




Monostationarity and Multistationarity in Tree Networks of Goldbeter–Koshland Loops

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Received: 21 July 2017 / Accepted: 21 May 2019
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Abstract

A major challenge in systems biology is to elicit general properties in the face of molecular complexity. Here, we introduce a class of enzyme-catalysed biochemical networks and examine how the existence of a single positive steady state (monostationarity) depends on the network structure, enzyme mechanisms, kinetic rate laws and parameter values. We consider Goldbeter–Koshland (GK) covalent modification loops arranged in a tree network, so that a substrate form in one loop can be an enzyme in another loop. GK loops are a canonical motif in cell signalling and trees offer a generalisation of linear cascades which accommodate network complexity while remaining mathematically tractable. In particular, they permit a modular, recursive proof strategy which may be more widely applicable. We show that if each enzyme follows its own complex reaction mechanism under mass action kinetics, then any network is monostationary for all appropriate parameter values. If the kinetics is non-mass action with a plausible monotonicity requirement, and each enzyme follows the Michaelis–Menten mechanism, then monostationarity is preserved. Surprisingly, a single GK loop with a complex enzyme mechanism under non-mass action monotone kinetics can have more than one positive steady state (multistationarity). The broader interplay between network structure, enzyme mechanism and kinetics remains an intriguing open problem.

Keywords Biochemical reaction network · Linear framework · Goldbeter–Koshland loop · Non-mass action kinetics · Monotone kinetics · Non-Michaelis–Menten reaction mechanism · Monostationarity · Multistationarity

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1 Introduction

One of the major challenges of systems biology is to elicit general properties of systems despite their overwhelming molecular complexity. Mathematics provides the most powerful tools for accomplishing this. However, mathematical models of systems at the molecular level are typically nonlinear and high dimensional and have most often been analysed by numerical simulation. While this has provided many insights (Beard 2011), simulation requires that all the details are fixed, including both the network of biochemical reactions and the mechanisms of the enzymes involved, and that numerical values are estimated for all the resulting parameters. This has made it difficult to generalise results to similar settings in which some of the details may vary and thereby to unearth general biological principles that transcend particular examples.

The possibility of avoiding numerical simulation and of proving general theorems about biochemical networks emerged from early studies in chemical physics and chemical engineering (Clarke 1980; Feinberg 1987; Horn and Jackson 1972) and the development of systems biology has given new impetus to such studies (Angeli et al. 2004; Banaji and Craciun 2010; Conradi et al. 2007; Craciun et al. 2009; Craciun and Feinberg 2005; Dexter et al. 2015; Feliu and Wiuf 2013a; Gunawardena 2014; Leenheer et al. 2007; Shinar and Feinberg 2010; Pérez Millán et al. 2012; Soulé 2003). These results suggest the possibility of rising above molecular complexity. However, notwithstanding these developments, certain simplifying assumptions, which are not always biochemically realistic, continue to be widely used.

For example, enzymes in biochemical networks are nearly always assumed to follow the standard Michaelis–Menten scheme,



in which the enzyme binds reversible to the substrate to form the intermediate enzyme-substrate complex, which then decays irreversibly to form P and release E . The great advantage of this assumption is that all enzymes are treated in the same way. However, this is far from the case in reality (Fersht 1985). Enzymes can exhibit complicated mechanisms with multiple intermediates (Johnson 2003). They can also exhibit product re-binding and the potential for reversibility. Michaelis and Menten were aware of such possibilities and took them carefully into account in their analysis; their use of Eq. 1 was limited to situations in which the concentration of P was negligible (Johnson and Goody 2011). Surprisingly, these issues have been largely ignored in systems biology, even in contexts where the concentration of P is substantial, although the dangers of doing so have been repeatedly pointed out (Blüthgen et al. 2006; Gunawardena 2014; Ortega et al. 2002).

A second example in which biochemical realism would be desirable concerns the assumption of mass action, which states that the rate of a reaction is proportional to the products of the concentrations of its substrates. This rule is widely applied to any biochemical reaction scheme, and it has the great advantage that it enables such a scheme to be immediately translated into mathematics. However, mass action can only be expected to hold for an elementary reaction, in which there are no intermediates (Mysels 1956). This is evident from the Michaelis–Menten scheme in Eq. 1, in which

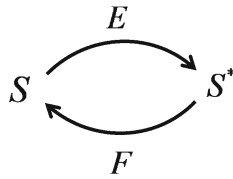


Fig. 1 A Goldbeter–Koshland loop in which substrate forms S and S^* are interconverted by a forward modifying enzyme E and a backward demodifying enzyme F . The substrate S may be a protein or a small molecule and the modifying group, such as a phosphate, is assumed to be provided by a donor molecule, such as ATP in the case of phosphate; see Sect. 2.1.2 for more details

the presence of an intermediate leads to saturation, which would not occur if P was obtained from S by an elementary reaction. However, it can be difficult to know if a reaction is truly elementary and a chemist would stress that, in general, the reaction rate cannot be deduced from the reaction scheme (Mysels 1956). This issue has also been largely ignored within systems biology.

These two issues set the context for the present investigation. In previous work, we analysed the behaviour of the Goldbeter–Koshland (GK) loop (Dasgupta et al. 2014; Xu and Gunawardena 2012), in which a substrate is interconverted between a modified and an unmodified form by a pair of enzymes (Fig. 1). In the original treatment by Goldbeter and Koshland, the substrate forms corresponded to the presence and absence of phosphorylation on a single amino acid site of a protein (Goldbeter and Koshland 1981). However, the same biochemistry applies more broadly to other kinds of covalent modification and demodification, including small molecules as well as proteins, and the GK loop has become a canonical motif in systems biology (Dasgupta et al. 2014; Gomez-Uribe et al. 2007; Heinrich et al. 2002; Ventura et al. 2008). In previous work, we exploited the “linear framework” for timescale separation (Gunawardena 2012, 2014), which enabled us to accommodate biochemically realistic enzyme mechanisms using only four aggregated parameters in place of the two needed for the Michaelis–Menten scheme (details in Sect. 2.1.2). We were able to prove theorems on the steady-state switching behaviour of GK loops with arbitrary complex reaction mechanisms (Xu and Gunawardena 2012; Dasgupta et al. 2014) and thereby provide insights into the role of bifunctionality in regulating glycolysis (Dasgupta et al. 2014).

In the present paper, we begin extending this analysis to networks of GK loops, arranged in the form of a tree. The definition of a tree network is given in Sect. 2.2.1, and Fig. 2 shows an example of such a network. The links in the network are such that either of the substrate forms in one loop can act as one of the enzymes in another loop. Such linking is found in linear enzymatic cascades, such as the mitogen-activated protein kinase (MAP kinase) cascade, which forms an evolutionarily conserved and widely duplicated motif in cellular signal transduction (Pearson et al. 2001). The extension to tree networks offers a tractable setting in which some of the broader complexity of cellular biochemistry can be mathematically explored.

Linear networks of GK loops have been previously studied (Feliu et al. 2012a, b), and tree-like cascades of multi-site modification, called “PTM” systems (short for Post-Translational Modification), have been recently introduced (de Freitas et al. 2016). The tree networks of GK loops considered are a special case of these PTM systems, but, in

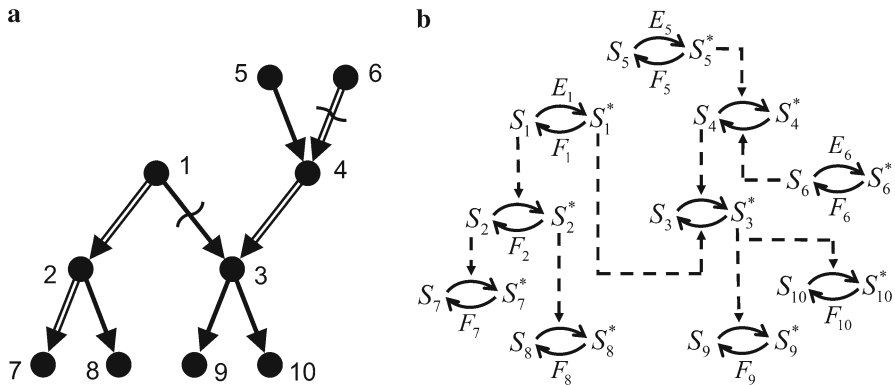


Fig. 2 **a** A tree network of GK loops. With the notation in Sect. 2.2.1, the unmodified substrate in loop 1 is the forward enzyme in loop 2 and the modified substrate in loop 1 is the backward enzyme in loop 3, and similarly for the other loops. **b** Expanded network showing the substrate forms and enzymes encoded by the representation in **a**

some applications, GK loops may have small molecules as substrates, not just proteins, so it would not be appropriate to refer to them as PTM systems.

Our focus here is on whether such a tree network of GK loops has only a single positive steady state (monostationarity). The basic strategy is that, if the network forms a tree, then it is possible to recursively alter the steady-state equations by removing root nodes or leaf nodes, in such a way that the equations retain general properties from which monostationarity can be deduced, once the tree has been reduced to a single node. In this way, we prove monostationarity for an infinite class of tree networks built up from GK loops in which each enzyme may have its own arbitrary, realistic mechanism under mass action kinetics (Theorem 1 in Sect. 2.2.3). Tree networks of this kind are examples of the Modifications of type Enzyme-Substrate or Swap with Intermediates (MESSI) systems introduced in Pérez Millán and Dickenstein (2018), about which we say more in Discussion. When the network is not a tree and has cycles, multistationarity can readily arise and it becomes much harder to determine the parametric conditions under which a given network exhibits a single steady state. These issues are reviewed further in the Discussion.

The properties required for the recursion are forms of continuity and monotonicity, which makes it feasible to attempt a similar analysis with non-mass action kinetics. However, while this yields monostationarity in tree networks of GK loops when the enzymes all follow the Michaelis–Menten reaction scheme (Theorem 2 in Sect. 2.3.3), we find an unexpected interaction between complex reaction mechanisms and non-mass action kinetics which leads, even in the case of a single GK loop, to multistationarity (Sect. 2.4).

Several powerful theorems have emerged in recent years from which monostationarity can be deduced for biochemical networks. To the best of our knowledge, none of these theorems can be applied to the tree networks considered here, but we review the relationship to our results in the Discussion.

The recursive proof strategy that we employ requires keeping track of steady-state functional relationships which change at each stage of the recursion along the tree, through eliminations which are only determined implicitly by existence results (such as Lemma 1). To make this procedure intelligible, we have used a lightweight formalism which focusses only on what is needed at each stage of the recursion. Because our tree networks have four kinds of edges between GK loops, depending on which substrate form acts as an enzyme and whether it acts in the forward or the backward direction, the proofs also involve a case-by-case analysis. This appears unavoidable. We have tried to structure the exposition to focus on the key steps and have placed several technical proofs and some redundant arguments in the ‘‘Appendix’’. We have also tried to clarify the general results on continuity and monotonicity on which we rely, as we believe that these may be helpful in other contexts. However, the devil, as always, lies in the details, and for these we ask the reader’s forgiveness.

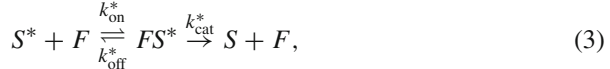
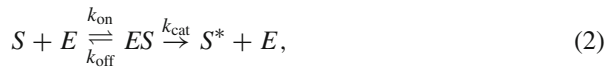
2 Results

2.1 A Single GK Loop with Complex Enzyme Mechanisms

2.1.1 Michaelis–Menten Mechanism of Enzyme Action

A GK loop (Fig. 1) consists of two substrate forms, S and S^* , and two enzymes, ‘‘forward’’ E , catalysing the reaction $S \rightarrow S^*$ and ‘‘backward’’ F , catalysing the reaction $S^* \rightarrow S$.

If we assume Michaelis–Menten mechanisms for the action of the enzymes



then it is easy to derive the equations describing the steady state under the assumption of mass action kinetics. In the first reaction, the intermediate complex with concentration $[ES]$ is formed at a rate $k_{\text{on}}[E] \cdot [S]$ and decays at a rate $(k_{\text{off}} + k_{\text{cat}})[ES]$, where $[E]$ and $[S]$ are concentrations of the forward enzyme and the first substrate form; similarly for the second reaction. At steady state, we have

$$\frac{d[ES]}{dt} = k_{\text{on}}[E] \cdot [S] - (k_{\text{off}} + k_{\text{cat}})[ES] = 0 \tag{4}$$

$$\frac{d[FS^*]}{dt} = k_{\text{on}}^*[F] \cdot [S^*] - (k_{\text{off}}^* + k_{\text{cat}}^*)[FS^*] = 0. \tag{5}$$

It is convenient to introduce the constants $\alpha = k_{\text{on}}/(k_{\text{off}} + k_{\text{cat}})$ and $\alpha^* = k_{\text{on}}^*/(k_{\text{off}}^* + k_{\text{cat}}^*)$ (the reciprocals of the usual Michaelis–Menten constants).

At steady state, the net flux from S to S^* , given by $k_{\text{cat}}[ES]$, must be balanced by the net flux from S^* to S , given by $k_{\text{cat}}^*[FS^*]$, so that,

$$k_{\text{cat}}[ES] = k_{\text{cat}}^*[FS^*].$$

Using Eqs. 4 and 5, we hence get the following equation for the concentrations of substrate forms $[S]$ and $[S^*]$ and enzymes $[E]$ and $[F]$,

$$k_{\text{cat}}\alpha[E] \cdot [S] = k_{\text{cat}}^*\alpha^*[F] \cdot [S^*].$$

We will use this kind of flux-balancing argument at steady state to derive similar “balance” equations for GK loops in more complicated contexts.

From now on, we are going to write the concentrations of the variables without brackets to reduce the notational overhead. Where confusion is possible, we will use a product sign “ \cdot ” to denote multiplication of concentrations. We will also use a product sign to distinguish multiplication from functional dependence.

Introducing parameters $\gamma = k_{\text{cat}}\alpha$, $\gamma^* = k_{\text{cat}}^*\alpha^*$, the balance equation can be rewritten as

$$\gamma E \cdot S = \gamma^* F \cdot S^*. \quad (6)$$

Since the total amounts of substrate S_T and of forward and backward enzymes, E_T and F_T , are conserved in our system, we obtain a further three conservation equations,

$$S + S^* + ES + FS^* = S + S^* + \alpha E \cdot S + \alpha^* F \cdot S^* = S_T, \quad (7)$$

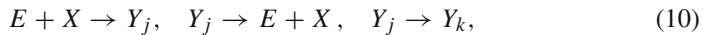
$$E + ES = E + \alpha E \cdot S = E_T, \quad (8)$$

$$F + FS^* = F + \alpha^* F \cdot S^* = F_T. \quad (9)$$

These four formulas (Eqs. 6–9) summarise the steady-state behaviour of a GK loop, and similar, albeit more complex, formulas will recur throughout the analysis that follows.

2.1.2 Complex Enzyme Mechanisms

We consider a more general class of enzyme mechanisms constructed using a “grammar” based on the reactions



where X denotes substrate forms, such as S or S^* , and the Y_j are intermediate complexes. Such a grammar allows each enzyme to follow a more realistic biochemical mechanism than that of Michaelis–Menten (Eqs. 2 and 3), in keeping with the known enzymology of forward modifying and backward demodifying enzymes (Fersht 1985). Donor molecules, such as ATP in the case of phosphorylation, and their hydrolysis products, such as ADP and inorganic phosphate, are not explicitly included in the reaction grammar. Their concentrations are assumed to be held constant by background metabolic processes and their effects absorbed into the rate constants. The methodology of using complex enzyme mechanisms in reaction networks was introduced

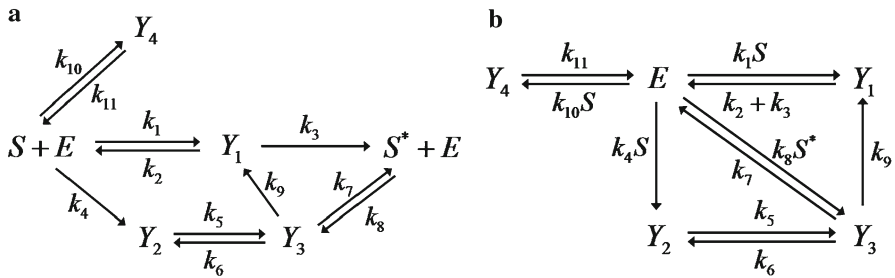


Fig. 3 **a** Example of an enzyme mechanism that is more complicated than that of Michaelis–Menten (Eq. 2). Here, there are four intermediate complexes Y_1 , Y_2 , Y_3 and Y_4 and the mechanism allows re-binding of the product as well as being reversible. **b** The directed graph with labelled edges constructed for the network in **a**, as described in Sect. 2.1.2

in Thomson and Gunawardena (2009) and incorporated into the graph-based linear framework for timescale separation (Gunawardena 2012; Mirzaev and Gunawardena 2013). It was further developed for Goldbeter–Koshland loops in Xu and Gunawardena (2012), Dasgupta et al. (2014) and reviewed in Gunawardena (2014). Reaction networks with multiple intermediate complexes have also subsequently been studied by several authors (Feliu and Wiuf 2013c; de Freitas et al. 2016; Pérez Millán and Dickenstein 2018).

A complex mechanism, an example of which is shown in Fig. 3a, may have multiple intermediates, Y_i ; may have multiple routes between substrate and product; may allow for the product of the mechanism to re-bind to the enzyme; and may be reversible, allowing for the product to be converted back into substrate. The Michaelis–Menten mechanism is neither reversible nor does it allow product re-binding; it is “strongly irreversible” (Xu and Gunawardena 2012; Dasgupta et al. 2014; Gunawardena 2014). Although product re-binding is obviously required for reversibility, these properties can otherwise vary independently for more complex mechanisms.

For an enzyme whose mechanism follows the grammar in Eq. 10, the steady state under mass action kinetics can be described in terms of four aggregated parameters. The enzyme mechanism may be arbitrarily complicated so long as it satisfies a simple condition. The procedure for calculating these aggregated parameters was previously developed in Thomson and Gunawardena (2009), Xu and Gunawardena (2012); see also Feliu and Wiuf (2013b) for a more general treatment. We will not use these details in the present paper, and will leave the mechanisms unspecified, because we can work with the aggregated parameters. However, we need to interpret the parameters, so the following brief explanation of how the parameters arise may be helpful.

Suppose given a mechanism for the enzyme E constructed from the grammar in Eq. 10, which catalyses the conversion of substrate form S into substrate form S^* , as in Fig. 3a. Note that such a mechanism may be reversible and also convert S^* into S . We assume that the mechanism obeys mass action kinetics. We can form a directed graph with labelled edges, in which the vertices are the enzyme, E , and intermediate complexes Y_j , the directed edges are the reactions and the edge labels are the reaction rates, with the concentrations of the substrate forms absorbed into the labels. Figure 3b shows the graph for the mechanism in Fig. 3a. The only condition imposed on the

mechanism is that this graph is strongly connected. It can then be shown that, for each intermediate, Y_i , there are non-negative quantities, $\mu_{i,S}$ and μ_{i,S^*} , which are defined only in terms of the reaction rates and which are not all zero, such that, at steady state,

$$Y_i = (\mu_{i,S}S + \mu_{i,S^*}S^*) \cdot E. \quad (11)$$

This result is established in Thomson and Gunawardena (2009, Prop.1); see also Xu and Gunawardena (2012, Appendix A) for a treatment based on the more general approach of the linear framework (Gunawardena 2012). We will follow the terminology of Thomson and Gunawardena (2009, Sect. 3.3) and refer to the quantities $\mu_{i,X}$ as “generalised Michaelis–Menten constants” (gMMCs). Note that they are reciprocal to the usual Michaelis–Menten constant, for much the same reason as the constants α and α^* in Sect. 2.1.1. The individual gMMCs for each intermediate may be added together to form a “total generalised Michaelis–Menten constant” (tgMMC) $\alpha = \sum_i \mu_{i,S}$, for conversion of S into S^* , and $\beta = \sum_i \mu_{i,S^*}$, for conversion of S^* into S . It follows from Eq. 11 that, at steady state, the total concentration of all intermediates in the mechanism is given by,

$$\sum_i Y_i = (\alpha S + \beta S^*) \cdot E. \quad (12)$$

The other two aggregated parameters determine the enzyme rate. If $Y_i \rightarrow E + S^*$ is any reaction in the mechanism which produces S^* and $k(Y_i \rightarrow E + S^*)$ is the corresponding reaction rate, then the “generalised catalytic efficiency” (gCE) for producing S^* from S , is given by

$$\gamma = \sum_{Y_i \rightarrow E + S^*} \mu_{i,S} \cdot k(Y_i \rightarrow E + S^*)$$

and that for producing S from S^* is given by,

$$\delta = \sum_{Y_i \rightarrow E + S} \mu_{i,S^*} \cdot k(Y_i \rightarrow E + S).$$

The gCEs give the contribution of the appropriate substrate to the rate of conversion,

$$\left. \frac{dS^*}{dt} \right|_{\text{from } S \text{ through } E} = \gamma S \cdot E, \quad \left. \frac{dS}{dt} \right|_{\text{from } S^* \text{ through } E} = \delta S^* \cdot E. \quad (13)$$

The tgMMC has dimensions of (concentration)⁻¹ and the gCE has dimensions of (time · concentration)⁻¹. For the forward enzyme, E , of the GK loop in Sect. 2.1.1, which satisfies the Michaelis–Menten reaction mechanism, the tgMMC and gCE for converting S into S^* simplify to the quantities called α and γ in Sect. 2.1.1. Since E is not reversible and does not bind product, gCE and the tgMMC for converting S^* into S are both zero.

For any complex enzyme mechanism defined within the grammar in Eq. 10, the aggregated parameters α , β , γ and δ can be explicitly calculated in terms of the rate constants, provided only that the graph defined above (Fig. 3b) is strongly connected. The key point is that, although complex mechanisms may differ widely in their biochemical details, the steady-state behaviour described in Eqs. 12 and 13 can be described with just four aggregated parameters.

In our context, in which two enzymes, E and F , participate in a GK loop between S and S^* , we will assume that each enzyme follows its own complex mechanism. We will use an asterisk to distinguish the parameters of the backward enzyme F . The following summary may help in keeping track of the notation and meanings.

forward enzyme E			backward enzyme F			(14)
param.	type	direction	param.	type	direction	
$\alpha > 0$	tgMMC	S into S^*	$\alpha^* > 0$	tgMMC	S^* into S	
$\gamma > 0$	gCE	S into S^*	$\gamma^* > 0$	gCE	S^* into S	
$\beta \geq 0$	tgMMC	S^* into S	$\beta^* \geq 0$	tgMMC	S into S^*	
$\delta \geq 0$	gCE	S^* into S	$\delta^* \geq 0$	gCE	S into S^*	

The sign conditions in Eq. 14 ensure that each enzyme is biased so that it always forms product from its substrate. Hence, for E , $\alpha, \gamma > 0$. The enzyme may also rebind to the product, if $\beta > 0$, and may be reversible, so that substrate is formed from product, if $\delta > 0$. However, neither of these latter behaviours occur for the Michaelis–Menten mechanism in Eqs. 2 and 3 and this more limited behaviour is permitted by allowing $\beta = 0$ and $\delta = 0$ in Eq. 14. The sign conditions thereby strictly generalise the Michaelis–Menten mechanism.

With the notation in Eq. 14, it is straightforward to see from Eq. 13 that the equation for the steady-state balance of production and consumption in the GK loop remains linear in the enzyme concentrations:

$$\gamma E \cdot S + \delta^* F \cdot S = \gamma^* F \cdot S^* + \delta E \cdot S^*,$$

which we will use in the form

$$E \cdot (\gamma S - \delta S^*) = F \cdot (\gamma^* S^* - \delta^* S). \tag{15}$$

Also, it follows from Eq. 12 that the equations of conservation of substrate and enzymes become

$$S + S^* + \alpha E \cdot S + \beta E \cdot S^* + \alpha^* F \cdot S^* + \beta^* F \cdot S = S_T, \tag{16}$$

$$E + \alpha E \cdot S + \beta E \cdot S^* = E_T, \tag{17}$$

$$F + \alpha^* F \cdot S^* + \beta^* F \cdot S = F_T. \tag{18}$$

If both E and F follow the Michaelis–Menten scheme, so that there is no reversibility, $\delta = \delta^* = 0$, and no product re-binding, $\beta = \beta^* = 0$, then Eqs. 15 to 18 revert to Eqs. 6 to 9, respectively.

In what follows, the enzyme mechanisms will have parameter values that conform to the restrictions in Eq. 14, with some parameter values positive and others only non-negative. To avoid unnecessary complexity of expression, we will refer to these as “appropriate” parameter values. Finally, we will always assume the total concentrations of all substrates and enzymes are positive, so that $S_T > 0$, $E_T > 0$ and $F_T > 0$.

We will be analysing steady-state equations without mentioning the intermediate complexes explicitly. The main question will be whether there exists a unique steady state for which concentrations of all components are positive. We will call this a “positive” steady state. Because we have used the linear framework to eliminate the steady-state concentrations of intermediate complexes, as described above, we will focus on steady-state concentrations of substrate forms and non-substrate enzymes, meaning those enzymes which are not also substrate forms, such as E_5 and F_5 in Fig. 2b. If these latter are shown to be positive, then it follows from Eq. 11 that the former are also positive.

2.1.3 Existence and Uniqueness of the Positive Steady State for a Single GK Loop

In this section, we show that for a single GK loop in which each enzyme has its own complex mechanism, the steady state equations, Eqs. 15 to 18, have a unique positive solution (Corollary 1). This has been shown previously (Xu and Gunawardena 2012), but the method of proof introduced here readily generalises to the more complicated situations considered subsequently.

The four steady-state equations for the GK loop, Eqs. 15 to 18, involve four variables, the two free enzyme concentrations, E and F , and the two substrate form concentrations, S and S^* . It is evident that E can be eliminated in terms of S and S^* by using Eq. 17 and similarly for F by using Eq. 18. Substituting for E and F in the balance equation, Eq. 15, yields the equation

$$p(S, S^*) \equiv \frac{E_T \cdot (\gamma S - \delta S^*)}{1 + \alpha S + \beta S^*} = \frac{F_T \cdot (\gamma^* S^* - \delta^* S)}{1 + \alpha^* S^* + \beta^* S} \equiv q(S, S^*) \quad (19)$$

between the two functions of S and S^* which we have called $p(S, S^*)$ and $q(S, S^*)$. Similarly, substituting for E and F into the substrate conservation equation, Eq. 16, yields an equation

$$g(S, S^*) \equiv S + S^* + \frac{E_T \cdot (\alpha S + \beta S^*)}{1 + \alpha S + \beta S^*} + \frac{F_T \cdot (\alpha^* S^* + \beta^* S)}{1 + \alpha^* S^* + \beta^* S} = S_T \quad (20)$$

for the function which we have called $g(S, S^*)$. The functions $p(S, S^*)$, $q(S, S^*)$ and $g(S, S^*)$ summarise the steady-state behaviour of the GK loop. The proof which follows depends only on certain properties of these functions which are evident from Eqs. 19 and 20, as we will explain next.

It will be helpful to introduce some abbreviations which will be repeatedly used in what follows. We shall be dealing with real functions of k variables $f(x_1, \dots, x_k)$: with each x_j belonging either to the infinite interval $[0, \infty)$, the finite, semi-open

interval $[0, a_j)$ or the finite, closed interval $[0, a_j]$, $j = 1, \dots, k$. For each variable on its interval of definition and independently of all the other variables,

- (c) means f is continuous.
- (i) means f is increasing: if $x < x'$ then $f(x) \leq f(x')$.
- (is) means f is strictly increasing: if $x < x'$ then $f(x) < f(x')$.
- (d) means f is decreasing: if $x < x'$ then $f(x) \geq f(x')$.
- (ds) means f is strictly decreasing: if $x < x'$ then $f(x) > f(x')$.
- (0) means $f(0) = 0$
- (n), or *normal*, which means that either
 - f is defined on $[0, a)$, where $a < \infty$, and is not bounded above;
 - or, f is defined on $[0, \infty)$ and may be bounded above or not.

In addition, we will sometimes require continuity as a function of several variables rather than continuity in individual variables. We will combine the abbreviations in an obvious way, with (cin) meaning a continuous, increasing, normal function and (cis) \times (cds) referring to a real function of two variables $f(x_1, x_2)$, which is continuous in each variable on the corresponding interval of definition, strictly increasing in the first and strictly decreasing in the second, for all values of the other variable. With this terminology in hand, the key properties of p, q and g , which follow directly from Eqs. 19 and 20, can be stated as follows.

1. $p(S, S^*) : [0, \infty) \times [0, \infty) \rightarrow \mathbb{R}$ is continuous with $p(0, 0)=0$ and p is (is) \times (d).
2. $q(S, S^*) : [0, \infty) \times [0, \infty) \rightarrow \mathbb{R}$ is continuous with $q(0, 0)=0$ and q is (d) \times (is). (21)
3. $g(S, S^*) : [0, \infty) \times [0, \infty) \rightarrow \mathbb{R}$ is continuous with $g(0, 0)=0, g(S, S^*) \geq S + S^*$ and g is (is) \times (is).

The asymmetrical requirements of strict versus non-strict monotonicity are introduced to deal with the sign conditions for the aggregated parameters, as specified in Eq. 14. For example, with Michaelis–Menten assumptions, when $\delta = \delta^* = \beta = \beta^* = 0$, but γ, γ^*, α and α^* are strictly positive, $p(S, S^*)$ does not depend on S^* and $q(S, S^*)$ does not depend on S , so strict monotonicity in these variables cannot be assumed.

With this terminology, in hand, we need some preparatory results which allow us to find implicit solutions of Eqs. 19 and 20 using only the properties of continuity and monotonicity that appear in Eq. 21. Let us denote by $U(\bar{x}^*, \bar{\xi})$ an open rectangular neighbourhood of a point $(x_1^*, \dots, x_n^*) \in \mathbb{R}^n$ such that $|x_i - x_i^*| < \xi_i, i = 1, \dots, n$ and by $U(y^*, \eta)$ a neighbourhood of a point $y^* \in \mathbb{R}$ such that $|y - y^*| < \eta$. The following lemma will be repeatedly used in the paper.

Lemma 1 *Let $\mathcal{F}(x_1, \dots, x_n, y)$ be a continuous function of independent variables in a rectangular domain $U(\bar{x}^*, \bar{\xi}) \times U(y^*, \eta)$, strictly monotonous in $y \in (y^* - \eta, y^* + \eta)$ for every $(x_1, \dots, x_n) \in U(\bar{x}^*, \bar{\xi})$, and $\mathcal{F}(x_1^*, \dots, x_n^*, y^*) = 0$. Then there exist neighbourhood $U(\bar{x}^*, \bar{\delta})$ of the point (x_1^*, \dots, x_n^*) and neighbourhood $U(y^*, \epsilon)$ of the point y^* such that for every $(x_1, \dots, x_n) \in U(\bar{x}^*, \bar{\delta})$ there exists unique solution $y =$*

$f(x_1, \dots, x_n)$ of the equation $\mathcal{F}(x_1, \dots, x_n, y) = 0$, $y \in U(y^*, \epsilon)$. $f(x_1, \dots, x_n)$ is continuous at (x_1^*, \dots, x_n^*) and $f(x_1^*, \dots, x_n^*) = y^*$.

This lemma provides a local form of the implicit function theorem but is distinctive in not requiring any differentiability assumptions. We give a proof in the ‘‘Appendix’’, where the origins of the result are discussed further. The following proposition will also be very helpful.

Proposition 1 *Let $\phi(x, y) : [0, \infty) \times [0, \infty) \rightarrow \mathbb{R}$ be continuous with $\phi(0, 0) = 0$. Suppose that $\phi(x, y)$ is (is) \times (ds), respectively, (i) \times (ds). Then there exists $0 < x_{max} \leq \infty$ such that $\phi(x, y) = 0$ has a solution function, $y(x)$, $y : [0, x_{max}) \rightarrow \mathbb{R}$, so that $\phi(x, y(x)) = 0$. Furthermore, $y(x)$ is (cis0n), respectively, (ci0n).*

The proof of Proposition 1 is given in the ‘‘Appendix’’.

Sometimes the function $\phi(x, y)$ is only defined on part of the non-negative quadrant. For these cases, the following modification of Proposition 1 will be helpful.

Proposition 2 *Let $\phi(x, y) : [0, \infty) \times [0, y_0] \rightarrow \mathbb{R}$, $y_0 > 0$ be continuous with $\phi(0, 0) = 0$. Suppose that $\phi(x, y)$ is (is) \times (ds), respectively, (i) \times (ds). Then $\phi(x, y) = 0$ has a solution function, $y(x)$: $\phi(x, y(x)) = 0$. $y(x)$ is either defined on an infinite interval $[0, \infty)$ or on a maximal finite interval $[0, x_{max}]$. Furthermore, $y(x)$ is (cis0), respectively (ci0), on the domain of definition.*

The proof of Proposition 2 is given in the ‘‘Appendix’’.

We are now in a position to actually solve the steady-state equations for a GK loop. It will be helpful to rewrite the balance and substrate conservation equations, Eqs. 19 and 20, more simply as,

$$p(S, S^*) = q(S, S^*), \tag{22}$$

$$g(S, S^*) = S_T. \tag{23}$$

Proposition 3 *Given functions $p(S, S^*)$, $q(S, S^*)$ and $g(S, S^*)$ with the properties stated in Eq. 21, there exists unique solutions $S, S^* \in (0, S_T)$ to Eqs. 22 and 23.*

Proof Let us rewrite the balance equation Eq. 22 in the form $\phi(S, S^*) = p(S, S^*) - q(S, S^*) = 0$. The function $\phi(S, S^*)$ satisfies the conditions of Proposition 1 with ϕ being (is) \times (ds). We therefore obtain a function $S^*(S)$ which is (cis0n) on some interval $[0, S_{max})$. We can now substitute this solution into Eq. 23. From the properties in Sect. 2.1.3, $g(S, S^*)$ is (cis) for $S \in [0, \infty)$, $g(0, 0) = 0$ and $g(S, S^*) \geq S + S^*$. Since the function $S^*(S)$ is normal, $g(S, S^*(S))$ is not bounded above on $[0, S_{max})$. Hence, for any $S_T > 0$, there exists a unique solution, $S \in (0, S_{max})$ to the equation $g(S, S^*(S)) = S_T$. Since $S^*(S)$ is (cis0n), the corresponding solution value of S^* also satisfies $S^* > 0$. Since $S_T = g(S, S^*(S)) \geq S + S^*$, it follows that $S, S^* \in (0, S_T)$, as required. \square

Corollary 1 *A GK loop in which each enzyme follows its own complex mechanism, as defined in Sect. 2.1.2, has a single positive steady state for each set of appropriate parameter values.*

Proof The GK loop gives rise to functions $p(S, S^*)$, $q(S, S^*)$ and $g(S, S^*)$, which satisfy properties 1, 2 and 3 in Eq. 21. Hence, Proposition 3 gives an unique solution $S, S^* \in (0, S_T)$. The values of the free enzymes are determined by those of S and S^* from the enzyme conservation equations 17 and 18 and are readily seen to be positive. Positivity for the concentrations of intermediate complexes then follows from Eq. 11. \square

2.2 Trees of GK Loops with Complex Enzyme Mechanisms and Mass Action Kinetics

2.2.1 Tree Networks of GK Loops

We define here the kind of tree networks on which we focus and introduce further notation. Consider a directed graph on N nodes in which each node, indexed $1 \leq i \leq N$, corresponds to a GK loop. Between any two nodes, i and j , there is at most one directed link, either from i to j or from j to i . A link signifies that a substrate form in the source node is an enzyme in the target node. There are four kinds of links, denoted as follows,

$$\begin{aligned} j \rightarrow i & \text{ if } S_j^* \text{ is a forward enzyme in loop } i \\ j \Rightarrow i & \text{ if } S_j \text{ is a forward enzyme in loop } i \\ j \rightrightarrows i & \text{ if } S_j^* \text{ is a backward enzyme in loop } i \\ j \Leftrightarrow i & \text{ if } S_j \text{ is a backward enzyme in loop } i \end{aligned}$$

We assume that there is only one forward and only one backward enzyme in any GK loop, so that there is at most one incoming link of the form \rightarrow or \Rightarrow and at most one incoming link of the form \rightrightarrows or \Leftrightarrow . The graph is asymmetric in that each node has at most two incoming links but can have a finite but arbitrary number of outgoing links. Each enzyme in the graph follows its own complex enzyme mechanism under mass action kinetics, as described in Sect. 2.1.2. Each node has 8 aggregated parameters as described in Eq. 14. We assume that the directed graph is connected, so that any two nodes are joined by a path of links, when directions are ignored, and that it forms a tree, so that it has no cycles when edge directions are ignored.

It is well known that substrate or enzyme sharing can be a cause of multistationarity; see, for instance, Feliu and Wiuf (2012). The only sharing permitted in our tree networks is when a substrate form in one GK loop acts as an enzyme in other GK loops. In particular, the $2N$ substrate forms are all distinct; the non-substrate enzymes are all distinct; the intermediate complexes which appear in enzyme mechanisms are all distinct; and non-substrate enzymes, substrate forms and intermediates are distinct from each other.

We will say that a node is a root if it has no incoming links and that a node is terminal if it has no outgoing links. An example of a tree network of GK loops is shown schematically in Fig. 2a. This graph has three root nodes and four terminal nodes. The expanded version in Fig. 2b shows the individual GK loops and the specific enzymatic connections.

2.2.2 Steady-State Equations for Tree Networks

Now let us present the steady state equations for the tree networks introduced in Sect. 2.2.1. It follows from Eq. 11 that the intermediate complexes can be eliminated in favour of the corresponding enzymes and substrate forms. If a non-substrate enzyme occurs at some node, i , of the tree, then the enzyme will satisfy a conservation equation, exactly as in Eqs. 17 or 18. The former equation will hold when there is no incoming link to node i of type \rightarrow or \Rightarrow and the latter equation will hold when there is no incoming link to node i of type \Rightarrow or \Leftrightarrow . In either case, it is clear that the concentration of enzyme not bound to substrate, which we will refer to as “free” enzyme, can be eliminated in terms of S_i and S_i^* in exactly the same way as was done in Sect. 2.1.3 for a single GK loop.

This elimination of the free enzymes in the network leaves only the $2N$ substrate forms, $S_1, S_1^*, \dots, S_N, S_N^*$. For each node in the network, there are two corresponding steady-state equations, which resemble the balance and substrate conservation equations for a single GK loop in Eqs. 22 and 23.

The balance equation for node i comes from the balance of flux between production and consumption of the substrate forms S_i and S_i^* . Either of these substrate forms may act as an enzyme in some other node in the network. However, an enzyme is a catalyst and is neither created nor destroyed during an enzymatic reaction, which involves only binding and unbinding to substrate forms. At steady state, these reactions contribute no flux of production or consumption of either S_i or S_i^* . Accordingly, at steady state, the same flux balance equation as in Eq. 15 holds, but the free enzyme terms, E and F , must be interpreted appropriately. There are several possibilities. If i is a root node, so that neither enzyme in that node is a substrate form in some other GK loop, then

$$p_i(S_i, S_i^*) = q_i(S_i, S_i^*), \quad (24)$$

where p_i and q_i have the same form as in Eq. 19 for a single GK loop. If the only incoming link is of the type \Rightarrow or \rightarrow , so that the forward enzyme is a substrate form of node j , then

$$U \cdot (\gamma_i S_i - \delta_i S_i^*) = q_i(S_i, S_i^*), \quad (25)$$

where $U = S_j$ if $j \Rightarrow i$ and $U = S_j^*$ if $j \rightarrow i$. If the only incoming link is of the type \Leftrightarrow or \Rightarrow , so that the backward enzyme is a substrate form of node k , then

$$p_i(S_i, S_i^*) = V \cdot (\gamma_i^* S_i^* - \delta_i^* S_i), \quad (26)$$

where $V = S_k$ if $k \Leftrightarrow i$ and $V = S_k^*$ if $k \Rightarrow i$. If both the forward enzyme is a substrate form of node j and the backward enzyme is a substrate form of node k , then

$$U \cdot (\gamma_i S_i - \delta_i S_i^*) = V \cdot (\gamma_i^* S_i^* - \delta_i^* S_i), \quad (27)$$

where U and V run through the same link-dependent options described above.

As examples of these possibilities, the balance equation for node 1 in Fig. 2 will be

$$p_1(S_1, S_1^*) = \frac{E_{1T} \cdot (\gamma_1 S_1 - \delta_1 S_1^*)}{1 + \alpha_1 S_1 + \beta_1 S_1^*} = \frac{F_{1T} \cdot (\gamma_1^* S_1^* - \delta_1^* S_1)}{1 + \alpha_1^* S_1^* + \beta_1^* S_1} = q_1(S_1, S_1^*),$$

which corresponds to Eq. 24, but for node 3 it will be

$$S_4 \cdot (\gamma_3 S_3 - \delta_3 S_3^*) = S_1^* \cdot (\gamma_3^* S_3^* - \delta_3^* S_3),$$

which corresponds to Eq. 27.

We will use p and q as notation for the functions of two variables that appear in the balance equation of a node when there is no corresponding incoming link, as described above. The variables which appear in p and q will then depend on the node in question. It is clear that these p and q satisfy the properties in Eq. 21. During the recursive proof strategy described below, we will generate new functions for which we will use the same notation, p and q . These functions will play a similar role in the new equations which emerge during the recursion and will continue to satisfy the properties in Eq. 21. In this sense, we will use the symbols p and q in a “generic” way, to capture what remains the same despite changes to the mathematical expressions which define them.

As for the substrate conservation equation for node i , making repeated use of Eq. 12, we see that it has the form,

$$\begin{aligned} g_i(S_i, S_i^*) &+ \left(\underbrace{S_p^* \cdot (\alpha_i S_i + \beta_i S_i^*)}_{\text{if } p \rightarrow i} \text{ or } \underbrace{S_q \cdot (\alpha_i S_i + \beta_i S_i^*)}_{\text{if } q \Rightarrow i} \right) \\ &+ \left(\underbrace{S_r^* \cdot (\alpha_i^* S_i^* + \beta_i^* S_i)}_{\text{if } r \rightarrow i} \text{ or } \underbrace{S_s \cdot (\alpha_i^* S_i^* + \beta_i^* S_i)}_{\text{if } s \Rightarrow i} \right) \tag{28} \\ &+ \sum_{i \rightarrow k} S_i^* \cdot (\alpha_k S_k + \beta_k S_k^*) + \sum_{i \Rightarrow l} S_i \cdot (\alpha_l S_l + \beta_l S_l^*) \\ &+ \sum_{i \rightarrow m} S_i^* \cdot (\alpha_m^* S_m^* + \beta_m^* S_m) + \sum_{i \Rightarrow n} S_i \cdot (\alpha_n^* S_n^* + \beta_n^* S_n) = S_{iT}. \end{aligned}$$

The alternatives on the first two lines of Eq. 28 reflect the constraint that each GK loop has only a single forward and a single backward enzyme. The term $g_i(S_i, S_i^*)$ involves only variables associated with the node i . The specific form of g_i depends on the same four cases as discussed for the balance equations, Eqs. 24 to 27, above. If node i is a root node, then g_i has the form in Eq. 20,

$$g_i(S_i, S_i^*) = S_i + S_i^* + \frac{E_{i,T} \cdot (\alpha_i S_i + \beta_i S_i^*)}{1 + \alpha_i S_i + \beta_i S_i^*} + \frac{F_{i,T} \cdot (\alpha_i^* S_i^* + \beta_i^* S_i)}{1 + \alpha_i^* S_i^* + \beta_i^* S_i}, \tag{29}$$

which arises after eliminating the forward enzyme for node i , E_i , and the backward enzyme for node i , F_i , as explained above, in favour of the enzyme totals, $E_{i,T}$ and $F_{i,T}$, respectively. By definition of a root node, neither the forward nor the backward enzyme is a substrate form. If the forward enzyme is a substrate form, but the backward enzyme is not, the summand involving $E_{i,T}$ in Eq. 29 is omitted; if the backward enzyme at node i is a substrate form but the forward enzyme is not, the summand involving $F_{i,T}$ in Eq. 29 is omitted; if both forward and backward enzymes at node i are substrate forms, then both the last two summands in Eq. 29 are omitted. In all cases, g_i includes as a summand the term $S_i + S_i^*$.

We will use g as notation for the function of two variables, $g_i(S_i, S_i^*)$, that appears in the substrate conservation equations of a node, as defined in Eq. 29 and the subsequent text. It is clear from its definition that g satisfies the properties in Eq. 21. During the recursive proof described below, we will use g in the same generic way as for the functions p and q introduced above.

As an example of Eq. 28, the substrate conservation equation for node 1 in Fig. 2 is

$$\underbrace{S_1 + S_1^* + \frac{E_{1T} \cdot (\alpha_1 S_1 + \beta_1 S_1^*)}{1 + \alpha_1 S_1 + \beta_1 S_1^*} + \frac{F_{1T} \cdot (\alpha_1^* S_1^* + \beta_1^* S_1)}{1 + \alpha_1^* S_1^* + \beta_1^* S_1}}_{g_1(S_1, S_1^*)} + \underbrace{S_1 \cdot (\alpha_2 S_2 + \beta_2 S_2^*)}_{1 \rightarrow 2} + \underbrace{S_1^* \cdot (\alpha_3^* S_3^* + \beta_3^* S_3)}_{1 \rightarrow 3} = S_{1T}.$$

For the single GK loop in Sect. 2.1.3, the balance equation for a root node, Eq. 24, reduces to Eq. 22 and the substrate conservation equation, Eq. 28, reduces to Eq. 23.

2.2.3 Proof Methodology

The argument in Sect. 2.2.2 has yielded a set of $2N$ steady-state equations for any tree with N GK loops. One equation arises from steady-state balance at each node, in one of the forms shown in Eqs. 24 to 27, and the other equation arises from conservation of substrate at each node, as described in Eq. 28. Our aim now is to use these equations to prove the following result.

Theorem 1 *Any tree of GK loops, for which each enzyme has its own complex mechanism under mass action kinetics, and thereby satisfies Eqs. 24 to 27 and 28, has a single positive steady state for each set of appropriate parameter values.*

The proof of this result occupies several subsections. We first explain the proof methodology. A common way to solve a system of equations of many variables is to try to express some variables in terms of the others using a subset of equations and substitute back into the remaining equations. It turns out that for a tree of GK loops we

can recursively remove nodes from the fringes of the tree and eliminate the substrate forms of the removed node in terms of the substrate forms of the remaining nodes. These eliminations modify the equations for the remaining substrate forms but do so in such a way as to retain the structure described in Eqs. 24 to 27 and 28, with the corresponding functions p , q and g continuing to satisfy the properties enumerated in Eq. 21. At the end of this procedure, when only a single node is left, the equations resemble that of a single GK loop in Eqs. 22 and 23, but the functions p , q and g are now potentially far more complicated because of the series of eliminations that have taken place. However, because p , q and g retain the properties enumerated in Eq. 21, we can appeal to Proposition 3 to show that the two remaining equations have a unique solution. Since the eliminated components are uniquely determined by those which remain, the equations of the original tree have a unique solution. Hence, the tree has a single steady state. Furthermore, it will become clear during the proof that the substrate form concentrations are all positive, from which it will also follow that the steady state is positive.

Node removal is accomplished by observing that, for any finite tree with more than one node, there is always either a terminal node which has only a single incoming link or a root node which has only a single outgoing link. To see why, suppose this is not the case. Choose any node in the tree. Since this is not the only node, there must be either an incoming or an outgoing link. Without loss of generality, suppose the latter and continue descending the tree along outgoing links until a terminal node is reached. By hypothesis, there must be an incoming link different from the one along which the terminal node was reached. Now begin ascending the tree along incoming links until a root node is reached. Again by hypothesis, there must be a link outgoing from the root node which is different from the one along which that node was reached. Now repeat the procedure by descending along this outgoing link. This zigzag traverse can never reach a repeated node because there are no cycles in the tree. Hence, the process does not terminate, which implies that the tree is infinite. This contradiction establishes the observation.

It is now easy to see that any tree can be systematically pruned by selecting such single-link terminal or root nodes until no more such nodes are available, when the tree consists of a single node, which is both a root node and a terminal node. It is helpful to think of this recursive removal process as giving rise at the k -th step to a new “current” tree T_k and an associated system of equations Σ_k . Here, T_0 is the original tree and Σ_0 is the system of equations given by Eqs. 24 to 27 and 28. If there are N nodes in the tree, then T_{N-1} will consist of a single node. We will prove the following properties for each step of the removal.

- A The equations Σ_k involve only the substrate forms of the nodes in T_k .
 - B The substrate forms of the remaining nodes in the original tree are uniquely expressed in terms of those in T_k .
 - C The equations Σ_k have the same structure for the tree T_k as described in Eqs. 24 to 27 and 28 with functions p , q and g at each node which retain the properties enumerated in Eq. 21.
- (30)

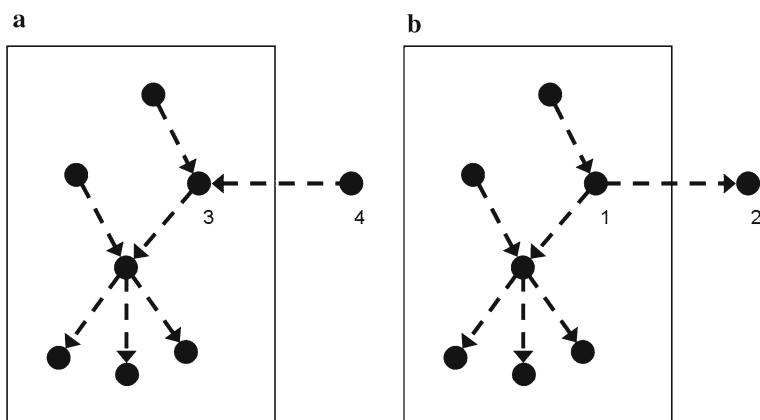


Fig. 4 **a** A hypothetical tree of GK loops showing a root node, labelled 4, with a single outgoing link to node 3. For this case, elimination of the root node is described in Sects. 2.2.4 and 2.3.3.1. **b** A hypothetical tree of GK loops showing a terminal node, labelled 2, with a single incoming link from node 1 in a tree of GK loops. For this case, elimination of the terminal node is described in Sects. 2.2.5 and 2.3.3.2. Each link can be of any of the four types, \rightarrow , \Rightarrow , \Rightarrow or \Rightarrow , provided that for each node there is at most one incoming link of the type \rightarrow or \Rightarrow and at most one incoming link of the type \Rightarrow or \Rightarrow

It is important to keep in mind that, when $k > 0$, the functions p , q and g which appear in Σ_k are not properties of T_k . In particular, they are not the functions which arise from considering T_k as a tree network in its own right. The functions p , q and g depend on the sequence of node removals which lead from the original tree, T_0 , to T_k .

Consider any tree $T = T_0$ and construct the system of equations Σ_0 as described in Eqs. 24 to 27 and 28. Properties A and B hold trivially while property C is true by construction. In the following two subsections, we show that properties A, B and C in Eq. 30 continue to hold for Σ_k as we remove nodes.

2.2.4 Eliminating the Variables of a Root Node with One Outgoing Link

Suppose that the removal process has reached stage k , where $0 \leq k \leq N - 2$, and that properties A, B and C in Eq. 30 hold for T_k and Σ_k . Suppose that we have a identified a root node of T_k with one outgoing link. To simplify the notation, let us assume that this is node 4 and that the single outgoing link goes to node 3, as shown in Fig. 4a. There are then four possibilities for the type of link from 4 to 3 and a case-by-case analysis becomes necessary.

Case 4 \Rightarrow 3. In this case, S_4^* is the backward enzyme in loop 3. By property C, the equations Σ_k satisfy Eqs. 24–27 and 28 for T_k . Accordingly, the balance and conservation equations for loop 3 have the form

$$\dots = S_4^* \cdot (\gamma_3^* S_3^* - \delta_3^* S_3), \tag{31}$$

$$g_3(S_3, S_3^*) + S_4^* \cdot (\alpha_3^* S_3^* + \beta_3^* S_3) + \text{other terms} = S_{3T}. \tag{32}$$

The left-hand side of Eq. 31 depends on whether there is an incoming link of the type \rightarrow or \Rightarrow to node 3 and will not be important here.

The corresponding equations for loop 4 have the form

$$p_4(S_4, S_4^*) = q_4(S_4, S_4^*), \tag{33}$$

$$g_4(S_4, S_4^*) + S_4^* \cdot (\alpha_3^* S_3^* + \beta_3^* S_3) = S_{4T}. \tag{34}$$

We are going to use the following.

Lemma 2 *Suppose the functions p, q and g have the properties enumerated in Eq. 21 and that σ is a parameter satisfying $\sigma \in [0, \infty)$, S_T is a positive constant and X is either S or S^* . Then the following system of equations in S and S^**

$$p(S, S^*) = q(S, S^*), \tag{35}$$

$$g(S, S^*) + X \cdot \sigma = S_T. \tag{36}$$

has an unique solution $(S(\sigma), S^*(\sigma))$ as a function of σ . Moreover, $S(\sigma)$ and $S^*(\sigma)$ are positive (cde) functions and $X(\sigma) \cdot \sigma$ is a bounded (cis0) function of $\sigma \in [0, \infty)$.

The proof of Lemma 2 is given in the ‘‘Appendix’’.

Let us apply Lemma 2 to the steady-state equations for node 4, Eqs. 33 and 34, which have the required form. We can assume that $S_3 \in [0, \infty)$ and $S_3^* \in [0, \infty)$, so that $\sigma = \alpha_3^* S_3^* + \beta_3^* S_3 \in [0, \infty)$ can be the parameter. Lemma 2 then gives us S_4^* as a function of S_3 and S_3^* , in the form of the implicit function $S_4^*(\alpha_3^* S_3^* + \beta_3^* S_3)$. We can now use this to eliminate S_4^* in the steady-state equations for node 3. In the substrate conservation equation for node 3, Eq. 32, we can augment g_3 to a new g function, g'_3 ,

$$g'_3(S_3, S_3^*) = g_3(S_3, S_3^*) + (\alpha_3^* S_3^* + \beta_3^* S_3) \cdot S_4^*(\alpha_3^* S_3^* + \beta_3^* S_3).$$

Lemma 2 tells us that the added term is (cis0), from which it follows that the new conservation equation for node 3, with g' in place g , satisfies the properties enumerated in Eq. 21.

In the balance equation for node 3, Eq. 31, eliminating S_4^* amounts to cutting the link from node 4 to node 3, so that node 3 no longer has an incoming link of the form \rightarrow or \Rightarrow . Accordingly, the balance equation should now acquire a function q , whose structure is evident from Eq. 31,

$$q_3(S_3, S_3^*) = (\gamma_3^* S_3^* - \delta_3^* S_3) \cdot S_4^*(\alpha_3^* S_3^* + \beta_3^* S_3).$$

Let us check the required properties of $q_3(S_3, S_3^*)$. It is continuous on $[0, \infty) \times [0, \infty)$ because $S_4^*(\sigma)$ is (cde) by Lemma 2. $q_3(S_3, S_3^*)$ is (is) in $S_3^* \in [0, \infty)$ because it can be written as

$$\left(\frac{\gamma_3^*}{\alpha_3^*} (\alpha_3^* S_3^* + \beta_3^* S_3) - \left(\frac{\gamma_3^* \beta_3^*}{\alpha_3^*} + \delta_3^* \right) S_3 \right) S_4^*(\alpha_3^* S_3^* + \beta_3^* S_3).$$

Also, if $\beta_3^* > 0$, $q_3(S_3, S_3^*)$ is (d) in $S_3 \in [0, \infty)$, because it can be written as

$$\left(\left(\gamma_3^* + \frac{\delta_3^* \alpha_3^*}{\beta_3^*} \right) S_3^* - \frac{\delta_3^*}{\beta_3^*} (\alpha_3^* S_3^* + \beta_3^* S_3) \right) S_4^* (\alpha_3^* S_3^* + \beta_3^* S_3).$$

If $\beta_3^* = 0$, then $q_3(S_3, S_3^*) = (\gamma_3^* S_3^* - \delta_3^* S_3) S_4^* (\alpha_3^* S_3^*)$ is also (d) in $S_3 \in [0, \infty)$. Finally, $q_3(0, 0) = 0$. Hence, q_3 satisfies the properties enumerated in Eq. 21.

It follows from Lemma 2 that S_4 and S_4^* are both positive when $S_3 \geq 0$ and $S_3^* \geq 0$.

We have now pruned the tree T_k to form the new tree T_{k+1} and constructed a new set of equations Σ_{k+1} on the variables in T_{k+1} . Properties A, B and C from Eq. 30 continue to hold for the new tree. Indeed, the equations Σ_{k+1} don't depend on variables S_4 and S_4^* , which are uniquely expressed in terms of S_3 and S_3^* . Variables that were expressed at earlier stages are still uniquely expressed in terms of those in Σ_{k+1} . Finally, the equations Σ_{k+1} have the same structure for the tree T_{k+1} as described in Eqs. 24 to 27 and 28 with functions p, q and g at each node which retain the properties enumerated in Eq. 21. At this step, the changes to the remaining equations occurred only in balance and substrate equations for node 3. One change was removal of the term depending on expressed variables S_4 and S_4^* from the substrate conservation equation (Eq. 32) for node 3 and replacement of the function g_3 by the new function g'_3 still with the same properties specified in Eq. 21. The other was the replacement of the right-hand side of the balance equation (Eq. 31) by the function q_3 with the properties specified in Eq. 21.

This completes the analysis for the case $4 \rightarrow 3$. The cases $4 \rightarrow 3, 4 \Rightarrow 3$ and $4 \Rightarrow 3$ can be reduced to the case $4 \rightarrow 3$, as shown in the ‘‘Appendix’’.

2.2.5 Eliminating the Variables of a Terminal Node with One Incoming Link

Suppose that the removal process has reached stage k , where $0 \leq k \leq N - 2$, and that properties A, B and C in Eq. 30 hold for T_k . Suppose that we have a identified a terminal node of T_k with one incoming link. To simplify the notation, let us assume that this is node 2 and that the single incoming link to node 2 comes from node 1 (Fig. 4b). There are then four possibilities for the type of link from 1 to 2 and a case-by-case analysis becomes necessary.

Case $1 \rightarrow 2$. By property C, the equations Σ_k satisfy Eqs. 24 to 27 and 28 for T_k . Since the forward enzyme in node 2 is S_1^* , the balance equation for node 2 satisfies Eq. 25, so that

$$S_1^* \cdot (\gamma_2 S_2 - \delta_2 S_2^*) = q_2(S_2, S_2^*). \tag{37}$$

Since node 2 is terminal and has no outgoing links, the conservation equation in Eq. 28 reduces to

$$g_2(S_2, S_2^*) + S_1^* \cdot (\alpha_2 S_2 + \beta_2 S_2^*) = S_{2T}. \tag{38}$$

In this case, we cannot obtain S_2 directly as a function of S_2^* , in the way we did when eliminating a root node. We now have two equations for S_2 and S_2^* which are

parameterised by S_1^* . We have to solve the equations and thereby eliminate S_2 and S_2^* in favour of S_1^* . The argument is correspondingly more involved than in the previous case and requires several stages.

We will need to use a property of the function q that goes beyond those enumerated in Eq. 21. To formulate it, let us remember that the function q_2 can appear either after expressing the free backward enzyme or after expressing a substrate acting as a backward enzyme in GK loop 2. If node 2 did not have an incoming link of the type \rightarrow or \Rightarrow in the original tree, then as in Eq. 19,

$$q_2(S_2, S_2^*) = \frac{(\gamma_2^* S_2^* - \delta_2^* S_2) F_{2T}}{1 + \alpha_2^* S_2^* + \beta_2^* S_2}. \tag{39}$$

Alternatively, if such a link was present in the original tree, but was removed at some step, then $q_2(S_2, S_2^*)$ also has a very specific form. Suppose a link from some node k of the type \rightarrow as in the previous subsection was removed (the case of a link of the type \Rightarrow is similar). Then, as shown in Sect. 2.2.4,

$$q_2(S_2, S_2^*) = (\gamma_2^* S_2^* - \delta_2^* S_2) S_k^* (\alpha_2^* S_2^* + \beta_2^* S_2).$$

Both these cases can be united in the following representation:

$$q_2(S_2, S_2^*) = (\gamma_2^* S_2^* - \delta_2^* S_2) \rho_2^* (\alpha_2^* S_2^* + \beta_2^* S_2).$$

It follows from Eq. 39 and Lemma 2 of the previous subsection that $\rho_2^*(x) > 0$ is (cds) on $[0, \infty)$ and $x\rho_2^*(x) \geq 0$ is bounded from above and (cis0) on $[0, \infty)$.

To proceed, the following lemma will be very helpful

Lemma 3 *Suppose the functions q and g have the properties enumerated in Eq. 21, parameter $\sigma \in [0, \infty)$ and, in addition, the following representation is valid:*

$$q(S, S^*) = (\gamma^* S^* - \delta^* S) \rho^* (\alpha^* S^* + \beta^* S),$$

where $\rho^*(x) > 0$ is (cds) on $[0, \infty)$ and $x\rho^*(x) \geq 0$ is bounded from above and (cis0) on $[0, \infty)$. Assume that $\alpha, \alpha^*, \gamma, \gamma^*$ and S_T are positive constants, β, β^*, δ and δ^* are non-negative constants. Then the following system of equations on S, S^*

$$\sigma \cdot (\gamma S - \delta S^*) = q(S, S^*), \tag{40}$$

$$g(S, S^*) + \sigma \cdot (\alpha S + \beta S^*) = S_T. \tag{41}$$

has a unique solution $(S(\sigma), S^*(\sigma))$. Moreover, $S(\sigma)$ and $S^*(\sigma)$ are positive for $\sigma > 0$ and $\sigma \cdot (\alpha S(\sigma) + \beta S^*(\sigma))$ is a (cis0) function of $\sigma \in [0, \infty)$.

The proof of Lemma 3 is given in the ‘‘Appendix’’.

Applying Lemma 3 to Eqs. 37 and 38, after the appropriate identifications, we see that the solution $(S_2(S_1^*), S_2^*(S_1^*))$ is unique for $S_1^* \in [0, \infty)$ and $S_2(S_1^*)$ and $S_2^*(S_1^*)$ are positive for $S_1^* > 0$. We now consider how this elimination alters the remaining

equations in Σ_k . The only equation which can be affected must come from a node which has a link to node 2. By hypothesis, the only such node is node 1. The balance equation for node 1 does not contain any term involving S_2 or S_2^* , but by Eq. 28 the conservation equation for node 1

$$g_1(S_1, S_1^*) + \dots + \underbrace{S_1^* \cdot (\alpha_2 S_2 + \beta_2 S_2^*)}_{1 \rightarrow 2} + \dots = S_{1T}$$

includes such a term. Let us substitute for S_2 and S_2^* in terms of S_1^* and incorporate the resulting term with $g_1(S_1, S_1^*)$ to form a new g -type function,

$$g'_1(S_1, S_1^*) = g_1(S_1, S_1^*) + S_1^* \cdot (\alpha_2 S_2(S_1^*) + \beta_2 S_2^*(S_1^*)). \tag{42}$$

Let T_{k+1} be the tree obtained from T_k by removing node 2. Let Σ_{k+1} be the system of equations obtained from Σ_k by removing the equations for node 2 and, in the conservation equation for node 1, replacing the summand on the right-hand side of Eq. 42 by the new g -type function $g'_1(S_1, S_1^*)$. The equations in Σ_{k+1} now depend solely on the substrate forms from the tree T_{k+1} . Furthermore, the remaining substrate forms of the original tree are uniquely determined by the substrate forms in T_{k+1} , as shown for S_2 and S_2^* above. Accordingly, properties A and B continue to hold for Σ_{k+1} . It remains only to confirm property C.

It is easy to see that $g'_1(S_1, S_1^*)$ is continuous on $(S_1, S_1^*) \in [0, \infty) \times [0, \infty)$ and strictly monotonously increasing in each variable. Indeed, $g_1(S_1, S_1^*)$ has this property by hypothesis and, by Lemma 3, the additional term $S_1^* \cdot (\alpha_2 S_2(S_1^*) + \beta_2 S_2^*(S_1^*))$ is a (*cis*0) function of S_1^* .

This completes the analysis for removal of a terminal node in the case $1 \rightarrow 2$. The cases $1 \Rightarrow 2$, $1 \rightarrow 2$ and $1 \Rightarrow 2$ can be reduced to the case $1 \rightarrow 2$, as shown in the “Appendix”.

2.2.6 Completing the Argument

By eliminating root and terminal nodes of the tree T , as described in Sects. 2.2.4 and 2.2.5, respectively, we reduce the tree in $N - 1$ steps to a single node for which the corresponding system of equations, Σ_{N-1} , satisfies properties A, B and C in Eq. 30. In the end, for the last remaining node we obtain two equations of the form 22 and 23 on the functions p , q and g with the properties specified in Eq. 21. Therefore, by Proposition 3, we conclude that the substrate-form concentrations for the last node are uniquely determined and positive. All other variables are determined uniquely by the substrate-form concentrations of the last node (property B). To see that all the other variables are also positive, it is sufficient to consider what happens at each stage of elimination along the tree.

In Sect. 2.2.4, we have seen that eliminating the root node with a single outgoing link expressed the concentrations of the two substrate forms S_{root} and S_{root}^* of the root node as positive (*cds*) functions of a non-negative linear combination of the concentrations of the remaining substrate forms. Therefore, S_{root} and S_{root}^* are both positive.

In Sect. 2.2.5, we have seen that eliminating the terminal node with a single incoming link left the concentrations of the two substrate forms S_{term} and S_{term}^* in $(0, S_{\text{term}}T)$, for positive values of the concentrations of the remaining substrate forms.

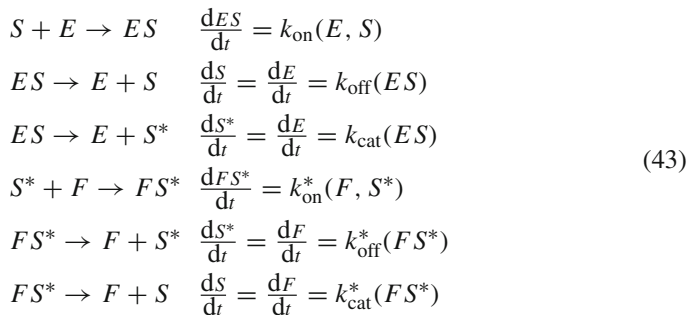
It follows by induction that the concentrations of all substrate forms in the tree are positive. The positivity of the concentrations of the free enzymes is an immediate consequence of Eqs. 17 or 18, where the substrate-form concentrations and parameters are for the same GK loop. The positivity of the concentrations of the intermediate complexes follows from the remarks in Sect. 2.1.2. This completes the proof of Theorem 1.

2.3 Trees of GK Loops with the Michaelis–Menten Mechanism and General Kinetics

2.3.1 The Case of a Single GK Loop

We have shown in the previous section that continuity and monotonicity properties were very useful in proving existence and uniqueness of the positive steady state for trees of GK loops, assuming the principle of mass action. A natural question is whether these results can be extended to more general kinetics. We show here that, when the enzymes follow the Michaelis–Menten reaction scheme, the recursive proof strategy used in Sect. 2.2 can be adapted with relatively little modification. We will see in Sect. 2.4 that the Michaelis–Menten restriction is important.

We start with a single GK loop. Consider the Michaelis–Menten reaction scheme in Eqs. 2 and 3, in which the rate of each reaction is now expressed by a function of the appropriate variables, as listed below,



Note that we continue to write concentrations without the usual brackets. We assume that the functions $k_{\text{off}}(x)$, $k_{\text{cat}}(x)$, $k_{\text{off}}^*(x)$ and $k_{\text{cat}}^*(x)$ are $(\text{cis}0)$ for $x \in [0, \infty)$. The functions $k_{\text{on}}(x, y)$ and $k_{\text{on}}^*(x, y)$ are continuous, $(\text{ci}0)$ for $x \in [0, \infty)$ and $(\text{ci}0)$ for $y \in [0, \infty)$. Moreover $k_{\text{on}}(x, y)$ and $k_{\text{on}}^*(x, y)$ are $(\text{cis}0)$ in each variable if the other variable is positive. Note that this includes the case of mass action kinetics, for which, for instance, $k_{\text{on}}(E, S) = k_{\text{on}}E \cdot S$. The kinetics considered here is an example of the weakly monotonic kinetics of Shinar and Feinberg (2012).

At steady state, we see that

$$\frac{dES}{dt} = k_{\text{on}}(E, S) - (k_{\text{off}} + k_{\text{cat}})(ES) = 0,
 \tag{44}$$

$$\frac{dFS^*}{dt} = k_{on}^*(F, S^*) - (k_{off}^* + k_{cat}^*)(FS^*) = 0. \tag{45}$$

If we wanted to eliminate intermediate complexes from these equations, we would need to assume that the functions $(k_{off} + k_{cat})(x)$ and $(k_{off}^* + k_{cat}^*)(x)$ are invertible. Instead, we will give a proof of existence and uniqueness without assuming invertibility. In the following, we will consider the “kinetic” equations, Eqs. 44 and 45, together with the usual balance, substrate and enzyme conservation equations. The balance equation can be obtained from the steady state equation for substrate S ,

$$\frac{dS}{dt} = -k_{on}(E, S) + k_{off}(ES) + k_{cat}^*(FS^*) = 0,$$

from which, using Eq. 44, we see that

$$k_{cat}(ES) = k_{cat}^*(FS^*). \tag{46}$$

In addition, we impose the conditions of the conservation of the substrate, forward and backward enzymes:

$$S + S^* + ES + FS^* = S_T, \tag{47}$$

$$E + ES = E_T, \tag{48}$$

$$F + FS^* = F_T. \tag{49}$$

To solve this system of six equations, Eqs. 44 to 49, for S, S^*, E, F, ES and EF , we can first uniquely express the concentrations E and ES in terms of S and F and FS^* in terms of S^* . The following lemma will be helpful.

Lemma 4 *Suppose k_{on}, k_{off} and k_{cat} are functions with the properties described in Sect. 2.3.1, σ is a non-negative parameter and E_T is a positive constant. Then the following system of equations on E and Y*

$$E + Y = E_T, \tag{50}$$

$$k_{on}(E, \sigma) = (k_{off} + k_{cat})(Y), \tag{51}$$

has an unique solution $E(\sigma)$, which is a positive (c)ds function, and $Y(\sigma)$, which is a (cis)0 function for $\sigma \in [0, \infty)$.

The proof of Lemma 4 is given in the “Appendix”.

We note that Eq. 48 has the same form as Eq. 50 and that the right-hand side of Eq. 44 can be placed in the same form as Eq. 51. Similarly, for Eqs. 49 and 45, respectively. Hence, it follows from Lemma 4 that E and ES can be eliminated in terms of S and that F and FS^* can be eliminated in terms of S^* . Moreover $E(S)$ and $F(S^*)$ are positive (c)ds functions and $ES(S)$ and $FS^*(S^*)$ are (cis)0 functions of their arguments on $[0, \infty)$.

We can now introduce the important functions, $p(S) \equiv k_{cat}(ES(S))$, $q(S^*) \equiv k_{cat}^*(FS^*(S^*))$, $g(S) \equiv S + ES(S)$ and $g^*(S^*) \equiv S^* + FS^*(S^*)$. The proof which

follows will depend only on the following properties of these functions, which are evident from these definitions.

1. $p(S)$ is a (cis0) function of $S \in [0, \infty)$.
 2. $q(S^*)$ is a (cis0) function of $S^* \in [0, \infty)$.
 3. $g(S)$ and $g^*(S^*)$ are (cis0) functions of $S \in [0, \infty)$ and $S^* \in [0, \infty)$, respectively, such that $g(S) \geq S$ and $g^*(S^*) \geq S^*$.
- (52)

Substituting in the balance and substrate conservation equations, Eqs. 46 and 47, we see that,

$$p(S) = q(S^*), \tag{53}$$

$$g(S) + g^*(S^*) = S_T. \tag{54}$$

Proposition 4 *Given functions $p(S)$, $q(S)$, $g(S)$ and $g^*(S^*)$ with properties 1, 2 and 3 stated in Eq. 52, there exists a unique positive solution in S and S^* to Eq. 53 and Eq. 54.*

Proof We can view $p(S)$ and $q(S^*)$ as the “old” functions $p(S, S^*)$ and $q(S, S^*)$ specified in Eq. 21, where only the argument in which they are strictly monotonous is kept and $g(S) + g^*(S^*)$ can be viewed as the “old” $g(S, S^*)$ in Eq. 21. Now Proposition 4 is an immediate consequence of Proposition 3. □

Corollary 2 *A GK loop in which the enzymes follow the Michaelis–Menten reaction scheme with the general kinetics specified in Sect. 2.3.1 has a single positive steady state.*

Proof The GK loop gives rise to functions $p(S)$, $q(S^*)$, $g(S)$ and $g^*(S^*)$, which satisfy properties 1, 2 and 3 in Eq. 52. Hence, Proposition 4 gives an unique solution $S, S^* \in (0, S_T)$.

Since $ES(S)$ and $FS^*(S^*)$ are (cis0) functions on $[0, \infty)$, the concentrations of the intermediate complexes ES and FS^* are positive and it follows from Lemma 4 that $E(S)$ and $F(S^*)$ are also positive on $[0, \infty)$. □

2.3.2 Trees of GK Loops

Consider a tree network of GK loops, as defined previously, in which each loop follows the Michaelis–Menten reaction scheme but with general kinetics, as specified in Sect. 2.3.1. The free enzyme concentrations and the concentrations of corresponding intermediate complexes can always be eliminated using Eqs. 44 and 48 or 45 and 49 and Lemma 4 as was done in Sect. 2.3.1. As in the derivation of Eqs. 53 and 54, this elimination leads naturally to functions $p_i(S_i)$, $q_i(S_i^*)$, $g_i(S_i)$ and $g_i^*(S_i^*)$ with the continuous, monotone properties enumerated in Eq. 52. We will make use of these types of functions to describe the steady-state equations, without defining the functions explicitly. From now on, the notation p , q , g or g^* signifies that these are any functions of one variable satisfying properties 1, 2 and 3 enumerated in Eq. 52; they no longer denote the specific functions defined in Eqs. 53 and 54.

Having eliminated the free enzymes and corresponding intermediate complexes, we still have balance and substrate equations for each node as well as kinetic equations for intermediate complexes in which the enzyme is a substrate form for each link in the graph. For a tree network of N nodes, this gives a system of $2N + N_{\text{links}}$ equations for the $2N$ variables $S_1, S_1^*, \dots, S_N, S_N^*$ and N_{links} intermediate complex variables $S_i^* S_j, S_i S_j, S_i^* S_j^*$ or $S_i S_j^*$. Note that, in contrast to trees with mass action kinetics, as described in Sect. 2.2.2, we cannot eliminate the intermediate complexes initially.

For the balance equation for node i , there are several possibilities. If i is a root node, then

$$p_i(S_i) = q_i(S_i^*). \tag{55}$$

If the forward enzyme is a substrate form of node j , so that the only incoming link is \rightarrow or \Rightarrow , then

$$k_{\text{cat},i}(U S_i) = q_i(S_i^*), \tag{56}$$

where $U = S_j$ if $j \Rightarrow i$ and $U = S_j^*$ if $j \rightarrow i$. If the backward enzyme is a substrate form of node k , so that the only incoming link is \Rightarrow or \rightleftharpoons , then

$$p_i(S_i) = k_{\text{cat},i}^*(V S_i^*), \tag{57}$$

where $V = S_k$ if $k \rightleftharpoons i$ and $V = S_k^*$ if $k \Rightarrow i$. If both the forward and backward enzymes are substrate forms, then,

$$k_{\text{cat},i}(U S_i) = k_{\text{cat},i}^*(V S_i^*), \tag{58}$$

where U and V run through the same options as described above.

For example, the balance equations for node 1 in Fig. 2 will be

$$p_1(S_1) = k_{\text{cat},1}(E_1 S_1(S_1)) = k_{\text{cat},1}^*(F_1 S_1^*(S_1^*)) = q_1(S_1^*),$$

Where $E_1 S_1(S_1)$ and $F_1 S_1^*(S_1^*)$ are constructed as in Sect. 2.3.1. The balance equation for node 3 will be

$$k_{\text{cat},3}(S_4 S_3) = k_{\text{cat},3}^*(S_1^* S_3^*).$$

For the substrate conservation equation for node i , we need to add appropriate terms arising from any incoming links to, and outgoing links from, node i ,

$$\begin{aligned}
 g_i(S_i) + g_i^*(S_i^*) + \underbrace{S_p^* S_i}_{\text{if } p \rightarrow i} + \underbrace{S_q S_i}_{\text{if } q \Rightarrow i} + \underbrace{S_r^* S_i^*}_{\text{if } r \rightarrow i} + \underbrace{S_s S_i^*}_{\text{if } s \Rightarrow i} + \\
 \sum_{i \rightarrow k} S_i^* S_k + \sum_{i \Rightarrow l} S_i S_l + \sum_{i \rightleftharpoons m} S_i^* S_m^* + \sum_{i \rightleftharpoons n} S_i S_n^* = S_i T
 \end{aligned} \tag{59}$$

Accordingly, the substrate conservation equation for node 1 in Fig. 2 will be

$$\underbrace{S_1 + E_1 S_1(S_1)}_{g_1(S_1)} + \underbrace{S_1^* + F_1 S_1^*(S_1^*)}_{g_1^*(S_1^*)} + \underbrace{S_1 S_2}_{1 \Rightarrow 2} + \underbrace{S_1^* S_3^*}_{1 \Rightarrow 3} = S_{1T}.$$

For a single GK loop, the balance equation in Eq. 53 corresponds to Eq. 55 and the conservation equation in Eq. 54 corresponds to Eq. 59.

Finally, the kinetic equations remaining after elimination of free enzymes and corresponding intermediate complexes have the form

$$k_{on,i}(U, S_i) = (k_{off,i} + k_{cat,i})(U S_i), \tag{60}$$

or

$$k_{on,i}^*(V, S_i^*) = (k_{off,i}^* + k_{cat,i}^*)(V S_i^*), \tag{61}$$

where U and V run through the same options as above.

2.3.3 Monostationarity for Trees of GK Loops

The main result that we will prove is the following.

Theorem 2 *Any tree of GK loops using the Michaelis–Menten reaction scheme with the general kinetics specified in Sect. 2.3.1 has a single positive steady state.*

The proof of this result follows a similar recursive proof strategy to that for Theorem 1, of removing nodes from the fringes of the tree and eliminating their variables in terms of those for the remaining nodes until a single node is left, to which Proposition 4 can be applied. There are, however, important differences between the two arguments, which mean that we cannot simply duplicate what was done previously. In particular, as noted in Sect. 2.3.2, the intermediate complexes cannot be eliminated initially but must be dealt with during the recursion, as will be evident from Eq. 62. While the general structure of the proof follows that for Theorem 1, and the arguments are, in fact, somewhat easier, the details are different.

As in the proof of Theorem 1, node removal is accomplished by selecting either a terminal node which has only a single incoming link or a root node which has only a single outgoing link. As before, it is helpful to think of this recursive removal process as giving rise at step k to a new “current” tree T_k and an associated system of equations Σ_k . Here, T_0 is the original tree and Σ_0 is the system of equations constructed as described in Sect. 2.3.2. If there are N nodes in the tree, then T_{N-1} will consist of a single node. We will prove the following properties for each step of the removal.

- A' The equations Σ_k involve only the substrate forms of the nodes in T_k and the corresponding intermediate complexes involving these substrate forms only.
- B' The substrate forms of the remaining nodes in the original tree, and the corresponding intermediate complexes involving these substrate forms are uniquely expressed in terms of those in T_k .
- C' The equations Σ_k have the same structure for the tree T_k as described in Eqs. 55 to 59 but with functions p, q and, possibly different, functions g and g^* , which retain the properties enumerated in Eq. 52. In addition, for each intermediate complex constructed from substrate forms of the T_k there is an appropriate kinetic equation 60 or 61.

As before, it is important to keep in mind that the functions p, q, g and g^* which appear in Σ_k are not properties of T_k but depend on the sequence of node removals which lead from the original tree to T_k . Equation 62 for general kinetics should be compared to Eq. 30 for mass action kinetics, to appreciate the difference in the two arguments.

Consider any tree $T = T_0$ and construct the system of equations Σ_0 as described in Eqs. 55 to 61. Properties A' and B' hold trivially, while property C' is true by construction. In the following two subsections we show that properties A', B' and C' continue to hold for Σ_k as we remove nodes.

2.3.3.1 Eliminating a Root Node with One Outgoing Link

Suppose that the removal process has reached stage k , where $0 \leq k \leq N - 2$, and that properties A', B' and C' hold for T_k . Suppose that we have identified a root node of T_k with one outgoing link. To simplify the notation, let us assume that this is node 4 and that the single outgoing link goes to node 3 (Fig. 4a). There are then four possibilities for the type of link from 4 to 3 and a case-by-case analysis becomes necessary.

Case 4 → 3. By property C', the equations Σ_k satisfy Eqs. 55 to 58 and 59 together with appropriate kinetic equations 60 or 61 for T_k . Since the forward enzyme in node 3 is S_4^* , the left side of the balance equation for node 3 is $k_{cat,3}(S_4^*S_3)$. Also, the term $S_4^*S_3$ enters the conservation of substrate equations for GK loops 3 and 4. Let us express the variables S_4, S_4^* and $S_4^*S_3$ for node 4 in terms of the variables of node 3. The balance and substrate conservation equations for node 4 and kinetic equation for node 3 can be written as follows:

$$p_4(S_4) = q_4(S_4^*), \tag{63}$$

$$g_4(S_4) + g^*(S_4^*) + S_4^*S_3 = S_{4T}, \tag{64}$$

$$k_{on,3}(S_4^*, S_3) = (k_{off,3} + k_{cat,3})(S_4^*S_3). \tag{65}$$

If node 4 is the root of the original tree, then we could obtain the functions $p_4(S_4), q_4(S_4^*), g_4(S_4)$ and $g_4^*(S_4^*)$ as was done in Sect. 2.3.1.

If node 4 had a solid (respectively, dashed) incoming link before, then after removal of such links the corresponding left (respectively, right) side of balance equation is

replaced by some function $p_4(S_4)$ (respectively, $q_4(S_4^*)$) having the properties specified in Eq. 52. In this section, we explain how the function $p_3(S_3)$ appears as we remove node 4.

The following Lemma will be helpful.

Lemma 5 *Suppose $p, q, g, g^*, k_{on}, k_{off}$ and k_{cat} are functions with the properties described in Sect. 2.3.1, σ is a non-negative parameter and S_T is a positive constant. Then the following system of equations*

$$p(S) = q(S^*), \tag{66}$$

$$g(S) + g^*(S^*) + Y = S_T, \tag{67}$$

$$k_{on}(S^*, \sigma) = (k_{off} + k_{cat})(Y). \tag{68}$$

has unique solution $S(\sigma) > 0$, (c ds), $S^*(\sigma) > 0$, (c ds) and $Y(\sigma)$, (cis0) for $\sigma \in [0, \infty)$.

The proof of Lemma 5 is given in the ‘‘Appendix’’.

Applying Lemma 5 to Eqs. 63, 64 and 65, after the appropriate identifications, we see that the concentrations S_4, S_4^* and $S_4^*S_3$ are uniquely expressed in terms of S_3 , for $S_3 \in [0, \infty)$, $S_4(S_3)$ and $S_4^*(S_3)$ being strictly positive. After removing node 4 (that is, after expressing all variables of node 4 in terms of variables of node 3), the (cis0) function $S_4^*S_3(S_3)$ will make a contribution to the new $g'_3(S_3) = g_3(S_3) + S_4^*S_3(S_3)$. Also, the left-hand side of the balance equation of node 3 will be replaced by the function p_3 given by $p_3(S_3) = k_{cat,3}(S_4^*S_3(S_3))$. Since $S_4^*S_3(S_3)$ is (cis0) from Lemma 5 and $g_3(S_3)$ is (cis0) and greater than S_3 by induction, it follows that $g'(S_3)$ has these same properties. The function $p_3(S_3)$ is (cis0) because $k_{cat,3}$ and $S_4^*S_3(S_3)$ are. Hence, the functions p_3 and g'_3 have the properties specified in Eq. 52.

The properties A', B' and C' from Eq. 62 continue to hold for the new tree T_{k+1} . Equations Σ_{k+1} involve only the substrate forms of the nodes in T_{k+1} and the corresponding intermediate complexes involving these substrate forms. The three variables S_4, S_4^* and $S_4^*S_3$ were expressed in terms of the others. The structure of the equations Σ_{k+1} is also preserved. The only changes in the equations that formed Σ_k were replacement of $g_3(S_3) + S_4^*S_3(S_3)$ by a new function $g'_3(S_3)$ in the conservation equation for substrate in node 3 and replacement of the left-hand side of the balance equation of node 3 by function $p_3(S_3)$. These new functions were shown to have the properties specified in Eq. 52.

This completes the analysis for the removal of a root node in the case $4 \rightarrow 3$. The cases $4 \Rightarrow 3, 4 \rightrightarrows 3$ and $4 \rightleftharpoons 3$ can be reduced to the case $4 \rightarrow 3$ as shown in the ‘‘Appendix’’.

2.3.3.2 Eliminating a Terminal Node with One Incoming Link

Suppose that the removal process has reached stage k , where $0 \leq k \leq N - 2$, and that properties A', B' and C' in Eq. 62 hold for T_k . Suppose that we have a identified a terminal node of T_k with one incoming link. To simplify the notation, let us assume that this is node 2 and that the single incoming link to node 2 comes from node 1 (Fig. 4b). There are then four possibilities for the type of link from 1 to 2 and a case-by-case analysis becomes necessary.

Case 1 \rightarrow 2. By property C', the equations Σ_k satisfy Eqs. 55 to 58 and 59 together with appropriate kinetic equations 60 and 61 for T_k . Since the forward enzyme in node 2 is S_1^* , the balance equation for node 2 satisfies Eq. 56, so that

$$k_{\text{cat},2}(S_1^* S_2) = q_2(S_2^*). \tag{69}$$

Since node 2 is terminal and has no outgoing links, the substrate conservation equation 59 reduces to

$$g_2(S_2) + g_2^*(S_2^*) + S_1^* S_2 = S_{2T}. \tag{70}$$

In addition, we have the following kinetic equation for node 2

$$k_{\text{on},2}(S_1^*, S_2) = (k_{\text{off},2} + k_{\text{cat},2})(S_1^* S_2). \tag{71}$$

We have three equations for S_2 , S_2^* and $S_1^* S_2$ which are parameterised by S_1^* . The first step is to solve the equations and thereby eliminate S_2 , S_2^* and $S_1^* S_2$ in favour of S_1^* . The following Lemma will be useful

Lemma 6 *Suppose q , g , g^* , k_{on} , k_{off} and k_{cat} are functions with the properties described in Sect. 2.3.1, σ is a non-negative parameter and S_T is a positive constant. Then the following system of equations*

$$k_{\text{cat}}(Y) = q(S^*). \tag{72}$$

$$g(S) + g^*(S^*) + Y = S_T. \tag{73}$$

$$k_{\text{on}}(\sigma, S) = (k_{\text{off}} + k_{\text{cat}})(Y). \tag{74}$$

has unique solution $S(\sigma) > 0$, (cis) , $S^*(\sigma)$ $(\text{cis}0)$ and $Y(\sigma)$ $(\text{cis}0)$ for $\sigma \in [0, \infty)$.

The proof of Lemma 6 is given in the ‘‘Appendix’’.

Applying Lemma 6 to Eqs. 69, 70 and 71, after the appropriate identifications, we see that the concentrations S_2 , S_2^* and $S_1^* S_2$ are uniquely expressed in terms of S_1^* , for $S_1^* \in [0, \infty)$, $S_2(S_1^*) > 0$ and $S_2^*(S_1^*)$ is $(\text{cis}0)$. After removing node 2 (that is, after expressing the variables S_2 , S_2^* and $S_1^* S_2$ in terms of variables of node 1), the $(\text{cis}0)$ function $S_1^* S_2(S_1^*)$ contributes to the new $g_1^{*'}(S_1^*) = g_1^*(S_1^*) + S_1^* S_2(S_1^*)$. Since $g_1^*(S_1^*)$ is $(\text{cis}0)$ and is greater than or equal to S_1^* by induction and $S_1^* S_2(S_1^*)$ is $(\text{cis}0)$ by Lemma 6, the function $g_1^{*'}(S_1^*)$ is also $(\text{cis}0)$ and $g_1^{*'}(S_1^*) \geq S_1^*$. Hence, $g_1^{*'}$ has the properties specified in Eq. 52.

The properties A', B' and C' from Eq. 62 continue to hold for the new tree T_{k+1} . Equations Σ_{k+1} involve only the substrate forms of the nodes in T_{k+1} and corresponding intermediate complexes involving these substrate forms. The three variables S_2 , S_2^* and $S_1^* S_2$ were expressed in terms of the others. The structure of the equations Σ_{k+1} is also preserved. Then only change in the equations that formed Σ_{k+1} was replacement of $g_1^*(S_1^*) + S_1^* S_2(S_1^*)$ by a new function $g_1^{*'}(S_1^*)$ in the conservation equation for substrate in node 1 which was shown to have the properties specified in Eq. 52.

This completes the analysis of the case $1 \rightarrow 2$. The cases $1 \Rightarrow 2$, $1 \rightarrow 2$ and $1 \Rightarrow 2$ can be reduced to the case $1 \rightarrow 2$ as shown in the ‘‘Appendix’’.

2.3.3.3 Completing the Argument

By eliminating root and terminal nodes as described in Sects. 2.3.3.1 and 2.3.3.2, respectively, we reduce the tree in $N - 1$ steps to a single node for which the corresponding system of equations, Σ_{N-1} satisfies properties A' , B' and C' . In the end, for the last remaining node we obtain two equations of the form 53 and 54 on the functions p , q , g and g^* with the properties specified in Eq. 52. Therefore by Proposition 4 we conclude that the substrate form concentrations for the last node are uniquely determined and positive. All other variables are determined uniquely by the substrate form concentrations of the last node (property B' in Eq. 62). It is now easy to confirm that these other variables are also positive. To do this, let us go in the opposite way to how the tree was deconstructed. Suppose we have shown that for an intermediate tree T_k , the concentrations of all substrate forms and intermediate complexes are positive.

In Sect. 2.3.3.1, by eliminating the root node with a single outgoing link we expressed the concentrations of the two substrate forms S_{root} and S_{root}^* of the root node and the only intermediate complex Y in terms of the remaining variables. Y was shown to be a (*cisO*) function on $[0, \infty)$ of the concentration of one of the remaining substrate forms (which is positive by hypothesis). Thus, $Y > 0$. It was also shown that S_{root} and S_{root}^* are positive for any non-negative concentrations of the remaining substrate form.

In Sect. 2.3.3.2, we have seen that eliminating the terminal node with a single incoming link from node k amounted to expressing the concentrations of the two substrate forms S_{term} and S_{term}^* and the only intermediate complex Y in terms of one of the substrate forms X_k (either S_k or S_k^*) in node k . One of the terminal node substrate forms was shown to be positive for all $X_k \geq 0$. Y and the other terminal node substrate form were shown to be (*cisO*) functions of $X_k \in [0, \infty)$, which is positive by hypothesis. Thus, S_{term} , S_{term}^* and Y are all positive.

Finally, let us see that concentrations of the free enzymes and the corresponding intermediate complexes are positive. The reasoning for a single GK loop in Sect. 2.3.1 applies here as well since the reactions involving enzyme, substrate form and intermediate complex are independent of the other reactions. From the corresponding kinetic and enzyme conservation equations (Sect. 2.3.1), the concentration of the intermediate complex Y is a (*cisO*) function of one of the concentrations of the substrate forms. The former is therefore positive, since we have already showed that substrate form concentrations for all nodes are positive. As for the free enzymes, it was shown in Sect. 2.3.1 that their concentration are positive for non-negative values of substrate forms. This completes the proof of Theorem 2.

2.4 Multistationarity for Complex Reaction Schemes and General Kinetics

Inspired by the results of the previous section, one might hope that the theorem for trees of GK loops is valid for a larger class of models with non-mass action dynamics. This turns out not to be true. In this subsection, we show that even a single GK loop with

$E_T = 1$ and $F_T = 1/8$, we get three positive solutions for (S, S^*) : (0.473, 9.075), (2.383, 6.641) and (8.122, 0.854). It follows from Eqs. 75 and 76 that the concentrations of free enzymes and intermediate complexes are positive in all three cases as well.

3 Discussion

Experimental biologists are continually striving to elicit general biological principles which hold irrespective of the molecular details of particular examples. This makes it all the more important, from the perspective of systems biology, to identify properties which hold, not just for individual molecular networks, but for classes of such networks in which certain details may vary between individual examples. Here, we have examined the property of monostationarity, the existence of a unique (positive) steady state, in respect of several kinds of “details”, network structure, enzyme reaction mechanism, kinetics and parameter values. We have found that monostationarity is preserved for any tree of GK loops with complex enzyme mechanisms and mass action kinetics and any allowable parameter values (Theorem 1). It is also preserved for any tree of GK loops with the Michaelis–Menten enzyme scheme and any general kinetics as specified in Sect. 2.3.1 (Theorem 2). However, a single GK loop with a complex enzyme mechanism and general kinetics can exhibit multiple positive steady states (Sect. 2.4). The GK loop has been a canonical motif in systems biology and it is surprising to find that such an apparently simple biochemical circuit exhibits multistationarity.

Monostationarity in molecular networks has been extensively studied, and several powerful methods have emerged for determining when it does, or does not, exist. These include, for example, Jacobian methods (Soulé 2003), deficiency theory (Feinberg 1987), the injectivity property (Craciun and Feinberg 2005) and concordance (Shinar and Feinberg 2012); for an overview, see Joshi and Shiu (2015). In particular, the Chemical Reaction Network Theory Toolbox, developed by Martin Feinberg and his students (Feinberg 1979), includes a variety of algorithms for checking monostationarity by some of these methods. We confirmed the behaviour of several examples of GK loop trees in the Toolbox, including the multistationarity described in Sect. 2.4. These tests further revealed a consistent pattern which leads us to conjecture that any tree of GK loops with the Michaelis–Menten reaction scheme is concordant and even strongly concordant. If this conjecture is true, then Theorem 2 could be deduced from the results of Shinar and Feinberg (2012) and, indeed, more would be known since concordance implies monostationarity for any weakly monotonic kinetics. However, Theorem 1 presents a different picture. The example in Sect. 2.4 shows that complex reaction mechanisms take us outside the class of concordant systems, even for a single GK loop. As mentioned in Introduction, trees of GK loops with complex enzyme mechanisms under mass action kinetics can be shown to be examples of the MESSI systems introduced in Pérez Millán and Dickenstein (2018). However, to exploit the results in Pérez Millán and Dickenstein (2018) to show monostationarity, it would be necessary to show that these particular MESSI systems are also “structurally toric”

and this appears not to be the case, in general. Trees of GK loops with general kinetics are not MESSI systems.

From a more general perspective, the reaction networks considered here satisfy mass balance and are therefore conservative (Feinberg 2019, Definition 4.1.1). There is, therefore, a steady state within each “stoichiometric compatibility class”, which is to say, for any positive values of the conserved quantities (Feinberg 2019, Theorem 10.5.21). In our context, it is not straightforward to show that this steady state is positive, especially for trees of GK loops with complex enzyme mechanisms. For trees of GK loops with the Michaelis–Menten reaction scheme, both positivity and uniqueness of the steady state would follow, as noted, if the concordance property were shown.

Complex reaction mechanisms and network structure have been two kinds of “detail” that we have tried to rise above. For the former, we have relied on the linear framework (Gunawardena 2012, 2014). As reiterated elsewhere (Gunawardena 2014), the reaction mechanisms of actual enzymes are complex and not at all like the Michaelis–Menten mechanism that is widely assumed in the literature. It is, therefore, especially important to address this complexity and we have shown here how the linear framework allows us to do this. As for network structure, the use of a GK loop as a repetitive motif has an important justification in the linear cascades that are ubiquitous in cellular signalling (Pearson et al. 2001), while the generalisation to trees offers a tractable context for an initial analysis of network effects.

Tree networks naturally suggest a modular and recursive approach, which is a central feature of the present paper. A GK loop (or module) on the periphery of the tree network, which is either a root node with one outgoing link or a terminal node with one incoming link, is removed, and the corresponding dynamical variables are eliminated in favour of those which remain. In order to prove monostationarity, some functional property of the variables has to be conserved during the removal step and we have relied on the weak requirements of continuity and monotonicity for these invariant functional properties. The advantage of weak requirements is that less needs to be proved at the recursive step. It may be possible to exploit such recursive methods for more complex networks.

The methods introduced here allow other questions about tree networks to be addressed. For instance, suppose that the total amount of some substrate form is changed, while the total amounts of all other substrates and enzymes are kept fixed. How do such modulations propagate through the network? For tree networks, either with complex mechanisms and mass action kinetics or with Michaelis–Menten mechanisms and general kinetics, we find that the direction of change in the total substrate, whether increasing or decreasing, is matched by the corresponding substrate forms which make up that total and also by those substrate forms that are an even number of loops upstream in the tree. However, the direction of change is reversed for those substrate forms that are an odd number of loops upstream. These results extend those of Feliu et al. (2012a, b) for linear GK loop networks with the Michaelis–Menten mechanism and mass action kinetics. For reasons of space, we have not included these and related results but hope to report on them elsewhere.

The tree structure is essential to the monostationarity results that we have proved. It is not hard to show that multistationarity can occur when there are loops in the

network. (We omit examples for reasons of space.) In other work, we have been developing ways to analyse cyclic GK loop networks and also hope to report on this elsewhere. It is interesting to speculate whether trees of “modules” other than the GK loop can exhibit similar properties and, if so, whether a more general form of recursive, tree-based analysis of biochemical networks may be possible. We hope in this way to have suggested new directions for the mathematical analysis of biochemical networks.

Acknowledgements We are most grateful to four anonymous reviewers for their extremely helpful and constructive comments, which contributed substantially to the paper’s structure and clarity. JG was supported by NSF Grant 1462629.

Appendix

Lemma 1

Courant proved a differentiable version of Lemma 1 in Courant (1936, pp. 119–121), but his differentiability assumptions are only used to show differentiability of the constructed implicit function. They can hence be dispensed with for our purposes. The proof below is adapted from a similar result in Kudryavtsev (1998).

Proof Consider $\mathcal{F}(\bar{x}^*, y)$. It is strictly monotonous in $y \in U(y^*, \eta)$ and we can assume that it is strictly monotonously increasing. (Otherwise, we could take $-\mathcal{F}$.) Choose $\epsilon \in (0, \eta)$. Since $\mathcal{F}(\bar{x}^*, y^*) = 0$, using monotonicity we get $\mathcal{F}(\bar{x}^*, y^* + \epsilon) > 0$ and $\mathcal{F}(\bar{x}^*, y^* - \epsilon) < 0$. From continuity of $\mathcal{F}(\bar{x}, y)$ on $U(\bar{x}^*, \bar{\xi}) \times U(y^*, \eta)$, it follows that there exists a neighbourhood $U(\bar{x}^*, \bar{\delta}) \subset U(\bar{x}^*, \bar{\xi})$ of the point \bar{x}^* such that $\mathcal{F}(\bar{x}, y^* + \epsilon) > 0$ and $\mathcal{F}(\bar{x}, y^* - \epsilon) < 0$ for any $\bar{x} \in U(\bar{x}^*, \bar{\delta})$. Since for a fixed $\bar{x} \in U(\bar{x}^*, \bar{\delta})$ the function $\mathcal{F}(\bar{x}, y)$ is continuous and monotonous in $y \in U(y^*, \epsilon)$, there exists an unique solution $y \in U(y^*, \epsilon)$ of $\mathcal{F}(\bar{x}, y) = 0$. Let us denote this correspondence $y = f(\bar{x})$, $\bar{x} \in U(\bar{x}^*, \bar{\delta})$.

So far we have proved existence and uniqueness of the function $y = f(\bar{x})$, $\bar{x} \in U(\bar{x}^*, \bar{\delta})$. Continuity of f at the point \bar{x}^* follows from the fact that for any $\epsilon \in (0, \eta)$ there exists $\bar{\delta}(\epsilon)$ such that if $\bar{x} \in U(\bar{x}^*, \bar{\delta})$ then $y \in U(y^*, \epsilon)$. Obviously $f(\bar{x}^*) = y^*$. This completes the proof of Lemma 1. □

Proof of Proposition 1

Proof Suppose the function $\phi(x, y)$ is continuous, $(is) \times (ds)$ on $[0, \infty) \times [0, \infty)$ and $\phi(0, 0) = 0$. The equation $\phi(x, y) = 0$ therefore defines a curve C going from the point $(0, 0)$ and increasing in both x and y . Choose $a, b > 0$. Since ϕ is strictly increasing in x and strictly decreasing in y , $\phi(a, 0) > 0$ and $\phi(0, b) < 0$. It is therefore evident on the grounds of continuity that the curve must cross the right or upper boundary of the box whose far right corner is at (a, b) . Suppose then that there exists some $b > 0$ such that there is no point on the curve with $y = b$, so that the curve is limited to lie in the region $y < b$. Then, there can be no such limitation in the x coordinate or there would exist a box whose boundary is not crossed. Hence, for $x \in [0, \infty)$, there exists some $y \in [0, b)$ such that $\phi(x, y) = 0$. Furthermore,

because of strict monotonicity, y must be unique, so this assignment defines a function $y(x) : [0, \infty) \rightarrow [0, b)$ such that $\phi(x, y(x)) = 0$. (The use of $y(x)$ is an abuse of notation but a convenient one which avoids the proliferation of function names.) $y(x)$ inherits the property of being strictly monotonously increasing from ϕ .

Alternatively, it is possible that the curve is not limited in any direction or it is limited to some $x < a$. In the former case, we get a *(is0)* function $y(x) : [0, \infty) \rightarrow [0, \infty)$ such that $\phi(x, y(x)) = 0$. In the latter case (denoting $x_{max} = \inf(a) > 0$), we get a *(is0)* function $y(x) : [0, x_{max}) \rightarrow [0, \infty)$ such that $\phi(x, y(x)) = 0$. This function is not bounded, otherwise we would have a box with right or upper boundary not crossed by the curve C . In the following, let us allow x_{max} to be ∞ to unite all three cases.

If we apply Lemma 1 to the function $\mathcal{F}(x, y) = \phi(x, y)$, we deduce the existence of a local implicit function which is locally continuous. Any implicit function must be unique by strict monotonicity, so the local function given by Lemma 1 coincides with $y(x)$. Hence, $y(x)$ is continuous for $x \in (0, x_{max})$. Let us show that it is also continuous at $x = 0$. Since $y(x)$ is strictly monotonously increasing and bounded from below, it must have a lower limit as $x \rightarrow 0^+$. Suppose that $\lim_{x \rightarrow 0^+} y(x) = y^+$. Since $y(x) > 0$ for $x > 0$, it must be that $y^+ \geq 0$. If $y^+ > 0$, then, since ϕ is continuous at $(0, 0)$, $\lim_{x \rightarrow 0^+} \phi(x, y(x)) = \phi(0, y^+) < 0$. But, by construction, $\phi(x, y(x)) = 0$. This contradiction shows that $y^+ = 0$ and so $y(x)$ is continuous at $x = 0$. We conclude that $y(x)$ is *(cis0)* for $x \in [0, x_{max})$ and that $y(0) = 0$. In fact, it is also normal, since it is not bounded if $x_{max} < \infty$.

The case of non-strict monotonicity in x of the function $\phi(x, y)$ is analogous. Strict monotonicity in y guarantees existence and uniqueness of $y(x)$ on some interval $[0, x_{max})$ and Lemma 1 guarantees continuity on $(0, x_{max})$. Continuity at $x = 0$ follows the same way. $y(x)$ inherits non-strict monotonicity in x from $\phi(x, y)$. \square

Proof of Proposition 2

Proof Let the continuous function $\tilde{\phi}(x, y)$ be an extension of the function $\phi(x, y)$ to the whole quadrant defined as follows. $\tilde{\phi}(x, y) = \phi(x, y)$, if $x \geq 0, y_0 \geq y \geq 0$ and $\tilde{\phi}(x, y) = \phi(x, y) + y_0 - y$ if $x \geq 0, y > y_0$. Note that $\tilde{\phi}(x, y)$ has the same monotonicity properties as $\phi(x, y)$. Applying Proposition 1 to $\tilde{\phi}(x, y) = 0$, we obtain a *(cis0n)*, respectively, *(ci0n)*, function $y(x)$, such that $\phi(x, y(x)) = 0$. $y(x)$ either does not exceed y_0 and therefore is defined on $[0, \infty)$ or there exists $a > 0$, such that $y(a) = y_0$ and $y(x) > y_0, x > a$. In the latter case, we can take $x_{max} = a$, so that the domain of definition is $[0, x_{max}]$.

Any other continuous extension of the function ϕ preserving monotonicity properties would be also acceptable, since it doesn't modify the curve in the region $y_0 \geq y \geq 0$ in which we are interested. \square

Proof of Lemma 2

Proof Consider first the case $X \equiv S^*$.

By the properties in Eq. 21 and using Eq. 35, the function $\phi(S^*, S) = -p(S, S^*) + q(S, S^*)$ satisfies the conditions of Proposition 1, with ϕ being continuous on $[0, \infty) \times$

$[0, \infty)$ and $(is) \times (ds)$. We therefore obtain a (*cis0n*) solution function $S(S^*)$ on $[0, S_{max}^*)$ such that $\phi(S^*, S(S^*)) = 0$.

Substituting $S(S^*)$ into Eq. 36, we get

$$g(S(S^*), S^*) + S^* \cdot \sigma = S_T.$$

It follows from the properties of g in Eq. 21 that the function on the left-hand side of this equation is also (*cis0n*) for $S^* \in [0, S_{max}^*)$ and that it grows at least as much as $S(S^*) + S^*$. It therefore has a unique solution function $S^*(\sigma) > 0$, with $\sigma \in [0, \infty)$, for which $S(S^*(\sigma)) > 0$.

Let us introduce the variable $\tilde{S}^* = S^*(0) - S^*$. Applying Proposition 2 to the continuous $(i) \times (ds)$ function $\phi(\sigma, \tilde{S}^*) = g(S(S^*(0) - \tilde{S}^*), S^*(0) - \tilde{S}^*) + (S^*(0) - \tilde{S}^*) \cdot \sigma - S_T$ defined on $[0, \infty) \times [0, S^*(0)]$, we get that $\tilde{S}^*(\sigma)$ is (*ci0*) on its domain of definition. Since $\phi(\sigma, S^*(0)) = -S_T \neq 0$, the curve $\tilde{S}^*(\sigma)$ cannot intersect $\tilde{S}^* = S^*(0)$ and therefore extends to infinity in the σ direction. In fact, it is (*cis0*) because ϕ is $(is) \times (ds)$ on $[0, \infty) \times [0, y]$, for any $0 < y < S^*(0)$. Thus, $S^*(\sigma) = S^*(0) - \tilde{S}^*(\sigma)$ and $S(S^*(\sigma))$ are (*cds*) function on $[0, \infty)$. Moreover, $S^*(\sigma) \cdot \sigma = S_T - g(S(S^*(\sigma)), S^*(\sigma)) \geq 0$ is a bounded (*cis0*) function of $\sigma \in [0, \infty)$.

The proof for $X \equiv S$ is similar. One way to see it is to express $S^*(S)$ from Eq. 35 and substitute it into Eq. 36. □

Eliminating a Root Node in Theorem 1

We show that the cases $4 \rightarrow 3$, $4 \Rightarrow 3$ and $4 \Leftrightarrow 3$ are equivalent to the case $4 \Rightarrow 3$ by redefining the variables. The crucial step in eliminating the variables of the root node 4 with a single outgoing link to node 3 (Sect. 2.2.4) is to prove that expression of variables S_4, S_4^* in favour of S_3 or S_3^* is unique and that, after elimination of S_4 and S_4^* , the new $g'_3(S_3, S_3^*) = g_3(S_3, S_3^*) + \Delta g_3(S_3, S_3^*)$ as well as the term $p_3(S_3, S_3^*)$ (or $q_3(S_3, S_3^*)$) have the required properties in Eq. 21. We will confirm these requirements in each case.

Case $4 \rightarrow 3$. The balance and substrate conservation equations for node 4 take the form

$$\begin{aligned} p_4(S_4, S_4^*) &= q_4(S_4, S_4^*), \\ g_4(S_4, S_4^*) + S_4^* \cdot (\alpha_3 S_3 + \beta_3 S_3^*) &= S_{4T}. \end{aligned}$$

These equations already have the same form as for the case $4 \Rightarrow 3$ in Sect. 2.2.4, except for the coefficients in the non-negative linear combination $(\alpha_3 S_3 + \beta_3 S_3^*)$. Thus, $S_4(\alpha_3 S_3 + \beta_3 S_3^*)$ and $S_4^*(\alpha_3 S_3 + \beta_3 S_3^*)$ are uniquely expressed, positive and (*cds*) functions of $(\alpha_3 S_3 + \beta_3 S_3^*) \in [0, \infty)$. $\Delta g_3(S_3, S_3^*) = (\alpha_3 S_3 + \beta_3 S_3^*) S_4^*(\alpha_3 S_3 + \beta_3 S_3^*)$ is (*cis0*) function of $(\alpha_3 S_3 + \beta_3 S_3^*)$, and $g'_3(S_3, S_3^*) = g_3(S_3, S_3^*) + \Delta g_3(S_3, S_3^*)$ has the required properties in Eq. 21. The left side of the balance equation for node 3, $S_4^* \cdot (\gamma_3 S_3 - \delta_3 S_3^*)$, becomes $p_3(S_3, S_3^*) = (\gamma_3 S_3 - \delta_3 S_3^*) S_4^*(\alpha_3 S_3 + \beta_3 S_3^*)$. If we make the following redefinition of variables, coefficients and functions, which preserve the properties of these functions, $S_3 = \tilde{S}_3, S_3^* = \tilde{S}_3^*, \gamma_3 = \tilde{\gamma}_3^*, \delta_3 = \tilde{\delta}_3^*, \alpha_3 = \tilde{\alpha}_3^*, \beta_3 = \tilde{\beta}_3^*$,

$p_3(S_3, S_3^*) = \tilde{q}_3(\tilde{S}_3, \tilde{S}_3^*)$, then the required properties of p_3 follow from the proven properties of q_3 in the case $4 \rightarrow 3$.

Case $4 \Rightarrow 3$. The balance and substrate conservation equations for node 4 take the form

$$p_4(S_4, S_4^*) = q_4(S_4, S_4^*),$$

$$g_4(S_4, S_4^*) + S_4 \cdot (\alpha_3 S_3 + \beta_3 S_3^*) = S_{4T}.$$

Consider the following redefinition of the variables and functions for node 4 which preserve the properties of these functions, $S_4 = \tilde{S}_4^*$, $S_4^* = \tilde{S}_4$, $p_4(S_4, S_4^*) = \tilde{q}_4(\tilde{S}_4, \tilde{S}_4^*)$, $q_4(S_4, S_4^*) = \tilde{p}_4(\tilde{S}_4, \tilde{S}_4^*)$, $g_4(S_4, S_4^*) = \tilde{g}_4(\tilde{S}_4, \tilde{S}_4^*)$. Then, the equations above take the form

$$\tilde{p}_4(\tilde{S}_4, \tilde{S}_4^*) = \tilde{q}_4(\tilde{S}_4, \tilde{S}_4^*),$$

$$\tilde{g}_4(\tilde{S}_4, \tilde{S}_4^*) + \tilde{S}_4^* \cdot (\alpha_3 S_3 + \beta_3 S_3^*) = S_{4T},$$

which are the same as for the case $4 \rightarrow 3$ above. Thus $\tilde{S}_4(\alpha_3 S_3 + \beta_3 S_3^*)$ and $\tilde{S}_4^*(\alpha_3 S_3 + \beta_3 S_3^*)$ are uniquely expressed, positive and *(c)ds* functions of $(\alpha_3 S_3 + \beta_3 S_3^*) \in [0, \infty)$. The expression $\Delta g_3(S_3, S_3^*) = (\alpha_3 S_3 + \beta_3 S_3^*) \tilde{S}_4^*(\alpha_3 S_3 + \beta_3 S_3^*)$ is a *(cis)0* function of $(\alpha_3 S_3 + \beta_3 S_3^*)$, and $g_3'(S_3, S_3^*) = g_3(S_3, S_3^*) + \Delta g_3(S_3, S_3^*)$ has the required properties in Eq. 21. The left side of the balance equation for node 3, $S_4 \cdot (\gamma_3 S_3 - \delta_3 S_3^*) \equiv \tilde{S}_4^* \cdot (\gamma_3 S_3 - \delta_3 S_3^*)$, becomes $p_3(S_3, S_3^*) = (\gamma_3 S_3 - \delta_3 S_3^*) \tilde{S}_4^*(\alpha_3 S_3 + \beta_3 S_3^*)$, and it follows from the case $4 \rightarrow 3$ that the required properties of p_3 are also satisfied.

Case $4 \Rightarrow 3$. The balance and substrate conservation equations for node 4 take the form

$$p_4(S_4, S_4^*) = q_4(S_4, S_4^*),$$

$$g_4(S_4, S_4^*) + S_4 \cdot (\alpha_3^* S_3^* + \beta_3^* S_3) = S_{4T}.$$

Consider the following redefinition of variables and functions for node 4 preserving the properties of these functions, $S_4 = \tilde{S}_4^*$, $S_4^* = \tilde{S}_4$, $p_4(S_4, S_4^*) = \tilde{q}_4(\tilde{S}_4, \tilde{S}_4^*)$, $q_4(S_4, S_4^*) = \tilde{p}_4(\tilde{S}_4, \tilde{S}_4^*)$, $g_4(S_4, S_4^*) = \tilde{g}_4(\tilde{S}_4, \tilde{S}_4^*)$. The equations above take the form

$$\tilde{p}_4(\tilde{S}_4, \tilde{S}_4^*) = \tilde{q}_4(\tilde{S}_4, \tilde{S}_4^*),$$

$$\tilde{g}_4(\tilde{S}_4, \tilde{S}_4^*) + \tilde{S}_4^* \cdot (\alpha_3^* S_3^* + \beta_3^* S_3) = S_{4T}$$

which are the same as for the case $4 \rightarrow 3$. Thus $\tilde{S}_4(\alpha_3^* S_3^* + \beta_3^* S_3)$ and $\tilde{S}_4^*(\alpha_3^* S_3^* + \beta_3^* S_3)$ are uniquely expressed, positive and *(c)ds* functions of $(\alpha_3^* S_3^* + \beta_3^* S_3) \in [0, \infty)$. The expression $\Delta g_3(S_3, S_3^*) = (\alpha_3^* S_3^* + \beta_3^* S_3) \tilde{S}_4^*(\alpha_3^* S_3^* + \beta_3^* S_3)$ is *(cis)0* function of $(\alpha_3^* S_3^* + \beta_3^* S_3)$ and $g_3'(S_3, S_3^*) = g_3(S_3, S_3^*) + \Delta g_3(S_3, S_3^*)$ has the required properties in Eq. 21. The right side of the balance equation for node 3, $S_4 \cdot (\gamma_3^* S_3^* - \delta_3^* S_3) \equiv \tilde{S}_4^* \cdot (\gamma_3^* S_3^* - \delta_3^* S_3)$, becomes $q_3(S_3, S_3^*) = (\gamma_3^* S_3^* - \delta_3^* S_3) \tilde{S}_4^*(\alpha_3^* S_3^* + \beta_3^* S_3)$ and it follows from the case $4 \rightarrow 3$ that the required properties of q_3 are also satisfied.

Proof of Lemma 3

Proof For positive σ , Eq. 40 can be written as

$$\chi(\sigma, S^*, S) = -\sigma \cdot (\gamma S - \delta S^*) + q(S, S^*) = 0.$$

The function $\chi(\sigma, S^*, S)$ is continuous on $(\sigma, S^*, S) \in (0, \infty) \times [0, \infty) \times [0, \infty)$, (is) in S^* and (ds) in S when other variables are fixed, by properties of the function q in Eq. 21 and $\chi(\sigma, 0, 0) = 0$ for any $\sigma > 0$. Applying Proposition 1, we get a family of (cis0n) functions $S(S^*, \sigma)$ of $S^* \in [0, S_{max}^*(\sigma))$, parameterised by $\sigma > 0$. It is possible to make a stronger statement for $S(S^*, \sigma)$ considered as a function of two variables.

Proposition 5 $S(S^*, \sigma)$ constructed above is defined on $[0, \infty) \times (0, \infty)$, continuous on $(0, \infty) \times (0, \infty)$ and (cis0n) in S^* .

Proof Let us first show that the domain of definition of $S(S^*, \sigma)$ contains $[0, \infty) \times (0, \infty)$ or, equivalently, that $S_{max}^*(\sigma) = \infty, \forall \sigma > 0$. Let us substitute the representation specified in Lemma 3 for the function q in Eq. 40 and rewrite it as

$$\sigma \gamma S + \delta^* S \rho^*(\alpha^* S^* + \beta^* S) = \sigma \delta S^* + \gamma^* S^* \rho^*(\alpha^* S^* + \beta^* S).$$

We see that the left side grows at least linearly in S for any choice of kinetic parameters and the right side can be bounded from above in both variables if $\delta = 0$. Indeed, $S^* \rho^*(\alpha^* S^* + \beta^* S)$ is a positive, decreasing function of S and an increasing and bounded function of S^* . Thus, the left side, and therefore also S , has to be bounded if $\delta = 0$. If S is bounded, then S^* cannot be (as follows from Proposition 1). On the other hand, if S goes to ∞ , when $\delta > 0$, then so does S^* (the right side can be unbounded only in S^*). Thus $S_{max}^*(\sigma) = \infty, \forall \sigma > 0$.

Let us apply Lemma 1 to the three-dimensional function $\mathcal{F}(S^*, \sigma, S) = \chi(\sigma, S^*, S)$. We get that the function $S(S^*, \sigma)$ defined on $[0, \infty) \times (0, \infty)$ is continuous on $(0, \infty) \times (0, \infty)$, (is) in $S^* \in [0, \infty)$ and $S(0, \sigma) = 0, \sigma > 0$. This completes the proof of Proposition 5. □

Continuing with the proof of Lemma 3, we can now substitute $S(S^*, \sigma)$ into Eq. 41 to get

$$g(S(S^*, \sigma), S^*) + \sigma \cdot (\alpha S(S^*, \sigma) + \beta S^*) = S_T.$$

The left side of the equation is (cis0) and unbounded in S^* . Therefore there is an unique solution $S^*(\sigma) > 0$ for any $\sigma > 0$. Applying Lemma 1 to the function $\mathcal{F}(\sigma, S^*) = g(S(S^*, \sigma), S^*) + \sigma \cdot (\alpha S(S^*, \sigma) + \beta S^*) - S_T$, we deduce that $S^*(\sigma)$ is continuous for $\sigma \in (0, \infty)$. The composition $S(S^*(\sigma), \sigma)$ is continuous for $\sigma \in (0, \infty)$ as well. $S(S^*(\sigma), \sigma)$ is positive for $\sigma \in (0, \infty)$ because $S^*(\sigma)$ is.

If $\sigma = 0$, then Eqs. 40 and 41 greatly simplify. The first equation becomes $q(S, S^*) = 0$. Applying Proposition 1 to the function $\phi(S, S^*) = -q(S, S^*)$, we

obtain a function $S^*(S)$ which is (*ci0n*) on some maximal interval $[0, \tilde{S}_{max})$. Substituting $S^*(S)$ in Eq. 41 at $\sigma = 0$, we get

$$g(S, S^*(S)) = S_T.$$

The left side is a (*cis0*) function of $S \in [0, \tilde{S}_{max})$ which is unbounded from above. Therefore there is a unique solution $S \in (0, S_T]$.

Having constructed unique solutions of Eqs. 40 and 41, $S(S^*(\sigma))$ and $S^*(\sigma)$, it will be convenient to combine them and denote the solutions simply as $S(\sigma)$ and $S^*(\sigma)$.

Next we show that $\sigma \cdot (\alpha S_2(\sigma) + \beta S^*(\sigma))$ is continuous and monotonously increasing for $\sigma \in [0, \infty)$. We already showed that $S(S^*(\sigma), \sigma)$ and $S^*(\sigma)$ are continuous for $\sigma \in (0, \infty)$. Also, clearly, $0 \leq S \leq S_T, 0 \leq S^* \leq S_T$ for any $\sigma \geq 0$. This means that $\lim_{\sigma \rightarrow 0^+} \sigma \cdot (\alpha S(\sigma) + \beta S^*(S(\sigma), \sigma)) = 0$, which implies continuity of $\sigma \cdot (\alpha S(\sigma) + \beta S^*(\sigma))$ at $\sigma = 0$.

Now let us prove that $\sigma \cdot (\alpha S(\sigma) + \beta S^*(\sigma))$ is monotonously increasing in $\sigma \in [0, \infty)$. Suppose σ is increasing to $\sigma + \Delta\sigma, \Delta\sigma > 0$. Then S and S^* will change to some $S + \Delta S$ and $S^* + \Delta S^*$, respectively. We need to consider four different cases:

1) $\Delta S \geq 0$ and $\Delta S^* \geq 0$ and at least one of them is strictly greater than zero. Since $\Delta\sigma > 0$, we get a contradiction because the left side of the substrate conservation equation 41 is increasing, while the right side is not changed.

2) $\Delta S \leq 0$ and $\Delta S^* \leq 0$ and at least one of them is strictly less than zero. Then, $\sigma \cdot (\alpha S + \beta S^*) = S_T - g(S, S^*)$ is indeed monotonously increasing from zero.

3) $\Delta S > 0$ and $\Delta S^* < 0$. Now the right side of Eq. 40 is decreasing (because of the properties of the function q in Eq. 21). Therefore $\sigma \cdot (\gamma S - \delta S^*)$ has to decrease as well. This is possible only if $\delta > 0$. If $\beta = 0$, we find that $\sigma \cdot \alpha S$ is indeed increasing. If $\beta > 0$, we can write

$$\sigma \cdot (\alpha S + \beta S^*) = \frac{\beta}{\delta} \left(\sigma \cdot (\delta S^* - \gamma S) + \left(\gamma + \frac{\delta}{\beta} \alpha \right) \sigma \cdot S \right)$$

which shows that it is monotonously increasing from zero as well.

4) $\Delta S < 0$ and $\Delta S^* > 0$. Now the right side of Eq. 40 is increasing (because of the properties of the function q in Eq. 21). Therefore $\sigma \cdot (\gamma S - \delta S^*)$ has to increase as well. But now our expression can be rearranged so that,

$$\sigma \cdot (\alpha S + \beta S^*) = \frac{\alpha}{\gamma} (\sigma \cdot (\gamma S - \delta S^*)) + \left(\delta + \frac{\gamma}{\alpha} \beta \right) \sigma \cdot S^*,$$

which shows that it is monotonously increasing from zero. This completes the proof of Lemma 3. □

Eliminating a Terminal Node in Theorem 1

We show that the cases $1 \Rightarrow 2, 1 \rightrightarrows 2$ and $1 \Leftrightarrow 2$ are equivalent to the case $1 \rightarrow 2$ by redefining the variables. The crucial step in eliminating the variables of the terminal node 2 with a single incoming link from node 1 (Sect. 2.2.5) is to prove that expression

of variables S_2, S_2^* in favour of S_1 or S_1^* is unique and that, after elimination of S_2 and S_2^* , the new $g_1'(S_1, S_1^*) = g_1(S_1, S_1^*) + \Delta g_1(S_1, S_1^*)$ has the required properties in Eq. 21. We confirm these requirements in each case.

Case 1 \Rightarrow 2. The balance and substrate conservation equations for node 2 take the form

$$S_1 \cdot (\gamma_2 S_2 - \delta_2 S_2^*) = q_2(S_2, S_2^*),$$

$$g_2(S_2, S_2^*) + S_1 \cdot (\alpha_2 S_2 + \beta_2 S_2^*) = S_{2T}.$$

These equations are the same as for the case 1 \rightarrow 2 in Sect. 2.2.5, with S_1^* replaced by S_1 . Thus $S_2(S_1)$ and $S_2^*(S_1)$ are uniquely expressed and positive for $S_1 > 0$. Furthermore, $\Delta g_1(S_1, S_1^*) = S_1 \cdot (\alpha_2 S_2(S_1) + \beta_2 S_2^*(S_1))$ has the same properties as a function of S_1 as $\Delta g_1(S_1, S_1^*) = S_1^* \cdot (\alpha_2 S_2(S_1^*) + \beta_2 S_2^*(S_1^*))$ has as a function of S_1^* for the case 1 \rightarrow 2 (Sect. 2.2.5).

Case 1 \Rightarrow 2. The balance and substrate conservation equations for node 2 take the form

$$p_2(S_2, S_2^*) = S_1^* \cdot (\gamma_2^* S_2^* - \delta_2^* S_2),$$

$$g_2(S_2, S_2^*) + S_1^* \cdot (\alpha_2^* S_2^* + \beta_2^* S_2) = S_{2T}.$$

Consider the following redefinition of parameters, variables and functions for node 2, which preserve the properties of these functions, $\alpha_2^* = \tilde{\alpha}_2, \beta_2^* = \tilde{\beta}_2, \gamma_2^* = \tilde{\gamma}_2, \delta_2^* = \tilde{\delta}_2, S_2 = \tilde{S}_2, S_2^* = \tilde{S}_2^*, p_2(S_2, S_2^*) = \tilde{q}_2(\tilde{S}_2, \tilde{S}_2^*)$ and $g_2(S_2, S_2^*) = \tilde{g}_2(\tilde{S}_2, \tilde{S}_2^*)$. The equations above now take the form

$$S_1^* \cdot (\tilde{\gamma}_2 \tilde{S}_2 - \tilde{\delta}_2 \tilde{S}_2^*) = \tilde{q}_2(\tilde{S}_2, \tilde{S}_2^*),$$

$$\tilde{g}_2(\tilde{S}_2, \tilde{S}_2^*) + S_1^* \cdot (\tilde{\alpha}_2 \tilde{S}_2 + \tilde{\beta}_2 \tilde{S}_2^*) = S_{2T},$$

which are the same as for the case 1 \rightarrow 2 (Sect. 2.2.5). Thus $S_2 = \tilde{S}_2^*(S_1^*)$ and $S_2^* = \tilde{S}_2(S_1^*)$ are uniquely expressed and positive for $S_1^* > 0$. Furthermore, $\Delta g_1(S_1, S_1^*) = S_1^* \cdot (\tilde{\alpha}_2 \tilde{S}_2(S_1^*) + \tilde{\beta}_2 \tilde{S}_2^*(S_1^*))$ has the same properties as a function of S_1^* as $\Delta g_1(S_1, S_1^*) = S_1^* \cdot (\alpha_2 S_2(S_1^*) + \beta_2 S_2^*(S_1^*))$ has as a function of S_1^* for the case 1 \rightarrow 2 (Sect. 2.2.5).

Case 1 \Rightarrow 2. The balance and substrate conservation equations for node 2 take the form

$$p_2(S_2, S_2^*) = S_1 \cdot (\gamma_2^* S_2^* - \delta_2^* S_2),$$

$$g_2(S_2, S_2^*) + S_1 \cdot (\alpha_2^* S_2^* + \beta_2^* S_2) = S_{2T}.$$

which are the same as for the case 1 \Rightarrow 2 above, with S_1^* replaced by S_1 . Thus $S_2(S_1)$ and $S_2^*(S_1)$ are uniquely expressed and positive for $S_1 > 0$. Furthermore, $\Delta g_1(S_1, S_1^*) = S_1 \cdot (\alpha_2^* S_2^*(S_1) + \beta_2^* S_2(S_1))$ has the same properties as a function of S_1 as $\Delta g_1(S_1, S_1^*) = S_1^* \cdot (\alpha_2^* S_2^*(S_1^*) + \beta_2^* S_2(S_1^*))$ has as a function of S_1^* for the case 1 \Rightarrow 2 above.

Proof of Lemma 4

Proof Expressing E from Eq. 50 and substituting in 51 we get

$$\phi(\sigma, Y) = k_{\text{on}}(E_T - Y, \sigma) - (k_{\text{off}} + k_{\text{cat}})(Y) = 0,$$

where $\phi(\sigma, Y)$ is a continuous $(i) \times (ds)$ function on $[0, \infty) \times [0, E_T]$. Applying Proposition 2 to $\phi(\sigma, Y)$, we obtain a $(ci0)$ function $Y(\sigma)$ on $[0, \infty)$. This follows from Proposition 2 and from the fact that $\phi(\sigma, E_T) < 0$ for any $\sigma \geq 0$, so that Y can never reach E_T . Therefore the curve is bounded by E_T and has to be unbounded in σ . In fact, $Y(\sigma)$ is $(cisd)$, because ϕ is $(is) \times (ds)$ on $[0, \infty) \times [0, y]$, for any $0 < y < E_T$. Since $Y < E_T$, $E(\sigma)$ is positive and $(cdis)$. \square

Proof of Lemma 5

Proof Applying Proposition 1 to the continuous function $\phi(S^*, S) = q(S^*) - p(S)$, which is $(is) \times (ds)$ on $[0, \infty) \times [0, \infty)$, and using Eq. 66, we obtain a $(cisd)$ function $S(S^*)$ on $S^* \in [0, S_{\text{max}})$. Substituting this solution in Eq. 67, we get

$$g(S(S^*)) + g^*(S^*) + Y = S_T.$$

Since $g(S(S^*)) + g^*(S^*)$ is a $(cisd)$ and unbounded from above function of $S^* \in [0, S_{\text{max}})$ from the last equation we obtain unique solution $S^*(Y)$, $Y \in [0, S_T]$. Obviously, $S_0^* = S^*(0) < S_{\text{max}}$. Let us define $\tilde{S}^* = S_0^* - S^*$. Applying Proposition 2 to the continuous $(is) \times (ds)$ function $\phi(Y, \tilde{S}^*) = g(S(S_0^* - \tilde{S}^*)) + g^*(S_0^* - \tilde{S}^*) + Y - S_T = 0$ defined on $[0, \infty) \times [0, S_0^*]$, we obtain a $(cisd)$ function $\tilde{S}^*(Y)$ on $[0, S_T]$, such that $\tilde{S}^*(S_T) = S_0^*$. Equivalently we have a $(cdis)$ function $S^*(Y) = S_0^* - \tilde{S}^*(Y)$ on $[0, S_T]$.

Substituting $S^*(Y)$ into Eq. 68, we get

$$k_{\text{on}}(S^*(Y), \sigma) = (k_{\text{off}} + k_{\text{cat}})(Y).$$

Applying Proposition 2 to the continuous $(i) \times (ds)$ function

$$\phi(\sigma, Y) = k_{\text{on}}(S^*(Y), \sigma) - (k_{\text{off}} + k_{\text{cat}})(Y)$$

defined on $[0, \infty) \times [0, S_T]$, we obtain a $(ci0)$ solution $Y(\sigma)$ on $[0, \infty)$. The corresponding curve C is not bounded in the direction σ because $\phi(\sigma, S_T) < 0$ for any $\sigma \geq 0$, so that Y cannot equal S_T . In fact, $Y(\sigma)$ is $(cisd)$, because ϕ is $(is) \times (ds)$ on $[0, \infty) \times [0, y)$, for any $0 < y < S_T$.

Since $Y(\sigma) < S_T$, it follows that $S^*(Y(\sigma))$ and $S(S^*(Y(\sigma)))$ are both $(cdis)$ and positive for $\sigma \in [0, \infty)$. \square

Eliminating a Root Node in Theorem 2

We show that the cases $4 \Rightarrow 3$, $4 \rightarrow 3$ and $4 \Rightarrow 3$ are equivalent to the case $4 \rightarrow 3$ by redefining the variables. The crucial step in eliminating the variables of the root node 4 with a single outgoing link to node 3 (Sect. 2.3.3.1) is to prove that expression of the variables S_4 , S_4^* and the corresponding intermediate complex, in favour of S_3 or S_3^* , is unique and that, after eliminating node 4, the new $g_3'(S_3) = g_3(S_3) + \Delta g_3(S_3)$ or $g_3'(S_3^*) = g_3^*(S_3^*) + \Delta g_3^*(S_3^*)$ and the terms $p_3(S_3)$ or $q_3(S_3^*)$ have the required properties in Eq. 52.

Case $4 \Rightarrow 3$. The balance and substrate conservation equations for node 4 and the kinetic equation for node 3 take the form

$$\begin{aligned} p_4(S_4) &= q_4(S_4^*), \\ g_4(S_4) + g_4^*(S_4^*) + S_4 S_3 &= S_{4T}, \\ k_{\text{on},3}(S_4, S_3) &= (k_{\text{off},3} + k_{\text{cat},3})(S_4 S_3). \end{aligned}$$

Consider the following redefinition of the variables and functions for node 4, which preserves the properties of these functions, $S_4 = \tilde{S}_4^*$, $S_4^* = \tilde{S}_4$, $p_4(S_4) = \tilde{q}_4(\tilde{S}_4^*)$, $q_4(S_4^*) = \tilde{p}_4(\tilde{S}_4)$, $g_4(S_4) = \tilde{g}_4(\tilde{S}_4^*)$, $g_4^*(S_4^*) = \tilde{g}_4^*(\tilde{S}_4)$ and $S_4 S_3 = Y$. The equations above now take the form

$$\begin{aligned} \tilde{p}_4(\tilde{S}_4^*) &= \tilde{q}_4(\tilde{S}_4^*), \\ \tilde{g}_4(\tilde{S}_4^*) + \tilde{g}_4^*(\tilde{S}_4^*) + Y &= S_{4T}, \\ k_{\text{on},3}(\tilde{S}_4^*, S_3) &= (k_{\text{off},3} + k_{\text{cat},3})(Y), \end{aligned}$$

which are the same as for the case $4 \rightarrow 3$ (Sect. 2.3.3.1). Thus $S_4(S_3) = \tilde{S}_4^*(S_3)$, $S_4^*(S_3) = \tilde{S}_4(S_3)$ and $S_4 S_3(S_3) = Y(S_3)$ are uniquely expressed in terms of S_3 , and S_4 and S_4^* are positive. Furthermore, $\Delta g_4(S_3) = S_4 S_3(S_3) = Y(S_3)$ and the term $p_3(S_3) = k_{\text{cat},3}(S_4 S_3(S_3)) = k_{\text{cat},3}(Y(S_3))$, which appears upon removal of node 4, are both (*cis*0).

Case $4 \rightarrow 3$. The balance and substrate conservation equations for node 4 and the kinetic equation for node 3 take the form

$$\begin{aligned} p_4(S_4) &= q_4(S_4^*), \\ g_4(S_4) + g_4^*(S_4^*) + S_4^* S_3^* &= S_{4T}, \\ k_{\text{on},3}^*(S_4^*, S_3^*) &= (k_{\text{off},3}^* + k_{\text{cat},3}^*)(S_4^* S_3^*), \end{aligned}$$

which are the same as for the case $4 \rightarrow 3$ (Sect. 2.3.3.1), except for the name of the functions in the last equation and replacement of S_3 and $S_4^* S_3$ by S_3^* and $S_4^* S_3^*$, respectively. Thus $S_4(S_3^*)$, $S_4^*(S_3^*)$ and $S_4^* S_3^*(S_3^*)$ are expressed uniquely and S_4 and S_4^* are positive. Furthermore, the term $\Delta g_4^*(S_3^*) = S_4^* S_3^*(S_3^*)$ and the term $q_3(S_3^*) = S_4^* S_3^*(S_3^*)$, which appears upon removal of node 4, are both (*cis*0).

Case 4 \Rightarrow 3. The balance and substrate conservation equations for node 4 and the kinetic equation for node 3 take the form

$$\begin{aligned} p_4(S_4) &= q_4(S_4^*), \\ g_4(S_4) + g_4^*(S_4^*) + S_4 S_3^* &= S_4 T, \\ k_{\text{on},3}^*(S_4, S_3^*) &= (k_{\text{off},3}^* + k_{\text{cat},3}^*)(S_4 S_3^*), \end{aligned}$$

which are the same as for case 4 \Rightarrow 3 above, except for the name of the functions in the last equation and replacement of S_3 and $S_4 S_3$ by S_3^* and $S_4 S_3^*$. Thus $S_4(S_3^*)$, $S_4^*(S_3^*)$ and $S_4 S_3^*(S_3^*)$ are expressed uniquely and S_4 and S_4^* are positive. Furthermore, the term $\Delta g_3^*(S_3^*) = S_4 S_3^*(S_3^*)$ and the term $q_3(S_3^*) = S_4 S_3^*(S_3^*)$, which appears upon removal of node 4, are both (*cis0*).

Proof of Lemma 6

Proof Applying Proposition 1 to the function $\phi(Y, S^*) = k_{\text{cat}}(Y) - q(S^*)$ using Eq. 72, we obtain a (*cis0n*) function $S^*(Y)$ on $[0, Y_{\text{max}})$. Substituting this solution in Eq. 73, we get

$$g(S) + g^*(S^*(Y)) + Y = S_T.$$

Since $g(S)$ is a (*cis0*) unbounded from above function of $S \in [0, \infty)$ and $g^*(S^*(Y)) + Y$ is a (*cis0*) unbounded from above function of $Y \in [0, Y_{\text{max}})$ from the last equation we obtain unique solution $Y(S)$ for $S \in [0, S_{\text{max}}]$, where S_{max} is the unique solution of $g(S_{\text{max}}) = S_T$. Clearly $Y(S_{\text{max}}) = 0$ and $Y_0 = Y(0) < Y_{\text{max}}$.

Let us introduce the variable $\tilde{Y} = Y_0 - Y$. Applying Proposition 2 to the continuous (*is*) \times (*ds*) function $\phi(S, \tilde{Y}) = g(S) + g^*(S^*(Y_0 - \tilde{Y})) + Y_0 - \tilde{Y} - S_T$ defined on $[0, \infty) \times [0, Y_0]$ using the above equation, we obtain a (*cis0*) solution $\tilde{Y}(S)$ on $S \in [0, S_{\text{max}}]$. Therefore $Y(S)$ is a (*cds*) function on this interval and the inverse $S(Y)$ is a (*cds*) function on $[0, Y_0]$, such that $S(0) = S_{\text{max}}$ and $S(Y_0) = 0$.

Substituting $S(Y)$ into Eq. 74, we get

$$k_{\text{on}}(\sigma, S(Y)) = (k_{\text{off}} + k_{\text{cat}})(Y).$$

Applying Proposition 2 to the continuous (*i*) \times (*ds*) function $\phi(\sigma, Y) = k_{\text{on}}(\sigma, S(Y)) - (k_{\text{off}} + k_{\text{cat}})(Y)$ defined on $[0, \infty) \times [0, Y_0]$, we obtain a (*ci0*) function $Y(\sigma)$. It is bounded and defined on $[0, \infty)$, because $\phi(\sigma, Y_0) < 0$, for all $\sigma \geq 0$, so that the corresponding curve cannot cross $Y = Y_0$. In fact, $Y(\sigma)$ is a (*cis0*) function, because ϕ is continuous (*is*) \times (*ds*) on $[0, \infty) \times [0, y]$, for any $0 < y < Y_0$.

Since $Y(\sigma)$ is a (*cis0*), so is $S^*(Y(\sigma))$. In addition, since $Y(\sigma) < Y_0$, it follows that $S(Y(\sigma))$ is a positive and (*cds*) function of $\sigma \in [0, \infty)$. □

Eliminating a Terminal Node in Theorem 2

We show that the cases $1 \Rightarrow 2$, $1 \Rightarrow 2$ and $1 \Rightarrow 2$ are equivalent to the case $1 \rightarrow 2$ by redefining the variables. The crucial step in expression of the variables of the

terminal node 2 with a single incoming link from node 1 (Sect. 2.3.3.2) is to prove that expression of variables S_2, S_2^* and the corresponding intermediate complex in favour of S_1 or S_1^* is unique and that, after elimination of node 2, the new $g_1'(S_1) = g_1(S_1) + \Delta g_1(S_1)$ or $g_1^{*'}(S_1^*) = g_1^*(S_1^*) + \Delta g_1^*(S_1^*)$ have the required properties in Eq. 52.

Case 1 \Rightarrow 2. The balance, substrate conservation and kinetic equations for node 2 take the form

$$\begin{aligned} k_{\text{cat},2}(S_1 S_2) &= q_2(S_2^*), \\ g_2(S_2) + g_2^*(S_2^*) + S_1 S_2 &= S_{2T}, \\ k_{\text{on},2}(S_1, S_2) &= (k_{\text{off},2} + k_{\text{cat},2})(S_1 S_2), \end{aligned}$$

which are the same as for the case 1 \rightarrow 2 (Sect. 2.3.3.2), with S_1^* and $S_1^* S_2$ replaced by S_1 and $S_1 S_2$, respectively. Thus, $S_2(S_1), S_2^*(S_1)$ and $S_1 S_2(S_1)$ are uniquely expressed and S_2 and S_2^* are positive for $S_1 > 0$. Furthermore, the term $\Delta g_1(S_1) = S_1 S_2(S_1)$ has the same properties as a function of S_1 as $\Delta g_1^*(S_1^*) = S_1^* S_2(S_1^*)$ has as a function of S_1^* for the case 1 \rightarrow 2 (Sect. 2.3.3.2).

Case 1 \Rightarrow 2. The balance, substrate conservation and kinetic equations for node 2 take the form

$$\begin{aligned} p_2(S_2) &= k_{\text{cat},2}^*(S_1^* S_2^*), \\ g_2(S_2) + g_2^*(S_2^*) + S_1^* S_2^* &= S_{2T}, \\ k_{\text{on},2}^*(S_1^*, S_2^*) &= (k_{\text{off},2}^* + k_{\text{cat},2}^*)(S_1^* S_2^*). \end{aligned}$$

Consider the following redefinition of variables and functions for node 2, which preserves the properties of the functions, $S_2 = \tilde{S}_2^*, S_2^* = \tilde{S}_2, S_1^* S_2^* = Y, p_2(S_2) = \tilde{q}_2(\tilde{S}_2^*), g_2(S_2) = \tilde{g}_2^*(\tilde{S}_2^*), g_2^*(S_2^*) = \tilde{g}_2(\tilde{S}_2), k_{\text{on},2}^*(S_1^*, S_2^*) = \tilde{k}_{\text{on},2}(S_1^*, \tilde{S}_2), k_{\text{off},2}^*(S_1^* S_2^*) = \tilde{k}_{\text{off},2}(Y)$ and $k_{\text{cat},2}^*(S_1^* S_2^*) = \tilde{k}_{\text{cat},2}(Y)$. The equations above now take the form

$$\begin{aligned} \tilde{k}_{\text{cat},2}(Y) &= \tilde{q}_2(\tilde{S}_2^*), \\ \tilde{g}_2(\tilde{S}_2) + \tilde{g}_2^*(\tilde{S}_2^*) + Y &= S_{2T}, \\ \tilde{k}_{\text{on},2}(S_1^*, \tilde{S}_2) &= (\tilde{k}_{\text{off},2} + \tilde{k}_{\text{cat},2})(Y), \end{aligned}$$

which are the same as for the case 1 \rightarrow 2 (Sect. 2.3.3.2). Thus $S_2 = \tilde{S}_2^*(S_1^*), S_2^* = \tilde{S}_2(S_1^*)$ and $S_1^* S_2^* = Y(S_1^*)$ are uniquely expressed and S_2 and S_2^* are positive for $S_1^* > 0$. Furthermore, the term $\Delta g_1^*(S_1^*) = Y(S_1^*)$ has the same properties as a function of S_1^* as $\Delta g_1^*(S_1^*) = S_1^* S_2^*(S_1^*)$ has as a function of S_1^* for the case 1 \rightarrow 2 (Sect. 2.3.3.2).

Case 1 \Rightarrow 2. The balance, substrate conservation and kinetic equations for node 2 take the form

$$\begin{aligned} p_2(S_2) &= k_{\text{cat},2}^*(S_1 S_2^*), \\ g_2(S_2) + g_2^*(S_2^*) + S_1 S_2^* &= S_{2T}, \\ k_{\text{on},2}^*(S_1, S_2^*) &= (k_{\text{off},2}^* + k_{\text{cat},2}^*)(S_1 S_2^*), \end{aligned}$$

which are the same as for the case $1 \rightarrow 2$ above, with S_1^* and $S_1^*S_2^*$ replaced by S_1 and $S_1S_2^*$, respectively. Thus, $S_2(S_1)$, $S_2^*(S_1)$ and $S_1S_2^*(S_1)$ are uniquely expressed and S_2 and S_2^* are positive for $S_1 > 0$. Furthermore, the term $\Delta g_1(S_1) = S_1S_2^*(S_1)$ has the same properties as a function of S_1 as $\Delta g_1^*(S_1^*) = S_1^*S_2^*(S_1^*)$ has as a function of S_1^* for the case $1 \rightarrow 2$.

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