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ORIGINAL ARTICLE

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New trends and perspectives in nonlinear intracellular dynamics: one century from Michaelis–Menten paper

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Abstract One century after the seminal work by Leonor Michaelis and Maud Menten devoted to the theoretical study of the enzymatic reactions, in this paper, we give an overview of the most recent trends concerning the mathematical modeling of several enzymatic mechanisms, focusing on its asymptotic analysis, which needs the use of advanced mathematical tools, such as center manifold theory, normal forms, and bifurcation theory. Moreover, we present some perspectives, linking the models here presented with similar models, arising from different research fields.

Keywords Michaelis–Menten kinetics · Quasi-steady-state approximations · Asymptotic expansions · Singular perturbations

Mathematics Subject Classification (2010) 92C40 · 92C45 · 41A99

1 Introduction

Mathematical modeling of chemical reactions is one of the bases of theoretical studies of actual biochemical research and has made important contributions to advances in biomedical and pharmaceutical researches.

The Michaelis–Menten–Briggs–Haldane approximation, or standard quasi-steady-state approximation (sQSSA) [21,62,73,109,151], represents a milestone in the mathematical modeling of enzymatic reactions.

The approximation, set forth by Henri in 1901 [69–71], systemized just one century ago by Michaelis and Menten in 1913 [73,109], and further developed by Briggs and Haldane [21], considers a reaction where a substrate S binds an enzyme E reversibly to form an unstable molecular complex C. The complex can then decay irreversibly to a product P and the enzyme, which is then free to bind another molecule of the substrate.

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The reaction can be described in terms of the so-called mass action principle, where the growth rates of each reactant are proportional to the instantaneous concentrations of the reactants themselves.

The hypothesis of quasi-steady state, which simplifies the above-described reaction, is crucial for the interpretation of the reaction and must be handled with much care. It is based on the assumption that the complex can be considered "substantially" constant, but this statement has led to many misinterpretations of the model. In fact, as shown by Heineken et al. [67], the exact mathematical interpretation of the quasi-steady-state assumption is that when we expand asymptotically the solutions of the ODEs governing the process with respect to an appropriate parameter, the sQSSA is the leading order approximation of the solution. As already observed by Briggs and Haldane from a chemical point of view, when the parameter of the expansion is sufficiently small, this approximation is valid. Heineken et al. used the parameter given by the ratio of the initial concentrations of enzyme E and substrate S, obtaining the well-known chemical requirement.

Mainly, in the fifties of the last century, several authors reconsidered the Michaelis–Menten kinetics, in order to understand the range of applicability of the QSSA and to determine other approximations and the related solutions. In particular, we want to cite [79,111,120,156,157].

In 1988 Segel [150] and in 1989 Segel and Slemrod [151] obtained the Michaelis–Menten approximation expanding the solutions in terms of a new parameter, including the so-called Michaelis constant and showing that the sQSSA is valid in a wider range of parameters than the one supposed before. However, it is well known that while in vitro the condition on the concentrations can be easily fulfilled, in vivo it is not always respected [1,51,152,153,155,159], in particular when the reaction is not isolated but is part of complex reaction networks. This means that, though very useful, this approximation not always can be applied, at least in several situations of medical and pharmaceutical interest.

Michaelis–Menten kinetics has recently become very popular thanks to the explosion of systems biology and in particular to the mathematical modeling of intracellular enzyme reactions, but in most literature any a priori analysis of the applicability of sQSSA is absent, even in very complex reaction networks. This fact has led to several problems concerning the study of particular phenomena, like oscillations [54,126], bistability [29], ultrasensitivity [127], or reverse engineering [125]. Following [79,111,120,143,156,157], recent papers [20,36,46,76,145,160–162] have introduced and explored a new approximation, called total quasi-steady-state approximation (tQSSA), which has been shown to be always roughly valid in the case of an isolated reaction.

The tQSSA has been applied to more complex reactions in a deterministic framework [7,29,76,78,124,126,127,162] and in a stochastic one [10,76,102,103], in order to better understand and interpret nonlinear phenomena like oscillations, multistability, and ultrasensitivity.

Nevertheless, since it is in any case an approximation, also the tQSSA can dramatically fail, as shown in [126], in more complex mechanisms, involving more than one reaction, but it is undoubtedly valid in a much wider range of parameters than the sQSSA [29,124–126,146].

One of the main problems of the mathematical treatment of the sQSSA is the misinterpretation of the hypothesis that the complex time concentration has zero derivative. Many papers and even monographs tend to indicate, probably for the sake of simplicity, the "substantial" equilibrium as a real equilibrium [64,81,130,169], which is obviously not true; in this case, any simplification can be definitely misleading. As observed in [67], p. 97, this use of the equations seems *scandalous* to any mathematicians and can bring to results that are absolutely inconsistent and false.

In this paper, we want to re-examine some mathematical aspects of Michaelis–Menten reaction and of the steady-state approximations, trying to clarify some aspects of the enzyme reactions. The paper is organized in the following way: in Sect. 2, we recall the most important notions and results on Michaelis–Menten kinetics and sQSSA; in Sect. 3, we discuss the biochemical and mathematical meaning of the tQSSA, comparing it with the sQSSA; in Sect. 4, we show some recent applications of tQSSA to more complex enzyme reactions; in Sect. 5, we quote some recent results on the study of oscillations and multistability in the double phosphorylation–dephosphorylation cycle and in the ubiquitous mitogen-activated protein kinase cascade (MAPK cascade), which is one of the most important mechanisms present in the great majority of the reaction networks in eukaryotic cells; in Sect. 6, we give a broad review of some open problems and perspectives for future developments.

2 The Henri–Michaelis–Menten–Briggs–Haldane kinetics: the standard quasi-steady-state approximation (sQSSA) and its mathematical meaning

One of the principal components of the mathematical approach to systems biology is the model of biochemical reactions set forth by Henri in 1901 [69–71] and Michaelis and Menten [109], and further developed by Briggs and Haldane [21]. This formulation considers a reaction where a substrate S binds an enzyme E reversibly to

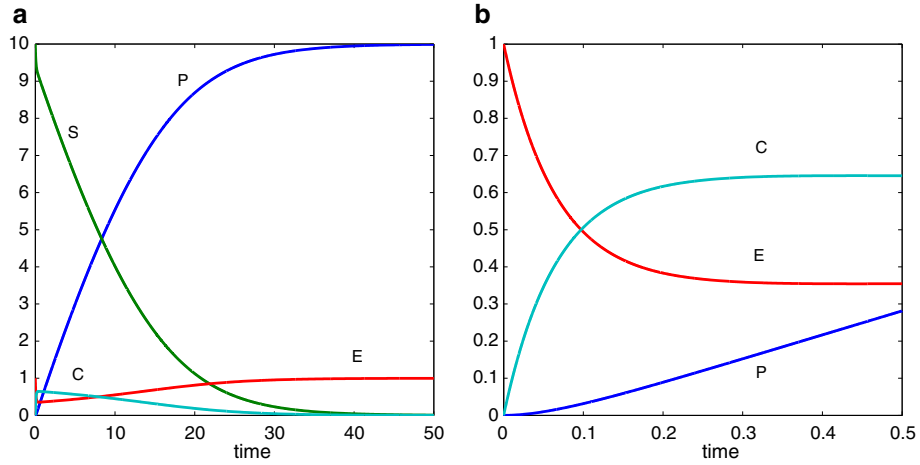

Fig. 1 **a** Time behavior of the reactants: S (*green*), C (*azure*), E (*red*), P (*blue*). **b** Zoom of the transient phase, where the initial convex behavior of P is clearly shown, together with the fast growth of C. Initial concentrations: $E(0) = E_T = 1,\ S(0) = S_T = 10,\ P(0) = 0,\ C(0) = 0$. Kinetic parameters: $a = k = 1,\ d = 4$ (color figure online)

form a complex C. The complex can then irreversibly decay to a product P and the enzyme, which is then free to bind another molecule of the substrate.

This process is summarized in the scheme

$$E + S \underset{d}{\overset{a}{\rightleftarrows}} C \xrightarrow{k} E + P, \tag{1}$$

where a, d and k are kinetic parameters (depending on temperature, but supposed constant during the reaction) associated with the reaction rates.

Each concentration is a dynamical variable.

The fundamental step is modeling all of the intermediate reactions, including binding, dissociation, and release of the product using mass action and conservation laws. This leads to an ODE for each involved complex and substrate concentration. We refer to this as the full system. For (1), the equations are

$$\frac{\mathrm{d}S}{\mathrm{d}t} = -a(E_T - C)S + dC, \tag{2}$$

$$\frac{\mathrm{d}C}{\mathrm{d}t} = a(E_T - C)S - (d + k)C. \tag{3}$$

with the initial conditions

$$S(0) = S_T, \quad C(0) = 0, \tag{4}$$

and the conservation laws

$$E + C = E_T, \quad S + C + P = S_T. \tag{5}$$

The initial conditions give the concentrations of S and C at the beginning of the reaction, and their time development is described by the ODEs, while E and P are linked to S and C through the conservation laws. Here, E_T is the total enzyme concentration assumed to be free at time $t = 0$. Also, the total substrate concentration, S_T, is free at $t = 0$. This is the so-called Michaelis–Menten (MM) kinetics [16,33,53,109,150]. Let us observe that the system (2), (3) admits an asymptotic solution for $t \to \infty$, obtained by setting the derivatives equal to zero. This solution is given by $C = S = 0$, so that from the conservation laws, $P = S_T$ and $E = E_T$. This means that all the substrate eventually becomes product due to the irreversibility, while the enzyme eventually is free and the complex concentration tends to zero, as expected by intuition.

The system (2), (3) does not admit any explicit solution; numerical integration of the system, together with (4), (5), leads to the time behavior of the reactants shown in Fig. 1, where the presence of two distinct phases is

evident: a transient one—much shorter than the second one—where the complex grows rapidly, and a second one, where all the reactant concentrations evolve in a much slower way.

In order to capture the qualitative behavior of the solutions, the most common technique is the so-called Henri–Michaelis–Menten–Briggs–Haldane, or simply Michaelis–Menten (MM) approximation, which assumes that the complex concentration is approximately constant after a short transient phase, during which the complex grows very rapidly, and on the other hand, the substrate does not vary significantly. This approximation is based on the *standard quasi-steady-state approximation* (standard QSSA, sQSSA) [16,21,74,109,112,118,146]. Since the pioneering papers by Bodenstein [19] and Chapman and Underhill [25], the quasi-steady-state approximation (QSSA) has represented a very important tool in the mathematical modeling of biochemical reactions. It brings to a simplification of the model and allows the qualitative analysis of the reaction, in terms of timescales separation, asymptotic behavior, etc., which any numerical analysis could not, in general, capture. The sQSSA assumptions lead to an ODE for the substrate, with initial condition $S(0) = S_T$, while the complex is assumed to be in a quasi-steady-state (i.e., $\frac{dC}{dt} \approx 0$ in (3)):

$$C(t) \approx -\frac{E_T \cdot S(t)}{K_M + S(t)} \tag{6}$$

$$\frac{dS}{dt} \approx -kC(t) \approx -\frac{V_{max} S(t)}{K_M + S(t)}, \quad S(0) = S_T, \tag{7}$$

where $K_M = \frac{d+k}{a}$ is the so-called *Michaelis constant* and $V_{max} = k\, E_T$.

The solution of problem (7) can be written in the form

$$t(S) = \left[S_T - S + K_M \log\left(\frac{S_T}{S}\right)\right] \cdot V_{max}. \tag{8}$$

Schnell and Mendoza [147] determined a closed form for the solution $S(t)$:

$$S(t) = K_M W\left(\frac{S_T}{K_M} \exp\left(\frac{-V_{max} t + S_T}{K_M}\right)\right), \tag{9}$$

where W is the *Lambert function* [32,59,147], which solves the equation

$$W(x)\exp(W(x)) = x. \tag{10}$$

Putting the initial value S_T in (6), we observe that C does not satisfy the initial condition $C(0) = 0$. This is because the sQSSA cannot follow the reaction from the very beginning, since it reproduces the second, slow, phase, just after C has rapidly reached its maximum value. Mathematically speaking, C shows a typical boundary layer phenomenon; thus, it is possible to interpret the sQSSA from a mathematical point of view, in terms of singular perturbations and asymptotic expansions [74,112,113,118].

Following [67,83,112], let us first non-dimensionalize the Eqs. (2), (3):

$$\begin{aligned} \frac{du}{d\tau} &= -u + (u + \kappa - \lambda)v \\ \epsilon \frac{dv}{d\tau} &= u - (u + \kappa)v \\ u(0) &= 1; \qquad v(0) = 0 \end{aligned} \tag{11}$$

where

$$\tau = a E_T t; \quad u(\tau) = \frac{s(t)}{S_T}; \quad v(\tau) = \frac{C(t)}{E_T}; \quad \lambda = \frac{k}{a S_T}; \quad \kappa = \frac{k+d}{a S_T} = \frac{K_M}{S_T}; \quad \epsilon = \frac{E_T}{S_T}. \tag{12}$$

Let us perform our analysis of (11) expanding the solutions in formal power series (asymptotic series) of the perturbation parameter ϵ, which must be supposed sufficiently small ($\epsilon \ll 1$) and taking into account that the presence of the term $\epsilon \frac{dv}{d\tau}$ predicts the existence of a boundary layer and the use of singular perturbation techniques.

Substituting

$$u(\tau;\epsilon)=\sum_{n=0}\epsilon^n u_n(\tau),\quad v(\tau;\epsilon)=\sum_{n=0}\epsilon^n v_n(\tau) \tag{13}$$

in (11), we have a sequence of ordinary differential equations in $u_n(\tau)$ and $v_n(\tau)$.

In particular, we find

$$O(1):\ \frac{\mathrm{d}u_0}{\mathrm{d}\tau}=-u_0+(u_0+\kappa-\lambda)v_0,\quad 0=u_0-(u_0+\kappa)v_0 \tag{14}$$

$$O(\epsilon):\ \frac{\mathrm{d}u_1}{\mathrm{d}\tau}=u_1(v_0-1)+(u_0+\kappa-\lambda)v_1,\quad \frac{\mathrm{d}v_0}{\mathrm{d}\tau}=u_1(1-v_0)-(u_0+\kappa)v_1. \tag{15}$$

It must be remarked that the zero-order equations reproduce the non-dimensionalized Eqs. (6) and (7), given by the sQSSA. This means that, mathematically speaking, the MM approximation can be viewed as the leading order of an asymptotic expansion with respect to a suitable perturbation parameter. Thus, the solutions can be determined following the same passages as for the solutions of (6) and (7).

However, Eq. (14) is a system of differential-algebraic equations (DAE), involving only one derivative; thus, we cannot expect that its solutions can satisfy both of the initial conditions $u_0(0)=1;\ v_0(0)=0$. Indeed, putting $u_0=1$ in (14), we obtain $v_0(0)=\frac{1}{1+\kappa}\neq 0$.

Thus, we solve the system ignoring the initial conditions, obtaining

$$u_0(\tau)+\kappa\ln u_0(\tau)=A-\lambda\tau,\quad v_0(\tau)=\frac{u_0(\tau)}{u_0(\tau)+\kappa}. \tag{16}$$

These must be interpreted as the *external solutions* of the problem, since they cannot adequately follow the analytic solutions for $\tau\to 0$.

In a neighborhood of $\tau=0$, we must define and use a more appropriate timescale, which can allow us to "expand" the short transient phase. Thus, we use $\sigma=\frac{\tau}{\epsilon}$.

With the transformations

$$\sigma=\frac{\tau}{\epsilon},\quad u(\tau;\epsilon)=U(\sigma;\epsilon),\quad v(\tau;\epsilon)=V(\sigma;\epsilon), \tag{17}$$

the problem becomes

$$\begin{aligned}&\frac{\mathrm{d}U_0}{\mathrm{d}\sigma}=0,\quad \frac{\mathrm{d}V_0}{\mathrm{d}\sigma}=U_0-(U_0+K)V_0,\\ &U_0(0)=1,\quad V_0(0)=0.\end{aligned} \tag{18}$$

Expanding U and V in asymptotic series in ϵ

$$U(\sigma;\epsilon)=\sum_{n=0}\epsilon^n U_n(\sigma),\quad V(\sigma;\epsilon)=\sum_{n=0}\epsilon^n V_n(\sigma), \tag{19}$$

and substituting in (18), we obtain

$$O(1):\ \frac{\mathrm{d}U_0}{\mathrm{d}\sigma}=0,\quad \frac{\mathrm{d}V_0}{\mathrm{d}\sigma}=U_0-(U_0+\kappa)V_0, \tag{20}$$

$$O(\epsilon):\ \frac{\mathrm{d}U_1}{\mathrm{d}\sigma}=-U_0+(V_0+\kappa-\lambda)V_0,\quad \frac{\mathrm{d}V_1}{\mathrm{d}\sigma}=(1-V_0)U_1-(V_0+\kappa)V_1, \tag{21}$$

and so on, with initial conditions given by

$$\begin{aligned}1=U(0;\epsilon)=\sum_{n=0}\epsilon^n U_n(0)\ &\Rightarrow\ U_0(0)=1,\quad U_{n\geq 1}(0)=0,\\ 0=V(0;\epsilon)=\sum_{n=0}\epsilon^n V_n(0)\ &\Rightarrow\ V_{n\geq 0}(0)=0.\end{aligned} \tag{22}$$

These can be considered the *internal solutions*, which "absorb" the initial conditions and must reproduce the initial fast transient phase, being valid only for the short timescale.

The solutions can be easily found:

$$U_0(\sigma) = 1, \quad V_0(\sigma) = \frac{1}{1+\kappa}\,(1 - \exp[-(1+\kappa)\sigma]). \tag{23}$$

The inner and the outer expansions must be both valid for intermediate times of the order $\epsilon \ll \tau \ll 1$ and must asymptotically agree in this regime, where $\sigma \to \infty$ and $\tau \to 0$ as $\epsilon \to 0$:

$$\lim_{\sigma\to\infty} [U_0(\sigma), V_0(\sigma)] = \left[1, \frac{1}{1+\kappa}\right] = \lim_{\tau\to 0} [u_0(\tau), v_0(\tau)] \quad \Rightarrow \quad A = 1. \tag{24}$$

The matching of the inner and outer solutions has brought to the determination of the unknown constants. Analogously, we can formally determine further terms u_i, v_i, U_i, V_i and perform their matching.

The procedure for obtaining a uniform approximation consists in adding the inner and the outer approximations and subtracting their common part:

$$\begin{aligned} u_0^{\text{unif}}(\tau) &= u_0(\tau) + U_0\left(\frac{\tau}{\epsilon}\right) - 1 = u_0(\tau); \\ v_0^{\text{unif}}(\tau) &= v_0(\tau) + V_0\left(\frac{\tau}{\epsilon}\right) - \frac{1}{\kappa+1} = \frac{u_0(\tau)}{u_0(\tau)+\kappa} - \frac{e^{\frac{-(\kappa+1)\tau}{\epsilon}}}{\kappa+1}. \end{aligned} \tag{25}$$

In [150,151], the authors use a different approach, based on the so-called reverse QSSA (rQSSA), obtained supposing that, after the transient phase, the substrate can reach a quasi-equilibrium $\left(\frac{\mathrm{d}s}{\mathrm{d}t} \approx 0\right)$ and expanding the solutions in asymptotic series in terms of a new perturbation parameter $\epsilon_{SS} = E_{\mathrm{T}}/(S_{\mathrm{T}} + K_{\mathrm{M}})$. They show that at the leading order the classical equations of the sQSSA are obtained again. This means that the Michaelis–Menten approximation is valid in a wider range of parameters and initial conditions, allowing E_{T} to be even greater than S_{T}, provided $\epsilon_{SS} = \frac{E_{\mathrm{T}}}{S_{\mathrm{T}}+K_{\mathrm{M}}} \ll 1$.

In these papers, the new parameter appears in a very natural way, linking the validity of any QSSA to the need of the separation between the two timescales characterizing, respectively, the transient phase (t_c) and the quasi-steady-state (t_{s}), as suggested in [83,143,149]. During the transient phase, the complex grows rapidly, while the substrate is supposed not to change substantially. Thus, an estimation of t_c can be determined by means of (23), where C has an exponential growth, whose timescale is

$$t_c \approx \frac{1}{a(S_{\mathrm{T}} + K_{\mathrm{M}})}. \tag{26}$$

In order to estimate t_s, characterizing the time interval in which $S(t)$ changes considerably after the fast transient, we consider the ratio between the maximum of S after the transient, i.e., S_{T}, and the maximal value of its rate of change, imposing that

$$t_{\mathrm{s}} \approx \frac{S_{\mathrm{T}}}{|\frac{\mathrm{d}S}{\mathrm{d}t}|_{\max}} \approx \frac{S_{\mathrm{T}} + K_{\mathrm{M}}}{kE_{\mathrm{T}}}. \tag{27}$$

Imposing that $t_c \ll t_{\mathrm{s}}$ brings to the condition

$$\frac{KE_{\mathrm{T}}}{(S_{\mathrm{T}} + K_{\mathrm{M}})^2} \ll 1, \tag{28}$$

which can be rewritten in the form

$$\frac{E_{\mathrm{T}}}{S_{\mathrm{T}} + K_{\mathrm{M}}} \ll \left(1 + \frac{K_D}{K}\right)\left(1 + \frac{S_{\mathrm{T}}}{K_{\mathrm{M}}}\right). \tag{29}$$

where $K_D = \frac{d}{a}$ is the so-called *dissociation constant*.

Equation (28) or, equivalently, Eq. (29) can be considered as a necessary condition for the validity of the sQSSA.

On the other hand, it is possible to determine a necessary condition for the validity of the assumption that the substrate concentration does not significantly change during the transient phase: this is the so-called reactant stationary approximation (RSA) [65,150], which yields

$$\left|\frac{\Delta S}{S_{\mathrm{T}}}\right| \approx \frac{1}{S_{\mathrm{T}}}\left|\frac{\mathrm{d}S}{\mathrm{d}t}\right|_{\max} \cdot t_c \approx \frac{E_{\mathrm{T}}}{S_{\mathrm{T}} + K_{\mathrm{M}}} = \epsilon_{SS} \ll 1. \tag{30}$$

Condition (30) is more restrictive than (29); thus, the RSA is a sufficient condition for the validity of the sQSSA.

Many authors have explored different features of the Michaelis–Menten kinetics and of the QSSA. We refer to [146] for a nice, general review of the kinetics and approximations of (1).

In particular, in [13,144], the case in which the initial concentration of the enzyme is much greater than S_T has been studied, bringing to QSSA equations similar in structure to (6), (7).

Moreover, in [14], being inspired by [117], the authors studied the asymptotic behavior of the reactant concentrations, by means of asymptotic expansions in terms of exponentials

$$S_{as}(t) = S_0 + S_1\, e^{-\alpha t} + S_2\, e^{-2\alpha t} + o\left(e^{-2\alpha t}\right) \tag{31}$$

$$C_{as}(t) = C_0 + C_1\, e^{-\alpha t} + C_2\, e^{-2\alpha t} + o\left(e^{-2\alpha t}\right) \tag{32}$$

obtaining

$$S_{as}(t) \approx S_1\, e^{-\alpha t} \tag{33}$$

$$C_{as}(t) \approx \frac{\alpha}{k-\alpha}\, S_1\, e^{-\alpha t} \tag{34}$$

where

$$\begin{aligned}\alpha &= \frac{k+d+aE_T - \sqrt{(k+d+aE_T)^2 - 4akE_T}}{2} \\ &= \frac{a}{2}\left[(K_M+E_T) - \sqrt{(K_M+E_T)^2 - \frac{4k}{a}E_T}\right] \\ &= \frac{a}{2}(K_M+E_T)\left[1 - \sqrt{1 - \frac{4kE_T}{a(K_M+E_T)^2}}\right]\end{aligned} \tag{35}$$

and S_1 is an unknown constant, which could be estimated from experimental data via a least-squares procedure.

Let us remark that (35) agrees with the results obtained in [117], in terms of the eigenfunction corresponding to the eigenvalue with smaller absolute value of the matrix associated with the linearization of (2) and (3).

In the paper, the authors solve an apparent incongruence remarked in several biochemistry monographs, determining the asymptotic value of the ratio $E(t)\,S(t)/C(t)$, showing that, for every choice of the kinetic parameters and of the initial conditions,

$$\frac{E_{as}\,S_{as}}{C_{as}}(t) \to \left(\frac{k-\alpha}{\alpha}\right) E_T =: K_W; \qquad (K_D < K_W < K_M) \tag{36}$$

differently from what is wrongly stated, for example, in [64,81,130,169]. Numerical simulations show the correctness of our conclusions.

The advantage of any quasi-steady-state approximation is that it reduces the dimensionality of the system, passing from two equations (*full system*) to one (*MM approximation or sQSSA*) and thus speeds up greatly numerical simulations, especially for large networks as found in vivo. Moreover, the kinetic constants in (1) are usually not known, or hard to determine [2,63,110,138,164,170], whereas finding the kinetic parameters K_M and V_{max} for the MM approximation is a standard in vitro procedure in biochemistry. See, e.g., [16] for a general introduction to this approach. Moreover, the approximation lowers the number of kinetic parameters from three to two, apparently simplifying the model, but in fact "hiding" the true meaning of the parameters, as observed in [14,126].

On the other hand, let us stress that the Michaelis–Menten approximation, which has shown to be valid whenever $E_T \ll S_T + K_M$ [150,151], is not always valid. For example, this condition is usually fulfilled for in vitro experiments, but often breaks down in vivo [1,51,152,153,155].

3 The total quasi-steady-state approximation (tQSSA) and its mathematical meaning

In 1955, Laidler [79,120] approached the Michaelis–Menten kinetics considering the equation for the product P instead of S, thus considering the problem

$$\begin{aligned}
&\frac{\mathrm{d}P}{\mathrm{d}t} = \mathrm{d}C,\\
&\frac{\mathrm{d}C}{\mathrm{d}t} = a(E_T - C)S - (d+k)C\\
&P(0) = 0, \quad C(0) = 0
\end{aligned} \tag{37}$$

with the conservation laws

$$E + C = E_\mathrm{T}, \quad S + C + P = S_\mathrm{T}. \tag{38}$$

then applying a quasi-steady-state approximation and determining sufficient conditions for the validity of the approximate solutions. The approach was followed also by Morales and Goldman [111] and Swoboda [156, 157]. The papers did not receive the due attention.

Only in 1996, Laidler's approach received new impulse, thanks to a paper by Borghans et al. [20], where a new approximation, called tQSSA, was introduced, considering the "lumped" variable $\overline{S}(t) = S(t) + C(t)$, already introduced by Swoboda in 1957 and in 1979 by Schauer and Heinrich [143], who determined approximated solutions for the transient and QSS phases. We will refer to [20], underlining that, thanks to the conservations laws, $P(t) = S_\mathrm{T} - \overline{S}(t)$, and the tQSSA can be viewed as the other side of the coin of Laidler's theory, though the approach followed in [20,143] implicitly contains much more information about the reliability of the approximation, as we will show in the following. In 2008, Khoo and Hegland [76] applied Tikhonov theorem [158] in order to study the tQSSA, obtaining similar results as in [20].

Let us consider again the classical Michaelis–Menten kinetics (1). Introducing the total substrate $\overline{S}(t) = S(t) + C(t)$, Eqs. (2–3) then become

$$\frac{\mathrm{d}\bar{S}}{\mathrm{d}t} = -kC \tag{39}$$

$$\frac{\mathrm{d}C}{\mathrm{d}t} = a\left[C^2 - (E_\mathrm{T} + \bar{S} + K_\mathrm{M})C + E_\mathrm{T}\bar{S}\right]. \tag{40}$$

with initial conditions

$$\overline{S}(0) = S_\mathrm{T}, \quad C(0) = 0, \tag{41}$$

and conservation laws

$$E + C = E_\mathrm{T}, \quad \overline{S} + P = S_\mathrm{T}. \tag{42}$$

Assuming that the complex is in a quasi-steady-state $\left(\frac{\mathrm{d}C}{\mathrm{d}t} \approx 0\right)$ yields the *total QSSA* (tQSSA) [20,79,143, 160], which is valid for a broader range of parameters covering both high and low enzyme concentrations:

$$\frac{\mathrm{d}\bar{S}}{\mathrm{d}t} \approx -k\, C_-(\bar{S}), \quad \bar{S}(0) = S_\mathrm{T}, \tag{43}$$

where

$$C_-(\bar{S}) = \frac{(E_\mathrm{T} + K_\mathrm{M} + \bar{S}) - \sqrt{(E_\mathrm{T} + K_\mathrm{M} + \bar{S})^2 - 4E_\mathrm{T}\bar{S}}}{2} \tag{44}$$

is the only biologically allowed solution of $\frac{\mathrm{d}C}{\mathrm{d}t} = 0$ in (40).

Tzafriri [160] showed that the tQSSA is valid whenever

$$\epsilon_{Tz} := \frac{K}{2S_\mathrm{T}}\left(\frac{E_\mathrm{T} + K_\mathrm{M} + S_\mathrm{T}}{\sqrt{(E_\mathrm{T} + K_\mathrm{M} + S_\mathrm{T})^2 - 4E_\mathrm{T}S_\mathrm{T}}} - 1\right) \ll 1, \tag{45}$$

where $K = \frac{k}{a}$, and that this is always roughly valid in the sense that

$$\epsilon_{Tz} \leq \frac{K}{4K_\mathrm{M}} \leq \frac{1}{4}. \tag{46}$$

The parameter K is the usual Van Slyke–Cullen constant.

As a first-order approximation to (43), Tzafriri [160] found the expression, obtained originally in [20] by different techniques,

$$\frac{\mathrm{d}\overline{S}}{\mathrm{d}t} \approx -\frac{V_{\max}\overline{S}}{K_{\mathrm{M}} + E_{\mathrm{T}} + \overline{S}}, \quad \overline{S}(0) = S_{\mathrm{T}}. \tag{47}$$

This approximation is valid at low enzyme concentrations $E_{\mathrm{T}} \ll S_{\mathrm{T}} + K_{\mathrm{M}}$, where it reduces to the MM expression (7), but holds moreover at low substrate concentrations $S_{\mathrm{T}} \ll E_{\mathrm{T}} + K_{\mathrm{M}}$ [160]. Thus, with minimal effort performing the substitutions of S by $\overline{S}$ and of K_{M} by $K_{\mathrm{M}} + E_{\mathrm{T}}$, one obtains a significantly improved MM-like approximation, with very simple algebra.

Tzafriri and Edelman [162] determined a closed form for the solution $\overline{S}(t)$ of (47), in terms of the Lambert function.

In the above quoted papers [20,79,111,120,143,156,157,160,162], the tQSSA has been approached requiring some conditions that simplify the equations, without any formal tool in terms of asymptotic expansions. In 2002, Schnell and Maini [145] studied the tQSSA by means of aggregation or lumping techniques, which reduce the number of differential equations describing the system. They non-dimensionalized the system of differential equations governing the reaction and introduced the perturbation parameter $\epsilon = \frac{K E_{\mathrm{T}}}{(K_{\mathrm{M}}+E_{\mathrm{T}}+S_{\mathrm{T}})^2}$. They consider a more general form of the total substrate $\overline{S}$ introduced by Swoboda. However, the leading order term of their expansion unexpectedly reproduces the sQSSA, instead of the tQSSA. In 2008, Dingee and Anton [46] developed a two-parameter singular perturbation analysis, which curiously, at the leading order, does not reproduce the approximated solutions given in [20,79,120,143], but the zero-order approximation of the tQSSA was obtained by Tzafriri [160] with respect to the parameter

$$\epsilon_{Tz} = \frac{K}{2S_{\mathrm{T}}}\left(\frac{E_{\mathrm{T}} + K_{\mathrm{M}} + S_{\mathrm{T}}}{\sqrt{(E_{\mathrm{T}} + K_{\mathrm{M}} + S_{\mathrm{T}})^2 - 4E_{\mathrm{T}}\,S_{\mathrm{T}}}} - 1\right),$$

which is valid in a more restricted range of parameters.

In [36], we find, as far as we know for the first time, the tQSSA as the leading order of an asymptotic expansion, obtained with respect to the "total" parameter $\epsilon = \frac{K E_{\mathrm{T}}}{(K_{\mathrm{M}}+E_{\mathrm{T}}+S_{\mathrm{T}})^2}$, the first-order corrections of the inner and the outer solutions (reproducing, respectively, the transient and the QSSA phase) and finally the uniform approximations.

Due to the first-order corrections, the parameter range of validity of our results improves that one obtained in [20].

Adopting the change of variables

$$\overline{S} = \alpha\,\bar{s}, \quad C = \beta\,c, \quad t = \gamma\,\tau, \tag{48}$$

we find that equations (39) become

$$\begin{aligned} \frac{\alpha}{\gamma}\frac{\mathrm{d}\bar{s}}{\mathrm{d}\tau} &= -k\beta\,c \\ \frac{\beta}{\gamma}\frac{\mathrm{d}c}{\mathrm{d}\tau} &= a\left[\beta^2 c^2 - (E_{\mathrm{T}} + K_{\mathrm{M}} + \alpha\,\bar{s})\,\beta\,c + E_{\mathrm{T}}\,\alpha\bar{s}\right] \end{aligned} \tag{49}$$

To ensure that all the terms on the right-hand side of (49) are of the same magnitude, we suppose that both c and $\bar{s}$ are $O(1)$. Proceeding as in [151] and [20], neglecting the term in c^2 and then setting for scaling purposes $\bar{s} = 1$ and $c = 1$, we find

$$\alpha = S_{\mathrm{T}}, \quad \beta = \frac{E_{\mathrm{T}}S_{\mathrm{T}}}{E_{\mathrm{T}} + K_{\mathrm{M}} + S_{\mathrm{T}}}, \quad \gamma_{\mathrm{IN}} = \frac{\beta}{a\,E_{\mathrm{T}}S_{\mathrm{T}}} = \frac{1}{a(E_{\mathrm{T}} + K_{\mathrm{M}} + S_{\mathrm{T}})}. \tag{50}$$

The parameter γ_{IN} corresponds to the timescale t_c of the complex formation [20,46,76].

Substituting in (49) the values obtained for α, β, and γ_{IN}, we have the inner equations:

$$\begin{aligned} \frac{\mathrm{d}\bar{s}}{\mathrm{d}\tau} &= -\epsilon\,c \\ \frac{\mathrm{d}c}{\mathrm{d}\tau} &= \sigma\,\eta c^2 - (\eta + \kappa_{\mathrm{M}})\,c - \sigma\,\bar{s}\,c + \bar{s} \end{aligned} \tag{51}$$

with initial conditions $\bar{s}(0) = 1$ and $c(0) = 0$, where

$$\sigma = \frac{S_T}{K_M + E_T + S_T}, \quad \eta = \frac{E_T}{K_M + E_T + S_T}, \quad \kappa_M = \frac{K_M}{K_M + E_T + S_T} \tag{52}$$

such that $\sigma + \kappa_M + \eta = 1$ and

$$\epsilon = \frac{K\, E_T}{(K_M + E_T + S_T)^2} \tag{53}$$

where $K = \frac{k}{a}$ is, as usual, the Van Slyke–Cullen constant.

The parameter ϵ, appearing in the right-hand side of the first equation (51), arises as the natural perturbation parameter of our asymptotic expansion.

Let us remark that, with our scaling argumentation, we obtain the same perturbation parameter as in [46, 76, 145]. Moreover, for any set of kinetic parameters and initial conditions, $\epsilon \leq \frac{1}{4}$ [46].

Expanding the solutions of (51) in the form

$$\bar{s} = \Sigma_0 + \epsilon\, \Sigma_1 + o(\epsilon), \quad c = \Gamma_0 + \epsilon\, \Gamma_1 + o(\epsilon), \tag{54}$$

substituting in (51) and taking into account the initial conditions, we find at order 0 that $\Sigma_0 = \text{const} = 1$ and

$$\frac{d\Gamma_0}{d\tau} = \sigma\, \eta\, \Gamma_0^2 - \Gamma_0 + 1 \tag{55}$$

whose solution, complying with (41), is easily found as

$$\Gamma_0(\tau) = \frac{\exp(\sqrt{1 - 4\sigma\, \eta}\, \tau) - 1}{\sigma\, \eta \left[\Gamma_0^+ \exp(\sqrt{1 - 4\sigma\, \eta}\, \tau) - \Gamma_0^-\right]} \tag{56}$$

where $\Gamma_0^{\pm} = \frac{1 \pm \sqrt{1 - 4\,\sigma\, \eta}}{2\,\sigma\, \eta}$.

Let us observe that in the classical treatment of the transient phase, in order to simplify equations, one of the most commonly accepted assumptions is that mostly when $S_T \gg E_T$, S can be considered constant, i.e., $\frac{dS}{dt} \approx 0$. Actually, taking into account the initial conditions, we have $\frac{dS}{dt}(0) = -k_1 E_T S_T$, which is clearly in contrast to the previous assumption, especially when $E_T\, S_T$ is high. On the contrary, using the total substrate, from (39) $\left(\frac{d\bar{s}}{dt}(0) = 0\right)$, the assumption $\frac{dS}{dt} \approx 0$ is much more realistic, since it mathematically reproduces the fact that, in the transient phase, the sum of S and C can be considered constant, because the product P has not been substantially created yet. The leading order approximation $\Sigma_0 = \text{const.} = 1$ is thus consistent with this assumption.

At order 1, we have

$$\frac{d\Sigma_1}{d\tau} = -\Gamma_0 \tag{57}$$

$$\frac{d\Gamma_1}{d\tau} = \Gamma_1\, (2\sigma\, \eta\, \Gamma_0 - 1) - \sigma\, \Sigma_1\, \Gamma_0 + \Sigma_1 \tag{58}$$

with homogeneous initial conditions, which give

$$\Sigma_1 = \frac{1}{\sigma\, \eta} \log\left(\frac{\Gamma_0^+ \exp(\sqrt{1 - 4\sigma\, \eta}\, \tau) - \Gamma_0^-}{\Gamma_0^+ - \Gamma_0^-}\right) - \Gamma_0^+\, \tau \tag{59}$$

and the corresponding Γ_1.

In the slow, quasi-steady-state phase, the total variable $\bar{s}$ cannot anymore be considered roughly constant: it decreases monotonically from S_T to zero. Hence, to balance the left-hand side with the right one, we set

$$\gamma_{OUT} = \frac{\alpha}{k\, \beta} = \frac{E_T + K_M + S_T}{V_{max}} \tag{60}$$

where $V_{\max} = k\, E_{\mathrm{T}}$ is the maximal reaction velocity. In this case, γ_{OUT} represents the timescale $t_{\bar{\mathrm{s}}}$, related to the total substrate depletion [20,46,76]. Note that, having denoted by t_c the timescale in the fast, pre-steady phase and by $t_{\bar{\mathrm{s}}}$ the timescale in the slow, quasi-steady phase, we get

$$\frac{t_c}{t_{\bar{\mathrm{s}}}} = \epsilon. \tag{61}$$

Thus, ϵ represents a measure of the separation between the two timescales.
Setting $T = \gamma_{\mathrm{OUT}} t$ and substituting (60) in (49), we obtain

$$\begin{aligned} \frac{\mathrm{d}\bar{s}}{\mathrm{d}T} &= -c \\ \epsilon \frac{\mathrm{d}c}{\mathrm{d}T} &= \sigma\, \eta c^2 - (\eta + \kappa_{\mathrm{M}})\, c - \sigma\, \bar{s}\, c + \bar{s} \end{aligned} \tag{62}$$

Since ϵ multiplies the highest derivative, we expect a boundary layer effect in the time evolution of c.

Expanding the solutions of (62) in the form

$$\bar{s} = \bar{s}_0 + \epsilon\, \bar{s}_1 + o(\epsilon), \quad c = c_0 + \epsilon\, c_1 + o(\epsilon), \tag{63}$$

upon substitution in (62), we find, at leading order,

$$\begin{aligned} \frac{\mathrm{d}\bar{s}_0}{\mathrm{d}T} &= -c_0 \\ \sigma\, \eta c_0^2 - (\eta + \kappa_{\mathrm{M}} + \sigma\, \bar{s}_0)\, c_0 + \bar{s}_0 &= 0 \end{aligned} \tag{64}$$

which corresponds to the equations obtained in the tQSSA [20,76,79,111,120,143,156,157].

The second equation above is algebraic in c_0 with solutions

$$c_0^{\pm} = \frac{\eta + \kappa_{\mathrm{M}} + \sigma\, \bar{s}_0 \pm \sqrt{(\eta + \kappa_{\mathrm{M}} + \sigma\, \bar{s}_0)^2 - 4\sigma\, \eta \bar{s}_0}}{2\,\sigma\,\eta} \tag{65}$$

and it is easy to see that only c_0^- is admissible.

Because of (65), the first equation in (64) becomes

$$\frac{\mathrm{d}\bar{s}_0}{\mathrm{d}T} = -c_0^-. \tag{66}$$

with the initial condition given by the matching condition $\bar{s}_0(0) = \lim_{\tau\to\infty} \Sigma_0(\tau) = 1$ for the leading order terms in the inner and outer expansions of $\bar{s}$; thus, we automatically have that $c_0(0) = c_0^-(0) = \Gamma_0^-$.

From (62), it is found that the first correction terms in the outer solutions are given by

$$\frac{\mathrm{d}\bar{s}_1}{\mathrm{d}T} = -c_1 \tag{67}$$

$$c_1 = \frac{c_0' + \bar{s}_1\,(\sigma\, c_0 - 1)}{2\,\eta\,\sigma\, c_0 - \sigma\, \bar{s}_0 - \eta - \kappa_{\mathrm{M}}}. \tag{68}$$

Note that, since for $\tau \to \infty$, we get

$$\Sigma_1(\tau) \approx \frac{1}{\sigma\,\eta} \log \frac{\Gamma_0^+}{\Gamma_0^+ - \Gamma_0^-} - \Gamma_0^-\, \tau, \tag{69}$$

then the matching condition for the first-order corrections of $\bar{s}$ gives the appropriate initial condition to solve numerically the first equation in (67), i.e.,

$$\bar{s}_1(0) = \frac{1}{\sigma\,\eta} \log \frac{\Gamma_0^+}{\Gamma_0^+ - \Gamma_0^-}. \tag{70}$$

It only remains to find the matching condition for the first-order correction of the complex concentration c.

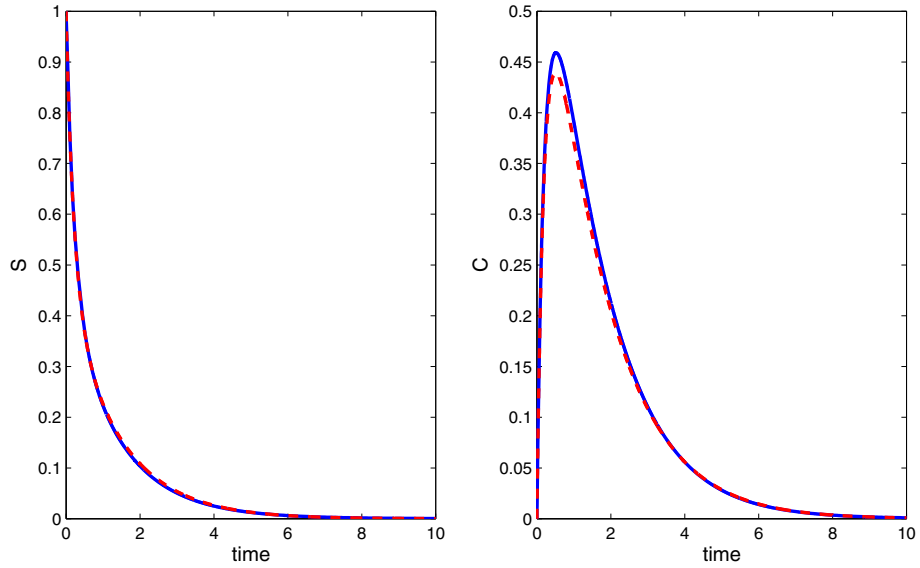

Fig. 2 Dynamics of S (*left panel*) and of C (*right panel*): full system (*solid*), uniform approximation (*dashed*). Initial concentrations: $E_T = 3,\ S_T = 1$. Kinetic parameters: $a = 1, d = 1, k = 1$. Taken from [36]

To this aim, we observe that Γ_1 behaves asymptotically as a straight line of the form $y = m\tau + q$, with

$$m = \frac{\Gamma_0^- (\sigma\, \Gamma_0^- - 1)}{\sqrt{1 - 4\sigma\eta}} \tag{71}$$

$$q = \frac{1}{\eta\sqrt{1 - 4\sigma\eta}} \left[\frac{\Gamma_0^-}{2} \left(1 - \frac{1 - 2\eta}{\sqrt{1 - 4\sigma\eta}} \right) + \frac{1 - \sigma\Gamma_0^-}{\sigma} \log\left(\frac{\Gamma_0^+}{\Gamma_0^+ - \Gamma_0^-} \right) \right] \tag{72}$$

In conclusion, we can write the (non-dimensional) uniform expansions as

$$c^{\mathrm{un}}(\tau) = c_0^{\mathrm{un}}(\epsilon\tau) + \epsilon c_1^{\mathrm{un}}(\tau); \qquad \bar{s}^{\mathrm{un}}(\tau) = \bar{s}_0^{\mathrm{un}}(\epsilon\tau) + \epsilon \bar{s}_1^{\mathrm{un}}(\tau) \tag{73}$$

where following [83], we obtain the uniform approximations adding the inner and outer solutions and subtracting their common part, i.e.,

$$\begin{aligned} c_0^{\mathrm{un}}(\tau) &= c_0(\epsilon\tau) + \Gamma_0(\tau) - \Gamma_0^- \\ \bar{s}_0^{\mathrm{un}}(\tau) &= \bar{s}_0(\epsilon\tau) + \Sigma_0(\tau) - 1 = \Sigma_0(\tau) \\ c_1^{\mathrm{un}}(\tau) &= c_1(\epsilon\tau) + \Gamma_1(\tau) - m\tau - q \\ \bar{s}_1^{\mathrm{un}}(\tau) &= \bar{s}_1(\epsilon\tau) + \Sigma_1(\tau) - \frac{1}{\sigma\eta} \log \frac{\Gamma_0^+}{\Gamma_0^+ - \Gamma_0^-} + \Gamma_0^-\, \tau \end{aligned} \tag{74}$$

where m and q are given by (71) and (72). In Fig. 2, we compare the approximation (74) with the numerical solution of the full system (2), (3), showing a good agreement, also in the matching layer.

The considerations made in the present section show that the tQSSA not only is much more efficient than the sQSSA, since it is valid in a much wider set of parameters and initial conditions, but also is a much more natural approximation. This fact can be confirmed considering the studies by Palsson and collaborators, where the authors are able to determine a sufficient condition for the validity of any QSSA in terms of the trace and the determinant of the Jacobian matrix J of the system (2), (3). Indeed, in [121,122], the authors state that a sufficient condition is given by $\frac{\det(J)}{\mathrm{tr}^2(J)} \ll 1$, where $\frac{\det(J)}{\mathrm{tr}^2(J)}$ corresponds to the perturbation parameter ϵ given in (53), which then arises as the natural parameter to be used in any efficient quasi-steady-state approximation.

4 Applications of the total quasi-steady-state approximation (tQSSA)

The tQSSA has been recently applied to more complex mechanisms, like the completely reversible enzyme kinetics [161], the antagonist toggle switch [141], the completely competitive inhibition [124,139,148], which consists of two reactions catalyzed by the same enzyme, i.e., a system with competing substrates, according to the scheme

$$\begin{aligned} S_1 + E &\underset{d_1}{\overset{a_1}{\rightleftarrows}} C_1 \xrightarrow{k_1} E + P_1, \\ S_2 + E &\underset{d_2}{\overset{a_2}{\rightleftarrows}} C_2 \xrightarrow{k_2} E + P_2, \end{aligned} \tag{75}$$

the double phosphorylation [124], the Goldbeter–Koshland switch, which models the single phosphorylation–dephosphorylation cycle [10,29,125,127], the double phosphorylation–dephosphorylation cycle, and the ubiquitous mitogen-activated protein kinase cascade (MAPK cascade), which is one of the most important mechanisms present in the great majority of the reaction networks in eukaryotic cells [15,28,37,38,103,126].

In all the cases studied, the tQSSA has shown to be much more efficient than the sQSSA, though, representing in any case an approximation, it can fail when the system undergoes oscillations, because in this case we cannot expect that the system can reach any equilibrium or quasi-equilibrium state.

Most of current literature uses the sQSSA also to describe the networks of enzyme reactions involved in the intracellular signal transduction.

However, in vivo, the reactions can be coupled in complex networks or cascades of enzymes, second messengers with successive reactions, competition between substrates, feedback loops, etc. In some cases, approximations of such scenarios have been carried out within the MM scheme, not only without any examination of its applicability, but also neglecting the complexes involved in the reactions (see for example [27,66,108]). Other authors [119] make use of conservation laws that account for the presence of the complexes. Nevertheless, the asymptotic values of the reactants do not yet correspond to the values obtained integrating numerically the full systems. In order to explain this apparent incoherence, we must underline that the sQSSA, as every QSSA, represents the system dynamics after a (in general short) transient phase, during which the substrates are partially bound and the complexes begin to form. The application of the sQSSA corresponds to ignore the initial, rapid transient phase, where complexes begin to be created, and the total amount of free (inactive) substrates is no more equal to S_T. Consequently, since the QSSA is applied considering the complexes (in which the enzymes are sequestered) substantially constant, the total concentration of free and active substrate(s) will be considered constant, but its value, due to the presence of complexes, cannot coincide with the initial substrate value, when all the complex concentrations were equal to zero. Setting S_T as initial value for the total amount of (inactive and active) substrate concentrations in the sQSSA naturally brings to wrong conclusions, since the system is forced to fulfill a conservation law that implicitly neglects all the complex concentrations. This means that even when the sQSSA holds for every non-competitive reaction in (75), the neglect of the complexes and of the competition leads to wrong estimations of the reactants time behavior.

On the other hand, the tQSSA does not have this drawback, because the substrates and the complexes formed by them are included in the same (total) variables and the system cannot distinguish between free substrates and bound ones. For this reason, differently from the sQSSA, the tQSSA saves the conservation laws and produces the same asymptotic values as the full system.

In particular, in the presence of a reaction cycle, it is natural to expect that the complexes are continuously depleted and created and that in a stationary state their concentrations cannot tend to zero.

This fact was already observed in [57] in the case of the phosphorylation–dephosphorylation cycle. Nevertheless, most of the current literature, when applying the sQSSA to complex schemes, imposes implicitly the depletion of all the complexes, seriously affecting the conservation laws and consequently the asymptotic values of the reactant concentrations.

The most dramatic consequence is that, when applied to well-known mechanism, like, e.g., the (double) phosphorylation–dephosphorylation cycle, or the MAPK cascade, the sQSSA predicts phenomena that do not appear when the mechanisms are studied by means of the full system of equations describing the systems or by means of the tQSSA.

A case where it is important to consider the contribution from intermediate complexes is the ubiquitous mechanism of covalent modification, or Goldbeter–Koshland switch [57], such as the cycle of phosphorylation and subsequent dephosphorylation of an enzyme. This reaction is very important in every intracellular pathway,

because the process of phosphorylation and dephosphorylation is one of the most relevant to activate and inactivate enzymes, by either the addition or the removal of a phosphate group, performed by two types of enzymes, called, respectively, kinases and phosphatases. The mechanism provides the building blocks of the MAPK cascade, one of the most relevant intracellular signal transduction pathways modeled since 1996 [72] and consists of a substrate S, which can be modified, for example, by phosphorylation, to the form P. Vice versa, P can be transformed, e.g., by dephosphorylation, back to S. The scenario investigated in the ground-breaking work [57] assumes that the enzymes follow the Michaelis–Menten reaction mechanism, given by

$$\begin{aligned} S + E_1 &\underset{d_1}{\overset{a_1}{\rightleftarrows}} C_1 \xrightarrow{k_1} E_1 + P, \\ P + E_2 &\underset{d_2}{\overset{a_2}{\rightleftarrows}} C_2 \xrightarrow{k_2} E_2 + S. \end{aligned} \tag{76}$$

In [10,125], the tQSSA was applied to the Goldbeter–Koshland switch. The reaction is governed by the coupled ODEs

$$\begin{aligned} \frac{\mathrm{d}S}{\mathrm{d}t} &= -a_1 E_1 \cdot S + d_1 C_1 + k_2 C_2, \qquad S(0) = S_T, \\ \frac{\mathrm{d}C_1}{\mathrm{d}t} &= a_1 E_1 \cdot S - (d_1 + k_1) C_1, \\ \frac{\mathrm{d}C_2}{\mathrm{d}t} &= a_2 E_2 \cdot P - (d_2 + k_2) C_2, \qquad C_i(0) = 0 \end{aligned} \tag{77}$$

and the conservation laws

$$S_T = S + C_1 + C_2 + P, \qquad E_{i,T} = E_i + C_i, \quad i = 1, 2. \tag{78}$$

Introducing the total substrates $\overline{S} = S + C_1$, $\overline{P} = P + C_2$, we rewrite equations (77) in the following way:

$$\begin{aligned} \frac{\mathrm{d}\overline{S}}{\mathrm{d}t} &= k_2 C_2 - k_1 C_1 = -\frac{\mathrm{d}\overline{P}}{\mathrm{d}t}, \qquad \overline{S}(0) = S_\mathrm{T} \\ \frac{\mathrm{d}C_1}{\mathrm{d}t} &= a_1 (E_{1,T} - C_1) \cdot (\overline{S} - C_1) - (d_1 + k_1) C_1, \\ \frac{\mathrm{d}C_2}{\mathrm{d}t} &= a_2 (S_\mathrm{T} - \overline{S} - C_2) \cdot (E_{2,T} - C_2) - (d_2 + k_2) C_2, \qquad C_i(0) = 0. \end{aligned} \tag{79}$$

Assuming the tQSSA $\frac{\mathrm{d}C_i}{\mathrm{d}t} \approx 0$ and considering only the biologically significant roots for C_i, we arrive at the following equation

$$\frac{\mathrm{d}\overline{S}}{\mathrm{d}t} \approx k_2 C_2^- - k_1 C_1^- \qquad \overline{S}(0) = S_\mathrm{T} \tag{80}$$

where

$$C_1^- = \frac{\left(\overline{S} + E_{1,T} + K_1\right) - \sqrt{(\overline{S} + E_{1,T} + K_1)^2 - 4\overline{S} E_{1,T}}}{2}, \tag{81}$$

$$C_2^- = \frac{(S_\mathrm{T} - \overline{S} + E_{2,T} + K_2) - \sqrt{\left(S_\mathrm{T} - \overline{S} + E_{2,T} + K_2\right)^2 - 4\left(S_\mathrm{T} - \overline{S}\right) E_{2,T}}}{2} \tag{82}$$

and $K_i = \frac{d_i + k_i}{a_i}$.

Differently from the case of a single phosphorylation reaction, in this situation, the quasi-steady state does not imply that the complexes tend to be negligible.

Thus, the supposed depletion of the complexes in the sQSSA brings to uncorrect asymptotic values of the inactive and active substrates in the Goldbeter–Koshland cycle, as predicted in [57]. On the other hand, the tQSSA reproduces in a very satisfactorily way not only the asymptotic values but also the dynamics of the reactants.

In [29,127], it is shown that the tQSSA reproduces zero-order ultrasensitivity in the Goldbeter–Koshland cycle, according to what was predicted in [57], while the sQSSA, for a wide range of parameter values, is not able to yield ultrasensitivity whenever it is expected by the theory.

Indeed, introducing total substrates, i.e., the sums of substrates and intermediate complexes, provides a reduction in the number of variables to consider but without neglecting the contribution from intermediate complexes, and the resulting tQSSA is known to be valid at almost all combinations of kinetic parameters, enzyme, and substrate concentrations. To illustrate the usefulness of such simplifications, in [127], we show how introducing the total substrates allows a simple analytical treatment of the relevance of significant enzyme concentrations for pseudo-first-order kinetics and reconciles two proposed criteria for the validity of the pseudo-first-order approximation. In addition, we show how the loss of zero-order ultrasensitivity in covalent modification cycles can be analyzed, in particular how approaches such as metabolic control analysis are immediately applicable to scenarios with intermediate or high enzyme concentrations described by the total substrates. A simple criterion, which excludes the possibility of zero-order ultrasensitivity, was presented in that paper.

The double phosphorylation process, as well as double dephosphorylation, was recently modeled in the context of the so-called mitogen-activated protein kinase cascade (MAPK cascade) (see, for example, [72,75, 108]).

Several authors have modeled this reaction. In [108], the authors describe the dephosphorylation reaction as a two-step mechanism. In [142], both phosphorylation and dephosphorylation are assumed to be two-step reactions. In [119], it is supposed that both phosphorylation and dephosphorylation happen in only one step, according to the scheme

$$\begin{aligned} S + E &\underset{d_1}{\overset{a_1}{\rightleftarrows}} C_1 \xrightarrow{k_1} E + S_{\mathrm{p}}, \\ S_{\mathrm{p}} + E &\underset{d_2}{\overset{a_2}{\rightleftarrows}} C_2 \xrightarrow{k_2} E + S_{\mathrm{pp}}, \end{aligned} \tag{83}$$

where the activating enzyme E is a kinase, which phosphorylates the substrate S. The phosphorylated molecule S_{p} can bind the same enzymes, producing the double-phosphorylated form S_{pp}. The reaction scheme can be seen as a special case of competitive system, because S and S_{p} compete for the same enzyme, E (though differently from the fully competitive reaction, it is usually assumed that at the beginning only S is present and, mainly, the concentration of the "inhibitor" S_{p} depends on the concentration of the substrate S).

Consequently, in [124], the tQSSA for competitive reactions was applied to this case, too, though it should be remarked that the theoretical investigation of the validity of the tQSSA does not work in the case of successive reactions.

Nevertheless, it seems like the conclusions concerning the validity of the tQSSA from [124] carry over to this scenario.

5 Steady-state approximations in reaction cascades: multistability and oscillations

In [141], it is shown that, in several mechanisms (like, e.g., the antagonistic toggle switch), the sQSSA can yield bistability even when the system, described by the full system of equations, is not bistable.

In [54] and [126], it is shown that, when the system undergoes oscillations, any QSSA, as in [56], risks to fail, because the central hypothesis for the quasi-steady-state assumption is a substantial equilibrium for the complexes, which cannot be guaranteed in the presence of the substrate oscillations. Flach and Schnell tested their considerations on the van Slyke–Cullen mechanism [163], while Pedersen et al. studied the MAPK cascade with feedback, as in [75].

In the MAPK pathway, the upstream kinase (denoted MKKK, i.e., MAP kinase kinase kinase; for example Raf), when activated, phosphorylates the immediately downstream target, which is also a kinase (MAPKK, i.e., MAP kinase kinase, for example MEK) successively on two specific sites, eventually activating it. This last double-phosphorylated kinase (MAPKK-PP) acts on the MAPK (for example ERK) through specific phosphorylation events on two distinct sites. The activated MAPK is then responsible for further downstream signalling.

The activated cascade is shut down by the reverse action of specific phosphatases, whose outcome is the time modulation of the signal, probably through the regulation of the active kinase (for example, transient

versus sustained activation). Moreover, the phosphatase controls the steady-state level of activated MAPK, which, in turn, controls downstream processes.

The novelty of this scheme is that, differently from the metabolic networks, for which the MM approximation is well suited, in this network, the enzymes are, by themselves, reversible substrates of other enzymes.

The double phosphorylation, as well as double dephosphorylation of MAPK, was recently modeled taking into consideration the competition between the pools of MAPK with different phosphorylation states [66,108]. In [15,37,38,126], we model this process by assuming that competition holds for both the phosphorylation as well as the dephosphorylation processes as in [66].

In [126], a comparison between the application of the sQSSA and of the tQSSA to the MAPK cascade computational model has been performed. Also, in this case, the double phosphorylation–dephosphorylation loops were approximated by the tQSSA for fully competitive reactions. The tQSSA was shown to be much more effective, though better approximations are needed for similar pathways, characterized by high complexity.

As already remarked, the main reason for the failure of the sQSSA lays in the fact that the complexes, far from asymptotically going to zero, are not accounted in the conservation laws. This implies the prediction, by the sQSSA, of lower total concentrations than what is expected or experimentally observed. This fact induced some authors [17,18,80,168], either to rediscover the sequestration of the substrates by the kinases or the phosphatases, or to postulate the existence of some substrate sequestration mechanism, by means of competition or inhibition, made by other enzymes. Actually, the use of the tQSSA not only does not need any additional sequestration hypothesis, but also correctly accounts for the exact asymptotic concentration values of inactive, active, and bound substrates.

We show on one side that the use of the sQSSA brings to wrong asymptotic values, and on the other side that the use of the tQSSA brings to correct predictions for the asymptotic concentrations of all the reactants, because the total variables take simultaneously into account substrates and complexes. In particular, in [15,37,38], we show that the sQSSA can predict bistability for large value ranges, whereas the full system shows monostability. The existence of multistability in futile cycles, as the single and double phosphorylation–dephosphorylation cycle, was investigated in [166] from a theoretical point of view, showing that in the Goldbeter–Koshland switch there exists only one steady-state point, while in the double cycle the system could show three stationary states.

We investigated the scheme where the double-phosphorylated kinase (MAPKK-PP) acts on the MAPK (for example ERK) through specific phosphorylation events on two distinct sites, while the phosphatase (MKP3) acts with a reverse action on MAPK-PP, inactivating it.

The reaction, as described in [119], can be summarized as follows

$$\begin{aligned}
M + E &\underset{k_{-11}}{\overset{k_{11}}{\rightleftarrows}} M - E = C_1 \overset{k_{12}}{\to} M_{\mathrm{p}} + E \\
M_{\mathrm{p}} + E &\underset{k_{-21}}{\overset{k_{21}}{\rightleftarrows}} M_{\mathrm{p}} - E = C_2 \overset{k_{22}}{\to} M_{\mathrm{pp}} + E \\
M_{\mathrm{pp}} + F &\underset{k_{-31}}{\overset{k_{31}}{\rightleftarrows}} M_{\mathrm{pp}} - F = C_3 \overset{k_{32}}{\to} M_{\mathrm{p}} + F \\
M_{\mathrm{p}} + F &\underset{k_{-41}}{\overset{k_{41}}{\rightleftarrows}} M_{\mathrm{p}} - F = C_4 \overset{k_{42}}{\to} M + F
\end{aligned} \tag{84}$$

where M, M_{p} and M_{pp}, respectively, represent the inactive, the monophosphorylated and the double-phosphorylated MAPK, E and F, are, respectively, the kinase MAPKK-PP, and the phosphatase MKP3 and C_i are the intermediate complexes.

Using the law of mass action, the full system of equations governing the dynamics of the system is therefore

$$\begin{aligned}
\frac{\mathrm{d}M}{\mathrm{d}t} &= -k_{11} M E + k_{-11} C_1 + k_{42} C_4 \\
\frac{\mathrm{d}M_{\mathrm{p}}}{\mathrm{d}t} &= -k_{21} M_{\mathrm{p}} E + k_{-21} C_2 + k_{-41} C_4 \\
&\quad -k_{41} M_{\mathrm{p}} F + k_{32} C_3 + k_{12} C_1 \\
\frac{\mathrm{d}M_{\mathrm{pp}}}{\mathrm{d}t} &= -k_{31} M_{\mathrm{pp}} F + k_{-31} C_3 + k_{22} C_2
\end{aligned}$$

$$\begin{aligned}
\frac{\mathrm{d}C_1}{\mathrm{d}t} &= k_{11} M E - (k_{-11} + k_{12}) C_1 \\
\frac{\mathrm{d}C_2}{\mathrm{d}t} &= k_{21} M_\mathrm{p} E - (k_{-21} + k_{22}) C_2 \\
\frac{\mathrm{d}C_3}{\mathrm{d}t} &= k_{31} M_\mathrm{pp} F - (k_{-31} + k_{32}) C_3 \\
\frac{\mathrm{d}C_4}{\mathrm{d}t} &= k_{41} M_p F - (k_{-41} + k_{42}) C_4
\end{aligned} \tag{85}$$

with initial conditions

$$M(0) = M_T, \quad M_\mathrm{p}(0) = M_\mathrm{pp}(0) = 0, \quad C_i(0) = 0, \tag{86}$$

where $i = 1, \ldots, 4$, and conservation laws

$$M + M_\mathrm{p} + M_\mathrm{pp} + C_1 + C_2 + C_3 + C_4 = M_T, \tag{87}$$

$$E + C_1 + C_2 = E_\mathrm{T}, \quad F + C_3 + C_4 = F_\mathrm{T}. \tag{88}$$

Setting $K_i = \frac{k_{-i1} + k_{i2}}{k_{i1}}$, $i = 1, \ldots, 4$, the sQSSA implies that

$$C_1 = \frac{M\,E}{K_1}, \quad C_2 = \frac{M_\mathrm{p}\,E}{K_2}, \quad C_3 = \frac{M_\mathrm{pp}\,F}{K_3}, \quad C_4 = \frac{M_\mathrm{p}\,F}{K_4} \tag{89}$$

which give

$$\begin{aligned}
\frac{\mathrm{d}M}{\mathrm{d}t} &= -\frac{k_{12}}{K_1} M\,E + \frac{k_{42}}{K_4} M_\mathrm{p}\,F \\
\frac{\mathrm{d}M_\mathrm{p}}{\mathrm{d}t} &= -\frac{k_{22}}{K_2} M_\mathrm{p}\,E - \frac{k_{42}}{K_4} M_\mathrm{p}\,F + \frac{k_{32}}{K_3} M_\mathrm{pp}\,F + \frac{k_{12}}{K_1} M\,E \\
\frac{\mathrm{d}M_\mathrm{pp}}{\mathrm{d}t} &= \frac{k_{22}}{K_2} M_\mathrm{p}\,E - \frac{k_{32}}{K_3} M_\mathrm{pp}\,F
\end{aligned} \tag{90}$$

where

$$E = \frac{E_\mathrm{T}}{1 + \frac{M}{K_1} + \frac{M_\mathrm{p}}{K_2}}, \quad F = \frac{F_\mathrm{T}}{1 + \frac{M_\mathrm{pp}}{K_3} + \frac{M_\mathrm{p}}{K_4}} \tag{91}$$

and

$$M(0) = M_\mathrm{T}, \quad M_\mathrm{p}(0) = M_\mathrm{pp}(0) = 0. \tag{92}$$

Let us observe that, when we apply the sQSSA, we set all the complexes constant. This means that the conservation law becomes

$$M + M_\mathrm{p} + M_\mathrm{pp} = \text{constant} \tag{93}$$

The constant in (93) is, in general, different from M_T but the application of both the sQSSA and the conservation law since the beginning of the reaction naturally brings to the equality constant $= M_\mathrm{T}$.

This leads to the complex depletion paradox: the application of the sQSSA implies that, while the complexes are related to the substrates by the equations (89), they are implicitly set equal to zero, because of (93).

The consequences are that the sQSSA predicts asymptotic values for the different substrate species which are higher than those ones predicted by the full system.

Let us introduce the *total* QSSA (tQSSA) by setting

$$\overline{M} = M + C_1, \quad \overline{M}_\mathrm{p} = M_\mathrm{p} + C_2 + C_4, \quad \overline{M}_\mathrm{pp} = M_\mathrm{pp} + C_3. \tag{94}$$

In terms of these new variables, supposing that the complexes are in a quasi-steady state, the set of equations (85) becomes

$$\begin{aligned}
\frac{\mathrm{d}\overline{M}}{\mathrm{d}t} &= k_{42} C_4 - k_{12} C_1, \\
\frac{\mathrm{d}\overline{M}_\mathrm{p}}{\mathrm{d}t} &= -k_{22} C_2 - k_{42} C_4 + k_{32} C_3 + k_{12} C_1, \\
\frac{\mathrm{d}\overline{M}_\mathrm{pp}}{\mathrm{d}t} &= -k_{32} C_3 + k_{22} C_2.
\end{aligned} \tag{95}$$

Table 1 Kinetic parameters of reaction (85)

k_{11}	k_{-11}	k_{12}	k_{21}	k_{-21}	k_{22}
0.02	1	0.01	0.032	1	15
k_{31}	k_{-31}	k_{32}	k_{41}	k_{-41}	k_{42}
0.0045	1	0.0092	0.01	1	0.5

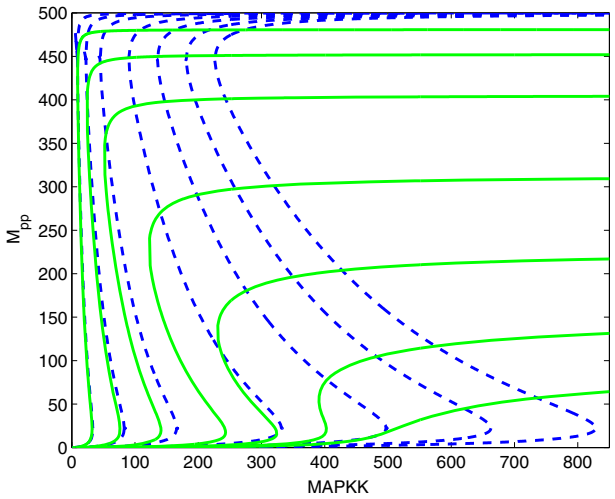

Fig. 3 Stationary branches of the double-phosphorylated MAPK (M_{pp}) in the full system (85) (*solid*) and in its sQSSA approximation (90) (*dashed*), obtained varying the initial concentration of the kinase MAPKK, for different values of the initial concentration of the phosphatase: MKP3 = 20, 50, 100, 200, 300, 400, 500 (*left–right*); kinetic parameters as in Table 1 and $M_{\mathrm{T}} = 500$. Taken from [37,38]

with same initial conditions of (86) and conservation law

$$\overline{M} + \overline{M}_{\mathrm{p}} + \overline{M}_{\mathrm{pp}} = M_{\mathrm{T}} \tag{96}$$

and where the complexes are given in function of the total substrates by

$$\begin{aligned} \left(\overline{M} - C_1\right)\,(E_{\mathrm{T}} - C_1 - C_2) - K_1\,C_1 &= 0 \\ \left(\overline{M}_{\mathrm{p}} - C_2 - C_4\right)\,(E_{\mathrm{T}} - C_1 - C_2) - K_2\,C_2 &= 0 \\ \left(\overline{M}_{\mathrm{pp}} - C_3\right)\,(F_{\mathrm{T}} - C_3 - C_4) - K_3\,C_3 &= 0 \\ \left(\overline{M}_{\mathrm{p}} - C_2 - C_4\right)\,(F_{\mathrm{T}} - C_3 - C_4) - K_4\,C_4 &= 0 \end{aligned} \tag{97}$$

The above system of equations has been obtained using the QSSA ($\frac{\mathrm{d}C_i}{\mathrm{d}t} = 0$), Eqs. (87) and (88) in the last 4 equations of (85).

Let us remark that, in this case, we do not observe any complex depletion paradox, because the initial conditions are given on the total substrates, no matter if the substrates are free or bound. Consequently, even after the application of the quasi-steady-state approximation, the conservation law is fully respected, without any additional condition on the complexes. Thus, the tQSSA yields the same asymptotic values for all the reactants (complexes included) as the full system.

The set of kinetic parameters shown in Table 1 was taken from [119], as well as the value of M_{T}.

In Fig. 3, we show the stationary branches of the double-phosphorylated MAPK (M_{pp}) in the full system (85) and in its sQSSA approximation (90), obtained varying the initial concentration of the kinase MAPKK, for different values of the initial concentration of the phosphatase. Note that the asymptotic values of the tQSSA are not shown because, as already remarked, the tQSSA gives the same asymptotic values of the full system.

In Fig. 4, we show the meaning of the complex depletion paradox: ignoring the initial, fast transient phase, the sQSSA imposes as initial condition for the sum of the substrate concentrations $M + M_{\mathrm{p}} + M_{\mathrm{pp}}$, the value M_{T} of the initial concentration of the unphosphorylated substrate. Actually, even in a fast transient phase, the

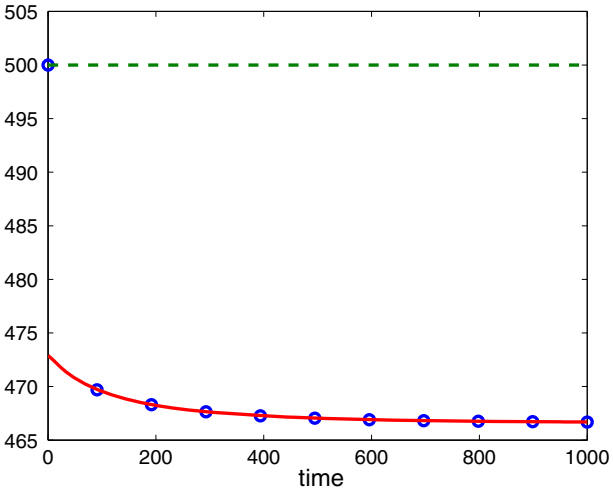

Fig. 4 Dynamics of $M + M_p + M_{pp}$ in the reaction (84): full system (*circles*), tQSSA approximation (*solid*), sQSSA approximation (*dotted*). Here, MAPKK = 30, MKP3 = 20, kinetic parameters as in Table 1 and $M_T = 500$. Differently from the full system and the tQSSA, the sQSSA predicts at any time a constant value M_T for $M + M_p + M_{pp}$ that would imply $C_1 + C_2 + C_3 + C_4 = 0$. Taken from [37,38]

substrates begin to be bound in the complexes and the initial condition for the quasi-steady-state phase should be set less than M_T. Nevertheless, relations (89) give at any time a concentration value different from zero for the total complexes $C_1 + C_2 + C_3 + C_4$, in contrast to the previous figure, where the fact that $M + M_p + M_{pp}$ is always equal to M_T would imply that $C_1 + C_2 + C_3 + C_4 = 0$ at any time.

The results clearly show the limits of the sQSSA when applied to cycles, where the central role of the intermediates cannot be neglected. Indeed, when the total amount of enzymes is sufficiently low, the sQSSA approximation shows discrete results while when the total concentration of enzymes grows, the sQSSA is completely wrong both for the asymptotic values and, more importantly, for the range of bistability. Note that for $MKP3 = 400, 500$, the sQSSA predicts a wide range of bistability, while the dynamics of the system is in a monostable regime, showing that, already in the case of the phosphorylation–dephosphorylation cycle, the application of the tQSSA or, better, of the full system of equations is in general much more appropriate.

6 Conclusions and perspectives

The Michaelis–Menten kinetics and the quasi-steady-state approximations have shown to be very efficient and accurate in reproducing experimental data of enzymatic reactions, provided the parameters and the initial conditions follow suitable hypotheses.

Due to the lack of any a priori study about the applicability of the approximations, many models governing complex reaction mechanisms can fail in reproducing experimental data, mainly when nonlinear phenomena, as bifurcations, oscillations, multistability, and ultrasensitivity, are concerned.

Many intracellular reactions are governed by threshold mechanisms. The wrong determination of the asymptotic reactant concentrations can heavily affect the mathematical model of the system and its predictions. Though the sQSSA represents a very important tool for the qualitative and quantitative study of single enzyme–substrate reactions, it has shown to be in general inadequate, for several reasons, when applied to complex reaction networks, like those ones occurring inside the cell.

One of the main advantages of the sQSSA is the simplification of the mathematical scheme describing the enzyme reactions, which allows us to capture many qualitative and quantitative features that could not be observed by means of the full system. Again, as shown in this paper, the application of the tQSSA brings to predictions that are much more accurate than the sQSSA ones, quantitatively and qualitatively. Thus, the use of the total substrates shows to be much more than a mere variable change, bringing to a correct explanation and prediction of the sequestration mechanism, which is not allowed by the sQSSA.

However, the importance of any steady-state approximation is related to the possibility to capture in a qualitative way the behavior of even complex systems. In particular, the QSSA can help the dimensional reduction in the differential systems governing the reaction networks.

Thus, deeper studies on the mathematical aspects of the QSSA are needed. The tools which seem to be more promising are the center manifold theory and the normal form theory (see, for example, [22,61,114,115,134,140,167], where the two theories are connected to each other and to the bifurcation theory and to chaos); the Tikhonov Theorem (see for example [47,67,76,158]); the renormalization group theory applied to singular perturbations, recently rediscovered (see, for example, [26,45,58,77,116,133]); the geometric singular perturbation theory (GSPT) [52,68,78]. All these theories cope on one side with small perturbation parameters, on the other side with multiscale phenomena and timescale separation, which both allow the dimensional system reduction and the simplification of the model, according to the innovative ideas and techniques introduced and applied in several recent works by Luongo et al. [55,85,87–101,123].

Some authors [26,58] proposed an alternative method to tackle the problem of singular perturbations, based on the renormalization group (RG) theory, observing that the problem of several singular perturbations is the presence of secular terms (i.e., terms diverging in time), physically incorrect, either in the inner or in the outer solution. The RG method is applied to eliminate the divergent terms, by the introduction of (even infinite) counterterms. In [77], this procedure is explained in a simplified form and applied to examples usually treated with multiple scale or other singular perturbation methods [9]. In a recent thesis [133], the author applied this method to the Michaelis–Menten kinetics, determining the $O(\epsilon)$ perturbation terms. In this case, the perturbation expansions lead to cumbersome equations and cannot be easily performed at orders higher than the first, where probably the physical content of the enzymatic scheme emerges. Indeed, the perturbation scheme pulls the singular terms toward higher orders; thus, a systematic study of the $O(\epsilon^2)$ terms would be desirable.

The dimensional reduction has already been approached in [22] (pp. 8–10) for the case of a single reaction, in a sQSSA framework, in terms of center manifold, while in [78] the authors obtain the center manifold of the same reaction in a tQSSA framework.

Let us also remark that, as shown in [126], in the presence of mechanisms, like feedbacks, generating oscillations, any QSSA is inadequate to approximate the full system, since substrate oscillations imply kinase and phosphatase oscillations, which are in contrast to the main quasi-steady-state assumption. In these cases, in order to obtain realistic predictions, the reactions can be described only by the full system, though it contains a much greater number of equations and variables.

Our aim is to extend our investigations to more complex reactions, governing the cell functioning. In fact, it is well known that the mathematical description of the double phosphorylation–dephosphorylation mechanism is a common feature not only of the MAPK cascade but of any reaction involving a double-step activation and the corresponding double-step deactivation. Even if we expect, in general, much more involved phenomena, like the presence of multiple timescales and thus more complex singular phenomena, we think that our mathematical tools will be able to model, explain, and predict their main characteristics. For example, preliminary studies on the application of optimal control techniques in still simple mechanisms, in order to simulate the effect of drugs on specific targets inside the intracellular reaction networks, were recently published [11,12].

On the other hand, theoretical studies concerning the presence of bifurcations and oscillations in some reaction cascades, as the MAPK cascade, have been recently proposed [84,131,171] by means of the so-called generalized modeling [60,154], showing, by means of Monte Carlo simulations, that a sufficient condition for oscillations in the MAPK cascade is the presence of bistability in the second layer of the cascade, characterized by the double cycle of phosphorylation–dephosphorylation studied in [37,38]. Moreover, the papers suggest the possible presence of complex Hopf bifurcations of generalized type [86,88–90,94,96] which must be deepened.

A theoretical study of the relations between bistability, oscillations, bifurcations, and chaos would be welcome, in order to extend the results to more complex scenarios.

For modeling of chemical reactions may be also important to take into account diffusion (since the different proteins move inside the cell, affecting the kinetics), delays, and transport phenomena, such as in the case of electrophoresis [6], where the action of the electrical field and fluid flow is very important. This makes systems describing the chemical reactions much more complicated. Such systems demonstrate various types of instabilities, oscillatory, and chaotic behavior. In addition, in such nonlinear systems, existence of material and non-material interfaces can be observed, such as in the case of modeling of phase transitions, see, e.g., [48–50,135–137].

The nonlinear system of differential equations, which has been described in the present paper, has some peculiar properties, which are related to the phenomena they are intended to describe. The authors are aware of the fact that many other coupled kinetic phenomena could be described by equations having similar structure.

Of course, the extensions and similarities are innumerable and could not be accounted for in a exhaustive way, even partially. Therefore, simply to give an idea of the possible extensions of the studies which we describe in this paper, we list some related researches and some interesting mathematical models, to which our attention was attracted mainly because we found several suggestive and even unexpected parallels with models presenting mathematical problems generalizing those dealt with here and we limit ourselves to shortly cite only them.

1. In the papers [3–5,8,40,128,129,165], the coupled systems are some structural elements (which could be studied also in nonlinear regimes) coupled by means of piezoelectric transducers (i.e., a system allowing for reversible energy exchange) to an electric circuit where an irreversible dissipation occurs. The systems considered in the previous list are governed by systems of PDEs, and therefore, the analysis could become much more complex: however, when some projection to a finite dimensional subspace of the state functions space is possible, the methods discussed in the present paper are more easily generalized.
2. In other papers, the considered coupling phenomena are those involving deformable porous matrices with connected or non-interconnected pores, completely filled with deformable fluids. In this case, the reversible coupling phenomenon involves an exchange of the deformation energy of the solid matrix and the compression energy of the fluid inside the pores, while the irreversible transformation may be identified with the dissipative phenomena occurring inside the fluid (as Darcy dissipation or Stokes–Navier dissipation). Also, in this case, some partial differential equations appear, but when the continuum models involve so-called internal variables, some of the coupled equations are indeed ordinary, circumstance that may simplify the study: we refer for instance to the papers [41,42,104,132].
3. Moreover, some other biological phenomena are governed by coupled nonlinear first-order differential equations. These phenomena occur in reconstructed bones, where bone mass density synthesis and bioresorbable scaffold material removal may occur. The system of ODEs presented in [82,105–107] has indeed a similar structure as those we have described in the present paper. However, the source terms appearing in them actually depend on the static solution of a coupled elastic problem: this makes the generalization of the presented results more difficult.
4. In the paper [39], an ordinary differential equation is coupled with two PDEs defined in two adjacent subdomains of $\mathbb{R} \times \mathbb{R}$. Indeed, the considered problem is a free-moving boundary problem, where a reversible phase transition occurs and the energy is produced or absorbed by arriving from far located fluid.
5. In the papers [23,24,30,31,34,35,43,44], some models for damaged and composite continua are introduced and studied. In these models, some strongly nonlinear ODEs are coupled to PDEs and the reversible changes in energy are related to elastic phenomena, while the irreversible transformations are related to damage or dissipation phenomena because of internal friction.

A deep analysis of the problems considered in the listed papers, and of the techniques applied for their resolution, could give fruitful scientific "contaminations" and suggestions for our future research.

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