BISTABILITY AND THE COMPLEX DEPLETION PARADOX IN THE DOUBLE PHOSPHORYLATION-DEPHOSPHORYLATION CYCLE

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Abstract: In this paper we discuss the applicability of the standard quasi steady-state approximation (sQSSA) to complex enzyme reaction networks, like the ones involved in intracellular signal transduction. In particular we focus on the dynamics of the intermediate complexes, which in common literature either are ignored or are supposed to rapidly become negligible in the quasi steady-state phase, differently from what really happens. This brings to what we call "complex depletion paradox", according to which complexes disappear in the conservation laws, in contrast with the equations of their dynamics. Applying the total quasi steady-state approximation (tQSSA) to the double phosphorylation-dephosphorylation cycle, we show how to solve the apparent paradox, without the need of further hypotheses, like, for example, the substrate sequestration.

1 INTRODUCTION - MICHAELIS-MENTEN KINETICS AND QUASI STEADY-STATE APPROXIMATION

Michelis-Menten kinetics (Henri, 1901a; Henri, 1901b; Michaelis and Menten, 1913; Briggs and Haldane, 1925) represents a fundamental milestone in biochemistry, as it gives a very good approximation of the dynamics of the different enzymes involved. Its formulation considers a reaction where a substrate S binds an enzyme E reversibly to form a 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.
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 (supposed constant) associated with the reaction rates.
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 ordinary differential equation (ODE) for each involved complex and substrate, where the concentration variation for each reactant is proportional to the reactant concentrations. We refer to this as the full system.
From now on we will use the same symbols for the names of the enzymes and their concentrations. For (1) the equations are

$$\frac{dS}{dt} = -a(E_T - C)S + dC \tag{2}$$

$$\frac{dC}{dt} = a(E_T - C)S - (d+k)C\,. \tag{3}$$

with initial conditions

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

and 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 dynamics is described by the ODEs, while E and P can be deduced from S and C via (5). 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 called Michaelis-Menten (MM) kinetics (Michaelis and Menten, 1913;

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Briggs and Haldane, 1925; Segel, 1988; Bisswanger, 2002). Let us observe that the system (2–3) admits only one asymptotic solution. This solution is given by $C = S = 0$, so that from (5) we get asymptotically $P = S_T$ 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.

Assuming that the complex concentration is approximately constant after a short transient phase leads to the usual Michaelis-Menten (MM) approximation, or *standard quasi steady-state approximation* (sQSSA). It leads to an ODE for the substrate while the complex is assumed to be in a quasi-steady state (i.e., $\frac{dC}{dt} \approx 0$):

$$\frac{dS}{dt} \approx -kC \approx -\frac{V_{max}S}{K_M + S}, \quad S(0) = S_T, \qquad (6)$$

where

$$V_{max} = kE_T, \quad K_M = \frac{d+k}{a}\ . \qquad (7)$$

Segel and Slemrod (Segel and Slemrod, 1989) showed that a necessary condition for the validity of the sQSSA approximation is

$$E_T \ll K_M + S_T$$

which is valid when the enzyme concentration is much lower than either the substrate concentration or the Michaelis constant K_M (Segel, 1988; Segel and Slemrod, 1989). This condition is usually fulfilled for *in vitro* experiments, but often breaks down *in vivo* (Straus and Goldstein, 1943; Sols and Marco, 1970).

However, to simulate physiologically realistic *in vivo* scenarios, one faces the problem that the MM approximation is no longer valid as mentioned above. Hence, even though the kinetic constants such as K_M are identical *in vivo* and *in vitro*, they need to be implemented in an approximation which is valid for the system under investigation.

Approximations such as the *total QSSA* (tQSSA) (Laidler, 1955; Borghans et al., 1996; Tzafriri, 2003),which is valid for a broader range of parameters covering both high and low enzyme concentrations, have been introduced recently. It arises by introducing the total substrate

$$\bar{S} = S + C.$$

(2)–(3) then become

$$\begin{aligned} \frac{d\bar{S}}{dt} &= -kC \\ \frac{dC}{dt} &= a[C^2 - (E_T + \bar{S} + K_M)C + E_T\bar{S}]\ . \end{aligned} \qquad (8)$$

Assuming that the complex is in a quasi steady-state $\left(\frac{dC}{dt} = 0\right)$ yields the tQSSA

$$\frac{d\bar{S}}{dt} \approx -kC_-(\bar{S}), \quad \bar{S}(0) = S_T, \qquad (9)$$

where

$$C_-(\bar{S}) = \frac{(E_T + K_M + \bar{S}) - \sqrt{(E_T + K_M + S)^2 - 4E_TS}}{2} \qquad (10)$$

is the only biologically allowable solution of $\frac{dC}{dt} = 0$ in (8).

Tzafriri (Tzafriri, 2003) showed that the tQSSA is valid whenever

$$\varepsilon_{Tz} := \frac{K}{2S_T}\left(\frac{E_T + K_M + S_T}{\sqrt{(E_T + K_M + S_T)^2 - 4E_TS_T}} - 1\right) \ll 1, \qquad (11)$$

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

$$\varepsilon_{Tz} \leq \frac{K}{4K_M} \leq \frac{1}{4}. \qquad (12)$$

The parameter K is known as the Van Slyke-Cullen constant.

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

However, *in vivo* the reactions are coupled in complex networks or cascades of intermediate, 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 (Hatakeyama et al., 2003; Markevich et al., 2004; Chickarmane et al., 2007)). Other authors (Ortega et al., 2006) 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. Consequently, since the QSSA is applied considering the complexes substantially constant, the total concentration of free and activated 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 activated) 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 is clearly shown in Figure 6.

In this paper we study the double phosphorylation-dephosphorylation, on one side showing that the use of the sQSSA brings to wrong asymptotic values, on the other showing 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, we show that the sQSSA can predict bistability for large value ranges, whereas the full system shows monostability.

2 THE COVALENT MODIFICATION CYCLE

A case where it is important to consider the contribution from intermediate complexes is the ubiquitous mechanism of covalent modification, 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 important to activate and inactivate enzymes. The mechanism, which provides the building blocks of the MAPK cascade, 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 (Goldbeter and Koshland, 1981) assumes that the enzymes follow the Michaelis-Menten reaction mechanism, given by

$$\begin{aligned} S + E_1 &\underset{d_1}{\overset{a_1}{\rightleftarrows}} C_1 \xrightarrow{k_1} E_1 + P \\ P + E_2 &\underset{d_2}{\overset{a_2}{\rightleftarrows}} C_2 \xrightarrow{k_2} E_2 + S \end{aligned} \tag{13}$$

The reaction is governed by the coupled ODEs

$$\begin{aligned} \frac{dS}{dt} &= -a_1 E_1 \cdot S + d_1 C_1 + k_2 C_2 \\ \frac{dC_1}{dt} &= a_1 E_1 \cdot S - (d_1 + k_1) C_1 \\ \frac{dC_2}{dt} &= a_2 E_2 \cdot P - (d_2 + k_2) C_2 \end{aligned} \tag{14}$$

with initial conditions

$$S(0) = S_T\,, \qquad C_i(0) = 0\,, \quad i = 1,2$$

and conservation laws

$$S_T = S + C_1 + C_2 + P, \tag{15}$$

$$E_{i,T} = E_i + C_i\,, \quad i = 1,2\,. \tag{16}$$

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 (Camps et al., 2000; Zhan et al., 2001), 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 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 (Hatakeyama et al., 2003; Markevich et al., 2004). We model this process by assuming that competition holds for both the phosphorylation as well as the dephosphorylation processes as in (Hatakeyama et al., 2003).

In 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 (Goldbeter and Koshland, 1981) in the case of the phosphorylation-dephosphorylation cycle. Nevertheless most of 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.

However, as observed in the previous section, even when the conservation law for substrates takes into account the complexes, 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, monophosphorylated and double phosphorylated) substrates is no more equal to S_T.

On the other hand the tQSSA cannot produce this situation, 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.

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 which 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.

For example, in (Pedersen et al., 2008) it is shown that the supposed depletion of the complexes in the sQSSA brings to uncorrect asymptotic values of the inactive and active substrate in the Goldbeter-Koshland cycle, as predicted in (Goldbeter and Koshland, 1981). 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 (Sabouri-Ghomi et al., 2008) 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 (Flach and Schnell, 2006) and (Pedersen et al., 2008) it is shown that when the system undergoes oscillations any QSSA 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 presence of the substrate oscillations. Flach and Schnell tested their considerations on the van Slyke-Cullen mechanism (van Slyke and Cullen, 1914), while Pedersen et al. studied the MAPK cascade with feedback, as in (Kholodenko, 2000).

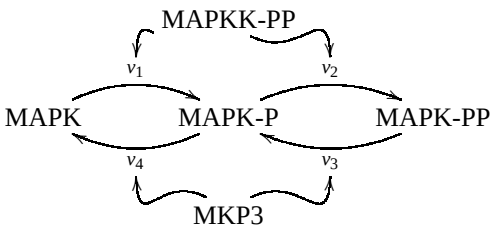
In (Ciliberto et al., 2007; Pedersen and Bersani, 2010) it is shown that the tQSSA reproduces zero-order ultrasensitivity in the Goldbeter-Koshland cycle, according to what was predicted in (Goldbeter and Koshland, 1981), while the sQSSA, for a wide range of parameter values, is not able to yield ultrasensivity whenever it is expected by the theory.

As already remarked, the main reason for the failures 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 (Bluthgen et al., 2006; Legewie et al., 2007; Xing and Chen, 2008), either to re-discover the sequestration of the substrates by the kinases or the phosphatates, or to postulate the existence of some substrate sequestration mechanisms, 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. Let us remark that one of the main advantages of any QSSA is the simplification of the mathematical scheme describing the enzyme reactions, which allows us to capture many qualitative and quantitative features that could not observed by means of the full system.

In the next section we will compare the application of the sQSSA and the tQSSA to the study of the double phosphorylation-dephosphorylation mechanism, showing that, while the latter brings to the same results as the full system, the former brings to consistent errors, mainly in the asymptotic concentration values, predicting bistability for large value ranges, whereas the full system (and the tQSSA) show monostability.

3 THE EQUATIONS

We want to study the scheme



(17)

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.

Several authors have modeled this reaction. In (Ortega et al., 2006) it is supposed that both phosphorylation and dephosphorylation happen in only one step. In (Markevich et al., 2004) the authors describe the dephosphorylation reaction as a two step mechanism. In (Salazar and Hofer, 2006) both phosphorylation and dephosphorylation are assumed to be two step reactions.

We will focus our analysis on the models described in (Ortega et al., 2006) and (Markevich et al., 2004).

The reaction, as described in (Ortega et al., 2006), can

be summarized as follows

$$\begin{array}{lll} M+E \underset{k_{-11}}{\overset{k_{11}}{\rightleftarrows}} & M-E=C_1 & \xrightarrow{k_{12}} M_p+E \\ M_p+E \underset{k_{-21}}{\overset{k_{21}}{\rightleftarrows}} & M_p-E=C_2 & \xrightarrow{k_{22}} M_{pp}+E \end{array} \tag{18}$$

$$\begin{array}{lll} M_{pp}+F \underset{k_{-31}}{\overset{k_{31}}{\rightleftarrows}} & M_{pp}-F=C_3 & \xrightarrow{k_{32}} M_p+F \\ M_p+F \underset{k_{-41}}{\overset{k_{41}}{\rightleftarrows}} & M_p-F=C_4 & \xrightarrow{k_{42}} M+F \end{array}$$

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

$$\begin{aligned} \frac{dM}{dt} &= -k_{11}ME + k_{-11}C_1 + k_{42}C_4 \\ \frac{dM_p}{dt} &= -k_{21}M_pE + k_{-21}C_2 + k_{-41}C_4 \\ &\quad -k_{41}M_pF + k_{32}C_3 + k_{12}C_1 \\ \frac{dM_{pp}}{dt} &= -k_{31}M_{pp}F + k_{-31}C_3 + k_{22}C_2 \\ \frac{dC_1}{dt} &= k_{11}ME - (k_{-11}+k_{12})C_1 \\ \frac{dC_2}{dt} &= k_{21}M_pE - (k_{-21}+k_{22})C_2 \\ \frac{dC_3}{dt} &= k_{31}M_{pp}F - (k_{-31}+k_{32})C_3 \\ \frac{dC_4}{dt} &= k_{41}M_pF - (k_{-41}+k_{42})C_4 \end{aligned} \tag{19}$$

with initial conditions

$$M(0)=M_T, \quad M_p(0)=M_{pp}(0)=0, \quad C_i(0)=0, \tag{20}$$

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

$$M+M_p+M_{pp}+C_1+C_2+C_3+C_4=M_T, \tag{21}$$

$$E+C_1+C_2=E_T, \quad F+C_3+C_4=F_T\,. \tag{22}$$

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

$$C_1=\frac{ME}{K_1}, \quad C_2=\frac{M_pE}{K_2}, \quad C_3=\frac{M_{pp}F}{K_3}, \quad C_4=\frac{M_pF}{K_4} \tag{23}$$

which give

$$\begin{aligned} \frac{dM}{dt} &= -\tfrac{k_{12}}{K_1}ME + \tfrac{k_{42}}{K_4}M_pF \\ \frac{dM_p}{dt} &= -\tfrac{k_{22}}{K_2}M_pE - \tfrac{k_{42}}{K_4}M_pF + \tfrac{k_{32}}{K_3}M_{pp}F + \tfrac{k_{12}}{K_1}ME \\ \frac{dM_{pp}}{dt} &= \tfrac{k_{22}}{K_2}M_pE - \tfrac{k_{32}}{K_3}M_{pp}F \end{aligned} \tag{24}$$

where

$$E=\frac{E_T}{1+\frac{M}{K_1}+\frac{M_p}{K_2}}, \quad F=\frac{F_T}{1+\frac{M_{pp}}{K_3}+\frac{M_p}{K_4}}$$

and

$$M(0)=M_T, \quad M_p(0)=M_{pp}(0)=0\,.$$

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

$$M+M_p+M_{pp}=constant \tag{25}$$

The constant in (25) 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 $constant=M_T$.

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 (23), they are implicitly set equal to zero, because of (25).

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

$$\overline{M}=M+C_1, \quad \overline{M}_p=M_p+C_2+C_4, \quad \overline{M}_{pp}=M_{pp}+C_3\,. \tag{26}$$

In terms of these new variables, supposing that the complexes are in a quasi steady-state, the set of equations (19) becomes

$$\begin{aligned} \frac{d\overline{M}}{dt} &= k_{42}C_4 - k_{12}C_1, \\ \frac{d\overline{M}_p}{dt} &= -k_{22}C_2 - k_{42}C_4 + k_{32}C_3 + k_{12}C_1, \\ \frac{d\overline{M}_{pp}}{dt} &= -k_{32}C_3 + k_{22}C_2\,. \end{aligned} \tag{27}$$

with same initial conditions of (20) and conservation law

$$\overline{M}+\overline{M}_p+\overline{M}_{pp}=M_T \tag{28}$$

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

$$\begin{array}{rcl}(\overline{M}-C_1)(E_T-C_1-C_2)-K_1C_1 &=& 0\\ (\overline{M}_p-C_2-C_4)(E_T-C_1-C_2)-K_2C_2 &=& 0\\ (\overline{M}_{pp}-C_3)(F_T-C_3-C_4)-K_3C_3 &=& 0\\ (\overline{M}_p-C_2-C_4)(F_T-C_3-C_4)-K_4C_4 &=& 0\end{array} \quad (29)$$

The above system of equations has been obtained using the QSSA ($\frac{dC_i}{dt}=0$), (21) and (22) in the last 4 equations of (19).

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.

Similarly, the reaction described in (Markevich et al., 2004) can be summarized as follows:

$$\begin{array}{lll} M+E \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} M-E=C_1 & \xrightarrow{k_2} M_p+E & \\ M_p+E \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} M_p-E=C_2 & \xrightarrow{k_4} M_{pp}+E & \\ M_{pp}+F \underset{h_{-1}}{\overset{h_1}{\rightleftarrows}} C_3 & \xrightarrow{h_2} C_4 & \underset{h_{-3}}{\overset{h_3}{\rightleftarrows}} M_p+F \\ M_p+F \underset{h_{-4}}{\overset{h_4}{\rightleftarrows}} C_5 & \xrightarrow{h_5} C_6 & \underset{h_{-6}}{\overset{h_6}{\rightleftarrows}} M+F \end{array} \quad (30)$$

which can be translated, as before, into differential equations by the law of mass action:

$$\begin{array}{rcl}\frac{dM}{dt} &=& -k_1ME+k_{-1}C_1+h_6C_6-h_{-6}MF\\ \frac{dM_p}{dt} &=& -k_3M_pE+k_{-3}C_2+h_3C_4-h_{-3}M_pF\\ && -h_4M_pF+k_2C_1+h_{-4}C_5\\ \frac{dM_{pp}}{dt} &=& -h_1M_{pp}F+h_{-1}C_3+k_4C_2\\ \frac{dC_1}{dt} &=& k_1ME-(k_{-1}+k_2)C_1\\ \frac{dC_2}{dt} &=& k_3M_pE-(k_{-3}+k_4)C_2\\ \frac{dC_3}{dt} &=& h_1M_{pp}F-(h_{-1}+h_2)C_3\\ \frac{dC_4}{dt} &=& h_{-3}M_pF+h_2C_3-h_3C_4\\ \frac{dC_5}{dt} &=& h_4M_pF-(h_{-4}+h_5)C_5\\ \frac{dC_6}{dt} &=& h_5C_5+h_{-6}MF-h_6C_6\end{array} \quad (31)$$

with initial conditions

$$M(0)=M_T,\quad M_p(0)=M_{pp}(0)=0,\quad C_i(0)=0, \quad (32)$$

where $i=1,\ldots,6$ and conservation laws

$$M+M_p+M_{pp}+C_1+C_2+C_3+C_4+C_5+C_6=M_T, \quad (33)$$

$$E+C_1+C_2=E_T,\quad F+C_3+C_4+C_5+C_6=F_T\,. \quad (34)$$

Setting

$$K_i=\frac{k_{-i}+k_{i+1}}{k_i}\,,\ i=1,2\,,\quad H_i=\frac{h_{-i}+h_{i+1}}{h_i}\,,\ i=1,4$$

the sQSSA approximation implies that

$$\begin{array}{l}C_1=\frac{ME}{K_1}\,,\quad C_2=\frac{M_pE}{K_2}\,,\quad C_3=\frac{M_{pp}F}{H_1}\,,\\ C_4=\frac{1}{h_3}\left[h_2\frac{M_{pp}}{H_1}+h_{-3}M_p\right]F\,,\quad C_5=\frac{M_pF}{H_4}\,,\\ C_6=\frac{1}{h_6}\left[h_5\frac{M_p}{H_4}+h_{-6}M\right]F\end{array}$$

which give

$$\begin{array}{rcl}\frac{dM}{dt} &=& -\frac{k_2}{K_1}ME+\frac{h_5}{H_4}M_pF\\ \frac{dM_p}{dt} &=& -\frac{k_4}{K_2}M_pE-\frac{h_5}{H_4}M_pF+\frac{h_2}{H_1}M_{pp}F+\frac{k_2}{K_1}ME\\ \frac{dM_{pp}}{dt} &=& \frac{k_4}{K_2}M_pE-\frac{h_2}{H_1}M_{pp}F\end{array} \quad (35)$$

where

$$E=\frac{E_T}{1+\frac{M}{K_1}+\frac{M_p}{K_2}},$$

$$F=\frac{F_T}{1+\frac{M_{pp}}{H_1}(1+\frac{h_2}{h_3})+H_5M_p+\frac{h_{-6}}{h_6}M}$$

with $H_5=\frac{h_{-3}}{h_3}+\frac{1}{H_4}+\frac{h_5}{h_6H_4}$ and

$$M(0)=M_T,\quad M_p(0)=M_{pp}(0)=0\,.$$

Let us introduce again the tQSSA by setting

$$\overline{M}=M+C_1+C_6,\quad \overline{M}_p=M_p+C_2+C_4+C_5,$$
$$\overline{M}_{pp}=M_{pp}+C_3\,.$$

In terms of these new variables, supposing that the complexes are in a quasi steady-state, the set of equations (31) becomes

$$\begin{array}{l}\frac{d\overline{M}}{dt}=-k_2C_1+h_5C_5,\\ \frac{d\overline{M}_p}{dt}=k_2C_1-k_4C_2+h_2C_3-h_5C_5,\\ \frac{d\overline{M}_{pp}}{dt}=k_4C_2-h_2C_3\,.\end{array} \quad (36)$$

with same initial conditions of (20) and conservation law

$$\overline{M} + \overline{M}_p + \overline{M}_{pp} = M_T \tag{37}$$

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

$$\begin{aligned}
&(\overline{M} - C_1 - C_6)(E_T - C_1 - C_2) - K_1 C_1 = 0 \\
&(\overline{M}_p - C_2 - C_4 - C_5)(E_T - C_1 - C_2) - K_2 C_2 = 0 \\
&(\overline{M}_{pp} - C_3)(F_T - C_3 - C_4 - C_5 - C_6) - H_1 C_3 = 0 \\
&h_2 C_3 + h_{-3}(\overline{M}_p - C_2 - C_4 - C_5)(F_T - C_3 - C_4 - \\
&\quad C_5 - C_6) - h_3 C_4 = 0 \\
&(\overline{M}_p - C_2 - C_4 - C_5)(F_T - C_3 - C_4 - C_5 - C_6) - \\
&\quad H_4 C_5 = 0 \\
&h_5 C_5 + h_6 (\overline{M} - C_1 - C_6)(F_T - C_3 - C_4 - C_5 - C_6) = 0
\end{aligned} \tag{38}$$

As before, the above system of equations has been obtained using the QSSA ($\frac{dC_i}{dt} = 0$), (33) and (34) in the last 6 equations of (31).

4 NUMERICAL SIMULATIONS AND FIGURES

We have numerically integrated (19) and (31), as well as their M-M approximations (24) and (35), with the standard MATLAB stiff integrator ODE15S. The set of kinetic parameters shown in Table (2) was taken from (Markevich et al., 2004), as well as the value of M_T. The set of kinetic parameters shown in Table (1) is almost the same, except in the two-step dephosphorylation phase, where we have eliminated the parameters related to the second step. Doing so, we tried to find the qualitative differences between a one-step and a two-step dephosphorylation process.

Table 1: Kinetic parameters of reaction (18).

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

The results are shown in Figures 1–7. 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. To find the stationary branches of the three substrate forms (M, M_p and M_{pp}) we have used an iterative algorithm, that can be explained briefly as follows. First, when looking for stationary points, i. e., when we set all the first members of (19) equal to zero, it is easy to express all variables in function of the complexes C_1 and C_2. Then, we begin the iteration on a stable branch,

Table 2: Kinetic parameters of reaction (30), taken from (Markevich et al., 2004).

k_1	k_{-1}	k_2	k_3
0.02	1	0.01	0.032
k_{-3}	k_4	h_1	h_{-1}
1	15	0.045	1
h_2	h_3	h_{-3}	h_4
0.092	1	0.01	0.032
h_{-4}	h_5	h_6	h_{-6}
1	0.5	0.086	0.0011

finding the solution of the full system of ODEs via numerical integration (performed with the standard MATLAB stiff integrator ODE15S). At the subsequent step, to find the solution of the two equations for C_1 and C_2 on the same stable branch, we use as starting guess in the MATLAB's routine FSOLVE the solution obtained at the previous step, and we repeat iteratively this procedure. As expected, stability problems appeared near threshold values, where stable and unstable stationary branches cross. Fine tuning mechanisms of input values of the iterative scheme allowed us to reconstruct efficiently and satisfactorily all the three stationary branches.
The computational costs for the simulations and the iterative algorithm, in terms of CPU time, are in general absolutely low, of the order of seconds for the former, and of few minutes for the latter.

The results show clearly 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 (first two branches on the left of Figures 1–4), 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.

In Figure 5 we show the dynamics of all the substrate and complex concentrations. The sQSSA fails its prediction of the behavior of M, because the initial conditions bring to ignore the presence of the complexes, while, as shown in the plot of C_1, complexes cannot be neglected, even if the initial kinase and phosphatase concentrations are much less than M_T.

In Figures 6–7 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 $M + M_p + M_{pp}$ the value M_T of the initial concentration of the unphosphorylated substrate. Actually, even in a fast transient phase the substrates begin to be bound in

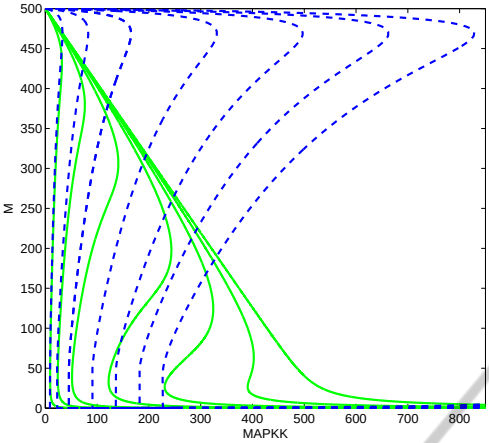


Figure 1: Stationary branches of the inactive MAPKK (M) in the full system (19) (solid) and in its sQSSA approximation (24) (dashed), obtained varying the initial concentration of the kinase MAPKK, for different values of the initial concentration of the phosphatase: MKP3=20,50,100,200,300,400,500 (left-right); kinetic parameters as in Table 1 and $M_T = 500$.

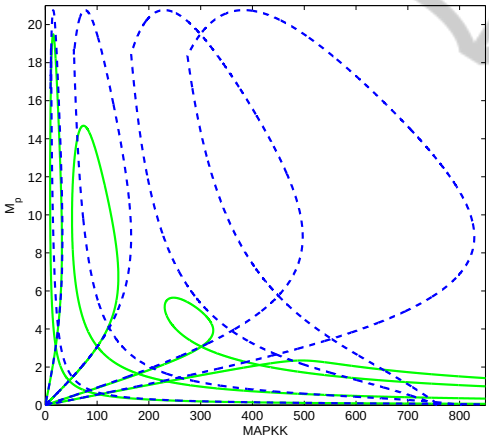


Figure 2: Stationary branches of the mono-phosphprylated MAPKK (M_p) in the full system (19) (solid) and in its sQSSA approximation (24) (dashed), obtained varying the initial concentration of the kinase MAPKK, for different values of the initial concentration of the phosphatase: MKP3=20,100,300,500 (left-right); kinetic parameters as in Table 1 and $M_T = 500$.

the complexes and the initial condition for the quasi steady-state phase should be set less than M_T. Thus, using (25), the total contribution of substrates remains constant, with value M_T.

Nevertheless, as shown in Figure 7, relations (23) give at any time a concentration value different from zero for the total complexes $C_1 + C_2 + C_3 + C_4$, in contrast with the previous figure, where the fact that $M + M_p + M_{pp}$ is always equal to M_T would imply

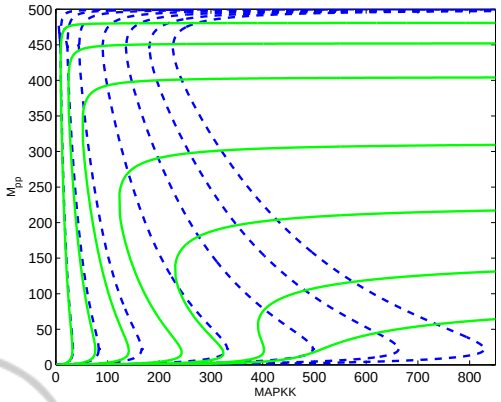


Figure 3: Stationary branches of the double phosphorylated MAPK (M_{pp}) in the full system (19) (solid) and in its sQSSA approximation (24) (dashed), obtained varying the initial concentration of the kinase MAPKK, for different values of the initial concentration of the phosphatase: MKP3=20,50,100,200,300,400,500 (left-right); kinetic parameters as in Table 1 and $M_T = 500$.

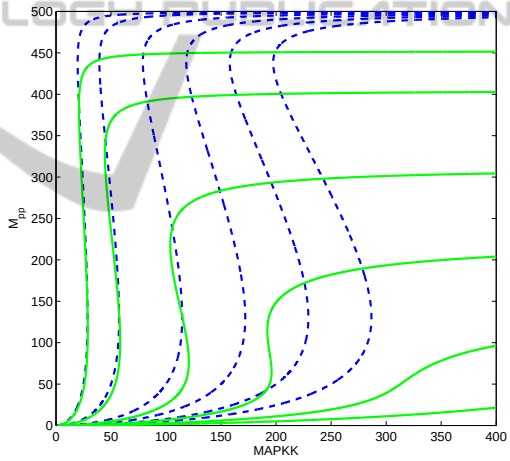


Figure 4: Stationary branches of the double phosphorylated MAPK (M_{pp}) in the full system (31) (solid) and in its sQSSA approximation (35) (dashed), obtained varying the initial concentration of the kinase MAPKK, for different values of the initial concentration of the phosphatase: MKP3=50,100,200,300,400,500 (left-right); kinetic parameters as in Table 2 and $M_T = 500$.

that $C_1 + C_2 + C_3 + C_4 = 0$ at any time.

On the other hand, in the tQSSA, considering as initial condition for the total substrate $\overline{M}(0) = M_T$ we obtain a very good approximation of the true (and in general unknown) initial condition after the transient phase, because in this phase we can reasonably suppose that only the binding of M in the complex C_1 has happened, without affecting the value of $\overline{M}$.

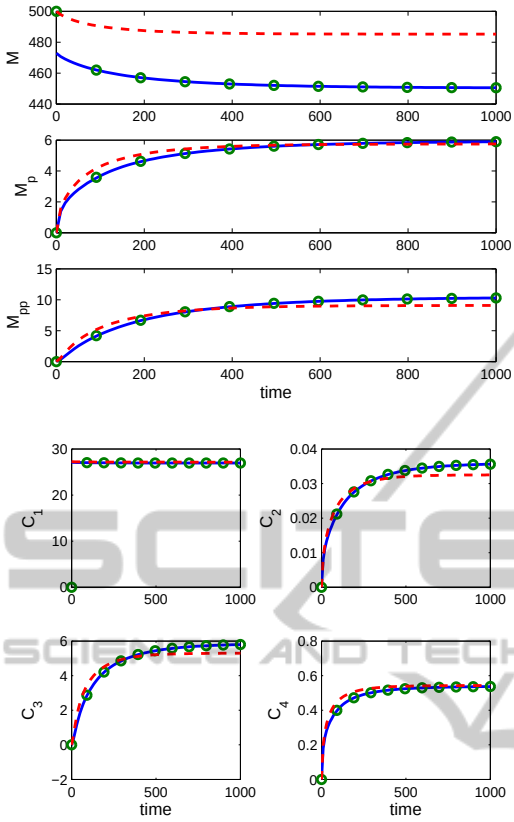


Figure 5: Dynamics of the system (19) with MAPKK=30, MKP3=20, kinetics parameters as in Table 1 and $M_T = 500$: full system (circles), tQSSA approximation (solid), sQSSA approximation (dashed).

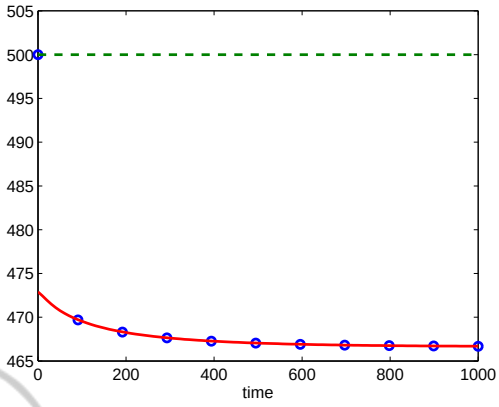


Figure 6: Dynamics of $M + M_p + M_{pp}$ in the reaction (18): full system (circles), tQSSA approximation (solid), sQSSA approximation (dotted). Here MAPKK=30, MKP3=20, kinetic parameters as in Table 1 and $M_T = 500$. Differently from the full system and the tQSSA, the sQSSA predicts at any time a constant value M_T for $M + M_p + M_{pp}$ that would imply $C_1 + C_2 + C_3 + C_4 = 0$.

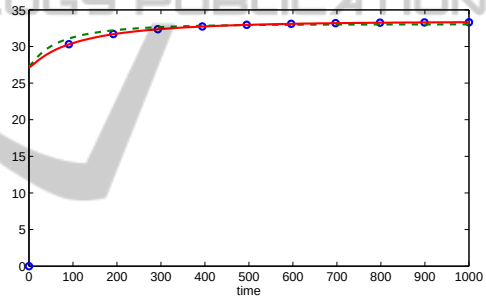


Figure 7: Dynamics of $C_1 + C_2 + C_3 + C_4$ in the reaction (18): full system (circles), tQSSA approximation (solid), sQSSA approximation (dashed). Here MAPKK=30, MKP3=20, kinetics parameters as in Table 1 and $M_T = 500$. In contrast with the previous figure, the sQSSA predicts at any time a value of $C_1 + C_2 + C_3 + C_4$ different from zero.

5 CONCLUSIONS

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.

In this paper we show that, 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.

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 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.

Let us also remark that, as shown in (Pedersen et al., 2008), in 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 with the main quasi steady-state assumption. In this 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 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, we think that our mathematical tools will be able to model, explain and predict their main characteristics.

The results that we present in this paper do need, of course, a validation through experimental data. For this purpose, we have got off the ground a collaboration with personnel from ISMAC–CNR, the Institute for Macromolecular studies (Genova,Italy), in order to test our predictions.

REFERENCES

Bisswanger, H. (2002). *Enzyme Kinetics. Principles and Methods*. Wiley-VCH.

Bluthgen, N., Bruggeman, F. J., Legewie, S., Herzel, H., Westerhoff, H. V., and Kholodenko, B. N. (2006). Effects of sequestration on signal transduction cascades. *FEBS J.*, 273:895–906.

Borghans, J., de Boer, R., and Segel, L. (1996). Extending the quasi-steady state approximation by changing variables. *Bull. Math. Biol.*, 58:43–63.

Briggs, G. E. and Haldane, J. B. S. (1925). A note on the kinetics of enzyme action. *Biochem. J.*, 19:338–339.

Camps, M., Nichols, A., and Arkinstall, S. (2000). Dual specificity phosphatases: a gene family for control of map kinase function. *FASEB J.*, 14:6–16.

Chickarmane, V., Kholodenko, B. N., and Sauro, H. M. (2007). Oscillatory dynamics arising from competitive inhibition and multisite phosphorylation. *J. Theor. Biol.*, 244:68–76.

Ciliberto, A., Capuani, F., and Tyson, J. J. (2007). Modeling networks of coupled anzymatic reactions using the total quasi-steady state approximation. *PLoS Comput. Biol.*, 3:463–472.

Flach, E. H. and Schnell, S. (2006). Use and abuse of the quasi-steady-state approximation. *IEE Proceed. Syst. Biol.*, 153:187–191.

Goldbeter, A. and Koshland, D. E. (1981). An amplified sensitivity arising from covalent modification in biological system. *Proc Natl Acad Sci*, 78:6840–6844.

Hatakeyama, M., Kimura, S., Naka, T., Kawasaki, T., Yumoto, N., Ichikawa, M., Kim, J. H., Saito, K., Saeki, K. M., Shirouzu, M., Yokoyama, S., and Konagaya, A. (2003). A computational model on the modulation of mitogen-activated protein kinase (mapk) and akt pathways in heregulin-induced erbb signalling. *Biochem. J.*, 373:451–463.

Henri, V. (1901a). Recherches sur la loi de l'action de la sucrase. *C. R. Hebd. Acad. Sci.*, 133:891–899.

Henri, V. (1901b). Über das gesetz der wirkung des invertins. *Z. Phys. Chem.*, 39:194–216.

Kholodenko, B. N. (2000). Negative feedback and ultrasensitivity can bring about oscillations in the mitogen-activated protein kinase cascades. *Eur. J. Biochem.*, 267:1583–1588.

Laidler, K. J. (1955). Theory of the transient phase in kinetics, with special reference to enzyme systems. *Can. J. Chem.*, 33:1614–1624.

Legewie, S., Schoeberl, B., N., B., and Herzel, H. (2007). Competing docking interactions can bring about bistability in the mapk cascade. *Biophys. J.*, 93:2279–2288.

Markevich, N. I., Hoek, J. B., and B.N., K. (2004). Signaling switches and bistability arising from multisite phosphorylation in protein kinase cascades. *J. Cell Biol.*, 164:353–359.

Michaelis, L. and Menten, M. L. (1913). Die kinetik der invertinwirkung. *Biochem. Z.*, 49:333–339.

Ortega, F., Garces, J. L., Mas, F., Kholodenko, B. N., and Cascante, M. (2006). Bistability from double phosphorylation in signal transduction. kinetic and structural requirements. *FEBS J.*, 273:3915–3926.

Pedersen, M. and Bersani, A. M. (2010). The total quasi-steady state approximation simplifies theoretical analysis at non-negligible enzyme concentrations: Pseudo first-order kinetics and the loss of zero-order ultrasensitivity. *J. Math. Biol.*, 60:267–283.

Pedersen, M. G., Bersani, A. M., and Bersani, E. (2008). Steady-state approximations in intracellular signal transduction – a word of caution. *J. Math. Chem.*, 43:1318–1344.

Sabouri-Ghomi, M., Ciliberto, A., Kar, S., Novak, B., and Tyson, J. J. (2008). Antagonism and bistability in protein interaction networks. *J. Theor. Biol.*, 250:209–218.

Salazar, C. and Hofer, T. (2006). Kinetic models of phosphorylation cycles: A systematic approach using the rapid equilibrium approximation for protein-protein interactions. *Biosystems*, 83:195–206.

Segel, L. (1988). On the validity of the steady state assumption of enzyme kinetics. *Bull. Math. Biol.*, 50:579–593.

Segel, L. A. and Slemrod, M. (1989). The quasi steady-state assumption: a case study in perturbation. *Siam Rev.*, 31:446–477.

Sols, A. and Marco, R. (1970). Concentrations of metabolites and binding sites, implications in metabolic regulation. *Curr. Top. Cell. Regul.*, 2:227–273.

Straus, O. H. and Goldstein, A. (1943). Zone behavior of enzymes. *J. Gen. Physiol.*, 26:559–585.

Tzafriri, A. R. (2003). Michaelis-Menten kinetics at high enzyme concentrations. *Bull. Math. Biol.*, 65:1111–1129.

van Slyke, D. D. and Cullen, G. E. (1914). The mode of action of urease and of enzymes in general. *J. Biol. Chem.*, 19:141–180.

Xing, J. and Chen, J. (2008). The Goldbeter-Koshland switch in the first-order region and its response to dynamic disorder. *PLOS ONE*, 3:e2140.

Zhan, X. L., Wishart, M., and L., G. K. (2001). Nonreceptor tyrosine phosphatases in cellular signaling: regulation of mitogen-activated protein kinases. *Chem. Rev.*, 101:2477–96.