed in the region where s and f are positive.

The key observation is that on the locus $A = (1+u)D$, $S_{\mathrm{T}} = E_{\mathrm{T}} + F_{\mathrm{T}}$, the numerator of $H(u) - S_{\mathrm{T}}$ is a quadratic form in $\ell = B/D - r$ and $v = r - u$ with discriminant $v^2(1+8u)$. In the variable $x = \sqrt{1+8u}$ it splits into two linear factors, and the steady-state equation becomes the *linear* condition

$$(x-1)\big(B(u) - rD(u)\big) = 2(r-u)D(u), \qquad u = \tfrac{x^2-1}{8}. \tag{1.4}$$

We then need polynomials B, D in u of degree $n-1$ with positive coefficients for which (1.4) holds at $2n-1$ prescribed values of x. Splitting $\prod_j (x - x_j)$ into its even and odd parts in x gives polynomials in u that solve a version of this interpolation problem with $r = \infty$; they are generated by a recurrence whose coefficients are visibly positive. A Bézout-type quotient moves the solution to finite r. Positivity of the quotient is where interlacing enters: the roots of N and J are real, negative, and interlace, so N/J has a partial-fraction expansion with positive residues, and this expresses D and B as positive combinations of polynomials with positive coefficients.

1.4 Formal verification

The existence statement underlying Theorem 1.2, that for every $n \geq 1$ there are positive rate constants and totals with $2n-1$ distinct positive steady states of the mass-action system (1.1), has been formalized and checked in Lean 4 [10] with Mathlib [26]. The formal statement refers to the vector field itself and not to a reduced equation. Section 9 states precisely what is and is not covered. All numerical statements in the paper are exact rational computations that are reproduced by a script distributed with the source.

1.5 Organization

Section 2 parametrizes the steady states and reduces them to a scalar equation. Section 3 introduces the square-root coordinate and the recurrence. Section 4 contains the conversion and the positivity theorem. Section 5 assembles the steady states and proves the counting part of Theorem 1.2. Section 6 proves nondegeneracy, the determinant formula, Corollary 1.3, and Theorem 1.5. Section 7 records further properties of the constructed steady states. Section 8 gives a complete three-site example and the stability computations. Section 9 describes the formal verification, and Section 10 discusses limitations and open problems.

2 Steady states of the mechanism

Let V be the mass-action vector field of (1.1) on $\mathbb{R}^{3n+3}$, with coordinates

$$(S_0, \dots, S_n,\ E,\ F,\ C_1, \dots, C_n,\ Y_1, \dots, Y_n),$$

and write $W \colon \mathbb{R}^{3n+3} \to \mathbb{R}^3$ for the linear map (1.2). The totals are conserved, so V takes values in $\ker W$. We shall see in Section 6 that $\ker W$ is the stoichiometric subspace; it has dimension $3n$. A *positive steady state* is a zero of V with all coordinates positive.

For given rate constants put

$$p_i = \frac{a_i}{b_i + c_i}, \qquad q_i = \frac{\alpha_i}{\beta_i + \gamma_i}, \qquad \lambda_i = \frac{c_i p_i}{\gamma_i q_i}, \qquad t_i = \prod_{k=1}^{i} \lambda_k \quad (t_0 = 1), \tag{2.1}$$

and define three polynomials with positive coefficients,

$$\begin{gathered} A(u) = \sum_{i=0}^{n} t_i u^i, \qquad B(u) = \sum_{i=0}^{n-1} B_i u^i, \qquad D(u) = \sum_{i=0}^{n-1} D_i u^i, \\ B_{i-1} = p_i t_{i-1}, \qquad D_{i-1} = q_i t_i \qquad (1 \leq i \leq n). \end{gathered} \tag{2.2}$$

The letter D always denotes this polynomial; the phosphatase complexes are called Y_i.

Proposition 2.1. (i) *The positive steady states of* (1.1) *are exactly the points*

$$\begin{aligned} &S_i = t_i u^i s \quad (0 \le i \le n), \quad E = uf, && F = f, \\ &C_i = B_{i-1} u^i s f, && Y_i = D_{i-1} u^i s f \quad (1 \le i \le n), \end{aligned} \tag{2.3}$$

with $(u, s, f) \in \mathbb{R}^3_{>0}$. *Their totals are*

$$E_{\mathrm{T}} = uf(1 + sB(u)), \qquad F_{\mathrm{T}} = f(1 + suD(u)), \qquad S_{\mathrm{T}} = sA(u) + usf(B(u) + D(u)). \tag{2.4}$$

(ii) *Conversely, let* $t_0 = 1$ *and let* $t_1, \dots, t_n$, $B_0, \dots, B_{n-1}$, $D_0, \dots, D_{n-1}$ *be arbitrary positive numbers. For any positive numbers* b_i, β_i, γ_i $(1 \le i \le n)$, *the rate constants*

$$c_i = \gamma_i \frac{D_{i-1}}{B_{i-1}}, \qquad a_i = (b_i + c_i)\frac{B_{i-1}}{t_{i-1}}, \qquad \alpha_i = (\beta_i + \gamma_i)\frac{D_{i-1}}{t_i} \tag{2.5}$$

are positive and have the given numbers as their coefficients (2.2).

Proof. The components of V for the complexes are $\dot{C}_i = a_i S_{i-1} E - (b_i + c_i) C_i$ and $\dot{Y}_i = \alpha_i S_i F - (\beta_i + \gamma_i) Y_i$. For $1 \le i \le n$ let

$$Z_i = \sum_{j=i}^{n} S_j + \sum_{j=i+1}^{n} C_j + \sum_{j=i}^{n} Y_j \tag{2.6}$$

be the amount of substrate carrying at least i phosphate groups, where C_j is counted with $j-1$ groups and Y_j with j. Binding and unbinding do not change Z_i, the kinase catalytic step at site i increases it, and the phosphatase catalytic step at site i decreases it; hence

$$\dot{Z}_i = c_i C_i - \gamma_i Y_i. \tag{2.7}$$

At a steady state, therefore, $C_i = p_i S_{i-1} E$, $Y_i = q_i S_i F$, and $c_i C_i = \gamma_i Y_i$, which gives $S_i = \lambda_i (E/F) S_{i-1}$. With $u = E/F$, $s = S_0$, $f = F$ this is (2.3). Conversely, at a point of the form (2.3) the complex components of V vanish and $c_i C_i = \gamma_i Y_i$ for every i. Since then $a_i S_{i-1} E - b_i C_i = c_i C_i$ and $\alpha_i S_i F - \beta_i Y_i = \gamma_i Y_i$, we get $\dot{S}_i = -c_{i+1} C_{i+1} + c_i C_i - \gamma_i Y_i + \gamma_{i+1} Y_{i+1} = 0$ (with the convention that terms with index 0 or $n+1$ are absent), and $\dot{E} = \dot{F} = 0$ because the complexes are stationary. Summation gives (2.4). Part (ii) is a direct substitution: (2.5) gives $p_i = B_{i-1}/t_{i-1}$, $q_i = D_{i-1}/t_i$, and $\lambda_i = c_i p_i / (\gamma_i q_i) = t_i / t_{i-1}$. □

Thus the set of positive steady states, and the number of them in each compatibility class, depend on the $6n$ rate constants only through the $3n$ coefficients of A, B, D. The remaining $3n$ degrees of freedom b_i, β_i, γ_i affect the dynamics, including stability, but not the steady states. For definiteness we shall use the normalization

$$\beta_i = \gamma_i = 1, \qquad b_i = c_i = \frac{D_{i-1}}{B_{i-1}}, \qquad a_i = \frac{2D_{i-1}}{t_{i-1}}, \qquad \alpha_i = \frac{2D_{i-1}}{t_i}, \tag{2.8}$$

which is (2.5) with $\gamma_i = \beta_i = 1$ and $b_i = c_i$.

The scalar equation

Fix positive totals and put $r = E_{\mathrm{T}}/F_{\mathrm{T}}$,

$$L(u) = B(u) - rD(u), \qquad M(u) = B(u) - uD(u). \tag{2.9}$$

Dividing the first two equations of (2.4) gives $r-u=suL(u)$. Hence a positive steady state with $u\neq r$ satisfies $(r-u)L(u)>0$, and then

$$s(u)=\frac{r-u}{uL(u)},\qquad f(u)=\frac{F_{\mathrm{T}}}{1+s(u)uD(u)}=\frac{F_{\mathrm{T}}L(u)}{M(u)}, \tag{2.10}$$

where we used $L+(r-u)D=M$; in particular $M(u)=L(u)\bigl(1+suD(u)\bigr)$ is nonzero and has the sign of $L(u)$. Conversely, for every $u>0$ with $(r-u)L(u)>0$ the formulas (2.10) define positive s,f such that (2.3) has enzyme totals $E_{\mathrm{T}},F_{\mathrm{T}}$. On the open set $\mathcal{U}=\{u>0:(r-u)L(u)>0\}$ define

$$H(u)=s(u)A(u)+u\,s(u)f(u)\bigl(B(u)+D(u)\bigr). \tag{2.11}$$

The positive steady states with totals $(E_{\mathrm{T}},F_{\mathrm{T}},S_{\mathrm{T}})$ and $u\neq r$ correspond bijectively to the solutions $u\in\mathcal{U}$ of $H(u)=S_{\mathrm{T}}$. This reduction is the one used in [29]; we need it only as a tool for constructing steady states and for computing slopes, and we take the upper bound from Theorem 1.1.

3 The square-root coordinate and a positive recurrence

3.1 A locus on which the scalar equation factors

Lemma 3.1. *Suppose $A(u)=(1+u)D(u)$ and $S_{\mathrm{T}}=E_{\mathrm{T}}+F_{\mathrm{T}}$, and normalize $F_{\mathrm{T}}=2$, so that $E_{\mathrm{T}}=2r$ and $S_{\mathrm{T}}=2(r+1)$. For $u\in\mathcal{U}$ put $\ell=B(u)/D(u)-r$, $v=r-u$, and $x=\sqrt{1+8u}$. Then*

$$H(u)-S_{\mathrm{T}}=\frac{(1+u)\bigl(v(\ell+v)-2u\ell^2\bigr)}{u\,\ell\,(\ell+v)}=-\frac{2(1+u)}{\ell(\ell+v)}\Bigl(\ell-\frac{2v}{x-1}\Bigr)\Bigl(\ell+\frac{2v}{x+1}\Bigr). \tag{3.1}$$

Proof. Since $L=D\ell$ and $M=D(\ell+v)$, (2.10) gives $s=v/(uD\ell)$ and $f=2\ell/(\ell+v)$. Therefore

$$H=\frac{(1+u)v}{u\ell}+\frac{2v(\ell+r+1)}{\ell+v},\qquad H-2(r+1)=\frac{(1+u)v}{u\ell}-\frac{2\ell(r+1-v)}{\ell+v}=(1+u)\Bigl(\frac{v}{u\ell}-\frac{2\ell}{\ell+v}\Bigr),$$

because $r+1-v=1+u$. This is the first expression. For the second, expand the product and use $x^2-1=8u$: $-2u\bigl(\ell^2-\frac{4v}{x^2-1}\ell-\frac{4v^2}{x^2-1}\bigr)=-2u\ell^2+v\ell+v^2$. □

On $\mathcal{U}\cap\{u<r\}$ we have $\ell,v>0$, so only the first linear factor in (3.1) can vanish. On this locus the steady-state equation is therefore equivalent to

$$\frac{B(u)}{D(u)}=r+\frac{2(r-u)}{x-1},\qquad x=\sqrt{1+8u}, \tag{3.2}$$

which is (1.4). The two conditions defining the locus have a simple meaning: $A=(1+u)D$ says that $t_i=D_i+D_{i-1}$, and $S_{\mathrm{T}}=E_{\mathrm{T}}+F_{\mathrm{T}}$ says that there is as much substrate as enzyme. They are used only to find a center for the construction; Corollary 1.3 removes both.

3.2 The recurrence

Put $m=n-1$. Let $x_0,\dots,x_{2m}$ be distinct real numbers greater than 1 and write

$$u(x)=\frac{x^2-1}{8},\qquad u_j=u(x_j),\qquad \Pi(x)=\prod_{j=0}^{2m}(x-x_j). \tag{3.3}$$

Start with $N = 1$ and $J = (x_0 - 1)/2$, and for $k = 0, \dots, m-1$, with $(a, b) = (x_{2k+1}, x_{2k+2})$, replace N, J simultaneously by

$$\begin{aligned} N_{\text{new}} &= \big[8u + (a+1)(b+1)\big]N + 2(a+b)J, \\ J_{\text{new}} &= 4(a+b)\,uN + \big[8u + (a-1)(b-1)\big]J. \end{aligned} \tag{3.4}$$

Lemma 3.2. *After m steps $N = \sum_{i=0}^{m} h_i u^i$ and $J = \sum_{i=0}^{m} d_i u^i$ have degree m, all coefficients h_i, d_i $(0 \le i \le m)$ are positive, and*

$$(x-1)\,N(u(x)) - 2\,J(u(x)) = \Pi(x). \tag{3.5}$$

Moreover

$$h_m = 8^m, \qquad d_m = 8^m\,\frac{\sum_j x_j - 1}{2}, \qquad d_0 = J(0) = \frac{1}{2}\prod_j (x_j - 1). \tag{3.6}$$

Proof. Let N, J have positive coefficients in degrees $0, \dots, k$ and none above. The coefficient of u^i in N_{new} is $8h_{i-1} + (a+1)(b+1)h_i + 2(a+b)d_i$ and in J_{new} it is $4(a+b)h_{i-1} + 8d_{i-1} + (a-1)(b-1)d_i$, with $h_{-1} = d_{-1} = h_{k+1} = d_{k+1} = 0$. Since $a, b > 1$, both are positive for $0 \le i \le k+1$. The leading coefficients obey $h \mapsto 8h$ and $d \mapsto 4(a+b)h + 8d$, which gives the first two formulas in (3.6). For (3.5), the initial pair gives $x - x_0$, and a direct expansion using $8u = x^2 - 1$ shows

$$(x-1)N_{\text{new}} - 2J_{\text{new}} = \big[x^2 - (a+b)x + ab\big]\big[(x-1)N - 2J\big]$$

at $u = u(x)$: the coefficient of N is $(x-1)(x^2 + ab + a + b) - (a+b)(x^2 - 1) = (x-1)(x-a)(x-b)$, and the coefficient of J is $2(a+b)(x-1) - 2(x^2 + ab - a - b) = -2(x-a)(x-b)$. Setting $x = 1$ in (3.5) gives d_0. □

Remark 3.3. Write $\Pi(x) = \Pi_{\text{e}}(x^2) + x\,\Pi_{\text{o}}(x^2)$. Replacing x by $-x$ in (3.5) and solving the two equations shows that, as functions of $w = x^2 = 1 + 8u$,

$$N = \Pi_{\text{o}}, \qquad J = -\tfrac{1}{2}(\Pi_{\text{e}} + \Pi_{\text{o}}). \tag{3.7}$$

So N and J are determined by (3.5) and depend only on the set $\{x_j\}$, not on its ordering. The recurrence is a convenient way to compute them and to see that their coefficients are positive.

4 Conversion and coefficient positivity

Let N, J be as in Lemma 3.2. For $r > 0$ write $N_r = N(r)$, $J_r = J(r)$ and define

$$Q(u) = \frac{u\,J_r N(u) - r\,N_r J(u)}{u - r}, \qquad D_{\text{raw}} = 4Q, \qquad B_{\text{raw}} = rD_{\text{raw}} + (4rN_r - 2J_r)N - 2J_r J. \tag{4.1}$$

The numerator of Q vanishes at $u = r$, so Q is a polynomial, of degree m with leading coefficient $J_r h_m$.

Lemma 4.1. *Let $c_* = J_r$ and $e_* = 4rN_r - J_r$. Then, with $u = u(x)$,*

$$(x-1)\big(B_{\text{raw}}(u) - rD_{\text{raw}}(u)\big) + 2(u-r)D_{\text{raw}}(u) = (c_* x + e_*)\,\Pi(x). \tag{4.2}$$

In particular (3.2) holds for $B_{\text{raw}}, D_{\text{raw}}$ at every u_j with $D_{\text{raw}}(u_j) \neq 0$.

Proof. We have $B_{\mathrm{raw}} - rD_{\mathrm{raw}} = (e_* - c_*)N - 2c_*J$ and $2(u-r)D_{\mathrm{raw}} = 8c_*uN - 2(c_*+e_*)J$, because $8rN_r = 2(c_*+e_*)$. With $8u = x^2 - 1$ the left side of (4.2) becomes $(x-1)\big[e_* - c_* + c_*(x+1)\big]N - 2(c_*x + e_*)J = (c_*x + e_*)\big[(x-1)N - 2J\big]$, and (3.5) applies. □

It remains to decide when D_{raw} and B_{raw} have positive coefficients. The answer comes from the location of the roots of N and J.

Lemma 4.2 (Interlacing)**.** *Let $m \geq 1$. The polynomials N and J have only real, simple roots $\nu_m < \cdots < \nu_1$ and $\zeta_m < \cdots < \zeta_1$, and*

$$\nu_m < \zeta_m < \cdots < \nu_2 < \zeta_2 < \nu_1 < \zeta_1 < 0, \qquad \nu_1 < -\tfrac{1}{8}. \tag{4.3}$$

Consequently

$$\frac{N(u)}{J(u)} = \alpha + \sum_{k=1}^{m} \frac{\sigma_k}{u - \zeta_k}, \qquad \alpha = \frac{h_m}{d_m} = \frac{2}{\sum_j x_j - 1} > 0, \qquad \sigma_k > 0. \tag{4.4}$$

Proof. For $y > 0$ evaluate (3.5) at $x = iy$, where $u = -(1+y^2)/8$ is real and runs over $(-\infty, -\frac{1}{8})$. Each factor is $iy - x_j = -(x_j^2 + y^2)^{1/2} e^{-i\phi_j}$ with $\phi_j = \arctan(y/x_j)$. The number of factors is odd, so $\Pi(iy) = R\,(-\cos\theta + i\sin\theta)$ with $R = \prod_j (x_j^2 + y^2)^{1/2} > 0$ and $\theta(y) = \sum_j \phi_j$. Comparing real and imaginary parts in $(iy-1)N - 2J = \Pi(iy)$ gives

$$N = \frac{R\sin\theta}{y}, \qquad J = \frac{R}{2}\Big(\cos\theta - \frac{\sin\theta}{y}\Big). \tag{4.5}$$

The function θ increases strictly and continuously from 0 to $(2m+1)\pi/2$, so it takes each of the values $\pi, 2\pi, \ldots, m\pi$ exactly once, at points $y_1 < \cdots < y_m$. These give m distinct roots $\nu_k = -(1+y_k^2)/8 < -\frac{1}{8}$ of N, which are all its roots since $\deg N = m$. By (4.5), $J(\nu_k) = (-1)^k R/2$. Hence J changes sign on each interval (ν_{k+1}, ν_k), and since $J(\nu_1) < 0 < J(0)$ by (3.6), also on $(\nu_1, 0)$. This accounts for all m roots of J and proves (4.3). The roots of J are simple, so N/J has the expansion (4.4) with $\sigma_k = N(\zeta_k)/J'(\zeta_k)$. Exactly $k-1$ roots of N and exactly $k-1$ roots of J exceed ζ_k, and both leading coefficients are positive; so $N(\zeta_k)$ and $J'(\zeta_k)$ both have sign $(-1)^{k-1}$, and $\sigma_k > 0$. □

For $1 \leq k \leq m$ let $J_k(u) = J(u)/(u - \zeta_k)$. Since all ζ_i are negative, J_k is a polynomial of degree $m-1$ with positive coefficients, and $J_k(r) > 0$ for $r > 0$.

Theorem 4.3 (Coefficient positivity)**.** (i) *For every $r > 0$, all coefficients of Q and D_{raw} in degrees $0, \ldots, m$ are positive.*

(ii) *Let $\rho = \sqrt{1+8r}$. If $\rho > \max_j x_j$ and $4r + \rho \geq \sum_j x_j$, then all coefficients of B_{raw} in degrees $0, \ldots, m$ are positive.*

Proof. For $m = 0$ we have $N = 1$, $J = d_0$, $Q = d_0$, and $B_{\mathrm{raw}} = 2(1+d_0)(2r - d_0)$. Here $d_0 = (x_0 - 1)/2$, and $\rho > x_0$ gives $2r = (\rho^2 - 1)/4 > (x_0 - 1)(x_0 + 1)/4 > d_0$. Let $m \geq 1$.

(i) By (4.4), $uN/J = \alpha u + \sum_k \sigma_k + \sum_k \sigma_k \zeta_k/(u - \zeta_k)$. Hence

$$Q(u) = J(u) J_r \,\frac{\frac{uN(u)}{J(u)} - \frac{rN_r}{J_r}}{u - r} = J(u) J_r \Big(\alpha + \sum_k \frac{\sigma_k |\zeta_k|}{(u - \zeta_k)(r - \zeta_k)}\Big) = \alpha J_r\, J(u) + \sum_{k=1}^{m} \sigma_k |\zeta_k|\, J_k(r)\, J_k(u).$$

Every term has nonnegative coefficients and the first has positive coefficients in all degrees $0, \ldots, m$.

(ii) From (4.4), $N = \alpha J + \sum_k \sigma_k J_k$. Substituting this and the formula for Q into $B_{\mathrm{raw}} = 4rQ + 4rN_rN - 2J_r(N+J)$ and using $J_r = (r+|\zeta_k|)J_k(r)$ gives

$$B_{\mathrm{raw}} = \alpha\Big[4rN_r - J_r\Big(\sum_j x_j + 1 - 4r\Big)\Big]J + \sum_{k=1}^{m} \sigma_k\Big[4rN_r - 2J_r\Big(1 - \frac{2r|\zeta_k|}{r+|\zeta_k|}\Big)\Big]J_k, \tag{4.6}$$

where we used $2+2/\alpha = \sum_j x_j + 1$. Evaluating (3.5) at $x = \rho$, where $u = r$, gives $(\rho-1)N_r - 2J_r = \Pi(\rho) > 0$, that is,

$$J_r < \tfrac{1}{2}(\rho-1)N_r. \tag{4.7}$$

Since $4r = (\rho-1)(\rho+1)/2$, each bracket in the sum in (4.6) is at least $4rN_r - 2J_r > \frac{1}{2}(\rho-1)^2 N_r > 0$. If $\sum_j x_j + 1 - 4r \le 0$, the first bracket is positive as well. Otherwise, by (4.7), it exceeds

$$\tfrac{1}{2}(\rho-1)N_r\Big[(\rho+1) - \Big(\sum_j x_j + 1 - 4r\Big)\Big] = \tfrac{1}{2}(\rho-1)N_r\Big(4r + \rho - \sum_j x_j\Big) \ge 0.$$

Thus B_{raw} is a combination of $J, J_1, \dots, J_m$ with positive coefficients, and J has positive coefficients in all degrees $0, \dots, m$. □

Since the J_k have degree $m-1$, the leading coefficient of B_{raw} is $h_m[4rN_r - J_r(\sum_j x_j + 1 - 4r)]$, by (4.6). The proof therefore shows that, for $r > \max_j u_j$, the polynomial B_{raw} has positive coefficients if and only if its leading coefficient is positive. The second condition in (1.3) is a convenient sufficient form of this, and it cannot simply be dropped: for the clustered roots $x = (1.059, 1.098, 1.003, 1.029, 1.038)$ one has $\max_j u_j \approx 0.0257$, but the leading coefficient of B_{raw} is negative at $r = 0.64$ and positive at $r = 0.66$, while (1.3) holds from $r \approx 0.675$ on.

Corollary 4.4. *For $x_j = j+2$ $(0 \le j \le 2n-2)$, so that $u_j = (j+1)(j+3)/8$, the polynomials D_{raw} and B_{raw} have positive coefficients for every $r > (4n^2-1)/8$.*

Proof. Here $\max_j x_j = 2n$ and $\sum_j x_j = (2n-1)(n+1)$. If $\rho > 2n$, then $(\rho+1)^2 > 4n^2 + 4n + 1 > 2(1 + \sum_j x_j) = 4n^2 + 2n$, and $(\rho+1)^2 \ge 2(1+\sum_j x_j)$ is the same as $4r + \rho \ge \sum_j x_j$. □

5 Common totals and the count

Fix r as in (1.3); the two conditions there are those of Theorem 4.3(ii), because $r > u_j$ if and only if $\rho > x_j$. Normalize and choose the totals by

$$D = \frac{D_{\mathrm{raw}}}{D_{\mathrm{raw}}(0)}, \qquad B = \frac{B_{\mathrm{raw}}}{D_{\mathrm{raw}}(0)}, \qquad A = (1+u)D, \qquad (E_{\mathrm{T}}, F_{\mathrm{T}}, S_{\mathrm{T}}) = (2r,\ 2,\ 2r+2). \tag{5.1}$$

Then $A(0) = D(0) = 1$, all coefficients of A, B, D in the ranges required by (2.2) are positive, and Proposition 2.1(ii) provides rate constants; with the normalization (2.8) they are rational functions of the x_j and r with rational coefficients. By Lemma 4.1, for every j,

$$\frac{B(u_j)}{D(u_j)} = r + \frac{2(r-u_j)}{x_j - 1} > r. \tag{5.2}$$

For $x = x_j$, $u = u_j$ define

$$s = \frac{x-1}{2uD(u)}, \qquad f = \frac{4}{x+1}, \qquad \text{so that} \qquad E = uf = \frac{x-1}{2}, \tag{5.3}$$

and let z_j be the point (2.3) with these (u, s, f). It is a positive steady state by Proposition 2.1(i). Its totals are computed from (2.4): first $suD = (x-1)/2$, so $F_{\mathrm{T}} = f(1+suD) = 2$; next, by (5.2), $suB = \frac{1}{2}r(x-1) + (r-u)$, so

$$E_{\mathrm{T}} = uf + f\big(\tfrac{1}{2}r(x-1) + r - u\big) = \tfrac{1}{2}f\,r\,(x+1) = 2r;$$

finally $sA = (1+u)(x-1)/(2u) = 4(1+u)/(x+1) = (1+u)f$, that is,

$$\sum_{i=0}^{n} S_i = E + F, \tag{5.4}$$

and since the bound substrate is $E_{\mathrm{T}} + F_{\mathrm{T}} - E - F$, we get $S_{\mathrm{T}} = E_{\mathrm{T}} + F_{\mathrm{T}} = 2r + 2$.

Proof of Theorem 1.2 (count, prescribed ratios, rationality) and of Theorem 1.4. Given distinct $u_j > 0$, the numbers $x_j = \sqrt{1+8u_j}$ are distinct and greater than 1. Condition (1.3) holds for all sufficiently large r. For any such r the points $z_0, \dots, z_{2n-2}$ are positive steady states of one and the same system with the same totals, and they are distinct because their ratios $E/F = u_j$ are. By Theorem 1.1 there are no others. If all x_j are rational, take r rational; then N, J, Q, B, D, the rate constants (2.8), and the concentrations (2.3) are rational. The statement about $x_j = j+2$ is Corollary 4.4. Nondegeneracy is proved in Section 6. □

Remark 5.1. (a) The map $x \mapsto (x^2-1)/8$ is increasing on $(1, \infty)$, so lists of ratios with rational x_j are dense among all lists; but we do not claim that an arbitrary list of rational ratios u_j can be realized by rational data. (b) Rescaling all concentrations by $\kappa > 0$ and the bimolecular constants a_i, α_i by $1/\kappa$ gives the totals $\kappa(2r, 2, 2r+2)$, and rescaling time multiplies all rate constants by a common factor; neither changes the count. The symmetry $E \leftrightarrow F$, $S_i \leftrightarrow S_{n-i}$ of (1.1) gives examples with $F_{\mathrm{T}}/E_{\mathrm{T}} = r$ and prescribed ratios F/E. (c) The hypothesis $r > \max_j u_j$ places all constructed steady states on the branch $u < r$, $L > 0$ of the chart (2.10).

6 Nondegeneracy, a determinant formula, and stability

6.1 Slopes

Proposition 6.1. *In the construction of Section 5, for every j,*

$$H'(u_j) = -\frac{16\,(1+u_j)\,(c_* x_j + e_*)}{(r-u_j)\,(x_j+1)^2\,D_{\mathrm{raw}}(u_j)}\;\Pi'(x_j), \qquad c_* x_j + e_* = 4rN_r + (x_j-1)J_r > 0. \tag{6.1}$$

In particular $H'(u_j) \neq 0$, and if $u_0 < \cdots < u_{2n-2}$ then $\operatorname{sign} H'(u_j) = (-1)^{j+1}$.

Proof. By (5.2), u_j lies in $\mathcal{U} \cap \{u < r\}$, where Lemma 3.1 applies. Dividing (4.2) by $(x-1)D_{\mathrm{raw}}(u)$ gives

$$g(u) := \ell - \frac{2v}{x-1} = \frac{(c_* x + e_*)\,\Pi(x)}{(x-1)\,D_{\mathrm{raw}}(u)},$$

which vanishes at u_j, and $H - S_{\mathrm{T}} = G\,g$ with $G = -2(1+u)(\ell + 2v/(x+1))/(\ell(\ell+v))$. Hence $H'(u_j) = G(u_j)g'(u_j)$. At u_j we have $\ell = 2v/(x-1)$, so $\ell + 2v/(x+1) = 4vx/(x^2-1)$, $\ell + v = v(x+1)/(x-1)$, and $G(u_j) = -4(1+u)x(x-1)/(v(x+1)^2)$. Since $\Pi(x_j) = 0$ and $dx/du = 4/x$, $g'(u_j) = 4(c_* x + e_*)\Pi'(x)/(x(x-1)D_{\mathrm{raw}}(u))$. Multiplying gives (6.1). All factors other than $\Pi'(x_j) = \prod_{k \neq j}(x_j - x_k)$ are positive, and $\Pi'(x_j)$ has sign $(-1)^{2m-j} = (-1)^j$ when the roots are ordered increasingly. □

6.2 The restricted Jacobian

We first fix coordinates. Put $y = (S_1, \dots, S_n, C_1, \dots, C_n, Y_1, \dots, Y_n)$ and $z = (S_0, E, F)$, and order the totals as $(S_{\mathrm{T}}, E_{\mathrm{T}}, F_{\mathrm{T}})$. Then $W = (W_y \;\; I_3)$ in the coordinates (y, z), so $\ker W$ is the graph of $z = -W_y y$ and y is a linear coordinate system on it. The $6n$ reaction vectors of (1.1) lie in $\ker W$ and span it: in the coordinates (C, Y, Z) on $\ker W$, with Z as in (2.6), a binding reaction changes only one complex coordinate, and the catalytic reactions at site i change Z_i and no other Z_k. So $\ker W$ is the stoichiometric subspace. Let $\mathcal{J}$ denote the matrix of $DV|_{\ker W}$ in the coordinates y, that is, $\mathcal{J} = V_{y,y} - V_{y,z}W_y$, where V_y are the y-components of V. A steady state is nondegenerate if and only if $\det \mathcal{J} \neq 0$.

Proposition 6.2 (Determinant formula)**.** *For arbitrary positive rate constants, at any positive steady state with $u = E/F \neq r = E_{\mathrm{T}}/F_{\mathrm{T}}$,*

$$\det \mathcal{J} = (-1)^{n+1} \Big(\prod_{i=1}^{n} (b_i + c_i)\, \alpha_i\, \gamma_i \Big)\, F^{\,n}\, u\, M(u)\, H'(u), \tag{6.2}$$

where H is formed with the enzyme totals of that steady state. In particular the steady state is nondegenerate if and only if $H'(u) \neq 0$.

Proof. By the Schur complement with respect to the block I_3,

$$\det \mathcal{J} = \det \begin{pmatrix} V_{y,y} & V_{y,z} \\ W_y & I_3 \end{pmatrix}.$$

Let $g = (g_Z, g_C, g_Y)$ consist of the $3n$ functions $g_{Z,i} = c_i C_i - \gamma_i Y_i$, $g_{C,i} = \dot{C}_i$, $g_{Y,i} = \dot{Y}_i$. By (2.6)–(2.7), $g = UV_y$ for a constant unitriangular matrix U, since $g_{Z,i}$ is $\dot{S}_i$ plus components of V_y that come later in the ordering of y. Hence the first $3n$ rows can be replaced by $(g_y \;\; g_z)$ without changing the determinant. In the block form belonging to $y = (S; C, Y)$,

$$g_y = \begin{pmatrix} 0 & * \\ * & \Delta \end{pmatrix}, \qquad \Delta = \operatorname{diag}\big(-(b_i + c_i);\, -(\beta_i + \gamma_i)\big),$$

and the Schur complement of Δ is the Jacobian with respect to $S_1, \dots, S_n$ of the reduced currents $c_i p_i S_{i-1} E - \gamma_i q_i S_i F$. It is lower bidiagonal with diagonal entries $-\gamma_i q_i F$. Therefore

$$\det g_y = \prod_i (b_i + c_i)(\beta_i + \gamma_i) \cdot (-1)^n \prod_i \gamma_i q_i F = (-1)^n F^n \prod_i (b_i + c_i)\alpha_i \gamma_i \neq 0.$$

In particular the zero set of g is locally the graph of a function $y = \varphi(z)$, in agreement with Proposition 2.1, and

$$\det \begin{pmatrix} g_y & g_z \\ W_y & I_3 \end{pmatrix} = \det g_y \cdot \det(I_3 - W_y g_y^{-1} g_z) = \det g_y \cdot \det \frac{\partial(S_{\mathrm{T}}, E_{\mathrm{T}}, F_{\mathrm{T}})}{\partial(S_0, E, F)},$$

where the last matrix is the derivative of the totals along the steady-state manifold $z \mapsto (\varphi(z), z)$, because $\varphi_z = -g_y^{-1} g_z$. We compute it in the coordinates $(u, s, f) = (E/F, S_0, F)$, for which $\det \partial(u, s, f)/\partial(S_0, E, F) = -1/F$. From (2.4),

$$\det \frac{\partial(E_{\mathrm{T}}, F_{\mathrm{T}})}{\partial(s, f)} = \det \begin{pmatrix} ufB & u(1 + sB) \\ fuD & 1 + suD \end{pmatrix} = uf\,(B - uD) = uf\, M(u) \neq 0, \tag{6.3}$$

so near the given steady state the enzyme totals determine (s, f) as functions of u, namely (2.10), and the Schur complement of the block (6.3) in $\partial(S_{\mathrm{T}}, E_{\mathrm{T}}, F_{\mathrm{T}})/\partial(u, s, f)$ is the derivative of S_{T} along that curve, which is $H'(u)$. Hence $\det \partial(S_{\mathrm{T}}, E_{\mathrm{T}}, F_{\mathrm{T}})/\partial(u, s, f) = ufM(u)H'(u)$, and collecting the factors gives (6.2). □

Proof of Theorem 1.2 (nondegeneracy). Combine Propositions 6.1 and 6.2. □

Proof of Corollary 1.3. Let k° and T° be the rate constants and totals of Theorem 1.2. In the coordinates y on the compatibility classes, the steady-state equations are $V_y(y, T - W_y y; k) = 0$, a system of $3n$ polynomial equations in $3n$ unknowns depending smoothly on $(k, T) \in \mathbb{R}^{6n+3}_{>0}$, and its derivative in y is $\mathcal{J}$. By the implicit function theorem each z_j continues to a smooth family of nondegenerate positive steady states for (k, T) in a neighborhood $\mathcal{O}_j$ of (k°, T°), and after shrinking the $\mathcal{O}_j$ the continued states remain pairwise distinct. On $\bigcap_j \mathcal{O}_j$ there are at least $2n - 1$ positive steady states, all nondegenerate, and by Theorem 1.1 there are no more. The perturbed parameters need not satisfy $A = (1 + u)D$ or $S_{\mathrm{T}} = E_{\mathrm{T}} + F_{\mathrm{T}}$. □

Corollary 6.3. *For arbitrary positive rate constants, a positive steady state with $u \neq r$ and $(r - u)H'(u) > 0$ is unstable; more precisely $\mathcal{J}$ has an odd number of positive real eigenvalues. If $(r - u)H'(u) < 0$, this number is even.*

Proof. When $\det \mathcal{J} \neq 0$, its sign is $(-1)^{e_-}$, where e_- is the number of negative real eigenvalues counted with multiplicity, because nonreal eigenvalues come in conjugate pairs. For the same reason $3n - e_-$ has the parity of the number e_+ of positive real eigenvalues. So $\operatorname{sign} \det \mathcal{J} = (-1)^n(-1)^{e_+}$. By (6.2) and since $M(u)$ has the sign of $L(u)$, hence of $r-u$, we also have $\operatorname{sign} \det \mathcal{J} = (-1)^{n+1} \operatorname{sign}\big((r - u)H'(u)\big)$. □

Proof of Theorem 1.5. All constructed steady states have $u_j < r$, and $\operatorname{sign} H'(u_j) = (-1)^{j+1}$ by Proposition 6.1. Apply Corollary 6.3. □

Corollary 6.3 is the form taken, for this network, by the general principle that the signs of $\det(-\mathcal{J})$ at the nondegenerate steady states of a dissipative network without boundary steady states add up to one [4]; here it is obtained by direct computation, together with the exact value of the determinant. It does not decide the stability of the even-indexed steady states, which depends on the $3n$ kinetic parameters that do not enter the steady-state equations.

7 Further properties of the constructed steady states

The formulas of Section 5 give closed expressions for several quantities of biochemical interest. We use the normalization (2.8) and order the ratios increasingly.

Proposition 7.1. *At the constructed steady state with auxiliary coordinate $x = x_j$:*

(i) *$E = (x - 1)/2$, $F = 4/(x + 1)$, and the total free substrate equals $E + F$. The fractions of kinase and phosphatase bound in complexes are $1 - (x - 1)/(4r)$ and $1 - 2/(x + 1)$.*

(ii) *The fully phosphorylated substrate is $S_n = \frac{1}{2} D_{n-1}\,(x - 1)\,u^{n-1}/D(u)$, and $S_n + Y_n = S_n\,(x + 5)/(x + 1)$. Both are strictly increasing functions of j.*

(iii) *The total phosphorylation flux is $\sum_i c_i C_i = \sum_i \gamma_i Y_i = 2(x - 1)/(x + 1)$, which is strictly increasing in j.*

Proof. (i) is (5.3), (5.4), and $\sum C_i = E_{\mathrm{T}} - E$, $\sum Y_i = F_{\mathrm{T}} - F$. (ii) Since $A = (1+u)D$, $t_n = D_{n-1}$, so $S_n = t_n u^n s$ and (5.3) give the first formula, and $Y_n = D_{n-1}u^n sf = fS_n$ gives the second. The function $D(u)/u^{n-1} = \sum_i D_i u^{i-(n-1)}$ is nonincreasing, $x-1$ is increasing, and $(x-1)(x+5)/(x+1) = x + 3 - 8/(x+1)$ is increasing. (iii) With $\gamma_i = 1$ the flux is $\sum Y_i = 2 - F$. □

So the $2n-1$ steady states are distinguished by an output that is commonly measured, the amount of fully phosphorylated substrate, and they are ordered in the same way by that output, by the free-enzyme ratio, and by the rate at which phosphate is turned over. The steady states are not thermodynamic equilibria: opposing catalytic currents are positive and equal, and in a model with explicit ATP the flux in (iii) would be the rate of ATP consumption needed to hold the state. By (i), in these examples the amount of enzyme equals the amount of substrate and most of the kinase is bound; they are far from the substrate-excess regime in which Michaelis–Menten reductions apply.

Remark 7.2 (Large r). For fixed x_j and $r \to \infty$, the coefficient formulas of Appendix A give $D \to J/d_0$ and $B/r \to (J+N)/d_0$ coefficientwise. Hence a_i and α_i converge to positive limits, while $b_i = c_i$ is asymptotic to $r^{-1}d_{i-1}/(d_{i-1}+h_{i-1})$. Increasing the kinase excess therefore slows the kinase catalytic step relative to the phosphatase in these examples, and the free concentrations and the slopes (6.1) have finite nonzero limits.

8 Examples and exact stability computations

8.1 A three-site example

Let $n = 3$ and $(x_0, \dots, x_4) = (2, 3, 4, 5, 6)$, so that the prescribed ratios are $u_j = \frac{3}{8}, 1, \frac{15}{8}, 3, \frac{35}{8}$. The recurrence gives

$$N = 1200 + 1256u + 64u^2, \qquad J = 60 + 1852u + 608u^2.$$

By Corollary 4.4 every $r > 35/8$ is admissible. For $r = 5$ we obtain

$$D = 1 + \frac{69002}{3405}u + \frac{9808}{3405}u^2, \qquad B = \frac{34797}{454} + \frac{308833}{2270}u + \frac{5236}{1135}u^2, \qquad A = (1+u)D, \tag{8.1}$$

the totals $(E_{\mathrm{T}}, F_{\mathrm{T}}, S_{\mathrm{T}}) = (10, 2, 12)$, and the rate constants of Table 1. The five steady states are listed in Table 2; all twelve concentrations of each are rational numbers given by (2.3) and (5.3), for instance $E = \frac{1}{2}, 1, \frac{3}{2}, 2, \frac{5}{2}$ and $F = \frac{4}{3}, 1, \frac{4}{5}, \frac{2}{3}, \frac{4}{7}$. Figure 1(a) shows the function $H - S_{\mathrm{T}}$.

Table 1: Rate constants of the three-site example ($r = 5$).

Site i	a_i	$b_i = c_i$	α_i	$\beta_i = \gamma_i$
1	2	454/34797	6810/72407	1
2	138004/72407	138004/926499	69002/39405	1
3	9808/39405	2452/3927	2	1

Nondegeneracy guarantees that the count persists under small perturbations but gives no size for the neighborhood. The following statement, for one parameter direction, is an example of what can be certified by exact evaluation.

Proposition 8.1. *With the rate constants of Table 1 and $(E_{\mathrm{T}}, F_{\mathrm{T}}) = (10, 2)$, the system has exactly five positive steady states for every $S_{\mathrm{T}} \in [12 - \frac{1}{500}, 12 + \frac{1}{500}]$.*

Table 2: The five positive steady states of the three-site example. Decimal entries are rounded values of exact rational numbers. The last column is the number of eigenvalues of $\mathcal{J}$ in the open right half-plane, obtained from an exact Routh array; no eigenvalue lies on the imaginary axis.

j	$u = E/F$	E	F	S_3	$S_3 + Y_3$	$H'(u_j)$	unstable
0	3/8	1/2	4/3	0.02249	0.05248	−0.13324	0
1	1	1	1	0.11930	0.23859	0.01315	1
2	15/8	3/2	4/5	0.30922	0.55660	−0.00561	0
3	3	2	2/3	0.59107	0.98512	0.00780	1
4	35/8	5/2	4/7	0.95195	1.49591	−0.06498	0

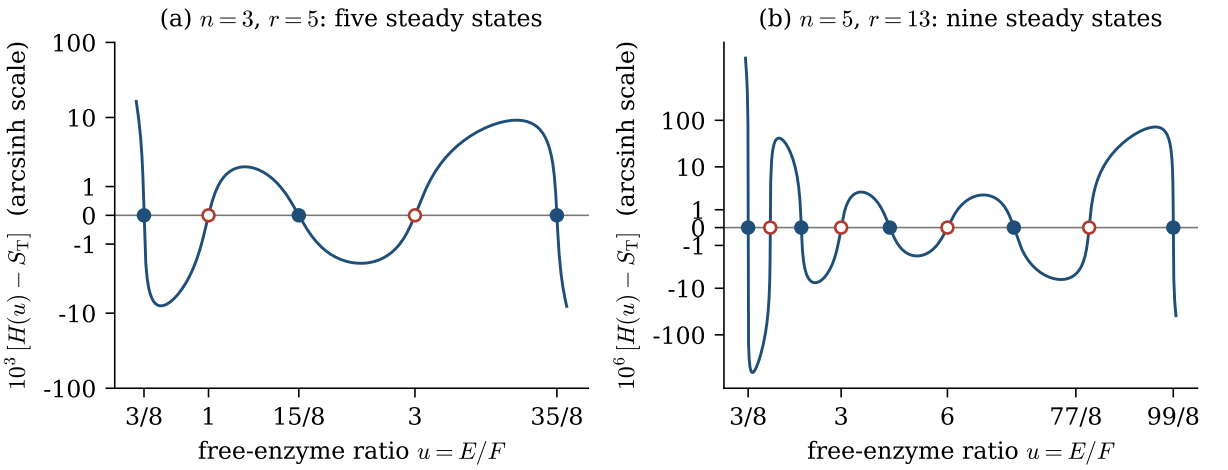

Figure 1: The scalar function $H(u) - S_T$ on the branch $u < r$ for the family $x_j = j + 2$: (a) $n = 3$, $r = 5$; (b) $n = 5$, $r = 13$. The vertical axis is arcsinh of the rescaled residual, labelled by the residual itself. Filled markers are asymptotically stable steady states, open markers are saddles (Proposition 8.2). The curves are drawn from exact rational evaluations; the count is certified by the formulas, not by the plot.

Proof. Here $L = B - 5D$ has a negative leading coefficient and $L(0), L(5) > 0$, so $L > 0$ on $[0, 5]$ by concavity, and H is continuous on $(0, 5)$. Exact evaluation at $u = \frac{1}{20}, \frac{69}{128}, \frac{4327}{3200}, \frac{7917}{3200}, \frac{6373}{1600}, \frac{399}{80}$ gives values of $H - 12$ that alternate in sign, starting with a positive one, and all exceed $\frac{1}{500}$ in absolute value (approximately 1.02, −0.0080, 0.0021, −0.0021, 0.0092, −2.6). The intermediate value theorem gives five solutions of $H(u) = S_T$ in $(0, 5)$, hence five positive steady states, and Theorem 1.1 excludes a sixth. □

The window is narrow, about 0.03% of S_T, because the three interior extrema of $H - S_T$ are small. Correspondingly the relative sensitivities $|d \log u / d \log S_T| = S_T/(u|H'(u)|)$ at the five steady states are approximately 240, 913, 1140, 513, 42, and the leading eigenvalues of $\mathcal{J}$, computed in floating point, are of order 10^{-4} (approximately -5.8×10^{-4}, 8.8×10^{-5}, -4.5×10^{-5}, 5.8×10^{-5}, -1.9×10^{-4}) while the remaining eigenvalues are of order 10^{-1} to 1. The example is a certified benchmark with exact data; it is not tuned for wide separation of the steady states, and we have not tried to optimize the x_j, r, or the $3n$ free kinetic parameters for that purpose.

8.2 Stability for $n \le 10$

Proposition 8.2 (Computer-assisted)**.** *Let $1 \le n \le 10$, $x_j = j + 2$, $r = \lfloor (4n^2 - 1)/8 \rfloor + 1$, and let the rate constants be given by (5.1) and (2.8). Then the n steady states $z_0, z_2, \dots, z_{2n-2}$ are asymptotically stable within their compatibility class: all $3n$ eigenvalues of $\mathcal{J}$ have negative real part. At each of the $n-1$ steady states $z_1, z_3, \dots, z_{2n-3}$, exactly one eigenvalue of $\mathcal{J}$ has positive real part and none lies on the imaginary axis.*

Proof. For each of the 100 steady states the matrix $\mathcal{J}$ is rational. Its characteristic polynomial was computed exactly by the Faddeev–LeVerrier recursion in integer arithmetic, with the Cayley–Hamilton identity as a check, and the Routh array [18, Ch. XV] was formed in rational arithmetic. In every case all $3n + 1$ entries of the first column are nonzero, so the array is regular, there are no eigenvalues on the imaginary axis, and the number of sign changes in the first column is the number of eigenvalues in the open right half-plane. It is 0 for even j and 1 for odd j. □

For $n = 3$ and $n = 4$, examples with $2n - 1$ steady states were first given in [17]. The numbers of right half-plane eigenvalues in Proposition 8.2 agree with the parities required by Theorem 1.5.

Conjecture 8.3. *For every $n \ge 1$ there are rate constants and totals for which (1.1) has $2n - 1$ positive steady states of which n are asymptotically stable. More specifically, the conclusion of Proposition 8.2 holds for all n.*

9 Formal verification and reproducibility

The lower bound has been formalized in Lean 4 [10] using Mathlib [26]. The development defines the state space, the six rate constants per site, the mass-action vector field of (1.1) componentwise, and the three totals, and proves

```
theorem PhosphorylationSharpness.sharp_lower_bound
(n : ℕ) (hn : 1 ≤ n) : Attains n (2*n-1)
```

where `Attains n k` states that there exist positive rate constants, positive totals $E_{\mathrm{T}}, F_{\mathrm{T}}, S_{\mathrm{T}}$, and an injective family of k states, each with all coordinates positive, at which every component of the vector field vanishes and the three totals take the given values. The statement thus concerns the full system of $3n + 3$ equations and contains no reduced equation, root count, or numerical input. The proof follows Sections 2–5 for the family $x_j = j+2$, with one difference: positivity of the coefficients of D_{raw} and B_{raw} is proved for all sufficiently large r by the elementary coefficient estimate of Appendix A, in the form of a limit as $r \to \infty$, and not by Theorem 4.3. It uses raw rather than normalized coefficients; multiplying A, B, D by a positive constant and dividing s by it changes neither the rate constants (2.5) nor the concentrations (2.3). The five modules (`Recurrence`, `Conversion`, `Source`, `Assembly`, `Resolution`; about 580 lines) compile without warnings under Lean `v4.30.0` with warnings treated as errors, and the theorem depends only on the axioms `propext`, `Classical.choice`, and `Quot.sound`. A further module, `PublicationAlgebra`, verifies the recurrence identity (3.5) and coefficient positivity for arbitrary lists of roots greater than one, the determinant identity (6.3), the factorization (3.1) of the numerator, and the sign of the prefactor in (6.1).

The following parts of the paper are proved only in the conventional way and are not covered by the formal development: the upper bound (Theorem 1.1, quoted from [29]), arbitrary prescribed ratios and rationality in Theorem 1.2, the interlacing lemma and Theorem 4.3, the slope and determinant formulas, Corollary 1.3, and Theorem 1.5. Propositions 8.1 and 8.2 are exact computations that are not formalized.

The source package of this paper contains the Lean modules and a Python script, `check_paper.py`, which uses only the standard library and rational arithmetic. It reconstructs the examples for $n \le 8$ for the family $x_j = j+2$ and checks, for all 64 steady states, that the vector field vanishes, that the totals agree, and that the slope formula (6.1) holds; it checks Lemma 4.2 by Sturm sequences and Theorem 4.3 at the boundary of the admissible range on several hundred random root configurations; it checks the determinant formula (6.2) exactly for the constructed steady states with $n \le 4$ and at random steady states of random rate constants; and it reproduces every number in Section 8, including Proposition 8.2 (for $n \le 6$ by default, and for $n \le 10$ when run with the option `--full`). These computations test the exposition and the implementation. The proofs above do not depend on them, with the exception of Propositions 8.1 and 8.2.

10 Discussion and open problems

The maximal number of positive steady states of the sequential distributive n-site phosphorylation cycle is $2n-1$ for every n, it is attained on an open set of parameters, and the locations of the steady states on the axis of the free-enzyme ratio can be prescribed arbitrarily. The proof is constructive and produces rational examples of any size in $O(n^2)$ arithmetic operations.

The construction has clear limitations, and we list the questions they suggest.

Stability. We prove that $n-1$ of the constructed steady states are unstable and verify for $n \le 10$ that the other n are stable. A proof of Conjecture 8.3 would require control of all $3n$ eigenvalues. The kinetic parameters b_i, β_i, γ_i of Proposition 2.1(ii) do not affect the steady states and may be chosen to help, for instance in a limit of fast binding, where the dynamics reduces to the n slow variables Z_i of (2.6). The argument of [15] relies on a regime that cannot contain more than $2\lfloor n/2 \rfloor + 1$ steady states, so a different reduction is needed.

Enzyme levels. In our examples $S_{\mathrm{T}} = E_{\mathrm{T}} + F_{\mathrm{T}}$, and the ratio $E_{\mathrm{T}}/F_{\mathrm{T}} = r$ allowed by (1.3) is large: of order n^2 for equally spaced x_j, and about $n/2$ when the x_j cluster near 1. Wang and Sontag showed that, generically, there are at most $n+1$ steady states when the enzymes are scarce relative to the substrate, and only one when they are in large excess [29, Theorems 4 and 5]. It would be interesting to know how small the ratios $E_{\mathrm{T}}/S_{\mathrm{T}}$ and $F_{\mathrm{T}}/S_{\mathrm{T}}$ can be in a system with $2n-1$ steady states, and whether the enzyme totals can be comparable to each other.

Size of the region. Corollary 1.3 gives an open set without an estimate of its size, and Proposition 8.1 shows that in the example it is small in at least one direction. The descriptions of multistationarity regions in [4, 1, 19, 13] are explicit but concern fewer steady states. A quantitative description of the region with $2n-1$ steady states, even for $n = 3$, is open. The freedom in the choice of the x_j and of r can presumably be used to enlarge it.

Other mechanisms. The method uses two features of (1.1): the monomial parametrization of the steady states, and a parameter locus on which the scalar steady-state equation factors after a quadratic change of variable. It is natural to ask whether a similar locus exists for related networks, such as cycles with several kinases or phosphatases, mixed processive–distributive mechanisms, or cascades, for which sharp bounds are not known.

A An elementary positivity bound

The following estimate gives the positivity of D_{raw} and B_{raw} for large r without the interlacing lemma. It is the route taken in the formal proof. Let $A_\Sigma = \sum_i h_i$, $D_\Sigma = \sum_i d_i$, $d_* = \min_i d_i$, and $\eta = h_m$.

Lemma A.1. *If*

$$r > \max\Big\{1,\ \frac{4A_\Sigma D_\Sigma}{\eta d_*},\ \frac{2D_\Sigma(A_\Sigma + D_\Sigma)}{\eta d_*}\Big\}, \tag{A.1}$$

then Q, D_{raw}, B_{raw} have positive coefficients in degrees $0, \dots, m$.

Proof. Write $Q = \sum_i q_i u^i$. Comparing coefficients in $(u - r)Q = uJ_rN - rN_rJ$ gives $q_0 = N_r d_0$ and $q_i = N_r d_i + (q_{i-1} - J_r h_{i-1})/r$, hence

$$q_i = N_r d_i + \sum_{k<i} r^{k-i}(N_r d_k - J_r h_k). \tag{A.2}$$

Since $r > 1$, $\eta r^m \le N_r \le A_\Sigma r^m$ and $J_r \le D_\Sigma r^m$, and $r^{k-i} \le r^{-1}$ for $k < i$. So the sum in (A.2) is at most $r^{-1}(N_r D_\Sigma + J_r A_\Sigma) \le 2A_\Sigma D_\Sigma r^{m-1}$ in absolute value, and the first bound in (A.1) gives $q_i > \frac{1}{2}\eta d_* r^m$. The coefficient of u^i in B_{raw} is $4rq_i + 4rN_r h_i - 2J_r(h_i + d_i) > 2\eta d_* r^{m+1} - 2D_\Sigma(A_\Sigma + D_\Sigma)r^m > 0$ by the second bound. □

The individual terms in (A.2) can be negative, so positivity of Q is not visible term by term; Theorem 4.3(i) shows that the sum is nevertheless positive for every $r > 0$. For the three-site example the right side of (A.1) is 6615, whereas Theorem 1.4 admits every $r > 35/8$. Formula (A.2) also gives the limits used in Remark 7.2: $q_i/r^m \to \eta d_i$ and $(B_{\mathrm{raw}})_i/r^{m+1} \to 4\eta(d_i + h_i)$, while $D_{\mathrm{raw}}(0) = 4N_r d_0$.

References

[1] F. Bihan, A. Dickenstein, and M. Giaroli. Lower bounds for positive roots and regions of multistationarity in chemical reaction networks. *J. Algebra*, 542:367–411, 2020. doi:10.1016/j.jalgebra.2019.10.002; arXiv:1807.05157.

[2] P. Cohen. The regulation of protein function by multisite phosphorylation – a 25 year update. *Trends Biochem. Sci.*, 25(12):596–601, 2000. doi:10.1016/S0968-0004(00)01712-6.

[3] C. Conradi, E. Feliu, and M. Mincheva. On the existence of Hopf bifurcations in the sequential and distributive double phosphorylation cycle. *Math. Biosci. Eng.*, 17(1):494–513, 2020. doi:10.3934/mbe.2020027; arXiv:1905.08129.

[4] C. Conradi, E. Feliu, M. Mincheva, and C. Wiuf. Identifying parameter regions for multistationarity. *PLoS Comput. Biol.*, 13(10):e1005751, 2017. doi:10.1371/journal.pcbi.1005751; arXiv:1608.03993.

[5] C. Conradi, D. Flockerzi, and J. Raisch. Multistationarity in the activation of a MAPK: parametrizing the relevant region in parameter space. *Math. Biosci.*, 211(1):105–131, 2008. doi:10.1016/j.mbs.2007.10.004.

[6] C. Conradi and M. Mincheva. Catalytic constants enable the emergence of bistability in dual phosphorylation. *J. R. Soc. Interface*, 11(95):20140158, 2014. doi:10.1098/rsif.2014.0158.

[7] C. Conradi and M. Mincheva. In distributive phosphorylation catalytic constants enable non-trivial dynamics. *J. Math. Biol.*, 89(2):20, 2024. doi:10.1007/s00285-024-02114-8; arXiv:2308.08524.

[8] C. Conradi, M. Mincheva, and A. Shiu. Emergence of oscillations in a mixed-mechanism phosphorylation system. *Bull. Math. Biol.*, 81(6):1829–1852, 2019. doi:10.1007/s11538-019-00580-6; arXiv:1809.02886.

[9] C. Conradi and A. Shiu. Dynamics of posttranslational modification systems: recent progress and future directions. *Biophys. J.*, 114(3):507–515, 2018. doi:10.1016/j.bpj.2017.11.3787; arXiv:1705.10913.

[10] L. de Moura and S. Ullrich. The Lean 4 theorem prover and programming language. In *Automated Deduction – CADE 28*, volume 12699 of *Lecture Notes in Comput. Sci.*, pages 625–635. Springer, 2021. doi:10.1007/978-3-030-79876-5_37.

[11] A. Dickenstein. Biochemical reaction networks: an invitation for algebraic geometers. In *Mathematical Congress of the Americas*, volume 656 of *Contemp. Math.*, pages 65–83. Amer. Math. Soc., 2016. doi:10.1090/conm/656/13076.

[12] A. Dickenstein. Algebraic geometry tools in systems biology. *Notices Amer. Math. Soc.*, 67(11), 2020. doi:10.1090/noti2188.

[13] E. Feliu, N. Kaihnsa, T. de Wolff, and O. Yürük. The kinetic space of multistationarity in dual phosphorylation. *J. Dynam. Differential Equations*, 34(2):825–852, 2022. doi:10.1007/s10884-020-09889-6; arXiv:2001.08285.

[14] E. Feliu, N. Kaihnsa, T. de Wolff, and O. Yürük. Parameter region for multistationarity in n-site phosphorylation networks. *SIAM J. Appl. Dyn. Syst.*, 22(3):2024–2053, 2023. doi:10.1137/22M1504548; arXiv:2206.08908.

[15] E. Feliu, A. D. Rendall, and C. Wiuf. A proof of unlimited multistability for phosphorylation cycles. *Nonlinearity*, 33(11):5629–5658, 2020. doi:10.1088/1361-6544/ab9a1e; arXiv:1904.02983.

[16] E. Feliu and C. Wiuf. Enzyme-sharing as a cause of multi-stationarity in signalling systems. *J. R. Soc. Interface*, 9(71):1224–1232, 2012. doi:10.1098/rsif.2011.0664; arXiv:1101.5799.

[17] D. Flockerzi, K. Holstein, and C. Conradi. N-site phosphorylation systems with $2N-1$ steady states. *Bull. Math. Biol.*, 76(8):1892–1916, 2014. doi:10.1007/s11538-014-9984-0; arXiv:1312.4774.

[18] F. R. Gantmacher. *The Theory of Matrices*, volume 2. Chelsea, New York, 1959.

[19] M. Giaroli, R. Rischter, M. Pérez Millán, and A. Dickenstein. Parameter regions that give rise to $2\lfloor n/2\rfloor+1$ positive steady states in the n-site phosphorylation system. *Math. Biosci. Eng.*, 16(6):7589–7615, 2019. doi:10.3934/mbe.2019381; arXiv:1904.11633.

[20] J. Gunawardena. Multisite protein phosphorylation makes a good threshold but can be a poor switch. *Proc. Natl. Acad. Sci. USA*, 102(41):14617–14622, 2005. doi:10.1073/pnas.0507322102.

[21] J. Hell and A. D. Rendall. A proof of bistability for the dual futile cycle. *Nonlinear Anal. Real World Appl.*, 24:175–189, 2015. doi:10.1016/j.nonrwa.2015.02.004; arXiv:1404.0394.

[22] K. Holstein, D. Flockerzi, and C. Conradi. Multistationarity in sequential distributed multisite phosphorylation networks. *Bull. Math. Biol.*, 75(11):2028–2058, 2013. doi:10.1007/s11538-013-9878-6; arXiv:1304.6661.

[23] N. I. Markevich, J. B. Hoek, and B. N. Kholodenko. Signaling switches and bistability arising from multisite phosphorylation in protein kinase cascades. *J. Cell Biol.*, 164(3):353–359, 2004. doi:10.1083/jcb.200308060.

[24] M. Pérez Millán, A. Dickenstein, A. Shiu, and C. Conradi. Chemical reaction systems with toric steady states. *Bull. Math. Biol.*, 74(5):1027–1065, 2012. doi:10.1007/s11538-011-9685-x; arXiv:1102.1590.

[25] C. Salazar and T. Höfer. Multisite protein phosphorylation – from molecular mechanisms to kinetic models. *FEBS J.*, 276(12):3177–3198, 2009. doi:10.1111/j.1742-4658.2009.07027.x.

[26] The mathlib Community. The Lean mathematical library. In *Proc. 9th ACM SIGPLAN Int. Conf. on Certified Programs and Proofs (CPP 2020)*, pages 367–381. ACM, 2020. doi:10.1145/3372885.3373824; arXiv:1910.09336.

[27] M. Thomson and J. Gunawardena. Unlimited multistability in multisite phosphorylation systems. *Nature*, 460:274–277, 2009. doi:10.1038/nature08102.

[28] A. Torres and E. Feliu. Symbolic proof of bistability in reaction networks. *SIAM J. Appl. Dyn. Syst.*, 20(1):1–37, 2021. doi:10.1137/20M1326672; arXiv:1909.13608.

[29] L. Wang and E. D. Sontag. On the number of steady states in a multiple futile cycle. *J. Math. Biol.*, 57(1):29–52, 2008. doi:10.1007/s00285-007-0145-z; arXiv:0704.0036.